The effect of transforming growth factor-β1 on the Nrf2 antioxidant signalling pathway in human aortic adventitial fibrolasts

Mughal, Tabasum

Awarding institution:
King's College London

The effect of transforming growth factor-β1 on the Nrf2 antioxidant signalling pathway in human aortic adventitial fibroblasts

A thesis submitted by Tabasum Mughal
For the degree of Doctor of Philosophy in the Faculty of Science
King's College, London

2013

Cardiovascular Division, BHF Centre of Research Excellence
School of Medicine
King's College, London

Abstract

Vascular remodelling in arterial disease is characterised by vascular smooth muscle cell and adventitial fibroblast (AF) proliferation and migration due to mechanical and oxidative stresses within the vessel wall, leading to enhanced growth factor signalling. Transforming growth factor β-1 (TGF-β1) is a pleiotropic growth factor that mediates vascular remodelling through eliciting phenotypic changes in AF, which may result from the generation of reactive oxygen species (ROS). Nrf2 is a key transcription factor that co-ordinates expression of endogenous antioxidant defense genes such as heme oxygenase-1 (HO-1) and plays an important role in cellular defense against the deleterious effects of ROS. This thesis has investigated whether TGF-β1 modulates redox-related signalling pathways, gene expression, cell phenotype and motility in AF derived from human aortas.

Results from this study have demonstrated that TGF-β1 treatment resulted in an increase in HO-1 protein expression in AF, an effect that was partially diminished in cells where Nrf2 had been knocked-down using siRNA. In addition, TGF-β1 also enhanced AF migration, measured using time-lapse video microscopy. In the presence of superoxide dismutase (SOD), migration of AF was significantly abrogated suggesting that TGF-β1-mediated AF migration may be mediated by the generation of superoxide. Measurement of ROS generation revealed an increase in AF treated with TGF-β1, an effect that was partially attenuated in the presence of a NAD(P)H oxidase inhibitor or SOD.

The ability of the dietary isothiocyanate, sulforaphane (SFN) to activate the Nrf2 pathway in AF was also investigated. Results revealed that SFN treatment increased HO-1 protein expression which was diminished in cells where Nrf2 had been silenced using siRNA, suggesting that HO-1 induction was Nrf2-dependent. SFN also caused increased nuclear accumulation of Nrf2 and binding to the antioxidant response element (ARE), suggesting the involvement of Nrf2 in ARE-mediated antioxidant gene expression. The ability of SFN to activate endogenous antioxidant defenses may be a potential therapeutic strategy to counteract pathophysiological processes following vascular injury and in the pathogenesis of atherosclerosis.

Findings from this study provide novel insights in to the mechanisms underlying the contribution of redox signaling in AF that lead to vascular diseases and for interventions to modulate TGF-β1-induced adventitial oxidative stress.

Acknowledgements

I would like to thank all of the people, past and present, in the Cardiovascular Division at King's College, London who have helped me to complete this PhD. A special thanks goes to my supervisors, Dr Richard Siow and Dr Maddy Parsons for all of their help, advice, guidance and patience. Their continued support and encouragement have contributed greatly to the completion of this PhD and it has been a great privilege to work with such focused, committed scientists. I would also like to thank the British Heart Foundation for funding this PhD and allowing me to undertake this research.

To all of the friends I have made during my PhD: thank you for giving me so many memories and for making my experience such an amazing one – each and every one of you have taught me that doing a PhD is more than just about doing science.

The biggest thanks go to my family; I couldn't have done it without you.

Table of Contents

CHAPTER 2 – MATERIALS AND METHODS

CHAPTER 3 – RESULTS

CHAPTER 4 – RESULTS

CHAPTER 5 – RESULTS

List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 6

List of Tables

CHAPTER 1

CHAPTER 2

CHAPTER 6

Abbreviations

(-SH)	Thiol
1^{O} AB	Primary antibody
2^{O} AB	Secondary antibody
4-HNE	4-hydroxynonenal
AAA	Abdominal aortic aneurysm
Ang-II	Angiotensin II
ANOVA	Analysis of variance
AoSMC	Aortic smooth muscle cells
AP-1	Activator protein-1
APC	Antigen presenting cells
ApoE	Apolipoprotein E
APS	Ammonium persulphate
ARE	Antioxidant response element
Arp2/3	Actin-related proteins 2/3
ASMC	Airway smooth muscle cell
ATF-3	Cyclic AMP-dependent transcription factor-3
B2M	Beta-2 microglobulin
Bach1	BACH1 BTB and CNC homology 1, basic leucine zipper transcription factor 1
BAEC	Bovine aortic endothelial cells
BCA	Bicinchoninic acid
BPB	Bromophenol blue
BSA	Bovine serum albumin
BTB	The Broad complex, Tramtrack and Bric-à-Brac
BZIP	Basic leucine zipper
$CACO_2$	Human epithelial colorectal adenocarcinoma cells
CDC42	Cell division control protein 42
Cyt_{b558}	Cytochrome B558
CCL2	Chemokine (C-C motif) ligand 2
CDK	Cyclin dependent kinase
cGMP	Cyclic guanosine monophosphate
CK2	Casein kinase 2
CdKI	Cyclin dependent kinase inhibitor
CNC	Cap'n'collar
CO_2	Carbon dioxide
CORM	Carbon monoxide releasing molecules
CBP	CREB-binding protein
CREB	cAMP-response element binding
CTF	Cell traction force
CTGF	Connective tissue growth factor
Ctrl	Control
CuO	Copper oxide
CVD	Cardiovascular disease

Cys	Cysteine residue
DCF	Dichlorodihydrofluorescein
DHE	Dihydroethidium
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethylsulfoxide
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelium nitric oxide synthase
Erk	Extracellular signal-regulated kinases
EtOH	Ethanol
FA	Focal adhesion
FACS	Fluorescent activated cell sorting
FAD	Flavin adenine dinucelotide
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
Gbr	Growth factor binding protein
GCLC	Glutathione cysteine ligase catalytic subunit
GCLM	Glutathione cysteine ligase modifier subunit
GPx	Glutathione peroxidase
GS•	Thiol radical
GSH	Glutathione
GSH	Reduced glutathione
GSR	Glutathione reductase
GSSG	Oxidized glutathione
GST	Glutathione s-transferase
GTP	Guanosine triphosphate
H_2O	Water
H_2O_2	Hydrogen peroxide
HAEC	Human aortic endothelial cells
HAoAF	Human aortic adventitial fibroblasts
HDL	High density lipoprotein
HEPG2	Human liver hepatocellular carcinoma cell line
HIF-1	Hypoxia inducible factor -1
HNE	4-hydroxynonenal
$HO^{\cdot}$	Hydroxyl radical
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
HT29	Liver hepatocellular cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor-I receptor

INRF-2	Inhibitor of Nrf-2
ITC	Isothiocyanate
JNK	C-Jun N-terminal kinases
K^+	Potassioum ion
Keap1	Kelch-like ECH-associated Protein 1
L-012	8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine- 1, 4(2H,3H)dione
LAP	Latency associated peptide
LDL	Low density lipoprotein
L-Glut	L-glutamine
LLC	Large latent complex
LTBP	Latent TGF-beta binding protein
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MEF	Mouse embryonic fibroblasts
MEK	Mitogen activating protein kinases
MHC	Myosin heavy chain
MI	Myocardial infarction
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase 2
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
MTT	(3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide)
Na^+	Sodium ion
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NAD(P)H-Oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAN_3	Sodium azide
NES	Nuclear export signal
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
NLS	Nuclear localising signal
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NAD(P)H oxidase
NOX2	NADPH oxidase isoform 2
NOX4	NADPH oxidase isoform 4
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	Nuclear factor erythroid 2 related Factor 2
NRK-49F	Normal rat kidney 49 fibroblasts
O_2	Singlet oxygen
$O_2^{\cdot -}$	Superoxide
$ONOO^{\cdot}$	Peroxynitrite
OPA	O-phthalaldehyde

oxLDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor-1
PAK	P21/Cdc42/Rac1-activated kinase
P/S	Penicillin / streptomycin
p38 MAPK	p38 mitogen activating protein kinase
PAB	Polyclonal antibody
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PHOX	Phagocyte oxidase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIC	Proteinase inhibitor cocktail
PEITC	Phenethyl isothiocyanate
PKC	Protein kinase C
PKCδ	Protein kinase c delta
PPARγ	Peroxisome proliferator-activated receptor gamma
PRX1	Peroxiredoxin-1
PVDF	Polyvinylidene difluoride
RAS	Rat Sarcoma gtpase
Redox	Reduction – oxidation reactions
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPL13A	Rbosomal protein L13a
RTK	Receptor tyrosine kinases
SARA	Smad anchor for receptor activation
S.E.M	Standard error of the mean
SDHA	Succinate dehydrogenase unit complex A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFN	Sulforaphane
sGC	Soluble guanylyl cyclase
siRNA	Small interference ribonucleic acid
Shc	Src homology domain 2-containing protein
SHR	Spontaneously hypertensive rat
Smad	Small mothers against decapentaplegic
small MAF	Musculoaponeurotic fibrosarcoma oncogene
SMC	Smooth muscle cell
SOD	Superoxide dismutase
Src	Sarcoma gene
S-S	Disulfide bond
Sulforaphane	1-isothiocyanato-(4R,S)(methylsulfinyl)butane
TAK	TGF-β-associated kinase 1
TATA BOX	Thymine, adenine, thymine, adenine box
T/E	Trypsin/Ethylenediaminetetraacetic acid

*t*BHQ	Tert-butylhydroquinone
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming Growth Factor-β1
Tβ1R1	TGF-β1 receptor type I
TH1/2	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
TRAF 6	TNF receptor associated factor-6
TRX	Thiroredoxin
TRXR	Thioredoxin reductase
Ty	Tyrosine
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
Veh	Vehicle
VSMC	Vascular smooth muscle cell
WASP	Wiskott-Aldrich syndrome family protein
XCT	Cysteine-glutamate transporter
α-SMA	Alpha-smooth muscle actin
α-Tubulin	Alpha-tubulin
γ-GCL	Gamma-glutamate cysteine ligase
γ-GCS	Gamma-glutamate cysteine synthetase
γ-GT	Gamma glutamyl transferase
15d-PGJ_2	15-deoxy-delta(12,14)-prostaglandin J2

CHAPTER 1:

Introduction

CHAPTER 1: Introduction

1.1 **Atherosclerosis**

Cardiovascular disease (CVD) is the leading cause of death in the world, accounting for 29% of all deaths globally in 2012 (World Health Organisation, 2012). Atherosclerosis, a progressive disease of the medium and large-size arteries is one of the most prevalent CVDs and a leading cause of mortality globally, with populations of middle to low income countries being most at risk (World Health Organisation, 2012). Despite advances in the knowledge about the pathogenesis of atherosclerosis, current therapies, although have helped to treat millions of patients, are unable to curb the rising numbers of individuals predisposed to this disease. Novel approaches to studying atherosclerosis from an alternative perspective may help to tackle this disease more effectively through better understanding of the mechanisms involved in its progression.

1.1.1 The 'inside-out' hypothesis of atherosclerosis

Cardiovascular disease and in particular, atherosclerosis, has traditionally been associated with the concept that the primary and predominant response to injury in a blood vessel is initiated in the lumen and the ensuing events occur in the sub-endothelial space (Lusis, 2000; Diaz et al., 1997; Quinn et al., 1987; see Fig 1.1). This well-established 'inside-out' hypothesis postulates that the endothelium plays a central role in mediating the inflammatory response by modulating monocyte adhesion and playing a pivotal role in the infiltration of oxidized lipids and the consequent formation and activation of atherosclerotic lesions (Diaz et al., 1997).

According to the "inside-out" hypothesis, cardiovascular risk factors, including those with a strong genetic component such as familial hypercholoesterolaemia, hypertension, diabetes and elevated levels of haemostatic factors as well as environmental factors such as a high-fat diet, lack of excercise and smoking, are thought to adversely alter the vascular endothelium and trigger a cascade of events (Hansson, 2005; Dzau et al., 2002; Lusis, 2000). Alterations in blood flow play a critical role in endothelial dysfunction which is central to this paradigm (Lovett and Rothwell, 2003; Ross, 1999; Nakashima et al., 1998; Gotlieb et al.,1996; MacMillan, 1985). Endothelial cells in the straight areas of arteries where flow is uniform and laminar are aligned in the direction of flow, however endothelial cells in and around areas of arterial branching where blood flow is disturbed and oscillatory, such as the aortic arch and bifurcations, have no

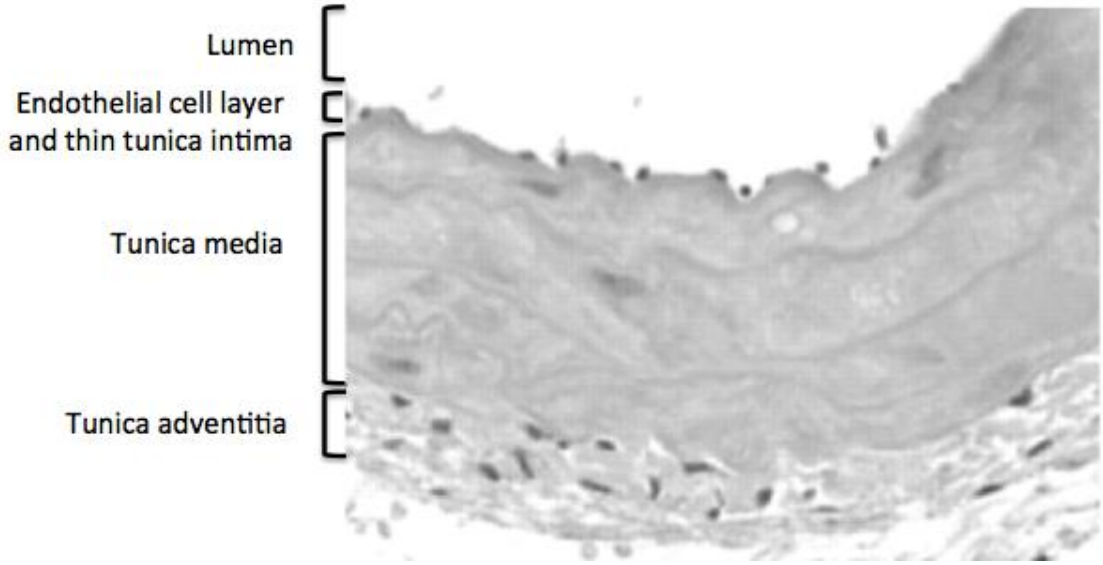


Figure 1.1 The structure of a normal large artery.
Atherosclerosis is a disease of large and medium-sized arteries. The normal morphological structure of a large artery consists of three layers (the intima, the media and the adventital layer) The luminal facing surface of the vessel consists of a monolayer of endothelial cells which rests on an internal elastic lamina. The intima which consists of extracellular connective tissue matrix, is a very thin layer and is mainly made up of proteoglycans and collagen. The media consists of smooth muscle cells and the outer layer, the adventitia, is mainly made up of connective tissue, some SMC, with the majority cell-type being fibroblasts. Adapted from Hu and Xu, 2011.

particular orientation and are likely to be more vulnerable to oxidarive and mechanical stresses, making them more permeable to macromolecules such as low density lipoprotein (LDL) and hence, lesion formation (Lusis 2000; Gimbrone, 1999).

Dysfunctional endothelium is an early manifestation of cardiovascular disease and in this context is usually characterised by impaired flow-mediated dilatation throughout the arterial tree due to the reduced bioactivity of nitric oxide (NO) (Forstermann, 2008; Davies, 2009). Under normal physiological conditions, NO biosynthesis from the endothelium is catalysed by the action of endothelial nitric oxide synthase (eNOS) which transfers electrons from NAD(P)H in its reductase domain to heme in its oxidase domain where the substrate, L-arginine is oxidized to L-citrulline and NO. This sequence of events is disturbed when the essential eNOS co-factor, tetrahydrobiopterin (BH_4) is depleted. Reactive oxygen species (ROS), likely to be generated via NAD(P)H oxidase (Landmesser et al., 2003), a hallmark of atherosclerosis, is thought to increase the degradation of NO by its reaction with superoxide leading to the formation of the peroxynitrite anion ($ONOO^{\cdot}$) which oxidizes BH_4 to the trihydrobioterin radical cation, BH_3 (Davies, 2009). BH_3 can be recycled to BH_4 by eNOS or by L-ascorbic acid, however increased levels of $ONOO^{\cdot}$, as a result of oxidation of BH_4 to biologically inactive products, overwhelm the cell's ability to perform this reduction resulting in eNOS uncoupling, that reduces oxygen to superoxide but no longer synthesises NO (Davis, 2008).

In addition to reduced bioavailability of NO during atherogenesis, another manifestation of altered redox state is the expression of genes that may directly or indirectly regulated by ROS (Kunsch and Medford, 1999). These adhesion molecules expressed by the dysfunctional endothelium such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1), increasing its adhesiveness and allowing the attachment and infiltration of inflammatory cells such as leukocytes and monocytes (Ross, 1999). Accumulation of oxidized LDL in the extracellular sub-endothelial matrix occurs passively through endothelial cell junctions and its retention in the vessel wall is thought to involve interactions between its protein constituent, apolipoprotein B and matrix proteaglycans (Boren et al., 1998). This causes endothelial cells to release proinflammatory molecules such as monocyte chemotactic protein-1 (MCP-1) and granulocyte colony stimulating factor (GCSF), consequently resulting in the recruitment

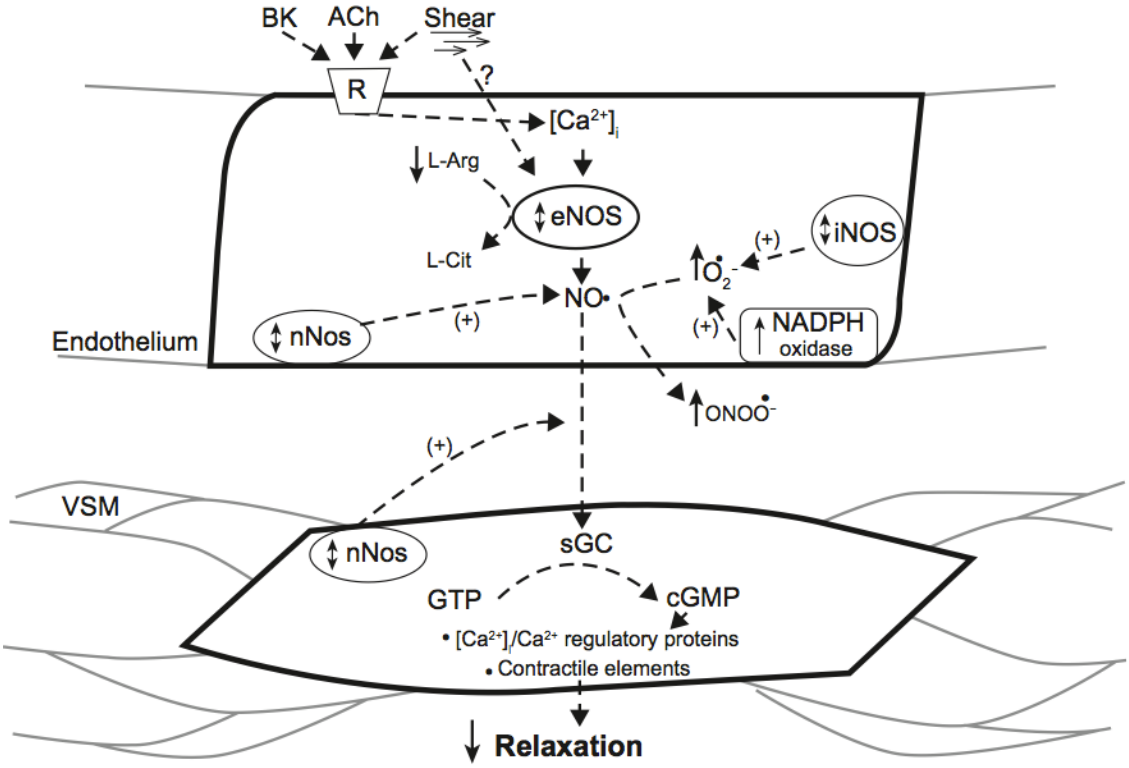


Figure 1.2 Endothelial nitric oxide-mediated vasodilation. In response to chemical and haemodynamic forces at the luminal surface of the blood vessel, eNOS production is stimulated. eNOS transfers electrons from NAD(P)H to the substrate, L-arginine which is then oxidized to L-citrulline and NO. NO diffuses into vascular smooth muscle cells in the media of the blood vessel and activates soluble guanylyl cyclase (sGC) which catalyses the production of cyclic GMP (cGMP) from GTP and causes vasorelaxation. Taken from Levy et al., 2009.

of monocytes to the arterial wall and their subsequent differentiation into macrophages (Diaz et al., 1997; Navab et al., 1996).

Further peroxidation of mildly oxidized LDL to highly oxidized LDL by monocytes and macrophages causes the modified apolipoprotein B component of LDL to become more negatively charged and allows scavenger receptors on macrophages to phagocytose accumulated LDL (Ross, 1999; Raines and Ross, 1993;. Goldstein et al., 1979).

The build-up of this oxidized LDL within the macrophage results in the formation of cytosolic lipid droplets and ultimately leads to the transformation of the macrophage into a foam cell (Hansson, 2005; Quinn et al., 1987). Activated macrophages also release pro-inflammatory cytokines and growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF-2) and transforming growth factor β1 (TGF-β1; Ross, 1999) which exacerbate the inflammatory response, causing the migration and proliferation of smooth muscle cells (SMC) and the synthesis of extracellular matrix (Hansson; 2005; Lusis, 2000), and results in the formation of a fibrous cap which covers a necrotic core consisiting of extracellular lipid, cholesterol crytstals and necrotic debris (Spagnoli et al., 2007; Hansson, 2005; Ross, 1999). For a time, the vessel may be able to compensate for this focal thickening by initiating outward remodelling in order to try and counteract the decrease in lumen size (Ward et al., 2000), however over time and due to a sustained inflammatory response accompanied by smooth muscle cell migration, proliferation and intimal thickening, the lumen of the vessel becomes occluded causing stenosis and restricting blood flow (Glagov et al., 1987), increasing haemodynamic forces acting on the vessel wall and further exacerbating endothelial dysfunction (Davies, 2009). Vascular smooth muscle cell (VSMC) migration is an essential element in the process of intimal hyperplasia and migration of VSMC from the media to the intima and their secretion of extracellular matrix (ECM) proteins is a major component of advanced complicated lesions (Willis et al., 2004, see Fig 1.3).

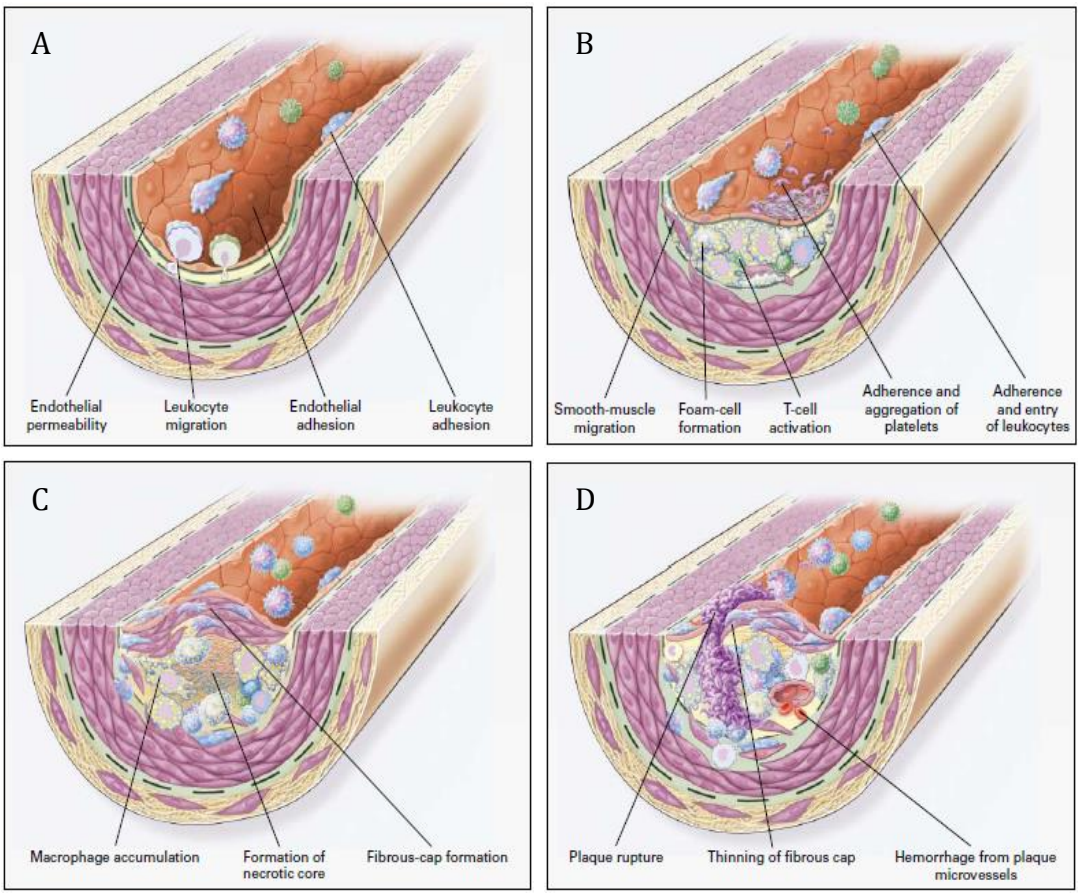


Figure 1.3 The progression of atherosclerosis from the 'inside-out'. (A) Endothelial dysfunction. The earliest hallmark of atherosclerosis is increased endothelial permeabilty, expression of adhesion molecules on the surface of endothelial cells and leukocyte adhesion (B) Fatty streak formation. Infiltration of monocytes, their transdifferentiation to macrophages within the vessel wall and their uptake of lipids results in fatty streak formation. (C) Formation of an advanced complicated lesion. A fibrous cap forms over the lesion, walling it off from the lumen of the vessel. (D) An unstable fibrous cap. Rupture or ulceration of the fibrous cap can lead to thrombosis and consequent occlusion of the lumen of the blood vessel. Adapted from Ross, 1999.

Although coronary artery stenosis contributes to localised ischemia and potentially myocardial infarction, it, in itself is not thought to precipitate these clinical events; most cases of infarction are preceded by the formation of an occluding thrombus on the surface of the plaque which consists of platelets and activated monocytes (Hansson, 2005; Zarifis, 2005). Thrombosis may be caused by plaque rupture, an event which can result in the seeping of the contents of the atheromatous plaque into the lumen of the blood vessel and consequent exposure of the thrombogenic core of the plaque to the blood (Hansson, 2005). The inflammatory response which is a result of the accumulation of mast cells, macrophages, platelets and T-cells at the site of the plaque, release molecules including inflammatory cytokines, proteases, coagulation factors, vasoactive molecules and radicals which contribute to plaque rupture; in particular two types of proteases, matrix metalloproteinases and cysteine proteases. Members of these families of enzymes, such as MMP-2, MMP-3 and MMP-9 affect the composition of the matrix and can lead to hemorrhage from the vasa vasorum or from the lumen of the artery with the final result often being acute coronary syndrome, and in most cases myocardial infarction (Hansson, 2005; Jones et al., 2003; Dzau et al., 2002; Ross, 1999).

The importance of looking at new strategies investigating other sites within the vessel wall, such as the adventitia, which is likely to play a role in atherogenesis, is apparent. Current therapeutic intervention such as aspirin, statins, calcium channel blockers (Willis et al., 2004) and sublingual nitroglycerine administration, though largely effective, cannot counteract the prevalence of this disease. Targeting other sites of inflammation may offer new insights into atherosclerosis as well as the opportunity for novel therapeutic intervention

1.1.2 The ‘outside-in’ hypothesis of atherosclerosis

The majority of evidence implicates the luminal surface as being the main site of the inflammatory response where atherosclerosis is initiated, however, there is a growing body of evidence which points to the previously ignored, outermost adventital layer of the blood vessel (Fig 1.4), which is composed mainly of fibroblasts (Maiellaro and Taylor, 2007). The ‘outside-in’ hypothesis postulates that the inflammatory reaction, involving complex interactions between an increased number of fibroblasts and myofibroblasts and inflammatory cells is one of the early hallmarks of vascular inflammation.

A key characteristic of the 'outside-in' hypothesis postulates that a denser vasa vasorum is thought to be implicated in the progression of the atherosclerotic plaque and eventual rupture by nourishing the plaque and potentially playing a role in the maintenance of vessel homeostasis during vascular remodelling in hypertensive arteries (Kuwahara et al., 2002). In addition to providing essential oxygen and nutrients to the media (Crawford et al., 1998), the vasa vasorum is associated with increased expression of adhesion molecules by the endothelial cells that line this network of microvessels, resulting in a high adventitial infiltration of various classes of leukocytes (Csanyi et al., 2009). Another characteristic of this hypothesis is an increased production of ROS (Maillero and Taylor, 2007; Pagano et al., 1997, see Fig. 1.4), which in turn further exacerbates the infiltration of inflammatory cells in the adventitial layer (Csanyi et al., 2009). Although the exact mechanisms underlying the early inflammatory response inititated in the adventitia are not clear, there is some evidence to suggest that ROS upregulate the transcription factor, NFκB, which causes the release of several pro-inflammatory cytokines, which contribute to adhesion molecule expression (Sarada et al., 2008).

Fibroblasts from different tissue beds display the same heterogeneous phenotype, and are morphologically characterised as adherent, flat, spindle-shaped cells with flat, oval nuclei (Enzerink and Vaheri., 2011). The origins of fibroblasts are thought to be of mesenchymal or neural crest origin, however little is known about their differentation into specific subsets during pathological processes (Enzerink and Vaheri., 2011). The adventitial fibroblast is a central tenet to the 'outside-in' hypothesis of atherosclerosis and its phenotypic switch to a myofibroblast within the vessel wall is a pivotal event (van den Borne et al., 2009; Powell, 2000). The phenotypic switch from a fibroblast to a myofibroblast during disease has been widely reported as being TGF-β1-dependent in several different types of fibroblast (Wang et al., 2010; van den Borne et al., 2009; Powell, 2000; Desmouliere, 1995; Desmouliere et al., 1993). When treated with TGF-β1, fibroblasts become 'activated' and express several proteins which give them the characteristic myofibroblast phenotype (Powell, 2000; see Fig 1.4).

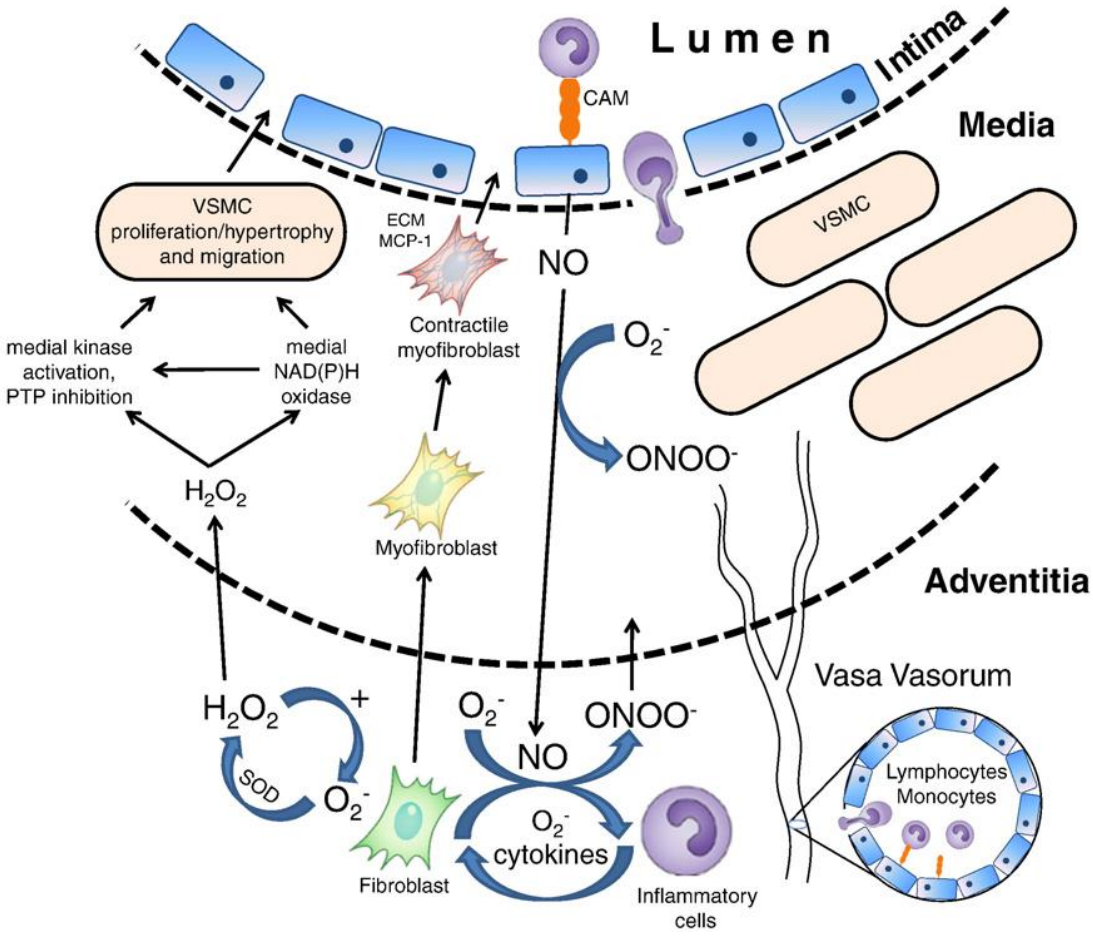


Figure 1.4 The mechanisms leading to the initiation and progression of vascular disease from the 'outside-in'. According to the 'outside-in' hypothesis, the adventitia plays a pivotal role in the initiation of the inflammatory response seen in atherosclerosis. Inflammatory cues such as cytokines signal adventitial fibrobolasts to undergo a phenotypic switch to a migratory myofibroblast. These myofibroblasts migrate to the medial and intimal layers and contribute to neointimal hyperplasia. The myofibroblast transforms into a contractile myofibroblast and generates cytokines, eventually undergoing apoptosis. Cytokine release causes a massive influx of exogenous leukocytes from the vasa vasorum and the subsequent production of superoxide which is enzymatically dismuted to H_2O_2 which may act as a paracrine mediator across the vessel wall. Under pathophysiological conditions, NO is rapidly inactivated by NAD(P)H-derived ROS and results in the highly reactive ROS, peroxynitrite ($ONOO^-$). VSMC proliferation and migration lead to further hypertrophy and constrictive medial remodelling characteristic of progeressive atherosclerotic disease. Taken from Csanyi et al., 2009.

Fully differentiated myofibroblasts are characterised by the expression of several smooth muscle cell proteins including α-smooth muscle actin, the actin polymerising protein, desmin as well as metavinculin and vimentin (Ehler et al., 1996; Powell, 2000; Zargham, 2008). Although they express many SMC proteins, myofibroblasts differ from smooth muscle cells in that they express very low levels of smooth muscle myosin heavy chain (SM-MHC) (Ehler et al., 1996; van den Borne et al., 2009). The contractile characteristic of a myofibroblast is mainly owing to the expression of α-smooth muscle actin and this cell type displays a secretory phenotype and produces an array of proinflammatory chemokines and prostaglandins (Eyden, 2001). It has been reported that at least three local events are required to generate α-smooth muscle actin-positive differentiated myofibroblasts; the accumulation of biologically active TGF-β1, the presence of certain ECM proteins such as the ED-A splice variant of fibronectin, and thirdly high extracellular stress arising from the mechanical properties of the ECM and cell remodelling activity (Hinz et al., 2007; Desmouliere et al., 2005; Tomasek et al., 2000; Fig 1.5).

During the progression of atherosclerosis, smooth muscle cells and adventitial fibroblasts proliferate and migrate into the intima and where adventitial fibroblasts secrete excess extracellular matrix proteins and contribute to neointima formation, constrictive remodelling and restenosis. Evidence of this adventital cell migration was seen in a model of transluminal coronary angioplasty where vascular lesion formation was assessed following balloon overstretch in porcine coronary arteries after days 1, 3, 7 and 14. It was found that BrdU-labelled adventitial cells had migrated into the intima of the injured vessel from 3 days after injury and after fourteen days, the majority of BrdU-labelled adventitial cells had migrated into the neointima suggesting that these cells contributed to vascular lesion formation in this disease model (Scott et al., 1996). The same study also detected an increase in the levels of α-smooth muscle actin in these cells after arterial injury, indicating that in addition to migration, the myofibroblasts had undergone differentiation and subsequently contributed to arterial remodelling and luminal occlusion (Scott et al., 1996). Further evidence of adventitial fibroblast migration into the neointima comes from a rat model of balloon injury, where adventitial fibroblasts transfected with beta-galactosidase (LacZ) were introduced into the adventita immediately after balloon injury.

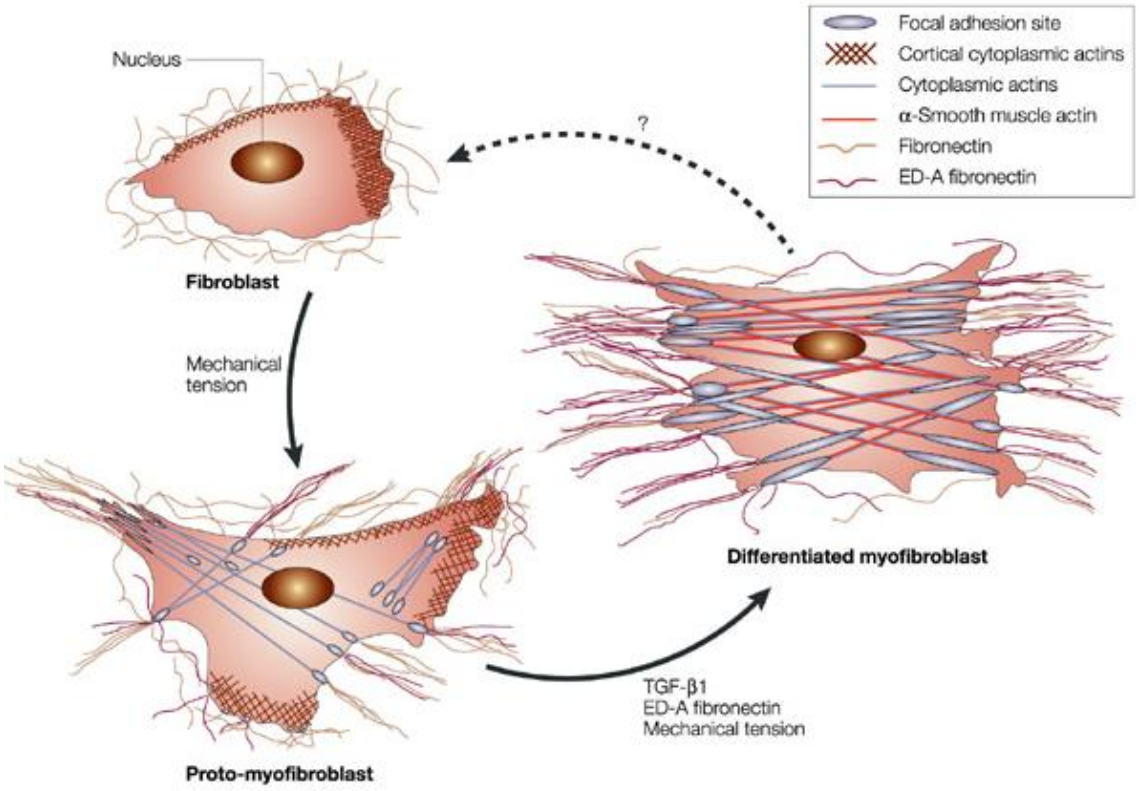


Figure 1.5 The process of fibroblast differentiation. Under mechanical stress, fibroblasts differentiate into proto-myofibroblasts which exhibit actin-containing stress fibres. In the presence of TGF-β1 and ED-A fibronectin, proto-myofibroblasts differentiate further into myofibroblasts. Myofibroblasts are characterised by the *de novo* expression of α-smooth muscle actin and vimentin as well as the presence of focal adhesions and fibronectin which aid cell migration and are responsible for the contractile nature of this cell type. Differentiated fibroblasts express many proteins also expressed by smooth muscle cells, however they differ from SMC in that they express very low levels of smooth muscle myosin heavy chain (SM-MHC). Taken from Tomasek et al., 2002.

Five days post-injury, LacZ-transfected cells were detected in the media and neointima at days 7, 10 and 14 (Li et al., 2000). In a similar study investigating arterial injury in rat carotid artery, TGF-β1 mRNA levels were increased fivefold after 14 days, during which there was significant neointimal formation indicating the importance of TGF-β1 in the process of adventitial cell-dependent neointimal thickening after injury (Majesky et al., 1991). Antagonism of TGF-β1 with the overexpression of inhibitory Smad7 in the adventitial layer of carotid artery exposed to balloon injury resulted in the attenuation of adventitial cells expressing β-galactosidase contributing to neointima formation (Mallawaarachchi et al., 2005) suggesting that neointimal formation is partly mediated by this growth factor.

Another growth factor released by platelets, mononuclear cells, endothelial cells and SMC in and in the surrounding area of vascular injury that has also been linked to enhancing vascular disease progression in concert with TGF-β1 during atherogenesis, is platelet-derived growth factor (PDGF; Levitzki, 2004). *In vitro*, PDGF has been found to increase the proliferation of VSMC, as well as increasing their migration; cells stimulated with this growth factor exhibited increased expression of actin filaments conducive to enhanced cell migration (Engel and Ryan, 1997). A possible mechanism by which PDGF may exert its effects on VSMC has been postulated by Sundaresan and colleagues who suggest that PDGF transiently increases intracellular levels of H_2O_2, which in-turn may act as a signalling molecule, causing tyrosine phosphorylation, mitogen-activated protein kinase activation and cell migration (Sundaresan et al., 1995). Furthermore, in a hamster vascular stenosis model in hypercholesterolemic hamsters, binding of PDGF to VSMC was higher than that seen in control animals and was associated with increased cell proliferation and increased neointima formation when compared to control animals (Matsuno et al., 2001). Due to its effect on VSMC proliferation in atherosclerotic disease, it is thought that PDGF targets the cyclin-dependent kinases 2 and 4 (Cdk2/Cdk4) in order to promote cell cycle progression and that targeting these may somewhat attenuate neointima formation (Chang et al., 1995; Abe et al., 1994).

In addition to adventitial cells contributing to neointimal formation; there is also recent evidence to suggest that progenitor cells arising from the adventitial layer may also play a role in luminal occlusion following injury. In order to investigate the possibility of vascular progenitor cells contributing to the atherosclerotic disease process, Hu and

colleagues examined tissue from ApoE-deficient mice (Hu et al., 2004). Immunohistochemical analysis of the adventitial layer from aortic roots revealed that large amounts of cell expressed stem cell markers, including Sca-1$^+$, c-kit$^+$, CD34$^+$ and Flk1$^+$ (Hu et al., 2004). Furthermore, the same study reported that when Sca-1$^+$ cells carrying the LacZ gene were transfected were transferred to the adventitial side of the vein grafts in these mice, β-gal$^+$ cells were found in the atherosclerotic lesion of the intima suggesting that progenitor cells do indeed contribute to atherosclerotic lesion formation (Hu et al., 2004). Interestingly, *in vitro,* in response to treatment with PDGF-BB, isolated Sca-1$^+$ cells were able to differentiate into SMC, suggesting that this growth factor may contribute to progenitor cell differentiation (Hu et al., 2004). Conversley, a study in mouse embryonic stem cells has shown that use of a PDGF inhibitor or TGF-β1-neutralizing antibodies did not prevent these cells from differentiating into SMC (Lindskog et al., 2006).

A more recent study has also reported similar findings; in injured femoral arteries in a mouse model, the number of cells expressing mesenchymal-stem cell markers were markedly higher when compared to uninjured control and originated from the adventitia (Tigges et al., 2013). In addition, this population of cells was found to contribute to neointimal formation (Tigges et al., 2013). Passman and colleagues have also postulated that the adventitia is the source of progenitor cells; immunohistochemical analysis revealed that Sca-1$^+$, c-kit$^+$, CD34$^+$, CD140$^+$ and Flk1$^+$ expressing cells clustered in a domain of sonic hedgehog signnalling (Shh) that was restricted solely to the adventitial layer (Passman et al., 2008). The above studies suggest that the adventitia is a possible source of progenitor cells which may act in concert with adventitia-derived fibroblasts and contribute to neointimal formation during atherosclerotic disease.

There is increasing evidence to support the hypothesis that the adventitia may be the 'first responder' and initiator of vascular remodelling in large and small arteries during atherogenesis, neointima formation following balloon injury and in restenosis. In addition to this, TGF-β1 seems to contribute to the activation, phenotypic switch and migration of adventital fibroblasts during these disease prosesses. Interactions between the outer layer of the blood vessel and this growth factor offer opportunity for novel therapeutic intervention from the outside-in (Siow and Churchman, 2007).

1.2 Reactive oxygen species in vascular disease

The term, ROS, refers to a group of biological molecules that are formed upon the incomplete reduction of oxygen making them highly reactive (D'Autreaux and Toledano, 2007). They include the superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) with each of these having distinct biological activities including lipid solubility, half-life and chemical reactivity (D'Autreaux and Toledano, 2007). Originally hypothesized to be the by-products of enzymatic reactions, and considered biological hazards, which caused gross cellular damage, mutagenesis, cancer and aging (Ray and Shah, 2005; Droge, 2002; Harman, 1956), upon closer observation it was revealed that ROS played complex roles in normal and pathophysiology. The balance between ROS generating systems and antioxidant defence systems is disturbed and results in oxidative stress where ROS generation overcomes the cells antioxidant defence system (Fig 1.6. B) and antioxidant enzymes (Fig 1.6. C).

Various enzyme systems have been implicated in the production of ROS in the cardiovascular system, including NAD(P)H oxidases, xanthine oxidase (XO), uncoupled eNOS as well as the leakage of activated oxygen from mitochondria during oxidative respiration (Forstermann, 2008). $O_2^{-\cdot}$ is one of the ROS implicated in the pathogenesis of vascular disorders and targets Fe-S clusters due to its high electrostatic attraction to them. However, due to its charge and its relatively short half-life (Paravicini and Touyz, 2006), this radical is unable to cross biological membranes making it a poor signalling molecule. $O_2^{-\cdot}$ is spontaneously dismuted to H_2O_2 by superoxide-dismutase (SOD; D'Autreaux and Toledano, 2007).

H_2O_2 is a milder oxidant in comparison to O_2^- and reacts with [Fe-S] and very slowly with glutathione and cysteine residues (D'Autreaux and Toledano, 2007). It has a longer half-life and is able to diffuse across lipid bilayers, making it an ideal candidate for signalling. H_2O_2 can undergo spontaneous conversion to the hydroxyl radical ($OH^{\cdot}$), the three-electron reduction state of molecular oxygen, via the Fenton reaction (Forstermann, 2008). This ROS is highly reactive and indiscriminate in its targets, however due to its extremely short half-life it is unlikely to mediate its effects distant from where it is produced (Paravicini and Touyz, 2006). These three main oxygen species are derived from various cellular enzyme systems, however those that are most relevant to vascular disease appear to be XO, uncoupled eNOS, the mitochondrial respiratory chain and NAD(P)H oxidase.

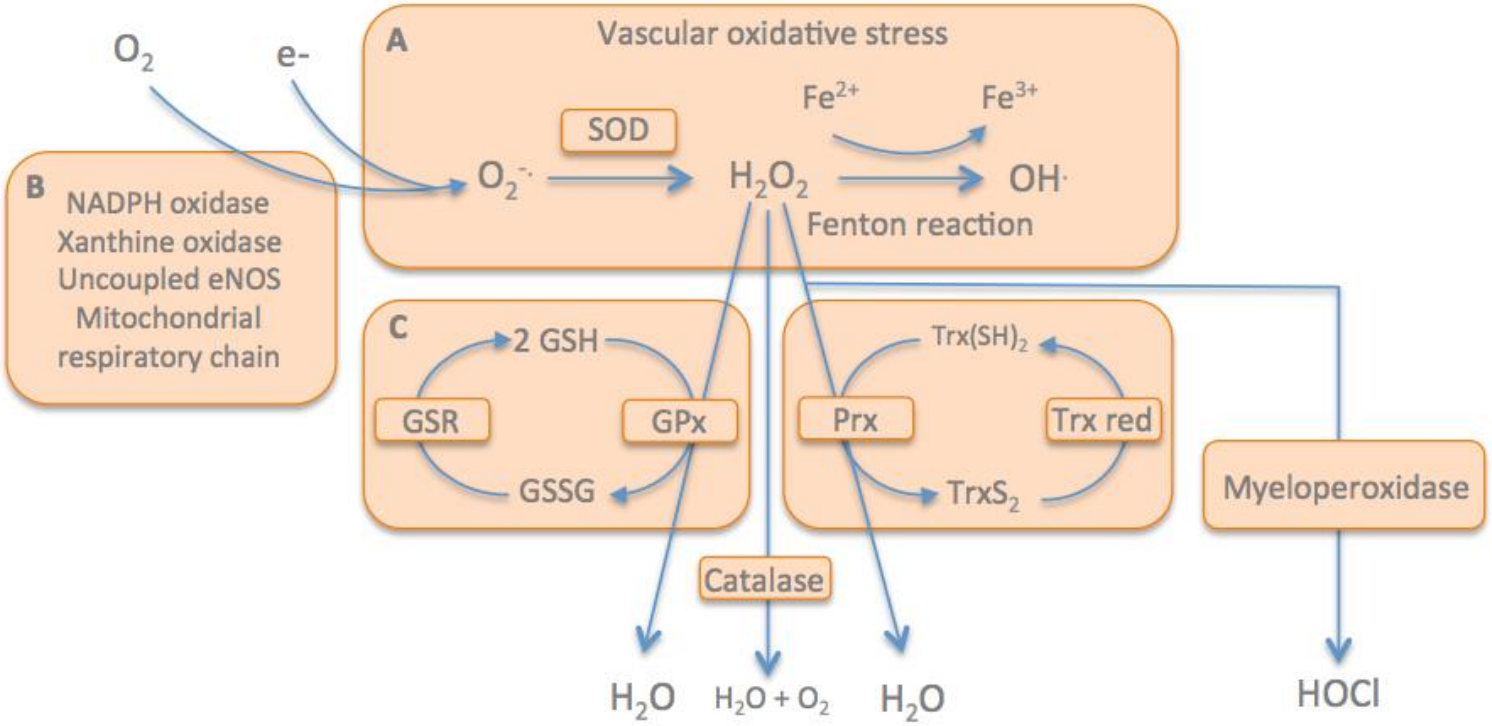


Figure 1.6 Enzyme systems involved in the generation and inactivation of ROS. (A) Vascular oxidative stress results from the overproduction of ROS, including O_2^- , H_2O_2 and $OH^·$ (B) O_2^- reacts with an impaired electron to form O_2^- (C) Enzyme systems set in place to detoxify ROS (GSR – glutathione reductase, GPx, gluthathione peroxidase, Prx – peroxiredoxin, Trx red- thioredoxin reductase). Adapted from D'Autreaux and Toledano, 2007.

1.2.1 ROS-generating enzyme systems

1.2.1(a) Xanthine oxidase

Xanthine oxidase (XO) is a metalloenzyme that catalyses the oxidation of hypoxanthine and xanthine to form $O_2^{-\cdot}$. Its ability to readily donate electrons to molecular oxygen to produce O_2^{-} has implicated it in vascular disease, in particular it is known to be present within the vascular endothelium and may play a role in endothelial dysfunction.

Several studies have shown that XO-generated $O_2^{-\cdot}$ plays a role in hypertension and perhaps atherosclerosis. *In vivo* evidence of XO involvement in atherosclerosis has been observed in spontaneously hypertensive rats (SHR) where the activity of the oxyradical producing form of XO was seen to be three-fold higher in comparison to the control group, with an associated increase in vascular tone in these animals. Administration of a tungsten-rich diet containing an inhibitor of XO eliminated detectable levels of enzyme activity, suggesting that this enzyme is responsible for oxyradical generation associated with increased vascular tone and may contribute to hypertension (Suzuki et al., 1998). In contrast to this, Laaksa and colleagues found that long-term treatment of SHR with the XO inhibitor, allopurinol reduced XO activity in a salt-induced hypertension model but did not reduce overall blood pressure, suggesting that in this case, XO–mediated ROS production was a consequence rather than a cause of hypertension (Laaksa et al., 1998).

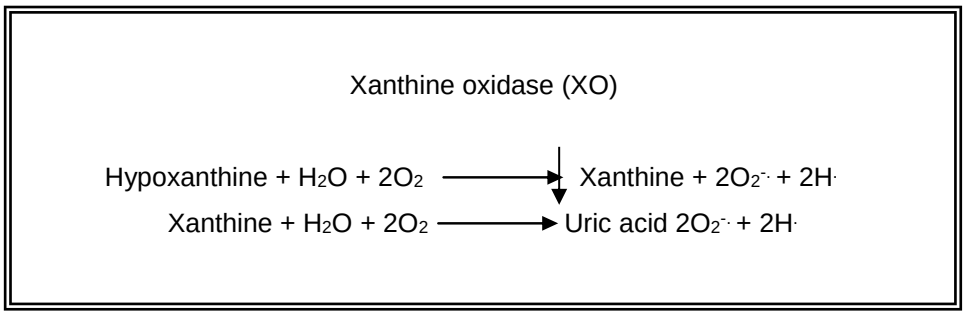


Figure 1.7 The XO reaction. Xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to form highly reactive $O_2^{-\cdot}$.

The source of XO in the plasma is unclear, but it is thought that increased levels of plasma cholesterol, as seen in atherosclerosis cause the release of this enzyme from the liver and into the circulation, whereupon it is able to bind to glycosaminoglycans on

endothelial cells which may be overexpressed as a result of hyperlipidemia (White et al., 1996). There is also evidence to suggest that endothelial cells may express xanthine dehydrogenase which is converted to XO (Forstermann, 2008), and that XO activity may depend upon the activity of NAD(P)H oxidase; endothelial cells lacking the p47phox subunit of NAD(P)H oxidase exhibited a markedly reduced production of $O_2^{-\cdot}$ and had minimal XO protein and activity (McNally et al., 2003).

Although there is some evidence to suggest that XO contributes significantly to vascular disease and to a certain extent, endothelial dysfunction, as demonstrated by studies where XO inhibitors caused an overall decrease in $O_2^{-\cdot}$ and an apparent restoration of endothelial cell function (Suzuki et al., 1998; Ohara et al., 1993), its role as a causative factor in vascular disease rather than a consequence (Laaksa et al., 1998) is still somewhat controversial. However, it is clear that XO-derived $O_2^{-\cdot}$ plays an important role in vascular pathophysiology.

1.2.1(b) Endothelial nitric oxide synthase

Nitric oxide (NO)-mediated vasodilatation is an important regulator of vascular homeostasis (Levy et al., 2009). Under normal physiological conditions, and in response to chemical and haemodynamic forces at the luminal surface of the blood vessel, endothelial nitric oxide synthase (eNOS) generates NO, which in-turn indices vasodilation, inhibits platelet aggregation and prevents oxidative modification of LDL cholesterol (Forstermann, 2008).

eNOS is a homodimeric enzyme with each subunit consisting of a reductase and oxygenase domain (Forstermann, 2008). Between these two domains is a zinc-thiolate cluster where the co-factor tetrahydrobiopterin (BH_4) and the substrate L-arginine bind (Forstermann, 2008). Electron transfer from the reductase domain enables ferric heme to bind to oxygen and form a ferrous dioxy-species, which can receive a second electron from either BH_4 or from the reductase domain. This reduction allows the oxidation of the substrate, L-arginine to L-citrulline and NO. NO is then able to diffuse from the endothelium into the adjacent SMC layer and cause vasodilatation via the activation of NO causes the elevation of cyclic-GMP and vasorelaxation via the activation of soluble guanylate cyclase (sGC) in the SMC layer.

Under pathophysiological conditions, such as hypertension and atherosclerosis, increased oxidative stress enhances the degradation of NO by its reaction with $O_2^{-\cdot}$ leading to the formation of the peroxynitrite anion ($ONOO^-$), which is able to oxidize BH_4 to form the radical BH_3 and results in the uncoupling of eNOS (Forstermann, 2008). This dysfunctional eNOS is no longer able to synthesize NO, and reduces oxygen to $O_2^{-\cdot}$ (Forstermann, 2008; Paravicini and Touyz, 2006). Endothelial dysfunction is the first step towards progressive atherogenesis (Ross, 1999; Lusis, 2000). Either the depletion of the co-factor BH_4, or decreased availability of L-arginine can contribute to eNOS uncoupling; however BH_4 supplementation can attenuate this process. In one study administration of BH_4 to normotensive and hypertensive patients resulted in augmentation of endothelium-dependent vasodilatation in both groups (Higashi et al., 2002). Uncoupled eNOS been implicated in many vascular diseases, in particular during the pathogenesis of diabetes, hypertension (Forstermann, 2008) and atherogenesis (Lusis, 2000), and its role in these diseases points to the pivotal role it may play in pathophysiology.

1.2.1(c) Mitochondria-derived ROS

Mitochondria are constant sources of superoxide, with NADH dehydrogenase (complex I) and ubiquinone-cytochrome b-c1 (complex III) being the primary sources of O_2^- production (Madamanchi and Runge, 2007). The amount of superoxide released from the mitochondria is dependent upon the activity of the antioxidant, manganese-containing superoxide dismutase 2 (SOD2) located in the mitochondrial matrix which protects against oxidative damage. Overexpression of SOD2 in a mouse model limited the infarct size following coronary artery ligation as well as rendering the heart more resistant to ischemia and reperfusion injury (Chen et al., 1998).

There is some evidence to suggest a link between atherosclerotic lesion development and mitochondrial dysfunction. Assessment of mitochondrial oxidant generation and DNA damage in human specimens showed that the degree of mitochondrial oxidant production corresponded with the degree of atherosclerotic lesion development. The same study found that in ApoE -/- mice, deficient in mitochondrial SOD2 exhibited early increases in mitochondrial DNA damage and a phenotype of accelerated atherosclerosis (Ballinger et al., 2002).

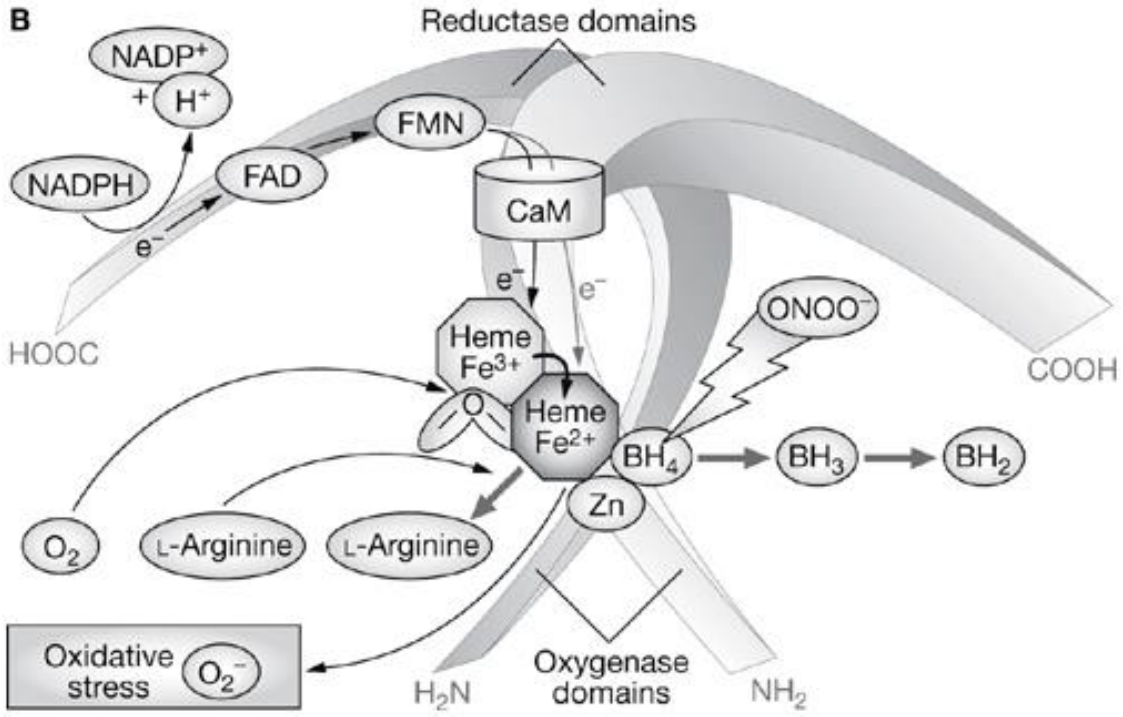


Figure 1.8 Uncoupling of endothelial nitric oxide synthase. Enhanced production of peroxynitrite as a result of oxidative stress causes the oxidation of BH_4 to biologically inactive products and overwhelms the cells capacity to re-reduce these back to BH_4, leading to an 'uncoupled' enzyme that reduces oxygen to superoxide but is no longer able to synthesize NO. Taken from Forstermann, 2008.

A recent study has implicated mitochondrial-derived ROS in the profibrotic effects of TGF-β1 in normal human lung fibroblasts; genetic disruption of mitochondrial complex III-generated ROS production was seen to attenuate TGF-β1-mediated profibrotic gene expression as well as attenuating myofibroblast dedifferentiation (Jain et al., 2013). Findings from this study highlight one of the potential sources of ROS which are thought to augment TGF-β1-driven vascular remodelling as seen during arterial repair (Shi et al. 1996).

Although direct evidence for the causal role of mitochondrial-derived ROS in vascular disease is yet to be elucidated, there is mounting evidence that mitochondrial dysfunction and consequent superoxide generation play a part in the development of vascular dysfunction, particularly in advanced atherosclerosis (Madamanchi and Runge, 2007).

1.2.1(d) NAD(P)H oxidase

NAD(P)H oxidases are multicomponent enzymes, originally found and characterised in phagocytes (Ray and Shah, 2005), are functional in membranes of vascular endothelial cells, SMCs and fibroblasts (Forstermann, 2008) and are thought to be the major source of ROS in the vascular wall (Birukov, 2009). There are seven different homologues; Nox1, Nox2, Nox3, Nox4, Nox5 as well as larger and more complex homologues, Duox1 and Duox2 (Ray and Shah, 2005). The NAD(P)H oxidase enzyme system reduces molecular oxygen to produce O_2^- by utilising NADH/NAD(P)H as an electron donor. Activation of NAD(P)H oxidases requires the assembly of several membrane-bound (gp91phox, Nox1/Nox4 and p22phox) and cytosolic subunits (p47phox and p67phox; see Fig 1.9). Vascular NAD(P)H oxidases produce constant low-levels of ROS under normal physiological conditions and are regulated by various vasoactive cytokines and growth factors as well as by physical factors such as shear stress, pulsatile strain and stretch (Birukov 2009; Haurani and Pagano, 2007).

In animal models of hypertension, NAD(P)H oxidase activation in the vasculature is apparent. In a rat model of angiotensin II (Ang II) -induced hypertrophy and subsequent measurement of the mRNA of the membrane bound subunit, p22phox showed that expression of this subunit increased three days following infusion (Fukui et al., 1997). Administration of SOD decreased both blood pressure and p22phox mRNA expression, suggesting that the NAD(P)H oxidase system is involved in the pathology of hypertension *in vivo* (Fukui et al., 1997). Interestingly, *in situ* hybridisation of aortic

tissue from these animals showed an increase in p22phox expression not only in the medial smooth muscle but also in the adventitia (Fukui et al., 1997). Further evidence in a Nox1 -/- murine model found that infusion of Ang II failed to increase blood pressure (Matsuno et al., 2005), suggesting a pivotal role for NAD(P)H oxidase-derived ROS in hypertension.

Though much evidence implicates Nox proteins in the development of atherosclerosis, recent evidence suggests that their role may be more complex than previously envisioned. Genetic deletion of p47phox in a model of diet-induced atherosclerosis in ApoE-/- mice found only a partial attenuation in the development of this disease (Martino et al., 2008) and a similar study found that p47phox deletion did little to stop the progression of atherosclerosis (Yang et al., 2004). Although the role of Nox proteins in atherosclerosis is contentious, their role in neointima formation and vascular remodelling seems to be more evident. Nox1 knockout mice displayed a significant reduction in neointima formation after wire-induced injury (Thomas et al., 2006) with a reduction in SMC migration and consequent neointima formation (Looi et al., 2008).

NAD(P)H-derived ROS production is clearly involved in atherosclerosis, but whether they are involved in promoting pro-atherosclerotic markers of this disease or play a greater role in the later stages including restenosis, neointima formation and vascular remodelling is yet to be clearly elucidated.

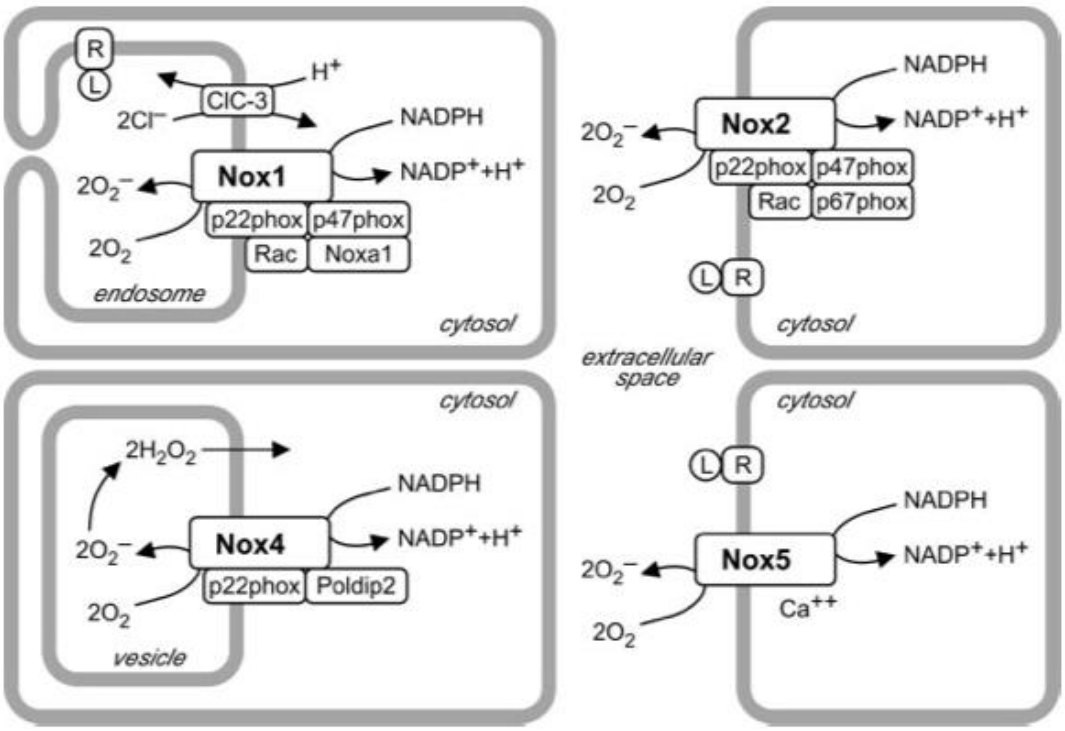


Figure 1.9 Spatial and molecular organization of vascular Nox enzymes. Nox 1, 2 and 5 are represented here in different cellular compartments but can be located either within cells or at the plasma membrane, thus releasing $O_2^{\cdot -}$ inside vesicles or extracellularly after activation of receptor (R) by ligand (L). $O_2^{\cdot -}$ may affect cytosolic signalling after crossing membranes via anion channels, reversible protonation, or conversion to H_2O_2. In contrast Nox4 is always intracellular and constitutively produces a higher proportion of membrane-permeable H_2O_2 than other oxidases. All oxidases, except Nox5, form a membrane complex with p22phox. Cytosolic activators vary with oxidase subtype: Rac, p47phox and Noxa1 for Nox1 in VSMC; Rac, p47phox and p67phox for Nox2; Poldip2 for Nox4; and Ca++ for Nox5. All vascular cells express multiple Nox subtypes simultaneously. Taken from Lassegue and Griendling, 2010.

1.2.2. Mechanical stretch and ROS

Exposure of blood vessels to cyclic stretch is essential in maintaining vascular homeostasis under normal physiological conditions as well as playing a significant role in vascular remodelling and maintenance of vascular cell phenotype, regulation of vascular tone and expression of vascular wall extracellular matrix constituents (Birukov, 2009). However, under pathophysiological conditions, such as hypertension, atherosclerosis and diabetes, cyclic stretch plays a detrimental role and contributes to disease progression (Birukov, 2009).

Chronic increases in cyclic stretch result in hypertension-induced vascular remodelling because of vascular cell proliferation and excess collagen synthesis. Ultimately, this remodelling leads to a decrease in luminal diameter and increased vascular resistance characteristic of clinical hypertension (Ali and Schumacker, 2002). Cell membranes and attachment sites are thought to serve as mechanosensors (Davies, 1995) which mediate mechanical signals via activation of signalling molecules such as tyrosine (focal adhesion kinase) kinases, growth factor receptors and via ion channels (Birukov, 2009).

Recent evidence has shown that ROS generated by stretch may act as signalling molecules to regulate gene expression. Endothelial cells exposed to cyclic stretch have been shown to generate ROS production which is attenuated by the use of the mitochondrial inhibitors diphenylene iodonium (DPI) or rotenone, indicating that the mitochondria may be the source of ROS during stretch. In addition to this, the same study has shown increased NFκB activation and VCAM-1 mRNA expression, which is absent in the presence of antioxidants (Ali et al., 2004). Similarly, in fibroblasts, cyclic stretch induced NFκB activation and translocation to the nucleus, which was abrogated in the presence of antioxidants suggesting the stretch caused ROS production and as a result, the translocation of this transcription factor to the nucleus and potential gene transcription of various antioxidant genes (Amma et al., 2005).

This stretch-mediated ROS production and consequent antioxidant gene expression is also seen in acute respiratory distress syndrome, where Nrf2/ARE pathway is activated resulting in the upregulation of several antioxidant enzymes, including HO-1 (Cho et al., 2006). *In vitro* investigation of the effect of cyclic stretch on Nrf2-dependent ARE-mediated transcriptional response in pulmonary epithelial and endothelial cells revealed that actin remodelling along with epidermal growth factor receptor (EGFR)-activated

PI3K-Akt signalling were necessary for the regulation of the Nrf2-regulated antioxidant genes, GCLM and GPx1 (Papaiahgari et al., 2007). Previously, actin remodelling has been postulated in playing a key role in the modulation of transcription factor activation and subsequent gene transcription in epithelial and endothelial cells (Birukova et al., 2005). As Keap1 is an actin binding protein and involved in the regulation of Nrf2, mechanosensing during stretch may be pivotal in activating the induction of antioxidant enzymes. There is much evidence for the stretch-induced activation signal transduction and gene expression in the vasculature (see Table 1.1). Stretch is particularly important in modulating changes in the outer layer of the blood vessel, the adventitia and may play a pivotal role in the generation of ROS. These ROS are likely to act as signalling molecules and cause changes in cellular differentiation, proliferation and migration and therefore constrictive remodelling in disease (Birukov, 2009; Cowan et al., 2003).

Title	Methods	Treatment	Key Findings	Reference
Stretch-induced regulation of angiotensinogen gene expression in cardiac myocytes and fibroblasts: Opposing roles of JNK1/2 and p38α MAP kinases.	Isolated neonatal rat ventricular myocytes and fibroblasts	20% equiaxial static-stretch (0-24 h)	Expression of angiotensinogen (Ao), was ↓ following four hours of mechanical stretch. After 8 hours Ao expression ↑. JNK1/2 negatively regulated stretch mediated Ao expression whereas p38α/β significantly ↑ stretch-induced (24 h) Ao gene expression.	Lal *et al.*, 2008
Role of integrins and focal adhesion kinase in the orientation of dermal fibroblasts exposed to cyclic strain.	Dermal fibroblasts	Cyclic stretch 0–24%	Focal adhesion kinase (FAK), p38 and Rho activated in fibroblasts exposed to cyclic stretch and incubation of cells with anti-integrin β1 before stretch abrogated fibroblast orientation. Fibroblast orientation in response to cyclic stretch is partly mediated by integrin β1 through phosphorylation of FAK, p38 and activation of Rho.	Wen *et al.*, 2009
Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-β1.	Cardiac myocytes or CMEC (cardiac microvascular endothelial cells)	10% stretch, 30 cycles/min TGF (1 or 10 ng/ml) for 4 h	TGF-β1 ↑ 2.5-fold after 1 h of stretch. Addition of TGF-β1 neutralizing antibodies inhibited the stretch-induced upregulation of VEGF. Stretch of CMEC ↑ VEGF mRNA and ↑ the levels of VEGF protein in the conditioned media.	Zheng *et al.*, 2001
Cyclic mechanical stretching modulates secretion pattern of growth factors in human tendon fibroblasts.	Human tendon fibroblasts	Cyclic biaxial mechanical stretching for 15 and 60 min	Mechanical stretching ↑ secretion pattern of TGF-β. ↑ PDGF and bFGF growth factor stimulate cell proliferation, differentitiation and matrix formation and encourage wound healing.	Skutek *et al.*, 2001
Cyclic mechanical stretch induces VEGF and FGF-2 expression in pulmonary vascular smooth muscle cells.	Ovine pulmonary arterial smooth muscle cells	Biaxial stretch at a frequency of 1 Hz for 24 h at amplitudes of 5, 15, and 25%	mRNA expression of VEGF and FGF peaked in stretched cells at 24 h and declined toward control levels at 48 h.	Quinn *et al.*, 2002
Cyclic stretch increases VEGF expression in pulmonary arterial SMCs via TGF-β1 and ROS: a requirement for NAD(P)H oxidase	Pulmonary arterial smooth muscle cells	20% stretch (0–24 h)	Cyclic stretch increased levels of ROS via TGF-β1, ROS was abrogated following treatment of cells with TGF-β1 neutralizing antibody.	Mata-Greenwood *et al.*, 2005

Table 1.1 Studies investigating the effect of mechanical stretch in vascular cells.

1.2.3. Adventitia-derived ROS

Fibroblast NAD(P)H oxidase production has received increasing attention in recent years, and there is controversy surrounding the location of NAD(P)H oxidases in the vascular wall. However, a large body of evidence in favour of NAD(P)H oxidases being the main culprits of ROS production in the vasculature has led to the idea that adventitial NAD(P)H oxidase is a harbinger and initiator of vascular disease. Interestingly, the ROS-generating system in adventitial fibroblasts shares a degree of homology to the phagocytic NAD(P)H oxidases, which surpasses that of any other vascular oxidase (Haurani and Pagano, 2007).

Several studies have implicated NAD(P)H oxidase-derived superoxide within the adventitia as being an important and pivotal event in the development of diabetes mellitus, hypertension, atherosclerosis and vascular injury (Chan ett al., 2007; Zhang et al., 2003; Azumi et al., 2002; Sorescu et al., 2002; Paravicini et al., 2000; Cifuentes et al., 1999; Wang et al., 1999). In a porcine model of stretozotocin-induced diabetes, increased NAD(P)H oxidase activity was observed in the coronary media and adventitia and was accompanied by an increased inflammatory response in the adventitia (Zhang et al., 2003). The NAD(P)H oxidase-dependent inflammatory response has also been reported by others; increased expression of adhesion molecules on the endothelial surface of the vasa vasorum was seen to lead to the subsequent infiltration of leukocytes into the adventitia (Li and Shah, 2004; Libby et al., 2002) leading to a proposal by Stenmark and colleagues that ROS-mediated cross-talk between adventitial fibroblasts and NFκB-dependent inflammatory processes may be a critical event during atherosclerotic disease (Stenmark et al., 2006).

In vivo it has been shown that delivery of the NAD(P)H oxidase inhibitor, gp91ds-tat (decoy peptide for cytosolic oxidase component p47phox) to the left carotid adventitia specifically blocked Ang-II-induced aortic NAD(P)H oxidase activity, as well as reducing Ang-II-induced medial hypertrophy, suggesting this process is mediated by adventitial fibroblast-derived ROS (Liu et al., 2004). In addition, Szöcs and colleagues have reported an increase in superoxide production 3 days after balloon injury in the adventitial and medial layer of coronary arteries (Szöcs et al., 2002), an event also reported in response to hypoxia in pulmonary artery adventitial fibroblasts, and postulated to be Nox4 dependent (Li et al., 2008).

Although there are a plethora of studies suggesting an important role of NAD(P)H oxidase-derived ROS in the adventitia, there is contention over the exact isoform that is predominantly expressed in the adventitia and several studies suggest that the isoform responsible for adventitia-derived ROS is Nox2; a study in Nox2-deficient mice found that AngII infusion for six days caused aortic superoxide production and an increase in the size of the medial layer in the wild-type (WT) animals that was absent in the knockout animals (Wang et al., 2001), with the aortic media increasing in size in the WT animals suggesting a role for Nox2–mediated SMC hyperplasia. Histological analysis of aortic sections from WT mice infused with Ang II revealed that the majority of Nox2 was located in the endothelium and adventitia (Wang et al., 2001). Abundant colocalization of Nox2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ has been observed in cultured adventitial fibroblasts of rabbit aorta (Pagano et al., 1998; Pagano et al., 1997). In addition, fluorescent immunohistochemistry has demonstrated that Nox2 was highly expressed in the adventitial layer of human coronary arteries whereas Nox4 was barely detectable (Sorescu et al., 2002). In contrast, in a separate study, qRT-PCR analysis of human cardiac fibroblasts has shown that Nox4 and Nox5 were abundantly expressed in cardiac fibroblasts whereas levels of Nox1 and Nox2 were barely detectable (Cucoranu et al., 2005).

Even under basal conditions, various findings have shown that the vascular adventitia is a major source of ROS (Wang et al, 2001; Pagano et al., 1995). Dihydroethidium (DHE) fluorescence *in situ* has shown that superoxide levels are greater in adventitial fibroblasts than in SMC (Chamseddine and Miller, 2003). It is thought that the predominant Nox isoform in the adventitia is Nox4 (Sorescu et al., 2002) and that in general, adventitial fibroblasts possess high specific activity of NAD(P)H oxidase relative to other vascular segments (Haurani and Pagano, 2007).

These recent investigations have shown that the adventitia, along with the media, is a potential source of ROS, which is likely to contribute to vascular remodelling, neointima formation and restenosis (see Table 1.1). Targeting ROS from these layers may attenuate this constrictive remodelling and lead to a decrease in consequent future vascular events.

Title	Cell type	Treatment	Key Findings	Reference
Mechanisms of vascular smooth muscle NAD(P)H oxidase 1 (Nox1) contribution to injury-induced neointimal formation.	Mouse vascular smooth muscle cells	-	↓ neointima formation, proliferation, apoptosis and migration in Nox1 KO mice in a wire-injury induced model of the femoral artery. Overexpression of Nox1 ↑proliferation, migration and fibronectin secretion.	Lee *et al.*, 2009
Nox4 oxidase overexpression specifically decreases endogenous Nox4 mRNA and inhibits angiotensin II-induced adventitial myofibroblast migration.	Rat adventitial fibroblasts	Angiotensin II (100 nmol/L)	Angiotensin II ↑adventitial fibroblast migration and ↓Nox4 and Nox1 levels. Co-transfection of cells with human Nox4 and p22-phox plasmids combined with angiotensin II ↓ endogenous Nox4 mRNA and ↓ angiotensin II-mediated myofibroblast migration	Haurani *et al.*, 2008
Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration.	Rat aortic smooth muscle cells	PDGF (2.5, 5 and 10 ng/ml)	PDGF ↑ VSMC migration in a dose-dependent manner. Attenuated by pretreatment with N-acetyl cysteine, DPI or ebselen and overexpression of catalase. Infection of VSMCs with dn PAK1 adenovirus attenuated migration. PDGF-induced VSMC migration is ROS dependent with the Src/PDK1/PAK1 signaling pathway being an important ROS-sensitive mediator of migration.	Weber *et al.*, 2004
Role of NADH/NAD(P)H oxidase-derived H_2O_2 in angiotensin II-induced vascular hypertrophy.	Vascular smooth muscle cells	Angiotensin II (100 nmol/L)	Angiotensin II significantly ↑ levels of intracellular H_2O_2, this ↑ was attenuated by extracellular catalase, treatment with DPI and the AT1 blocker, losartan. In cells transfected with antisense p22-phox, angiotensin II-mediated H_2O_2 production was completely inhibited.	Zafari *et al.*, 1998
Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction.	Rat vascular smooth muscle cells	PDGF (2.5 ng/ml)	PDGF ↑ intracellular concentrations of H_2O_2, which was blunted by ↑the intracellular concentration catalase or N-acetyl cysteine. The response of VSMCs was inhibited when PDGF-mediated ↑ in intracellular H_2O_2 was blocked.	Sundaresan *et al.*, 1995

Table 1.2 Studies implicating ROS in the growth and migration of vascular smooth muscle cells and fibroblasts.

1.3 Transforming Growth Factor- β

The transforming growth factor β (TGF-β) family is responsible for eliciting a plethora of cellular processes including cell proliferation, differentiation, extracellular matrix synthesis and apoptosis (Wrighton et al., 2009; Zhang, 2009; Pardali and Moustakas, 2007; Bierie and Moses, 2006), as well as important signalling during embryonic development (Shi and Massagué, 2003). Conservation of this superfamily across species indicates the intrinsic role it plays in biological processes and consequently, perturbation of TGF-β signalling can play a significant role in cancer metastasis, fibrotic, cardiovascular and autoimmune disease (Goumans et al., 2009; Padua and Massagué, 2009; Watabe and Miyazono, 2009; Grainger, 2007; Zhiang et al., 2007). Its synthesis and activation are dependent upon various extracellular factors which regulate its transcription and consequent expression.

1.3.1 TGF-β1 activation

The TGF-β family of cytokines contains two subfamilies; the TGF-β/Activin Nodal subfamily and the BMP (Bone morphogenic protein) subfamily (Shi and Massagué, 2003). Members of this superfamily elicit distinct cellular responses, are structurally similar and all signal through the activation of heteromeric receptor complexes of type II and type I transmembrane threonine/serine kinases (Wrighton et al., 2009).

TGF-β1 mRNA expression is increased in response to various extracellular factors, including Ang II, mechanical stress, high glucose concentration or ET-1 (Ruiz Ortega et al., 2007) and as a result there is an increase in the production of TGF-β1 protein. TGF-β1 is produced as a large latent complex (Annes et al., 2003) which is inactive and consists of a main region and a latency associated peptide (LAP). This inactive form is anchored in the extracellular matrix by latent TGF-β binding proteins (Ruiz-Ortega et al., 2007) and can be activated by proteolytic cleavage by matrix metalloproteinases (MMP-2 and 9) and acidic microenvironments (Ruiz-Ortega et al., 2007). In addition, ROS have also been implicated in the activation of latent TGF-β; Baricellos-Hoff and colleagues showed that ROS generated by irradiation caused oxidation of several amino acids in the latency conferring peptide leading to a conformational change in the latent complex, allowing release of TGF-β (Barricellos-Hoff and Dix, 1996). Integrin-mediated physical forces can also activate TGF-β1; it has been shown that distorting the large latent complex via single-molecule force spectroscopy resulted in the release of active TGF-β1 (Hubmacher and Apte, 2013). This release is as a result of the cells

pulling at the LAP within the large latent complex/latent TGF-β1 binding proteins, 'squeezing' out the active TGF-β1 (Humacher and Apte, 2013). This suggests that movement of the cell can augment the release of further TGF-β1, which is of particular relevance during vascular remodelling. During the pathophysiology of atherosclerosis, fibroblasts and SMC become activated and move from the adventitial and medial layer toward the lumen of the vessel, partly due to the chemotactic effect of TGF-β1 and other growth factors released from platelets and endothelial cells at the lumen of the vessel (Grainger, 2007; Silverstein and Rifkin, 1987) and from macrophages and T-cells recruited from the circulation into the intima, forming the beginnings of a plaque (Gong et al., 2012; Grainger, 2007). The high levels of activated TGF-β1 at the site of vascular injury accelerate the inflammatory disease process (Stenmark et al., 2012). Interestingly, several studies into serum levels of TGF-β1 have reported an increase in the levels of latent TGF-β1 and a marked decrease in active TGF-β1 levels amongst patients with severe atherosclerosis (Grainger, 2007; Grainger et al., 1995) although other studies have reported that serum levels of active TGF-β1 are actually increased in these individuals (Wang et al., 1997). This is in contrast to the high concentration of active TGF-β1 within the neointima, released from platelets, endothelial cells, inflammatory cells, as well as from migrating fibroblasts and SMC (Stenmark et al., 2012; Grainger, 2007, Grainger et al., 1995).

The active form of TGF-β is a 25 kDa dimer, with the two polypeptides interacting via a disulphide bond and hydrophobic links. Once active TGF-β is released, it binds to the TGF-βII receptor (TβRII) resulting in activation of TGF-β signalling cascades.

There are seven known mammalian type I receptors termed ALK1-7 (activin receptor like-kinase and five type II receptors (Rahimi and Leof, 2007) in the vast majority of cell types. The receptor serine/threonine kinase family is made up of seven type I (TβRI) and five type II (TβRII) receptors (Manning et al., 2002) both types of which consist of approximately 500 amino acids organised into an N-terminal extracellular ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain (Shi and Massagué, 2003; Huse et al., 1999). The receptor complex usually comprises of two type II receptors and two type I receptors. Upon ligand binding, the two receptor types are brought together, and the type II receptor transphosphorylates the GS region of the type I receptor, causing its activation and allowing it to catalyse the phosphorylation of receptor-regulated SMADs (R-Smads). Phosphorylated R-Smads form complexes involving two R-Smads and one Co-smad, in

the cytoplasm and these complexes translocate to the nucleus to regulate gene transcription (Lonn et al., 2009).

1.3.2 The Smad family of proteins

The first intracellular mediator of TGF-β signalling, mothers against decapentplegic (MAD; Sekelsky et al., 1995) was identified in Drosophila and consequent studies revealed orthologs in C.elegans and vertebrates (Derynck et al., 1996) that were given the name 'SMA' from gene *sma* for small body size. Smad is a portmanteau of the two.

The Smad family comprises of eight Smad proteins, which can be classified into three functional groups. The first group are the receptor-activated Smads (R-Smads; Smad 1/2/3/5/8), which are phosphorylated by the type I receptor kinases on the Ser-Ser-X-Ser (SSXS) motif at their extreme C-termini (Hill, 2009). This phosphorylation is facilitated by auxillary proteins including SARA (Smad anchor for receptor activation) and HRS/HGS (hepatocyte growth factor-regulated tyrosine kinase substrate; Miura et al., 2000; Tsukkazaki et al., 1998).

Both SARA and HRS/HGS contain the phospholipid binding FYVE domainand function as adaptors to aid recruitment of the receptor activated Smads, Smads2 and 3 to the TGF-β receptor complex (Rahimi and Leof., 2007). In particular SARA immobilizes Smad2 and 3 near the cell surface in order to aid phosphorylation of these proteins by the type I receptor (Tsukazaki et al., 1998). In the basal state, the R-Smads are associated with SARA in the cytoplasm, however phosphorylation of Smad2/3 by type I receptors on C-terminal residues is thought to decrease their affinity for the binding site on SARA (Xu et al., 2000; Tsukazaki et al., 1998). As a result, Smads2/3 dissociate from SARA (Tsukazaki et al., 1998) and associate with co-Smad4. The need for adaptor proteins to generate the active receptor complex is also coupled to receptor endocytic activity, which directly impacts on downstream TGF-β signalling (Hayes et al., 2002; Peinheiter et al., 2002). Smad anchor for receptor activation (SARA) is thought to be an important accessory protein in the facilitation of Smad signalling, however there is evidence to suggest that this protein is not an essential requirement for Smad3 activation (Goto et al., 2001). COS7 cells transfected with mutant Smad3 which lacked the binding site for SARA was phosphorylated by TβRI in a similar manner to the wild type Smad3. In addition, mutant Smad3 was also able to form complexes with Smad4 and translocate to the nucleus in the absence of its SARA binding site (Goto et al., 2001).

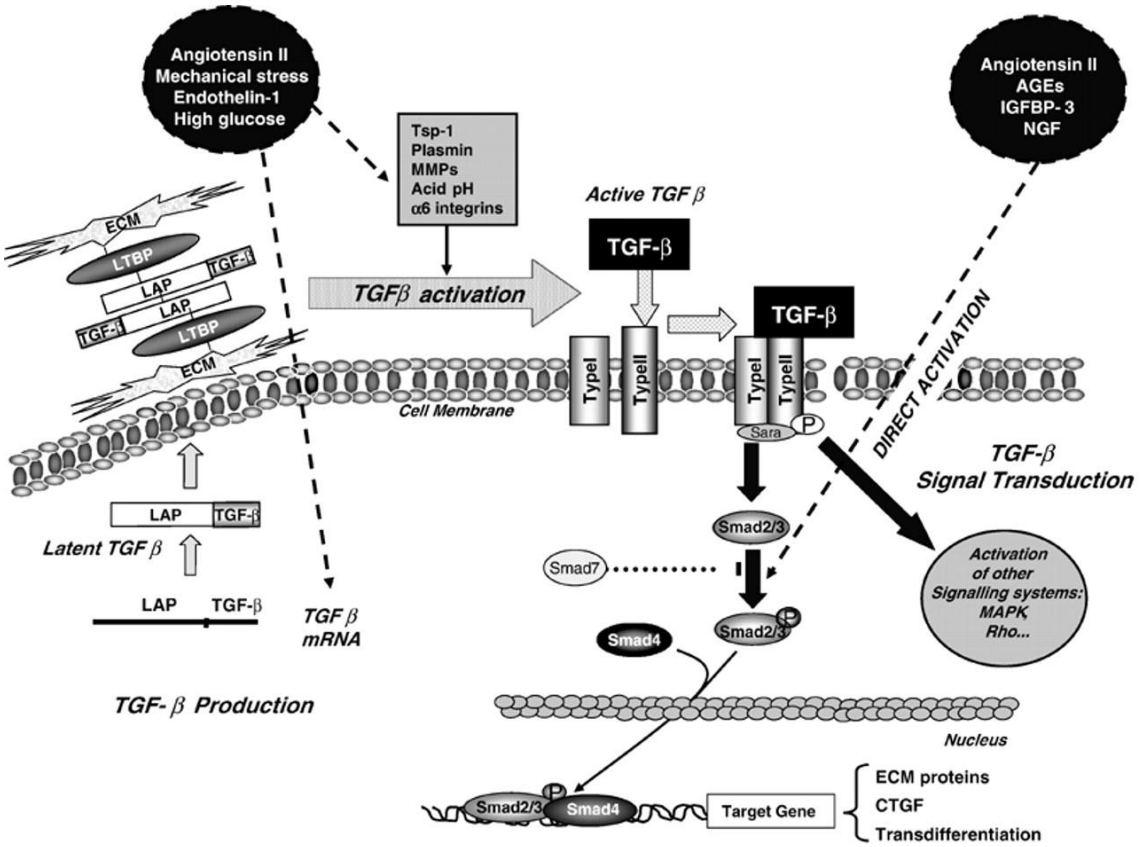


Figure 1.10 TGF-β synthesis and signalling. Several extracellular stimuli increase TGF-β mRNA expression, leading to synthesis of this protein. TGF-β is synthesized as an inactive pro-peptide consisting of a main region and a latency associated peptide (LAP), which is targeted to the ECM. TGF-β is activated upon cleavage and binds to the TβR II which inturn transphosphorylates TβRI leading to downstream TGF-β signalling-mediated target gene expression. Adapted from Ruiz-Ortega et al., 2007.

Furthermore, mutant and wild-type Smad3 equally enhanced TGF-β-induced transcription (Goto et al., 2001), indicating that in contrast to SARA/Smad2 interaction, SARA/Smad3 interaction is not essential for TGF-β/Smad3-mediated signalling (Goto et al., 2001).

Once phosphorylated, the R-Smads dissociate from the receptor/SARA complex and form either homomeric or heteromeric complexes with Smad4, which makes up the second functional group of Smad proteins, known as the common or co-Smad (Hill, 2009, Wrighton et al., 2009). This R-Smad/Co-Smad complex translocates to the nucleus and interacts with Smad-binding elements (SBE: GTCT) or GC-rich sequences present in certain promoters (Shi et al., 1998; Zawel et al., 1998). Due to the close proximity of SBEs to other transcription factors, Smad proteins have often been found to cooperate with a wide variety of transcription factors to regulate gene expression such as the members of the forkhead box O family (FoxO1, FoxO3, and FoxO4) (Gomis et al., 2006; Massague et al., 2005).

Interestingly the differential functional roles of Smad have also been demonstrated. In a pancreatic ductal adenocarcinoma cell line (PDAC), siRNA-mediated silencing of Smad3 induces growth inhibition but increases migratory response. Conversely, silencing of Smad2 enhanced enhances growth inhibition but decreases migratory response (Ungfroroen et al., 2011). This balance between the different R-Smads and their consequent responses is obviously a critical factor in the downstream effects on gene expression.

There is increasing evidence to suggest nucleocytoplasmic shuttling of Smad proteins (Hill 2009). Studies in mouse oocytes revealed that treatment of cells with leptomycin B, a specific inhibitor of CRM1 or Exportin 1 led to the rapid nuclear accumulation of Smad4 suggesting that CRM1 was required for the nuclear export of Smad4 (Pierreux et al., 2000; Watanabe et al., 2000; Fonerod et al., 1997). This inhibition did not affect Smad2/3 shuttling, however later studies in zebrafish embryos using GFP-tagged Smad2/3 have provided visual evidence of the continuous shuttling of the R-Smads from the cytoplasm to the nucleus (Batut et al., 2007; Schmierer et al., 2005; Nicolas et al., 2004). Smad2/3 and Smad4 nuclear accumulation of a relatively slow process which reaches maximum response 45 min following ligand stimulation by TGF-β1 (Pierreux et al.. 2000) and the majority have been reported to remain in the nucleus for 4-5 h after

which they begin to be exported back to the cytoplasm (Inman et al., 2002). In addition it is thought that Smad2/3 and Smad4 complexes dissociate in the nucleus as a result of Smad2/3 dephosphorylation and are then exported out of the nucleus separately by distinct mechanisms (Chen et al., 2005). Thus continuous Smad4 shuttling requires the activity requires the activation of nuclear export proteins, whilst Smad2/3 require low level dephosphorylation (Hill, 2009).

The third functional group of Smad proteins comprises of the inhibitory Smads (I-Smads), Smad6 and Smad7 (Wrighton et al., 2009). These proteins negatively regulate the TGF-β pathway by mediating receptor inactivation through recruiting the E3 ubiquitin ligases; Smurf1/2 (Smad ubiquitination regulatory factor-1) or recruiting the catalytic subunit of protein phosphatase-1 to the receptors (Shi et al., 2004) which lack the C-terminal sites for phosphorylation by TβRI (Rahimi et al., 2007). Smad6 and 7 inhibit TGF-β signalling via different mechanisms; Smad6 appears to only inhibit the BMP pathway whereas Smad7 inhibits the TGF-β/activin and BMP pathways (Hayashi et al., 1997; Imamura et al. 1997). Smad6 competes with Smad4 for receptor phosphorylated Smad1 binding whereas Smad7 competes with R-Smads for interacting with TβRI (Hayashi et al., 1997) as well as directly mediating TGF-β receptor ubiquitination by recruiting Smurf ubiquitin ligases (Kavsak et al., 2000). The transcription of inhibitory Smad mRNA is induced by TGF-β and therefore it is likely that they act as part of an autoregulatory negative-feedback mechanism during TGF-β signal transduction (Heldin et al., 1997).

The importance of the regulation of the TGF-β signalling pathway is evident where dysregulation of signalling results in metastatic cancers, fibrotic and cardiovascular disease including hypertension, restenosis, atherosclerosis, cardiac hypertrophy, and heart failure (Ruiz-Ortega et al., 2007). As a result, various regulatory processes are set in place, which, under normal conditions police this pathway.

1.3.3 Non-Smad pathways in TGF-β signalling

Although it is well established that canonical TGF-β signalling occurs downstream via Smad proteins, it is becoming increasingly evident that TGF-β also signals via Smad-independent pathways that are activated by the TGF- β receptors through either phosphorylation or direct interaction. These pathways include branches of MAP kinase

(MAPK) pathways, Rho-like GTPase signalling pathways, and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (Zhang. 2009).

TGF- β mediated Erk-pathway activation was first identified in rat intestine and mink lung epithelial cells (Mulder et al. 1992; Yan et al., 1994). The kinetics of Erk activation in response to TGF-β stimulation is an entirely cell and culture dependent event; rapid activation has been observed in epithelial cells (Hartsough and Mulder., 1995) breast cancer cells (Frey and Mulder., 1996) and fibroblasts (Musci et al., 1996) whereas more delayed activation has been observed in pancreatic acinar cells (Simeone et al., 2001).

Recent discovery of the dual specificity of TβRs acting as both tyrosine and serine/threonine kinases resulted in the elucidation of the mechanism whereby TGF-β activates the Ras-Erk-MAPK pathway (Zhang, 2009). Phosphorylation of both serine and threonine residues as well as at tyrosine residues has been shown to be an initial event which results in the recruitment of several adaptor proteins that leads to the activation of the Erk-MAPK signalling pathway in response to TGF-β stimulation. Tyrosine autophosphorylation on three sites of the cytoplasmic domain of TβRII (Lawler et al., 1997) can result in the recruitment of Src homology domain 2-containing protein (Shc) and consequent phosphorylation of TβRII Ty284 and recruitment of the Shc domain of growth factor binding protein 2 (Grb2) and p38 MAPK activation (Galliher and Schiemann, 2007). The tyrosine kinase activity of TβRII suggests that it could promote non-Smad signalling pathways, further supported by the finding that selective activation of the Erk 1/2 in dermal cells has been attributed to the high expression of TβRII, whereas in contrast, in epidermal cells, canonical TβRI-Smad signalling is dominant and Erk activation is non-existent due to the high expression of TβRI (Bandyopadhay et al., 2011).

Activated TβRI uses its intrinsic tyrosine kinase activity to phosphorylate Shc directly on its tyrosine kinase and serine residues (Mu et al., 2012). Phosphorylated Shc then associates with TβRI and recruits Grb2 and SOS, and in the process, activates Erk-MAPK (Lee et al., 2007). Dephosphorylation of TβRI by PP2a results in the inhibition of further signalling downstream of this receptor, indicating that its activity is highly dependent upon its phosphorylation status; decreased recruitment of PP2a to TβRI results in increased TβRI signalling in a manner dependent on Erk-MAPK (Yu et al., 2010).

In addition to Erk-MAPK activation, TGF-β has also been demonstrated to activate the JNK/p38 pathway, which is perhaps the best characterised Smad-independent pathway. JNK and p38 are activated by MAP kinase kinases (MKK); TGF-β is able to activate JNK and p38 through MKK4 and MKK3/6 respectively (Engel et al., 1999; Hocever et al. 1999; Frey and Mulder, 1997) and p38 MAPK in the C2C12 fibroblast cell line (Hanausa et al., 1999), in HEK293 cells (Sano et al., 1999), and in murine epithelial cells (Bhowmick et al., 2001). The Smad proteins have been shown to be dispensable in the TGF-β-induced activation of JNK; experiments carried out using a dominant-negative form of Smad3 or use of cells deficient in Smad3 or Smad4 have demonstrated the induction of genes downstream of TGF-β as a result of activation of JNK signalling (Hocevar et al., 1999; Engle et al., 1997). Furthermore, use of a mutant TβRI with a mutation in the L45 loop rendering it defective in Smad binding and activation but without the loss of its kinase activity, demonstrated activation of the JNK and p38 MAPK pathways in response to TGF-β ligand binding (Itoh et al., 2003; Yu et al., 2002).

One component that is important in the upstream activation of p38-MAPK is TGF-β-activated kinase 1 (TAK1) (Yamaguchi et al., 1995). TAK1 has been postulated as being indispensible for TGF-β-induced activation of JNK; deletion of the *Tak1* gene in mice led to earlt embryonic lethality and impaired JNK activation (Shim et al., 2005). Recently, it has been reported that TGF-β activates TAK-1 through the catalytic activation of the ubiquitin ligase TNF-receptor-associated factor 6 (TRAF6, Landstrom et al., 2010). TRAF6 was found to be a crucial component in the activation of TAK1-KNK/p38 pathways; phosphorylation of p38 by TβRI was severely curtailed by a TRAF6 deletion mutant (Yu et al., 2002). In a mouse hepatocyte cell line, knockdown of TRAF6 using siRNA resulted in the inhibition of TGF-β activation of JNK and p38, however upon reintroduction of TRAF6 into these cells using TRAF6 cDNA restored the activation of these kinase pathways in response to TGF-β (Yamashita et al., 2008).

The TRAF6-TAK1-JNK/p38 cascade occurs in conjunction with the Smad-dependent pathway to regulate cellular responses downstream of TGF-β such as the induction of apoptosis, during which TRAF6-TAK1-JNK/p38 pathway is essential; knockdown of TRAF6 or using a p38 inhibitor resulted in the abrogation of TGF-β-mediated apoptosis in mouse hepatocytes and mouse epithelial cells (Yu et al., 2002; Yamashita et al., 2008). Furthermore, using a dominant negative form of MKK3 or p38 blocked TGF-β-

induced changes in the cytoskeleton and EMT (Yu et al., 2002). Similarly, knockdown of TRAF6 resulted in inhibition of TGF-β-mediated EMT (Yamashita et al., 2008).

Another pathway implicated in the TGF-β signalling pathway is PI3K which has been shown to rapidly activate PI3K by phosphorylating its downstream effector, Akt. In mouse mammary epithelial cells, treatment with exogenous TGF-β1 resulted in the phosphorylation of Akt at Ser-473 and consequent changes in cell morphology, including the acquisition of spindle cell morphology, an effect which was inhibited in the presence of the Akt inhibitor, LY294002 (Bakin et al 2000). The ability of TGF-β1 to induce cell survival and induce angiogenesis is also PI3K/Akt dependent; inhibition of this pathway resulted in decreased cell survival and impairment of angiogensis in mouse capillary endothelial cells (Vinals and Pouyssegur, 2001).

There is much evidence in the literature to demonstrate the importance of Smad-independent pathways in TGF-β1-mediated cell responses in a wide range of cell types. It is important to note that these pathways often work in conjunction with the Smad proteins to elicit various cellular mechanisms and also highlights the complexity of TGF-β1-mediated signalling, the regulation of which is extremely important and perturbation of which can result in pathological conditions.

1.3.4 Regulation of TGF-β receptors

An early step in TGF-β signalling is the ligand-induced formation of a tetrameric receptor complex comprising of two TβRII and TβRI units (Lonn et al., 2009). This activated tetrameric receptor complex is internalized via either clathrin-coated pits or clathrin-independent caveolin-1-positive lipid rafts (Guglielmo et al., 2003). The mechanism of endocytosis at the cell membrane can have direct downstream effects on signal transduction. Clathrin-mediated endocytosis results in receptor re-cycling back to the cell surface subsequent to transportation of receptors to early endosomes. Conversely caveolae-dependent internalisation results in receptor ubiquitination and lysosomal degradation (Lonn et al., 2009). Receptor endocytosis via these two pathways is at equilibrium; TGF-β mediated receptor activation is not thought to favour one of these pathways of internalisation over the other (Lonn et al., 2009). Receptor endocytosis is still a poorly understood phenomenon and specific factors, which result in either of these two pathways of receptor internalisation have not been determined. Furthermore, It has been proposed that in the serine-threonine kinase system, ligands do

not regulate trafficking but rather act to recruit the type I receptor and stabilize the heterotetrameric receptor complexes during constitutive trafficking (Guglielmo et al., 2003).

In broad terms, clathrin-mediated endocytosis increases TGF-β signalling by carrying the receptor complex to early endosomes where sustained signalling may take place after which the receptors are recycled back to the cell surface (McClean amd Guglielmo, 2010; Guglielmo et al., 2003). In this pathway, the Smad2 anchoring protein, SARA is bound to receptors at the plasma membrane but it is also highly enriched in early endosomes (Guglielmo et al., 2003). Although it was previously thought that the clathrin-mediated pathway was responsible for bringing the receptor to SARA-bound Smad2 and therefore resulting in receptor-mediated Smad2 phosphorylation, it is now believed that this pathway functions to sequester receptors away from the caveolin-1-positive lipid rafts, which can inhibit TβRI and its downstream signalling (Guglielmo et al., 2003).

Caveolin-1-positive lipid rafts are involved in the negative feedback of TGF-β signalling as internalisation via caveolae results in the association of Smurf2 with the recepors and consequent ubiquitination of the receptor complex and lysosomal degradation (McClean and Guglielmo, 2010; Lonn et al., 2009). Whereas SARA-bound Smad2 localises to early endosomes rich in phosphatidyl inositol 3 phosphate (PtdIns(3)P), the inhibitory Smad7 and the E3 Ubiquitin ligase, Smurf1/2 prefer association with the cholesterol and sphingolipid-rich composition of lipid rafts which aid inhibition of downstream signalling (Guglielmo et al., 2003). It has been proposed that the Smurf proteins enhance the interaction of inhibitory Smad7 with the TGF-β receptor complex, allowing Smad7 to compete with R-Smads for receptor activation before it begins to degrade the receptor complex (Suzuki et al., 2002). Various proteins, including heat shock protein 90, the adaptor serine-threonine kinase receptor-associated protein (STRAP) and several E2 and E3 ubiquitin ligases control the overall process of receptor downregulation (Lonn et al., 2009).

Equilibrium between each of these receptor-regulatory pathways determines whether the pathway results in Smad activation and gene transcription (clathrin-dependent pathway) or receptor degradation (raft-caveolin route) and therefore inhibition of TGF-β signalling. The endocytic mechanism of TβRs can therefore have profound effects on

TGF-β signalling and a number of pathologies, including metastatic cancers and fibrotic diseases show aberrant TGF-β signalling indicating the importance of receptor endocytosis as a method by which downstream TGF-β signalling is regulated.

1.3.5 Dysegulation of TGF-β signalling

Due to the wide-ranging effects of TGF-β1 on cellular processes, its signalling is a tightly regulated process which, when it becomes dysregulated, can result in pathophysiological situations. Notably, dysregulation is often associated with epithelial-mesenchymal transition (EMT) and myofibroblastic differentiation, where excess production of TGF-β1 can result in a pathological cell type that can compound and accelerate vascular disease (Bobik, 2006). These initial local changes can have far-reaching implications during disease development with excessive fibrocellular proliferation and inward arterial remodelling, which can eventually result in a clinical event (Davies et al., 2003; Wu et al., 2003; Ward et al., 1997).

1.3.5a TGF-β1 and vascular disease

TGF-β has a multitude of effects on the different cell types that make up the vessel wall (Grainger, 2007). In culture, TGF-β1 stimulates ECM production and the expression of contractile proteins and in endothelial cells it inhibits cell proliferation, migration and the expression of adhesion molecules (Grainger et al., 2000). Dysregulation of TGF-β1 signalling ultimately results in pro-atherogenic changes in the blood vessel (Ruiz-Ortega et al., 2007).

Several animal models have highlighted the importance of TGF-β1 in the maintenance of normal vessel physiology; deletion of a single allele of the *tgfb1* gene caused a 50% reduction in TGF-β1 protein in the vessel media in mice. A high-fat diet caused these mice to express high levels of ICAM-1 and VCAM-1 on their vascular endothelium, which was accompanied by the infiltration of macrophages and fatty streak formation in the aortic wall, characteristic of early inflammatory changes in lesion formation in human atherosclerotic disease (Grainger et al., 2000). TGF-β1 is thought to be involved in plaque stability by promoting the secretion of ECM proteins such as collagen. In order to investigate this further, Mallat and colleagues used a neutralizing TGF-β antibody in ApoE-/- mice which led to the acceleration of lesion formation. These lesions contained more inflammatory components and a decreased collagen content, making them more prone to rupture (Mallat et al., 2001). This is supported by findings

from human coronary arteries in which there was a decreased expression of TGF-β1 in unstable plaques when compared to TGF-β1 expression in stable plaques, where its expression was invariably higher (Jiang et al., 2004; Reckless et al., 2001). mRNA expression analysis of human aortas found that in branch-points of the aorta that were more likely to develop atherosclerotic plaques, TGF-β1 mRNA expression was very low, whereas in straight parts of the aorta where the probability of plaque development was low, higher levels of TGF-β1 mRNA expression were detected (Borkowski et al., 1995). In spite of the body of evidence which suggests that impaired TGF-β1 signalling accelerates atherosclerosis, it is not clear whether increased expression of TGF-β1 protects against atherosclerosis. However, one study has reported that the overexpression of TGF-β1 in the aortae of ApoE-/- hyperlipidemic mice resulted in fewer T-lymphocytes, more collagen, less lipid and lower expression of inflammatory cytokines which may offer possible therapeutic potential (Frutkin et al., 2009).

Further animal studies have yielded similar results; in mice, in areas of arterial injury following surgery, the expression of active TGF-β1 was lower in the medial layer than that seen in non-injured arteries, with expression of SM-MHC and type IV collagen also significantly depressed in the media, suggesting that TGF-β1 modulates arterial remodelling by causing the differentiation of cells in the medial layer (Grainger et al., 1998).

Initial studies looking at the role of TGF-β1 in arterial injury produced by balloon angioplasty revealed that mRNA levels of TGF-β1 were significantly increased within 6 h of carotid injury (Majeskt et al., 1991). Furthermore, immunohistochemical studies showed that the majority of SMC in the neointima stained for TGF-β1 and was related to neointima formation implicating it in neointima thickening following balloon angioplasty (Majesky et al., 1991). More recently, study into LTBPs which govern TGF-β1 release has shown that LTBP-1 and 2 are detected in the neointima and neoadventitia of a porcine model of coronary angioplasty, in a similar fashion to TGF-β1 (Sinha et al., 2001). The same study demonstrated a higher level of LTBP proteolysis that correlated with increased active and latent levels of TGF-β1 and increased arterial injury response suggesting a pivotal role for TGF-β1 during angioplasty-induced injury (Sinha et al., 2001). Results in neoadventital cells from this study are supported by those in a balloon catheter denudation model in which rats were treated with vehicle or TGF-β1 inhibitor (Ryan et al., 2003). Inhibition of TGF-β1 was seen to promote vessel

enlargement, however lumen size remained unchanged, despite neointima formation suggesting a role for TGF-β1 in injury-induced reduction of lumen area since its inhibition prevented this (Ryan et al., 2003). Intra-arterial infection of adenovirus-expressing Smad3 (AdSmad3) in Sprague-Dawley rats that had undergone left carotid balloon injury found that contribution of TGF-β1 to neointima formation may be via the proliferation of vascular SMC resulting in intimal hyperplasia, an event that was found to be dependent upon the phosphorylation of the cyclin-dependent kinase inhibitor p27 by Smad3 (Tsai et al., 2009). This critical role for Smad3 in remodelling has been further compounded by its medial gene transfer in a rat carotid angioplasty model in which it caused adventitial changes including myofibroblast transformation, proliferation and collagen production, which are all hallmarks of adaptive remodelling (Kundi et al., 2009). In light of these findings that implicate TGF-β1, it is not surprising that antagonising its actions has proven to be beneficial in preventing fibrosis and scarring. The use of locally administered TGF-β1 receptor antagonists following angioplasty/stenting are therefore a promising therapeutic avenue for the prevention of restenosis following angioplasty-induced injury (Agrotis et al., 2005).

Although the majority of data on the biological role of TGF-β in the vascular wall shows that it is protective against disease by promoting plaque stability, there is some data that contends this (Grainger, 2007). A number of studies have shown that TGF-β1 is implicated in disease pathogenesis (Table 1.3). In the intima of normal human non-atherosclerotic aortic segments there was very little expression of TGF-β1, however fibro-fatty/fatty streak lesions a high expression of TGF-β1 was detected, indicating that TGF-β1 could contribute to lesion formation (Grainger, 2007). In particular, in smooth muscle cells *in vitro,* TGF-β1 stimulates the production of the ECM proteins, fibronectin and type I collagen (Pennttnen et al., 1988; Ignotz et al., 1987), characteristics of adverse vessel remodelling that is associated with coronary artery occlusion (Ross, 1999). Transfection of the human *tgf-β1* gene into porcine arteries *in vivo* demonstrated that this increased TGF-β1 expression was associated with ECM synthesis accompanied by intimal hyperplasia, implicating TGF-β1 in the development of vascular lesions (Nabel et al. 1993). A more recent study has also reported similar findings; delivery of a TGF-β1 antisense-expressing construct prevented TGF-β1 expression in a rat model of intimal hyperplasia, and consequently also significantly decreased intimal thickness when compared to saline-treated control animals (Sun et al., 2012).

The regulation of gene expression in cardiovascular disease by microRNAs (miRNAs), and in particular, those that regulate the downstream actions of the TGF-β1 signalling pathway are a highly topical area of research. miRNAs are strands of short non-coding RNAs that regulate gene expression by modulating the translation and/or ability of target mRNAs (Boyd, 2008). Primary transcripts of miRNAs are transcribed by RNA polymerase II, subsequently processed within the nucleus by the RNaseIII endonuclease, Drosha into approximately 70 nt hairpin intermediates known as pre-miRNAs. These are subsequently cleaved by the cytoplasmic RNaseIII, Dicer to release a double-stranded duplex comprising the mature miRNA and its antisense complement. miRNAs are incorporated into the RNA-induced silencing complex (RISC) which recognises its target genes by partial sequence complementarity between the miRNA and motifs typically within the 3'untranslated regions (UTRs) of its target gene then promote mRNA degradation or inhibit translation (Dimmeler and Nicotera, 2012).

Recent investigation into miRNA regulation of TGF-β1 has highlighted several miRNAs that are involved in vascular disease progression, including miR-26a, miR-143 and miR-145 (Hata and Davis, 2009; Thum et al., 2008; see General discussion section 6.5.1). These miRNAs have been implicated in vascular disease progression in response to the production of TGF-β1, though the mechanisms behind this relationship are still to be fully elucidated (Thum et al., 2008). Preliminary data regarding the cross-talk between TGF-β1 and several miRNAs is displayed in the General Discussion (section 6.5.1).

The importance of local changes in TGF-β activity at sites of lesion formation and its subsequent role in the balance between a stable and unstable plaque phenotype ultimately dictates the progression of atherosclerosis and disease outcome. The significance of this cytokine in the vessel wall during atherogenesis is evident and its effects on the various vascular cell types make it a key target for therapeutic intervention.

Title	Cell Type	Treatment	Key Findings	Reference
Hypoxia-generated superoxide induces the development of the adhesion phenotype.	Human fibroblasts and myofibroblasts	Xanthine (0-2.4 µM), xanthine oxidase (100 mU/ml), SOD (0-20 U/ml)	Hypoxia ↑ mRNA levels of TGF-β and type I collagen in a time-dependent manner. Xanthine/xanthine oxidase system enhanced the myofibroblast phenotype by ↑ TGF-β and type I collagen mRNA levels which was abrogated after treatment with SOD.	Fletcher *et al.*, 2008
Pro-oxidant effect of transforming growth factor-β_1 mediates contractile dysfunction in rat ventricular myocytes.	Rat ventricular myocytes	TGF-β1 (1 ng/ml)	TGF-β1 elicited production of ROS. ↑ was blocked when cells were treated with a ROS scavenger or NAD(P)H oxidase inhibitor. TGF-β_1 ↑ levels of $gp91^{phox}$ and $p22^{phox}$. A time dependent depletion of GSH was also observed.	Li *et al.*, 2008
NAD(P)H oxidase-4 mediates transforming growth factor-ß1–induced differentiation of cardiac fibroblasts into myofibroblasts.	Myofibroblasts	TGF-β1 (10 ng/ml)	TGF-β1 induced NAD(P)H –derived superoxide production by upregulating Nox4. Nox4 mediates differentiation of cardiac fibroblasts to myofibroblasts by regulating Smad2/3 activation.	Cucoranu *et al.*, 2005
Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts.	Mesenchymal cells isolated from patients with idiopathic pulmonary fibrosis. Small airway epithelial cells (SAECs)	TGF-β (2ng/ml) and DPI (10 µM)	TGF-β ↑ the extracellular production of H_2O_2 in IPF. In DPI-treated IPF there was complete inhibition of H_2O_2 production. TGF-β1 stimulated apoptosis of SAECs which was reversed with the addition of catalase to the co-culture system.	Waghray *et al.*, 2005
TGF-β1-induced suppression of glutathione antioxidant defenses in hepatocytes: caspase-dependent post-translational and caspase-independent transcriptional regulatory mechanisms.	Murine TAMH hepatocyte cell line	TGF-β1 (5 ng/ml)	TGF-β1 ↑ in cell death in a time-dependent manner with upregulation of caspases 3 and 9 and downregulation of the anti-apoptotic protein Bcl-X_L. TGF-β1-induced cleavage and loss of GCLC protein is associated with a ↓ in GCL enzymatic activity and the depletion of intracellular GSH.	Franklin *et al.*, 2003

Table 1.3 Studies implicating TGF-β in the production of ROS.

Title	Cell Type	Treatment	Key Findings	Reference
Hydrogen peroxide increases extracellular matrix mRNA through TGF-β in human mesangial cells.	Human mesangial cells	Glucose oxidase (GOx) (1 mU/ml) Catalase (80 U/ml)	GOx significantly ↑ levels of TGF-β1 and ECM proteins. Catalase pre-treatment prevented the GO-induced stimulation of TGF-β1 mRNA. GO-induced H_2O_2 production induced TGF-β1 synthesis and ↑ ECM gene expression.	Iglesias-De La Cruz *et al.*, 2001
Requirement of hydrogen peroxide generation in TGF-β1 signal transduction in human lung fibroblast cells: involvement of hydrogen peroxide and Ca^{2+} in TGF-β1-induced IL-6 expression	Human lung fibroblasts	TGF-ß1 (1 ng/ml)	TGF-ß1 induced [ROS]i levels and ↑ IL-6 mRNA concentrations in HLF. Catalase, NAC, and GSH ↓ the TGF-ß1-stimulated IL-6 mRNA expression significantly. Results indicate ↑ ROS is required for TGF-β1-induced IL-6 gene expression.	Junn *et al.*, 2000

Table 1.3 Studies implicating TGF-β in the production of ROS cont'd.

1.4 Sulforaphane

Sulforaphane (SFN) is one of a large number of naturally occurring isothiocyanates (ITC), first identified and isolated from hoary cress (Prochdzka, 1959) and later from broccoli florets (Zhang et al., 1992). Its isolation from broccoli was also accompanied by the observation that SFN was able to induce both quinine reductase and glutathione-*S*-transferase activities in liver, stomach, small intestine and lung tissue derived from mice fed SFN extracts (15 μM, 5 days). Later studies showed that it is a potent chemopreventative (Keum, 2011; Munday et al., 2008; Traka et al., 2008; Zhang and Tang, 2007; Kwak et al., 2002) and is also a known activator of the Nrf2/ARE pathway and consequently, the induction of Nrf2-target antioxidant genes (Oh et al., 2012; Chen et al., 2011; Chang et al., 2010).

1.4.1 Occurrence/source(s) and isolation

ITCs are found in cruciferous vegetables such as broccoli florets, cabbage and cauliflower. SFN is an ITC and in its natural form, exists as the glucosinolate precursor, glucoraphanin. ITC are known to be synthesized and stored as glucosinolates in plants and are released when damage to plant tissues occurs (Zhang and Tang, 2007). The conversion of glucosinolate to ITC is catalysed by myrosinase, which, in plants, co-exists with glucosinolates but is stored separately (Fenwick et al., 1983). Glucosinolate can be partially hydrolysed in the intestinal tract and local enteric flora is known to possess myrosinase activity allowing the coversion of glucosinolates to ITC (Bheemreddy and Jeffery, 2007).

1.4.2 Metabolism and excretion

In vivo, SFN is rapidly metabolized through the mercapturic acid pathway (Zhang and Tang, 2007) and conjugated with GSH, a reaction that is catalysed by GST and results in a conjugate which undergoes further enzymatic modifications, catalysed by Y-glutamyltranspeptidase and results in the formation of a cysteinylglycine-SFN conjugate. Following further modification, which is catalysed by cysteinylglycinase, results in the formation of a cysteine conjugate. A final modification by *N*-acetyltransferase results in a *N*-acetylcysteine-SFN conjugate (Zhang and Tang, 2007).

SFN is rapidly eliminated and this is correlated with its rapid absorption and short plasma half-life; in one human study, plasma concentrations were reported to be in the

range of 0.94 – 2.27 μM one hour following consumption of 200 μM ITC extract (Petri et al., 2003). Other pharmacokinetic studies in humans have revealed that consumption of a single portion of broccoli cress or mature broccoli can generate plasma SFN concentrations of approximately 1 μM (Riedl et al., 2009; Ye et al., 2002) and 60 nM (Hanlon et al., 2009) respectively. Further studies have gone on to confirm the breakdown and excretion of SFN to be mainly via the mercapturic acid pathway (Al Janobi et al., 2006; Gasper et al., 2005).

In cell culture, the intracellular accumulation of SFN, particularly in the cytoplasm, is a rapid process and has been reported in several different cell types (Callaway et al., 2004; Zhang and Callaway; 2002; Zhang, 2001). In murine hepatoma cells, treatment with SFN (5 μM) resulted in rapid intracellular accumulation after just 30 min of exposure which continued to increase for up to 6 h, reaching peak levels of 350 μM and decreasing thereafter (Zhang and Talalay, 1998). Similarly in mouse fibroblast cells, rapid intracellular accumulation of SFN was observed after 30 min, with intracellular concentrations attained being 40 – 180 times higher than the initial extracellular concentration (5 μM) (Zhang and Talalay, 1998). The rapid accumulation of SFN is thought to be a GSH-driven process where ITC-GSH conjugates accumulate in cells and as a result, cause phase II enzyme induction. Therefore this suggests that cells with higher GSH concentrations have a higher level of intracellular accumulation of ITC (Zhang, 2001; Callaway et al., 2004). ITC that are already conjugated with thiols are unable to accumulate in cells with accumulation also being dependent on the continuous presence of ITC in the extracellular space (Zhang, 2000). In order to counteract ITC-GSH build-up inside the cell, conjugates are rapidly exported by a membrane transport protein, MRP1. In human leukaemia and human myeloma cells, use of an inhibitor for MRP1 resulted in continued intracellular accumulation of SFN-GSH conjugates (Zhang and Callaway., 2002). The balance between the accumulation and export of ITC-GSH conjugates is an important process, and one which results in the induction of phase II detoxifying enzymes, thereby enhancing the cells ability to protect itself against further extracellular insults.

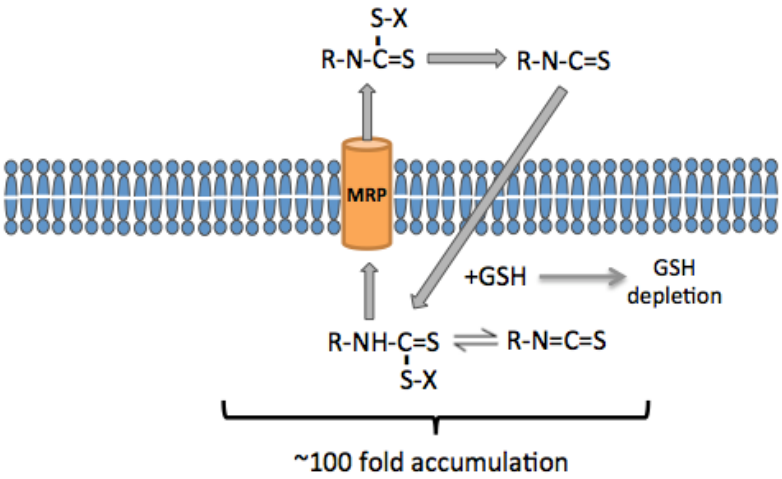


Figure 1.11 Cellular accumulation and export of SFN. SFN (R-N=C=S) penetrates the cell membrane by diffusion and upon entering the cell is rapidly conjugated with intracellular thiols, predominantly GSH. Intracellular accumulation of SFN-GSH conjugates results in the rapid export of these conjugates and is mediated by the membrane transporter, multidrug resistance protein (MRP).

1.4.3 SFN and therapeutic benefits

A number of epidemiological studies have revealed the beneficial effects of diet in preventing the pathophysiological events that can lead to cardiovascular disease (CVD) (Cornelis et al., 2007; Genkinger et al., 2004; Hung et al., 2004; Yochum et al., 1999) and more recently, several studies have reported the beneficial effects of the consumption of SFN-rich vegetables in reducing the risk of CVD-related complications. Although rapidly cleared from plasma, it is plausible that SFN is able to induce antioxidant enzymes for up to at least 24 h in healthy individuals, and levels that have been reported in plasma through consumption of Brassica vegetables have been demonstrated to influence the physiology of vascular and inflammatory cells (Evans, 2011). Several studies have investigated the effects of Brassica vegetable consumption on vascular physiology and pathophysiology. In one such study, ingestion of 200 mg/day of broccoli sprouts in a hypertensive-stroke prone rat model resulted in significantly lower oxidative stress levels in cardiovascular and kidney tissues as demonstrated by increased levels of reduced glutathione (GSH) content and decreased oxidized GSH in addition to an increase in endothelial-dependent relaxation of the aorta and significantly lower blood pressure, demonstrating the beneficial effects of its consumption during hypertension (Wu et al., 2004). Another study investigating the effects of SFN on the murine aorta from wild-type and Nrf2- knockout animal found

that SFN was able to abrogate the expression of the adhesion molecule VCAM-1 in atheroprone sites within the aorta whilst also activating the Nrf2/ARE pathway (Zakkar et al., 2009).

Evidence from *in vitro* studies has also confirmed the beneficial and potentially therapeutic effects of SFN on pathophysiology. In rat aortic smooth muscle cells, treatment with SFN (0.25 – 5 μM) has been shown to result in a concentration-dependent induction of GSH, glutathione peroxidase, glutathione *S*-transferase and NQO1 (Zhu et al., 2008). The same study also showed that pre-treatment with SFN protected aortic smooth muscle cells from oxidative and electrophilic cytotoxicity induced by xanthine oxidase or H_2O_2, giving cells resistance to extracellular stresses (Zhu et al., 2008). These results correlate with those found in a later study in endothelial cells where a one hour of pre-treatment with SFN (1 – 4 μM) suppressed TNF-α-induced monocyte chemotactic protein (MCP-1) and VCAM-1 mRNA and protein levels. Expression of dominant negative Nrf2 inhibited SFN-induced ARE-driven promoter activity in the same study, though this had no effect on SFN-mediated inhibition of VCAM-1 and MCP-1 (Chen et al., 2009). GSH is one of the first lines of defence cells employ in order to protect against oxidative insults and measurement of intracellular GSH and its associated enzymes is often used as an indicator of a cell's redox status; the oxidation and reduction of proteins which can often occur in a complementary fashion in order to maintain cellular homeostasis. In vascular SMC from spontaneously-hypertensive rats, basal levels of GSH were significantly lower than those observed in normotensive rats. This was accompanied by higher levels of oxidative stress that were attenuated by treatment with SFN, which also enhanced HO-1 protein levels in these animals (Wu and Juurlink, 2001).

As well as being cardio-protective, SFN is also a widely known chemopreventative agent and has been demonstrated as being a potent natural inhibitor of the cancer disease pathology. In animal models of chemical carcinogenesis, SFN has been shown to abrogate, and in some cases, inhibit the disease process. Zhang and colleagues have shown that administration of SFN (75 - 150 μM, 5 days) around the time of exposure to the carcinogen, 9,10-dimethyl-1,2-benzanthracene, resulted in a significant decrease in the incidence, multiplicity and weight of mammary tumours in a Sprague-Dawley model of chemical carcinogenesis (Zhang et al., 1994). Similar results have been reported in a mouse model of benzo[a]pyrene-induced carcinogenesis formation where

SFN (7.5 μmol/day for 20 weeks) blocked tumour formation in the forestomach region (Fahey et al., 2002). The same study reported that brief exposure to SFN was shown to be bacteriacidal and was reported to eliminate intracellular *H- pylori* from a human epithelial cell line (Fahey et al., 2002). Both the chemopreventative and antibacteriacidal effects of SFN in this study have been postulated to be attributed to the induction of phase II antioxidant enzymes via the Nrf2/ARE pathway (Fahey et al., 2002) (see below). The chemopreventative action of SFN has been attributed to its ability to inactivate histone deacetylase-6, thereby interfering with the expression of androgen receptor genes which are important in facilitating signalling and therefore disease progression during cancer (Gibbs et al., 2009).

1.4.4 SFN and the Nrf2/ARE pathway

The activation of the Nrf2/ARE pathway by SFN has been shown to be as a result of the modification of critical cysteine residues on Keap1 (Dinkova-Kostova et al., 2002). One study has shown that SFN-mediated ARE-luciferase activation is abrogated when NIH3T3 cells are co-transfected with plasmids containing an ARE-luciferase reporter gene for Nrf2 and a mutant Keap1 protein containing deletions of several domains including C151, suggesting this cysteine is essential for SFN-mediated activation of the Nrf2 pathway (Zhang and Hannink, 2003). Work carried out by Hong and colleagues has shown that exposure of recombinant Keap1 protein to SFN results in the formation of a Keap1-SFN adduct (Hong et al., 2005). Further work by another group has confirmed the formation of a Keap1-SFN adduct and have postulated that this interation occurs between SFN and the Cys151 residue on Keap1 (Hu et al., 2011). In contrast, one study has suggested that the modification of specific cysteine residues on Keap1 may be insufficient for Nrf2 activation and that this activation may occur through alternative mechanisms, possibly via the ubiquitination of Keap1, which is followed by the nuclear accumulation of Nrf2 (Eggler et al., 2005). Other studies have postulated the importance of the mitogen-activated protein kinase pathway in SFN-mediated Nrf2 activation; treatment of human hepatoma (HepG2) and murine hepatoma (Hepc1c1c7) cells with SFN stimulated the activity of Erk2 and resulted in the induction of NQO1, which was abolished after the use of an Erk2 inhibitor (Yu et al., 1999). Furthermore, in HepG2 cells, the same treatment stimulated the activity of p38 MAPK, and its inhibition using a specific inhibitor resulted in enhanced activity of quinone reductase while overexpression of dominant-negative mutant of p38 MAPK potentiated the activation of the ARE reporter gene suggesting that p38 MAPK is important in the

regulation in ARE-mediated induction of phase II detoxifying enzymes following SFN treatment (Yu et al., 2000).

Although the mechanisms behind the activation of Nrf2 by SFN remain to be fully elucidated, it is well established that the Nrf2/ARE pathway is an important downstream target of SFN and several studies have reported the modulatory effects of SFN on this antioxidant defence pathway. Initial *in vivo* evidence came from a murine Nrf2 knockout model which concluded that the protective effects of SFN administration against tumour formation were abrogated in Nrf2-deficient animals and strongly suggested that this protective effect was due to the activation of the Nrf2/ARE pathway (Fahey et al., 2002). This led to further studies examining the protective effects of SFN as a chemopreventative agent and results from Fahey and colleagues were confirmed in a skin tumorigenesis mouse model study. Here, the topical application of SFN (100 nmol/day for 14 days) prior to exposure to a carcinogen resulted in a decrease incidence of skin tumour in the WT animals whilst no chemoprotective effect was observed in Nrf2 knockout animals (Xu et al., 2006). Furthermore, microarray analysis of tissue samples from the small intestine of wild-type and Nrf2-knockout mice treated with SFN, revealed that a number of phase II genes were upregulated, including NQO1, GST, GCS, GPx and biosynthetic enzymes of the GSH and glucoronidation conjugation pathways as well as the induction of Nrf2 protein in wild-type animals, an effect that was abrogated in knockout animals (Thimulappa et al., 2002). Interestingly, the study also reported that Nrf2 mRNA levels remained unchanged, confirming previous findings that SFN may affect Nrf2 post-translationally by mediating the activity of a variety of intracellular kinases pathways and thereby causing its phosphorylation (Keum, 2011; Zhang and Hannink, 2003).

Due to its beneficial effects during cancer pathogenesis, there has been growing interest in the potential therapeutic benefits of SFN during other disease pathologies. In a study in a streptozocin-induced model of diabetic nephropathy, SFN was able to significantly attenuate oxidative damage and prevent the fibrotic changes such as hypertrophy and extracellular matrix accumulation in the kidney by suppressing the protein expression of TGF-β1 in wild-type animals whilst having little or no effect in Nrf2-deficient animals. The same study also reported increases in Nrf2 protein levels and an inverse decrease in TGF-β1 protein expression in human renal mesangial cells treated with SFN (Zheng et al., 2011). The effects and possible cross-talk between SFN and the TGF-β1 signalling

pathways has also been demonstrated in a hepatic fibrosis mouse model. SFN was reported to prevent hepatic fibrosis in this model, whilst also reducing the expression of α-SMA and type I collagen. This effect was postulated to be due to the Nrf2-dependent inhibition of TGF-β1 signalling where Nrf2 was thought to prevent the phosphorylation of Smad2/3, thereby preventing TGF-β1-mediated gene expression and fibrosis (Oh et al., 2012).

The inconclusive evidence from clinical trials looking at the potential therapeutic effects of exogenous antioxidant administration has led to the investigation of ways in which endogenous antioxidants may be upregulated in order to protect against oxidative stress and disease (Kris-Etherton et al., 2004; BiondiS-Zaccai et al., 2002; Steinberg, 2000). The dietary intake of SFN may help to protect against disease by activating the endogenous antioxidant Nrf2/ARE pathway and therefore may be used as a preventative measure against various disease pathologies (Annabi et al., 2008; Noyan-Ashraf et al., 2006).

1.4.5 SFN and reactive oxygen species

Several studies have postulated that SFN may cause the activation of endogenous antioxidant pathways by increasing the transient generation of intracellular ROS. In human bronchial epithelial cells, SFN (10 μM) has been shown to increase ROS levels as measured by the DCF-DA assay, 10 min after treatment and these levels continued to increase up until 8 h after treatment. This increase in ROS levels was accompanied by the activation of the Nrf2/ARE pathway and increased protein expression of HO-1 after 4 h, an observation that was abrogated in cells transfected with Nrf2 siRNA (Lee and Lee, 2011). Although this particular study highlights the possibility that SFN activates Nrf2-mediated gene expression through the generation of ROS, it did not investigate the potential source of ROS in this cell type (Lee and Lee, 2011), however it has been suggested that the source of ROS may be mitochondria-dependent.

Previously, Singh and colleagues have reported that in a human prostate cancer cell model, SFN increases intracellular ROS levels by disrupting the mitochondrial membrane potential as SFN-induced ROS generation was significantly attenuated on pre-treatment with mitochondrial respiratory chain complex 1 inhibitors including diphenyleneiodonium and rotenone (Singh et al., 2005). Furthermore, SFN treatment also resulted in GSH depletion and a decrease in cell viability which was attenuated in

cells pre-treated with N-acetylcysteine in this cell type (Singh et al., 2005). Similar results were found in a study where endothelial cells were exposed to SFN (4 μM) in culture, resulting in an increase in intracellular ROS levels as assessed by the DCF-DA assay, and was accompanied by an increase in the activation of Nrf2 and related ARE-linked gene expression which protected cells from further oxidative damage and cytotoxicity (Xue et al., 2008). A more recent study in human lung cells has also reported an increase in Nrf2-regulated gene expression (NQO1, glutmate-cysteine ligase and thioredoxin reductase) at 24 h following the nuclear accumulation of Nrf2 in response to a rise in intracellular ROS levels follwowing exposure of cells to SFN (Poerschke et al., 2012).

The ability of SFN to activate the endogenous antioxidant Nrf2/ARE pathway highlights it as an important, possible preventative and therefore therapeutic agent during cardiovascular disease. Its ability to rapidly enhance intracellular ROS levels and therefore cause an elevated and sustained increase in antioxidant gene expression even after its metabolism and excretion may offer it up as a preventative measure to attenuate disease progression.

1.5 Endogenous antioxidant enzyme systems

In order to counter-balance oxidative stress, the cell has a number of antioxidant defence systems, which protect it against the excess production of ROS and maintain cellular homeostasis. In particular, glutathione, is the first line of defence and is responsible for the conjugation, metabolism and excretion of most stress-inducing insults facing the cell. Antioxidant genes are also important in providing the cell with longer-term defence and these include heme-oxygenase (HO-1) and the phase II detoxifying enzyme, NQO1. These cellular defence systems are tightly regulated and under the control of Nrf2, a transcription factor that plays an important role in the protection of the cell against oxidative stress.

1.5.1 Nrf2-Keap 1 pathway

The Nrf2-Keap1 pathway regulates the oxidative stress-protective responses in mammals (D'Autreaux and Toledano, 2007). Nrf2 (nuclear factor (erythroid-derived-2)-like2) is a cap'n'collar basic leucine zipper (CNC-bZIP) transcription factor which binds to the antioxidant response element (ARE) found in promoter regions of genes

encoding phase II detoxification enzymes and antioxidant proteins (Lee and Johnson, 2004) and which is thought to be ubiquitously expressed. However, despite its widespread expression, disrupting the *nrf2* gene in mouse embryonic stem cells has revealed that it may be dispensable for normal development (Chan et al., 1996). Regulation of Nrf2 is via six highly conserved domains known as Nrf2-ECH homology (Neh 1-6) domains; the Neh1 domain contains a CNC-type basic leucine zipper domain, which allows Nrf2 to heterodimerize with small Maf proteins and is essential for DNA binding as well as containing a nuclear localization signal (Motohashi et al., 2004; Itoh et al., 1999). The N-terminal Neh2 domain binds to the Kelch domain of the Nrf2-cytosolic repressor, Keap1 (Itoh et al., 1999) and contains seven lysine residues to allow for the negative regulation of Nrf2 via ubiquitin conjugation and subsequent proteasome-mediated degradation of Nrf2 (Zhang et al., 2004). The C-terminal Neh3 domain is essential for the transcriptional activity of Nrf2 Zhang, 2006) whilst Neh4 and 5 are two independent transactivation domains that interact with CREB-binding protein (Katoh et al., 2001). The Neh6 domain contains an abundance of serine residues, though the exact function of this domain has not been clearly identified (Zhang, 2006).

The activity of Nrf2 is regulated by its inhibitory protein Keap1 (Zhang, 2006). Its discovery was as a result of using the N-terminal Neh2 domain of Nrf2 as bait in a yeast two-hybrid assay (Itoh et al., 1999) during which it was found that the C-terminal Kelch domain of Keap1 was found to bind to the Neh2 domain of Nrf2 (Li et al., 2004). There is much controversy over the exact location of Keap1. Initially described as being located primarily in the cytoplasm, bound to the actin cytoskeleton and with its main role being to retain Nrf2 in this subcellular compartment until exposure of cells to oxidative stress (Kang et al., 2004), there is now evidence to suggest that Keap1 may not be as passive a protein as initially thought. Several studies have reported that Keap1 plays a pivotal role in actively targeting Nrf2 for proteasomal degradation rather than passively sequestering it in the cytoplasm (Nguyen et al., 2009; Kobayashi et al., 2004; Zhang et al., 2004). It has been suggested that under basal conditions, the constitutive activity of Keap1 promotes the ubiquitylation of Nrf2 through the cullin-3-dependent pathway (Kobayashi et al., 2004; Zhang et al., 2004), however oxidative stress enables Nrf2 escape Keap1-dependent degradation, leading to stabilization of Nrf2, its nuclear localization and activation of ARE-driven genes (Zhang and Hannink, 2003). In particular, two critical cysteine residues important for Keap1-dependent ubiquitylation have been found on Keap1; C273 and C288 and serine substitution at either disables

Keap1-dependent ubiquitination of Nrf2 (Zhang and Hannink, 2003). A third cysteine residue, C151, located on the BTB domain of Keap1 has been reported to be required for the stabilization of Nrf2 and for the activation of Nrf2-regulated gene transcription (Tian et al., 2012). It has also been suggested that antioxidant-induced modification of Keap1 C151 leads to a conformational change in Keap1, allowing Nrf2 to be phosphorylated at Ser40, leading to Nrf2 release from Keap1 (Tian et al., 2012). Other studies suggest that due to the constitutive expression of Nrf2-regulated genes, the Nrf2-Keap1 complex is not localized within the cytoplasm but rather that it shuttles in and out of the nucleus to allow for basal expression of genes in order to maintain redox homeostasis within the cell (Petri et al., 2012). Many studies report increases in Nrf2 protein levels but no change in Nrf2 mRNA levels suggesting that some oxidants may regulate Nrf2 in a post-translational manner (Purdom-Dickinson et al., 2007).

Several kinases have been reported to directly phosphorylate Nrf2, thereby affecting its cellular location and stability (Keum, 2011). Initial studies looking at possible kinases identified protein kinase C (PKC) as being a possible candidate responsible for this phosphorylation; Huang and colleagues found that exposing HepG2 cells to oxidative stress in the form of phorbol 12-myristate 13-acetate (PMA) resulted in the nuclear localization of Nrf2, an effect which was abrogated in the presence of a PKC inhibitor suggesting PKC-mediated phosphorylation is important in the nuclear translocation of this transcription factor (Huang et al., 2000). Further work by the same group revealed the phosphorylation site as being Ser40 on Nrf2; Nrf2 bearing a Ser to Ala mutatuion at amino acid 40 was not phosphorylated by PKC and partially impaired Nrf2 activation og ARE-driven transcription in a reporter gene assay (Huang et al., 2002). However, the observation that Nrf2 activation was only partially impaired suggested that there may be other factors that may modify Nrf2 and thereby downstream gene transcription. Treatment of rat cardiomyocytes with H_2O_2 showed an increase in Nrf2 protein after 1 h as well as increased Nrf2-ARE binding activity, both of which were blocked by treatment of cells with the PI3K inhibitor, LY294002 (Purdom-Dickinson et al., 2007). To further confirm that the increase in Nrf2 protein was not due to increased levels of Nrf2 RNA, cells were treated with the RNA synthesis inhibitor, actinomycin D, which failed to block H_2O_2 from increasing levels of Nrf2 protein (Purdom-Dickinson et al., 2007). This study demonstrates that in addition to PKC, PI3K may also regulate Nrf2 by phosphorylation by increasing its stability and thereby allowing it accumulation in the cell.

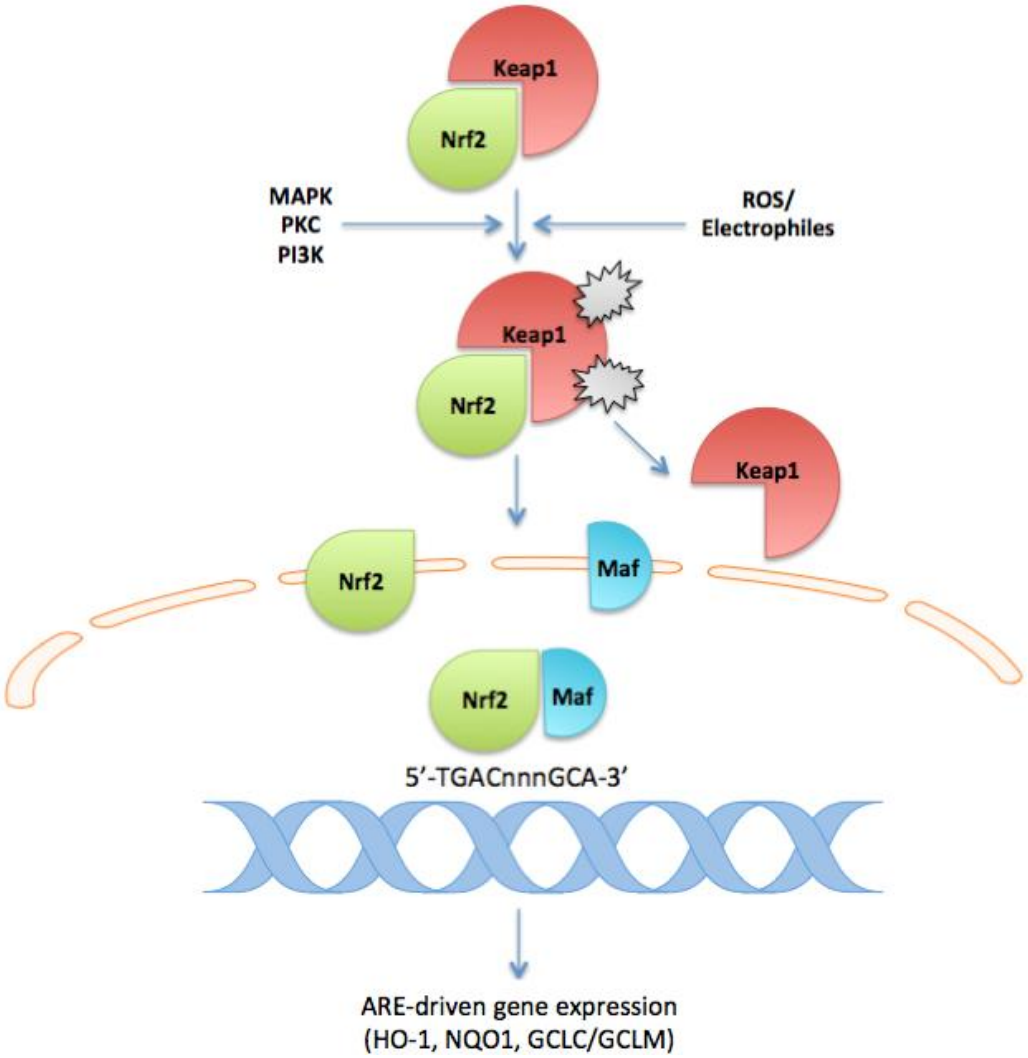


Figure 1.12 The Nf2-Keap1 pathway of ARE-driven gene expression. Under normal physiological conditions, Nrf2 is sequestered in the nucleus by Keap1 and targeted for degradation via the ubiquitin-proteasome pathway. However, in response to oxidative stress, cysteine residues on Keap1 become oxidized and Keap1 is no longer able to mediate the degradation of Nrf2. Nrf2 translocates to the nucleus, and along with small Maf protein binds to the ARE of target genes to drive their expression.

In addition to PKC and PI3K, there is evidence to suggest that p38 MAPK also directly phosphorylates Nrf2; in HepG2 cells, p38 MAPK directly phosphorylated the recombinant GST-tagged Nrf2 protein and promoted the interaction of this recombinant protein with endogenous Keap1 *in vitro*, preventing Nrf2 nuclear translocation (Keum et al., 2006). In the same study, the isothiocyanate, SFN was found to promote the nuclear translocation of Nrf2 by blocking the p38 MAPK pathway, suggesting that this pathway is involved in the negative regulation of Nrf2 (Keum et al., 2006). A study in a hamster cancer cell line treated the isothiocyanate, phenethyl isothiocyanate (PEITC), found that this compound increased the phosphorylation of ERK1/2 and JNK1/2, causing the release of Nrf2 from Keap1 and its subsequent translocation to the nucleus, indicating the importance of these kinases in ARE-driven gene expression (Xu et al., 2006). The import and export of Nrf2 is pivotal in driving antioxidant gene expression; sustained and excessive nuclear accumulation of Nrf2 has been shown to be lethal in mice (Wakabayashi et al., 2003) and *in vitro* has been shown to be lead to apoptotic cell death (Strachan et al., 2005) therefore it can be assumed that the regulation of the nuclear export of Nrf2 is also tightly regulated. Jain and Jaiswal have proposed that Fyn, a tyrosine kinase that phosphorylates Nrf2 at Tyr568, and promotes its nuclear export and degradation (Jain and Jaiswal, 2006). Further work by the same group proposes that GSK-3β acts as adirect upstream regulatory kinase of Fyn, contributing to Nrf2 phosphorylation at Tyr568 (Jain and Jaiswal, 2007). This is further compounded by evidence froma study carried out by Rojo and colleagues in which SFN is thought to activate ARE-dependent gene expression by regulating Fyn/ GSK-3β activity (Rojo et al., 2008).

As well as phosphorylation, the acetylation and deacetylation of Nrf2 has also been postulated to play a role in its nuclear localization and transactivation. In HEK293T cells exposed to sodium arsenite, Nrf2 was shown to undergo reversible acetylation in a number of lysine residues in its Neh1 DNA-binding domain under the action of CREB-binding protein (CBP). This acetylation was reported to encourage Nrf2 transcriptional activity (Sun et al., 2009). Similarly, in another study, in HepG2 cells, CBP induced the acetylation of Nrf2, increasing its binding to the ARE and thereby increasing Nrf2-driven gene transcription (Kawai et al., 2011). Furthermore, the histone deacetylase, sirtuin 1 (SIRT1) was shown to decrease acetylation of Nrf2 and subsequent gene transcription; briefly the study reported that acetylation of Nrf2 resulted in its increased nuclear localization whereas deacetylation conditions favored its cytoplasmic

localization (Kawai et al., 2011). It is now widely reported that the post-translational modification of Nrf2 is pivotal in determining its sub-cellular localization and consequently its ability to induce antioxidant genes. Phosphorylation and acetylation events can enhance Nrf2 nuclear localization, however as the above studies have shown, these events can change depending on the oxidative stressor or the cell type in question.

ARE-driven enzymes and antioxidants include glutathione peroxidase (GSH), NADPH:quinone oxidoreductase-1 (NQO1), heme-oxygenase-1 (HO-1) and Peroxiredoxin (Prx). Nrf2-regulated expression of antioxidant enzymes in relation to cardiovascular disease has been widely reported. Treating murine aortic smooth muscle cells and macrophages with the lipid peroxidation product, 4-hydroxynonenol (4-HNE) resulted in the nuclear translocation of Nrf2. Nrf2 also caused the up-regulation of CD36 on macrophages, an important scavenger receptor mediating the uptake of oxidized LDL (oxLDL) and playing an important role in foam cell formation. This was accompanied by Nrf2-mediated HO-1 and Prx protein expression in the macrophage (Ishii et al., 2004). The Nrf2-mediated induction of antioxidant enzymes human aortic smooth muscle cells in response to oxidized lipids was also seen in another study where Nrf2 induced HO-1 protein expression in response to moderately oxidized LDL (Anwar et al., 2005). In a similar study, Nrf2-expressing adenovirus was transfected into vascular smooth muscle cells (VSMCs) following angioplasty, resulting in an increase in the expression of several antioxidant enzymes, including HO-1 (Levonen et al., 2007) as well as effectively reducing oxidative stress in response to oxLDL and vascular inflammation as assessed by macrophage cell count (Levonen et al., 2007).

The importance of this pathway has also been seen in endothelial cells exposed to shear stress. Laminar shear stress is thought to be atheroprotective and exposure of endothelial cells to laminar shear stress revealed that Nrf2 was strongly activated and mediated downstream antioxidant protein expression, offering some of the first evidence that the Nrf2/ARE pathway was key in protecting certain areas of the artery from atherosclerotic lesion formation (Dai et al., 2007). Shear stress also caused activation of the Nrf2/ARE pathway in human umbilical vein endothelial cells (HUVECs) exposed to laminar shear stress. These cells exhibited an upregulation of several Nrf2-mediated genes, including HO-1 and NQO1 and inhibition of Nrf2 with the antioxidant, N-acetyl cysteine, also caused a downregulation in the expression of HO-1 and NQO1 (Warabi et al., 2007).

These studies demonstrate the significance of the Nrf2/ARE pathway in protecting against oxidative insults and regulating redox balance in the vasculature.

1.5.2 Glutathione

Glutathione (GSH) is one of the first and most important antioxidant defence systems in mammalian cells (Meister, 1983 Meister 1988a & b). An ubiquitous intracellular tripeptide, it is composed of glutamate, cysteine and glycine and its synthesis in the cytosol is a tightly regulated process (Lu, 2009) and it is also here that the majority of cellular GSH is located as well as within several intracellular organelles including the mitochondria, which contains a much smaller proportion of cellular GSH, with a small percentage located in the endoplasmic reticulum (ER) and the remainder in the nucleus (Hwang et al., 1992; Meredith and Reed, 1982). GSH is the thiol-reduced form and exists in millimolar concentration in most mammalian cells (fibroblasts 0.5 – 5 mM, Zucker et al., 1997, liver 5 – 10 mM, Lu., 2009), with the disulphide-oxidised (GSSG) form making up less than 1% of total cellular GSH (Akerboom et al., 1982). The importance of this intracellular thiol is demonstrated in the number of vital functions it serves within the cell; detoxification of electrophiles; scavenging free radicals; maintainence of protein thiol status; acting as a reservoir for cysteine as well as modulating critical cellular processes such as DNA synthesis, immune function and cell proliferation (Kaplowitz et al., 1985; Meister and Anderson 1983).

The most important and well-known function of GSH is antioxidant defence; GSH can detoxify H_2O_2 and lipid peroxide, reactions which are catalysed by glutathione peroxidase (GPx) as well as conjugating with and detoxifying electrophiles through glutathione *S*-transferase-catalysed reactions (Liu and Pravia, 2010). The small redox enzymes glutaredoxin (Grx) and sulfiredoxin (Srx) catalyse reactions, which keep protein cysteine residues in their reduced form, which when oxidised can be converted to the sulfenic (RSOH), sulfinic (RSO_2H) or sulfonic (RSO_3H) acid (see Fig 1.13). The oxidative modification of protein cysteine residues, including those on the cytosolic inhibitor of Nrf2, Keap1 (Kobayashi and Yamamoto, 2005) are important in ROS-mediated regulation of protein function (Lui and Pravi, 2010). The ratio of GSSG:GSH is an indicator of a cell's redox status and maintenance of intracellular GSH homeostasis is vital for normal cellular functions; GSH redox cycling, catalysed by GSSG reductase prevents loss of GSH in the form of GSSG during various reduction reactions with oxidants, however most cells maintain their redox status by *de novo*

synthesis of GSH. This synthesis is a two-step process, catalysed by glutamate-cysteine ligase (GCL) and GCL synthetase and is explained further below.

The availability of cysteine, the sulphur amino acid precursor and the activity of the rate limiting enzyme, GCL, composed of a catalytic (GCLC) and modifier (GCLM) subunit, are the main determinants of GSH synthesis (Liu and Pravia., 2010; Lu., 2009). The rate of GSH synthesis is highly dependent upon the levels of GCL activity; oxidative stress caused by a variety of agents can result in increased GCL activity, increased GCLC mRNA and GCLC gene transcription and as a consequence, an increase in total cellular levels of GSH (Wild et al., 1999; Zipper and Mulcahy 2003; Zipper and Mulcahy 2000). TGF-β1 has also been shown to regulate GSH synthesis at the level of GCLC; in type II alveolar epithelial cells, TGF-β1 was found to inhibit the expression of GCLC whilst having no effect on GCLM (Jardine et al., 2002) and this was further confirmed in hepatocytes where TGF-β1-mediated apoptosis resulted in decreased GCLC mRNA and cleavage of GCLC protein and consequently a decrease in GCL activity but had no effect on GCLM protein levels (Franklin et al., 2003). Furthermore, in rat hepatic stellate cells, TGF-β1 lowered levels of GSH, which consequently facilitated fibrogenesis (Fu et al., 2008).

The transcriptional regulation of GCLC has been closely studied and consensus NFκB, Sp-1, AP-1, metal response and antioxidant response (ARE)/electrophile responsive (EpRE) elements have been identified in the human GCLC promoter (Moinova and Mulcahy, 1998; Mulcahy et al., 1997; Mulcahy and Gipp,, 1995). In particular, identification of ARE led to the finding that Nrf2 plays an important role in the regulation of GSH synthesis by transactivating the human GCLC promoter (Mulcahy et al., 1997). To further confirm this, transcriptional regulation of rat GCLC using Nrf2 knockout fibroblasts showed that Nrf2 knockout cells had lower levels of GCLC (Yang et al., 2005a). Okouchi and colleagues have also shown that Nrf2 is important for GCLC subunit expression; exposure of human brain endothelial cells to chronic hyperglycemic stress resulted in apoptosis, increased activation of PI3K/Akt/mTOR signalling and increased phosphorylation and nuclear translocation of Nrf2 and consequently increased GCLC subunit expression (Okouchi et al., 2006).

Nrf2 is also important for the induction of GCLM; the rat GCLM promoter has a functional ARE element (-295 to -285) which is important for the basal promoter

activity as well as TNF-α-mediated induction of GCLM (Yang et al., 2005b). Mulcahy and colleagues have demonstrated that upregulation of GCLM by beta-naphthoflavone involved binding of Nrf2 to a functional ARE/EpRE site located at -302 of human GCLM (Moinova and Mulcahy, 1998). However, not all inducers of oxidative stress affect levels or induction of GCLM and the discordance between the relative expression of GCLC and GCLM may depend upon the expression of the two subunits in different cell/tissue types (Lu, 2009).

Increased levels of GSH are associated with an early proliferative response and are essential for the cell to enter the S phase (Iwata et al., 1994; Messina and Lawrence, 1989; Shaw and Chou, 1986). Increased levels of GSH have been reported in rat hepatocytes (Huang et al. 2008) and following partial hepatectomy; blocking the increase in GSH resulted in impaired liver regeneration (Huang et al., 2001). It is known that GSH modulates DNA synthesis by maintaining reduced glutaredoxin or thioredoxin, both of which are required for the activity of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (Holmgren, 1981) and this may be one way by which GSH modulates cell proliferation. Changes in the thiol-redox status may also affect the expression or activity of factors important for cell cycle progression and can also modulate cell death; GSH depletion has been reported during apoptosis secondary to increased ROS, enhanced GSH efflux and decreased GCL activity (Lu, 2009).

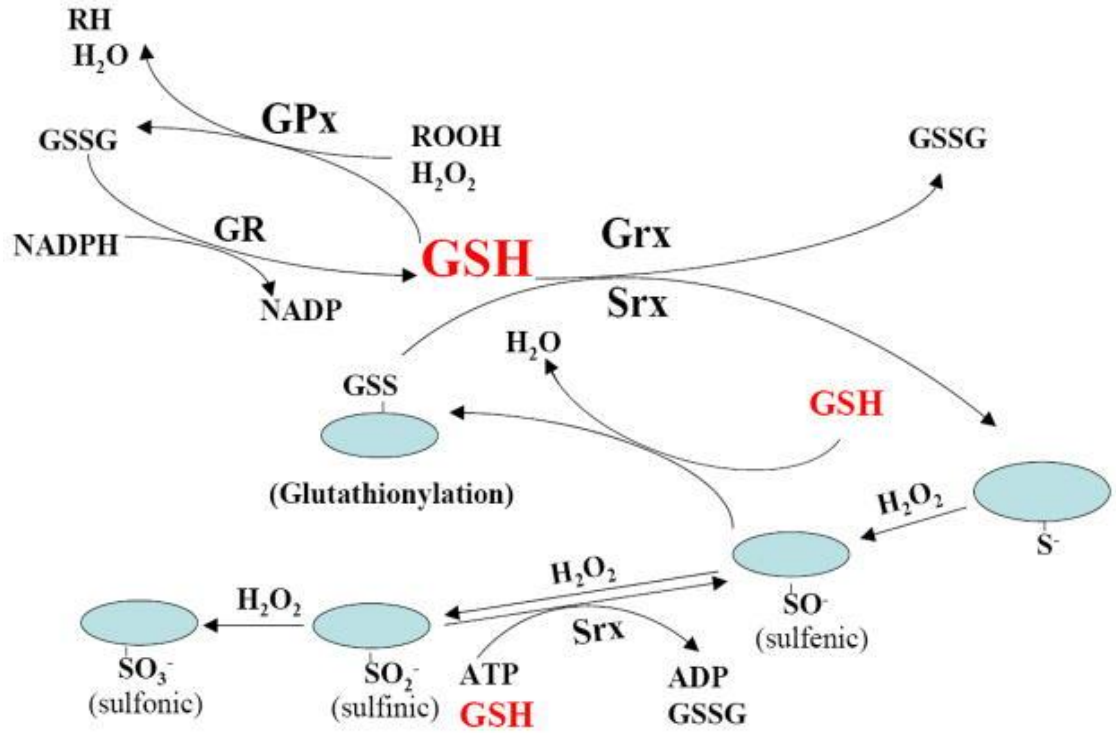


Figure 1.13 GSH synthesis and cycling in cells
GSH reduces hydrogen peroxide (H_2O_2) and lipid peroxide (LOOH) through glutathione peroxidase (GPx) catalysed reactions resulting in oxidised glutathione (GSSG). GSSG is then reduced back to GSH in a reaction catalysed by glutathione reductase (GRS) and which requires NAD(P)H. GSH is also important in protein redox signalling; depending upon the intensity of the oxidative insult, protein cysteine residues can be oxidised to sulfenic (RSOH), sulfinic (RSO_2H) and sulfonic (RSO_3H) acid. Conversion to sulfonic acid form is considered to be irreversible, however sulfinic acid can be reduced back to sulfenic acid through an Srx catalyzed reaction. Sulfenic acid then reacts with GSH to form protein mixed disulfides (glutathionylation), which can be reduced back to free-thiol form (deglutathionylation) through Grx or Srx catalyzed reactions using GSH as a reductant. Taken from Liu and Gaston-Pravia, 2009).

1.5.3. Heme oxygenase-1

Various risk factors for the development of coronary heart disease and other cardiovascular diseases mediate HO-1 gene expression, including increased blood pressure, altered laminar flow, advanced glycation end products (AGEs), oxidized lipids and a number of inflammatory processes (Stocker and Perella, 2006). HO-1 catalyses the first rate-limiting step of heme to its metabolites carbon monoxide (CO), which causes mild vasodilation, ferrous iron (Fe^{3+}) which is stored by ferritin and biliverdin which is further broken down to bilirubin and which acts as an antioxidant (Fig 1.14).

High levels of bilirubin and biliverdin are thought to be inversely related to the progression of atherosclerosis (Idriss et al., 2008). The protective effects of bilirubin may be due to its ability to protect plasma lipids, such as LDL from peroxidation by strongly inhibiting the formation of hydrogen peroxide (Neuzil and Stocker, 1994). Bilirubin has also been shown to be protective, in the post-ischemic myocardium in a Langendorff model of ischemia/reperfusion, where exogenously applied bilirubin significantly restored myocardial function, limited infarct size and mitochondrial damage. This attenuation in myocardial function was completely abolished in the presence of a heme-oxygenase inhibitor (Clarke et al., 2000), suggesting that HO-1-derived bilirubin is involved in cardio-protection against reperfusion injury. Further evidence for the protective role of bilirubin in the vasculature comes from studies that show that there is an inverse relationship between plasma bilirubin and risk of coronary artery disease (Idriss et al, 2008). Evidence for the cardio-protective role of bilirubin in the prevention of atherosclerosis is supported detection of decreased bilirubin activity in atherosclerosis (Hopkins et al., 1996). More recent evidence has shown that the cardio-protective effects of bilirubin may be due to its ability to directly inhibit NAD(P)H oxidase by interrupting the assembly and activation of this enzyme (Jiang et al., 2006), thereby attenuating ROS generation.

Another product of HO-1 that is cardio-protective is CO, which has anti-proliferative and anti-inflammatory properties (Morita, 2005). Its action as a vasodilator is due to its ability to increase levels of cGMP in VSMCs; inhibition of CO using haemoglobin reduced VSMC cGMP (Morita and Kourembanas, 1995). The same study found that the additional effects of CO included inhibition of endothelin-1 (ET-1) and platelet-derived growth factor (PDGF-B) which in turn abrogated VSMC proliferation. Therefore CO

may play a role in attenuating neointima formation and medial hyperplasia in atherosclerosis

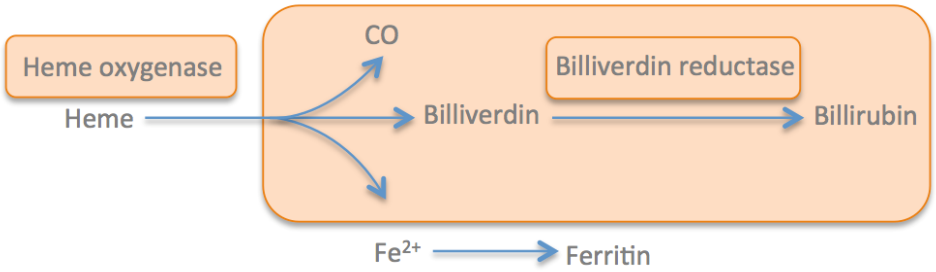


Figure 1.14 The heme-oxygenase enzyme system. Heme oxygenase is the first rate limiting enzyme that catalyses the breakdown of heme to CO (mild vasodilator), ferrous iron (which is stored by ferritin) and biliverdin which is further broken down to bilirubin by biliverdin reductase (antioxidant properties).

The third product of heme metabolism, ferrous iron, can act as an electron donor and therefore contribute to ROS generation. However, there is some evidence to show that it can also be cardioprotective (Idriss et al, 2008). The HO-1-dependent release of ferrous iron is thought to increase resistance to oxidative stress (Ossola et al., 2000) by enhancing cellular capacity for sequestering iron (Tomaro and Batlle, 2002). In addition to this, in endothelial cells, increases in ferritin keep iron pools low and reduce oxidant-induced lipid peroxidation (Selmeci et al., 2000). This experimental evidence highlights the important role of HO-1 and its metabolites in protecting the vasculature against the damaging effects of oxidative stress.

1.5.4 NADPH:quinone oxidoreductase 1

NQO1 is an enzyme belonging to the family of phase II detoxification enzymes, which are responsible for the conjugation of xenobiotics into water-soluble compounds, which may be excreted in urine or bile. NQO1 is thought to be involved in the cellular defence against the electrophilic and oxidizing metabolites of xenobiotic quinones, such as vitamin E, which results in the generation of the antioxidant forms of these molecules (Ross, 2004). Although initial study revealed that its primary function is chemoprotection, recent evidence has shown that it is involved in superoxide

scavenging with the cell (Ross, 2004). NQO1 works by mediating the two-electron reduction of quinones to hydroquinones, in particular its antioxidant role involves reduction of ubiquinone to ubiquinol (Ross, 2004). A two-electron reduction removes a reactive electrophilic quinone from a biological system and bypasses one-electron reduction reactions that can generate reactive oxygen species (Ross, 2004). NQO1 knockout mice have revealed the importance of this enzyme against quinone toxicity; NQO1 knockout mice administered with the superoxide and quinone generator, menadione were more susceptible to oxidative stress and toxicity when compared to wild-type animals (Radjendirane et al., 1998). Under levels of high oxidative stress, NQO1 is induced in cells to high levels (Dinkova-Kostova and Talalay, 2010), and although the rate of interaction of this enzyme with superoxide is less than a magnitude above the rate of chemical dismutation of superoxide (Dinkova-Kostova and Talalay, 2010), its activity in tissues with decreased activity of SOD could provide an additional layer of protection against oxidative stress.

1.5.5. Superoxide dismutase

As previously mentioned, NO, which has important anti-inflammatory and anticoagulant properties as well as its vasodilatory effect, can be rapidly inactivated by reacting with O_2^- leading to the production of the unstable anion $ONOO^-$, a process commonly occurring in hypercholesterolemia, hypertension, diabetes and ageing (Fukai and Fukai, 2011; Madamanchi and Runge, 2007; Guzik and Harrison, 2006). The main cellular defence against this reaction are a group of oxidoreductase enzymes, known as superoxide dismutases (SODs). The SOD system is one of the major antioxidant systems in the cell, catalysing the dismutation of the highly reactive O_2^- to oxygen and H_2O_2. Three forms of the enzyme exist in humans, SOD1 (copper-zinc SOD located in the cytoplasm), SOD2 (manganese SOD, found in the mitochondria) and SOD3 (an extracellular form of copper-zinc SOD) (Forstermann, 2008) each of which are contained in distinct subcellular localisations; SOD1 is localised in the cytosol and mitochondria, SOD2 is localised to the mitochondrial matrix and SOD3 is anchored to the extracellular matrix (Nguyen et al., 2004; Petersen et al., 2004; Fukai et al., 2002). Although their cellular localisations are distinct, all three isoforms exert various biological effects through H2O2, which can function as a signalling molecule and stimulate cellular responses such as hypertrophy, proliferation and migration via the oxidation of Akt, Src, and MAPKs or the inactivation of protein tyrosine kinases (Fukai and Fukai, 2011; Rhee, 2006).

Due to its localisation in the cytosol, SOD1 plays an important role in protecting NO-mediated vasorelaxation; SOD1 knockout mice exhibit increased levels of vascular O_2^- and $ONOO^-$, increased myogenic tone and vasoconstrictor responses (Didion et al., 2002). Furthermore, overexpression of SOD1 in transgenic mice has been shown to improve vascular dysfunction in a model of subarachnoid haemorrhage (Kamii et al., 1999). Adenoviral gene-transfer of SOD1 to the aortas of Watanabe heritable hyperlipidemic rabbits resulted in a significant decrease in endothelial O_2^- (Miller et al., 1998). A similar study in diabetic rabbits found that the adenoviral gene-transfer of SOD to the aorta significantly reduced O_2^- production and thereby improved endothelium-dependent relaxation, suggesting that SOD prevented the oxidation of NO to $ONOO^-$ in this disease model (Zanetti et al., 2001) and mice with a deleted SOD1 gene were more susceptible to ischemia/reperfusion injury in comparison with wild-type animals (Forstermann, 2008). Due to the ability of SOD to stimulate cellular responses such as migration, several studies have examined the role of SOD in reducing neointima formation. Ozumi and colleagues have reported that adenoviral transfer of extracellular SOD reduced ROS generation as well as reducing proliferation of VSMC and associated neointima formation in a cuff-injury model in rats (Ozumi et al., 2005). Similarly, catheter-mediated gene transfer to the arterial wall of balloon-denuded rabbit aortas reduced restenosis by decreasing the number of macrophages and enhancing the recovery of endothelial layer in this disease model (Laukkanen et al., 2002) whilst liposomal SOD delivery reduces redox-dependent expression of TGF-β1 and collagen in dermal myofibroblasts (Vozenin-Brotons et al., 2001). This evidence points to the critical regulation of O_2^-, H_2O_2, and $ONOO^-$, by SOD as being one of the most important defence systems in the vasculature that protects against increased oxidative stress during vascular physiologies and pathophysiologies.

1.6 Cell migration

Cell migration is a highly regulated process, involving the spatiotemporal reorganization of a number of structural proteins which make up the actin cytoskeleton as well as coordinating a myriad of signaling events that come together to mediate migration during embryogenesis, wound healing, immune response and tissue development (Kole et al., 2005). Due to the diverse role it plays in normal physiological processes, its dysregulation can contribute to the development of various pathological processes, including cardiovascular disease and cancer (Ridley, 2001).

Depending upon the stimulus, cell migration can be directional or random, however it is not entirely clear how the cells basic motility machinery is coupled to a steering mechanism that is able to integrate environmental cues with polarized signalling to promote persistent migration. Directional cell migration is thought to have two sources: intrinsic cell directionality and external regulation. Intrinsic directionality is as a result of the cell responding to a non-directional motogenic signal, such as the uniform application of PDGF that can trigger the motility machinery in the absence of an external guiding factor and is often known as chemokinesis (Giannone et al., 2009; Petrie et al., 2009). During chemokinesis, In contrast, chemotaxis occurs when a soluble factor, such as TGF-β1 is applied asymmetrically and dictates the direction of cell migration. This directional migration is as a result of regulated formation of lamellopodia, which are observed as a consequence of external guidance cues, topography of the ECM, intracellular polarity machinery and adhesion receptors (Petrie et al., 2009). Directional migration is as a result of new protrusions forming at the pre-existing leading edge, rather than in different directions around the cell periphery (Andrew and Insall, 2007). TGF-β1 has been shown to be a chemotactic agent for fibroblasts and application of this growth factor can result in the directional movement of this cell type toward the increasing gradient of this chemotactic agent (Acharya et al., 2009; Postlethwaite et al., 1987). In addition to TGF-β1, fibroblasts have also been shown to migrate towards chemotactic agents, including PDGF, FGF and epidermal growth factor (Archarya et al., 2009; Giannone et al., 2009; Petrie et al., 2009). Transwell migration assays have shown that the addition of a PDGF to the lower chamber of the well results in the directional migration of fibroblasts, which increases with increasing PDGF concentration (Engel and Ryan, 1997). *In vivo*, growth factors that can act as chemotactic agents are numerous, especially during disease, when the production of several of these is increased and can work in synergy to cause the

directional movement of fibroblasts toward the lumen of the vessel, where they are usually concentrated (Stenmark et al., 2012; Grainger et al., 2007).

An initial event seen during cell migration is the establishment of spatial polarity accompanied by the extension of membrane protrusions, known as lamellipodia in the direction of movement. *In vitro*, study of fibroblasts cultured on a 2D matrix has revealed that cells exhibit a polarized morphology; the leading edge displays a fan-like front called the lamella and a tapered tail end (Suraneni et al., 2012). Observation of the leading edge has revealed membrane ruffling, a process that is facilitated by the formation of newly polymerized actin filaments which make up the two of the structures seen at the leading edge; filopodia and lamellipodia (Ridley, 1994; Fig 1.15). Both structures lie on top of the more stable lamellal actin network and probe the cellular environment ahead of a migrating cell, detecting the stiffness of the surrounding ECM. In particular, the lamellopodia and the lamella are thought to play a pivotal role during migration and both of these actin-rich structures are known to work together in order to drive the forward movement of the cell (Giannone et al., 2009). Adhesions located at the leading edge are transduced by the actin network to the rear of the lamellipodium where myosin II-dependent contractile forces are generated, eventually pulling at the lamellipodial actin bundles resulting in a variation of migratory behavior dependent upon the strength of the adhesions (Burnette et al., 2011). An alternative model of cell migration has been postulated where actin filaments that are gathered in the lamellipodia as ‘arcs’ are thought to be pulled back into the lamella by myosin II and adhesions at the leading edge. In this model, a single arc may contact a number of adhesion sites, acting as a break to slow down migration or alternatively, using it as a site of future protrusion resulting in a net gain in forward movement of the cell as long as adhesions at the leading edge remain strong (Burnette et al., 2011; Giannone et al., 2007).

Actin polymerization is a critical event in the initial step of lamellipodial extension during cell migration and requires the activity of the small signaling G-protein, Rac. Branching of the existing actin filament networks that make up lamellipodial extensions is thought to be through the Rac-dependent activation of actin-related proteins 2/3 (Arp2/3) and this complex acts as a nucleation site for new actin filaments to form at 70° from existing, ‘mother’ filaments (Lauffenberger and Horwitz, 1996) downstream of Rac activation. The Rac-driven interaction of IRSp53, an adaptor protein necessary for Rac1-mediated membrane ruffling, with WAVE, a member of Wiskott-Aldrich

syndrome family protein (WASP), further drives filament branching as this complex binds to the C-terminal acidic domain of the Arp2/3 complex, resulting in its nucleation (Ridley, 2001). An additional mechanism by which Rac promotes actin polymerization is by 'uncapping' actin filaments at their barbed ends or by affecting the rate of actin depolymerization by stimulating the activity of LIM-kinase which then phosphorylates and inactivates cofilin, a protein that severs actin filaments thereby enhancing actin depolymerization by (Pavlov et al., 2007; Stanyon and Bernard, 1999; Arber et al., 1998). The initial induction of Rac has been reported to be as a result of growth factors, including TGF-β1, cytokines as well as extracellular components, and whose activation is mediated by tyrosine kinases and G-protein coupled receptors, both of which are dependent upon PI3K activity (Ridley, 2001).

Evidence implicating Rac in the phosphorylation of both myosin heavy chain (MHC) and myosin light chain (MLC) via p21-activated kinase (PAK) with one study reporting enhanced phosphorylation of MLC in the lamellipodial region of cells (Matsumura et al., 1998).

The attachment of the extending lamellipodia to the extracellular matrix (ECM)/substrate is modulated by the small focal complex structures and is thought to activate Rac and the small GTPase, Cdc42 (Horwitz and Parsons, 1999). The speed of migration and the level of activity of Rac and Cdc42 are dependent upon the composition of the surrounding ECM/substrate (Ridley, 2001). In slow moving cells, such as fibroblasts, focal complexes can mature into Rho-induced focal adhesions (Velichkova and Hasson, 2003). Under normal physiological conditions, the continuous assembly and disassembly of focal complexes is an important event during migration, a process that is also regulated by Rac, directly through PAK and indirectly by its ability to antagonize Rho activation (Sander, 1999). As well as Rac, Src and focal adhesion kinase (FAK) have also been reported to induce the disassembly of focal adhesions (Ridley, 2001).

As the cell moves forward, its body contracts in order to facilitate movement, an event that is dependent upon actomyosin contractility and can be regulated by Rho. Rho-activation of Rho-kinases results in the inhibition of phosphorylation of MLC which is mediated by MLCK, and is activated by calcium and stimulated by Erk MAPKs (Sander 1999) (Fig 1.15). Reducing Rho activity can have two converse effects; enhanced migration due to reduction in the adhesion of cells to the ECM or alternatively, a reduction in cell contractility which inevitably attenuates the process of cell migration

(Sander et al., 1999; Arber et al., 1998).

A fourth and final event during cell migration is tail detachment and is generally considered the ‘rate-limiting’ step of cell migration, with the rate of tail detachment being dependent upon the type of cell and the number of focal adhesion attachments to the ECM (Palecek et al., 1998). In slow moving cells such as fibroblasts, this process is regulated by calpain, a cysteine protease responsible for facilitating the degradation of focal adhesion complexes at the rear of a moving cell (Palecek et al., 1998). The importance of calpain in mediating tail detachment has been demonstrated by the use of pharmacological inhibitors of this protease in goldfish fin fibroblasts where it was observed that inhibiting it resulted in an inhibition of adhesion complex disassembly, suggesting a critical role for it during cell migration (Bhatt et al., 2002). Src and FAK may also mediate focal adhesion disassembly and therefore mediate tail detachment (Ridley, 2001).

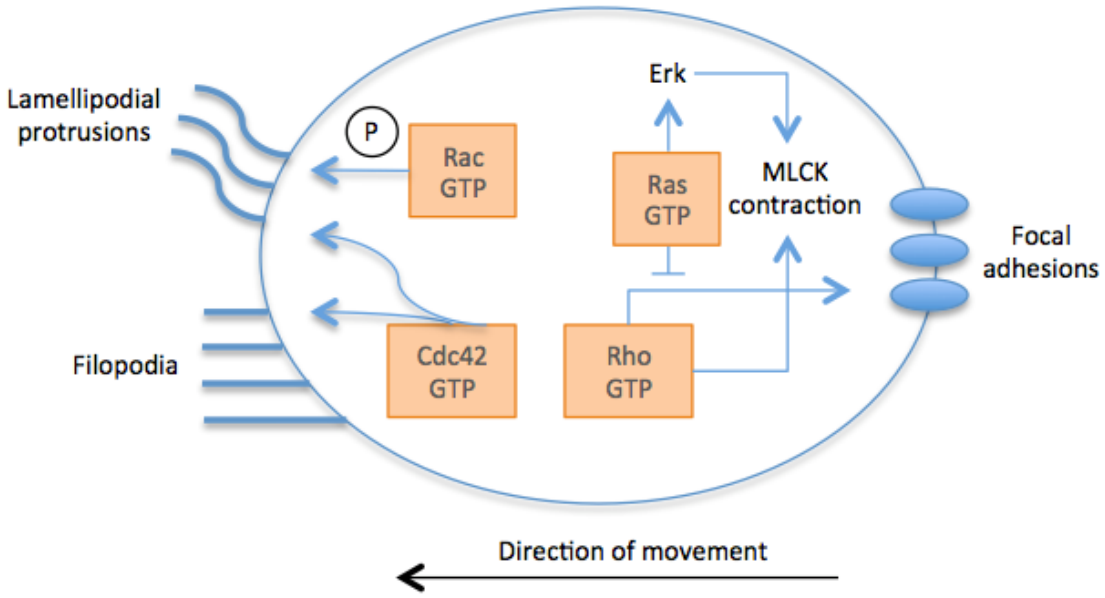


Figure 1.15 Role of Ras and Rho GTPases in cell migration
The formation of protrusions at the leading edge of a cell requires the activity of the small GTPase, Rac whilst Cdc42 induces filopodia formation and provides a polarity signal, ensuring that Rac-induced protrusions are restricted to the leading edge. Focal adhesions form at the trailing edge of the cell and undergo continuous assembly and disassembly, often occurring as a result of Ras-dependent inhibition of Rho. Contraction at the rear of the cell requires the activity of Erk-dependent phosphorylation of myosin light chain kinase (MLCK).

1.7 Hypothesis and Aims

TGF-β1 can modulate fibroblast phenotype leading to enhanced expression of myofibroblast marker proteins and increased cell motility. Previous studies have also reported that TGF-β1 causes the upregulation of antioxidant enzymes, possibly by causing the generation of reactive oxygen species (ROS). We hypothesise that TGF-β1 may cause activation of the Nrf2 signalling pathways and increase the activity and protein expression of HO-1 and NQO1 as well as depleting total cellular GSH. Therefore the aims of this thesis were to examine whether TGF-β1, a growth factor which is involved in atherogenesis, differentially modulates the expression and activity of proteins and enzymes downstream of the antioxidant stress pathway, Nrf2, including mRNA and protein levels of HO-1 and NQO1 and total intracellular glutathione (GSH) in cultured human aortic adventitial fibroblasts (HAoAF).

In addition, the effects of the dietary isothiocyanate, sulforaphane (SFN) were also investigated in HAoAF. SFN has been reported as a known activator of the Nrf2/ARE pathway and an inducer of phase II enzyme activity and several studies have demonstrated its potential therapeutic benefit when administered either prophylactically or during cardiovascular disease. We hypothesise that SFN activates the Nrf2 pathway and increases the protein expression and activity of HO-1 and NQO1 in HAoAF as well as causing the depletion of GSH levels. The current study sought to investigate the effect of SFN on the activation of the Nrf2/ARE pathway and its downstream transcriptional targets. The effect of SFN on HO-1and NQO1 mRNA and protein levels were determined and its effect on total cellular GSH was also measured, the activation of which may be of potential therapeutic benefit in this cell type.

TGF-β1 and ROS in the vessel wall may contribute to neointimal hyperplasia in atherogenesis, partly by the modulation of vascular remodelling as a result of enhanced cell migration from the adventitial and medial layers towards the intima. We hypothesise that TGF-β1 casues an increase in HAoAF migration and that this may occur in a ROS-dependent manner. The aim of this thesis was to further elucidate the effect of TGF-β1 on HAoAF migration and to determine whether this effect was a ROS-modulated process. Furthermore, investigation into the potential source of ROS was also investigated. The findings from this study may provide novel insights for future

therapeutic intervention to modulate antioxidant defence genes with TGF-β1 signalling or SFN during the pathogenesis of cardiovascular disease.

CHAPTER 2:

Materials and Methods

CHAPTER 2: Materials and Methods

2.1 Human aortic adventitial fibroblasts

2.1.1 Culture of human aortic adventitial fibroblasts

Cryopreserved human aortic adventitial fibroblasts (HAoAF) were commercially obtained (Lonza) at passage 3. Manufacturer's information indicated that cells had been routinely characterized using immunofluorescence staining and stained negative for smooth muscle α-actin, a marker used to indicate that HAoAF had not differentiated into smooth muscle cells. Quality control information provided by the manufacturer stated that all cells were performance assayed for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi and that cell viability, morphology and proliferative capacity were measured after recovery from cryopreservation.

Subsequently, cells were defrosted and cultured in a T25 (25 cm^2) flask. Cells were cultured in phenol red-free Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich UK) containing 1000 mg/L glucose and supplemented with 10% fetal calf serum (FCS), L-glutamine (5 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml). Cells were maintained at 37°C in an atmosphere of air and 5% CO_2. Experiments were conducted with cells from passages 4-9. Once cells had reached confluence in the T25 flask, they were detached using trypsin/EDTA (0.1% trypsin and 0.02% EDTA) in sterile phosphate-buffered saline (PBS). Detachment of cells was checked using an inverted light microscope (Nikon, TMS). The trypsin/EDTA was inactivated using phenol red-free DMEM supplemented with 10% FCS (37°C). The cells were then resuspended and transferred to a T75 (75 cm^2) flask. Once confluence was reached, cells were further subcultured into T75 flasks or in 6, 24 or 96 well plates for experiments (see section 2.1.3). The media was replaced every 2 days until an 80% confluent monolayer was observed.

2.1.2 Determination of fibroblast seeding density

To seed the requisite number of fibroblasts for experiments (Table 2.1), cell density was determined using a Neubauer chamber (Fig 2.1). Confluent fibroblasts at passage 4-8 were trypsinized and resuspended in DMEM supplemented with 10% FCS. For counting, 10 μL of cell suspension was transferred onto a haemocytometer chamber. The number of cells overlying four 1 mm^2 areas of the haemocytometer were counted

using an inverted light microscope (4× objective magnification) and the number of cells per millilitre of cell suspension determined from average of cell numbers in all four areas using the following formula; concentration = number of cells x 10,000/number of squares. The media was replaced every other day until the monolayer was confluent.

Culture plate	Seeding area (cm^2)	Working volume/well (ml)	Average cell yield at confluency
6-well plate	9.5	1.9 – 2.9	9.5×10^5
24-well plate	1.9	0.38 – 0.57	1.9×10^5
96-well plate	0.32	0.1 – 0.2	0.32×10^4

Table 2.1 Number of fibroblasts seeded per culture plate

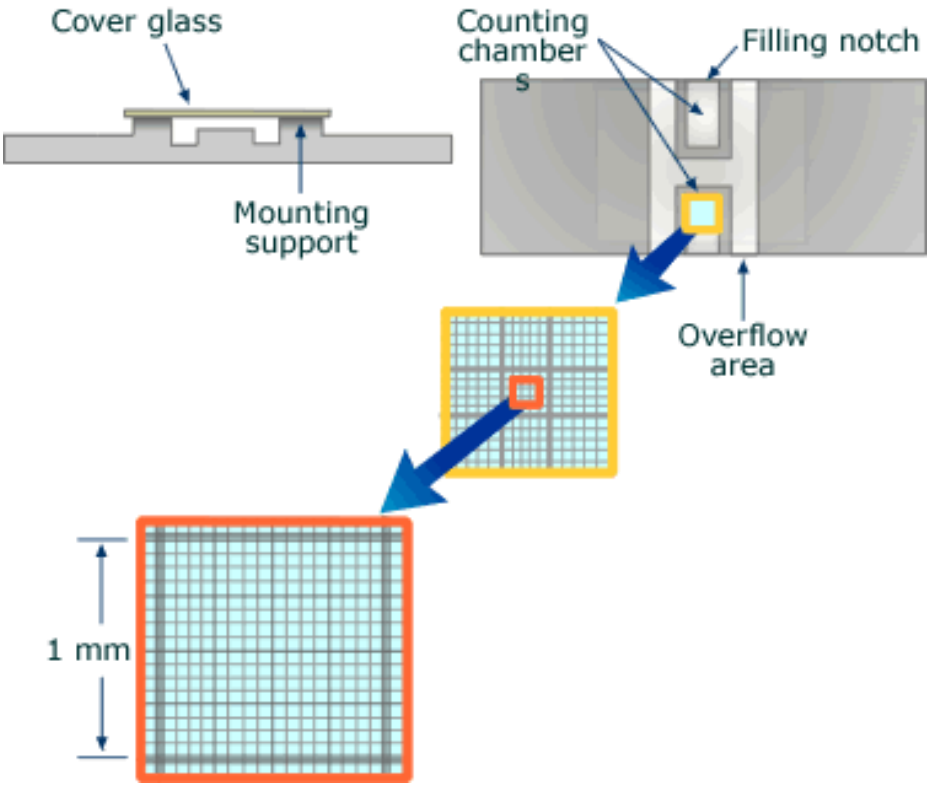


Figure 2.1 Cell counting using a haemocytometer
Cells in the corner areas were counted under a microscope. Cell density was calculated from the average of the cell numbers in four corner areas using the following formula; concentration = number of cells x 10, 000/number of cells.

2.1.3 Treatment of human aortic adventitial fibroblasts

Confluent monolayers of HAoAF were equilibrated in DMEM supplemented with 1% FCS, L-glutamine (5 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) 18-24 h prior to incubating with treatments. Treatments (transforming growth factor beta-1 [TGF-β1, 0-10 ng/ml], sulforaphane [SFN, 0-10 µM] or vehicle [0.01% dimethyl-sulphoxide, DMSO v/v] were diluted in DMEM supplemented with 1% FCS. Cells were incubated with treatments for 0-72 h. In studies investigating migration in HAoAF and in studies investigating ROS generation, cells were treated in the absence or presence of superoxide dismutase (SOD, 200 U/ml) in addition to treatment with TGF-β1. Furthermore, in ROS-generation studies, cells were pre-incubated with the NAD(P)H oxidase inhibitor, VAS2870 (10 µM) or SOD (200 U/ml) for 30 min and then acutely co-treated with TGF-β1 and VAS or SOD for 40 min during measurement of ROS generation.

2.2 Determination of protein expression in HAoAF by Western blot analysis

2.2.1 Extraction of total cellular proteins

After treatment periods were completed, HAoAF cultured in 6 or 24-well plates were gently washed twice with ice cold PBS. Cells were then lysed in 125 µl for 6-well plates or 75 µl for 24-well plates of sodium-dodecyl sulphate (SDS) lysis buffer (50 mM Tris base pH 6.8, 10% glycerol and 2% SDS and with the addition of 0.02% protease inhibitor cocktail [protease inhibitor I and II, see Appendix II]). Lysis buffer used for the extraction of phosphorylated proteins contained phosphatase inhibitors I and II (0.1 % v/v, Sigma-Aldrich, UK, see Appendix II). The cells were incubated on ice for 10 min and lysates subsequently transferred to microcentrifuge tubes and heated for 5 min at 95°C and stored at -20°C until further analysis.

2.2.2 Extraction of nuclear proteins

HAoAF were cultured in T75 cm^2 flasks until confluent and nuclear protein was extracted using a nuclear fraction extraction kit (Active Motif, USA). After treatment with TGF-β1 (2.5 or 5 ng/ml), SFN (2.5 or 5 µM) or vehicle control (DMSO 0.01% v/v), confluent monolayers were washed with ice cold PBS/phosphatase inhibitor solution once and detached by scraping in 3 ml of this buffer. The cell suspension was centrifuged at 500 rpm for 5 min and cell pellets were resuspended in 500 µl kit

hypotonic buffer and incubated on ice for 15 min in order to rupture cell membranes. 25 µl of kit detergent was added to the cell pellet and the total solution was vortexed for 10 s. The cell pellet was centrifuged at 13,000 rpm for 30 s and supernatants containing the cytosolic fraction were collected and stored at -80 °C until required for western blot analysis. Pellets containing nuclear proteins were resuspended in 50 µl kit complete lysis buffer (containing protease inhibitor cocktail, lysis buffer and supplemented with 10 mM dithiothreitol [DTT]) and subsequently placed on a rotating platform (150 rpm) for 30 min. Pellets were then vortexed for 30 s and centrifuged at 13,000 rpm for a further 10 min. Nuclear extracts in the supernatant were then collected and stored at -80 °C until further analysis by western blot or DNA binding ELISA (TransAM, Activ Motif, USA). Fractionation of nuclear and cytosolic lysates was confirmed by western blot analyses of specific nuclear and cytosolic proteins.

Protein concentration of nuclear lysates was determined using the BCA protein assay (see section 2.2.3).

2.2.3 Determination of protein concentration using the bicinchoninic acid protein assay

The protein concentration of each sample was determined using the bicinchoninic acid (BCA) protein assay as described by Smith et al., 1985. The assay was performed using a commercial kit (BCA Protein, Novagen) in a clear 96-well plate. Samples were heated at 95 °C for 5 min before being diluted 1:1 with ddH_2O. The protein concentration of unknown samples was determined by using bovine serum albumin (BSA) protein standards (0.15 - 2 mg/ml BSA in distilled water). 5 µl of each standard and 5 µl of SDS lysis buffer was added to duplicate wells in order to generate a standard curve. A blank duplicate consisted of 5 µl ddH_2O and 5 µl SDS lysis buffer.

200 µl of BCA reagent, made up of reagent A (bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 M NaOH, pH 11.25) and reagent B (4% cupric sulphate) in a ratio of 50:1 was added to each well. The plate was then covered and incubated at 37°C for 30 min before absorbance was measured at 562 nm using a plate reader (Spectramax 190, Molecular devices). Data was analysed using the SoftMaxPro programme and protein concentrations were calculated based on mean absorbance values. In order to ensure an accurate readout was being taken, only standard curves with a correlation coefficient of $R^2 > 0.995$ were accepted (see Fig. 2.2).

Protein concentration of nuclear lysates was determined using the BCA protein assay (see above). Due to the high reactivity of reducing agents within the lysis buffer with the cupric ions in the BCA reagents, nuclear lysate samples were diluted five-fold in ddH_2O. Nuclear extracts were then diluted 1:1 with SDS lysis buffer and heated for 5 min at 95 °C before SDS-PAGE and subsequent assessment of nuclear protein expression by western blot analysis.

2.2.4 SDS-PAGE and Western blotting

Expression of specific proteins in total and nuclear lysates was determined by western blot analysis. Samples were separated on either 10% or 12% polyacrylamide minigels using the Laemmli SDS-PAGE buffer system (Laemmli, 1970). Polyacrylamide minigels were prepared (375 mM Tris, 0.1% SDS at pH 8.8, 0.04% ammonium persulphate [APS] and 0.08% N,N,N',N'-tetramethylethylenediamine [TEMED]) and overlaid with a 3% stacking gel (0.125 M Tris, 0.1% SDS at pH 6.8, 0.05% APS and 0.2% TEMED). Before loading, all samples were first reduced by the addition of 2-Mercaptaethanol and 0.2% bromophenol blue and then treated for 5 min at 95°C. Samples (10 µg per lane) were loaded onto the stacking gel and a voltage gradient of 150 volts was applied for 1 h in a mini gel electrophoresis tank (Biorad). Following SDS-PAGE, the separated proteins were transferred onto a methanol-activated polyvinylidene (PVDF) membrane using a semi-dry electrophoretic transfer cell (Biorad) and transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol), as described by Towbin et al., 1979, at 20 V for 1.5 h.

In order to determine whether proteins had been adequately transferred onto polyvinylidene difluoride (PVDF) membranes, the membranes were stained using the azo dye, Ponceau red (Sigma) and subsequently washed several times in ddH_2O in order to remove residual staining. Membranes were then incubated in 5% non-fat dried milk in PBS 0.1% Tween for 1 h at room temperature in order to minimise non-specific primary antibody binding, followed by washing in PBS 0.1% Tween and incubation with appropriate primary antibody diluted in 3% BSA (w/v) in PBS 0.1% Tween (Table 2.1) for a further hour. After the transfer, the polyacrylamide minigels were stained using coomassie blue gel staining solution (50% methanol, 0.05% Coomassie Brilliant

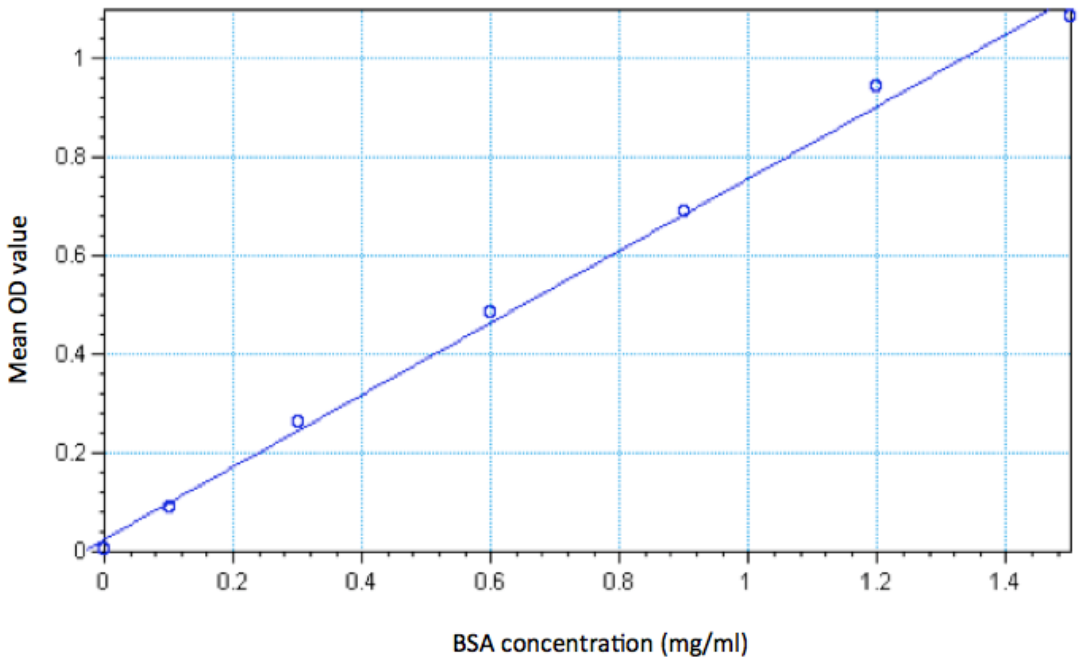


Figure 2.2 Protein concentration standard curve
Bovine serum albumin (BSA) protein standards (0.15-2.0 mg/ml) were added in duplicate to a 96 well plate. 200 µl of BCA protein assay reagent was added to each well and the plate was incubated for 30 min at 37°C. The absorbance read on a plate reader (Spectramax 190, Molecular devices) at a wavelength of 562 nm. Correlation coefficient, $r^2 = 0.996$.

Blue R and 10% acetic acid) overnight at room temperature. The following day, gels were destained using destain solution (7% acetic acid and 5% methanol) in order to visualise protein bands.

After primary antibody incubation, membranes were washed in PBS 0.1% Tween for 15 min and subsequently for 3 x 5 min washes and incubated with appropriate HRP-conjugated secondary antibody made up in 3% (w/v) non-fat milk (Table 2.2) for 1 h at room temperature. Membranes were washed as before and enhanced chemiluminescence was performed in order to visualise protein expression (Thorpe and Kricka., 1986).

2.2.5 Enhanced Chemiluminescence Detection

Enhanced chemiluminescence (ECL) was performed after incubation of the PVDF membrane with HRP-conjugated secondary antibody using a commercially available detection kit (Pierce). The ECL solution was prepared by diluting the reagents provided by the manufacturer to a ratio of 1:1. The membrane was placed on saran wrap and the protein side was incubated with ECL solution for 1 min at room temperature. Excess ECL solution was removed and the protein bands visualised using the G:Box gel imaging system from SynGene (Cambridge, UK). Relative band densities were quantified using the densitometry software, ImageJ (version 1.42, National Institutes of Health) and the ratio of the protein of interest to the loading control, α-tubulin, for whole cell extracts or lamin C for nuclear extracts, was determined and expressed a ratio and plotted using Graphpad Prism (v6.0).

Target Protein	MW (kDa)	Species	Dilution	Supplier	Catalog No.
HO-1	32	Mouse mAb	1:500	BD Transduction	610713
NQO1	31	Goat pAb	1:500	Santa Cruz	Sc-16464
Nrf2 (c20)	110	Rabbit pAb	1:500	Santa Cruz	Sc-722
Nrf2	66	Rabbit pAb	1:500	Spring Biosciences	E12501
Smooth muscle α-actin	43	Mouse mAb	1:1000	Santa Cruz	sc-32251
Vimentin	57	Rabbit mAb	1:1000	Abcam	ab15248
Caldesmon	120-150	Mouse mAb	1:1000	Millipore	mab3576
Calponin	36	Rabbit mAb	1:1000	Millipore	46794
Phospho Smad2 (Ser465/467)	55-60	Rabbit mAb	1:500	Millipore	04953
Smad 2/3	50 - 60	Rabbit pAb	1:500	Millipore	07-408
Phospho-Akt	60	Rabbit	1:500	Cell Signalling	9271S
pan-Akt	60	Rabbit	1:500	Cell Signalling	4685S
Phospho-JNK	46, 54	Rabbit	1:500	Cell Signalling	9251L
pan-JNK	46, 54	Rabbit	1:500	Cell Signalling	9252L
Phospho-Erk 1/2	44, 42	Rabbit	1:500	Cell Signalling	4370L
pan-Erk1/2	60	Rabbit	1:500	Cell Signalling	4695
$p38^{MAPK}$	43	Rabbit	1:500	Cell Signalling	9212S
Lamin A/C	69, 62	Goat pAb	1:500	Santa Cruz	sc-6254
α-Tubulin	55	Rat mAb	1:5000	Chemicon	mab1864

Table 2.2 Primary antibodies used for the determination of specific proteins on polyvinylidene membrane. Following semi-dry transfer of proteins onto nitrocellulose membrane, the membrane was blocked for 1 h and subsequently incubated with 10 ml of appropriate primary antibody, diluted in PBS containing 3% BSA and 0.1% Tween-20, for a further hour. The membrane was then washed in PBS 0.1% Tween for 30 min in order to remove any non-specific binding and incubated for a further hour with HRP-conjugated secondary antibody. Antibodies for phosphorylated proteins were diluted in TBS-T containing 5% BSA and 0.05% Tween-20.

Antibody	Dilution	Supplier	Catalog No.
Donkey anti-goat	1:2000	Santa Cruz	sc-2020
Goat anti-mouse	1:2000	Autogen	abn106 HRP
Goat anti-rabbit	1:2000	Autogen	abn117 HRP
Goat anti-rat	1:2000	Autogen	abn192 HRP

Table 2.3 Secondary antibodies used for the determination of specific proteins on polyvinylidene membrane. Following incubation with primary antibody for 1 h, the membrane was washed in PBS 0.1% Tween for 30 min and subsequently incubated for a further hour with HRP-conjugated secondary antibody. After incubation with secondary antibody, the membrane was washed for 30 min to remove any non-specific binding and analysed using enhanced chemiluminescence.

2.3 Tetrazolium assay to measure HAoAF viability

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as an index to measure HAoAF viability. Mitochondrial dehydrogenases in viable cells reduce the tetrazolium dye, MTT to the water insoluble dark blue/purple formazan (Denizot and Lang., 1986). A solubilisation solution, such as dimethyl sulphoxide (DMSO) is added to dissolve the insoluble formazan solution to give a visible dark purple colour which acts as an indicator of cell viability and which can be quantified by measuring the intensity of absorbance and correlating it with the mitochondrial dehydrogenase activity and cell viability.

Confluent cultures of HAoAF were seeded at a density of $1x10^3$ cells per well in a 96 well plate in DMEM supplemented with 10% FCS. Cells were equilibrated in DMEM supplemented with 1 % FCS 18 h prior to treatment with TGF-β1 (0 – 5 ng/ml), SFN (2.5 – 10 μM) or H_2O_2 (50 – 100 μM) for 24 h. Cells were treated in 4 replicate wells in each experiment. After this incubation period, the treatment conditions were aspirated and 50 μl of 10% MTT solution (made up in DMEM supplemented with 1% FCS) was dispensed into each well. The cells were then further incubated at 37°/5% CO_2 for 4 h after which 200 μl of DMSO was added to each well and left on a rotating platform for 10-15 min to aid dissolution of the formazan crystals. The absorbance was then measured at 562 nm using a spectrophotometer (SpectraMax 190, Molecular Devices) and data was expressed as a percentage of the absorbance in untreated control cells.

2.4 Measurement of nuclear Nrf2 accumulation and DNA binding

To investigate the effect of TGF-β1 on the transcription factor, Nrf2, and its nuclear accumulation in HAoAF and the Nrf2 mediated antioxidant gene activation was determined by western blotting, immunofluorescence (He et al., 2009; Churchman et al., 2009) and a DNA binding ELISA (TransAM, Activ Motif, USA).

2.4.1 DNA binding ELISA for Nrf-2-DNA binding activity

The capacity of nuclear Nrf2 to bind ARE sequences was quantified using a DNA binding ELISA kit (TransAM, Active Motif, USA) in order to detect and quantify Nrf2 activation. Oligonucleotides containing the ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG -3') immobilized on an ELISA plate bind the active form of Nrf2 contained in the nuclear extracts. Following incubation of the nuclear extracts with the immobilized oligonucleotide, a primary antibody used to detect Nrf2 upon DNA binding is added to the wells followed by the addition of an HRP-conjugated secondary antibody. The result is colorimetric change that can be quantified by spectrophotometry at 450 nm (Spectramax 190, Molecular devices).

HAoAF nuclear extracts were thawed at room temperature and subsequently placed on ice. As recommended by the manufacturers protocol, 7.5 µg of nuclear extract diluted in complete lysis buffer (provided by the kit) to give a total volume of 10 µl was added to the ELISA plate in duplicate followed by the addition of 40 µl complete binding buffer. In addition, 10 µl complete lysis buffer as blank and 7.5 µg of Nrf2-transfected COS (**C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material) nuclear extract (provided by the kit) and used as a positive control were also assessed in addition to HAoAF nuclear extracts.

After incubation of nuclear extracts with immobilized oligonucleotides at room temperature for 1 h with mild agitation, the wells were washed twice with washing buffer (provided with the kit) and incubated with Nrf2 specific primary antibody (1:1000 diluted in antibody binding buffer, 100 µl/well) at room temperature without agitation. HRP-conjugated secondary antibody (1:1000 diluted in antibody binding buffer, 100 µl/well) was then added to the wells and incubated for a further 1 h. Subsequent to antibody incubations, wells were washed twice using buffer and incubated with developing solution (provided with the kit, 100 µl/well) for ~15 min in the dark until a colour change from yellow to medium-dark blue was observed. A stop

solution (provided with the kit, 100 µl/well) was then immediately added to the wells in order to prevent further colour development. The absorbance was measured at 450 nm using a spectrophotometer (Spectramax 190, Molecular devices) within 5 min of the addition of the stop solution with a reference reading taken at 655 nm. All readings were corrected for background.

2.4.2 Immunofluorescent staining of Nrf2 in HAoAF

To evaluate Nrf2 nuclear translocation, cells were incubated with TGF-β1 (5 ng/ml) or SFN (5 µM) for 1, 2 or 4 h, washed twice with PBS, and fixed with 4% formaldehyde for 30 min at room temperature. Cells were left in permeabilization solution (0.1% Triton X-100 in PBS) for 10 min followed by antibody blocking solution (3% BSA in PBS) for 30 min, and subsequent overnight incubation with primary antibody anti-Nrf2 (rabbit, 1:400; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted in PBS (1% BSA) at 4 °C. After being washed with PBS, the cells were incubated with Alexa 488-conjugated secondary antibody (goat anti-rabbit IgG–488; 1:200; Invitrogen, Paisley, UK) for 2 h at room temperature and then washed twice with PBS. In order to viualise F-actin, cells were further incubated with rhodamine phalloidin conjugated to tetramethylrhodamine (TRITC, 200 nM) diluted in PBS for 1 h at room temperature. Coverslips were mounted upside down onto SuperFrost® microscope slides (Menzel Glaser, Braunschweig, Germany) using Vectasheild® Mounting Medium containing 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI) for nuclear staining. Coverslips were viewed using a fluorescent inverted microscope (Nikon Diaphot adapted for fluorescence, Japan) fitted with appropriate filters and linked to image processing software (Hamamatsu HC Image Live v1.2 , Japan).

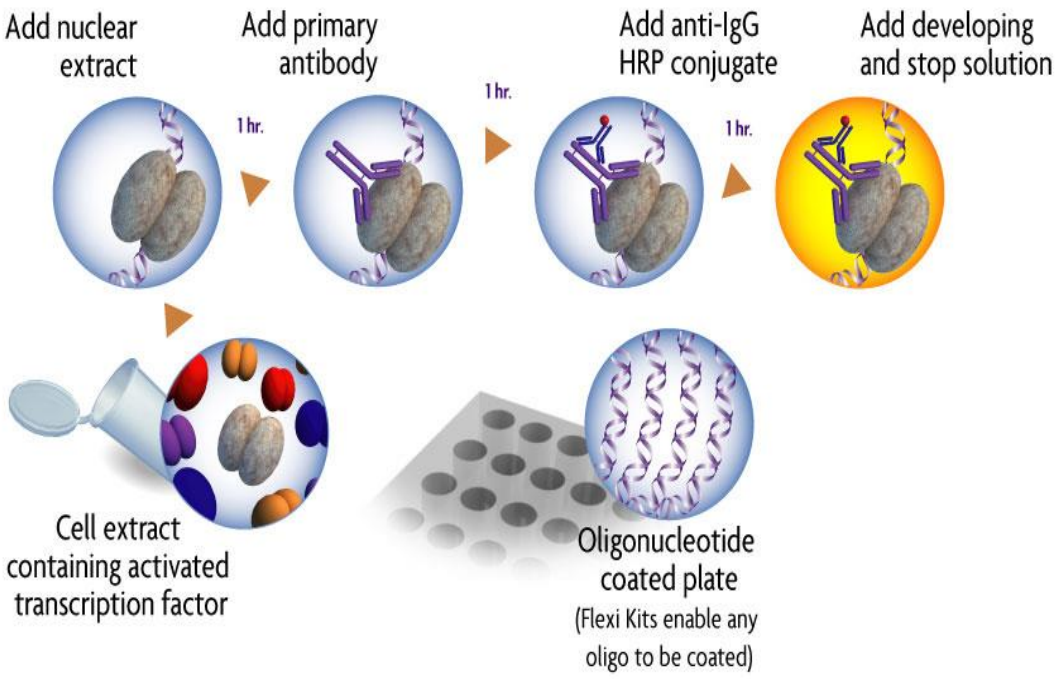


Figure 2.3 DNA binding ELISA assay for the determination of nuclear Nrf2 activity
Nuclear lysates containing activated Nrf2 from HAoAF were incubated with the ELISA plate coated with immobilized oligonucleotides containing an ARE sequence. Following incubation, primary antibody which recognises epitopes on Nrf2 upon DNA binding was added to the wells followed by the addition of HRP conjugated secondary antibody. The colorimetric readout was detected at 450 nm using a spectrophotometer and indicated the levels of active Nrf2 in the nuclear extracts (schematic taken from manufacturer's manual).

2.5 Expression of marker proteins to characterise HAoAF phenotype

2.5.1 Immunfluorescent staining of human aortic adventitial fibroblasts

Cultures of human aortic adventitial fibroblasts were stained for α- smooth muscle actin (Santa Cruz) and vimentin (Abcam) in order to determine their phenotype. Immunofluorescent staining was performed following treatment of cells with control (DMEM supplemented with 1% FCS) or TGF-β1 (2.5 – 5 ng/ml, 0-24 h). Cells were cultured on glass coverslips (VWR International) in 6 well plates. Cells were adapted in DMEM supplemented with 1% FCS for 18-24 h prior to commencement of treatment(s). Following treatment with TGF-β1 for appropriate time period, treatments were aspirated and cells were gently washed twice with PBS (37°C) and fixed with 4% formaldehyde in PBS (1 ml/well) for 30 min at room temperature. Following formaldehyde fixation, cells were gently washed again twice with PBS and permeabilised with Triton X-100 (0.1 % in PBS) for 10 min at room temperature. In order to minimise non-specific primary antibody binding, cells were incubated with blocking buffer containing 3% BSA in PBS for 30 min at room temperature before being incubated with appropriate primary antibody in 1% BSA for 4 h at room temperature. Following incubation of cells with primary antibody (α- smooth muscle actin or vimentin), cells were incubated with an Alexafluor 488 labelled secondary antibody (1:400, ex/em 495/519 nm, Invitrogen) for 2 h at room temperature in the dark and subsequently washed. To stain for F-actin fibres, cells were stained with rhodamine phalloidin conjugated to tetramethylrhodamine (TRITC, 200 nM) (200 nm, Sigma Aldrich) diluted in PBS for 1 h at room temperature. Coverslips were mounted upside down onto SuperFrost® microscope slides (Menzel Glaser, Braunschweig, Germany) using Vectasheild® Mounting Medium containing 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI) for nuclear staining. Coverslips were viewed using a fluorescent inverted microscope (Nikon Diaphot adapted for fluorescence, Japan) fitted with appropriate filters and linked to image processing software (Hamamatsu HC Image Live v1.2 , Japan).

2.6 Determination of intracellular glutathione

2.6.1 Cell extraction for glutathione assay

After incubation with treatments (see section 2.1.3), medium was aspirated and cells were washed twice with ice-cold PBS and incubated with 400 µl/well of 6.5% trichloroacetic acid (TCA) to lyse cells. The plates were then left on ice and gently agitated occasionally to ensure even coverage of the cells with 6.5% TCA. After 10 min, supernatant containing GSH was collected and transferred for storage at -80°C for further analyses. The remaining cells in the well plate were solubilised by adding 300 µl of 0.5 M NaOH to each of the wells and left on the bench for 2-4 h before storage at -20°C until further required for analysis of protein content. Protein concentrations were determined using the BCA assay (Pierce) as previously described in section 2.3.1 to correlate with the GSH levels.

2.6.2 Determination of intracellular reduced glutathione by fluorometric assay

Intracellular glutathione levels were measured as originally described by Hissin & Hilf (1976) and as modified by Senft *et al.*, (2000). Samples stored at -80°C, previously extracted as described above, were defrosted on ice for determination of glutathione (GSH) content using a fluorometric assay. Conjugation of total GSH to a fluorophore reagent O-Phthaldialdehyde (OPA) leads to the formation of a highly fluorescent isoindole (Senft et al., 2000).

Microcentrifuge tubes containing the extracted sample in 6.5% TCA or GSH standards prepared in 6.5% TCA (0-30 nmol) were vortex mixed and 7.5 µl each added to 277.5 µl of phosphate-EDTA (0.1 mol^{-1} KH_2PO_4, 5 $nmol^{-1}$ EDTA, pH>8) in triplicate to a black 96 well plate. This was followed by the addition of 15 µl of freshly prepared 0.1% w/v *o*-Phthaldialdehyde (OPA, Sigma-Aldrich, UK) in methanol which fluoresces upon reacting with the GSH in the cell extracts. The plate was incubated in the dark for 25 min at room temperature and fluorescence measured at 350 nm excitation and 420 nm emission in a microtitre plate reader (Hidex Chameleon V). Glutathione concentrations in each sample were determined by correlating with the glutathione standard curve obtained (Fig 2.4) and normalised for protein content in each well to express glutathione concentration as nmol/mg protein.

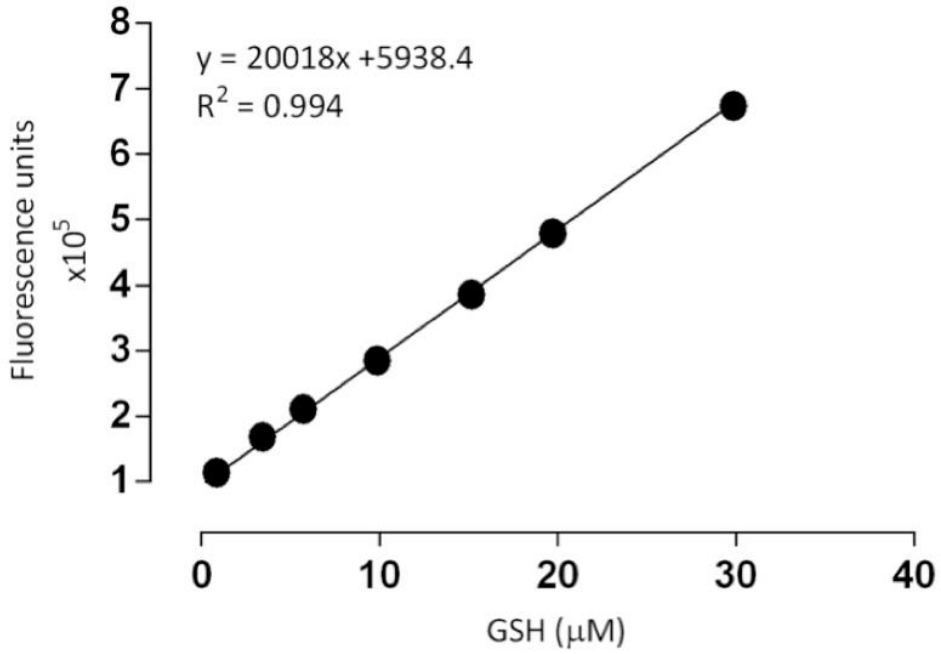


Figure 2.4 Representative GSH standard curve
Fluorescence readings of the GSH standards were expressed relative to their known concentrations (0-30 μM) to generate a standard curve. The equation obtained from the standard curve was then used to calculate the concentration of GSH in samples read on the same plate with the standards. Only the standard curves with $R^2 > 0.95$ were used for experiments.

2.7 Reactive oxygen species detection

2.7.1 Determination of reactive oxygen species generation using luminescence

Detection of reactive oxygen species (ROS) generation in live cells was carried out using the luminol analogue, LO12 (20 μM, Wako Chemicals, USA), which has been shown to detect extracellular superoxide generation (Daiber et al., 2004). Cells were seeded in sterile white 96 well plates with a clear-bottomed base and equilibrated in phenol red-free DMEM supplemented with 1% FCS 18-24 h prior to treatment. All treatments were in quadruplicate and were diluted in Krebs buffer (131 mM NaCl, 5.6 mM KCl, 25mM $NaHCO_3$, 1mM $NaH_2PO_4.H_2O$, 2.5mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, 5mM glucose and 100uM L-arginine) and the plate was read in a luminescence plate reader (Hidex Chameleon V) for 1 sec/well over 40 min at 37°C. For long-term treatments, cells were incubated with treatments diluted in phenol red-free DMEM supplemented with 1% FCS and after the appropriate incubation times, the treatments were removed and replaced with the same treatment conditions diluted in Krebs buffer and subsequently luminescence was measured over 40 min. Luminescence was determined as mean light units (MLU) and were indicative of superoxide generation. After readings were obtained, the Krebs buffer was removed from the plate and replaced with 50 μl NaOH (0.5 M) overnight at room temperature for the determination of protein (μg) content per well. The BCA protein assay was performed (see section 2.3.1) and chemiluminescence readings were corrected for protein content of each well. An average of the MLU for each condition was taken in order to determine the changes in superoxide in response to various treatments.

2.8 Transfection of HAoAF with Nrf2 siRNA

In order to determine whether the transcription factor, Nrf2 is responsible for the upregulation of specific antioxidant proteins in response to various treatments, cells were transfected with short interfering (siRNA) targeted against Nrf2 (Piao et al., 2012) and then challenged with either TGF-β1 or SFN. Short RNAs area group of intracellular regulatory RNAs (usually <30 bp) including siRNA, micro interfering (miRNA) and repeat associated short interfering (rasiRNA) (Hannon and Rossi, 2004; Meister and Tuschl, 2004). The mechanism of small RNA interference is depicted in Fig 2.6. once delivered into mammalian cells, double strand short RNA templates undergo several maturation steps to form a single strand short RNA. This single strand short RNA is complementary to the target mRNA and its binding initiates the degradation of the target mRNA, mediated by the RNA-induced silencing complex (RISC) (Meister and Hannon, 2004). Specific gene silencing provides an important method to investigate the downstream effects of the protein of interest. In the current study, comparison of antioxidant responses in HAoAF to TGF-β1 or SFN were determined in cells transfected with siRNA targeted against Nrf2. Whole cell protein lysates were then collected and protein expression determined using SDS-PAGE and western blot analysis.

Cells were seeded at a density of 50,000 cells per well in a 24-well plate and were maintained in phenol red-free DMEM supplemented with 10 % FCS. Cells were allowed to adhere for 24 h and were subsequently transfected with siRNA. 2 µl of DharmaFECT4® (Dharmacon, Thermo Scientific, UK) was diluted into 498 µl of serum-free Optim-MEM® (Roche). Scrambled and Nrf2 siRNA was also prepared by diluting 8 µl scrambled siRNA or 8 µl Nrf2 siRNA with 100 µl Optim-MEM® to give a final concentration of 4 picomoles/well. The transfection reagent was then added to the scrambled and Nrf2 siRNA tubes and incubated for 20 min to allow the siRNA to form a complex with the transfection reagent. The siRNA:transfection complex was then added drop-wise to wells containing phenol red-free DMEM supplemented with 10% FCS and the plate was gently rotated to ensure equal distribution of siRNA over the centre surface of the well. Eight hours following transfection, the transfection mixture was aspirated from wells and replaced with DMEM supplemented with 1% FCS for overnight equilibration. Cells were then treated with TGF-β1 or SFN for 8 h. Control wells contained only DMEM supplemented with 1% FCS. Following incubation with appropriate treatment, culture medium was aspirated and cells were gently washed twice

with ice cold PBS (4°C). Cells were lysed in 75 µl SDS-lysis buffer and the collected sample was boiled at 95°C for 5 min. Samples were stored at -20°C until required for analysis.

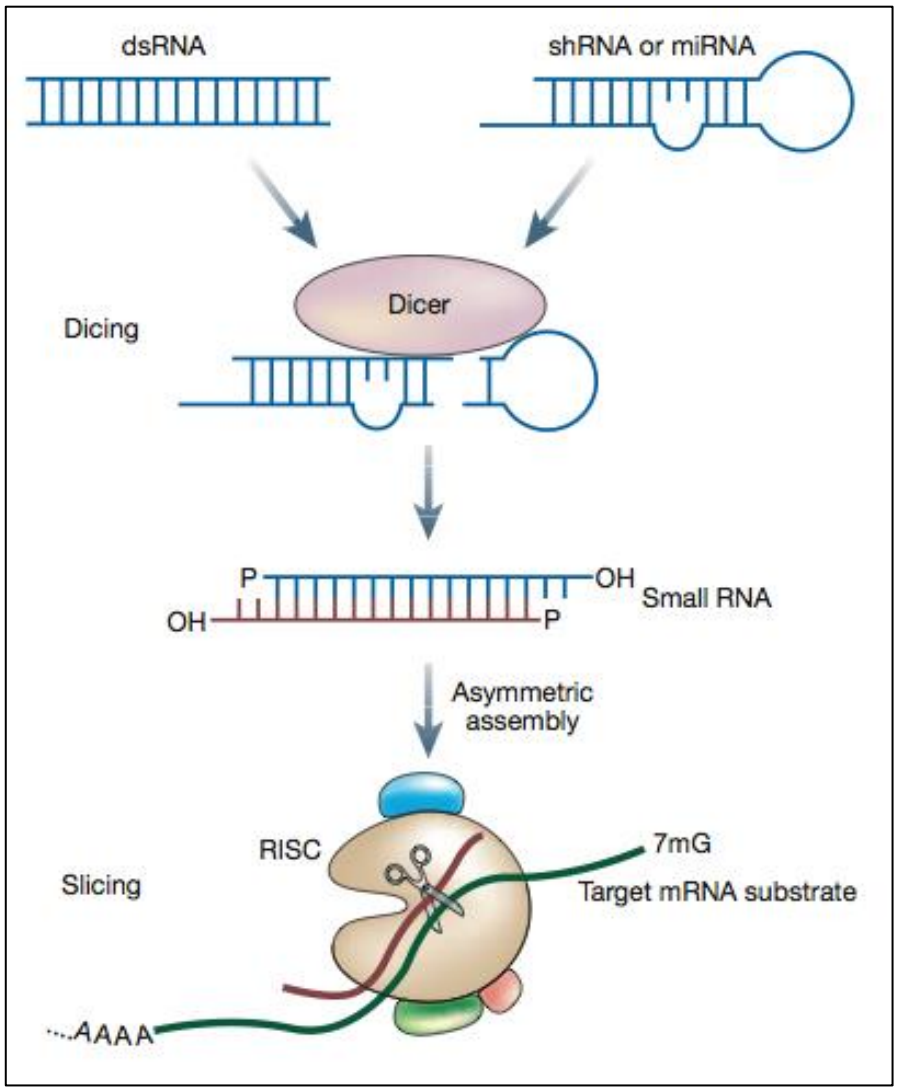


Figure 2.5 Gene silencing by short RNA in mammalian cells
Exogenous short RNAs such as dsRNA (siRNA) and short hairpin RNA (shRNA) and endogenous miRNA in mammalian cells undergo the same maturation process to a single strand small RNAwhich is complementary to the target mRNA. The binding of small RNA to the target mRNA initiates its degradation mediated by RNA-induced silencing complex (RISC). Figure adapted from Hannon and Rossi, 2004.

2.9 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for detection of mRNA and miRNA levels in HAoAF

Levels of messenger RNA and microRNA of genes of interest in HAoAF were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and mRNA and miRNA was extracted using the Qiagen miRNeasy spin column kit and the RNeasy Plus mini kit respectively, following the manufacturer's protocol. All experimental procedures were carried out using DNase and RNase-free consumables.

2.9.1 Extraction and purification of total RNA

Total RNA was extracted from cells using the miRNeasy spin column kit (Qiagen) following the manufacturers protocol. The kit provided all materials required for the isolation of total RNA <200 nucleotides using spin columns and their patented phenol-guanidine-based QIAzol Lysis Reagent. All experimental procedures were carried out using DNase and RNase-free consumables. Briefly, after adapting cells to DMEM supplemented with 1% FCS for 18-24 h, confluent HAoAF were treated with either TGF-β1 (0-5ng/ml) or SFN (0-5 μM) for 4 or 8 h. Following incubation, cells were washed with ice-cold PBS twice prior to the addition of QiaZol lysis reagent (300 μl/well for 6 well plate) and incubated on ice for 10 min before being transferred to RNase-free microcentrifuge tubes for homogenizing by vortex-mixing. The homogenate was then incubated at room temperature (15-25°C) for 5 min followed by the addition of 140 μl chloroform (Sigma) after which it was shaken vigorously for 15 s and incubated at room temperature for 2-3 min. The homogenate was then spun down and the resulting precipitated RNA was sequentially purified and eluted using the spin column procedure where the sample was loaded onto the spin column and spun down followed by several wash steps resulting in the adsorption of the RNA onto the silica gel membrane. Total RNA was then eluted in nuclease-free water and RNA concentration was determined by using a spectrophotometer.

2.9.2 Extraction and purification of microRNA

Total miRNA was purified from total RNA in cells using the RNeasy Plus mini kit (Qiagen) following the manufacturers protocol. The kit provided all materials for the isolation of small RNA (containing miRNA, ~22 nucelotides in length) using gDNA Eliminator spin columns. All experimental procedures were carried out using DNase and RNase-free consumables. Briefly, after adapting cells to DMEM supplemented with

1% FCS for 18-24 h, confluent HAoAF were incubated with either DMEM supplemented with 1% FCS (control), TGF-β1 (5ng/ml), HNE (10 μM) or glucose oxidase (2.5 U/ml), for 6 h. Following incubation, cells were washed with ice-cold PBS twice prior to the addition of Buffer RLT Plus (350 μl/well for 6 well plate) before disrupting and homogenizing the sample. The homogenate transferred to a gDNA Eliminator spin column and centrifuged for 30 s at ≥10,000 rpm. 1.5 volumes of 70% ethanol was added to the flow-through and mixed thoroughly by vortexing. The sample, including any precipitate that may have formed, was then transferred to an RNeasy spin column and centrifuged for 15 s at ≥10,000 resulting in the adsorption of the RNA onto the silica gel membrane. 0.65 volumes of 100% ethanol was added to the flow-through and mixed thoroughly by vortexing before transferring to a RNeasy MiniElute spin column and centrifuging for 15 s at ≥10,000 rpm. Following a wash step, 500 μl of 80% ethanol was added to the RNeasy MiniElute spin column and then spun for 15 s at ≥10,000 rpm. The flow-through was discarded and the RNeasy MiniElute spin column was placed in a 1.5 ml collection tube. Small RNA (containing miRNA) was then eluted in RNase-free water.

2.9.3 Measurement of RNA quality

RNA concentration in the extract (1 μl) was determined by absorbance read at 260 nm (A_{260}) using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Concentrations (ng/μl) were calculated based on the assumption that 1 unit absorbance at 260 nm represents 40 ng/μl of mRNA in the sample. RNA purity was checked by the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}). A pure RNA extraction with minimum genomic DNA contamination is indicated by a ratio of ~2. The ratio of 18S to 28S (ribosomal peaks) was used as a reference for RNA integrity. Only samples with A260/A280 > 2 were used in this study according to recent guidelines (Bustin et al., 2009).

2.9.4 Reverse Transcription

In order to perform qRT-PCR, isolated RNA was first transcribed into cDNA using the High Capacity RNA-to-cDNA Kit from Applied Biosystems according to the manufacturer's instructions. The kit provides removal of any contaminating genomic DNA as well as the achievement of a high yield of cDNA. 500 ng of extracted RNA was used for RT and added to 10 μl of RT mix. The total mixture was made up to 20 μl with RNase-free water and RT was run using a thermal cycler (Applied Biosystems)

pre-set according to manufacturers guidelines. The cycle was as follows; 37°C for 60 min, 95°C for 5 min and a subsequent hold at 4°C for 5 min. Following RT, stock concentration was then diluted 1 in 5 for qRT-PCR.

2.9.5 qRT-PCR

cDNA in the RT samples was assessed by real time quantitative polymerase chain reaction using a SyberGreen-based PCR mix (Sensi-Mix® SYBR-green no ROX, Bioline). A master mix containing forward primer, reverse primer, Quantifast SYBR green and nuclease free H_2O was first prepared on ice (see 2.9.1 composition) and added to 100 well gene discs (Qiagen) using an automated robot, CAS-1200 (Corbett Life Sciences, Australia) into tubes containing 2 µl of sample cDNA or of standard cDNA (10^{-1} - 10^{-9} copies of target genes). On each disc, 2 blank (nuclease free water only) and two RT negative samples were also assessed to check contamination of genomic DNA. Loaded discs were run for PCR amplification in a Corbett Rotorgene which determines copy numbers via relative fluorescence generated by SYBR-green following its excitation. The polymerase enzyme in the samples was activated at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s (denaturing), 57°C for 15 s (primer annealing) and at 72°C for 5 s (amplification). To assess the quality and efficiency of the PCR reaction, a melting point analysis was carried out after each run to distinguish target amplicons from PCR artefacts such as misprimed products; samples were heated from 65°C to 95°C at 1 degree/cycle. Rotorgene PC software (Corbett, UK) was used to evaluate PCR efficiency and transform the absorbance values to a logarithmic scale which allowed gene copy number in samples to be obtained, relative to the standard curve. The copy number of target genes was adjusted relative to the geometric mean of 3 stably expressed house-keeping genes: ribosomal protein L13a (RPL13A), succinate dehydrogenase unit complex A (SDHA) and TATABOX (thymine, adenine, thymine, adenine box). The stability of these genes was determined as an M value using GeNorm Excel software (http://medgen.ugen.be/ivdesomp/genorm/), which analyses the variation in the expression of a reference gene compared with that of other reference genes (Vandesomplele et al., 2002). The M value is inversely proportional to the variation and any reference gene with an M value $\leq$ 1.5 was rejected for use for the pairing analysis, with the final combination of 3 reference genes having the lowest M value indicating their relative stability in this cell system. A normalisation factor for each sample was then calculated by the software based on the geometric mean of these reference genes, the expression of target genes (copy numbers) in each sample was then

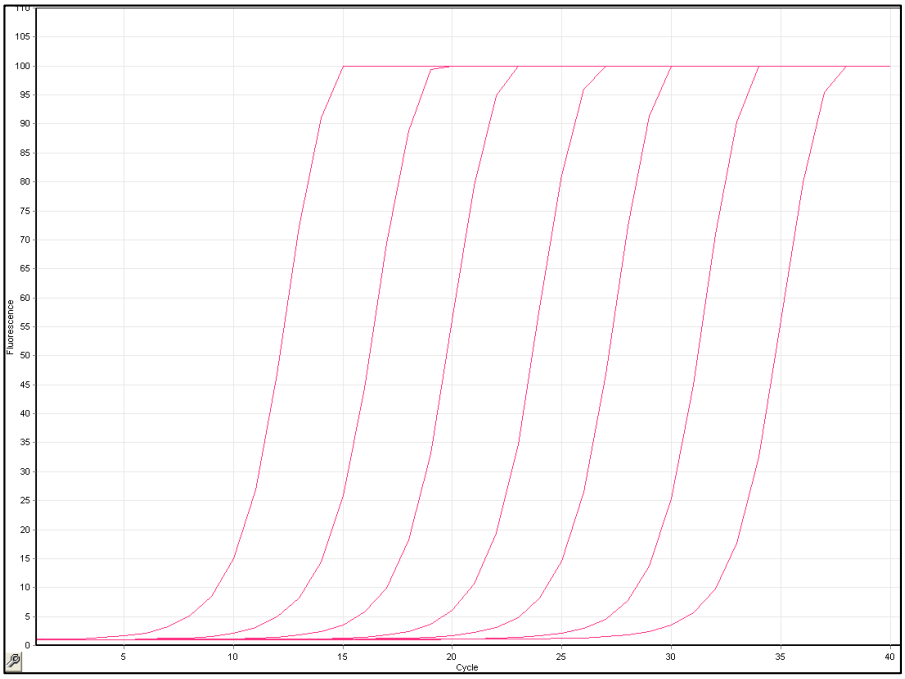
corrected by the corresponding normalisation factor using the Rotorgene program to minimize non-biological variations between the samples (Vandesompele et al., 2002; Dikalov et al., 2007).

Reagent	Volume (µl)
Forward primer	0.4
Reverse primer	0.4
SYBR-Green	5
H_2O	3.6
cDNA	1
Total	10

Table 2.4 Relative components of mastermix used for RT-qPCR

During PCR, the number of cycles required to enter the exponential phase of the PCR is dependent upon the initial amount of target sequence at the beginning of the reaction and shifts in PCR curves are expressed as the differences between the crossing thresholds (CT) between each sample (Guenin et al., 2008). Following the assessment of PCR efficiency (Fig 2.5), which is specific for each primer pair and therefore indicative of the suitability of the primers chosen, the difference between the expression of a target gene between two samples can be calculated and then normalized to a reference gene.

A



B

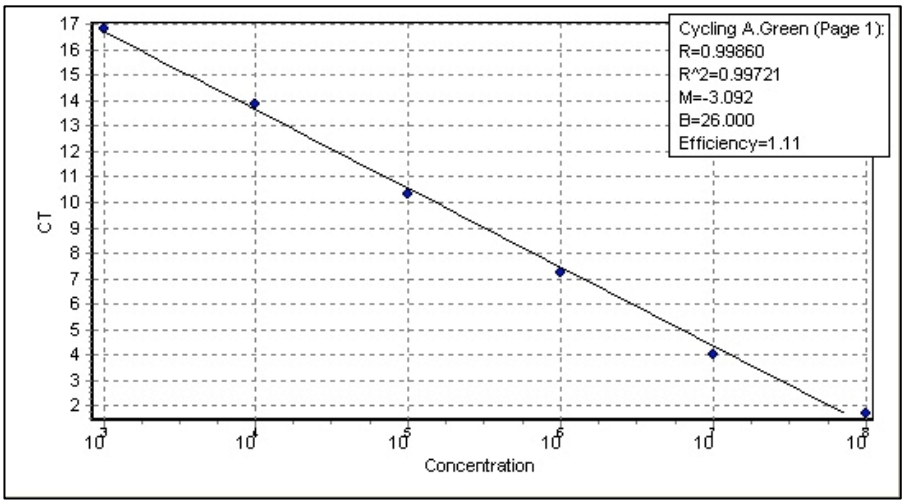


Figure 2.6 Representative qPCR standards to determine gene copies in samples
(A) Logarithmic-transformed amplification profile of a typical standard curve, demonstrating that gene copy number is inversely proportional to the point at which the amplification plot crosses the cycle number threshold. (B) Logarithmic-transformation of the standard curve plotting log concentration against the cycle number in which the amplification plot (A) crosses the threshold. Linear regression analysis shows the correlation coefficient (R^2) and the efficiency of the reaction, which gives a measure of both the accuracy of the qPCR step and the suitability of the primers chosen.

2.10 Time-lapse Video Microscopy

Time-lapse video microscopy was performed in cells cultured in 12 well plates. Cells were seeded in phenol red-free DMEM supplemented with 10% FCS at a density of 30,000 cells/well and allowed to adhere for 24 h. Culture media was changed to phenol red-free DMEM supplemented with 1% FCS 18 h prior to incubation with the treatments (see Methods section 2.1.3) in DMEM supplemented with 1% FCS. Some cells were incubated with SOD (200 U/ml) for 30 min prior to the commencement of imaging. [i]Multifield live cell imaging was performed on a Zeiss Axiovert 100 microscope using an automated scanning stage. Phase contrast images were acquired by using a 10x/0.25 N-Achroplan phase contrast objective. Images were collected using a Sensicam (PCO Cook) charge coupled device (CCD) camera. A frame was captured every 10 min over 8 h using AQM acquisition software (Andor Bioimaging, Belfast, U.K.). Nuclei of migrating cells from the time-lapse sequence were tracked using ImageJ software. Movement of the nuclei was taken as an indication of the distance migrated per cell and the average of the total distance travelled per cell was calculated. All cells in a particular field were tracked and the average distance of all cells was determined in that particular field of view and taken as the average distance migrated in response to the treatment administered. Migration speed and distance was quantified by importing cell-tracking data into Excel and calculating the average distance migrated per treatment group.

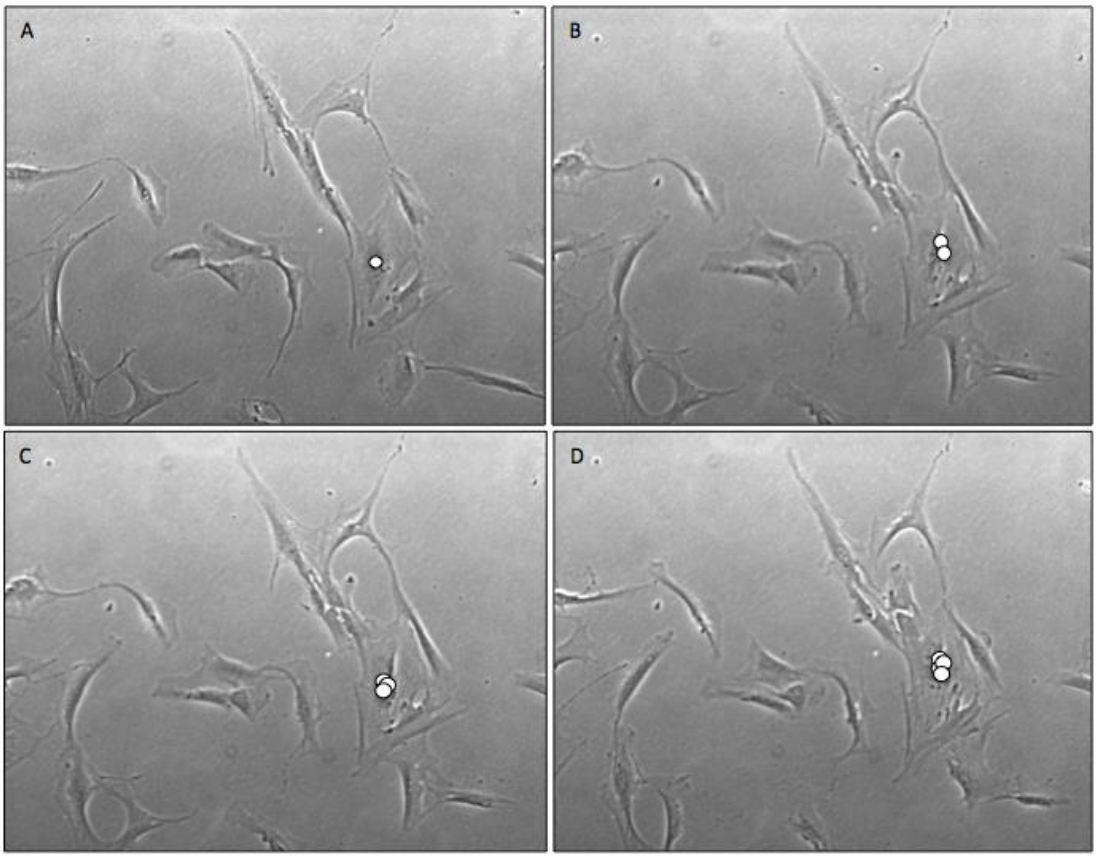


Figure 2.7 Representative image of cell tracking using ImageJ
Cells migration was analysed using ImageJ. Tracking the movement of nuclei was used as an indicator of cell migration and all cells in the field of view were tracked over 8 h. Frame (A) is the first frame taken from a single well and (B), (C) and (D) are subsequent frames over an 8 h period. Circles indicate the movement of the nucleus of a single cell being tracked. Tracking data was then imported into Excel and the total distance migrated by cells was calculated.

2.11 Statistical analysis

All data are expressed as means ± standard error of the mean (SEM) from a minimum of at least 3 different experiments, unless stated otherwise. Statistical variance from the mean was determined using the normal distribution, tested using a two-tailed students *t*-test in Microsoft Excel. Confidence limits were established using an unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with the Tukey's post hoc correction for multiple comparisons, to give a statistical significance between data sets established at $p < 0.05$ to $p < 0.001$. All results that are statistically significant are marked, and in the absence of such indication can be assumed to not be significant.

CHAPTER 3:

Results

CHAPTER 3: Phenotypic characterisation of human aortic adventitial fibroblasts

3.1 Introduction

The adventitial fibroblast, previously assumed to be an inert cell type, whose main role was thought to provide support and contribute to the structure of the outermost layer of the blood vessel, is now a known key player in the pathology of vascular diseases. First described in experimental wound healing (Gabbiani et al., 1971), fibroblast to myofibroblast differentiation is initiated during tissue injury, when the local release of cytokines, in particular, TGF-β1 (Eyden, 2004) results in the activation of fibroblasts, which, as a consequence, acquire a migrating (Coen et al., 2011) and contractile phenotype (Desmouliere et al., 2005), typically seen after stretch injury due to balloon angioplasty and during wound healing. This differentiation process is characterised by the acquisition of a contractile apparatus (Desmouliere et al., 2005; Gabbiani et al., 1971) during which the *de novo* expression of the cytoskeletal proteins, α-smooth muscle actin and vimentin, occurs (Enzerink and Vaheri, 2011; Tomasek et al., 2002). These series of events eventually lead to the synthesis of several components of ECM and the organisation of a mechanically supportive structure, which is able to produce significant contractile force (Hinz et al., 2007; Tomasek et al., 2002).

There is much evidence to implicate of TGF-β1 in the pathogenesis of fibrosis during disease and wound healing. Release of biologically active TGF-β1 enhances the synthesis of extracellular matrix (ECM) proteins, exerting its effects via the intracellular Smad proteins, which have been postulated to play an essential role in ECM protein gene expression (Dobaczewski et al., 2010) as demonstrated in a mouse model of bleomycin-induced pulmonary fibrosis, where Smad3 deficiency attenuated collagen deposition (Zhao et al., 2002). Similar results were observed in a murine model of renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction (Sato et a., 2003), where the TGF-β1/Smad3 signalling pathway was disrupted resulting in a decrease in the levels of renal fibrosis and epithelial to mesenchymal transition.

The incorporation of α-smooth muscle actin into stress fibres augments the contractile activity of fibroblastic cells and is a pivotal step in the contraction phase of connective tissue remodelling (Hinz et al., 2001); interfering with α- smooth muscle actin significantly reduces tension generated by myofibroblasts (Hinz et al., 2002).

Furthermore, in the infarct area of post-myocardial infarcted heart, there was an upregulation of α-smooth muscle actin protein expression when hearts were subjected to pressure or volume overload (Santiago et al., 2010; Borg et al., 1996).

In addition to α-smooth muscle actin, a second cytoskeletal protein is expressed by myofibroblasts. Vimentin, first described as being the only intermediate filament protein to be expressed by myofibroblasts (Schurch et al., 1984), and initially a widely used marker for the identification of myofibroblast phenotype (Coulson-Thomas et al., 2010; de Matos et al., 2010; Firth et al., 2010; Ji et al., 2010; Touhami et al., 2005; Muchaneta-Kubara et al., 1997), is expressed by mesenchymal cells (Shi et al., 1996). The primary function of vimentin is to provide structure to cells and is thought to be potentially involved in cell movement (Strauss et al., 2000). Several studies have reported an increase in the expression of vimentin in myofibroblasts (Ji et al., 2010). In tissue from patients with chronic thromboembolic pulmonary hypertension, there was an increase in vimentin in areas of fibrotic thrombus formation with resident fibroblasts expressing vimentin and exhibiting a myofibroblast phenotype (Firth et al., 2010). In addition to this, thoracic aortas transplanted into the abdominal aortas of Wistar rats in order to assess the role of adventitial fibroblasts in transplant vasculopathy, revealed adventitial fibroblast activation and differentiation into myofibroblasts was characterized by immunostaining of vimentin and α-smooth muscle actin (Ji et al., 2010). Furthermore, the treatment of pulpal fibroblasts with TGF-β1 enhanced the expression of vimentin and resulted in a contractile myofibroblast phenotype (Martinez et al., 2007). Therefore this chapter aimed to determine the phenotype of HAoAF in culture by assessing the expression of these two myofibroblast markers and the response of this cell type to exogenous TGF-β1 treatment.

TGF-β1 is known to be promitogenic in several different cell types (Bhowmick et al., 2003), it is also a potent growth inhibitor in epithelial and hematopoietic cells (Akhurst, 2002), whilst in fibroblasts it causes proliferation and migration (Strutz et al., 2001). The ability of TGF-β1 to cause proliferation and migration in some cell types and growth inhibition in others is still only partly understood; its growth arrest properties are though to be attributed to its association with a group of proteins known as cdks (Strutz et al., 2001) which associate with cell-cycle regulating cyclins. These proteins form a dimer which drives cell cycle progression and can be inhibited by the action of cdk inhibitors that can be mediated by TGF-β1. In addition to this, TGF-β1 also opposes

the effects of genes that affect vascular remodelling (Bobik, 2006), mitogenic growth factors and proinflammatory cytokines; the binding of Smad3 to the CCAT/enhancer-binding protein-β and reducing the expression of CCAT/enhancer-binding protein-δ results in the activation of vascular smooth muscle cells (VSMC; Feinberg at el., 2005). Despite evidence to suggest that TGF-β1 can act as a growth inhibitor, it is well established that its autocrine and paracrine action on VSMC during neointima formation results in their activation and proliferation (Kundi et al., 2009). At low concentrations it can stimulate proliferation of VSMC by inducing platelet-derived growth factor (PDGF, Kundi et al., 2009)

The effects of TGF-β1 on HAoAF are poorly defined in the literature, and due to the pleiotropic nature of this growth factor it is important to establish the various actions it may have on this cell type in terms of phenotype, proliferation, toxicity and activation of downstream signalling.

3.2 Morphology of HAoAF

3.2.1 Expression of α-smooth muscle actin in HAoAF

α-Smooth muscle actin is a widely used marker for the characterisation of fibroblast to myofibroblast differentiation, (Hinz et al., 2012; Dabiri et al., 2006; Cucoranu et al., 2005; Rocic and Lucchesi, 2005). In order to determine whether exposure to TGF-β1 contributes to HAoAF differentiation, HAoAF were treated with TGF-β1 (0-5 ng/ml, 12-24 h) and subsequently incubated with a primary antibody against the cytoskeletal protein α-smooth muscle actin and a secondary fluorescent antibody (see Methods section 2.5). Immunofluorescence analysis revealed that there was basal expression of α-smooth muscle actin in untreated cultured HAoAF (Fig 3.1, panel D). This expression was not significantly increased following treatment of cells with TGF-β1 for 12 or 24 h (Fig 3.1, panel E and F), indicating that these cells had undergone differentiation in culture and were exhibiting a myofibroblast phenotype.

In addition to immunofluorescence analysis, to confirm that HAoAF expressed α-smooth muscle actin, Western blot analysis of protein expression was carried out in cells treated with TGF-β1 (0-5 ng/ml,) for 4-72 h to assess the long-term effects of TGF-β1 on HAoAF phenotype. Confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF- β1. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis (see Methods section 2.2). Results revealed that there was a basal expression of α-smooth muscle actin in HAoAF and that this expression was not significantly augmented following exposure to TGF-β1 (0 – 5 ng/ml) over 72 h (Fig 3.2 A & B). This finding further confirms the results from immunofluorescence analysis and suggests that HAoAF had acquired a myofibroblast phenotype *in vitro*. In addition, cells treated with SFN exhibited no change in the expression of α-smooth muscle actin, suggesting that their phenotype did not alter in response to this isothiocyanate (Fig 3.2, C).

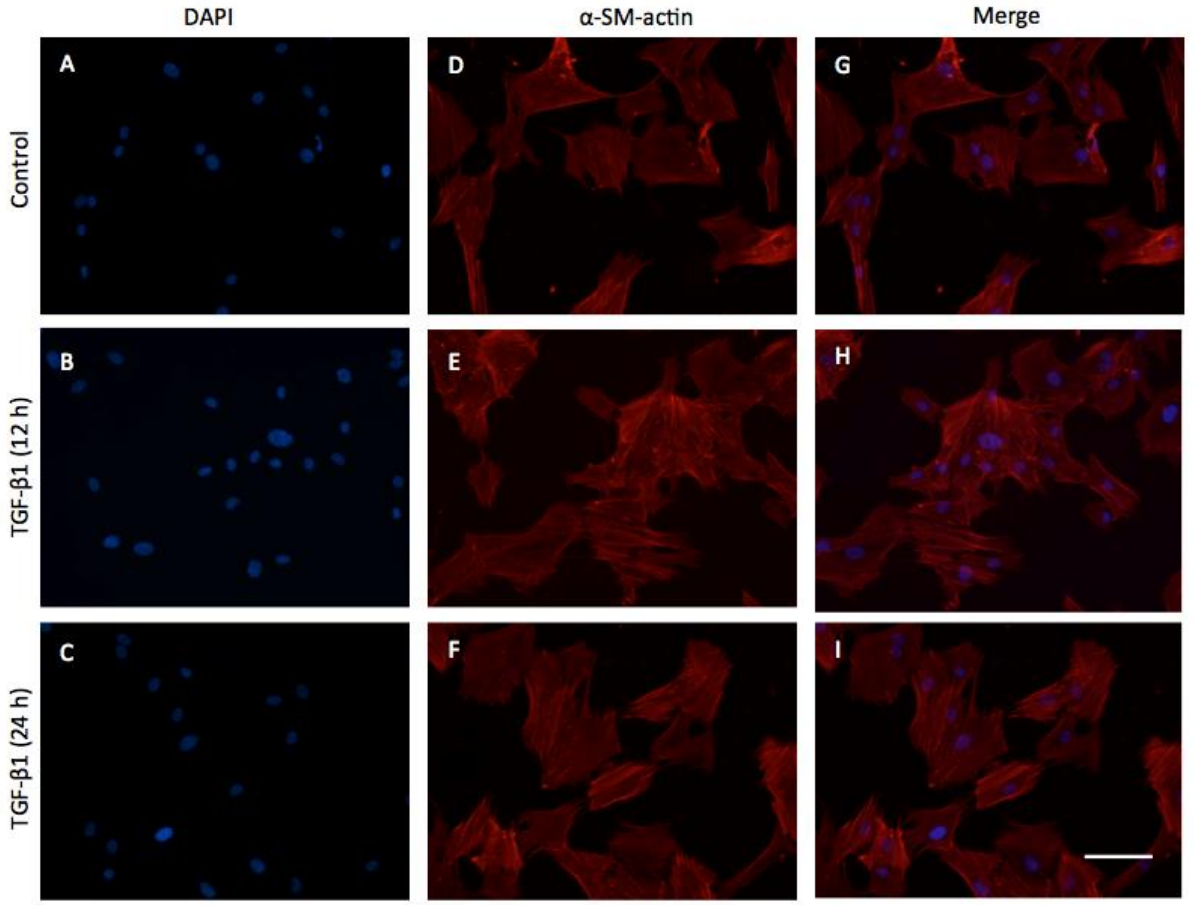


Figure 3.1 α- Smooth muscle actin staining of HAoAF treated with TGF-β1. Sub-confluent cultures of HAoAF seeded onto glass coverslips were equilibrated in phenol red-free DMEM supplemented with 1% FCS 18 h prior to incubation with TGF-β1 (5 ng/ml) for 12 or 24 h. Following incubation with treatment conditions, cells were fixed and incubated with specific antibody against α-smooth muscle actin (D-F) and an AlexaFluor-488 conjugated secondary antibody. Coverslips were mounted using Vectasheild® Mounting Medium containing DAPI for nuclear staining (A-C) and viewed using a fluorescent microscope (scale bar = μm). Representative image from 3 fields of view per treatment condition from 3 independent experiments.

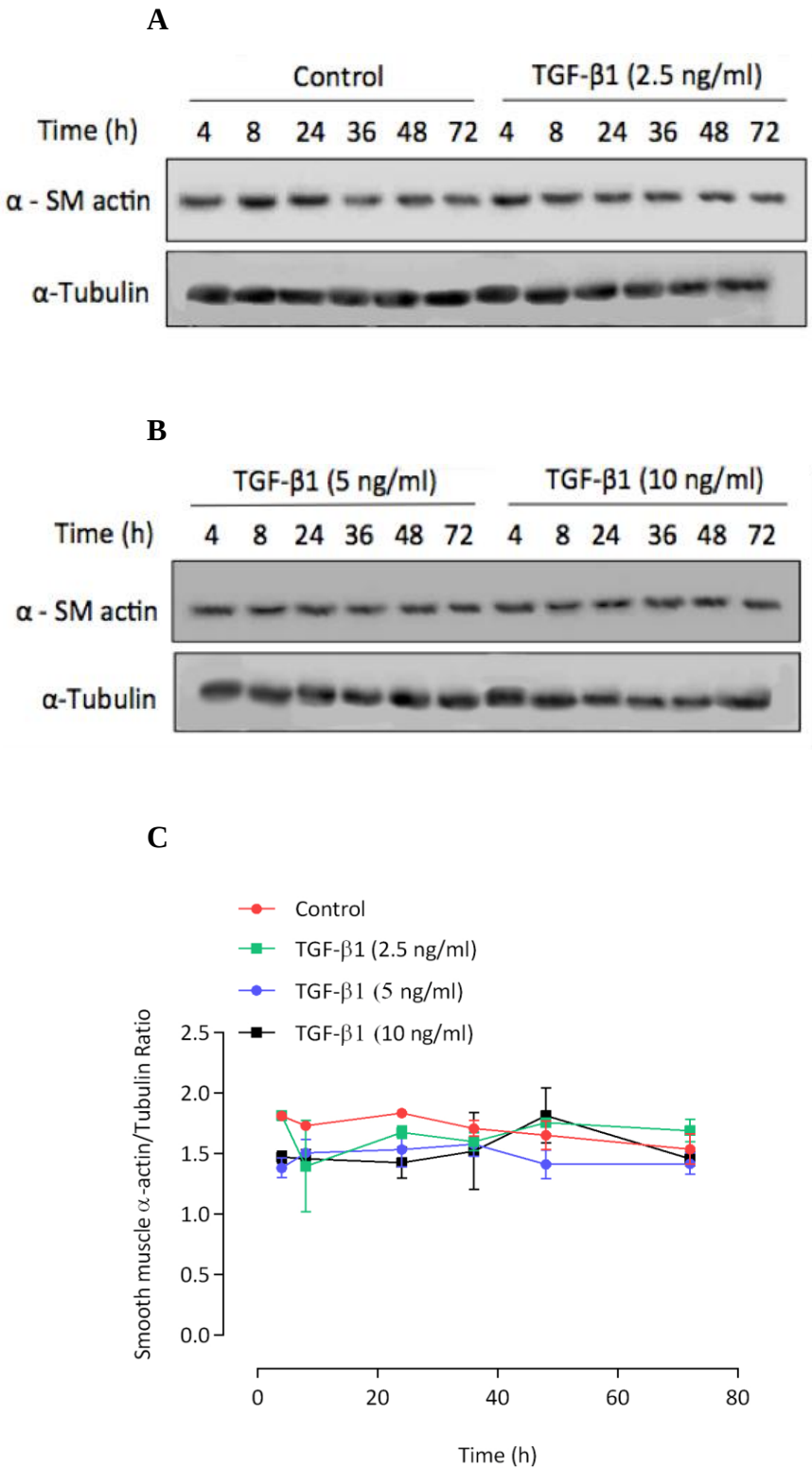


Figure 3.2 α- Smooth muscle actin protein expression in HAoAF after treatment with TGF-β1. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -10 ng/ml) for 4 - 72 h prior to whole cell protein extraction. α-Smooth muscle actin protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A and B). Results were analysed by densitometry with smooth muscle α-actin corrected for the loading control, α-Tubulin (C). Values denote means ± SEM, n = 3 independent experiments.

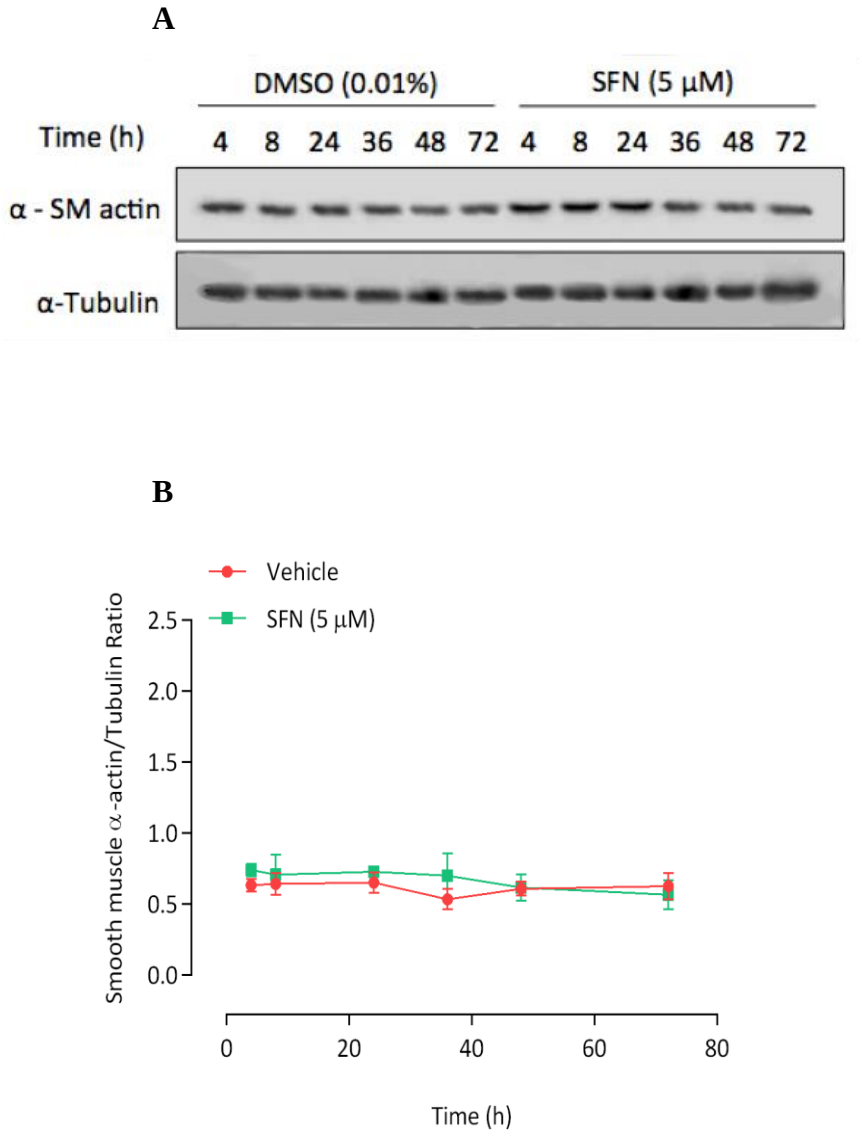


Figure 3.3 α-Smooth muscle actin protein expression in HAoAF after treatment with SFN. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle control (DMSO 0.01%) or SFN (5 µM) for 4 - 72 h prior to whole cell protein extraction. α- Smooth muscle actin protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with α- smooth muscle actin corrected for the loading control, α-Tubulin (B). Values denote means ± SEM, n = 3 independent experiments.

3.2.2 Expression of vimentin in HAoAF

Another molecular marker that is characteristic of the phenotypic switch of a fibroblast to a myofibroblast is the intermediate filament protein vimentin, the expression of which has been widely used as an index of myofibroblast differentitation (Roelofs et al., 1998; Sampson et al,. 2011). In order to determine whether exposure to TGF-β1 contributes to HAoAF differentiation, cells were treated with TGF-β1 (0-5 ng/ml, 12-24 h) and subsequently incubated with a primary antibody against vimentin and a secondary fluorescent antibody (see Methods section 2.5). Basal expression of vimentin was observed in untreated cells (Fig 3.4, panel D) and this was comparable to vimentin expression exhibited by cells treated with TGF-β1 (5 ng/ml, 12 - 24 h, Fig 3.3, panels E and F). This effect was independent of the time period of incubation with the treatment conditions, as indicated by the cytoplasmic staining of vimentin in all treatment groups (Fig 3.3). This immunofluorescence data suggests that HAoAF exhibit basal expression of vimentin and that this expression is not further enhanced or altered by treatment with TGF-β1 suggesting that HAoAF exhibited a myofibroblast phenotype at the time of experiments.

In addition, vimentin protein expression was also assessed by Western blot analysis. Cells were treated with TGF-β1 (0-5 ng/ml,) for 4-72 h to assess the long-term effects of TGF-β1 on HAoAF phenotype. Results revealed that there was a basal expression of vimentin in HAoAF in untreated control cells and that this expression was not enhanced in the presence of TGF-β1. Treatment of cells with SFN also revealed that SFN did not alter the expression of vimentin in HAoAF. These findings confirm results from immunofluorescent analysis and further indicate that HAoAF had differentiated into myofibroblasts (Fig 3.4, A & B).

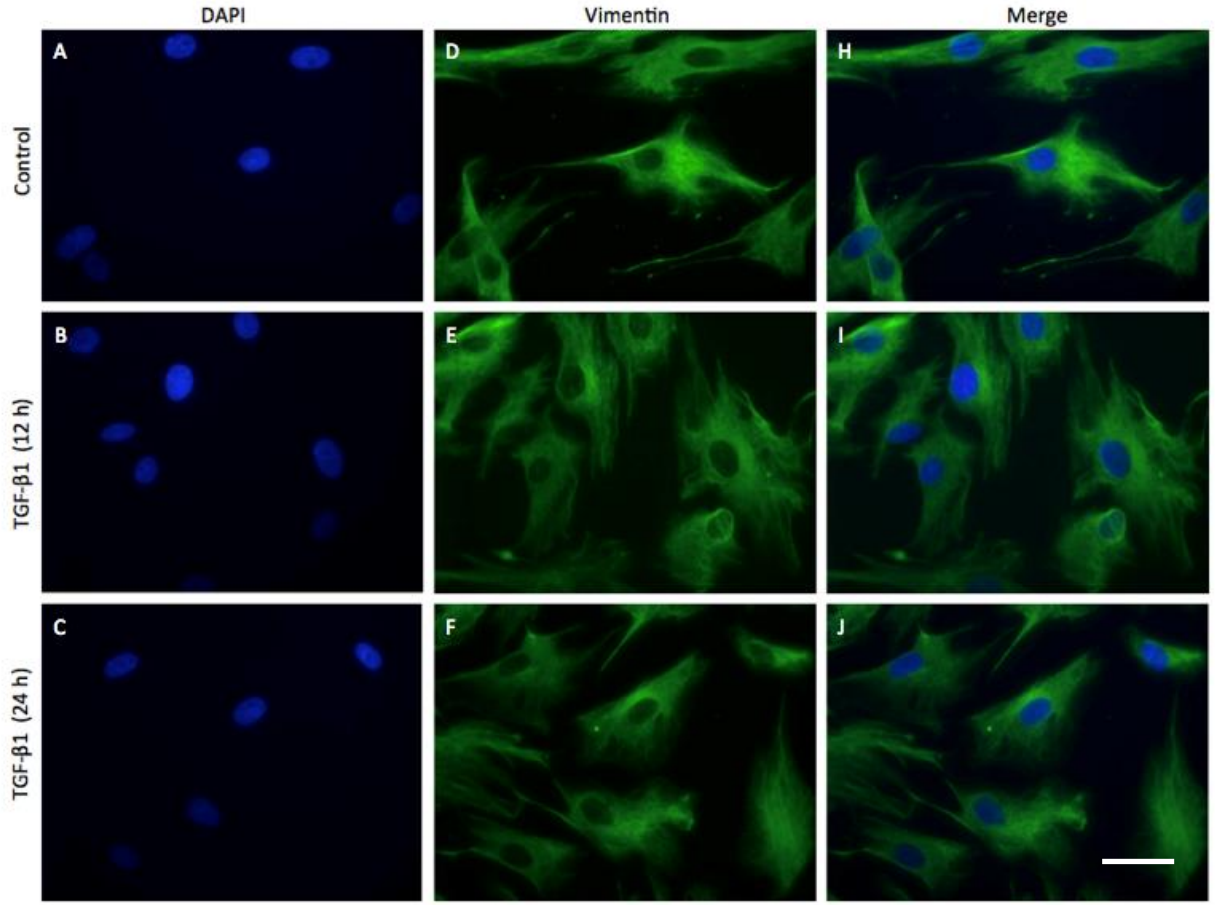


Figure 3.4 Vimentin staining of HAoAF treated with TGF-β1. Sub-confluent cultures of HAoAF seeded onto glass coverslips were equilibrated in phenol red-free DMEM supplemented with 1% FCS 18 h prior to incubation with TGF-β1 (5 ng/ml) for 12 or 24 h. Following incubation with treatment conditions, cells were fixed and incubated with specific antibody against vimentin (D-F) and an AlexaFluor-488 conjugated secondary antibody. Coverslips were mounted using Vectasheild® Mounting Medium containing DAPI for nuclear staining (A-C) and viewed using a fluorescent microscope. Representative image from 3 fields of view per treatment condition from 3 independent experiments (scale bar = 10 μm).

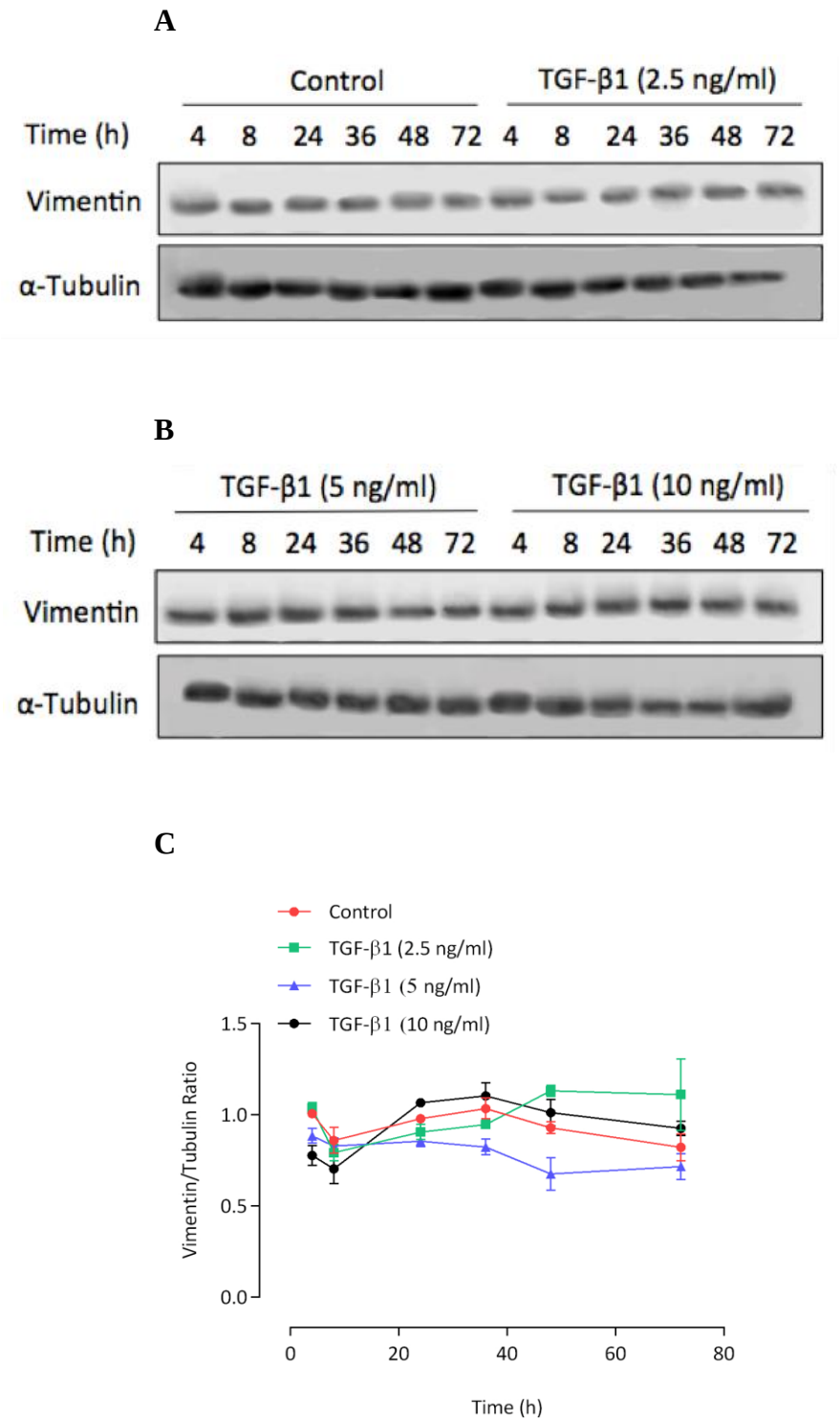


Figure 3.5 Protein expression of vimentin in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -10 ng/ml) for 4 - 72 h prior to whole cell protein extraction. Vimentin protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A and B). Results were analysed by densitometry with vimentin corrected for the loading control, α-Tubulin (C). Values denote means ± SEM, n = 3 independent experiments.

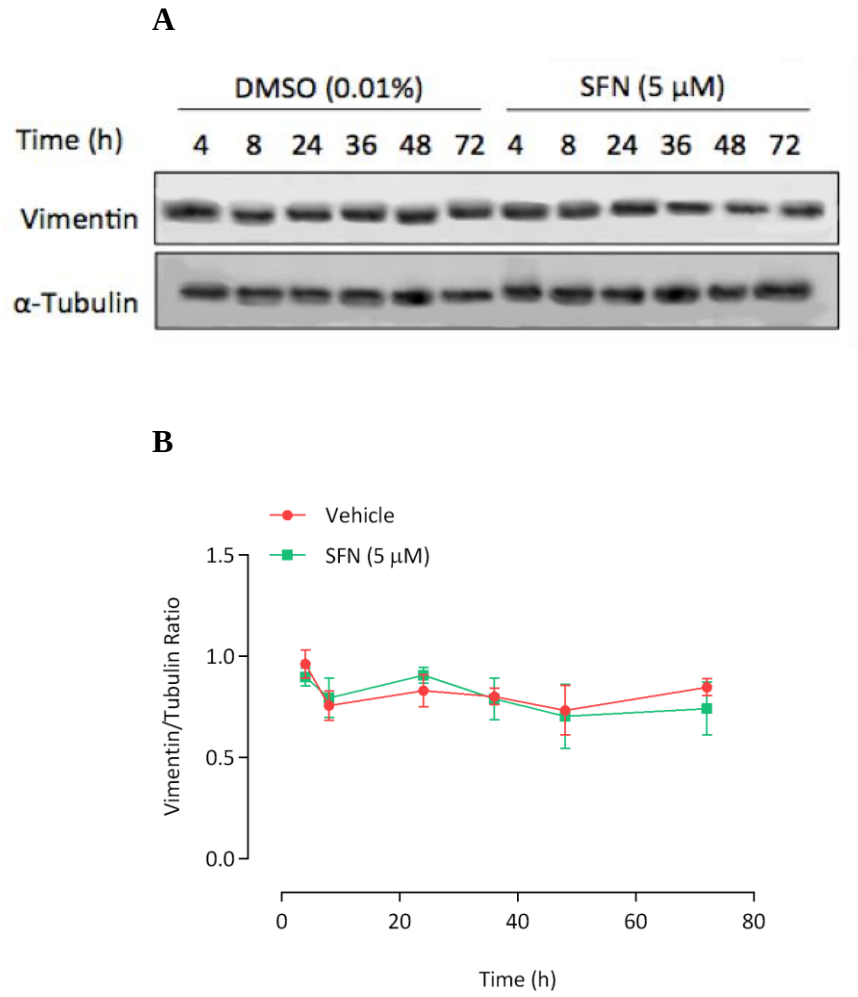


Figure 3.6 Protein expression of vimentin in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle control (DMSO 0.01%) or SFN (5 µM) for 4 - 72 h prior to whole cell protein extraction. Vimentin protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with vimentin corrected for the loading control, α-Tubulin (B). Values denote means ± SEM, n = 3 independent experiments.

3.3 Effect of SOD on expression of α-smooth muscle actin and vimentin in HAoAF

In order to determine whether the differentiation of HAoAF was dependent upon ROS, in particular, $O_2^{-\cdot}$ (Vozenin-Brotons et al., 2001), HAoAF were treated with TGF-β1 in the absence or presence of superoxide dismutase (SOD). Confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (0 - 5 ng/ml) in the absence or presence of SOD (200 U/ml), for 4 – 72 h. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis (see Methods section 2.2).

Results revealed that basal expression of vimentin and α-smooth muscle actin in HAoAF was not affected in the presence of SOD. Exposure of cells to TGF-β1 (0 - 5 ng/ml, 4-72 h) did not alter their phenotype and no differences were observed when cells were incubated with SOD. These results suggest that the differentiation of HAoAF to myofibroblasts may not be altered by scavenging $O_2^{-\cdot}$ which further suggests that cells are already terminally differentiated myofibroblasts.

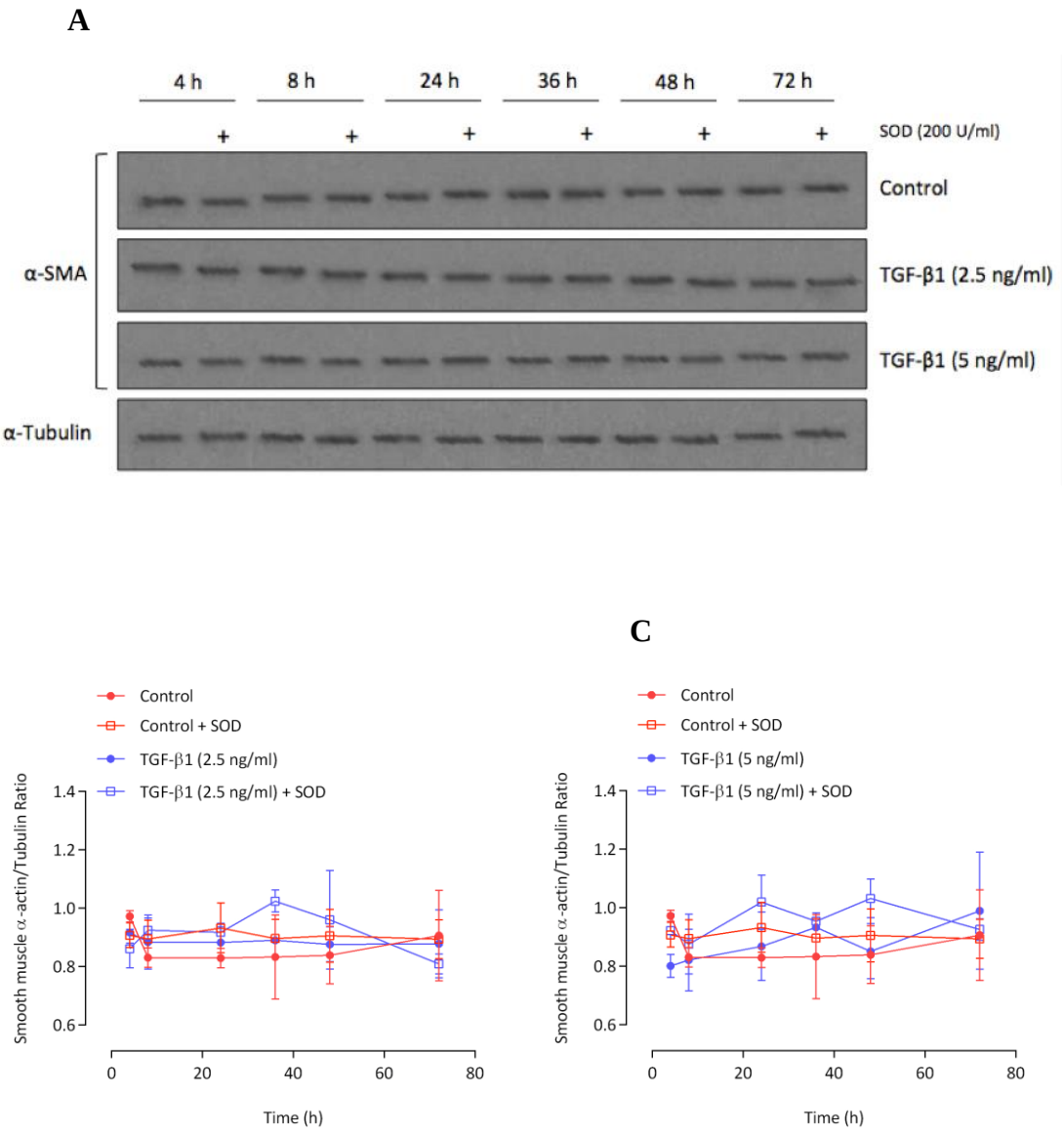


Figure 3.7 α- Smooth muscle actin protein expression in HAoAF after treatment with TGF-β1 and SOD. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) in the absence or presence of SOD (200 U/ml) for 4 - 72 h prior to whole cell protein extraction. α- Smooth muscle actin protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with α- smooth muscle actin corrected for the loading control, α-Tubulin (B and C). Values denote means ± SEM, n= 3 independent experiments.

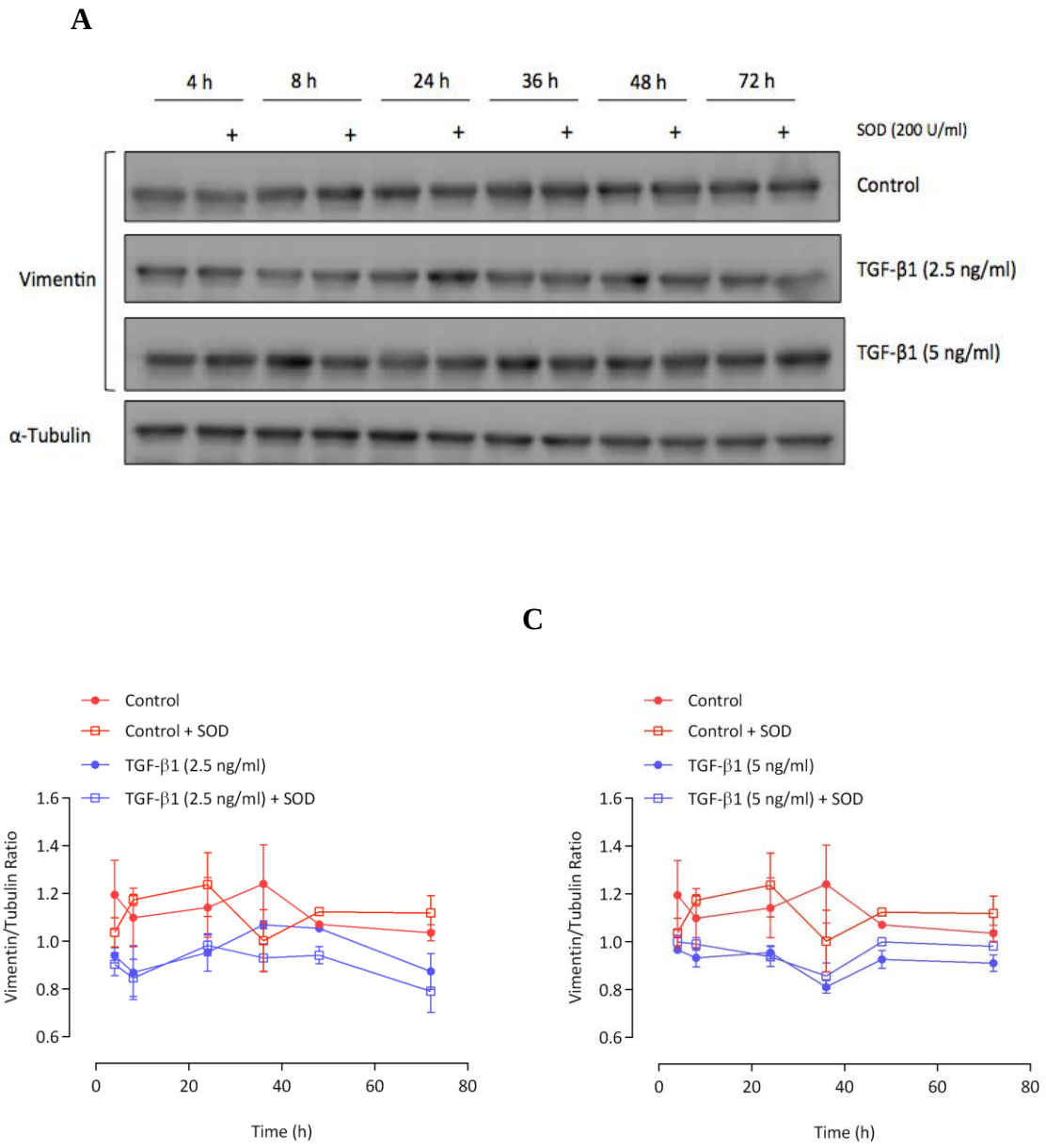


Figure 3.8 Vimentin protein expression in HAoAF after treatment with TGF-β1 and SOD
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) in the absence or presence of SOD (200 U/ml) for 4 - 72 h prior to whole cell protein extraction. Vimentin expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with α- smooth muscle actin corrected for the loading control, α-Tubulin (B and C). Values denote means ± SEM, n= 3 independent experiments.

3.4 Effect of TGF-β1 on mitochondrial dehydrogenase activity in HAoAF

Cell viability was assessed by measuring mitochondrial dehydrogenase activity using the MTT assay (see Methods section 2.3), a widely used method to assess cytotoxicity in cultured cells (Fotakis & Timbrell, 2006; Bjorkerud & Bjorkerud, 1996). HAoAFs were seeded at a density $1x10^3$ cells per well in a 96 well plate in DMEM supplemented with 10% FCS. Once confluent and 18 h prior to incubation with treatment conditions, cells were equilibrated in DMEM supplemented with 1 % FCS and subsequently treated with TGF-β1 (2.5 – 10 ng/ml), vehicle control (DMSO 0.01%), SFN (2.5 -10 μM) or H_2O_2 (50 – 100 μM). Cells treated with TGF-β1 exhibited no significant change in mitochondrial dehydrogenase activity when compared to untreated control (Fig 3.7, A), indicating that TGF-β1 has no effect on HAoAF mitochondrial dehydrogenase activity. H_2O_2, which was used as a positive index of MTT formazan development; cells treated with 50 and 100 μM exhibited a 93% decrease in mitochondrial dehydrogenase activity, suggesting that H_2O_2 caused a significant decrease in the viability of HAoAF.

In addition to TGF-β1, cells were also treated with SFN. Following treatment of HAoAF with SFN (2.5 μM), there was a slight decrease in mitochondrial dehydrogenase activity when compared to vehicle control and treatment with SFN (10 μM) also resulted in a decrease in mitochondrial dehydrogenase activity (Fig 3.7, B), however this decrease was not statistically significant. These results suggest that SFN does not cause a significant decrease in HAoAF viability at the concentrations used in this study.

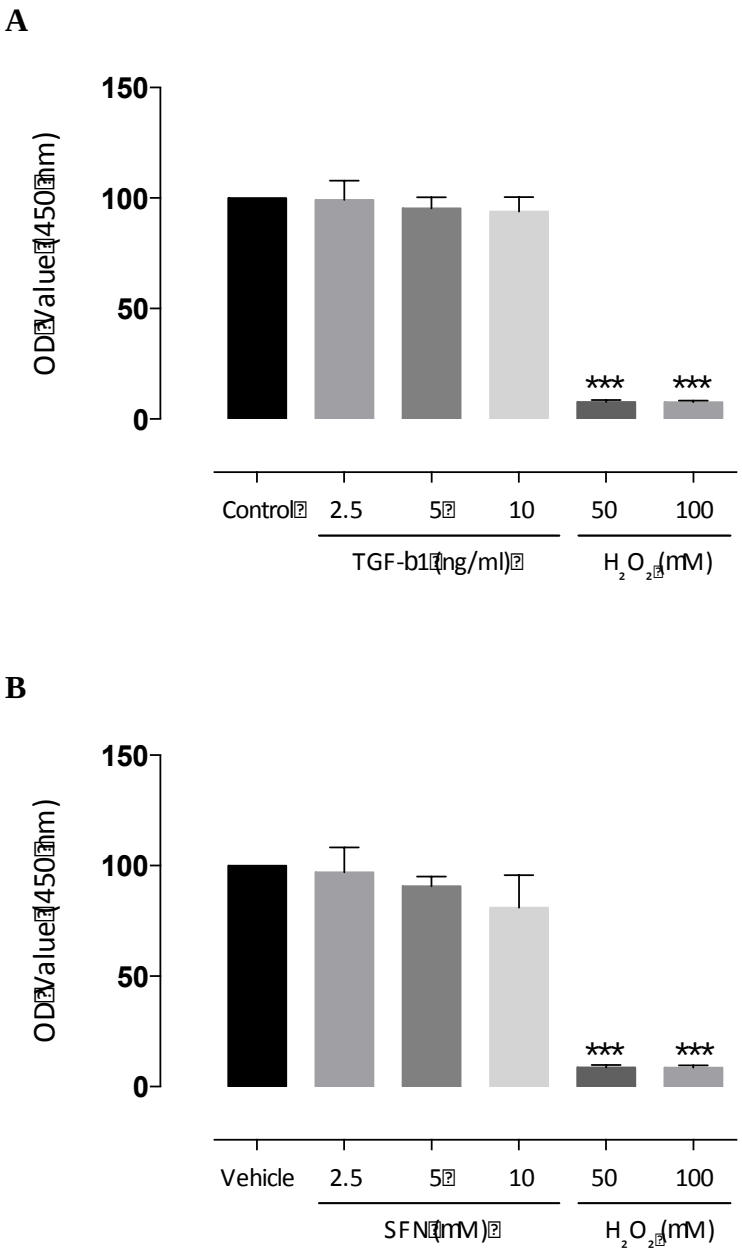


Figure 3.9 Effect of TGF-β1 and SFN on HAoAF viability
Confluent cultures of HAoAF were seeded at $1x10^3$ cells per well in a 96 well plate in DMEM supplemented with 10% FCS. Cells were equilibrated in DMEM supplemented with 1 % FCS 18 h prior to treatment with (A) TGF-β1 (2.5 – 5 ng/ml), (B) SFN (2.5 – 10 μM) or H_2O_2 (50 – 100 μM) for 24 h. Mitochondrial dehydrogenase activity was determined by measuring the formation of MTT formazan dye as described in Methods and was used as an indicator of cell viability. Values denote mean ± SEM, n = 4 independent experiments. ****p* <0.001 vs. control or vehicle control (DMSO 0.01%) using ANOVA.

3.5 Effect of TGF-B1 on Smad phosphorylation in HAoAF

The phosphorylation of Smad2 (pSmad2) investigated in HAoAF to confirm the activation of TGF-β1 signalling in these cells. Confluent HAoAF (P_6 - P_9) were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (0 - 5 ng/ml), for 0.5 – 4 h. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis (see Methods section 2.2). Basal expression of pSmad2 protein was observed in untreated HAoAF (0.5 – 4 h). This effect was significantly augmented during the treatment of HAoAF with TGF- β1 (2.5 ng/ml, 2 – 4 h, $p<0.05$), and maximum expression of pSmad2 was observed after 2 h (5 ng/ml, $p<0.05$; Fig 3.8, A). These results corroborate those observed during immunofluorescence analysis and suggest that cells are responsive to TGF- β1 and that the Smad signalling pathway is activated in this cell type.

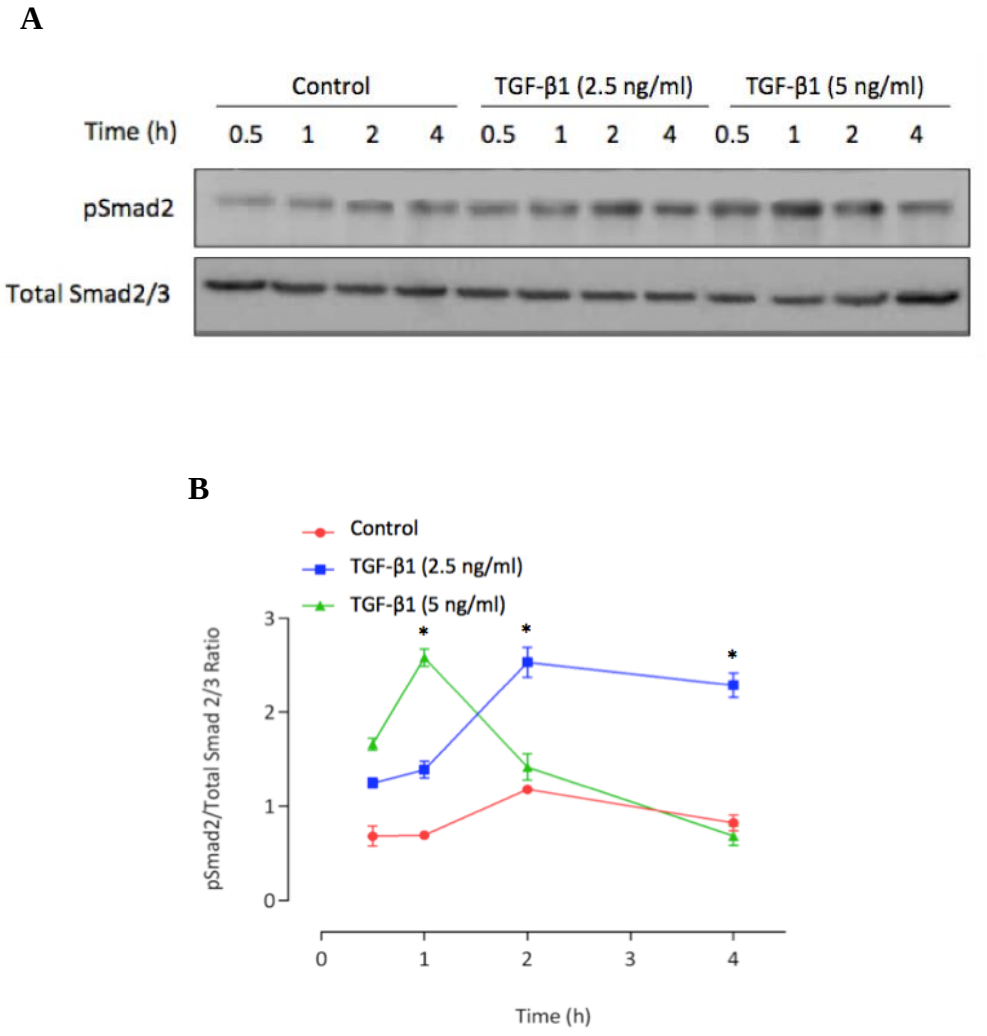


Figure 3.10 Phosphorylated Smad2 protein expression in HAoAF after treatment with TGF-β1. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) for 30 min – 4 h prior to whole cell protein extraction. pSmad2 protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with pSmad2 protein levels corrected for the loading control, total Smad2/3. Values denote means ± SEM, *p<0.05 vs. untreated control using unpaired Student's t-test. n = 3 independent experiments.

3.6 Effect of TGF-β1 on pSmad localisation in HAoAF

Phosphorylation of Smad2 and its nuclear translocation in response to TGF-β1, which typically occurs between 30 min - 4 h (Stockwell and Schreiber, 1998; Xu et al., 2000; Watkins et al., 2012), is an important and definitive step in the activation of the TGF-β1 pathway (Guo et al., 2009; Lu et al., 2009) and is often used as an index to assess the activation of this pathway (Watkins et al., 2012; Ungefroren et al., 2011; Mavrakis et al., 2007; Bitzer et al., 2000). HAoAF were seeded onto glass coverslips in DMEM supplemented with 10% FCS. Once appropriate confluence was achieved, cells were equilibrated in DMEM supplemented with 1% FCS 18 h prior to incubation with TGF-β1 (2.5 - 5 ng/ml) for 2 h. Cells were subsequently fixed and stained for pSmad2. Immunofluorescence staining of HAoAF revealed that pSmad2 is localized diffusely in the cytoplasm as well as the nucleus. However, upon treatment with TGF-β1 (2.5 – 5 ng/ml) for 2 h, cells exhibit increased fluorescence within the nuclear compartment indicating that there is an increase in the levels of pSmad2 in the nuclei of TGF-β1-treated cells. These results suggest that the TGF-β1 signaling pathway may already be acutely activated in HAoAF, perhaps due to the autocrine production of TGF-β1 by these cells, however when incubated with exogenous TGF-β1, pSmad2 nuclear translocation is enhanced and therefore indicative of the activation of this signaling pathway by exogenous administration of TGF-β1 in these cells.

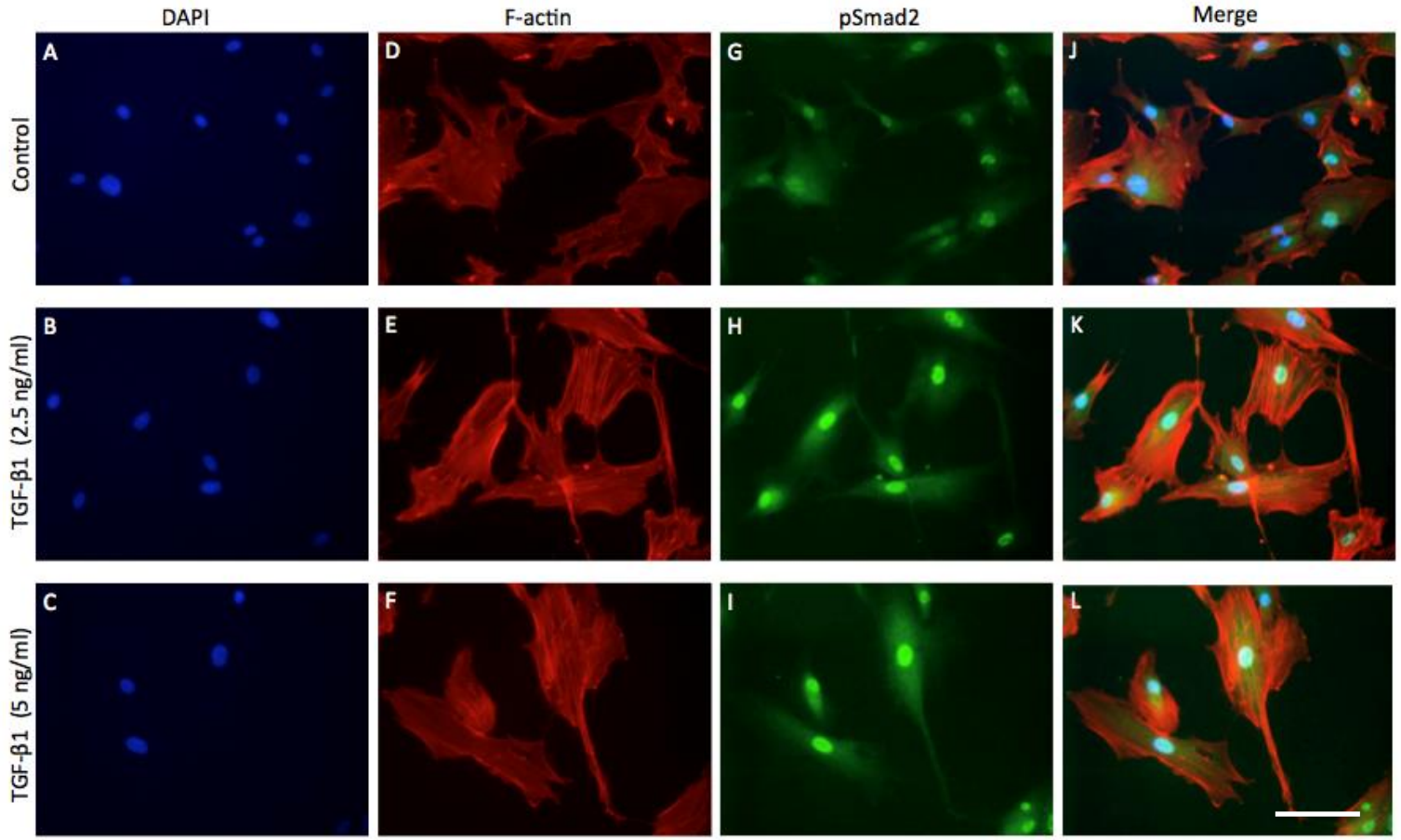


Figure 3.11 Localisation of pSmad2 in HAoAF treated with TGF-β1. Sub-confluent cultures of HAoAF seeded onto glass coverslips were equilibrated in phenol red-free DMEM supplemented with 1% FCS 18 h prior to incubation with TGF-β1 (2.5 – 5 ng/ml) for 2 h. Following incubation with treatment conditions, cells were fixed and incubated with specific antibody against pSmad2 (G-I) and an AlexaFluor-488 conjugated secondary antibody. Coverslips were mounted using Vectasheild® Mounting Medium containing DAPI for nuclear staining (A-C). In order to visualise F-actin, cells were incubated with rhodamine phalloidin conjugated to TRITC and viewed using a fluorescent microscope. Representative image from 3 fields of view per treatment condition from 3 independent experiments (scale bar = 20 μm).

3.7 Effect of TGF-β1 on HAoAF proliferation

TGF-β1 is well known to increase proliferation in mesenchymal cells and in fibroblasts (Frolik et al., 1983), whilst in SMC, epithelilal and endothelial cells it acts as a growth inhibitor (Chen et al., 2006). To assess the effect of TGF-β1 on HAoAF proliferation, a cell proliferation assay was carried out. Cells were seeded at a density of 1 x 10^4 cells/well in a 24-well plate in DMEM supplemented with 10% FCS. 18 h prior to incubation with treatment conditions, cells were equilibrated in DMEM supplemented with 1% FCS and subsequently incubated with either low serum media (DMEM supplemented with 1% FCS), high serum media (DMEM supplemented with 10% FCS) or with TGF-β1 (5 ng/ml) in DMEM supplemented with 1% FCS for a time period of 0-4 days. Cell number and protein content was determined every 24 h over this time period; Figures 3.10, A and B show the differential increase in protein concentration and cell number over time. Cells treated with high serum media, exhibited a significant increase in cell number over four days ($p<0.001$); this was correlated with protein concentration, which also increased over four days. Similarly, cells incubated with TGF-β1 exhibited a significant increase in cell proliferation as measured by cell number and protein after 4 days ($p<0.001$). In contrast to this, cells in low serum media exhibited no change in cell number or in protein concentration. When cell number and protein concentration were plotted, data exhibited a positive linear correlation coefficient of $r^2 = 0.967$ (Fig 3.11).

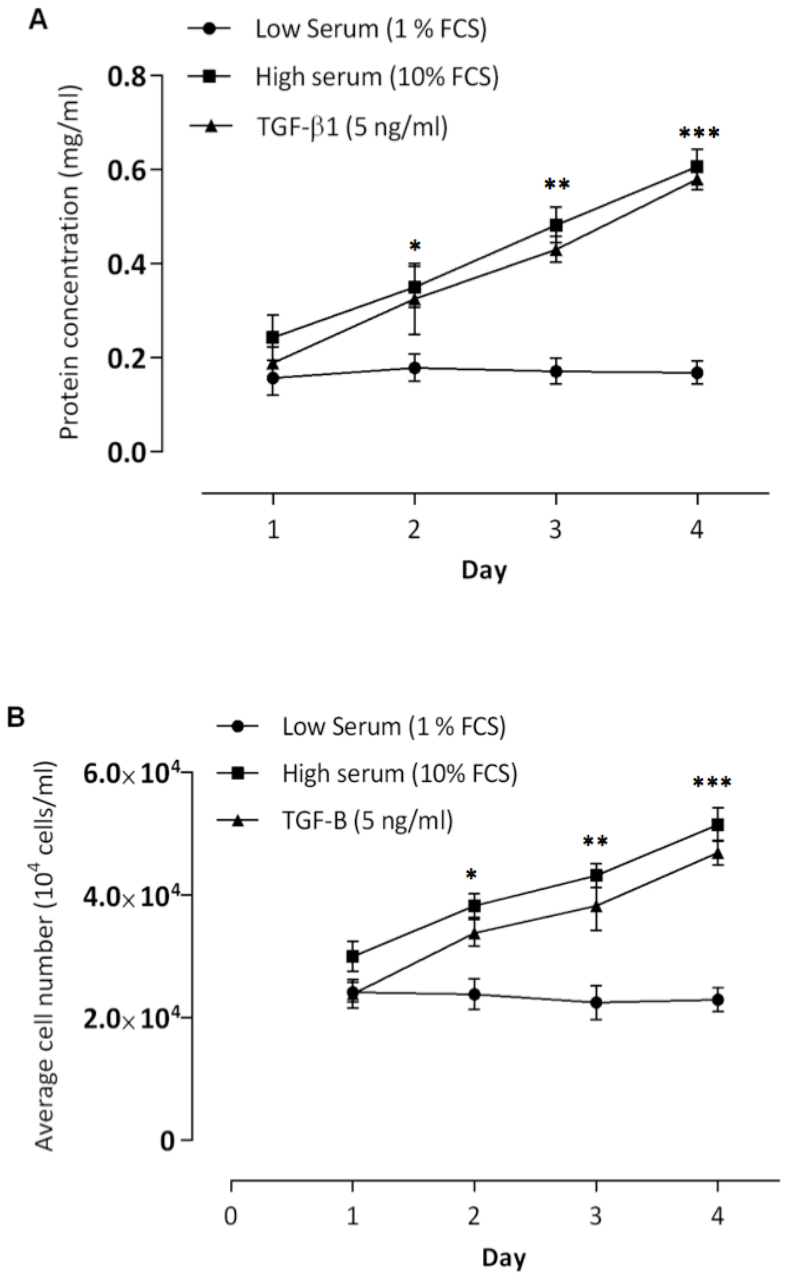


Figure 3.12 HAoAF cell proliferation curve following treatment with TGF-β1
Cells were seeded in 24 well plates in phenol red-free DMEM supplemented with 10% FCS. Cells were adapted to DMEM supplemented with 1% FCS for 18 h, 24 h after seeding. Once adapted, cells were treated with either phenol red-free DMEM supplemented with 1% FCS, phenol red-free DMEM supplemented with 10% FCS or with TGF-β1 (5 ng/ml) added to DMEM supplemented with 1% FCS for four days. The (A) average protein concentration and (B) cell number was determined every 24 h over four days. Total protein concentrations were determined following lysis of cells with NaOH (0.5 M) using the BCA protein assay. Values denote means ± SEM. *p <0.05 vs. control, **p <0.01 vs. control, ***p <0.001 vs. control using unpaired Student's t-test. n = Representative of 4 independent cell cultures.

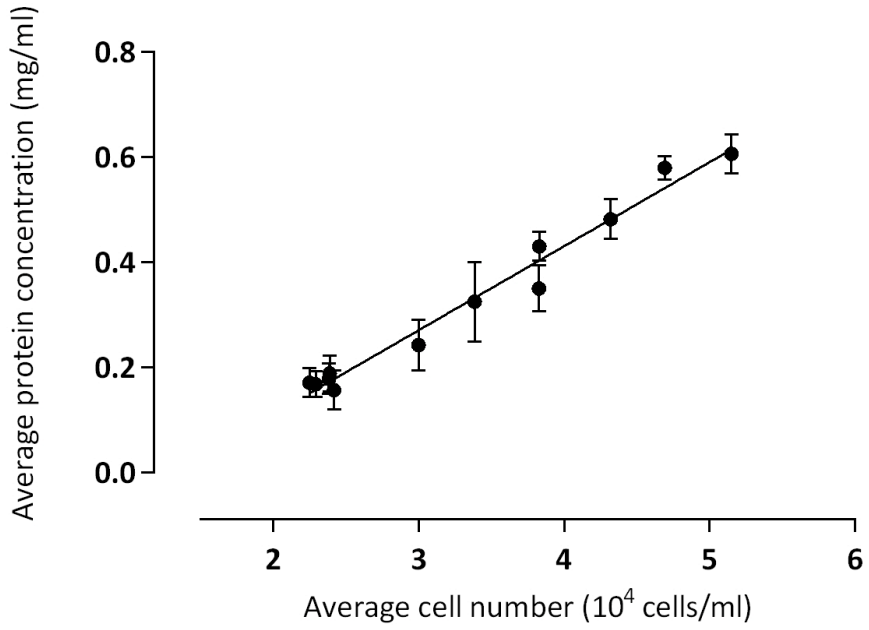


Figure 3.13 Correlation of cell number with protein concentration in cultured HAoAF
Cells were seeded in 24-well plates in phenol red-free DMEM supplemented with 10% FCS. Cells were adapted in DMEM supplemented with 1% FCS for 18 h, 24 h after seeding. Once adapted, cells were treated with either phenol red-free DMEM supplemented with 1% FCS, phenol red-free DMEM supplemented with 10% FCS or with TGF-β1 (5 ng/ml) for four days. Values represent correlation of mean data shown in Fig 3.10. n = 6 independent experiments, correlation coefficient $r^2 = 0.967$.

3.8 Effect of TGF-β1 on activation of Smad-independent signalling pathways

In addition to Smads, TGF-β1 also modulates other downstream cellular signalling pathways through either phosphorylation or via direct interaction, including various branches of MAP kinase and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (Bakin et al., 2000; Simeone et al., 2001; Zhang et al., 2009). It has also been postulated that the activation of the Nrf2/ARE signalling pathway may be regulated by several kinases including phosphorylation of Erk 1/2 (Cullinan et al., 2003; Araujo et al., 2010), $p38^{MAPK}$ and Akt/PI3K pathways (Zipper et al., 2000; Zipper et al., 2003; Papaiahgari et al., 2006). Therefore, in order to determine whether TGF-β1 activates Smad-independent pathways in HAoAF and whether activation of these may lead to phosphorylation events that may trigger activation of the Nrf2/ARE pathway, phosphorylation of kinases was investigated in HAoAF. Confluent HAoAF (were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (5 ng/ml) or SFN (5 μM) for 1 – 4 h. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis (see Methods section 2.2).

Following treatment of HAoAF with TGF-β1 for 1 – 4 h, there was a significant increase in pAkt protein expression when compared to untreated control ($p<0.05$). This increase was observed 1 h after treatment and continued to increase with maximum protein expression was observed after 2 h ($p<0.01$). At 4 h, this decreased slightly, however remained significantly upregulated when compared to untreated control ($p<0.05$). There was no increase in pAkt seen in untreated control cells (Fig 3.12). SFN (5 μM) also caused an increase in pAkt, ($p<0.05$) and continued to increase after 4 h ($p<0.01$). This suggests that both TGF- β1 and SFN activate the PI3K/Akt signalling pathway in HAoAF.

The protein expression of pErk 1/2 was also investigated. Basal expression of protein expression of pErk was observed in HAoAF in untreated control after 30 min. This expression decreased over time, and was seen to decrease in a time-dependent manner after 1 h. Similarly, after 30 min of TGF- β1 treatment, protein expression of pErk was observed after 30 min, an effect which had markedly decreased after 4 h. This response to TGF- β1 was not statistically significant when compared to untreated control (Fig

3.13). HAoAF treated with SFN expressed pErk 30 min after treatment which was sustained for 2 h following treatment but which decreased after 4 h, however this decrease was not statistically significant.

There was also some basal protein expression of p-p38^{MAPK} in HAoAF that was sustained for up to 2 h and had increased after 4 h in untreated control cells. In TGF- β1 treated cells, there was an increase in p-p38^{MAPK} 30 min following treatment which continued to decrease after 2 h, though this decrease was not statistically significant. SFN treated cells exhibited an increase in p-p38^{MAPK} after 30 min and this increase was maintained in cells for up to 4 h after 60 min and remained elevated after 4 h (Fig 3.16).

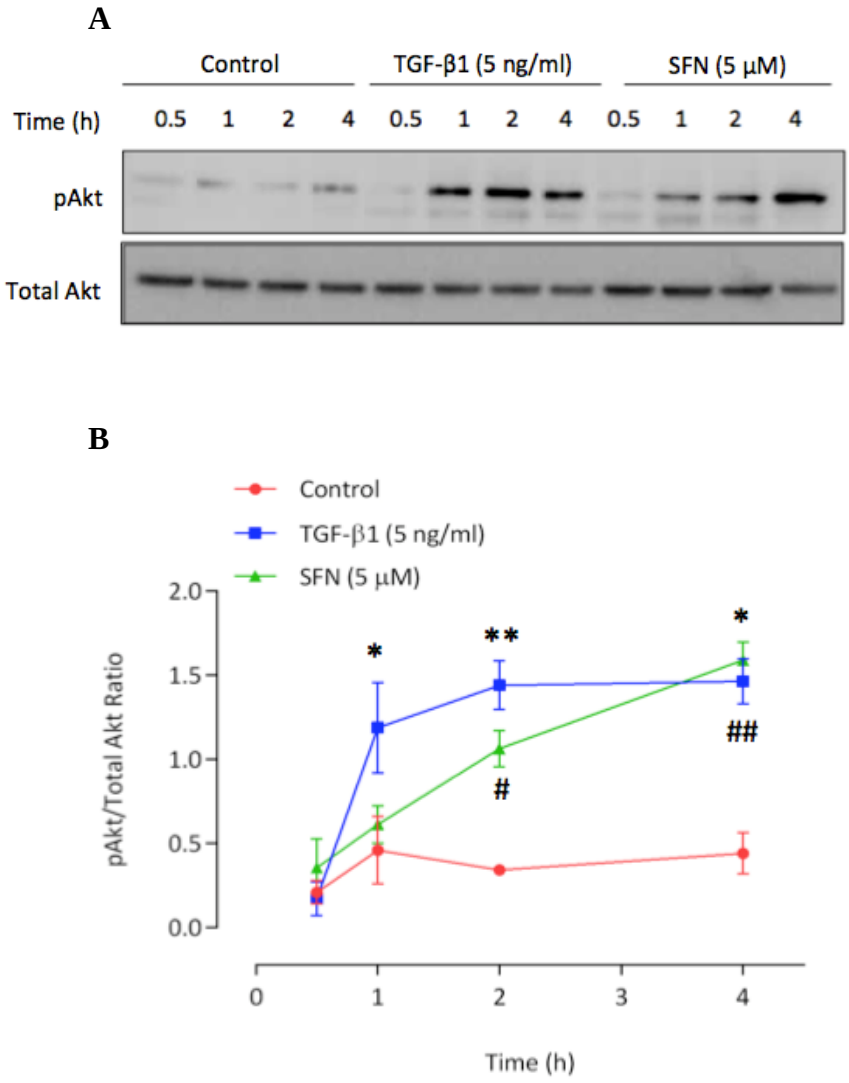


Figure 3.14 Phosphorylated Akt protein expression in HAoAF after treatment with TGF-β1 or SFN. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) or SFN (5 µM) for 30 min – 4 h prior to whole cell protein extraction. Phospho-Akt protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with pAkt protein levels corrected for the loading control, total Akt (B). Values denote means ± SEM, **p* <0.05 TGF- β1 vs. control, ***p* <0.01 TGF- β1 vs. control, #*p* <0.05 TGF- β1 vs. control, ##*p* <0.001 SFN vs. control using unpaired Student's t-test. n= 3 independent experiments.

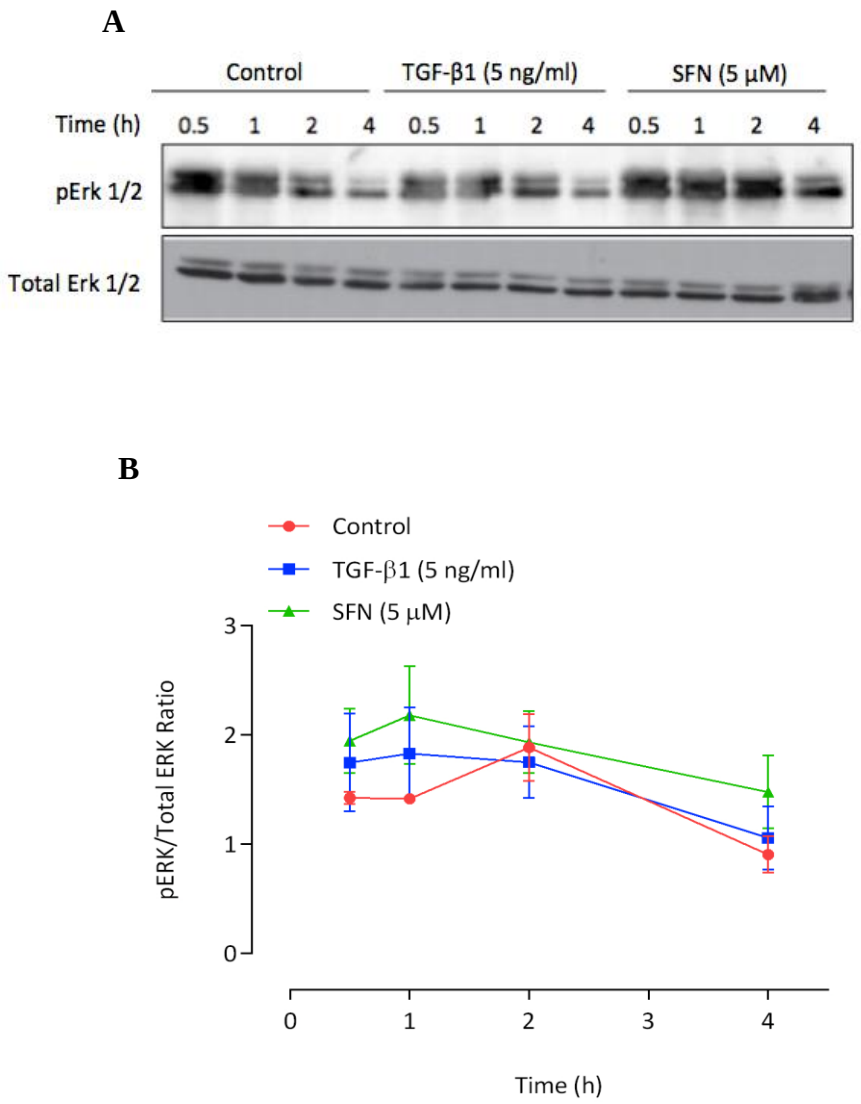


Figure 3.15 Phosphorylated Erk protein expression in HAoAF after treatment with TGF-β1 or SFN. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) or SFN (5 µM) for 30 min – 4 h prior to whole cell protein extraction. Phospho-Erk protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with pErk protein levels were corrected for the loading control, total Erk (B). Values denote means ± SEM, n= 3 independent experiments.

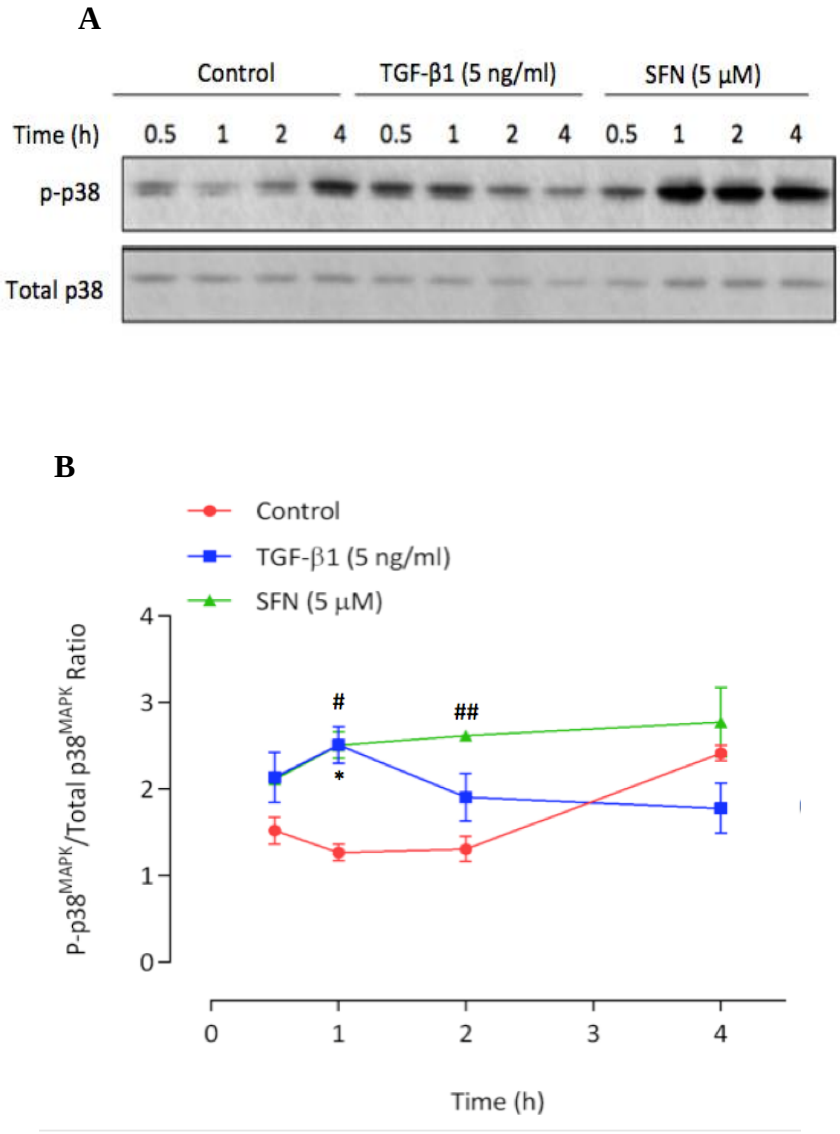


Figure 3.16 Phosphorylated p38 MAPK protein expression in HAoAF after treatment with TGF-β1 or SFN. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 -5 ng/ml) or SFN (5 μM) for 30 min – 4 h prior to whole cell protein extraction. Phospho-p38 protein expression levels in these samples was assessed using SDS-PAGE and Western blot analysis (A). Results were analysed by densitometry with P-p38 protein levels corrected for the loading control, total p38 (B). Values denote means ± SEM. *p<0.05 TGF-β1-treated vs. control (1 h), #p<0.05 SFN-treated vs. control (1 h), ##p<0.001 SFN-treated vs. control (2 h) using unpaired Student's t-test. n= 3 independent experiments.

3.9 Discussion

Vascular disease is partly characterised with changes in the cellular microenvironment that actively promote disease development, one characteristic feature of this process being fibroblast differentiation (Sampson et al., 2011). Differentiation of fibroblasts to a myofibroblast phenotype is a well established sequence of events (Hu and Xu, 2011) and is initiated upon vascular injury, causing the release of cytokines, including TGF-β1, from resident cells (Werner and Grose, 2003). The process takes place in the arterial adventitia and results in myofibroblast formation, a cell type that has migratory and contractile properties partly owing to the *de novo* expression of α-smooth muscle actin and vimentin and consequent actin cable formation and the expression of focal adhesions, events which, in concert, facilitate the pathophysiological role of this cell type (Engel et al., 1997; Stephens et al., 1997). During vascular disease and in response to TGF-β1, myofibroblasts migrate towards the neointima and contribute to adverse vascular remodelling (Enzerink et al., 2011), therefore in this chapter, HAoAF were characterised and the effect of TGF-β1 on HAoAF was investigated.

3.9.1 Expression of α-smooth muscle actin and vimentin in HAoAF

Fibroblasts acquire contractile stress fibres in response to mechanical challenge, which are composed of the cytoplasmic actins, β and γ-actins, a feature that is characteristic of the 'protomyofibroblast'. This phenotype is an intermediate step in *in vivo* vascular models of disease and usually proceeds toward the 'differentiated myofibroblast' (Hinz et al., 2007). Further differentiation results in a cell type that has migratory and contractile properties and is known as the myofibroblast. Several studies have reported the myofibroblastic transformation of vascular fibroblasts *in vitro* (Li et al., 2007; Shephard et al., 2004; Masur et al., 1996). Therefore, in order to establish the phenotype and morphology of cells being used for this study, the protein expression of α-smooth muscle actin and vimentin in HAoAF was investigated using immunofluorescence and Western blot analysis. Immunofluorescence analysis revealed the basal expression of both of these myofibroblast marker proteins by HAoAF. (Fig 3.1 and 3.4). Similarly, Western blot analysis revealed the basal expression of both α-smooth muscle actin and vimentin, the expression of which did not change with treatment of cells with exogenous TGF-β1 (Fig 3.2 and 3.4). These results suggest that cells had differentiated into myofibroblasts, possibly through the process of cell culture (Hinz et al., 2009; Wipiff et al., 2007).

The release of endogenous TGF-β1 by fibroblasts in culture has been widely reported in the literature and has been shown to result in fibroblast differentiation (Hinz et al., 2007; Li et al., 2007; Shephard et al., 2004). Masur and colleagues postulate that although the presence of TGF-β1 is necessary for the differentiation of fibroblasts to myofibroblasts in culture, it alone is not sufficient since, in addition to the presence of this growth factor, the lack of cell-cell contacts is also required for myofibroblastic transformation to occur (Masur et al., 1996). Their study in corneal fibroblasts revealed that cells seeded at lower density (5 cells per mm^2) produced a cell culture population consisting of 70-80% myofibroblasts, 5-7 days after seeding however, cells seeded at a high density (500 per mm^2) produced cultures with only 5-10% myofibroblasts (Masur et al., 1996). In the current study, HAoAF were cultured at a sub-confluent density; although cells in culture were not completely devoid of cell-cell contact, in comparison to *in vivo* conditions, and coupled with other culture conditions, as discussed below, it may, in part, explain the myofibroblast phenotype exhibited under basal conditions by these cells.

Other studies have also reported differentiation of cells through the cell culture process. Investigation of rat hepatic fibroblasts in culture revealed that this cell type also underwent differentiation *in vitro*, with increased staining of α-smooth muscle actin observed on day 4 of culture which progressively increased over time. Further analysis using qRT-PCR also demonstrated the development if α-smooth muscle actin expression over time in culture (Li et al., 2004). This differentiation was seen to be TGF-β1-dependent since use of a TGF-β1 receptor kinase inhibitor which antagonised autocrine TGF-β1 signalling, resulted in the attenuation of the differentiation of fibroblasts into myofibroblasts, as measured by α-smooth muscle actin expression (Li et al., 2007). The same study postulated that this differentiation was a function of mechanical stiffness, i.e. the stiffness of the substrate upon which cells were plated. It was observed that fibroblasts plated on gels of increasing stiffness underwent myofibroblastic differentiation, with α-smooth muscle actin being more readily incorporated into stress fibres, accompanied by cell spreading and increased expression of pro-collagens I and III (Li et al., 2007).

In culture, fibroblasts may become ‘activated’ by rigid cell culture vessel plastic and acquire α-smooth muscle actin-positive stress fibres, an effect which is not exhibited by

cells cultured on soft culture substrates (Hinz, 2012). The first report of this phenomenon came from a study in NIH3T3 fibroblasts that observed that cells detected and responded to soft and stiff substrates (Pelham and Wang, 1997) with cells cultured on a soft substrate generating well-separated myotubes with actomyosin appearing diffuse, however, cells grown on a stiff substrate began to acquire abundant stress fibres and strong focal adhesions after only a few days in culture (Discher et al., 2005; Pelham and Wang., 1997). Cell shape was also distinctly different; on the more rigid substrate, cells were well spread, however, those grown on increasingly flexible substrates were less so and displayed an irregular shape, different to the atypical spindle shape displayed by undiffentiated fibroblasts (Pelham and Wang, 1997). These studies suggest that substrate stiffness may be partly responsible for the cytoskeletal re-organisation seen in fibroblasts undergoing differentiation and may be an important factor with further implications on cell migration in culture conditions (Buxboim et al., 2010; Discher et al., 2005; Engler et al., 2004). These reports partly explain the myofibroblast phenotype exhibited by HAoAF observed in the current sudy, a phenomenon which may be due to the conditions in which cells were cultured.

In contrast, other studies have reported that in culture, cells remain undifferentiated and only differentiate upon exposure to TGF-β1. Results from this study are not consistent with those observed in cultured human colon-derived fibroblasts, where untreated cells exhibited no myofibroblast markers, however upon treatment of cells with TGF-β1, cells underwent myofibroblastic transformation and exhibited an increased expression of α-smooth muscle actin and myofibroblast contractility which were accompanied by the release of collagen type I when compared to untreated cells (Denys et al., 2008). However, other studies have revealed that TGF-β1 may not completely account for the process myofibroblastic transformation and that mechanical force generation, culture medium containing high levels of serum and other growth factors may also be involved as demonstrated by a keratinocyte-fibroblast co-culture model (Shephard et al., 2004). In this co-culture model, TGF-β1 alone was insufficient in causing fibroblast differentiation, however in the presence of a stiff substrate, and in synergy with granulocyte-macrophage colony stimulating factor, fibroblasts began to express α-smooth muscle actin (Shephard et al., 2004). Results reported in the literature and work from the current study suggest that the culture conditions, substrate stiffness and length

of time in culture may all have played a role in the differentiation of HAoAF into myobroblasts in this study.

It must be noted that the origin of the cells used in the current study carries several experimental limitations. The manufacturer (Lonza, USA) stated that cells had undergone routine characterization and had stained negative for smooth muscle α-actin, a common marker used for the identification of a smooth muscle cell morphology (Enzerink and Vaheri, 2011; Tomasek et al., 2002; see Chapter 3 discussion). However, although this is the case, it was also stated that the cultures generated from these cells could be used for the study of disorders of fibrosis, scleroderma, fibrosarcoma, xeroderma pigmentosum and histiocytoma. This may indicate that the results reported in this study regarding the effects of TGF-β1 may not be HAoAF specific and that TGF-β1 may have similar effects on non-aortic fibroblasts. Future work in non-aortic fibroblasts, such as dermal fibroblasts, in order to determine an experimental comparison would need to be carried out.

3.9.2 Effect of TGF-β1 and SFN on mitochondrial dehydrogenase activity in HAoAF

Studies have previously reported that TGF-β1 is able to increase the proliferation of fibroblasts *in vitro* (Zang et al., 2006) whereas others have reported that TGF-β1 is an antiproliferative cytokine (Kim et al., 2002). SFN has also been shown to be cytoprotective in mouse embryonic fibroblasts (Akhtar et al., 2012; Higgins et al., 2009) whilst also demonstrated as being a cytotoxic agent by other studies (Doudican et al., 2010; Sestili et al., 2010). Therefore in order to determine the effect of TGF-β1 and SFN on HAoAF viability, the colorimetric MTT assay was employed. Cells were treated with TGF-β1 (2.5 – 10 ng/ml) or SFN (2.5 – 10 μM) for 24 h and subsequently the MTT assay was carried out. Results indicated that treatment with TGF-β1 resulted in no changes in mitochondrial dehydrogenase activity when compared to untreated control. HAoAF treated with SFN also displayed no significant changes in mitochondrial dehydrogenase activity when compared to untreated control.

Previous studies using the MTT assay have also shown that TGF-β1 does not suppress cell growth; in nasopharyngeal carcinoma cells treated with TGF-β1, there was no effect on growth despite observed nuclear translocation of pSmad2, indicating that

TGF-β1 signalling pathways were activated in this cell type (Xiao et al., 2010). Similar results have been reported in ovarian cancer cells, where cells exhibited no significant growth inhibition in response to TGF-β1 (5 ng/ml) as measured by the MTT assay, despite activation of the TGF-β1 signalling pathway, as determined by phosphorylation of the type I TGF-β1 receptor (Yamada et al., 1999). A more recent study in keloid fibroblasts has shown that exposure to TGF-β1 (5 ng/ml) for 24 h resulted in no change in cell viability or alteration in proliferation when compared to untreated control (Wu et al., 2012). In contrast to these findings, the anti-proliferative activity of TGF-β1 has been reported in other cell types. In NRK-49F fibroblasts, a fibroblast cell line derived from a mixed population of fibroblasts, TGF-β1 caused a dose-dependent decrease in cell number as measured by the MTT assay following exposure to TGF-β1 for 6 – 96 h (Weyhe et al., 2007). The authors attributed this phenomenon as being cell type-dependent as well as being partly due to culture conditions such as substrate material and noted that it was in contrast with the existing literature (Weyhe et al., 2007). However, the concentration of TGF-β1 that fibroblasts were exposed to in this particular study was supra-physiological (25 ng/ml – 50 ng/ml), and similar levels that would only be seen in pathophysiological conditions such as in end-stage systemic sclerosis or advanced atherosclerotic lesions and may in-part explain these contrasting findings (Dziadzio et al., 2005; McCaffery, 2000).

Additionally, in human limbal epithelial cells, TGF-β1 (1 ng/ml) has been shown to decrease cell viablility and was thought to do so by antagonising the ability of epidermal growth factor to stimulate corneal cell proliferation as well as by upregulating the expression of the cyclin-dependent kinase inhibitors, p57 and p15 (Chen et al., 2006). There is evidence in the literature which points to TGF-β1 being a growth suppressor by decreasing cell viability as well as increasing cell growth as measured by the MTT assay, however, the effect of TGF-β1 appears to be a cell-type and TGF-β1 concentration and time-dependent event. Existing evidence in the literature suggests that TGF-β1 is a growth-inhibitor for epithelial cell cultures whilst acting as a powerful growth factor for mesenchymal cells (Bhowmick et al., 2003; Wenner and Yan, 2003; Moses et al., 1992). In VSMC and endothelial cells, TGF-β1 has been reported to inhibit proliferation (Halloran et al., 1995; Kirschenlohr et al., 1995; Mii et al., 1993; Bjorkerud, 1991; Takehara et al., 1987; Baird and Durkin et al., 1986). One study has postulated that the antiproliferative effects of TGF-β1 in SMC are due to the

ability of TGF-β1 to prolong the G2 phase during proliferation of SMC *in vitro* (Grainger et al., 1994). In the current study, it has been found that TGF-β1 (2.5 -5 ng/ml) does not increase cell proliferation or decrease cell viability in HAoAF over a 24 h time-period.

SFN has been demonstrated to cause cytotoxicity in several cell types. In B16F-10 cells, a melanoma cell line, increasing concentrations of SFN (1 – 50 μg/ml) caused a concomitant increase in cytotoxicity and significantly reduced the rate of proliferation in these cells (Thejass and Kuttan, 2006). Further investigation in mouse embryonic fibroblasts from wild-type and Nrf2 -/- animals revealed that SFN caused a significant increase in cell death at concentrations above 3 μM in cultures from wild-type animals. This effect was markedly enhanced in knockout animals where challenge with SFN caused a significant decrease in cell viability, suggesting that the activation of the Nrf2/ARE is protective against SFN-induced cytotoxicity in this cell type (Higgins et al., 2009). A similar study carried out in a pancreatic tumour cell line has also reported that treatment of these cells with SFN resulted in an increase in apoptotic cell death, thought to be as a result of SFN antagonising the NFκB pathway, downregulating apoptotic inhibitors, and thus decreasing cell viability (Kallifatidis et al., 2009). Conversely, one study looking at the effect of SFN against the toxic response of copper oxide (CuO) nano-particles in mouse embryonic fibroblasts found that in cells exposed to these nanoparticles, there was a dose-dependent decrease in cell toxicity in cells treated with SFN (Akhtar et al., 2012). The study postulated that the protective effect of SFN against CuO nano-particles was due to the ability of SFN to activate the Nrf2/ARE pathway and consequently up-regulate levels of glutathione reductase and GSH in this cell type (Akhtar et al., 2012). The effect on SFN as a cytotoxic agent may be harnessed and its use as a theraputic agent in diseases such as cancer may be of high therapeutic value.

Due to its ability to activate phase II enzymes, SFN may be used as a therapeutic agent to prevent or attenuate vascular disease processes such as hypertension. Previously, Wu and colleagues have reported that treatment of SMC from spontaneously hypertensive rats (SHR) displayed higher oxidative stress when compared to wild-type animals, as determined by the expression of HO-1 protein (Wu et al., 2001). However, when treated with SFN (0.05 – 1 μM) for 24 h, SMC exhibited an increase in cellular GSH levels and

experienced a decrease in oxidative stress levels (Wu et al., 2001). Recent evidence has also reported the ability of SFN to inhibit neointima formation by targeting adhesion molecules such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) through the suppression of NFκB and another transcription factor, GATA6, the mRNA and protein expression of both of which was reduced in SFN-treated injured carotid artery sections (Kwon et al., 2012). SFN treatment of SMC from these animals revealed that both proliferation and migration of SMC was inhibited by SFN (Kwon et al., 2012). These results were confirmed in a separate study that also suggests that SFN may attenuate SMC proliferation and therefore neointima formation by suppressing VCAM-1, the increased expression of which is associated with increased proliferation of SMC which eventually leads to enhanced atherosclerotic lesion formation (Kim et al., 2012). However, in the current study, SFN (2.5 – 10 μM) did not cause a significant decrease in cell viability following exposure of cells for 24 h, but it must be noted that the ability of SFN to induce cytotoxicity may be a function of cell type, concentration and length of time of exposure to this isothiocyanate.

3.9.3 TGF-β1 elicits Smad phosphorylation and nuclear translocation in HAoAF

In order to demonstrate that TGF-β1 was able to activate Smad signalling in HAoAF, the phosphorylation of Smad2 was investigated using Western blot analysis. Cells were treated with TGF-β1 (2.5 – 5 ng/ml) for 0.5 – 4 h and whole cell protein was extracted and Western blot analysis was carried out (Massague, 2012; Hill, 2009; Feinberg et al., 2005, Fig 3.10). Results demonstrated that exposure to TGF-β1 resulted in the significant phosphorylation of Smad2 when compared to untreated control, indicating that the Smad signalling pathway is activated downstream of TGF-β1 in HAoAF. In order to further confirm the activation of this pathway, immunofluorescence analysis was carried out in order to determine the nuclear translocation of pSmad2 in HAoAF, an event which is required for the induction of genes downstream of TGF-β1 (Fig 3.11). Results showed that in response to TGF-β1, there was enhanced nuclear translocation of pSmad2, further confirming the activation of the Smad signalling pathway by TGF-β1 in HAoAF.

The phosphorylation of Smad2 downstream of TGF-β1 is well established signalling event, and in particular, the phosphorylation of two serine residues in the C-terminus of

Smad2, Ser465 and 467, in an obligate manner, are required for TGF-β1 signalling to occur (Souchelnytskyi et al., 1997). A recent study in keloid fibroblasts that were exposed to TGF-β1 (5 ng/ml) for 48 h showed that cells exhibited a sustained activation of the Smad signalling pathway in response to this growth factor as demonstrated by the phosphorylation of Smad2 and 3 (Wu et al., 2012). In nasal polyp-derived fibroblasts, the phosphorylation of Smad2/3 has been shown to be a ROS-dependent process as exposure of cells to TGF-β1 treatment of cells resulted in the production of ROS as measured by 2″,7″-dichlorfluorescein-diacetate (DCF-DA) in addition to the phosphorylation of pSmad2/3, however treatment of cells with the flavoprotein inhibitor diphenyliodonium, or the antioxidants *N*-acetylcysteine, and ebselen resulted in the abrogation Smad2/3 phosphorylation, suggesting that the activation of this pathway downstream of TGF-β1 may be partly mediated by ROS (Park et al., 2012). In the present study, the potential regulation of Smad2 phosphorylation by ROS was not investigated, however, further work investigating this possibility of ROS in affecting signalling downstream of TGF-β1 would be novel in HAoAF.

In HAoAF, a basal expression and nuclear translocation of pSmad2 was observed in untreated control cells. Several studies have proposed that the activation of the Smad signalling pathway occurs during myofibroblast differentiation (Usuki et al., 2012; Gu et al., 2007; Hu et al., 2003). In a murine lung fibroblast cell line, exposure to TGF-β1 for 24 h resulted in an increase in mRNA levels of α-smooth muscle actin and a concomitant increase in mRNA levels of type I collagen, which is typically downstream of TGF-β1/Smad signalling. Double immunostaining of cells showed phosphorylation of Smad2 as well as an increase in α-smooth muscle actin expression suggesting that differentiation of this cell type was closely related to Smad2 phosphorylation and its nuclear translocation (Usuki et al., 2012). An additional study in human fetal lung fibroblasts demonstrated that the overexpression of Smad3 markedly increased TGF-β1-induced α-smooth muscle actin promoter activity and α-smooth muscle actin expression *in vitro*, an effect that was abrogated in the presence of the overexpression of a dominant negative mutant Smad3 or Smad7 (Gu et al., 2007). Gu and colleagues postulate the involvement of Smad3 in fibroblast differentiation as cells had undergone differentiation and exhibited the expression of myofibroblast markers in culture, as well as the basal expression of pSmad2 and its presence in the nucleus in untreated control

cells which may suggest that these two events may be related (Gu et al., 2007). These results are consistent with those reported in the current study.

3.9.4 TGF-β1 results causes HAoAF proliferation

To determine the effects of TGF-β1 on proliferation in HAoAF, cells were treated with TGF-β1 for 1-4 days and cell number was recorded and total cell protein was extracted and used as a an indicator of proliferation. Results showed that TGF-β1 caused a significant increase in the average cell protein concentration ($p<0.001$) and an associated increase in average cell number (Fig 3.12) over 4 days when compared to untreated control. Since its discovery, TGF-β1 has been widely associated with the proliferation of various cell types (Frolik et al., 1983) but in particular, during vascular pathophysiology, it is seen as being a key player in the proliferation of fibroblasts. *In vivo,* during transplant vasculopathy performed in rats, qRT-PCR revealed a significant upregulation of TGF-β1 and further analysis using immunohistochemistry revealed proliferation of fibroblasts and myofibroblasts in the adventitial layer and their subsequent migration towards the neointima, an event that was TGF-β1-dependent (Ji et al., 2010). Furthermore, in rats, pressure overload induced by a suprarenal aortic constriction resulted in the production of myocardial fibrosis as a result of fibroblast proliferation in response to increased levels of TGF-β1 mRNA (Kuwahara et al., 2002).

Assessment of myocardial mRNA revealed increased TGF-β1 levels after 3 days, but intraperitoneal administration of an anti-TGF-β1 neutralizing antibody subsequently prevented myocardial fibrosis, implicating TGF-β1 in driving proliferation in this disease model (Kuwahara et al., 2002). In addition to *in vivo* studies, *in vitro* studies have also confirmed the role of TGF-β1 in enhancing cell proliferation. In fibroblasts from a transgenic mouse model in which the TGF-β1 type II receptor is upregulated, it was found that exogenous treatment with TGF-β1 resulted in enhanced fibroblast proliferation, a finding that was confirmed with immunohistochemistry (Derret-Smith et al., 2010). Yijing and colleagues have also demonstrated similar findings in adventitial fibroblasts derived from rat aorta where exposure of cells to TGF-β1 for 24 h resulted in a two-fold increase in proliferation when compared to untreated control (Yijing et al., 2012). This increase was coupled with an increase in the protein expression of pErk 1/2, known to be upregulated during proliferation of cells (Mebratu and Tesfaigzi, 2009). In another study, targeted knockdown of Erk2 using a lentivirus resulted in a marked

decrease in proliferation in rat joint-adhesion tissue fibroblasts, suggesting that TGF-β1 activation of Erk1/2 is an important pathway involved in TGF-β1-mediated proliferation of fibroblasts (Li et al., 2009). This is of particular relevance to the current study as basal upregulation of pErk was observed in HAoAF and may explain the increase in cell proliferation following exposure of cells to TGF-β1.

Although in fibroblasts, TGF-β1 is known to cause cell proliferation, in several different other cell types including epithelial, endothelial and hematopoietic cells, it can potently inhibit cell proliferation (Elliot and Blobe, 2005). This inhibition of growth, although poorly understood, is thought to be due to the ability of TGF-β1 to prevent progression through the cell cycle by inducing the expression of cyclin kinase inhibitors p15INK4b (Hannon et al., 1994), p21CiP1 (Datto et al., 1995) and p27KIP1 (Polyak et al.,1994) which block cyclin and cyclin-dependent kinases from phosphorylating the tumour suppressor protein, retinoblastoma protein, allowing the hypophosphorylated form of this protein to bind and sequester the E2F transcription factor, as well as directly suppressing *c-myc* expression (Pietenpol et al., 1990).

An increase in oxidative stress can act as a pro-oncogenic-associated stress that promotes proliferation and motility (Rachkonda et al., 2010). Loss of MnSOD in mouse embryonic fibroblasts increased ROS levels, stimulating cyclins D1 and B1, making cells insensitive to antigrowth signals whereas overexpression of MnSOD, resulted in the suppression of superoxide levels and facilitated the transition of proliferating cells into a quiescent phase (Sarsour et al. 2008). Conversely, in lung fibroblasts, enhanced proliferation following exposure to TGF-β1 was not attenuated after treatment with the antioxidant *N*-acetyl-cysteine (Moses et al., 1994). In the current study, cell proliferation in the presence of TGF-β1 and an antioxidant was not assessed. Further studies looking at the potential of antioxidants to attenuate adventitial fibroblast proliferation in the presence of TGF-β1 may offer a window of therapeutic opportunity since TGF-β1 has been reported to increase the production of ROS, therefore its attenuation may be clinically beneficial (Samson et al., 2011; Sturrock et al., 2006; Rocic et al., 2005).

3.9.5 Effect of TGF-β1 on activation of Smad-independent signalling pathways

It is becoming increasingly evident that in addition to the classical Smad pathway, TGF-β1 also activates non-Smad signalling pathways including Erk, p38 ^{MAPK} and PI3K/Akt, which were investigated in this study.

The mechanism by which TGF-β1 causes the activation of the Erk pathway has recently been reported as being attributed to the dual specificity of the TGF-β1 type I and II receptors that are thought to act as both tyrosine and serine/threonine kinases and phosphorylation of tyrosine residues in the activated receptors results in the recruitment of several adaptor proteins, possibly Smad7, which have been reported to be involved in the Erk signalling pathway (Mu et al., 2012). Erk-MAPKs are thought to interact with the canonical Smad signalling pathway by preventing the nuclear translocation of Smad proteins which occurs when Erk phosphorylates the linker region in Smads, consequently preventing TGF-β1-dependent gene expression (Feng and Derynck, 2005). In the current study, Erk phosphorylation was observed basally in untreated control cells and in TGF-β1 treated cells, it was observed after 30 min of stimulation. The kinetics of Erk phosphorylation have been postulated as being cell-type and culture-condition dependent with some studies reporting peak Erk phosphorylation hours after TGF-β1 stimulation as seen in pancreatic acinar cells (Simeone et al., 2001) whilst others have reported phosphorylation after 5-10 min (Olsson et al., 2001; Musci et al., 1996). In the current study, Erk phosphorylation was observed basally in untreated control cells and in TGF-β1 and SFN-treated groups after 30 min Fig. 3.15). A recent study carried out in epithelial and mesenchymal cells and in fibroblasts has reported similar findings. Basal phosphorylation of Erk was reported in mesenchymal cells, however this activation was not observed in epithelial cells, which required exposure to TGF-β1 for it to occur (Hough et al., 2012). The same study reported that Erk phosphorylation was necessary for TGF-β1 –dependent fibroblast proliferation (Hough et al., 2012). Results from proliferation studies in HAoAF demonstrate that proliferation in this cell type is TGF-β1-dependent, and the activation of Erk signalling may be a mechanism by which these cells proliferate. These results are supported by further evidence in NIH3T3 cells in which fibroblast proliferation and differentiation induced by TGF-β1 was an Erk-dependent process as use of an Erk 1/2 inhibitor completely abrogated this response in cells (Xiao et al., 2012).

$p38^{MAPK}$ has also been implicated in mediating cell proliferation in conjunction with Erk1/2. Airway SMC treated with TGF-β1 (1 ng/ml) exhibited increased [3H]-thymidine incorporation and cell number over 48 h whilst also exhibiting a significant increase in phosphorylated p38 and Erk1/2 after 1 h of treatment. Treatment of cells with p38 or Erk1/2 inhibitors significantly abrogated TGF-β1-induced DNA synthesis in these cells suggesting p38 and Erk1/2 phosphorylation is necessary for TGF-β1-dependent proliferation in this cell type (Chen and Kalil, 2006).

There is also a plethora of evidence to suggest a role of PI3K in TGF-β1 signalling. In the current study, the downstream effector of PI3K, Akt was observed to be rapidly and significantly phosphorylated in HAoAF exposed to TGF-β1 after 1 h of treatment (Fig 3.12, $p<0.05$ TGF- β1 vs. control and $p<0.01$ TGF- β1 vs. control at 2 h). This phosphorylation by TGF-β1 has been previously reported as being due to the constitutive association of the TGF-β1 type II receptor with p85, a regulatory subunit of PI3K (Yi et al., 2005). Upon TGF-β1 binding to the receptor, p85 also associates with the TGF-β1 type I receptor and activates PI3K consequently activating a downstream signalling cascade (Yi et al., 2005). The downstream PI3K/Akt pathway has been implicated in being involved in TGF-β1-induced EMT with one study reporting that it was necessary for the TGF-β1-mediated actin filament reorganisation and consequent cell migration seen during epithelial-mesenchymal transition (Bakin et al., 2000). As well as this, PI3K/Akt activation has also been postulated to play a role in TGF-β1-mediated fibroblast proliferation as pharmacological inhibition of this kinase results in an abrogation in TGF-β1-mediated morphological alterations and cell proliferation in fibroblasts (Wang et al., 2005; Daniels et al., 2004). These studies suggest that PI3K/Akt activation is an important downstream pathway by which TGF-β1 exerts its mitogenic effects in fibroblasts. In the context of the current study, this pathway may be involved in the TGF-β1-dependent proliferation of HAoAF, however, further studies examining the effects of pharmacological inhibition of PI3K/Akt are required in order to confirm this.

A number of studies have reported the ability of SFN to effect the activation of Erk and p38 pathways. In the present study, SFN was observed to significantly increase the protein levels of phosphorylated Akt ($p<0.05$ SFN vs. control and $p<0.01$ SFN vs. control) as well as increasing phosphorylated p38 in a time-dependent manner;

however, Erk phosphorylation was upregulated after 30 min of exposure to SFN (Figs 3.12 and 3.13). It is apparent from previous studies that the effect of SFN on these various kinase pathways is dependent upon several different factors, including cell type, period of time of exposure and the concentration of SFN that cells are exposed to. In human HepG2 cells, SFN (20 μM) has been reported to activate Erk1/2 phosphorylation whilst strongly suppressing phosphorylation of p38 (Keum et al., 2006). The authors also postulated that stimulation of p38 in these cells resulted in a greater interaction of Nrf2 with its inhibitory protein, Keap1, suggesting that SFN-mediated activation of this antioxidant pathway was partly regulated by SFN-mediated suppression of p38 (Keum et al., 2006). The ability of SFN to augment antioxidant signalling downstream of Nrf2 may be of therapeutic relevance as this modulation of an endogenous antioxidant pathways can offer increased cytoprotection against oxidative stress, something that exogenous antioxidants have not been able to achieve (Kris-Etherton et al., 2004; Steinberg 2000). However the disparity of the effects of SFN in different cell types highlights the complexity of its actions on cell signalling and must be taken into account when considering it for therapeutic use. This disparity is reflected in a study investigating its effects on adipocytes exposed to SFN (0 – 20 μM) for up to 48 h where SFN was reported to decrease the phosphorylation of Erk 1/2 and Akt, an effect that resulted in cell cycle arrest at the G0/G1 phase (Choi et al., 2012). A more recent study carried out in VSMC has reported that 2 h pre-treatment of cells with SFN (1-5 μg/ml) resulted in a dose-dependent inhibition of TNF-α-induced adhesion of THP-1 monocytic cells and protein expression of VCAM-1 coupled with an associated suppression of TNF-α-induced production of intracellular ROS and suppression of phosphorylation of p38, Erk and JNK (Kim et al., 2012). The anti-inflammatory actions of SFN observed in this study further point to the potential therapeutic use of this ITC in modulating endogenous antioxidants with its use being of particular relevance to cardiovascular disease.

Chuang and colleagues have shown that SFN inhibited PI3K signalling in platelets, and in culture was able to significantly reduce thrombus formation on a collagen-coated surface under flow conditions (Chuang et al., 2013). The underlying mechanism of this inhibition was postulated to be the ability of SFN to cause the ubiquitination of the p85 regulatory subunit of PI3K, thereby preventing its translocation to the membrane for signalling events to occur, as well as causing the ubiquitination and degradation of

phosphoinositide-dependent kinase 1 which is required for Akt activation (Chuang et al., 2013). The underlying mechanisms of MAPK activation by SFN were not investigated in this study but further work examining these may offer a clearer understanding of how SFN modulates the activation of various signalling cascades in HAoAF. The increase in pAkt in response to SFN in HAoAF may suggest a possible alternative route by which SFN may phosphorylate Nrf2, thereby activating the Nrf2/ARE pathway, as has also been reported elsewhere (Zheng et al., 2011).

This chapter has provided evidence of the effects of TGF-β1 on HAoAF in culture conditions. Exposure of cells to TGF-β1 results in the activation of the Smad signalling pathway in this cell type as well as the activation of several Smad-independent signalling pathways. Stimulation of HAoAF with TGF-β1 did not appear to alter the phenotype of these cells in culture and it was demonstrated that these cells had fully differentiated into a myofibroblast cell type. TGF-β1 has also been shown to increase cell proliferation and activate several Smad-independent pathways which were also activated by exposure to SFN in this cell type.

CHAPTER 4:

Results

CHAPTER 4: Activation of redox signalling in human aortic adventitial fibroblasts treated with TGF-β1 and SFN

4.1 Introduction

Adventitial fibroblast (AF) migration and proliferation contributes to medial hypertrophy following vascular injury (Siow et al., 2003; Li et al., 2000) and there is increasing evidence to suggest that adventitia-derived reactive oxygen species (ROS) can act as signalling molecules to enhance this migration (Cascino et al., 2011; Haurani and Pagano, 2007). The cytokine, TGF-β1 is a key regulator of remodelling in vascular diseases, contributing to AF migration, a process that may occur in concert with enhanced ROS generation (Siow and Churchman, 2007). The Nrf2/ARE pathway is one of the most important antioxidant pathways within a cell that acts to restore redox balance following excess ROS production. Under normal physiological conditions, Nrf2 is sequestered in the cytoplasm by its inhibitory protein, Keap1 and is subject to proteasomal degradation (Itoh et al., 1999; McMahon et al., 2003). However Nrf2 activation, as a result of disruption of the complex formed between Keap1 and Nrf2 is an important mechanism of defence against oxidative and xenobiotic stress (Motahashi et al., 2004) and results in the accumulation of Nrf2 in the nucleus, dimerization with small Maf protein and its binding to the ARE in the promoter region of target genes and their consequent transcriptional activation (Osburn and Kensler, 2008).

Heme oxygenase-1 (HO-1) is one of the many Nrf2-regulated genes and is a rate limiting, stress inducible enzyme that has previously been shown to confer protection against oxidative injury whilst maintaining tissue homeostasis (Morita, 2005; Ning et al., 2002). Its role in catalysing the degradation of heme to yield bilirubin, carbon monoxide and free ferrous iron has been reported as being protective in rodent models of ischemia reperfusion injury and intimal hyperplasia following balloon injury (Otterbein et al., 2003). The importance of this antioxidant enzyme has been exhibited in HO-1 knockout mice where absence of HO-1 results in increased cell death and accelerated neointima formation due to enhanced vascular smooth muscle cell migration (Yet et al., 2003).

The flavoenzyme, NAD(P)H-quinone oxireductase (NQO1) is another Nrf2-regulated antioxidant gene and the depletion or knockdown of NQO1 is associated with increased

oxidative stress and predisposition to disease (Li et al., 2012). *In vitro* and *in vivo* studies carried out in human and rodent models have consistently reported that NQO1 is one of the most robustly inducible genes amongst the battery of Nrf2-regulated antioxidant enzymes and a number of pharmacological inducers of the Nrf2/ARE pathway have been shown to induce NQO1 mRNA and protein levels (He et al., 2011; Higgins et al., 2011; Suh et al., 2004). Recently, a number of studies in animal models have reported the potential protective role NQO1 plays in cardiovascular injury and atherogenesis, therefore investigating its induction in HAoAF in response to TGF-β1 and SFN is an important indicator of the induction of the Nrf2/ARE pathway (Zhu and Li, 2012).

Glutathione (GSH) is one of the first lines of defence cells employ in order to protect against oxidative stress (Lui et al., 2011). This tripeptide is able to prevent damage to important cellular components caused by ROS by serving as an electron donor, itself becoming reversibly oxidised. Its reversion back to its reduced form is catalysed by glutathione reductase (GSR; Englemann et al., 2005). The ratio of reduced to oxidised glutathione within cells is often an indicator of cellular toxicity and measurement of GSH depletion is widely used method of assessing the degree of oxidative stress that a pharmacological compound induces (Chen et al., 2006; Hissin and Hilf, 1976). Glutamate cysteine ligase (GCL) is a heterodimeric enzyme critical in the first step of glutathione synthesis and is composed of two proteins; glutamate cysteine ligase catalytic subunit (GCLC) and glutamate cysteine ligase modifier subunit (GCLM), both of which are transcriptionally regulated by Nrf2 (Yang et al., 2005). The levels of GSH and the expression of the component parts of its pathway are accurate indicators of levels of stress in a cell or tissue and are a routine measure of cellular toxicity (Hissin and Hilf, 1976).

Due to its inducible nature and its biological effects, the Nrf2/ARE pathway plays a protective role in the vascular wall against atherogenesis (Morita, 2005) and interventions aimed at modulating the activation of this pathway in the vascular wall may therefore provide a novel way in which to treat or prevent vascular injury or disease. Previously it has been shown that TGF-β1 activates the Nrf2 antioxidant pathway in human aortic smooth muscle cells, resulting in HO-1 protein expression (Churchman et al., 2009) as well as causing the depletion of GSH during fibrosis in

various disease models (Kang et al., 20011; Lui et al., 2004). SFN is a known phase II enzyme inducer, as well as causing glutathione depletion in various cell culture and animal models and in the current study it was used to activate the Nrf2/ARE pathway (Krehl et al., 2012; Oh et al., 2012; Vomhof-Dekrey et al., 2012; McMahon et al., 2001). Therefore, following the characterisation of HAoAF, as described in Chapter 3, this chapter sought to explore the effect of TGF-β1 and SFN on the transcriptional activation of the Nrf2 pathway in HAoAF.

4.2 Nrf2 expression in HAoAF treated with TGF-β1 or SFN

Total protein and mRNA levels of Nrf2 in whole cell lysates from HAoAF treated with TGF- β1 (0-5 ng/ml, 4-8 h) were determined by Western blot analyses and qRT-PCR respectively. HAoAF lysates were probed with a Nrf2 specific antibody (Spring Bioscience, USA, see Methods section 2.2 and 2.9) which detected two bands at 110 kDa and 66 kDa. There is evidence to suggest that the mobility of the Nrf2 protein on denaturing Tris/glycine buffered SDS gels does not correspond directly to its molecular mass of approximately 66 kDa (Nguyen et al., 2009; Li et al., 2005) and may be due to an abundance of acidic residues found in Nrf2, causing it to migrate anomalously (Moi et al., 1994) to give a band at 110 kDa. Furthermore, in siRNA knockdown experiments carried out in this study, a decrease in Nrf2 expression detected at the 66 kDa band has been observed. Therefore, all western blot analysis of Nrf2 in HAoAF was based on the densitometry of the 66 kDa band (Nguyen et al., 2009).

4.2.1 Nrf2 mRNA levels in HAoAF following treatment with TGF- β1 or SFN

For the determination of Nrf2 mRNA levels following treatment of HAoAF with TGF-β1 (0-5 ng/ml, 4-8 h), confluent HAoAF were equilibrated in DMEM supplemented with 1% fetal calf serum (FCS) 18 h prior to treatment for 4 and 8 h, mRNA was extracted (see Methods section 2.9) at two different time-points and qRT-PCR was performed. After treatment with TGF- β1 (2.5 ng/ml) for 4 h, there was no change in Nrf2 mRNA levels when compared to untreated control. Treatment with TGF-β1 (5 ng/ml) resulted in a slight decrease in Nrf2 mRNA levels, however this decrease was not significant (Fig 4.1) After 8 h of treatment with TGF- β1 (2.5 and 5 ng/ml) there was also little change in Nrf2 mRNA levels when compared to untreated control (Fig 4.1.1b).

As there was little or no significant change in Nrf2 mRNA levels following treatment of HAoAF with TGF- β1, suggesting it is a very mild insult in this cell type, HAoAF were also treated with the dietary isothiocyanate, sulforaphane (SFN), as a positive control and Nrf2 mRNA levels were examined. At 4 h of treatment with SFN, there was a slight but not significant increase in Nrf2 mRNA levels when compared to vehicle treated cells (DMSO 0.01 %, Fig 4.2). Interestingly, at 8 h, vehicle treated cells had higher Nrf2 mRNA levels when compared to SFN-treated (2.5 – 5 μM) cells (Fig 4.2) and levels were lower than those observed at 4 h.

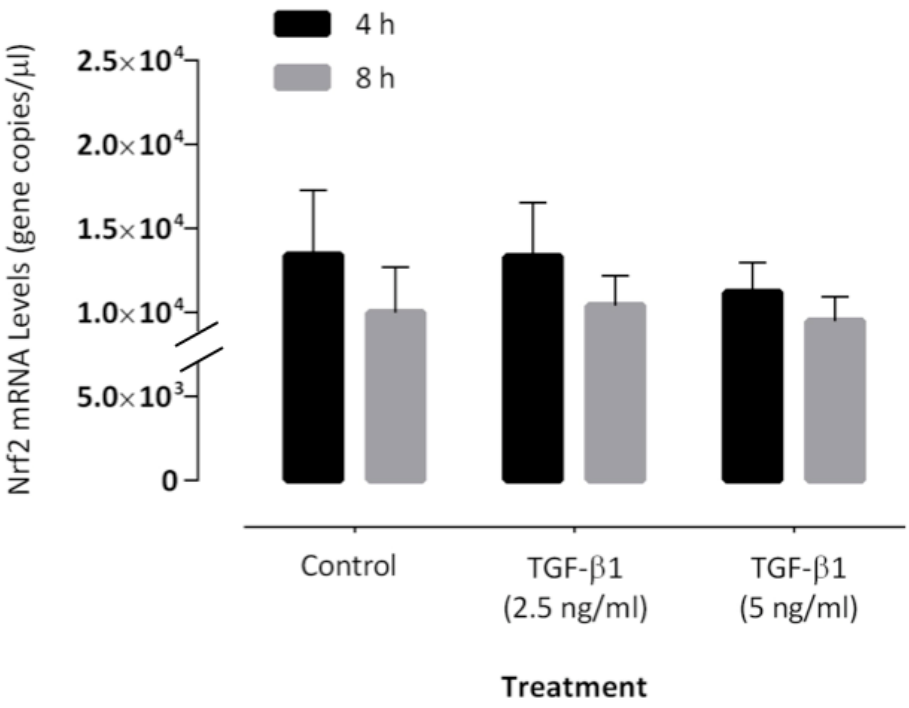


Figure 4.1 Effect of TGF-β1 on Nrf2 mRNA levels in HAoAF
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 – 5 ng/ml) for 4 or 8 h. Levels of Nrf2 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

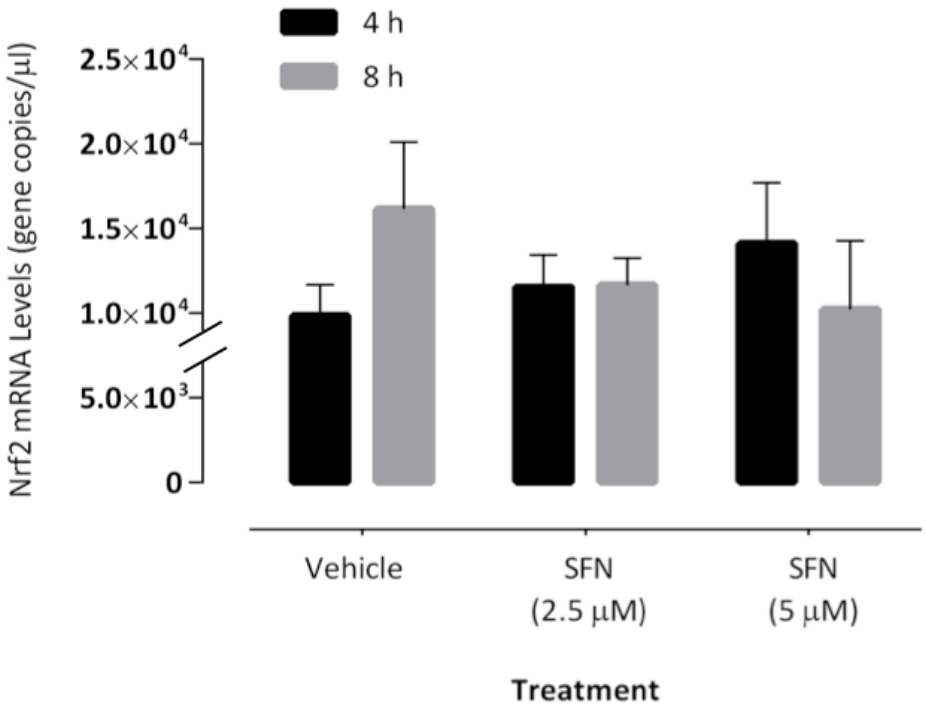


Figure 4.2 Effect of SFN on Nrf2 mRNA levels in HAoAF
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle control (DMSO 0.01%) or SFN (2.5 – 5 µM) for 4 or 8 h. Levels of Nrf2 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

4.2.2 Protein expression of total Nrf2 in HAoAF following treatment with TGF- β1 or SFN

To determine whether treatment of HAoAF with TGF- β1 or SFN elicited changes in the levels of Nrf2 protein in cells, confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF- β1 (0 - 5 ng/ml), vehicle control (DMSO 0.01%) or SFN (2.5 – 5 μM) for 4 – 24 h. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis (see Methods section 2.2). In untreated cells, there was basal Nrf2 protein expression (Fig 4.3a) at 4 – 24 h. Interestingly, treatment with TGF- β1 (2.5 ng/ml) resulted in a decrease in total Nrf2 protein expression when compared with control, with total Nrf2 declining after 8 h and then gradually increasing, but remaining below the levels observed in untreated control cells. Cells treated with TGF- β1 (5 ng/ml) exhibited a further decrease in Nrf2 protein expression at 24 h (Fig 4.1.3b). These immunoblot results indicate that TGF- β1 does not significantly alter total Nrf2 protein levels in HAoAF when compared with untreated control.

Treatment of cells with SFN (2.5 μM) did not appear to result in an increased protein expression of Nrf2 when compared with vehicle control. When treated with a higher concentration of SFN (5 μM), cells exhibited increased Nrf2 protein expression after 12 h which appeared to plateau after 12 h (Fig 4.1.4b). Although protein expression in response to SFN (5 μM) was higher than that seen in untreated control, it was not a significant increase.

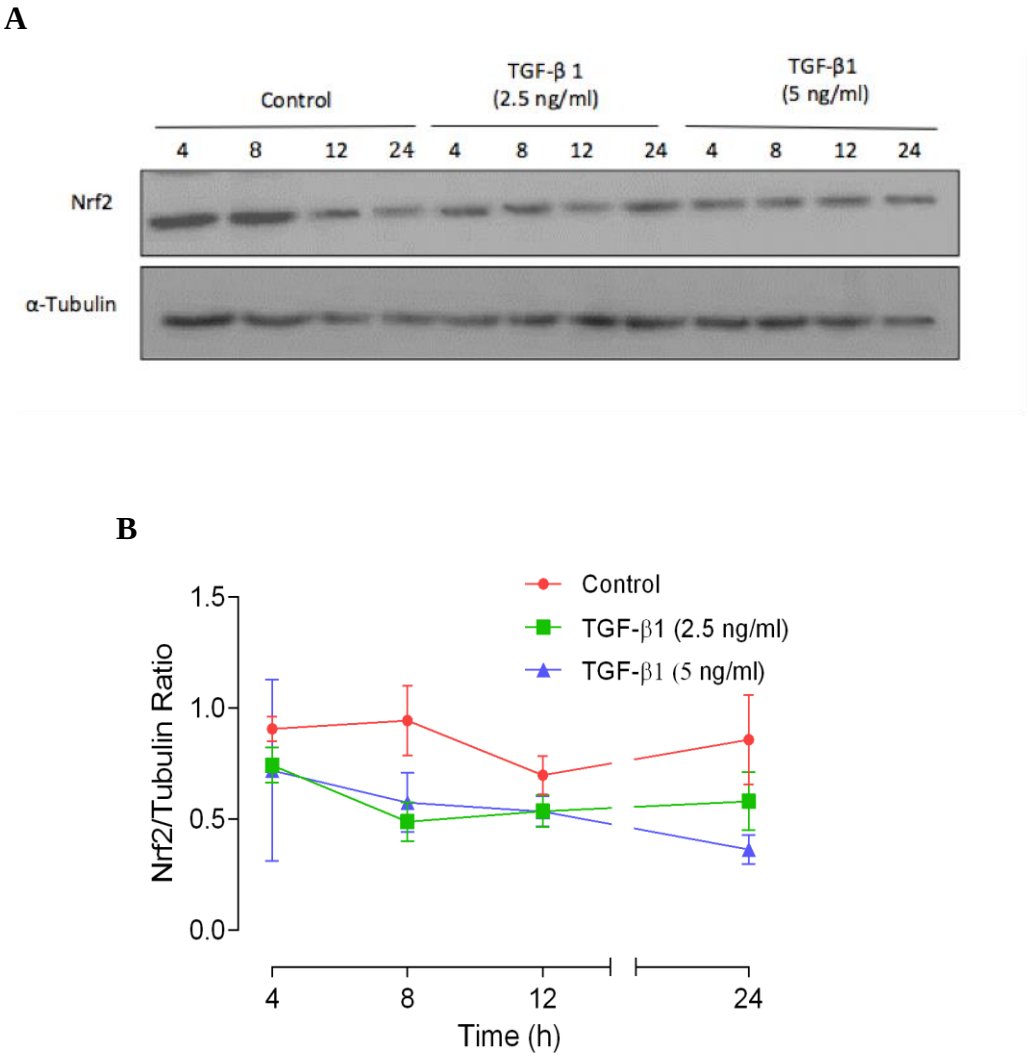


Figure 4.3 Nrf2 protein expression in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 – 5 ng/ml) for 4 - 24 h prior to whole cell protein extraction. Total Nrf2 protein levels in these samples were assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with Nrf2 corrected for the loading control, α-Tubulin. Values denote means ± SEM, n= 3-5 independent experiments.

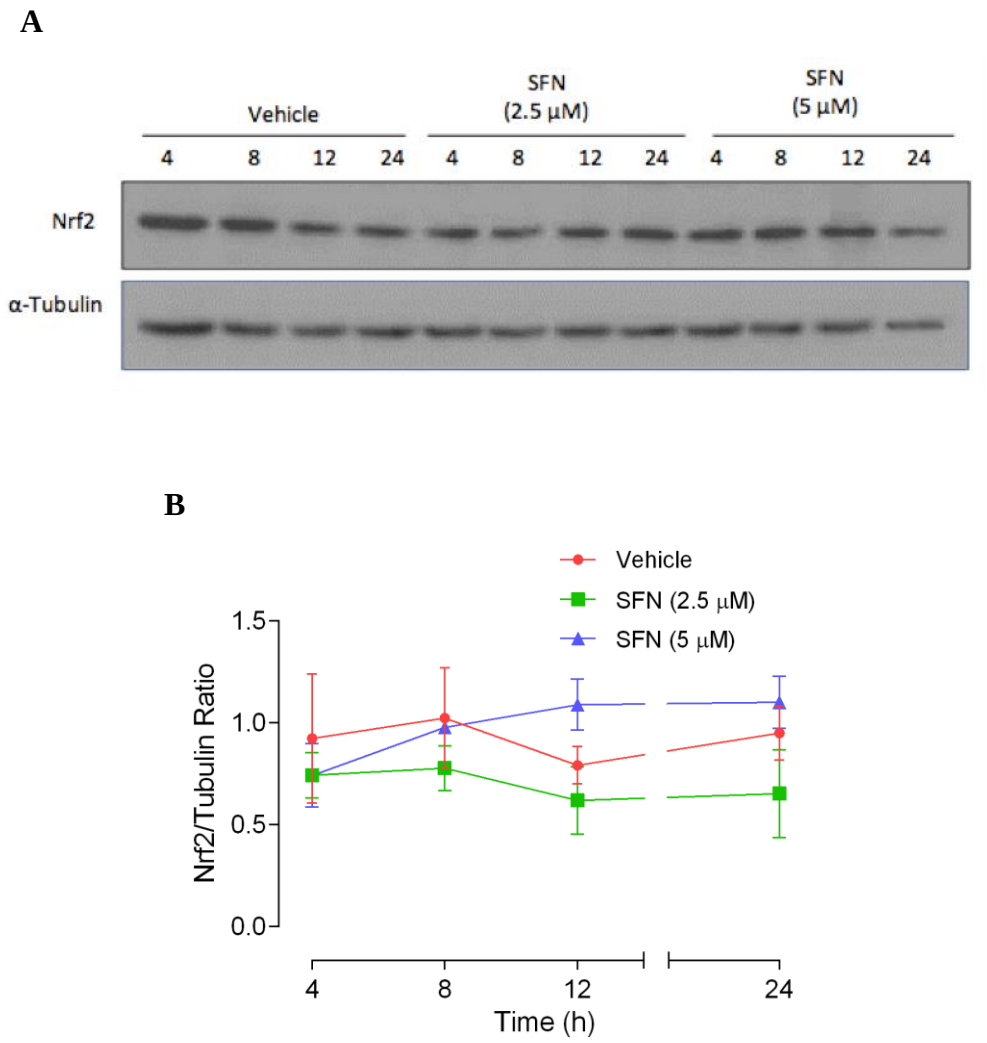


Figure 4.4 Nrf2 protein expression in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle control (DMSO 0.01%) or SFN (2.5 – 5 μM) for 4 - 24 h prior to whole cell protein extraction. Total Nrf2 protein levels in these samples were assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with Nrf2 corrected for the loading control, α-Tubulin. Values denote means ± SEM, n= 3-5 independent experiments.

4.3 HO-1 expression in HAoAF treated with TGF-β1 or SFN

4.3.1 HO-1 mRNA levels in HAoAF following treatment with TGF- β1 or SFN

As HO-1 is an antioxidant enzyme and is transcriptionally regulated by Nrf2, amongst other transcription factors (Jadhav et al., 2008; Lin et al., 2007; Morita, 2005), the levels of HO-1 mRNA following treatment of HAoAF with TGF- β1 (0 – 5 ng/ml) for 4 and 8 h were examined. After 4 h of treatment with TGF- β1 (2.5 and 5 ng/ml) there was no change in HO-1 mRNA levels when compared to untreated control (Fig 4.5). Similar observations were made following exposure of HAoAF to TGF- β1 (2.5 and 5 ng/ml) for 8 h, however, interestingly, there was a decrease in HO-1 mRNA levels across all treatments after 8 h of treatment (Fig 4.5), although this was not significant.

In cells treated with SFN (2.5 and 5 μM), a dose dependent increase in HO-1 mRNA levels was observed when compared with vehicle control (DMSO 0.01%), however this increase was non-significant (Fig 4.6). After 8 h, HAoAF treated with vehicle (DMSO 0.01%) or SFN (2.5 μM) exhibited increased HO-1 mRNA levels when compared to cells treated with the same treatments for 4 h. Conversely, treatment of HAoAF with SFN (5 μM) for 8 h resulted in a decrease in HO-1 mRNA levels when compared to 4 h treatment (Fig 4.6).

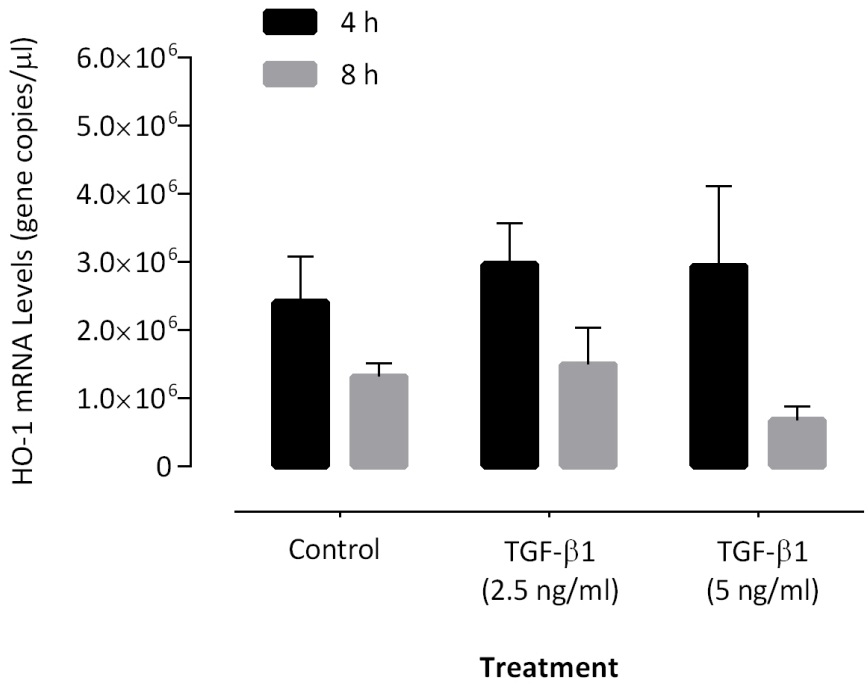


Figure 4.5 HO-1 mRNA levels in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS for 18 h and subsequently treated with TGF-β1 (0 – 5 ng/ml) for 4 or 8 h. Levels of HO-1 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

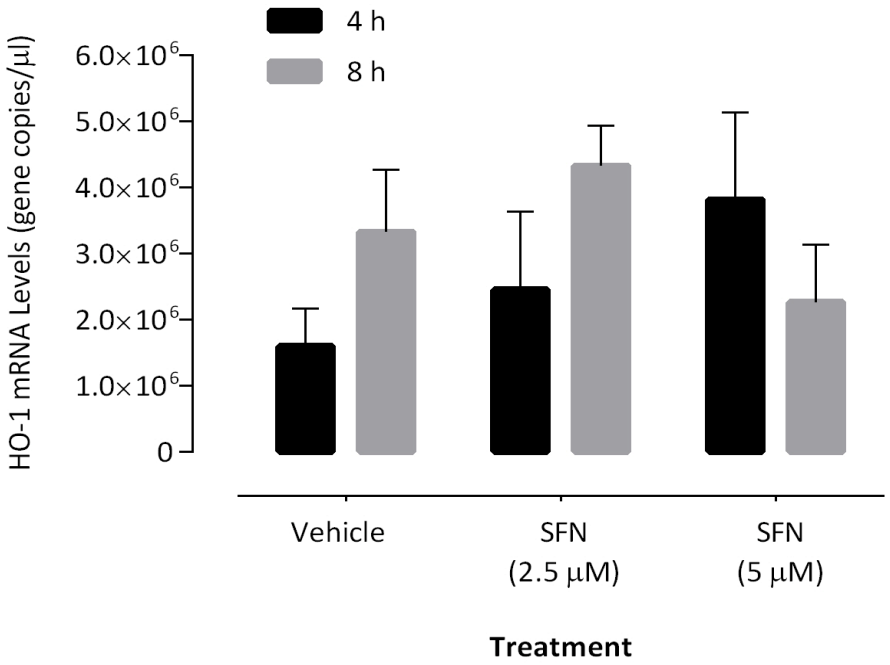


Figure 4.6 HO-1 mRNA levels in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle (DMSO 0.01%) or SFN (2.5 – 5 μM) for 4 or 8 h. Levels of HO-1 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

4.3.2 Protein expression of HO-1 in HAoAF following treatment with TGF- β1 or SFN

In addition to assessing the changes in mRNA levels of HO-1 in response to TGF- β1 or SFN, HO-1 protein levels were determined in HAoAF cell lysates treated with TGF- β1 or SFN. Confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF- β1 (0 - 5 ng/ml), vehicle control (DMSO 0.01%) or SFN (2.5 – 5 μM) for 4 – 24 h. Subsequently, cells were lysed and total cell protein was extracted and assessed using SDS-PAGE and Western blot analysis. Lysates were probed for a HO-1 specific antibody (see Methods section 2.2) and expressed relative to α-tubulin.

As shown in Fig 4.7, treatment with three different concentrations of TGF- β1 (2.5 - 10 ng/ml) resulted in the increased protein expression of HO-1 after 8 h when compared with untreated control. Results indicate that 4 h following treatment TGF- β1 there was little or no increase in HO-1 protein expression when compared with untreated control. After 8 h there was a significant increase in HO-1 protein expression ($p<0.05$) when compared with untreated control and this decreased after 24 h. When the three different concentrations of TGF- β1 are compared, 5 ng/ml was the concentration at which there was the highest induction of HO-1 protein expression (Fig 4.7B), with 8 h being the timepoint at which this induction was statistically significant. These results suggest that TGF- β1 (5 ng/ml) caused a significant increase in HO-1 protein expression after 8 h in HAoAF.

Treatment with three different concentrations of SFN (2.5 – 10 μM) resulted After 8 h there was a significant increase in HO-1 protein expression ($p<0.05$) when compared with untreated control and this decreased after 24 h. Peak induction of HO-1 protein expression occurred at 8 h for all concentrations of SFN used to treat cells. These results suggest that SFN (5 and 10 μM, 8 h) caused a significant increase in HO-1 protein expression after 8 h in HAoAF (Fig 4.8A and B).

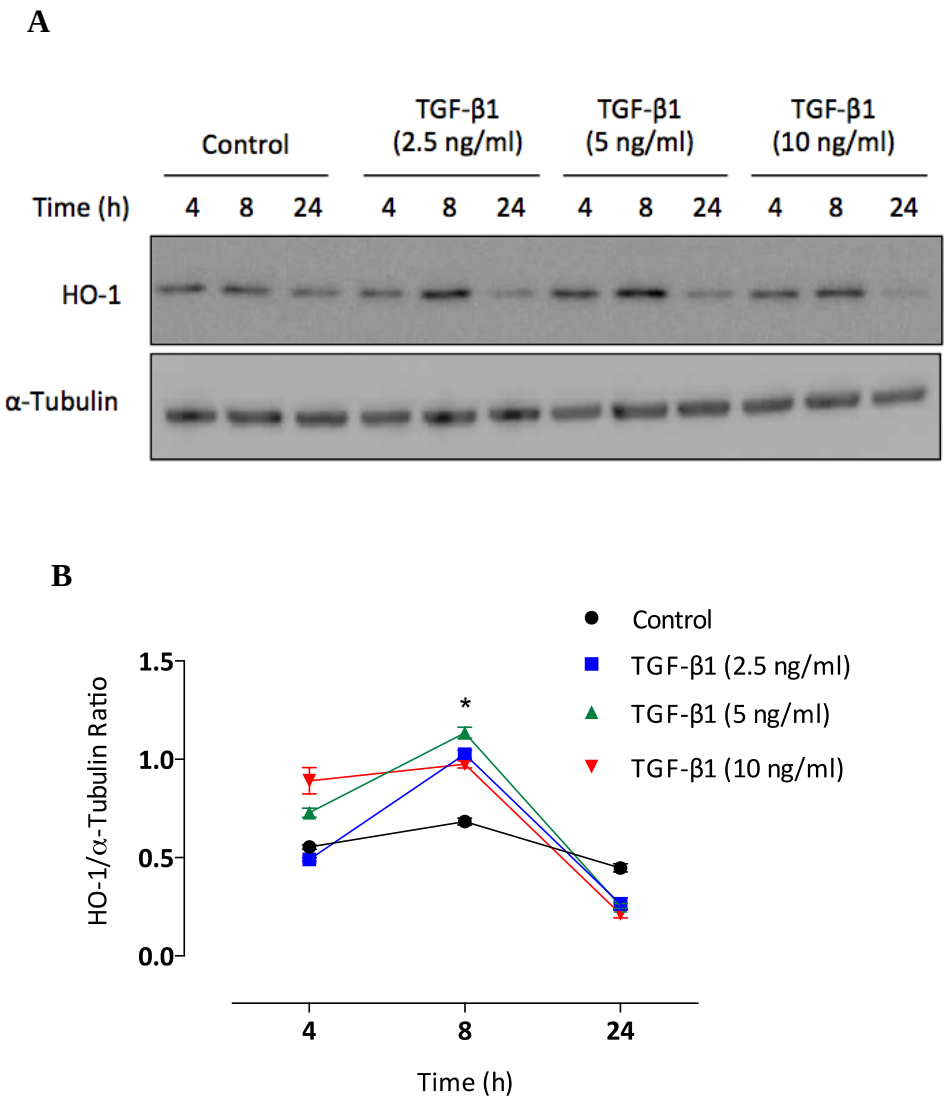


Figure 4.7 HO-1 protein expression in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 – 10 ng/ml) for 4 - 24 h prior to whole cell protein extraction. Total HO-1 protein levels in these samples were assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with HO-1 corrected for the loading control, α-Tubulin. Values denote means ± SEM, *p<0.05 TGF- β1 (5 ng/ml) vs. untreated control using unpaired Student's t-test. n = 3-5 independent experiments.

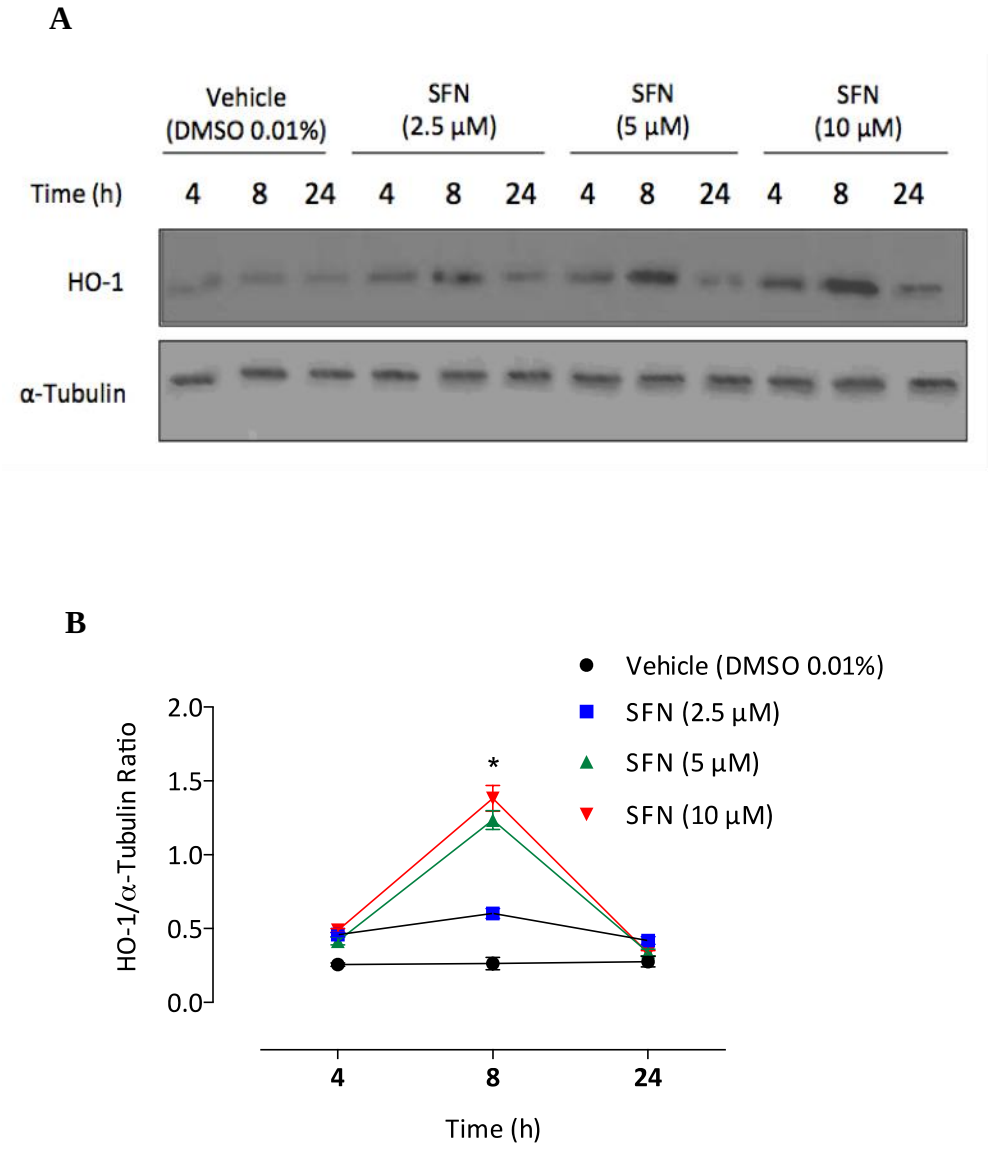


Figure 4.8 HO-1 protein expression in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle (DMSO, 0.01%) or SFN (2.5 µM – 10 µM) for 4 - 24 h prior to whole cell protein extraction. Total HO-1 protein levels in these samples were assessed using SDS-PAGE and western blot analysis. Results were analysed by densitometry with HO-1 corrected for the loading control, α-Tubulin. Values denote means ± SEM, **p*<0.05 SFN (5 and 10 µM) vs. untreated control using unpaired Student's t-test. n = 3-5 independent experiments.

4.4 NQO1 expression in HAoAF treated with TGF-β1 or SFN

4.4.1 NQO1 mRNA levels in HAoAF following treatment with TGF- β1 or SFN

The transcriptional activation of NQO1 is regulated by Nrf2 (Miller et al., 2012; Oh et al., 2012), therefore levels of NQO1 mRNA in HAoAF in response to treatment with TGF- β1 (0 – 5 ng/ml) and SFN (2.5 and 5 μM) were determined. The same samples that were used to determine Nrf2 and HO-1 mRNA levels were also used to determine NQO1 mRNA levels. Following treatment with TGF-β1 (2.5 and 5 ng/ml) for 4 h, there was no change in levels of NQO1 mRNA when compared to untreated control (Fig 4.9). After 8 h, similar observations were made, however treatment with TGF- β1 (2.5 ng/ml) resulted in a decrease in NQO1 mRNA levels, although this was not significant (Fig 4.9).

NQO1 mRNA levels in cells treated with SFN (2.5 and 5 μM) did not change after 4 h in comparison with vehicle control (DMSO 0.01%). This was also observed in cells treated for 8 h; although all treatment groups displayed a slight increase in NQO1 levels after 8 h of exposure to either vehicle control or SFN when compared to 4 h, this increase was not statistically significant. Treatment with SFN (2.5 and 5 μM) for 8 h did not increase NQO1 mRNA levels in HAoAF in comparison to vehicle control (DMSO 0.01%) at 8 h (Fig 4.10).

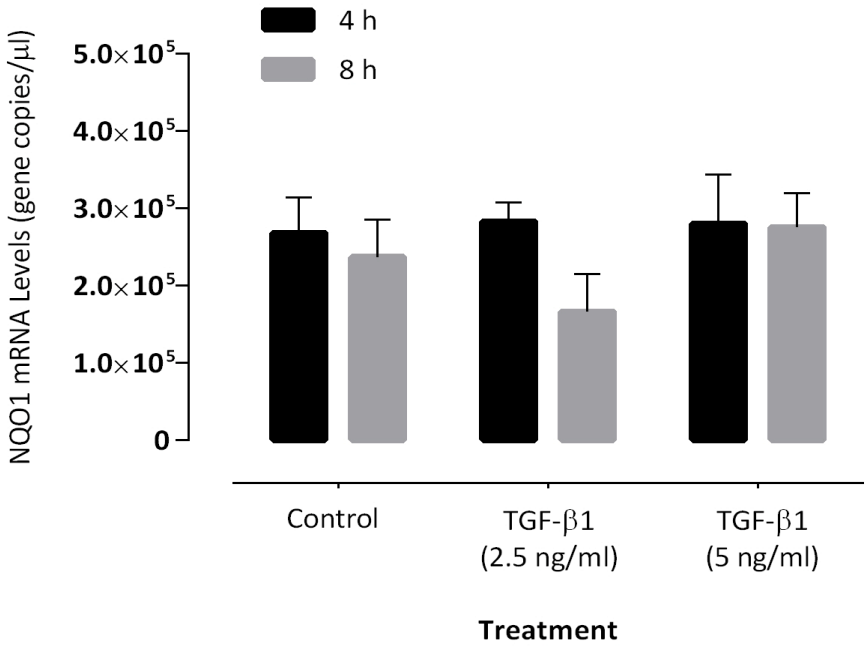


Figure 4.9 NQO1 mRNA levels in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 – 5 ng/ml) for 4 or 8 h. Levels of NQO1 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

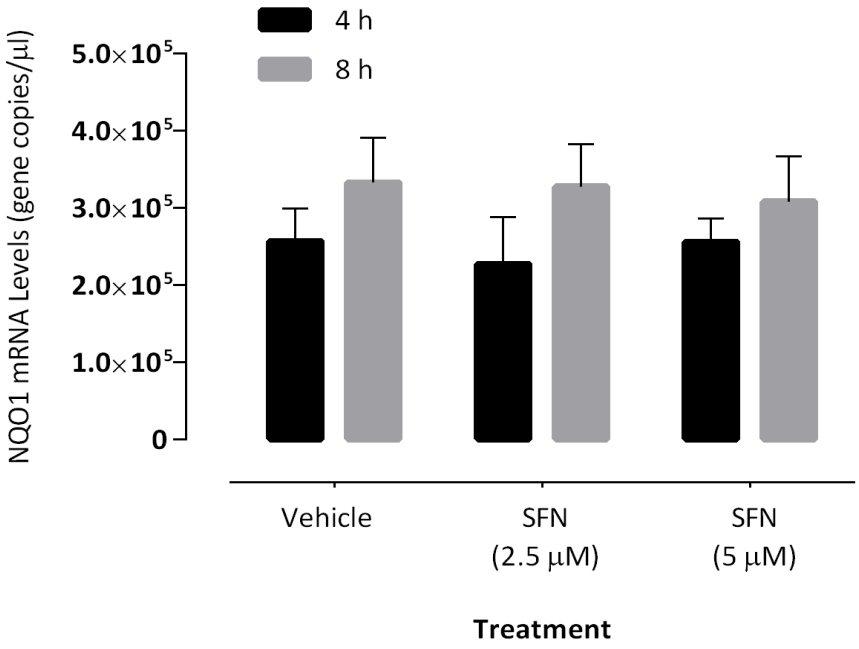


Figure 4.10 NQO1 mRNA levels in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with vehicle (DMSO 0.01%) or SFN (2.5 – 5 µM) for 4 or 8 h. Levels of NQO1 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = 6 independent experiments.

4.4.2 Protein expression of NQO1 in HAoAF following treatment with TGF- β1 or SFN

Expression of NQO1 protein was also determined in HAoAF treated with TGF-β1. After 4 and 8 h, treatment of HAoAF with TGF-β1 (2.5 and 5 ng/ml) resulted in a slight increase in NQO1 protein expression when compared with untreated control, and these levels appeared to decrease after 12 h of treatment (TGF-β1 5 ng/ml). After 24 h, levels of NQO1 protein in TGF-β1-treated cells was below that observed in untreated cells (Fig 4.11). Changes in NQO1 protein expression in cells following exposure to TGF-β1 were not statistically significant.

Cells treated with SFN exhibited lower levels of NQO1 protein expression than vehicle control (DMSO 0.01%) after 4 and 8 h of treatment (Fig 4.3.4a). This increased after 8 h and continued to rise after 12 h. After 24 h, SFN-treated cells had higher levels of NQO1 protein expression than vehicle control, though this was not statistically significant (Fig 4.12).

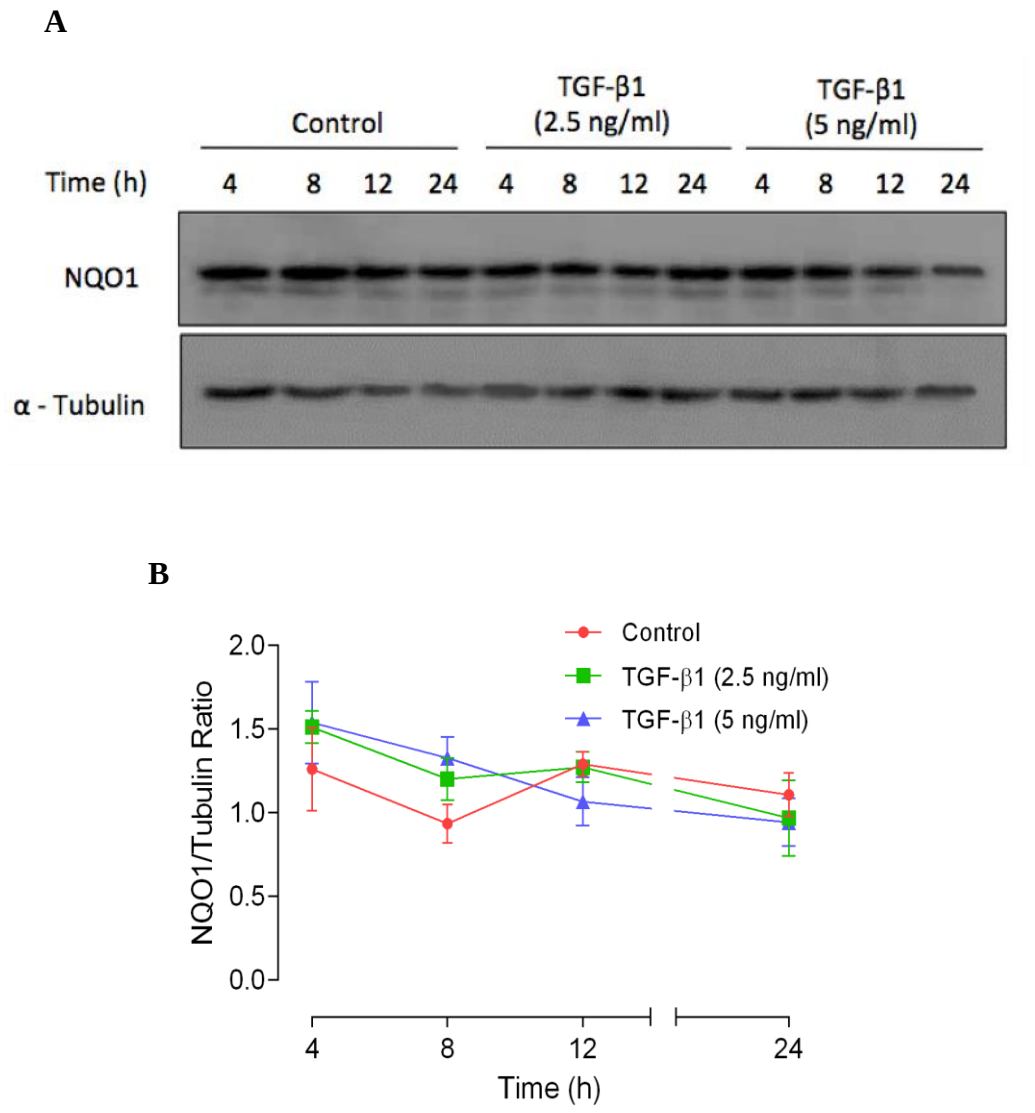


Figure 4.11 NQO1 protein expression in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 – 5 ng/ml) for 4, 8, 12 or 24 h prior to whole cell protein extraction. Total NQO1 protein levels in these samples were assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with NQO1 corrected for the loading control, α-Tubulin. Values denote means ± SEM, n = 3-5 independent experiments.

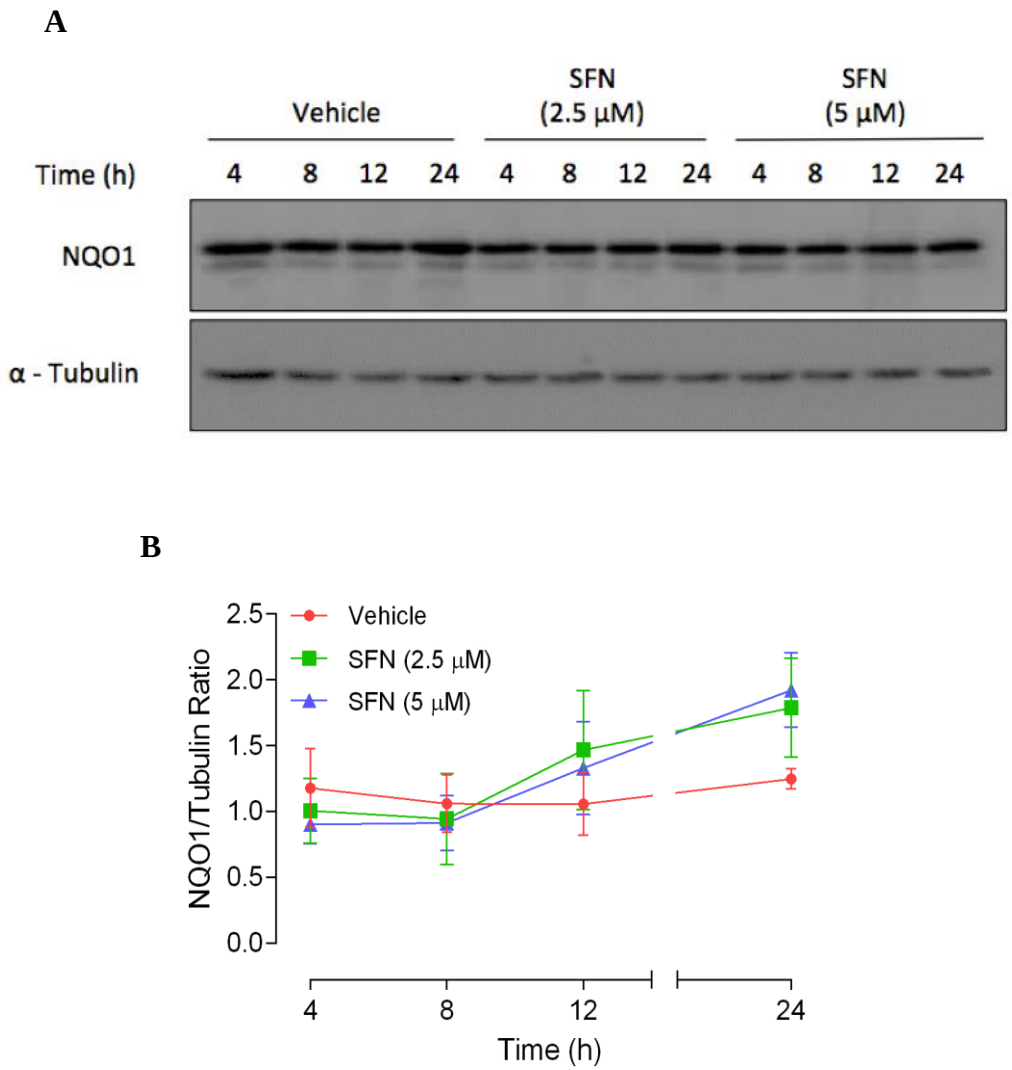


Figure 4.12 NQO1 protein expression in HAoAF after treatment with SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with either vehicle control (DMSO 0.01%) or SFN (2.5 – 5 µM) for 4, 8, 12 or 24 h prior to whole cell protein extraction. Total NQO1 protein levels in these samples were assessed using SDS-PAGE and Western blot analysis. Results were analysed by densitometry with NQO1 corrected for the loading control, α-Tubulin. Values denote means ± SEM, n = 3-5 independent experiments.

4.5 Levels of intracellular GSH in HAoAF treated with TGF-β1 or SFN

Intracellular GSH levels are an important indicator of a cell's redox status and are often used as a determinant of whether the cell/tissue has been exposed to oxidative stress. Several studies have reported that that TGF-β1 causes a depletion in intracellular GSH levels and as a consequence, induces oxidative stress during tissue remodelling or fibrotic processes observed in bronchial asthma, chronic pulmonary disease, idiopathic pulmonary fibrosis and vascular remodelling (Liu et al., 2012; Sugiura et al., 2009; Bakin et al., 2005; White et al., 1992). However, others have reported that TGF-β1 attenuates glutathione depletion and protects against oxidative stress (Pauly et al., 2011). Similarly, SFN has also been shown to deplete intracellular GSH levels by initially and rapidly conjugating with glutathione-*S*-transferases (GST; Munday et al., 2008; Dinkova-Kostova et al., 2007). SFN has also been observed as increasing the expression of glutathione reductase by activating the Nrf2/ARE pathway (Xue et al., 2008) and when administered in the diet, it has been associated with a lower risk of myocardial infarction (Cornelis et al., 2007). In order to elucidate the effects of TGF-β1 and SFN on intracellular GSH levels in HAoAF, cells were treated with TGF-β1 or SFN and intracellular GSH levels were measured.

Confluent cultures of HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 -24 h prior to incubating with TGF-β1 (5 ng/ml), vehicle control (DMSO 0.01%) or SFN (5 μM) for a further 0 – 24 h. Intracellular reduced GSH was analysed using a fluorescence assay as described in Methods (see Methods section 2.6.1 and 2.6.2). GSH levels in cells treated with TGF-β1 remained unchanged when compared to untreated control (Fig 4.13). After 8 h, there was an increase in intracellular GSH levels in both untreated control and TGF-β1 treated cells, which returned to baseline after 24 h.

When treated with SFN, there was an initial and significant depletion in intracellular GSH levels in SFN-treated cells after 2 – 4 h ($p<0.05$ SFN vs. control, 2 and 4 h) of when compared to control. This initial depletion recovered after 8 h and returned to just above baseline after 24 h (Fig 4.14).

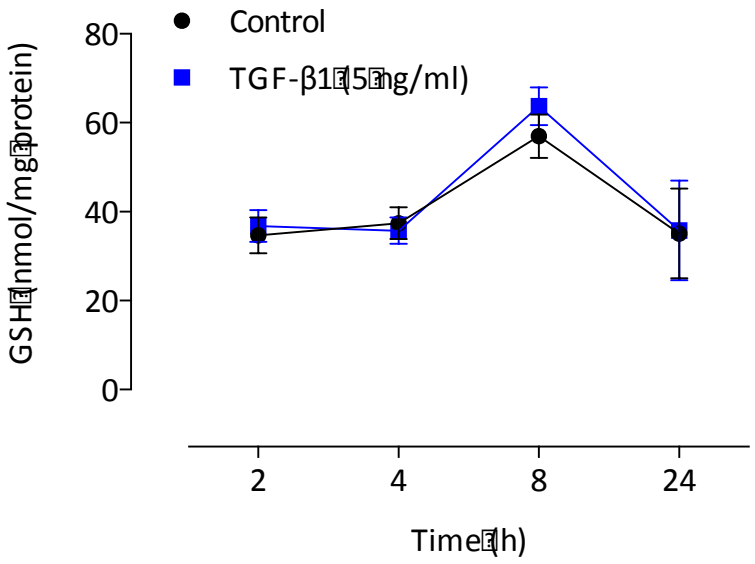


Figure 4.13 Glutathione levels in HAoAF treated with TGF-β1
Cells were treated with TGF-β1 (5 ng/ml) for 2, 4, 8, 24 and subsequently incubated with 6.5% TCA. Following incubation, the supernatant was removed from cells and total glutathione levels were determined using the opthalaldehyde assay. Glutathione was expressed relative to cellular protein content. Values denote means ± SEM. n = 3 independent cell cultures.

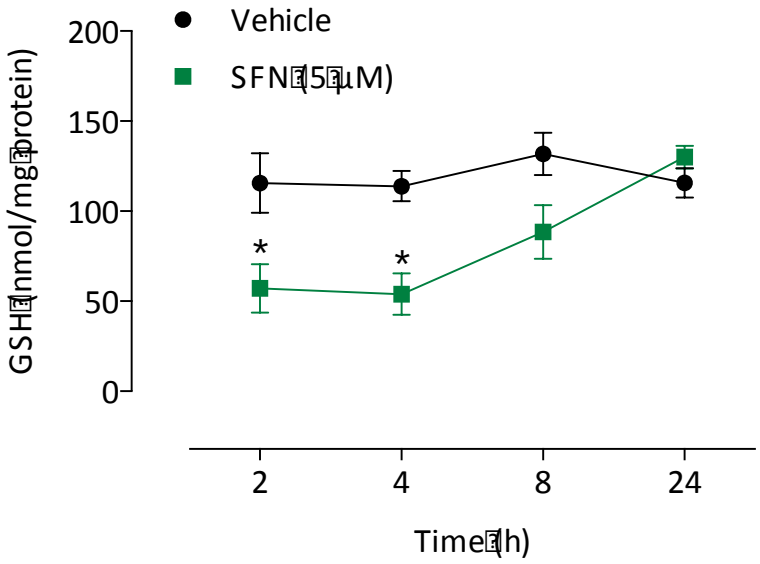


Figure 4.14 Glutathione levels in HAoAF treated with SFN
Cells were treated with vehicle (DMSO 0.01%), SFN (5 μM) for 2, 4, 8, 24 and subsequently incubated with 6.5% TCA. Following incubation the supernatant was removed from cells and total glutathione levels were determined using the opthalaldehyde assay. Glutathione was expressed relative to cellular protein content. Values denote means ± SEM, *p<0.05 SFN vs. vehicle control using unpaired Student's t-test. n = 3 independent cell cultures.

4.6 Nuclear levels of Nrf2 in HAoAF treated with TGF- β1 or SFN

Activation of the Nrf2 pathway by oxidants and the consequent downstream transcription of antioxidant enzymes is a process that is largely dependent on the translocation of Nrf2 to the nucleus and its subsequent binding to the ARE in the promoter region of target genes. Therefore, changes in the levels of Nrf2 protein in the nucleus can be indicative of activation of the Nrf2/ARE signalling pathway. The following set of experiments sought to determine whether treatment of HAoAF with either TGF- β1 or SFN resulted in Nrf2/ARE activation and were assessed using Western blot and an ELISA-based binding activity assay (TransAM) of nuclear extracts from treated HAoAF. Nrf2-specific immunofluorescence was also carried out in order to determine Nrf2 subcellular localisation. It has been reported that nuclear import of Nrf2 following stimulation with a biological challenge is a rapid process and can occur within 15 min of exposure (Kaspar and Jaiswal., 2010). Furthermore, there is evidence to suggest that this initial nuclear import of Nrf2 begins to decline 4 hours after exposure to the challenge (He at al., 2011). Therefore, nuclear levels of Nrf2 were assessed between 1 – 4 hours of exposure to either TGF- β1 or SFN.

4.6.1 Expression of nuclear Nrf2 in HAoAF following treatment with TGF- β1 or SFN

To determine whether TGF- β1 or SFN elicited an increase in the translocation of Nrf2 to the nucleus in HAoAF, confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment for 1 – 4 h. Subsequently cells were lysed and cell fractionation was carried out using a commercially available kit in order to obtain nuclear extracts (see Methods section 2.4) which were then analysed using SDS-PAGE and Western blot. Nuclear Nrf2 levels were expressed relative to lamin C. After 1 h, there was no increase in the protein expression of nuclear Nrf2 with either TGF- β1 or SFN treatment. There was basal protein expression of Nrf2 in the cytoplasm at 1 h and this was significantly higher than Nrf2 protein observed in the nucleus (Fig 4.15; $p<0.05$, untreated control, nuclear vs. cytoplasmic fraction; $p<0.05$, TGF- β1-treated, nuclear vs. cytoplasmic fraction; $p<0.01$, SFN-treated, nuclear vs. cytoplasmic fraction). At 2 h, there was an increase in nuclear Nrf2 levels with both TGF- β1 and SFN treated cells when compared to cytosolic levels; an effect that was statistically significant (Fig 4.16; $p<0.05$, untreated control, nuclear vs. cytoplasmic fraction; $p<0.01$, TGF- β1-treated, nuclear vs. cytoplasmic fraction; $p<0.001$, SFN-treated, nuclear vs. cytoplasmic

fraction) as analysed by densitometry. Levels of cytoplasmic Nrf2 appeared to decrease when compared to those seen at the 1 h timepoint. After 4 h, levels of nuclear Nrf2 had returned to baseline in all groups treated, whilst levels in the cytosol increased significantly when compared to nuclear levels (Fig 4.17; $p<0.01$, untreated control, nuclear vs. cytoplasmic fraction; $p<0.01$, TGF-β1-treated, nuclear vs. cytoplasmic fraction; $p<0.001$, SFN-treated, nuclear vs. cytoplasmic fraction) suggesting that Nrf2 had transclocated out of the nucleus after 4 h.

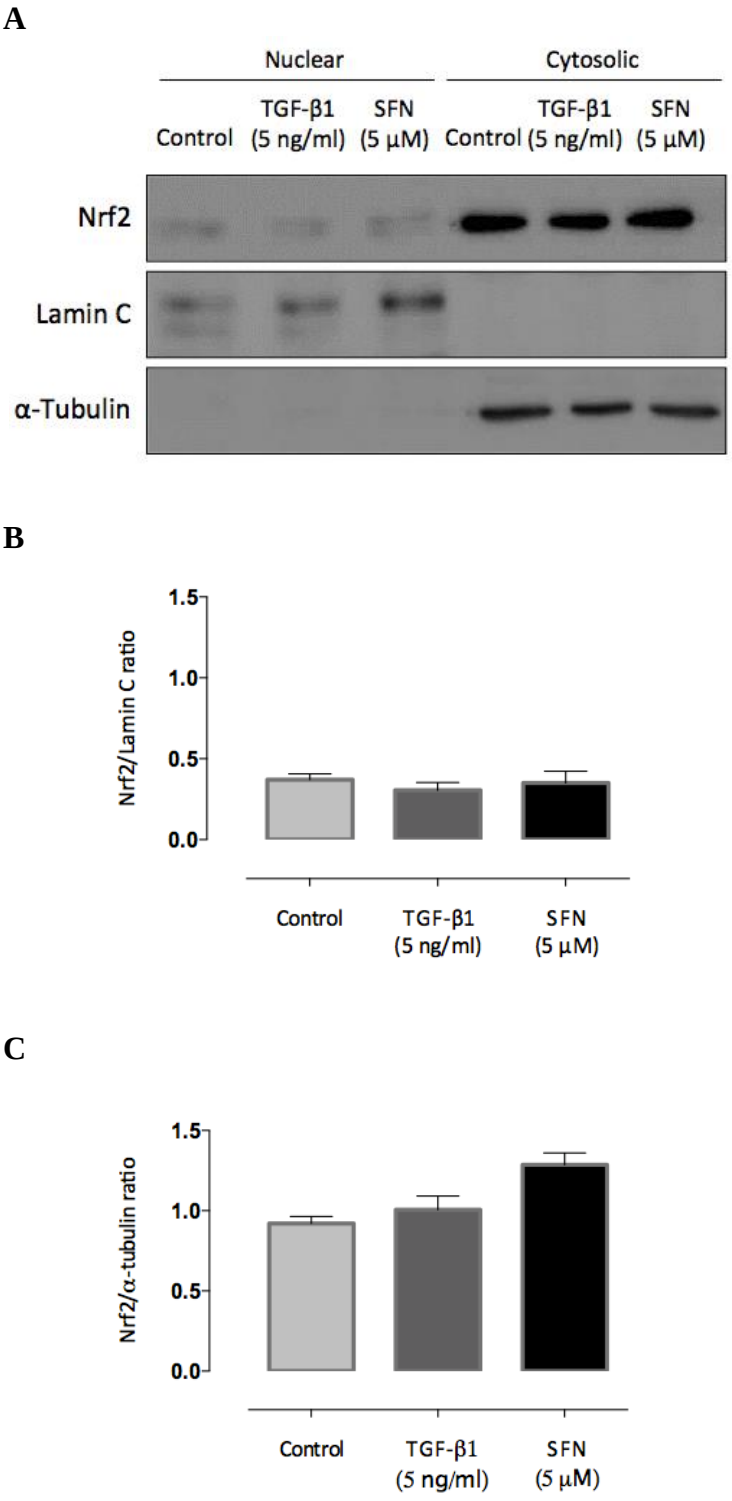


Figure 4.15 Nuclear and cytosolic Nrf2 levels in HAoAF following TGF-β1 or SFN treatment for 1 h. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with either TGF-β1 (5 ng/ml) or SFN (5 μM) for 1 h. Levels of Nrf2 (66 kDa) in (B) nuclear and (C) cytosolic extracts was determined by Western blotting. Results were analysed by densitometric analysis and nuclear Nrf2 was corrected for the nuclear reference protein, lamin C (62 kDa). Cytosolic Nrf2 levels were corrected for α-Tubulin (52 kDa). Values denote means ± SEM. No significant changes were observed in Nrf2 levels in treatment vs. control.

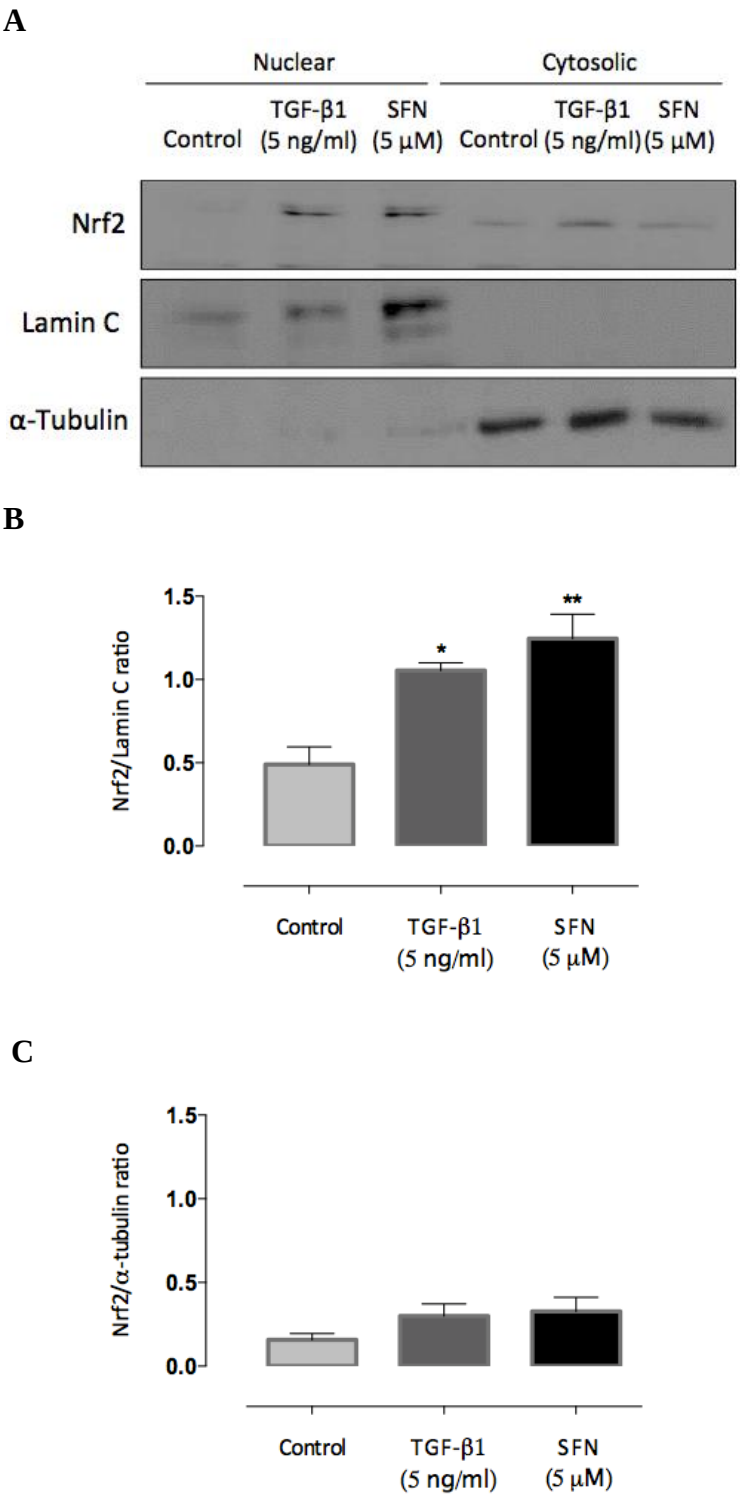


Figure 4.16 Nuclear and cytosolic Nrf2 levels in HAoAF following TGF-β1 or SFN treatment for 2 h. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with either TGF-β1 (5 ng/ml) or SFN (5 μM) for 1 h. Levels of Nrf2 (66 kDa) in (B) nuclear and (C) cytosolic extracts was determined by Western blotting. Results were analysed by densitometric analysis and nuclear Nrf2 was corrected for the nuclear reference protein, lamin C (62 kDa). Cytosolic Nrf2 levels were corrected for α-Tubulin (52 kDa). Values denote means ± SEM. *p<0.05, TGF-β1-treated nuclear fraction vs. control-treated nuclear fraction; **p<0.01, SFN-treated nuclear fraction vs. control-treated nuclear fraction using unpaired Student's t-test. n = 3-5 independent experiments.

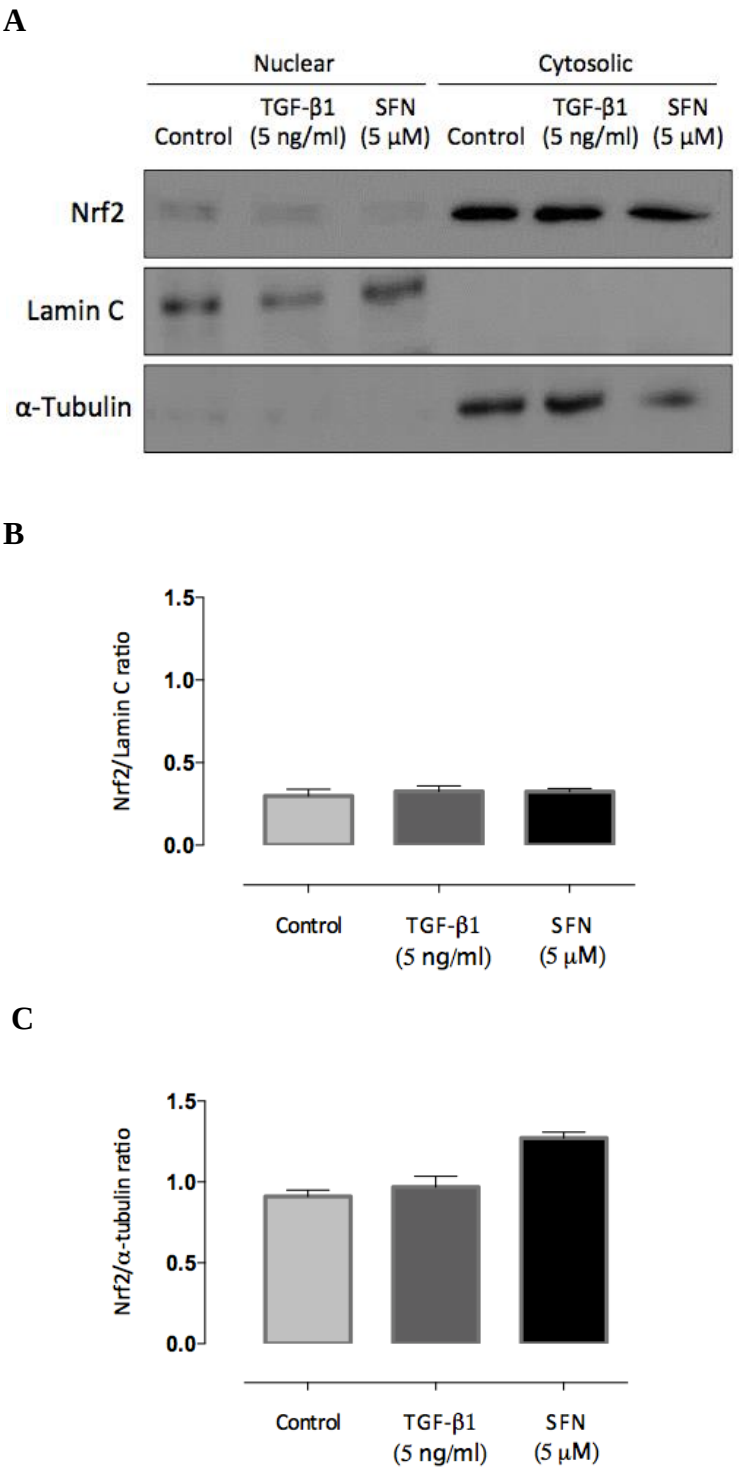


Figure 4.17 Nuclear and cytosolic Nrf2 levels in HAoAF following TGF-β1 or SFN treatment for 4 h. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with either TGF-β1 (5 ng/ml) or SFN (5 μM) for 1 h. Levels of Nrf2 (66 kDa) in (B) nuclear and (C) cytosolic extracts was determined by Western blotting. Results were analysed by densitometric analysis and nuclear Nrf2 was corrected for the nuclear reference protein, lamin C (62 kDa). Cytosolic Nrf2 levels were corrected for α-Tubulin (52 kDa). Values denote means ± SEM. No significant changes were observed in Nrf2 levels in treatment vs. control. n = 3-5 independent experiments.

4.6.2 Nrf2 immunofluorescence in HAoAF treated with TGF-β1 or SFN

In addition to Western blot analysis, immunofluorescent staining of Nrf2 was performed in HAoAF in order to assess Nrf2 subcellular localisation in response to TGF-β1 (5 ng/ml) or SFN (5 μM). Immunofluorescence is a widely used technique to determine the subcellular localisation of Nrf2 in various cell types (Zhang et al., 2012; Tanaka et al., 2011; Papaiahgari et al., 2006; Papaiahgari et al. 2004), which allows the simultaneous visualisation of changes within the cytosol and nuclei of cells that have been treated with different stimuli. Sub-confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (5 ng/ml) or SFN (5 μM) for 1, 2 or 4 h and subsequently fixed and incubated with an Nrf2-specific antibody (see Methods section 2.4.2). Nuclei were stained with DAPI and F-actin was stained with rhodamine-conjugated phalloidin. Nrf2 was localised primarily in the cytosol of cells treated with either TGF-β1 (5 ng/ml) or SFN (5 μM) for 1 h and was comparable to untreated control (Fig 4.18A, panels G-I). After 2 h of treatment with TGF-β1 (5 ng/ml), there was an increase in nuclear Nrf2 (Fig 4.4.2b, panel H) when compared to that seen in the nuclei of untreated control cells (Fig 4.18B panel G). SFN (5 μM) also caused an increase in the nuclear accumulation of Nrf2 after 2 h. After 4 h, in TGF-β1 (5 ng/ml) treated cells, Nrf2 had moved out of the nucleus and was primarily located in the cytosol (Fig 4.18C, panel H); SFN (5 μM) treated cells displayed more diffuse staining with Nrf2 located in the cytosol with some remaining in the nucleus (Fig 4.18C, panel I). This immunofluorescence data is consistent with Western blot analysis (Figs. 4.13-4.15) and indicates that Nrf2 nuclear translocation occurs 2 h following treatment of HAoAF with TGF-β1 (5 ng/ml) or SFN (5 μM).

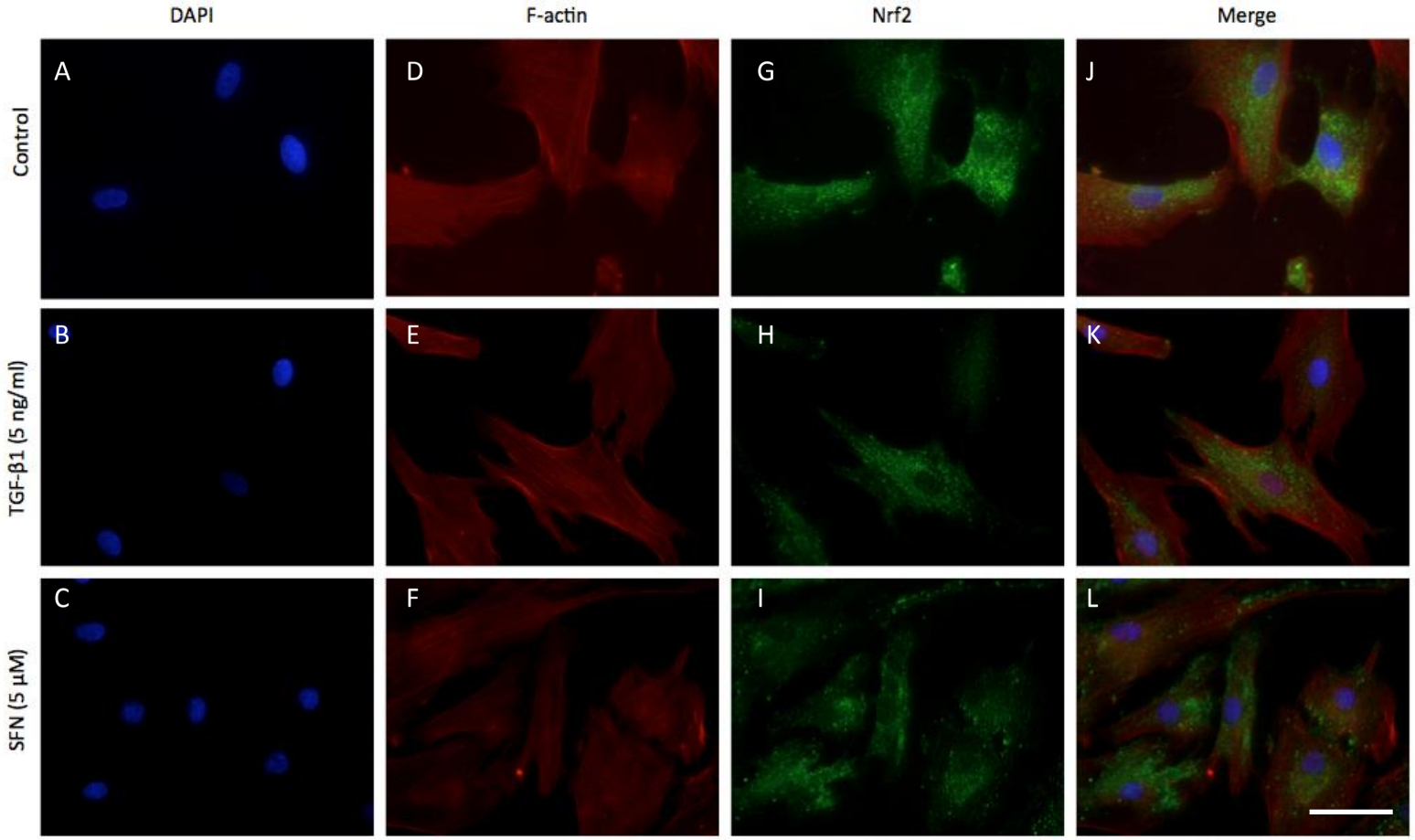


Figure 4.18 (A) Immunofluorescent staining of HAoAF showing Nrf2 nuclear translocation after treatment with TGF-β1 or SFN for 1 h.
Sub-confluent cultures of HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml) or SFN (5 µM) for 1 h. Following treatment, cells were fixed and incubated with specific antibody against F-actin and Nrf2 and a fluorescent secondary antibody (green, excitation/emission 475/455) and viewed using a fluorescence microscope (scale bar = 20 µm). n = 3 independent experiments.

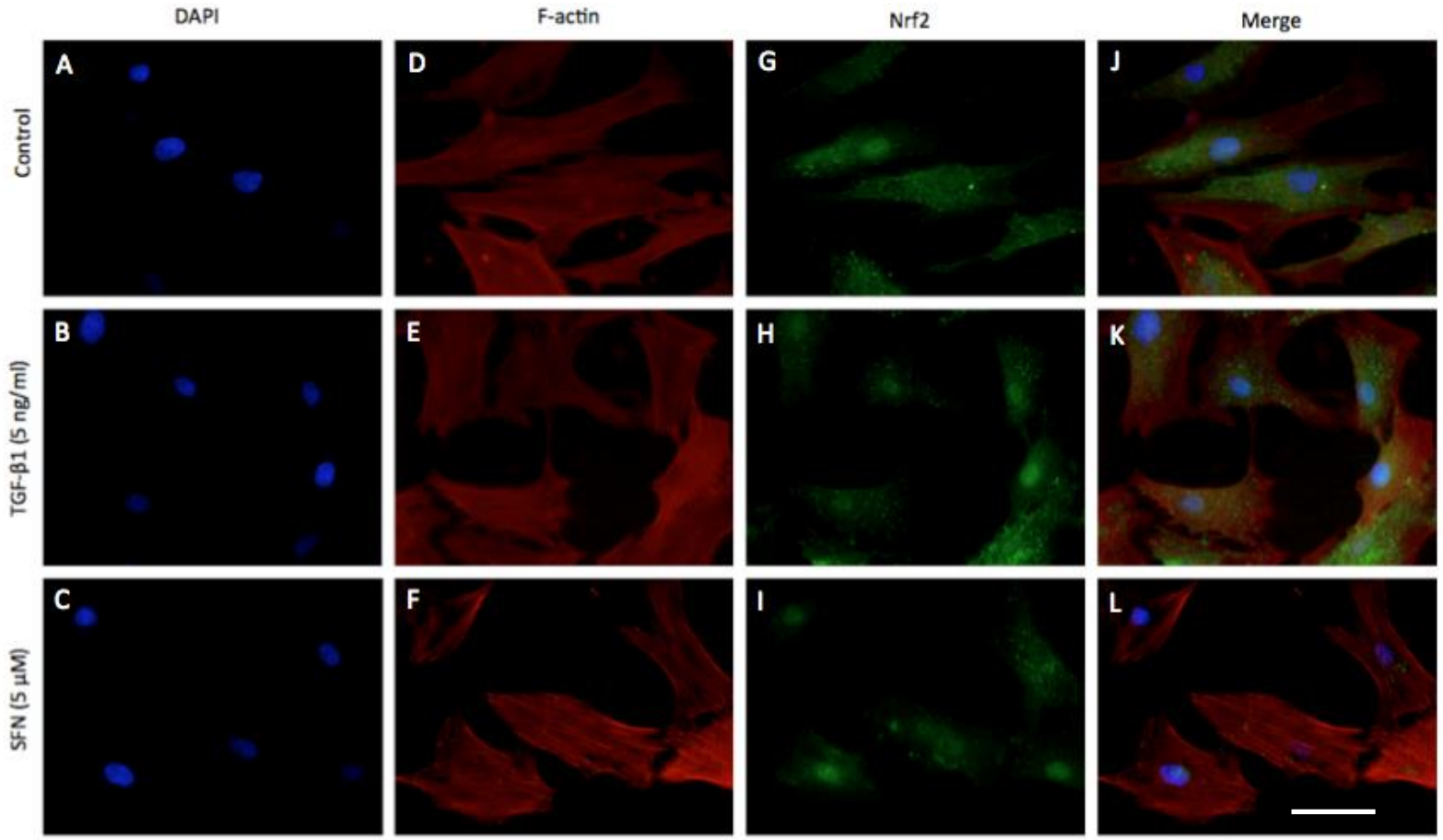


Figure 4.18 (B) Immunofluorescent staining of HAoAF showing Nrf2 nuclear translocation after treatment with TGF-β1 or SFN for 2 h.
Sub-confluent cultures of HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml) or SFN (5 μM) for 2 h. Following treatment, cells were fixed and incubated with specific antibody against F-actin and Nrf2 and a fluorescent secondary antibody (green, excitation/emission 475/455) and viewed using a fluorescence microscope (scale bar = 20 μm). n = 3 independent experiments.

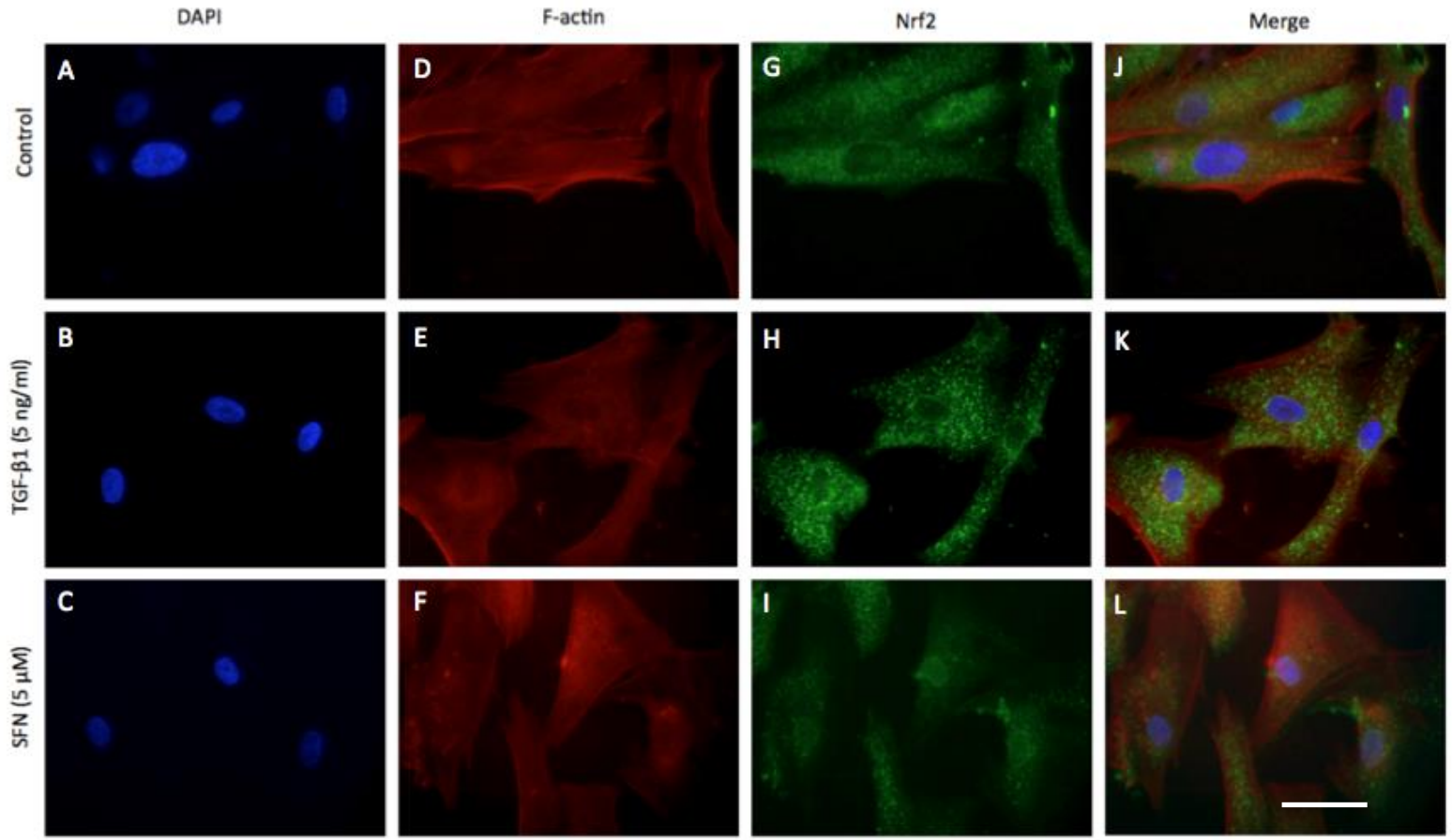


Figure 4.18 (C) Immunofluorescent staining of HAoAF showing Nrf2 nuclear translocation after treatment with TGF-β1 or SFN for 4 h.
Sub-confluent cultures of HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml) or SFN (5 µM) for 4 h. Following treatment, cells were fixed and incubated with specific antibody against F-actin and Nrf2 and a fluorescent secondary antibody (green, excitation/emission 475/455) and viewed using a fluorescence microscope (scale bar = 20 µm). n = 3 independent experiments.

4.6.3 Nuclear Nrf2-ARE binding activity in HAoAF treated with TGF-β1 or SFN

Although nuclear translocation of Nrf2 is associated with the induction of downstream antioxidant enzymes, it does not necessarily directly correlate with Nrf2-mediated gene transcription (Kratschmar et al., 2012). Activation of the Nrf2 pathway requires the binding of Nrf2 to the ARE in the promoter region of antioxidant genes in order to elicit gene transcription. Nrf2 nuclear accumulation occurs 2 h after treatment with TGF-β1 or SFN; therefore, the binding of Nrf2 in nuclear lysates obtained from cells treated with TGF-β1 (5 ng/ml) or SFN (5 μM) for 2 h was investigated using a commercially available ELISA assay (Active Motif, see Methods section 2.4). The kit allows measurement of binding of the transcription factor in the nuclear extracts to consensus ARE oligonucleotides (Afonyushkin et al., 2010). In nuclear extracts from cells treated with TGF-β1 (2.5 ng/ml and 5 ng/ml), there was no significant change in nuclear binding of Nrf2 to the ARE when compared to untreated control (Fig 4.19A). In SFN (2.5 – 5 μM) treated cells appeared to be an increase in Nrf2 binding activity in a dose dependent manner. SFN-treated extracts had a significantly increased Nrf2 binding activity (Fig 4.19B; $p<0.05$, SFN (2.5 μM) vs. vehicle control (DMSO 0.01%), $p<0.05$, SFN (5 μM) vs. vehicle control (DMSO 0.01%)). These results indicate that although TGF-β1 may cause an accumulation of Nrf2 in the nuclei of HAoAF, this may not be indicative of Nrf2-ARE binding and therefore activation of the Nrf2 signalling pathway, however SFN treatment results in the binding of Nrf2 to the ARE and is therefore indicative of activation of this pathway.

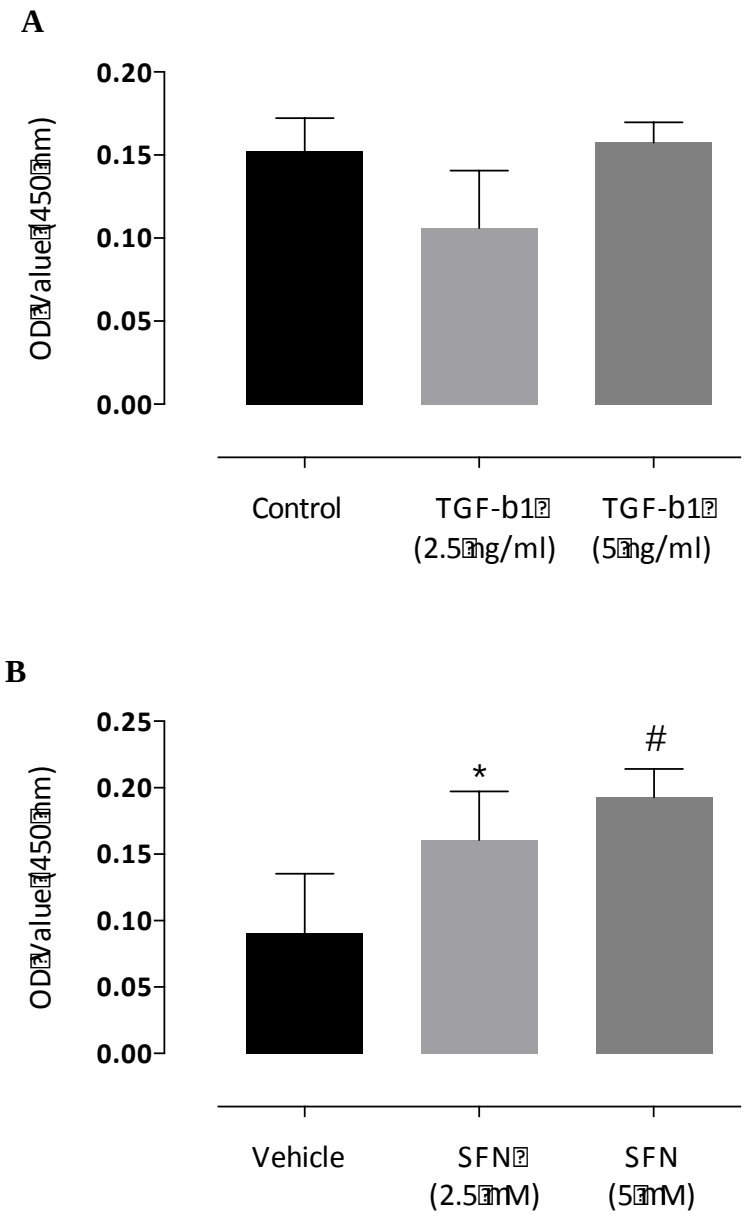


Figure 4.19 ARE-binding activity of nuclear Nrf2 in HAoAF treated with TGF-β1 or SFN
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (2.5 - 5 ng/ml) or SFN (2.5 – 5 μM) for 2 h. Nuclear extracts containing active Nrf2 were then collected using the TransAM nuclear extraction kit and binding activity of Nrf2 was investigated using an ELISA based assay for duplicate samples. Nrf2 that was bound to the immobilized ARE on the ELISA plate was expressed as absorbance values read at 450 nm. Values denote means ± SEM. *p<0.05, SFN (2.5 μM) vs. vehicle and #p<0.05, SFN (5 μM) vs. vehicle using unpaired Student's t-test. n = Representative of 3 - 5 independent experiments.

4.7 Induction of HO-1 in HAoAF transfected with Nrf2 siRNA

In order to determine whether TGF-β1-mediated HO-1 protein expression was as a result of the activation of the Nrf2/ARE signalling pathway, siRNA was performed. Cells were seeded and maintained in DMEM supplemented with 10 % FCS. Cells were allowed to adhere for 24 h and were subsequently transfected with siRNA. Eight hours following transfection, the transfection mixture was aspirated from wells and replaced with DMEM supplemented with 1% FCS for overnight equilibration. Cells were then treated with TGF-β1 (5 ng/ml) or SFN (5 μM) for 8 hours. HAoAF transfected with scramble siRNA and then treated with TGF-β1 or SFN, exhibited an increase in Nrf2 and HO-1 protein expression when compared to untreated control, however this increase was not significant. In cells transfected with Nrf2 siRNA and subsequently treated with TGF-β1, there was a significant decrease in Nrf2 and HO-1 protein expression ($p<0.05$) when compared to those cells transfected with scramble siRNA (Fig 4.20 B and C). A significant decrease in Nrf2 and HO-1 protein expression was also observed in cells transfected with Nrf2 siRNA and subsequently treated with SFN ($p<0.05$ vs. scramble and $p<0.01$ vs. scramble respectively, Fig 4.20 A, B and C). Although cells exhibited a significant decrease in HO-1 protein expression, it was not completely abrogated suggesting that TGF-β1 and SFN-induced HO-1 protein expression may only be partly mediated by Nrf2.

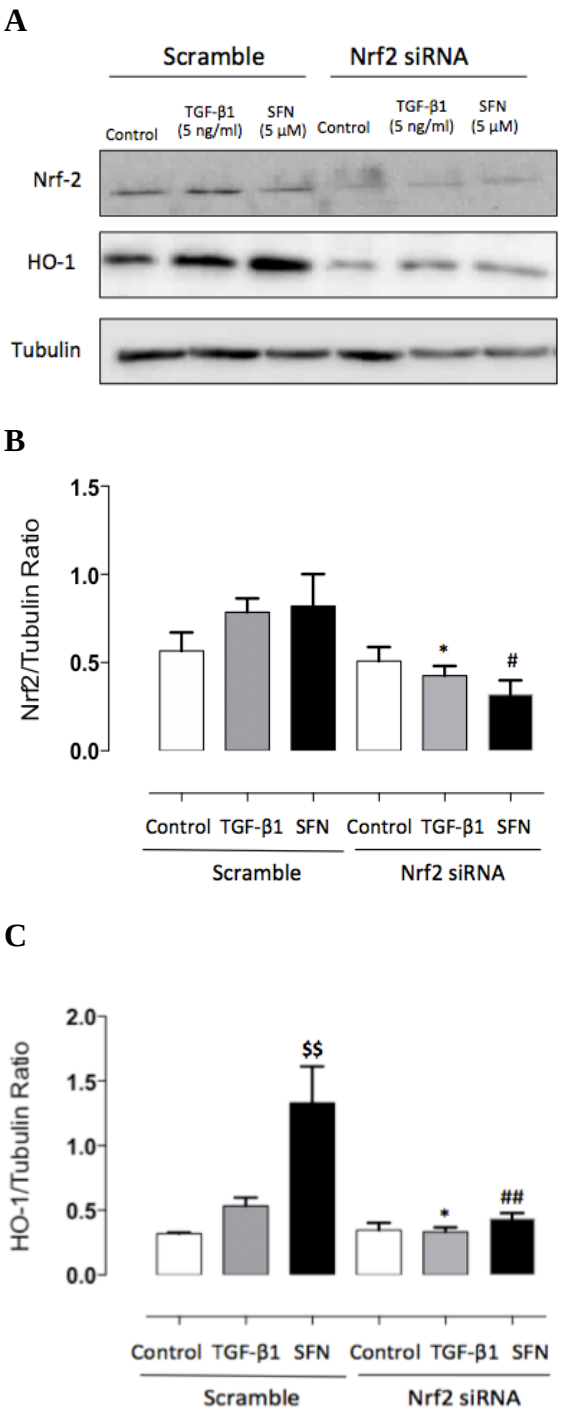


Figure 4.20 Effect of Nrf2 siRNA knockdown on TGF-β1 and SFN-mediated HO-1 protein expression. HAoAF transfected with Nrf2 or scrambled siRNA were seeded and maintained in 10% FCS. 24 h after seeding, cells were transfected for 8 h, then equilibrated in 1% FCS for 18 h prior to treatment with either TGF-β1 (5 ng/ml) and SFN (5 μM) for 8 h. Nrf2 and HO-1 protein expression levels in whole cell lysates were determined by western blot analysis, (A). Representative western blot and quantification of (B) Nrf2 and (C) HO-1 levels were determined by densitometry and expressed relative to α-tubulin. Values denote ± SEM, $$<p0.01 SFN-treated scramble vs. control-treated scramble, *p<0.05 TGF-β1-treated scramble vs. TGF-β1-treated Nrf2 transfected, #p<0.05 SFN-treated scramble vs. SFN-treated Nrf2-transfected, ##p<0.01 SFN-treated scramble vs. SFN-treated Nrf2-transfected using ANOVA. n = 7 independent experiments.

4.8 Discussion

Recent investigations into the pathophysiology of atherosclerosis and restenosis have revealed that the previous understanding of the disease processes involved was incomplete (Haurani and Pagano, 2007). Early evidence in this area of research suggested that the intimal layer and smooth muscle cell (SMC) layer was the main site where the inflammatory process was initiated (Schwartz et al., 1997; Ross, 1999; Lusis, 2000); however, later studies have revealed that the adventitia may in fact be one of the sites of the initiation of diseases of the medium and large arteries (Liu et al., 2010; Lusis, 2000). The role of growth factors, in particular of TGF-β1 in SMC migration and proliferation have been reported in the literature over the past two decades. In particular, TGF-β1 has been reported to play a pivotal role in atherogenesis and vascular remodelling following angioplasty in humans and models of arterial injury in animals (Khan et al., 2007; Chung et al., 2002; Schulick et al., 1998; Nabel et al. 1993) and in mediating fibrotic kidney disease as seen during diabetic nephropathy and hypertension (Lan and Chung, 2012; Zarjou and Agarwal, 2012). Interestingly, dietary supplementation of the isothiocyanate, SFN has been shown to be beneficial in increasing basal levels of GSH, glutathione peroxidase (GPx) and glutathione reductase (GSR) as well as increasing the protein levels of these Nrf2–regulated antioxidant enzymes in vascular SMC (VSMC) from spontaneously hypertensive rats (Juurlink et al., 2001). Therefore, this study sought to investigate the effect(s) of both TGF-β1 and SFN on HAoAF and the possible role they may play in mediating cellular redox status in this cell type. Results from this study provide novel evidence that TGF-β1 and SFN cause the induction of the cytoprotective antioxidant enzyme, HO-1 and nuclear translocation of the transcription factor Nrf2 in HAoAF. Furthermore, SFN causes a significant increase in Nrf2-ARE binding in HAoAF implicating it in the activation of this redox-sensitive antioxidant pathway in this cell type.

4.8.1 Effects of TGF-β1 and SFN on HO-1 and NQO1 induction in HAoAF

The upregulation of TGF-β1 in atherosclerotic disease has been widely reported with the adventitia being implicated as one of the sites of disease initiation (Haurani and Pagano, 2007). Dietary SFN has been reported as being beneficial during cardiovascular disease, owing to its ability to upregulate several Nrf2-regulated phase II antioxidant enzymes. A hallmark of atherosclerotic disease is oxidative stress therefore the purpose of this study was to investigate the effect of TGF-β1 and SFN on the redox status of

HAoAF. Recently, it has been postulated that ROS are released during the phenotypic transformation of fibroblasts to myofibroblasts and are important mediating collagen deposition that is widely associated with vascular remodelling following injury (Rocic and Lucchesi, 2005). TGF-β1 mediates myofibroblastic transformation but it is not known whether induction of antioxidant genes, such as HO-1 and NQO1 via Nrf2 is involved in this process.

TGF-β1 is widely implicated in a variety of cardiovascular diseases and is secreted by all vascular cell types, including endothelial cells, VSMC, macrophages and myofibroblasts (Ruiz-Ortega et al., 2007). In culture, VSMC have been reported to produce TGF-β1 for their own growth and proliferation (Majesky et al., 1991) and in aortic tissue of hypertensive rats, TGF-β1 mRNA levels have been reported to be threefold higher than those seen in normotensive animals (Sarzani et al., 1989). Similar observations have been made in a model of carotid artery injury where mRNA levels of TGF-β1 increased six hours after injury and remained elevated for two weeks following injury. This observation was coupled by an increased production of TGF-β1 by SMC found in the neointima (Majesky et al., 1991).

Thus this study provides an important insight into the mechanisms underlying adaptive responses to TGF-β1 in HAoAF, a cell type of direct relevance to atherogenesis (Liu and Kong, 2010; Haurani and Pagano, 2007). When treated with TGF-β1, HAoAF did not exhibit an increase in NQO1 mRNA or protein levels (Fig 4.9) and there was also no change in HO-1 mRNA levels after 4 h when compared to untreated control, though interestingly, levels decreased after 8 h (Fig 4.5). Despite these observations, a time-dependent increase in HO-1 protein expression was seen in response to TGF-β1 (5 ng/ml), which peaked after 8 h and which was a statistically significant effect ($p<0.05$); levels returned to slightly below baseline after 24 h (Fig 4.7). Although these results are not entirely consistent with evidence in the literature, similar findings have been reported in a study in airway SMC, where cells treated with TGF-β1 (1 ng/ml) exhibited significant increases in HO-1 protein expression but with no change in NQO1 protein levels and little change in HO-1 and NQO1 mRNA levels at 4 and 8 h (Michaeloudes et al., 2011).

The underlying mechanisms responsible for the increase in HO-1 protein induction are likely to vary between cell-types. Kutty and colleagues first reported the alteration of HO-1 mRNA and protein by TGF-β1 (5 ng/ml) in four different cell types; HeLa cells, bovine choroid fibroblasts, bovine corneal fibroblasts and human retinal pigment epithelial cells. A maximal increase in HO-1 protein expression was observed after 8 h of treatment in bovine choroid fibroblasts, a finding that is consistent with the present study, but which was absent in HELA, bovine corneal fibroblasts and human retinal pigment epithelial cells in the same study (Kutty et al., 1994). Other studies have also reported a peak increase in HO-1 protein levels after 8 h in response to TGF-β1, for example in lung epithelial cells, HO-1 induction in response to a range of TGF-β1 (0-30 ng/ml) concentrations, peaked at 8 h and was attributed to the activation of the transcription factor, NFkB via the PI3K/Akt pathway (Lin et al., 2007).

Although the present study did not observe any significant changes in mRNA levels of HO-1, others have shown increases in HO-1 mRNA levels that correlate with a concomitant increase in protein. Kapturczak and colleagues have reported that treatment with TGF-β1 (2 ng/ml) led to a significant increase in HO-1 mRNA levels after 4 h which peaked at 8 h and returned to baseline after 48 h and was accompanied by an increase in HO-1 protein expression (Hill-Kapturczak et al., 2000). Although TGF-β1 elicited increases in HO-1 protein in HAoAF, in contrast to the findings of Hill-Kapturczak and colleagues, TGF-β1 had no significant effect on HO-1 mRNA levels in this present study.

In addition, a study carried out in human aortic smooth muscle cells (HAoSMC), Churchman and colleagues reported that in response to treatment with TGF-β1 (2.5 and 5 ng/ml), there was a significant increase in HO-1 protein expression after 4 h which remained elevated for up to 12 h following treatment (Churchman et al., 2009). It was also observed that TGF-β1-mediated HO-1 induction was in-part regulated by the Erk 1/2 and JNK signalling pathways in addition to the classical Smad signalling pathway in this cell type. Interestingly, antagonism of TGF-β1 signalling by adenoviral over-expression of Smad7 (Nakao et al., 1997) resulted in a downregulation of HO-1 protein expression, strongly suggesting that there is possible cross-talk between the TGF-β1 signalling pathway and the induction of HO-1 in HAoSMC (Churchman et al., 2009). Similar results have been observed in human proximal tubule cells, where the use of an

anti-TGF-β1 antibody resulted in a marked decrease in HO-1 mRNA levels when compared to cells treated with TGF-β1 alone (1 ng/ml, 8 h) further suggesting that these two signalling pathways may interact (Hill-Kapturczak et al., 2000).

Although the majority of studies investigating the effect of TGF-β1 on HO-1 levels are consistent with the results reported here in HAoAF, it has also been reported that TGF-β1 can decrease levels of HO-1 mRNA and protein. In a rat model of endotoxemia, LPS-induced HO-1 mRNA and protein were suppressed after the administration of TGF-β1 (Pellacani et al., 1998). The same study also reported that rat aortic SMC stimulated with IL-1β and then treated with TGF-β1, exhibited a downregulation of IL-1β-mediated HO-1 mRNA and protein levels as well as a decrease in HO-1 enzymatic activity (Pellacani et al., 1998). The present study in HAoAF has found that although TGF-β1 treatment results in an increase in HO-1 protein levels, it had no effect on mRNA levels. This may be due to the time-point at which these measurements were made as other studies have shown changes in HO-1 mRNA levels after treatment with TGF-β1 over 2 – 48 h (Hill-Kapturczak et al., 2000; Kutty et al., 1994). The underlying mechanism by which TGF-β1 induces HO-1 protein expression is unclear, however measuring the enzymatic activity of HO-1 (Moterlini et al., 1996) in response to TGF-β1 could provide further useful information and allow elucidation of the pathway and mechanism by which this induction occurs.

In this study, use of the isothiocyanate, SFN was used as a positive control as its role as an Nrf2-inducer has been characterised in several different cell types (Keum et al., 2006; Keum et al., 2011; Michaeloudes et al., 2011). However, emerging evidence recently reported in the literature highlights the possible effects of SFN on the Smad signalling pathways (Oh et al., 2012; Chin et al., 2011) highlighting the potential role of SFN as an important therapeutic agent in fibrosis, diabetes, chemoprevention and vascular disease (Kaminski et al., 2010, Negi et al., 2011; Oh et al., 2012; Keum et al., 2011). Therefore, induction of antioxidant enzymes by SFN in myofibroblasts may be a way of conferring protection from further and on-going oxidative stress in vascular diseases and may even be used as a clinically beneficial means of intervention against vascular remodelling. In this study, HAoAF were treated with SFN and HO-1 and NQO1 mRNA levels and protein expression were analysed.

Observation of NQO1 mRNA (2.5 – 5 μM, 4 – 8 h) and protein (2.5 – 5 μM, 4 – 24 h) levels revealed that these did not change in response to SFN in HAoAF (Figs. 4.10 and 4.12). This finding is in contrast to results previously reported in other cell types, in particular, in airway SMC and mouse embryonic fibroblasts where SFN increased NQO1 mRNA levels and protein expression (Michaeloudes et al., 2011; Keum et al., 2006). Analysis of SFN treatment on HO-1 mRNA levels in HAoAF revealed that it had no significant effect on these levels (Fig 4.6), however cells exhibited a dose and time-dependent increase in HO-1 protein expression, with the highest significant increase observed at 8 h after SFN treatment, which returned to basal levels after 24 h (Fig 4.8). Similar observations have been made by Keum and colleagues, who reported that exposure of mouse embryonic fibroblasts to SFN (5 μM) resulted in an induction of HO-1 protein levels 12 h after treatment, which decreased after 24 h. The same study showed that treatment of mouse embryonic fibroblasts with SFN was accompanied by a significant increase in Nrf2 protein levels and increased ARE-luciferase activity, suggesting that HO-1 induction was mediated by the Nrf2-ARE pathway in this cell type (Michaeloudes et al., 2011; Keum et al., 2006).

In both TGF-β1 and SFN-treated cells, there was little change in Nrf2 mRNA levels and basal expression of Nrf2 protein was unaltered (Figs. 4.1-4.4). This is in-keeping with some reports which suggest that Nrf2 is constitutively expressed and whole cell protein levels remain unaltered in some cell types (Nguyen et al., 2005). Although this was the case, several factors are able to affect the activity of proteins. The quantity of an mRNA, its stability or degradation and rates of ribosome initiation all directly impact on protein output; degradation of mRNA results in the steady state decrease in the expression of the protein (Hargrove and Schmidt, 1989). Without changes at the transcription level, mRNA steady-state levels can vary dramatically by small changes in mRNA stability, which has recently been shown to be prone to regulation by microRNA, which can repress or target degradation resulting in gene silencing (Eades et al., 2011). mRNA stability may be a reason for the unchanged levels of Nrf2 in response to TGF-β1 and SFN treatment in this study. Other possible post-translational modifications include hypermethylation of proteins; hypermethylation of the Keap1 promoter has been shown to result in Keap1 protein expression and the accumulation of nuclear Nrf2 (Kansanen et al., 2013). In addition, the acetylation of Nrf2 by the transcription factor CREB, has been observed as increasing it's binding to its cognate

response element in a target gene promoter and increasing Nrf2-dependent transcription from target gene promoters (Kawai et al., 2011). Post-translational modifications of proteins were not investigated in the current study, however future work regarding these may shed further light on the levels of protein expression observed here.

Results from other studies suggest that alternatively, the transcription factor, NFκB, may regulate HO-1 protein as opposed to Nrf2, as was reported in a study where human gingival fibroblasts were stimulated with cyclosporine A (Chin et al., 2011). Analysis of alternative transcription factors was not investigated in the present study, however future work focusing on other potential transcription factors that regulate HO-1, and that have been reported as being activated by TGF-β1 and SFN, including AP-1 and CREB (Kawal et al., 2011; Zhang et al., 2006; Sano et al., 1999) would shed further light on the molecular pathway(s) involved in HO-1 induction in HAoAF.

4.8.2 Effect of TGF-β1 and SFN on intracellular GSH levels in HAoAF

The dysregulation of GSH has been reported in aging (Lui et al., 2004) and various disease models including diabetes mellitus (Okouchi et al., 2006), cholestasis (Serviddio et al., 2004), cancer, drug-resistant tumours (Huang et al., 2001) and fibrosis amongst others (Roum et al., 2003; Rahman et al., 1999; Beeh et al., 2002). TGF-β1 has been implicated in as being involved in these disease processes and has been found to reduce intracellular GSH (Lui et al., 2004) in various cell types, including epithelial cells (Bakin et al., 2005), hepatocytes (Fu et al, 2008), endothelial cells (White et al., 1992) and fibroblasts (Lui et al., 2004) *in vitro*. Therefore, in order to assess the effects of TGF-β1 on GSH levels in HAoAF, a fluorometric assay was performed (Hissin and Hilf, 1976). It was found that treatment of cells with TGF-β1 did not elicit any changes in intracellular GSH in HAoAF over 24 h.

Previous studies have indicated that TGF-β1 elicits GSH depletion. In NIH3T3, a murine embryonic fibroblast cell line, depletion of intracellular GSH as a result of TGF-β1 exposure was accompanied by an increase in collagen accumulation, an effect which was blocked following replenishment of intracellular GSH with exogenous GSH or *N*-acetyl cysteine (NAC), indicating that GSH plays a pivotal role in TGF-β1-mediated fibrogenesis (Sanchez et al., 1997). A more recent study has postulated a possible cross-talk between the mechanisms involved in TGF-β1-induced profibrotic responses and GSH where treatment of human fetal lung fibroblasts with TGF-β1 resulted in the transformation of cells to a pro-fibrotic phenotype whilst also augmenting fibronectin production and enhancing gel contraction (Sugiura et al., 2009). However, when co-treated with NAC, these effects were significantly attenuated suggesting that NAC inhibited not only TGF-β1 signalling but also the TGF-β1-mediated wound healing process and fibroblast differentiation (Liu et al., 2004). They have also suggested that NAC is able to modify TGF-β1, rendering it inactive as well as inhibiting its binding to its receptor (Sugiura et al., 2009). This finding suggests a possible mechanism by which ROS are involved in TGF-β1-mediated effects in fibroblasts and that use of an antioxidant such as NAC may abrogate these effects. Such changes may include the activation of certain MAPK phosphatases, leading to the sustained activation of various MAPK pathways and subsequent activation of downstream signalling pathways, including antioxidant pathways such as Nrf2 and NFκB (Liu and Pravia, 2010).

It has been postulated that the mechanism by which TGF-β1 causes the depletion of intracellular GSH is via the inhibition of glutamate cysteine ligase catalytic subunit (GCLC) gene transcription and the suppression of GCLC promoter activity, with many studies suggesting that this may be the process by which TGF-β1 causes intracellular GSH depletion in fibrotic diseases. (Jardine et al., 2002). Jardine and colleagues have reported this observation as being due to the TGF-β1-mediated activation of the activator protein (AP-1) pathway; their study in alveolar epithelial cells has reported an increase in the DNA binding activity of both AP-1 and ARE in TGF-β1-treated epithelial cells (Jardine et al., 2002). This finding has been further confirmed by a more recent one in mammary epithelial cells, which has found that TGF-β1 suppresses of the activity of the human GCLC proximal promoter which bears an ARE (Bakin et al., 2005). Others studies have suggested TGF-β1 is able to cleave the catalytic subunit of GCLC and downregulate GCLC gene expression resulting in a reduction in the activity of GCL and consequent depletion of intracellular GSH (Franklin et al., 2003). Findings from these studies suggest that TGF-β1 may elicit an increase in oxidative stress by depleting the antioxidant protection afforded by GSH which plays an important role in ROS detoxification. However, in the present study, and in contrast to findings from the literature, TGF-β1 treatment did not affect intracellular GSH levels in HAoAF, which may indicate a possible adaptive response in cells, similar to that observed by Pauly and colleagues who found that TGF-β1 stimulates cysteine uptake leading to the maintenance of cellular glutathione (Pauly et al., 2011). This study may also potentially explain the increase in intracellular GSH seen 8 h after treatment, and may be indicative of normal redox cycling events during GSH synthesis.

Animal studies and several epidemiological studies suggest that regular consumption of cruciferous vegetables lower the risk and incidence of diseases including cancer of the prostate, lung, breast and colon (Liu et al., 2008; Juge et al., 2007) as well as conferring protection against hyperglycaemia, hypertension and myocardial infarction (Xue et al., 2008; Cornelis et al., 2007; Noyan-Ashraf et al., 2006; Wu et al., 2001). Therefore, in order to determine the potential beneficial effects that SFN could confer to the adventitial layer, which is known to be involved in atherosclerotic disease progression (Ruiz-Ortega et al., 2007), HAoAF were treated with SFN and intracellular GSH levels measured. Results revealed that SFN causes an initial depletion in GSH levels after 4 h of treatment (see Fig 4.14), which recovered after 8 h and returned to just above baseline after 24 h. Several studies have also reported the depletion of GSH in cells

treated with SFN. Similar findings in endothelial cells were observed where treatment with SFN resulted in a transient depletion of GSH which recovered over 12 h of treatment and which was abrogated when cells were pre-incubated with NAC (Liu et al., 2008). In a study in VSMC from spontaneously hypertensive rats, cells treated with SFN (0 – 0.5 μM) displayed an initial decrease in intracellular levels, however cells recovered over time and levels of GSH returned to baseline (Wu and Juurlink et al., 2001). A more recent study carried out in a bronchial epithelial cell line demonstrated similar findings to the current study where SFN treatment resulted in the depletion of intracellular GSH after 2 h followed by a GSH rebound and elevation of GCL owing to an increase in the levels of nuclear Nrf2 after 4 h of SFN treatment (Poerschke et al., 2012).

The recovery of intracellular GSH levels has been attributed to the ability of SFN to induce the expression of the Nrf2/ARE-regulated genes GCLC/GCLM; initial exposure to SFN results in the conjugation of SFN with GST resulting in the observed GSH depletion, however SFN has also been reported as modifying cysteine residues on Keap1, allowing the release of Nrf2, its translocation to the nucleus and consequently the increased expression of GST and glutamate cysteine ligase (GCL) which in turn results in the synthesis of newly formed GSH, replenishing intracellular levels and returning them to baseline (Xue et al., 2008). Interestingly, vehicle-treated cells displayed higher levels of GSH than those treated with SFN. NMR studies have revealed that GSH is highly flexible in an aqueous solutions and is able to transition to the extended, semi-folded and folded states. However, in DMSO, the stronger hydrogen bonds and the hydrophobic interactions make GSH stable in the folded state, allowing it to accumulate in high levels. In the absence of an agent which causes its depletion, GSH may continue to accumulate in the cells in a time-dependent manner, as observed here (Fig. 4.14; Zhang et al., 2011). A more accurate gauge of cellular redox state could be ascertained with the use of an enzymatic or HPLC assay which would give the GSH:GSSG ratio (Rousar et al., 2012). In addition, due to the commencement of measurement at 2 h following treatment, measurements were not obtained at time zero and so conclusions pertaining to an increase or decrease in the levels of GSH in either treatment group, from time zero cannot be drawn. The current study has found that SFN treatment results in the initial depletion of intracellular GSH, however this later recovers

and returns to baseline at 24 h and may offer potential of SFN as prophylaxis, affording protection to cells from ongoing oxidative stress during disease.

The method used to analyse cellular GSH levels in the present study was the fluorometric method, first proposed by Hissin and Hilf, 1976 which measures reduced glutathione (GSH). However, it has been suggested that this is not an accurate measure of the redox state of the cell, especially when the cell is under oxidative stress and that the ration of reduced to oxidized glutathione (GSH:GSSG) is a more accurate gauge of cellular stress (Floreani et al., 1997). Furthermore, when compared to a variety of other glutathione assays, levels measured by the fluorometric method were not comparable with those measured by the other assays (Floreani et al., 1997).

Work into the methodology of the original protocol (Hissin and Hilf, 1976) has allowed the optimisation of this method to produce a more accurate readout of GSH (Rousar et al., 2012). Rousar and colleagues found that adjusting the original wavelengths used for excitation from 350 to 340 nm enhanced the intensity of fluorescence by 20% as did decreasing the temperature during measurement (Rousar et al., 2012). In the current study, the excitation wavelength used was 350 nm and the samples were incubated at room temperature before fluorometric reading was commenced, both of which may have affected the values obtained of the levels of GSH in HAoAF.

Irrespective of these factors, measurement of the ratio of GSH:GSSG remains a more accurate measure of cellular oxidative stress, which is not taken into account when using the fluorometric method. Although this method is easy to perform and with the potential of a high throughput of samples, it is limited by its inability to determine the GSH:GSSG ratio which is important when investigating oxidative stress in a cell system. Future work using an enzymatic method or HPLC may provide a more accurate readout with regards to the effect of TGF-β1 and SFN on glutathione levels in HAoAF.

4.8.3 Effect of TGF-β1 and SFN on Nrf2 nuclear accumulation

As activation of the Nrf2 pathway requires its translocation to the nucleus and subsequent binding to the ARE, the effect of TGF-β1 and SFN treatment on the cellular localisation of Nrf2 in HAoAF was investigated. Nrf2 heterodimerizes with Jun and small Maf proteins in the nucleus and binds to the ARE in the promoter region of antioxidant enzymes to regulate their expression; including the cytoprotective enzyme, HO-1 and the phase-II detoxification enzyme NQO1 (Jaiswal, 2004; Jain et al., 2005). The distribution and activity of Nrf2 in response to chemical/electrophillic stress is variable between different cell types (Nguyen et al., 2009; Watai et al., 2007; Jain et al., 2005; Velichkova and Hasson, 2005), however in all cell types, its nuclear translocation is associated with the transcription of antioxidant genes following binding to the ARE of antioxidant genes (Keum, 2011), therefore HAoAF were treated with TGF-β1 (5 ng/ml) or SFN (5 μM) and nuclear fractions extracted. Immunofluorescence analysis revealed that in response to TGF-β1 (5 ng/ml), there was enhanced nuclear accumulation of Nrf2, which was also seen in response to SFN (5 μM) after 2 h of treatment (Fig. 4.18 B). These results are in agreement with previous data from HAoSMC where Nrf2 nuclear accumulation was seen after 2 h of TGF-β1 (5 ng/ml) treatment; an accumulation that increased in a dose-dependent manner (Churchman et al., 2009). Initially, these results suggest that TGF-β1 and SFN may reduce the Keap1-directed degradation of Nrf2, therefore allowing its accumulation in the cell. SFN has been postulated as activating Nrf2 via an alternative method; Keum and colleagues have proposed that it reacts with protein thiols to form thionacyl adducts which affect cysteine residues in Keap1, allowing the release of Nrf2, and its translocation to the nucleus (Keum et al., 2006). In addition, SFN is thought to affect intracellular kinases to phosphorylate Nrf2, thereby affecting its ability to translocate to the nucleus (Keum et al., 2011).

However, the nuclear accumulation of a transcription factor is not necessarily an indicator of its transcriptional activity. Although Nrf2 nuclear translocation was observed in response to TGF-β1 treatment after 2 h, when analysed by immunofluorescence, when the TransAM DNA binding ELISA was carried out in order to assess Nrf2 transcriptional activity, no change in ARE binding of Nrf2 was observed (Fig. 4.19). Baseline levels of nuclear Nrf2 appeared to be higher than levels observed in TGF-β1-treated cells, which may indicate that cells were already under some stress

and that redox pathways, in particular, the Nrf2 pathway, had been activated perhaps due to the experimental procedure.

However, similar findings that suggest that TGF-β1 has little or no effect on Nrf2-ARE activation have also been reported in airway SMC, where TGF-β1 caused an initial increase in HO-1 mRNA levels after 2 h, however there was no increase in Nrf2-ARE driven luciferase activity or a change in whole-cell protein levels of Nrf2 (Michaeloudes et al., 2011), observations that are consistent with the current study. Interestingly, the same study also reported that treatment of airway SMC with SFN resulted in an increase in whole cell Nrf2 levels which was accompanied by a concentration-dependent increase in ARE-driven luciferase activity (Michaeloudes et al., 2011). Whilst increases in whole-cell Nrf2 were not observed in HAoAF in response to SFN, there was a dose-dependent (2.5 – 5 μM) increase in Nrf2-DNA binding activity. The modulation of Nrf2 by TGF-β1 may be a more complicated process than simple activation and nuclear translocation. Michaeloudes and colleagues argue that although initial increases in the mRNA of Nrf2-regulated genes are observed in response to TGF-β1 in airway SMC, TGF-β1 may inactivate Nrf2 by inducing the expression of the Nrf2 transcriptional repressor, ATF-3 which results in the later decrease of HO-1 mRNA levels after 4 h (Michaeloudes et al., 2011). They argue that TGF-β1 increases the mRNA of ATF-3, thereby blocking Nrf2-mediated transcription; ATF-3 is postulated to do this by displacing the Nrf2 co-activator, CREB binding-protein from the ARE complex (Michaeloudes et al., 2011). This may also be a mechanism by which HO-1 mRNA levels decrease over time in HAoAF, however, in order to determine this, further work to investigate other mechanisms, including ATF-3 activity and possibly other transcriptional repressors such as Bach1 and DJ-1(Im et al., 2012; Kang et al., 2011; Clements et al., 2006; Shan et al., 2006), would need to be carried out. The evidence in the present study regarding SFN-mediated HO-1 induction suggests that SFN may activate the Nrf2/ARE pathway in HAoAF as reported previously (Michaeloudes et al., 2011; Keum et al., 2011; Keum et al., 2006).

4.8.4 Effect of knockdown of Nrf2 on TGF-β1 and SFN induction of antioxidant genes

To investigate whether Nrf2 regulated HO-1 induction response in HAoAF, knockdown of this transcription factor using short interfering RNA transcript was carried out. Following transfection, cells were treated with TGF-β1 or SFN. Western blot analyses show that HAoAF transfected with Nrf2 siRNA displayed abrogated HO-1 protein expression when treated with both TGF-β1 and SFN (Fig 4.20). This further confirms that SFN mediated-HO-1 expression in HAoAF is an Nrf2-dependent process. However, with regards to TGF-β1, these results do not correlate with those found in the Nrf2-DNA binding assay, where it was found that TGF- β1 does not increase Nrf2-ARE binding activity, however siRNA studies have demonstrated that in cells transfected with Nrf2 siRNA, TGF- β1-induced HO-1 protein expression was abrogated. These data suggest that TGF-β1 mediated - HO-1 protein induction may be partly mediated by Nrf2.

Previously, Itoh and colleagues reported some of the first evidence that Nrf2 induced the expression of phase-II detoxifying enzymes by disrupting the *nrf2* gene *in vivo*. By using butylated hydroxyanisole (BHA), a selective phase-II enzyme inducing stress agent, in both wild-type (WT) and Nrf2-deficient mice they were able to compare the induction of the antioxidant enzymes, HO-1 and NQO1. Findings showed that induction of phase-II enzymes in Nrf2-deficient mice was largely eliminated in isolated liver and intestinal tissue, whilst WT mice displayed a normal expression pattern of these enzymes (Itoh et al., 1997). Similarly another study found a 50-80% reduction in the constitutive induction of NQO1 in hepatic and gastric tissue from Nrf2-deficient mice in comparison to WT (Ramos-Gomez, 2001).

Interestingly, in the present study, although induction of HO-1 was clearly decreased following siRNA knockdown of Nrf2, and Nrf2 protein expression in Nrf2 siRNA-transfected cells was significantly lower than in non-transfected control cells, some Nrf2 protein expression was still detected in transfected cells (Fig. 4.18). This can be explained by the 'Hinge and Latch' model proposed by Tong and colleagues (see Fig. 4.17) which postulates that after exposure to electrophillic stress, newly synthesized Nrf2 molecules bypass the 'Keap1 gate', (which traps Nrf2 and causes its degradation via the 26S proteasome) (Tong et al., 2006a; Tong et al., 2006b; Tong et al., 2007;

Watai et al., 2007). This newly synthesized Nrf2 then translocates to the nucleus where it binds to the ARE of antioxidant genes and causes their transcription (Watai et a., 2007). In this model, instead of causing disruption of the Keap1-Nrf2 association, electrophillic stress simply represses Keap1-mediated ubiquitination of Nrf2, so that Nrf2 is still associated with Keap1 but not degraded. In the context of the current study, it is possible that Nrf2 siRNA caused the knockdown of any newly synthesized Nrf2, which would have otherwise bypassed the Keap1 pathway and resulted in transcription of antioxidant enzyme(s), whilst affecting Nrf2 already present in the cell. In addition to this, only the induction of antioxidant enzymes is abrogated, however their ubiquitous and constitutive activation is still present, albeit at lower levels (Nguyen et al., 2009).

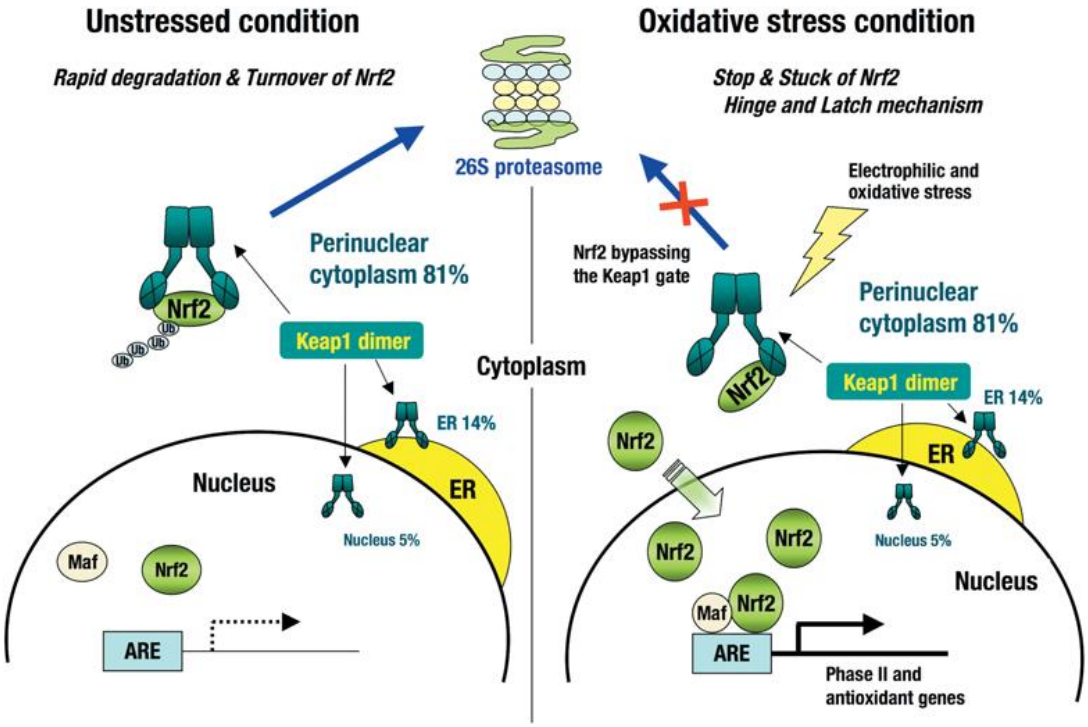


Figure 4.21 The 'Hinge and Latch' model of Keap1-Nrf2 degradation.
Under unstressed conditions, Nrf2 is sequestered in the cytoplasm by Keap1 homodimer and rapidly degraded via the ubiquitin-proteasome pathway. Upon exposure to oxidants or electrophiles, Keap1 is inactivated and becomes 'stuck' with the Nrf2 it is associated with, allowing *de novo* synthesized Nrf2 to bypass the cytoplasmic Keap1-proteasome pathway, translocate to the nucleus and bind to the ARE of phase-II and antioxidant genes. Taken from Watai et al., 2007.

Several studies have reported that activation and transcription of HO-1 can be mediated by the NFκB pathway. The TGF-β1 gene promoter contains binding sites for NFκB (Lee et al., 2006) as does the gene promoter for HO-1 (Jadhav et al., 2008) and this may be an alternative pathway in which TGF-β1 mediates HO-1 induction.

An interaction between HO-1, NFκB and TGF-β1 has been proposed by Jadhav and colleagues in a rat model of hypertension where the HO system has been postulated to critical for the regulation of fibrotic events mediated by TGF-β1 and the available binding sites for NFκB in the TGF-β1 promoter as well as in the HO-1 promoter suggest an intricate relationship between both TGF-β1 and HO-1 and their regulation by NFκB (Jadhav et al., 2008). Upregulation of HO activity as well as the administration of hemin reduced hypertension, suppressed oxidative stress and reduced atherosclerotic lesion formation as analysed by immunohistochemistry characteristic of cardiac lesions (Jadhav et al., 2008). Furthermore, A459 cells, induction of HO-1 by TGF-β1 was thought to be dependent on PI3K/Akt and NFκB signalling as use of both a PI3K inhibitor and an NFκB inhibitor inhibited TGF-β1-induced HO-1 expression and HO-1-ARE luciferase activity (Lin et al., 2007). In human hepatoma HepG2 cells transfected with a NFκB reporter plasmid, treatment with TGF-β1 led to a 50% increase in NFκB-luciferase reporter activity. This was mediated by TRAF-6-induced polyubiquitination of TGF-β1-associated kinase 1, thereby activating it and inducing a prosurvival pathway via NFκB. This may be a potential mechanism by which HO-1 is induced via the TGF-β1 signalling pathway (Hamidi et al., 2012). In addition, another study using A459 cells demonstrated the importance of NFκB and PI3K in TGF-β1-mediated migration; the use of inhibitors for both of these resulted in abrogation of cell migration in response to TGF-β1 (Fong et al., 2009).

These studies suggest that other alternative pathways such as NFκB may mediate the TGF-β1 mediated HO-1 induction in HAoAF. Although other transcription factors such as NFκB were not considered in this present study, it would be important to investigate these in any future work to shed light upon the mechanisms behind the lack of Nrf2-DNA binding activity in HAoAF in response to TGF-β1. The cross-talk between SFN and the TGF-β1 signalling pathway was not investigated by the current study, however an interaction between the two was first reported by Traka and colleagues in a study carried out in NIH-3T3 cells where it was found that SFN can indirectly increase TGF-

β1-mediated activation of the Smad signalling pathway when it is pre-incubated with SFN (2 μM) for 30 min. The binding of SFN to TGF-β1 resulted in enhanced Smad-mediated transcription, inhibiting cell proliferation and the authors went on to propose that this was the underlying mechanism by which SFN may act as an anticarcinogenic agent (Traka et al., 2008). Similarly, in an adenocarcinoma cell line, pre-incubation of cells with SFN (5-50 μM) resulted in an increase in TGF-β1 mRNA levels after 1-3 h which was accompanied by an increase in the protein levels of both TGFβ-RI and TGF-βRII owing to the ability of SFN to suppress ornithine decarboxylase, an enzyme required for cell proliferation.

This chapter has provided novel evidence that TGF-β1 causes the induction of HO-1 in HAoAF, as well as causing the nuclear translocation of Nrf2, however, it does not increase Nrf2-DNA binding activity suggesting that the induction of HO-1 may be via an alternative pathway. It has also been demonstrated that SFN also causes the induction of HO-1 in HAoAF, GSH depletion and nuclear translocation of Nrf2 as well as increasing Nrf2-DNA binding activity, suggesting that SFN is an activator of the Nrf2/ARE pathway in HAoAF.

CHAPTER 5:

Results

CHAPTER 5: Effect of TGF-β1 on HAoAF migration

5.1 Introduction

Cell migration is a critical process during embryonic development as well as during tissue repair and immune function (Valster et al., 2005). Migration is usually triggered by a signal, often chemotactic, that results in the polarisation and extension of cellular protrusions, such as lamellipodia, in the direction of movement and as the cell moves forward, the protrusions, containing focal adhesions (FA) adhere to the substrate and the trailing edge of the cell retracts (Hurd et al., 2012). Cell motility is a highly regulated process and requires timely assembly, disassembly and reorganisation of the actin cytoskeleton, a process that is mediated by members of the Rho family of small GTPases (Kole et al., 2005). Membrane protrusions and formation of wide lamellipodia, which make up membrane ruffles at the leading edge of the cell, are dependent upon the activity of the small GTPases, Rac and Cdc42 (Nobes and Hall, 1999). There is increasing evidence to suggest that reactive oxygen species (ROS) play a role in directing changes required for cell motility with one study carried out in zebrafish suggesting that hydrogen peroxide (H_2O_2) produced during injury acts as a chemoattractant to attract immune cells to the site of the wound (Niethammer et al., 2009).

Early studies carried out *in vitro* have suggested that ROS are important during cell migration; this was first demonstrated in human fibroblasts where chemotactic response to conditioned medium and platelet-derived growth factor (PDGF) was inhibited in the presence of superoxide dismutase (SOD), implicating the superoxide anion ($O_2^{\cdot -}$) in playing a role during migration in this cell type (Wach et al., 1989). Other studies have reported similar findings; in human umbilical vein endothelial cells, exposure to Angiotensin-I triggered the production of ROS via NAD(P)H oxidase as well as causing a threefold increase in cell migration, however overexpression of SOD and catalase significantly inhibited this effect (Harfouch et al., 2005). Furthermore, in mouse embryonic fibroblasts, exposure of cells to PDGF increased the production of H_2O_2 and subsequently led to an increase in cell migration and proliferation, both of which were abrogated in the presence of antioxidants (Choi et al., 2005). In the same study, mice lacking PrxII exhibited enhanced neointimal lesion formation as a result of increased vascular smooth muscle cell (VSMC) migration (Choi et al., 2005). It has been

postulated that the source of $O_2^{-\cdot}$ and H_2O_2 are the NAD(P)H oxidase isoforms, Nox2 and Nox4 (Haurani and Pagano, 2007). In VSMC, knockdown of Nox4 resulted in a marked decrease in ROS production, loss of focal adhesions and inhibition of migration, implicating this Nox isoform in all of these processes (Lyle et al., 2009). Furthermore, inhibition of Nox4 using diphenyleneidonium (DPI) or knockdown using siRNA in a fibroblast cell line abolished the ability of these cells to migrate (Tobar et al., 2010).

TGF-β1 has been widely implicated in mediating increases in ROS production, which can cause alterations in actin cytoskeleton organisation and fillipodia formation (Hu et al., 2005). Treatment of endothelial cells with TGF-β1 induced an increase in ROS production, which was attributed to increased Nox4 activity (Hu et al., 2005). Similar findings have been reported in VSMC, where TGF-β1 induced the expression of Nox4 and consequently increased ROS production, which was reduced when cells were treated with the flavoprotein inhibitor, DPI (Sturrock et al., 2006). Furthermore, stimulating human lung fibroblasts with TGF-β1 led to a transient but rapid increase in H_2O_2 production.

There is much evidence to suggest that ROS are important in mediating cell migration and that TGF-β1 is able to drive this process in various cell types, therefore, in this chapter, the effect of TGF-β1 on HAoAF migration was examined. Furthermore, the ability of TGF-β1 to mediate ROS generation in HAoAF was also investigated and finally, the effect of TGF-β1 on the migration of cells with impaired antioxidant defences was determined.

5.2 Effect of TGF-β1 on HAoAF phenotype during migration

During cell migration, cells undergo various structural changes, such as actin re-organisation, which enable them to carry out processes such as establishing spatial polarity and extension of membrane protrusions in the direction of movement (Kole et al., 2005; Ridley et al., 2001). As discussed in Chapter 3, HAoAF had differentiated into myofibroblasts and expressed the cytoskeletal proteins, α-smooth muscle actin and vimentin, and had acquired a migratory phenotype. In order to determine whether cytoskeletal changes had affected the morphology of cells, sub-confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (2.5 - 5 ng/ml) or DMEM supplemented with 10% FCS and time-lapse video microscopy was carried out. A frame was captured every 10 min over 8 h and phenotypic changes were observed. HAoAF incubated with 1 % FCS appeared less confluent and were smaller in size displaying an absence of ruffled edges, a hallmark of a migrating cell, and therefore were indicative of a less migratory phenotype (Fig. 5.1, panel A). HAoAF incubated with TGF-β1 (2.5 ng/ml; Fig. 5.1, panel B) and TGF-β1 (5 ng/ml; Fig. 5.1, panel C) appeared more confluent and displayed ruffled leading edges indicative of cell motility. Cells were elliptical in shape, exhibited cell spreading and a flattened morphology. This indicates that TGF-β1 drives HAoAF migration with some effect on cell phenotype and behavior. As a positive control, HAoAF were also incubated with DMEM supplemented with 10 % FCS (Fig. 5.1, panel D), a known inducer of migration. Cells appeared more confluent and exhibited ruffled leading edges, indicative of a migratory phenotype and were visibly more motile than cells incubated with DMEM supplemented with 1% FCS. These results indicate that TGF-β1 affects cell morphology and consequently drives cell migration in HAoAF.

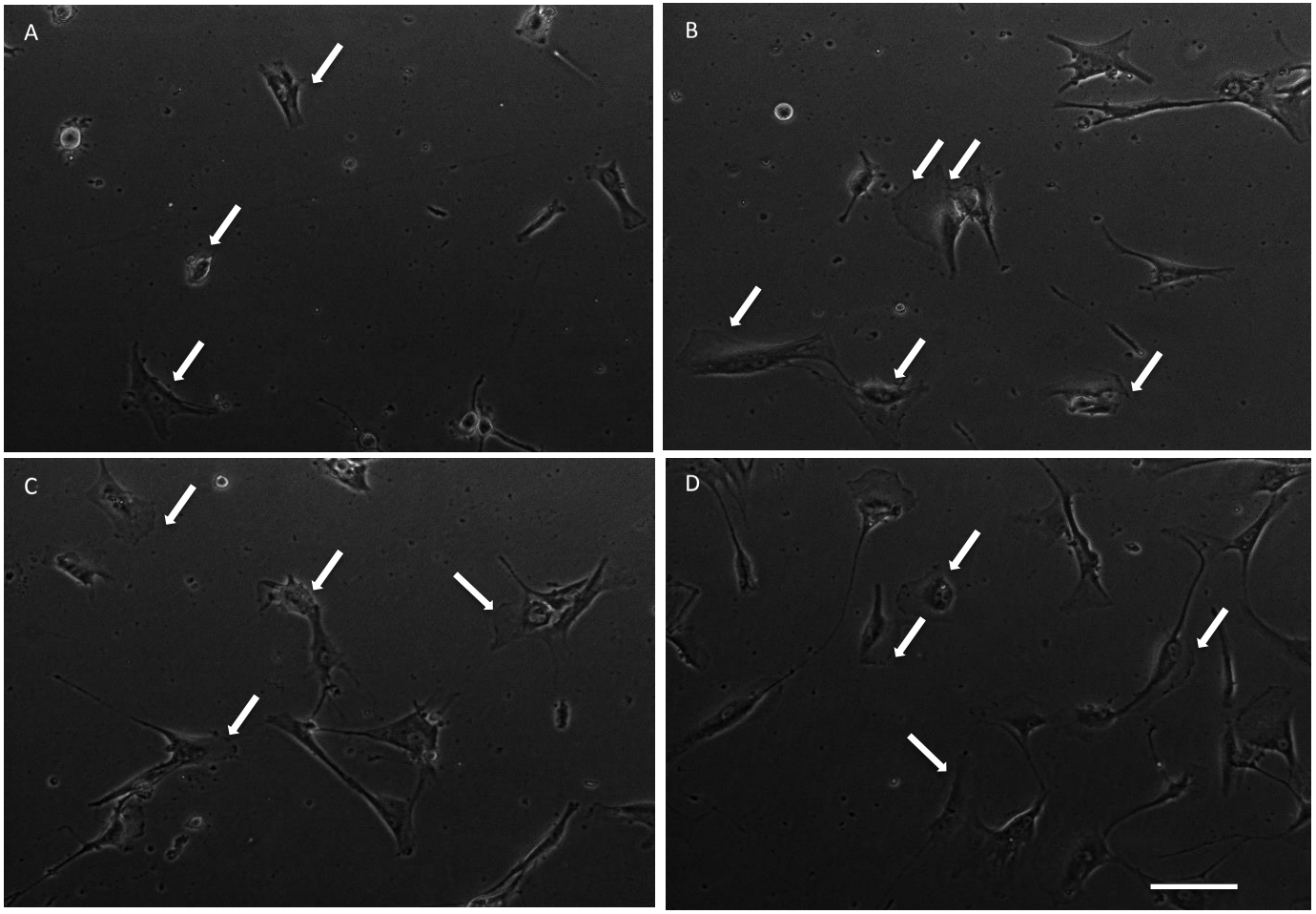


Figure 5.1 Cellular morphology of HAoAF in response to incubation with TGF-β1. HAoAF were seeded at 2 x 10^4/well in a 12-well culture plate and allowed to adhere overnight. 18 h prior to incubation with TGF-β1, cells were adapted in DMEM supplemented with 1% FCS and subsequently incubated in (A) 1 % FCS (B) TGF-β1 (2.5 ng/ml) (C) TGF-β1 (5 ng/ml) and (D) 10% FCS for 8 h. Arrows indicate leading ruffled edges, indicative of migration. Image is representative of 4 independent experiments (scale bar = 15 μm).

5.3 Effect of TGF-β1 on HAoAF migration and velocity

TGF-β1 is a well known inducer of migration in fibroblasts, with some reports suggesting that this process may be driven by enhanced ROS generation, however the underlying mechanism by which this occurs has not been fully elucidated (Ren et al., 2011; Riedy et al., 1999; Shi et al., 1996). In order to determine the effect of TGF-β1 on HAoAF migration, sub-confluent HAoAF were equilibrated in DMEM supplemented with 1% FCS 18 h prior to treatment with TGF-β1 (5 ng/ml). Some cells were incubated with SOD (200 U/ml) 30 min prior to the commencement of imaging in order to determine whether ROS mediate TGF-β1-driven migration. A frame was captured every 10 min over an 8 h period and in the presence or absence of SOD (200 U/ml). Cells treated with TGF-β1 caused a significant increase in HAoAF migration ($p<0.001$) when compared with untreated control (Fig 5.2A). This increase in migration was abrogated when cells were pre-incubated with SOD and subsequently treated with TGF-β1, indicating that this migration may be driven by ROS. Cells treated with TGF-β1 alone also migrated with significantly greater velocity when compared to untreated control ($p<0.01$). This was also abrogated in the presence of SOD, which further indicates that TGF-β1-driven HAoAF migration may be mediated by ROS (Fig 5.3 and Table 5.1).

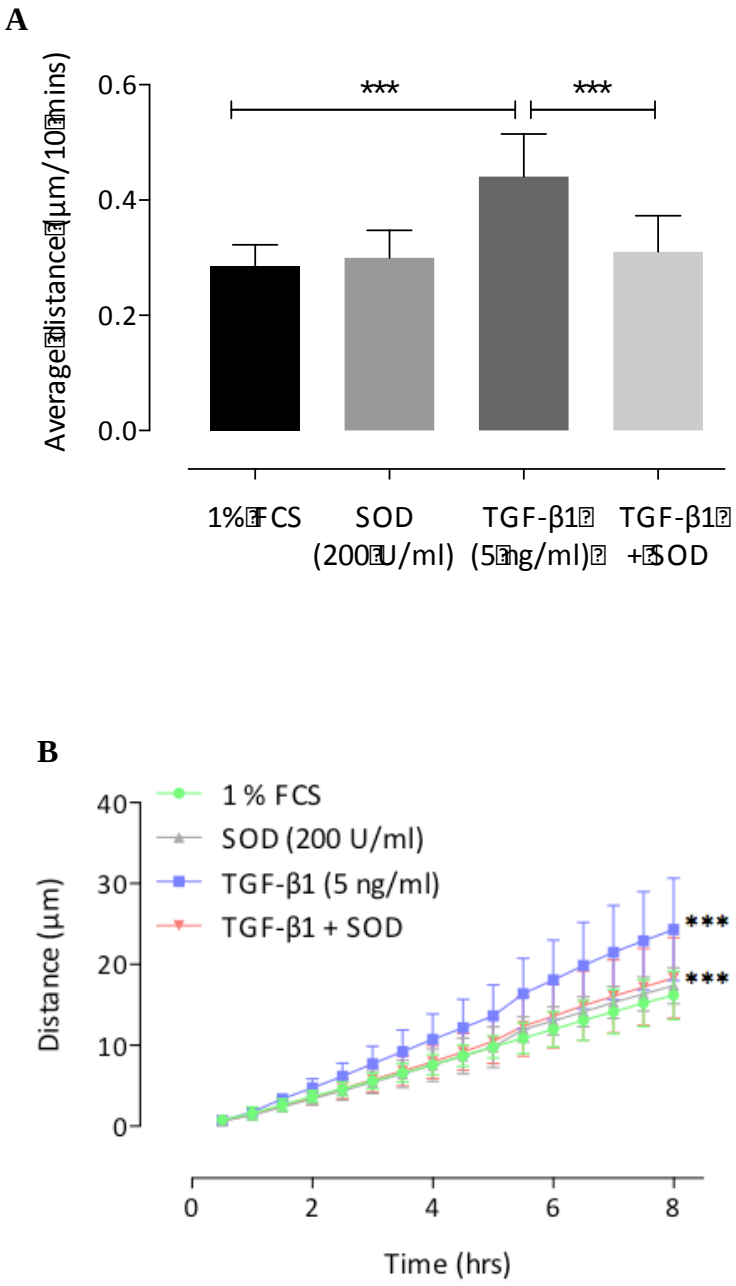


Figure 5.2 Effects of ROS on TGF-β1-mediated HAoAF migration
HAoAF were seeded at a density of 2 x 10^4/well in a 12-well culture plate and allowed to adhere overnight. 18 h prior to imaging, cells were adapted in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml). 30 min prior to commencement of imaging, some cells were treated with SOD (200 U/ml). Multifield live cell imaging was performed with a single frame being taken every 10 min for 8 h. Cell tracking (A) was performed using ImageJ. Migration speed (B) was quantified by importing cell-tracking data into Excel. Values denote means ± SEM ***p<0.001 TGF-β1 vs. untreated control and TGF-β1 treated vs. TGF-β1 + SOD using unpaired Student's t-test. n = Representative of 4 independent experiments.

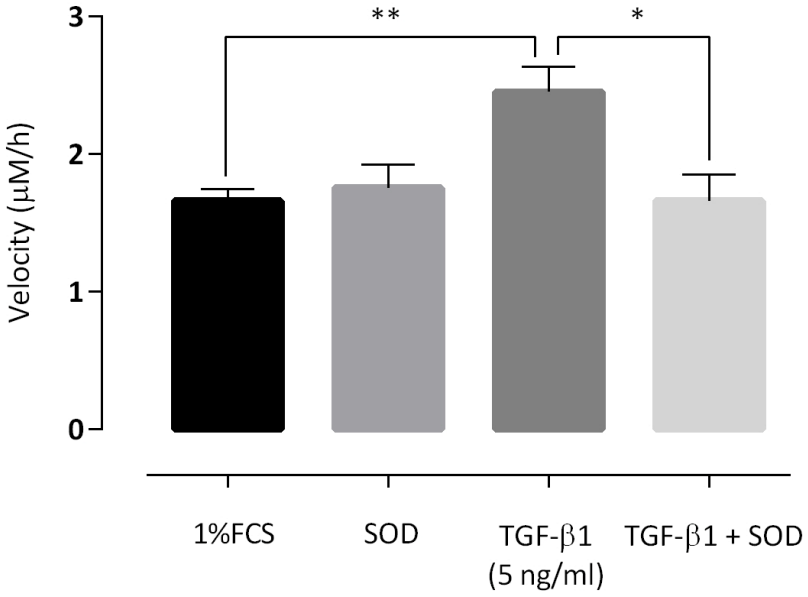


Figure 5.3 Effect of TGF-β1 on velocity of HAoAF migration
HAoAF were seeded at a density of 2 x 10^4/well in a 12-well culture plate and allowed to adhere overnight. 18 h prior to imaging, cells were adapted in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml). 30 min prior to commencement of imaging, some cells were treated with SOD (200 U/ml). Multifield live cell imaging was performed with a single frame being taken every 10 min for 8 h. Cell tracking was performed using ImageJ and migration speed was quantified by importing cell-tracking data into Excel. Values denote means ± SEM $^{**}p<0.01$ TGF-β1 vs. untreated control, $^{*}p<0.05$ TGF-β1 vs. TGF-β1 + SOD using unpaired Student's t-test. n = Representative of 4 independent experiments.

5.4 Effect of Nrf2 on HAoAF migration and velocity

To determine whether Nrf2 was involved in TGF-β1-mediated HAoAF migration, sub-confluent HAoAF were seeded and allowed to adhere overnight. Subsequently cells were transfected with scramble or Nrf2 siRNA as validated in Chapter 4 (see Fig 4.20). Eight hours following transfection, the transfection mixture was aspirated from wells and replaced with DMEM supplemented with 1% FCS for overnight equilibration. Cells were treated with TGF-β1 (5 ng/ml) or DMEM supplemented with 10% FCS and live imaging was commenced. A frame was captured every 10 min for 8 h. Incubation of cells with DMEM supplemented with 10% FCS increased migration, suggesting that cells were responsive to a chemotactic stimulus. However, knockdown of Nrf2 had no marked effect on migration distance or on the velocity of migration in HAoAF (Fig 5.4A and B).

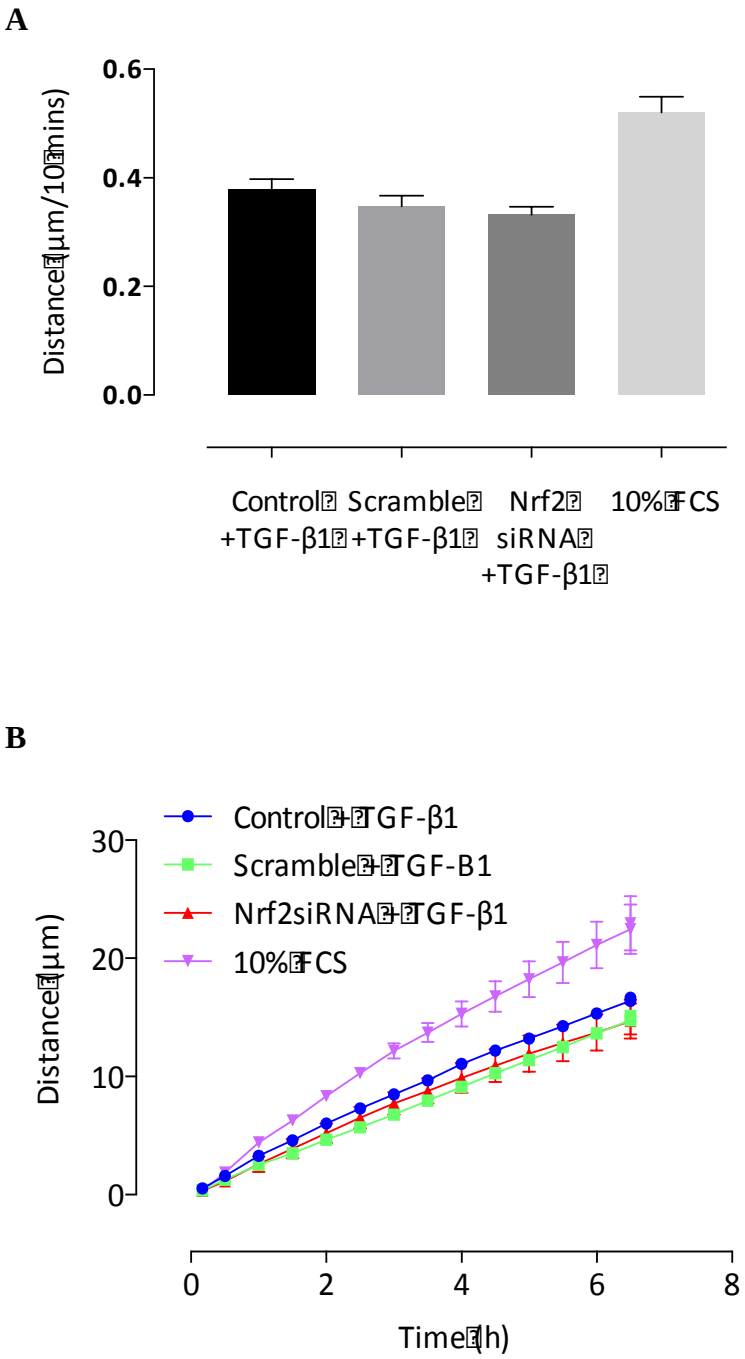
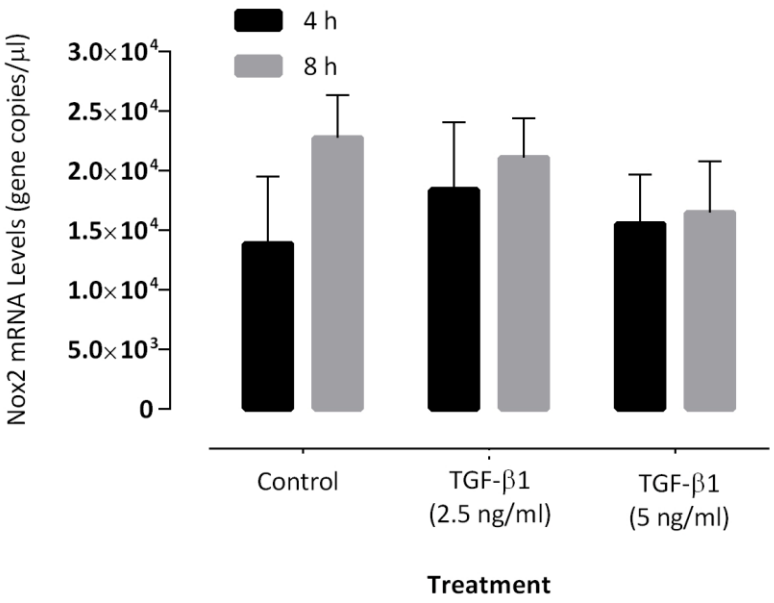


Figure 5.4 Effects of Nrf2 siRNA on TGF-β1-mediated HAoAF migration
HAoAF were seeded at 2 x 10^4/well in a 12-well culture plate and allowed to adhere overnight and were subsequently transfected with scramble or Nrf2 siRNA. Eight hours following transfection, the transfection mixture was aspirated from wells and replaced with DMEM supplemented with 1% FCS for overnight equilibration. Subsequently cells were treated with TGF-β1 (5 ng/ml) or DMEM supplemented with 10% FCS. Multifield live cell imaging was performed with a single frame being taken every 10 min for 8 h. Cell tracking (A) was performed using ImageJ. Migration speed (B) was quantified by importing cell-tracking data into Excel. Values denote means ± SEM. n = Representative of 2 independent experiments.

5.5 Effect of TGF-β1 on Nox mRNA levels

As NAD(P)H oxidases have been implicated in mediating the migration of vascular smooth muscle cells and adventitial fibroblasts, often in response to TGF-β1 (Hurd et al., 2012; Tobar et al., 2010; Lyle et al., 2009; Guzik et al., 2006; Pagano et al., 1998), mRNA levels of two Nox isoforms reported to be expressed by adventitial fibroblasts (Haurani and Pagano, 2007) were determined. Cells were treated with TGF-β1 (0 – 5 ng/ml) for 4 and 8 h and levels of Nox2 and Nox4 mRNA were analysed. Treatment with TGF-β1 for 4 or 8 h did not alter levels of Nox2 mRNA in HAoAF when compared to untreated control (Fig 5.5a). Levels of Nox4 mRNA were also not altered in HAoAF in response to treatment with TGF-β1 for 4 or 8 h when compared to untreated control (Fig 5.5b).

A



B

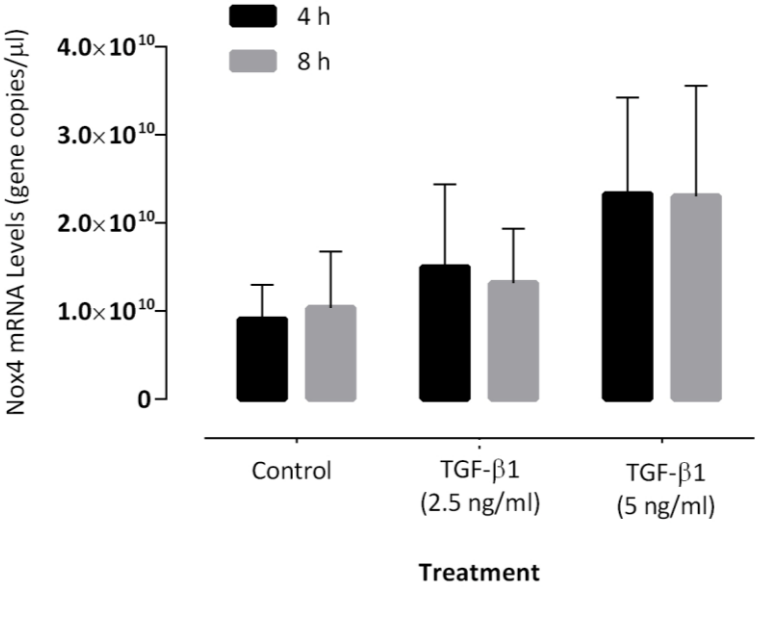


Figure 5.5 Nox2 and Nox4 mRNA levels in HAoAF after treatment with TGF-β1
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (0 - 5 ng/ml) for 4 or 8 h. Levels of Nox2 and Nox4 mRNA in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes (RPL13A, SDHA and TATABOX). Values denote means ± SEM. n = Representative of 6 independent experiments.

5.6 Effect of TGF-β1 on ROS generation in HAoAF

In order to determine the effect of TGF-β1 on ROS generation in HAoAF, cells were equilibrated in phenol red-free DMEM supplemented with 1% FCS 18-24 h prior to pre-treatment with SOD (200 U/ml) or the NAD(P)H oxidase inhibitor, VAS2870 (VAS; 10 μM) before co-treatment with TGF-β1 (0 - 5 ng/ml) over 40 minutes of ROS measurement. The luminol analogue, LO12 (20 μM) was used as a probe for the detection of ROS generation. Treatment with TGF-β1 (2.5 and 5 ng/ml) resulted in enhanced ROS generation when compared to untreated control (Fig 5.6). In order to determine the type of ROS generated in response to TGF-β1, cells were treated with extracellular SOD, which catalyses the dismutation of $O_2^{\cdot -}$ to H_2O_2. In the presence of SOD, ROS generation was significantly abrogated in cells and comparable to basal levels seen in untreated control, suggesting that $O_2^{\cdot -}$ may be the predominant ROS generated in response to TGF-β1 treatment ($p<0.01$ TGF-β1 (2.5 ng/ml) + SOD vs. TGF-β1 alone and $p<0.001$ TGF-β1 (5 ng/ml)+ SOD vs. TGF-β1 alone). Furthermore, use of VAS, a NAD(P)H oxidase inhibitor, also resulted in a significant abrogation of ROS levels when compared to cells treated with TGF-β1 (2.5 and 5 ng/ml) alone suggesting that $O_2^{\cdot -}$ generation seen in response to TGF-β1 treatment was NAD(P)H-dependent ($p<0.05$ TGF-β1 (2.5 ng/ml) + VAS vs. TGF-β1 alone and $p<0.01$ TGF-β1 (5 ng/ml) + VAS vs. TGF-β1 alone).

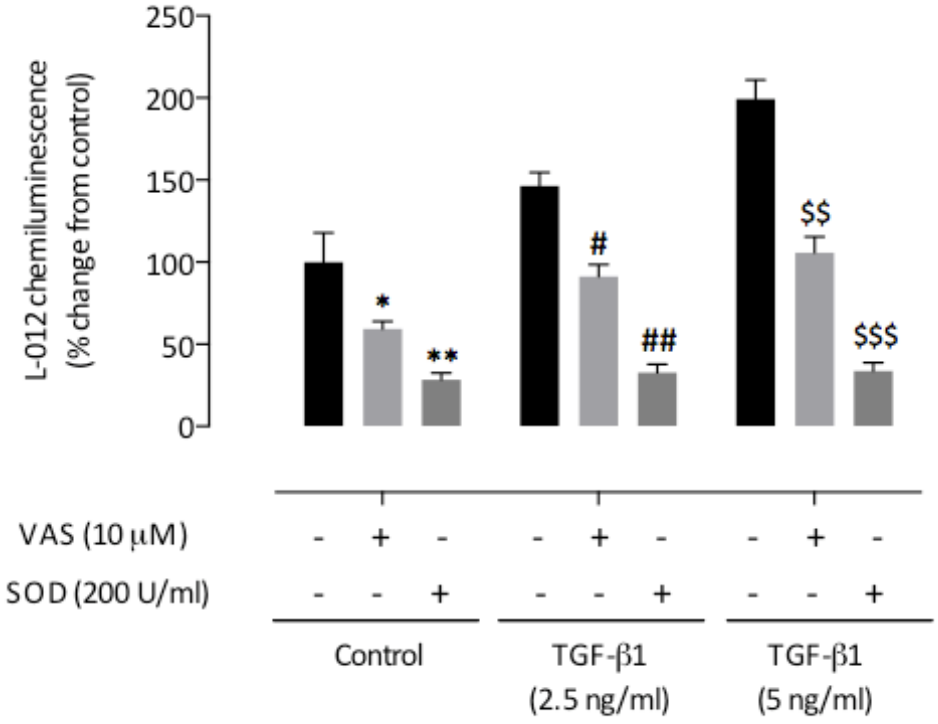


Figure 5.6 Effect of TGF-β1 treatment on ROS generation in HAoAF
Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently pre-treated with SOD (200 U/ml) or the NAD(P)H oxidase inhibitor, VAS2870 (VAS; 10 μM) for 30 min prior to co-treatment with TGF-β1 (0 - 5 ng/ml). ROS production was determined over 40 min using the luminescence probe, LO12 (20 μM) as described in Methods. Representative percentage change from control was determined over 40 min. Values denote means ± SEM *p<0.05 Untreated control + VAS vs. untreated control alone, **p<0.01 Untreated control + SOD vs. untreated control alone; # p<0.05 TGF-β1 (2.5 ng/ml) + VAS vs. TGF-β1 alone, ## p<0.01 TGF-β1 (2.5 ng/ml)+ SOD vs. TGF-β1 alone; $$ p<0.01 TGF-β1 (5 ng/ml) + VAS vs. TGF-β1 alone, $$$ p<0.001 TGF-β1 (5 ng/ml) + SOD vs. TGF-β1 alone. n = Representative of 4 independent experiments.

5.7 Discussion

Inimal thickening and adverse vascular remodelling are key events in advanced vascular disease and injury (Pels et al., 1999). Under the autocrine and paracrine actions of TGF-β1, released in the adventitial layer, adventitial fibroblasts are activated and can migrate from the adventitial layer of arteries to the intima, where they subsequently proliferate, therefore contributing to this pathology (Zalewski and Shi, 1997). Recent investigation into this process has revealed evidence of the pivotal role of adventitia-derived ROS with some evidence to suggest that this may be a TGF-β1-mediated event (Csanyi et al., 2009; Lyle et al., 2009). Understanding the underlying mechanism of this process in HAoAF has not been fully elucidated in HAoAF. The following experiments sought to explore the mechanisms by which TGF-β1 may cause HAoAF migration in culture.

5.7.1 Effect of TGF-β1 on HAoAF migration

As discussed in Chapter 3, treatment of HAoAF with TGF-β1 resulted in HAoAF proliferation, an event widely reported to occur *in vivo* in models of vascular injury (Ren et al., 2011; Liu et al., 2008; Siow et al., 2003; Shi et al., 1996). As the processes of proliferation and migration often occur in parallel in vascular disease (Sivakumar et al., 2006), migration of HAoAF in response to TGF-β1 (2.5 - 5 ng/ml, 8 h) treatment was analysed using time-lapse video microscopy. Results revealed that the average distance of migration was significantly higher in HAoAF treated with TGF-β1 than that measured in untreated control (Fig 5.2). These results are in keeping with previous studies that have shown that cell migration is a TGF-β1-driven process. It has been reported that siRNA against the downstream facilitators of the TGF-β1 signalling pathway, phopsho-Smad2 and 3 significantly decrease adventitial fibroblast migration *in vitro*, coupled with a concomitant decrease in proliferation of these cells and a decrease in the mRNA levels of the TGF-β1-target genes; α-smooth muscle actin and collagens I and II (Ren et al., 2011).

Furthermore, transfection of vessels with an adenovirus coordinating the expression of the secreted form of TGF-β type II receptor in order to antagonise TGF-β1 signalling in a porcine model of restenosis following angioplasty, revealed a decrease in adventitial collagen deposition and decreased constrictive remodelling, suggesting that TGF-β1 facilitated these pathological events (Kingston et al., 2001). Similarly, TGF-β1 has been shown to increase proliferation and migration in the medial layer of the vessel in a rat model of carotid balloon injury. Administration of neutralizing anti-TGF-β1 antibodies

significantly reduced the size of intimal lesions as well as decreasing cell proliferation in the medial layer (Wolf et al 1994). Furthermore, the injection of recombinant soluble TGF-β1 type II receptor localised preferentially at the adventitial layer and developing neointima in an injured rat carotid artery resulted in a reduction in intimal lesion formation of up to 65% and coincided with reduced adventitial fibrosis and collagen deposition (Smith et al., 1999). Mallawaarachchi and colleagues reported that gene transfer of an adenovirus overexpressing Smad7, an inhibitor of the classical TGF-β1 signalling pathway, specifically to the adventitial layer, attenuated adventitial cell migration to the neointima following balloon injury in rat carotid artery (Mallawaarachci et al., 2005). This evidence and results from the current study strongly suggest the involvement of TGF-β1 in driving cell migration and *in vivo,* may be implicated in HAoAF-driven migration and consequent vascular remodelling during disease. In particular, TGF-β1-mediated adventitial cell migration plays an important role in neointima formation and constrictive remodelling, a process, which may be attenuated by antagonising TGF-β1 signalling (Kato et al., 2001).

5.7.2 Effect of SOD on TGF-β1–mediated HAoAF migration

Although it is well established that TGF-β1 causes cell migration during vascular disease and injury, the exact mechanisms underlying this process remain to be fully elucidated. Redox imbalance is a tenet of the pathogenesis seen during the progression of many vascular diseases (Haurani and Pagano, 2007; Rocic and Lucchesi, 2005). This imbalance in oxidant-generating *vs.* oxidant-catalysing systems leads to a build-up of ROS, including $O_2^{-\cdot}$ and H_2O_2. Much of this redox imbalance is first seen in the adventitial layer where it has been reported that adventitial NAD(P)H oxidase-derived ROS plays a role in neointimal growth and medial hypertrophy by causing cell migration (Haurani and Pagano, 2007) and there is mounting evidence to suggest that ROS are used to relay signals from activated cell surface receptors to direct changes necessary for cell movement (Hurd et al., 2012). Due to existing studies postulating that ROS are involved in driving migration of cells from the media and adventitia of injured vasculature and that the predominant species implicated is $O_2^{-\cdot}$, the migration of HAoAF was investigated in the presence of TGF-β1 with or without the $O_2^{-\cdot}$ scavenger, SOD. Findings show that when co-treated with TGF-β1 and SOD, the total distance that cells migrated was significantly attenuated and was similar to that observed in untreated cells (Fig. 5.2). Furthermore, this was accompanied by a

significant attenuation in the velocity of migration, from 2.451 ± 0.366 μm/h in cells treated with TGF-β1 to 1.661 ± 0.382 μm/h in cells treated with TGF-β1 in the presence of SOD (Table 5.1). These results suggest that ROS may play a role in TGF-β1-mediated HAoAF migration.

The first study to suggest that fibroblast migration may be reduced by administration of SOD was carried out by Wach and colleagues. Fibroblasts exhibited increased cell proliferation and migration in response to phorbol myristate acetate, an inducer of superoxide generation, however, addition of SOD abrogated this response suggesting that $O_2^{-}\cdot$, in small amounts, is involved in fibroblast migration and possibly proliferation (Wach et al., 1987). An earlier study looking at the effects of SOD on neutrophil migration in plasma samples from healthy adults was assessed in the absence or presence of SOD and it was reported that SOD inhibited the migratory response of neutrophils to sites of inflammatory challenge, suggesting that the process was in part, $O_2^{-}\cdot$ dependent. In order to rule out the possibility that H_2O_2 may be partly responsible for neutrophil migration, the enzyme that catalyses the breakdown of H_2O_2 to water and oxygen, catalase, was used in place of SOD. Results showed that catalase did not affect neutrophil migration, suggesting that this migration was a H_2O_2-independent event (Petrone et al., 1980). Similarly, a recent study in human lung fibroblasts also reported that SOD and not catalase was involved in the regulation of TGF-β1 release and subsequent activation of fibroblasts (Qi et al., 2009). The study also demonstrated that a chloride channel blocker was able to inhibit the increase in intracellular $O_2^{-}\cdot$ induced by exposure to extracellular $O_2^{-}\cdot$ suggesting that this may be a possible mechanism whereby extracellular $O_2^{-}\cdot$ may regulate the release of TGF-β1 and cause the subsequent activation of fibroblasts (Qi et al., 2009). The findings from the current study support existing evidence in the literature and demonstrate for the first time that TGF-β1 may drive HAoAF migration and this may be partly through mediating an increase in ROS production in this cell type. In particular, this process may be due to TGF-β1-driven generation of $O_2^{-}\cdot$.

Several studies have shown that extracellular SOD makes up approximately 50% of total SOD activity in the human aorta (Luoma et al., 1998; Stralin et al., 1995), whereas in other tissues represents only a small proportion of the total SOD activity (Marklund, 1984), indicating that extracellular SOD is physiologically relevant and plays a

significant role in the redox balance of the vascular wall. In the present study, cells were treated with exogenous SOD as opposed to the cell permeable form of SOD typically linked with polyethylene glycol (PEG-SOD). This ensured that any attenuation of migration that was observed in the presence of SOD was likely to be a result of the dismutation of $O_2^{-\cdot}$ released into the extracellular space. Results from HAoAF treated with TGF-β1 in the presence of SOD show that these cells have markedly reduced distance of migration and decreased velocity, suggesting that migration in this cell type is driven by $O_2^{-\cdot}$ released into the extracellular space. However, although these results demonstrate a significant abrogation in migration distance and speed, it would be interesting to observe the effects of administration of PEG-SOD on migration of HAoAF as it has been postulated that intracellular $O_2^{-\cdot}$ may also be important in mediating cell motility as has been demonstrated by a study in VSMC in which mitochondrial-derived intracellular $O_2^{-\cdot}$ has been shown to activate the PI3K/Akt pathway which in turn was able to drive VSMC migration during neointima formation (Artaud-Macari et al., 2013; Wang et al., 2012; Lyle et al., 2009).

Due to the effect of SOD administration on the migratory behaviour of cells, many studies have investigated the effect of this antioxidant on pathophysiological cell migration. There is a large body of evidence that postulates that the expression of extracellular SOD using gene transfer can reduce neointima formation by decreasing the migration of cells from the medial and adventitial layers of the blood vessel towards the lumen (Wang et al., 2012). This has been observed in a rabbit model where the adenoviral gene transfer of extracellular SOD in the aorta after endothelial denudation was shown to result in a decrease in VSMC migration and consequently a decrease in intimal thickening as well as an accompanied decrease in $O_2^{-\cdot}$ production as determined by the dihydroethidium assay was observed (Laukkenen et al., 2002). Another study has reported a decrease in restenosis following angioplasty in rabbit atherosclerotic iliac arteries after adenovirus-mediated gene transfer of extracellular SOD, with a decrease in collagen accumulation, inflammation and improved endothelial function when compared to those arteries injected with adenovirus carrying no transgene (Durand et al., 2005). Furthermore, Brasen and colleagues have also demonstrated the beneficial effects of the gene transfer of extracellular SOD and reported a 61% decrease in neointima formation 28 days after endothelial denudation and stent insertion in Watanabe heritable hyperlipidemic rabbits as well as a decrease in

observed oxidant production following SOD administration (Brasen et al., 2007). These studies demonstrate that extracellular SOD may decrease neointima formation by abrogating the proliferation of cells in the medial and adventitial layers of the blood vessel and their subsequent migration towards the lumen of the vessel. This correlates with findings from this study, which have shown that HAoAF migration induced by TGF-β1 is abrogated in the presence of extracellular SOD.

The potential source of superoxide in response to vascular injury has been postulated to be the membrane-bound NAD(P)H oxidase system (Csanyi et al., 2009) which is one of the major sources of $O_2^{-\cdot}$ implicated in compounding vascular injury (Kanellakis et al., 2004; Jacobson et al., 2003; Szocs et al., 2002). In one study, measurement of Nox homologs after carotid injury showed an increase in p22phox, gp91phox and Nox4 mRNA levels after 3 days. These levels remained elevated for 15 days with an accompanied increase in $O_2^{-\cdot}$, observed in neointimal smooth muscle and adventitial fibroblasts (Szocs et al., 2002).

The presence of growth factors in and around the neointima has led to investigation into whether they contribute to the production of ROS during vascular injury. Initial work in VSMC led to the observation that TGF-β1 enhanced $O_2^{-\cdot}$ production in VSMC with an accompanied increase in the protein expression of p22phox (Churchman et al., 2009). Previously, Thannickal and colleagues proposed a mechanism whereby TGF-β1 may induce the expression of membrane bound NAD(P)H oxidase subunits in human lung fibroblasts by protein tyrosine phosphorylation (Thannickal et al., 1998). Blocking the TGF-β1-mediated tyrosine phosphorylation using an inhibitor resulted in the abrogation of NAD(P)H oxidase activation and H_2O_2 production, suggesting that this was the primary method by which TGF-β1 induced ROS production in this cell type (Thannickal et al., 1998). In contrast to findings from Thannickal et al., in fibroblasts from the human stroma, TGF-β1 is thought to activate Nox4 as opposed to the membrane-bound Nox2 (Sampson et al., 2011). TGF-β1 induction of Nox4 and the consequent ROS production is required for downstream cytoskeletal remodelling which was postulated to accelerate fibroblast-to-myofibroblast differentiation (Sampson et al., 2011) and may also be an important mechanism during TGF-β1-mediated cell migration (Thannickal et al., 2003). TGF-β1 can also activate several MAPK pathways such as PKC, independent of Smad signalling, as previously discussed in Chapter 3.

A possible mechanism for this enhanced $O_2^{-\cdot}$ production has been suggested by Lyle and colleagues who have demonstrated that Nox4 is involved in cell migration of VSMC when in complex with p22phox and an accessory protein known as polymerase (DNA-directed) delta-interacting protein (Poldip2). Poldip2 has been shown to associate with p22phox, Nox1 and Nox4 and co-localises with p22phox at sites of Nox4 localisation, increasing its enzymatic activity and positively regulating the basal production of ROS in VSMCs (Lyle et al., 2009). Interestingly, overexpressing Poldip2 results in activation of RhoA, which is known to mediate the process of cell migration by regulating actin polymerisation and depolymerisation (Ridley, 2001). Proteins of the Rho subfamily are well known to regulate the cytoskeleton, cell growth and migration (Meyer et al., 2006; Smith et al., 2006) and Rho-stimulated contractility drives stress fibre and focal adhesion formation and up-regulation of α-smooth muscle actin in fibroblasts (Hinz et al., 2001) and epithelial cells (Zhong et al., 1997).

The Poldip2/p22phox/Nox4 complex has been reported to regulate focal adhesion turnover in VSMC, a process which is pivotal for cell migration to occur (Lyle et al., 2009). Moreover the small GTPase Rac1 has been shown to play a key role in vascular superoxide generation by NAD(P)H oxidase, regulating myofibroblast migration via assembly of adhesion complexes and actin re-organisation alongside ROS-mediated Ras activity (Parsons et al. 2008; Alexandrova et al., 2006). These results show that changes in ROS production and consequent downstream cytoskeletal re-arrangement may result in increased or decreased cell migration and may go some way in explaining the underlying molecular processes of migration during neointima formation as a function of elevated ROS levels typically seen during this stage of the disease process.

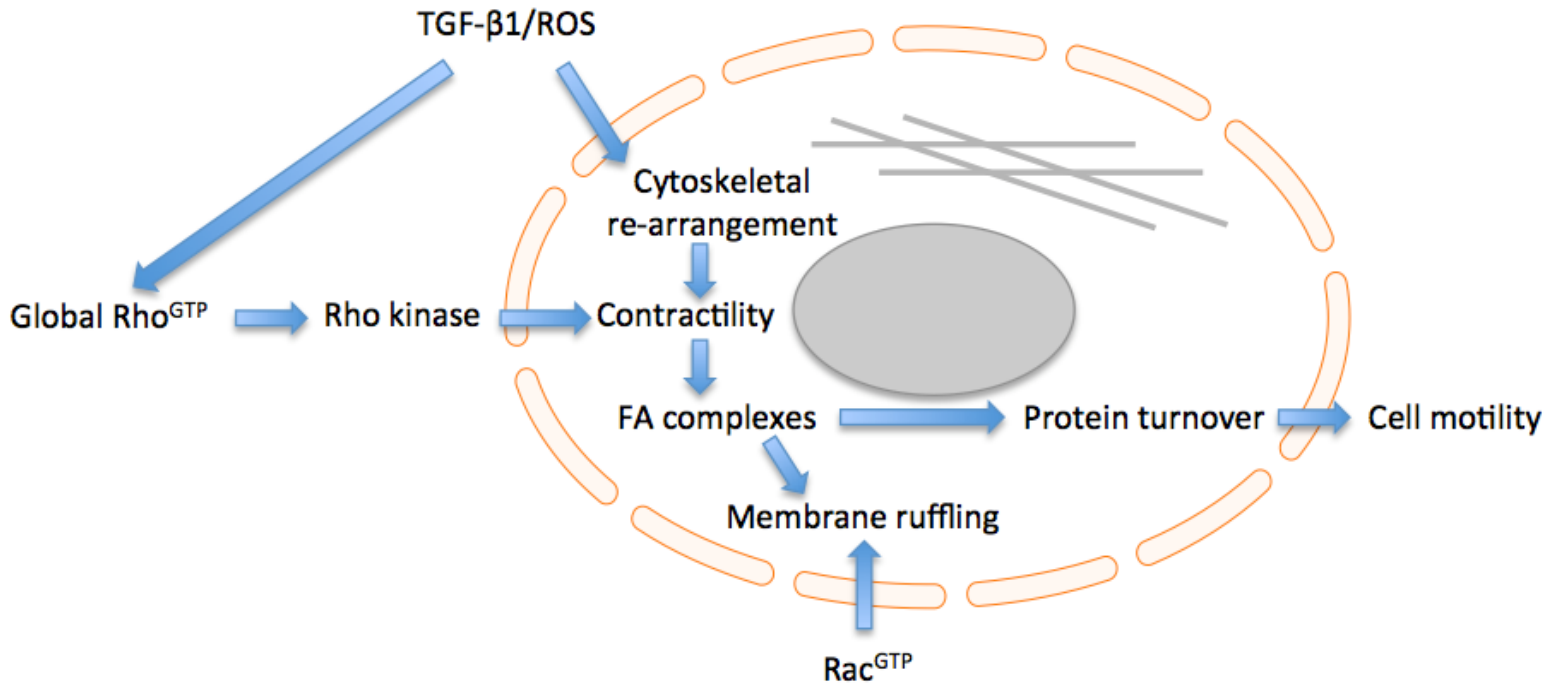


Figure 5.7 Possible mechanism by which TGF-β1 or ROS may mediate cell motility. Rho-stimulated contractility drives stress fibre and focal adhesion formation and up-regulation of α-smooth muscle actin and results in contractility and formation of focal adhesion (FA) complexes and consequent membrane ruffling, a process also driven by RacGTP. FA complexes have been implicated in cell motility and require constant protein turnover. In culture, these events are affected by substrate rigidity; increased rigidity is proportional to cell traction force, acquisition of α-smooth muscle actin
positive stress fibres, cell differentiation and migration.

5.7.3 Effect of TGF-β1 on Nox2 and Nox4 mRNA levels in HAoAF

Actin cytoskeleton reorganisation is pivotal in promoting cell migration and there is much experimental evidence to suggest that during this process both Ras and Rho GTPases make an essential contribution (Bar-Sagi and Hall, 2000), possibly in a ROS-mediated manner (Parsons et al., 2008; Alexandrova et al., 2006). In endothelial cells, these ROS-driven changes in migration are thought to be mediated by p21-activated kinase-1 (PAK1), the small GTPase Rac1 and NAD(P)H oxidase regulatory subunits (Fukai, 2006). In contrast to findings in VSMC where Nox4 is thought to drive migration of cells, in endothelial cells, Nox2 is thought to mediate this process by colocalising with its regulatory subunit, $p47^{phox}$ at the leading edge of the cells, where it associates with actin to promote migration; an effect which is significantly abrogated following Nox2 knockdown (Ikeda et al., 2005). Rac1 and PAK1 are equally important as dominant negative forms of both of these proteins block ROS production, membrane ruffling and as a consequence, cell migration (Wu et al., 2005).

HAoAF express both Nox2 and Nox4 (Haurani and Pagano, 2007), therefore in order to determine which of these NAD(P)H oxidase isoforms were responsible for TGF-β1-mediated ROS production in HAoAF, levels of Nox2 and Nox4 mRNA in cells treated with TGF-β1 were investigated. It was found that cells exhibited no significant changes in Nox2 or Nox4 mRNA following treatment with TGF-β1 after 4 or 8 h.

Assembly of Nox has been reported to occur for up to 24 h after exposure to an oxidant (Serrander et al., 2007), however this is largely dependent upon the Nox isoform; Nox4 is constitutively active isoform and Nox4 mRNA has been reported to increase in the absence of a stimulant (Serrander et al., 2007). One study suggests that new protein synthesis was not required for TGF-β1 regulated induction of Nox4 in human pulmonary SMC (HPSMC) as use of the protein synthesis inhibitor, cycloheximide failed to inhibit TGF-β1 induction of Nox4 (Sturrock et al., 2006). The same study reported a significant increase in Nox4 mRNA levels following treatment with TGF-β1 (2 ng/ml) for 24 h and Nox4 protein levels were also increased after incubation of cells with TGF-β1 (1 ng/ml) for 72 h as well as there being a significant increase in $O_2^{-\cdot}$ production (Sturrock et al., 2006). Similar results have been reported in endothelial cells where acute TGF-β1 (10 ng/ml, 5 min) treatment has been observed to increase the production of H_2O_2, an increase which was abrogated in cells transfected with dominant negative Nox4 (Hu et al., 2005). ROS produced by Nox4 is still to be fully elucidated - although some argue that Nox4 primarily produces H_2O_2 (Lyle et al., 2009), others

suggest that Nox4 may generate $O_2^{-\cdot}$ which dismutates to the membrane-permeant H_2O_2 (Serrander et al., 2007). Although the above evidence suggests that TGF-β1 increases Nox4 mRNA in other cell types, the current study was not able to demonstrate this in HAoAF where TGF-β1 (2.5 – 5 ng/ml) treatment for 4 or 8 h did not affect levels of Nox4 mRNA, however further work looking at the effect of TGF-β1 on Nox4 expression over longer time-points may provide clearer evidence of the underlying source of ROS in this cell type.

The adventitia has also been widely reported as expressing Nox2, and its component parts, p22phox, p47phox and p67phox which make up a multiprotein complex required for the activation of this Nox isoform (Haurani and Pagano, 2007; Pagano et al., 1998; Pagano et al., 1997). The role of this isoform in cardiovascular disease progression has also been widely reported; Wang and colleagues have shown that AngII infusion into mice for 6 days resulted in intimal and adventitial NAD(P)H oxidase-derived superoxide production and subsequent aortic medial thickening, however upon deletion of Nox2, intimal and adventitial superoxide production was attenuated and there was a marked reduction in aortic medial thickening (Wang et al., 2001). These findings were confirmed in a more recent study where targeted inhibition of Nox2 to the adventitial layer resulted in an attenuation of AngII-induced medial hypertrophy (Liu et al., 2004). The ROS primarily implicated as being released by Nox2 is $O_2^{-\cdot}$ the production of which has been reported as being significantly attenuated in neointima formation following targeted delivery of an adenovirus expressing a specific Nox2 inhibitor, nox2ds, to the adventitia (Dourron et al., 2005). The observation that Nox2 is also able to contribute to ROS production in the adventitial layer and result in the activation and migration of fibroblasts from this layer to contribute to restenosis is widely established in the literature. However, in the current study, mRNA levels of this Nox isoform were relatively low and did not change significantly in response to treatment with TGF-β1 (2.5 – 5 ng/ml) treatment for 4 or 8 h when compared with untreated control. It should be noted that although this data is interesting, mRNA levels of Nox 2 and 4 are not entirely reflective of the activity of these isoforms in a cell system when taken alone and that post-translational modifications should also be taken into account in future work.

5.7.4 Effect of knockdown of Nrf2 on HAoAF migration

Cellular redox homeostasis is a highly regulated process and is maintained, in part, by the Nrf2/ARE signalling pathway; ROS has been shown to increase cell migration and interestingly the Nrf2 inhibitor, Keap1 is an actin binding protein which may play a role in mechanosensing (Dai et al., 2007; Hosoya et al., 2005) and cell migration via its localisation with focal adhesion complexes (Velichkova and Hasson, 2003).

Therefore in order to determine whether the Nrf2/ARE signalling pathway was acting as a mechano-sensor for oxidative stress, cells were transfected with Nrf2 siRNA. Subsequently migration studies were carried out, however, knockdown of Nrf2 had no effect on HAoAF migration in response to TGF-β1 (Fig 5.4). Previously, others have reported that silencing of Nrf2 results in enhanced cell migration; Rachkonda and colleagues found that cell migration was increased in A549 cells transfected with Nrf2 siRNA or in HepG2 cells in which Keap1 had been overexpressed (Rachkonda et al., 2010). As cell motility is closely related with TGF-β1 signalling, the same study investigated the relationship between Nrf2 and TGF-β1 in HepG2 cells; immunprecipitation assays demonstrated that Nrf2 forms a nuclear complex with phospho-Smad2/3 and Co-Smad4 and that it suppresses CAGA-directed reporter activity. RNAi-mediated suppression of Nrf2 enhanced the expression of the Smad target genes, plasminogen activator inhibitor-1 (PAI-1) and E-cadherin. In addition, loss of Nrf2 in cells was associated with an increase in Smad-mediated motility suggesting that loss or suppression of Nrf2 contributes to increased motility as a result of dysregulated Smad signalling (Rachkonda et al., 2010).

Similar results have been observed in a recent study in mouse lung fibroblasts where Nrf2 knockdown resulted in fibroblast differentiation and an increase in TGF-β1-regulated genes such as α-smooth muscle actin when compared to control cells as well as an increase in oxidative stress (Artaud-Macari et al., 2013) suggesting that Nrf2/ARE and TGF-β1 signalling may be closely related pathways. Further evidence comes from the rat renal fibroblast cell line (NRK-49F) in which over-expression of Nrf2 using an adenovirus decreased the expression of TGF-β1-target genes, PAI-1, α-smooth muscle actin, fibronectin and type I collagen as well as inhibiting TGF-β1-mediated Smad3 phosphorylation, which was restored by siRNA-mediated knockdown of Nrf2 (Oh et al., 2012). This evidence suggests that Nrf2 negatively regulates the TGF-β1-Smad pathway and potential therapeutic intervention may be beneficial during neointima formation, restenosis and vascular remodelling seen during disease.

The present study provides novel evidence that TGF-β1 may mediate cell migration in HAoAF by increasing ROS production. There is much evidence in the literature to suggest a close relationship between the TGF-β1 signalling pathway and the Nrf2/ARE antioxidant pathway exists and therefore the potential cross-talk between these pathways may offer a way of mediating pathophysiological cell migration. Evidence from this study did not indicate that Nrf2 knockdown had any effect on cell motility in HAoAF, although this is not entirely unexpected since Nrf2 does not lie directly downstream of the TGF-β1 signalling pathway established in this cell type. Nevertheless, in order to generate more conclusive evidence in this cell type, further work would need to be carried out. In particular, it would be interesting to investigate the effects of TGF-β1 on migration of fibroblasts from Nrf2 knockout animals. Cells from knockout animals have the advantage over Nrf2 siRNA transfected cells as the transfection can be inefficient and transient and more importantly, the process of transfection itself can be toxic enough to activate stress-response pathways within the cell (Seth et al., 2012), a factor which is especially relevant to the current study. *In vivo* studies looking specifically at adventitial cell migration into the neointima of an atherosclerotic artery or one that has undergone stenting in Nrf2 knockout animals would also provide some clarity about the cross-talk between these two signalling pathways and their effects on cell migration. Elucidating the exact mechanism by which TGF-β1 causes HAoAF migration may be an important step towards successful therapeutic intervention during vascular injury and disease.

5.7.5 Effect of inhibition of NAD(P)H oxidase on TGF-β1-mediated ROS generation in HAoAF

Generation of ROS in response to TGF-β1 has been widely reported in endothelial cells (Hu et al., 2005) and cardiac fibroblasts (Cucoranu et al., 2005), however whether this growth factor had a similar effect on HAoAF has not been previously elucidated. In order to determine whether TGF-β1 treatment resulted in ROS generation in this cell type, cells were treated with TGF-β1 and luminescence was measured using the luminol analogue, L-012. In addition, to elucidate the type and potential source of ROS, cells were also co-treated with SOD, which catalyses the dismutation of $O_2^{-\cdot}$ to H_2O_2 and VAS2870, a specific NAD(P)H oxidase inhibitor.

Results demonstrated that in response to TGF-β1 (2.5 and 5 ng/ml), there was enhanced ROS generation when compared to untreated control and that this increase was abrogated in the presence of SOD, suggesting that the primary species of ROS detected in HAoAF following TGF-β1 treatment was $O_2^{-}\cdot$. Similar findings have been reported in a study in human umbilical vein endothelial cells (HUVEC) in which treatment of cells with TGF-β1 (10 ng/mg) led to ROS production as measured by the probe, dichlorodihydrofluorescein (DCF), and which was attributed to a NAD(P)H oxidase isoform. The observed ROS generation was attenuated in the presence of the flavoprotein inhibitor, diphenyl iodinium (DPI, Hu et al., 2005). Interestingly, the same study reported cytoskeletal changes in HUVEC in response to TGF-β1 that were blocked following transfection of cells with a dominant- negative Nox4 adenovirus which suggests that these changes were mediated by ROS production (Hu et al., 2005). Similarly, in cardiac fibroblasts, Cucoranu and colleagues have shown that exposure of cells to TGF-β1 (10 ng/ml) resulted in $O_2^{-}\cdot$ release from cardiac fibroblast cell membranes and further investigation revealed a significant increase in Nox4 mRNA levels after 24 h with a concomitant increase in protein levels of Nox4, suggesting that this isoform was the source of ROS in this cell type (Cucoranu et al., 2005). Their findings also went on to confirm those from the study by Hu et al., that changes in the cytoskeleton of cells in response to TGF-β1, in particular in the upregulation of α-smooth muscle actin, required ROS; treatment of cells with DPI abrogated this effect (Cucoranu et al., 2005).

There is much contention surrounding the species of ROS produced by Nox4 with some studies suggesting that this isoform generates predominantly H_2O_2 at low levels as opposed to $O_2^{-}\cdot$, largely determined by its third extracytosolic loop which contains an extra 28 amino acids more than Nox1 and Nox2, isoforms which generate $O_2^{-}\cdot$ (Takac et al., 2011). Indeed, previous studies have postulated that TGF-β1 exposure results in the NAD(P)H-dependent production of H_2O_2 in lung fibroblasts, although the particular isoform responsible for this was not elucidated (Thannickal et al., 1998; Thannickal et al., 1995). The probe used to detect ROS generation in the current study was the luminol analogue, L-012 which, upon its discovery, was proposed as a specific probe for $O_2^{-}\cdot$ (Nishinaka et al., 1993) but has since been discovered as detecting various species of ROS, including H_2O_2 and $ONOO^{-}\cdot$ (Dikalov et al., 2007). In addition,

although initially reported to not undergo redox cycling, investigation of its structure revealed that it has the ability to do so (Dikalov et al., 2007). Therefore, specific scavengers were used in order to assess the contribution of various species of ROS, including SOD as well as the NAD(P)H specific inhibitor, VAS. Alternative methods of measuring ROS production are available, in particular and with regards to $O_2^{-}\cdot$, production, electron spin resonance (ESR) is the current 'gold standard' of measuring ROS (Wang et al., 2012) and future work using ESR to look at TGF-β1-mediated ROS production in HAoAF would be important.

VAS compounds work by inhibiting the assembly of NAD(P)H oxidases and differ from the conventionally used pharmacological Nox inhibitors, including DPI and apocynin, in that they do not exhibit intrinsic antioxidant activity and do not inhibit other flavoproteins such as eNOS and xanthine oxidase (Altenhofer et al., 2012). Although widely used, DPI and apocynin often have off-target effects which can affect other oxidant-generating systems in the cell (Aldieri et al., 2008). Currently, there is the lack of an isoform-specific Nox inhibitor, and VAS compounds are the closest alternative as specific-NAD(P)H oxidase inhibitors, however, use of a appropriate scavengers in conjunction with these compounds would present a more accurate of the Nox isoform responsible for ROS generation in a particular model system. Results from the current study suggest that the species of ROS generated in HAoAF in response to TGF-β1 treatment is $O_2^{-}\cdot$, however the Nox isoform (Nox2 or 4) responsible for this was not determined.

The current chapter has presented novel data which suggests that in HAoAF, TGF-β1 treatment results in the generation of ROS, in particular $O_2^{-}\cdot$, the source of which is NAD(P)H oxidase. Furthermore, TGF-β1-mediated HAoAF migration is a ROS-dependent mechanism and requires the activity of $O_2^{-}\cdot$. Therefore, targeting the TGF-β1 signalling pathway in the adventitia may be a potential avenue of therapeutic intervention during the progression of cardiovascular disease, as demonstrated by Mallawarachichi and colleagues where Smad7 gene transfer attenuated vascular remodelling following balloon injury (Mallawaarachichi et al., 2005). Targeting this pathway prophylactically may also offer the opportunity as a potential method of preventing the initiation of cardiovascular disease.

CHAPTER 6:

General Discussion

CHAPTER 6: General Discussion

As described in Chapter 1, atherosclerosis is a progressive vascular disease, which can eventually lead to a plethora of cardiovascular complications, including, angina, myocardial infarction and stroke. It has been implicated as being one of the most prevalent causes of death in the developed world and despite the development of a number of therapies to combat this disease, the number of patients reported to have developed atherosclerosis is on the rise (World Health Organisation, 2012). Initial studies into the underlying molecular mechanisms of this disease led to the 'response to injury' hypothesis (Ross, 1999) which postulated that the endothelium plays a central role in mediating the inflammatory response by modulating monocyte adhesion and playing a pivotal role in the infiltration of oxidized lipids and the consequent formation and progression in development of atherosclerotic lesions (Diaz et al., 1997).

An increasing body of evidence now suggests that this 'inside-out' hypothesis of atherosclerosis does not take into account the outermost adventitial layer of the blood vessel, largely consisting of fibroblasts (Maillaro and Taylor, 2007). Termed the 'outside-in' hypothesis (Zalewski and Shi, 1997), it suggests that early in the development of vascular disease, the adventitia plays an important role in the initiation and progression of vascular inflammation, characterized by a denser vasa vasorum which is responsible for delivering oxygen and nutrients to an increasingly hypoxic and nutrient deficient media as well as enhancing the delivery of inflammatory cells (Csanyi et al., 2009; Kuwuhara et al., 2002). A key player in the 'outside-in' hypothesis is the adventitial fibroblast and its phenotypic switch to a myofibroblast cell type which is able to subsequently migrate and augments the progression of disease by contributing to the formation of a neointima. This phenotypic switch has been reported to be a TGF-β1-dependent event (van den Borne et al., 2009; Mallawaarachchi et al., 2007; Powell, 2000). In particular, one method by which TGF-β1 is thought to elicit phenotypic changes and migration of fibroblasts is from the generation of reactive oxygen species (ROS), an effect which is counter-balanced by the cell's innate antioxidant defense pathways, and in particular by the Nrf2/ARE signalling pathway.

There is increasing emphasis on the activation of endogenous antioxidant defense systems in order to counteract pathophysiological disease processes since evidence from clinical trials in which exogenous antioxidants such as tocopherol, were administered to patients, has provided somewhat inconsistent outcomes (Steinhubl, 2008; Kris-Etherton et al., 2004; Steinberg, 2000; Steinberg, 1993). One dietary activator of endogenous antioxidant pathways and in particular of the Nrf2/ARE antioxidant pathway is the isothiocyanate, sulforaphane (SFN). Found in cruciferous vegetables, its bioavailability and rapid absorption and metabolism make it an ideal candidate for use as a therapeutic agent in order to activate endogenous phase II enzyme detoxification systems (Petri et al., 2003). Its ability to decrease oxidative stress has been demonstrated in spontaneously hypertensive rats where it was shown to ameliorate oxidative stress and tissue inflammation (Noyan-Ashraf et al., 2006). Its potential to prevent neointima formation and inflammation during atherosclerosis, to which adventitia-derived cells contribute, is therefore a highly promising avenue of research.

It is important to note that all HAoAF used for the duration of the study came from a total number of four human donors. Assurance from the manufacturer stated that all individuals had passed away suddenly and without any major disease pathology. Despite this, it is important to regard the data gathered in this study with caution. Cells were taken from the adventitial layer of the aorta of donors, however the manufacturer was not clear about whether the donors suffered from any risk factors that would increase the progression or contribute to the development of cardiovascular disease. It is well known that all members of the general population exhibit atherosclerotic plaque formation to some extent (Ross, 1999), and it is likely that the donors from whom these cells were taken also exhibited some atherosclerotic plaque development. This variability between donors is likely to have affected the behaviour of the fibroblasts isolated from their aortas and ultimately the outcomes of the current study. Future work using a larger sample size and perhaps with the medical history of the donors would help to reduce this variability and provide more accurate data with the potential of less variability.

The present study aimed to elucidate whether TGF-β1 modulates phenotype and motility due to enhanced ROS generation or altered antioxidant defense gene expression via the Nrf2/ARE pathway. The effect of the isothiocyanate, SFN, an inducer of the Nrf2/ARE

pathway, on HAoAF was also investigated in order to determine whether it plays a role in the induction of phase II enzymes via the activation of the Nrf2/ARE pathway in this cell type and therefore be used as a method of intervention in cardiovascular disease.

6.1 Cellular localisation of the Nrf2/Keap1 complex

This study has investigated the effect of TGF-β1 and SFN treatment on the expression pattern of Nrf2 in HAoAF. Immunofluorescence and Western blot analysis suggests that in response to exposure to TGF-β1 or SFN, Nrf2 localises to the nucleus after 2 h and by 4 h after exposure, Nrf2 is largely localised in the cytoplasm. These results are consistent with findings from a previous study carried out in human aortic smooth muscle cells where nuclear accumulation of Nrf2 was seen after 2 h in response to TGF-β1 (Churchman et al., 2009). Another study in a prostate cancer cell line also confirms results found in the present study where exposure of cells to SFN resulted in the translocation of newly synthesized Nrf2 to the nucleus, whilst its binding protein, Keap1 remained in the cytoplasm (Xu et al., 2006). The location of the Nrf2 binding protein, Keap1, was not investigated during the present study, however the intracellular localisation of the Nrf2-Keap 1 complex has gained much interest in recent years and various studies contend with the initial hypotheses, which suggested that under basal conditions, the complex resides mainly in the cytoplasm, bound to F-actin, under basal conditions.

The mechanisms by which Keap1 is thought to regulate Nrf2 levels are controversial as is the exact subcellular localisation of the Keap1/Nrf2 complex. The majority of evidence suggests that Keap1 sequesters Nrf2 in the cytosol where it mediates the proteasomal degradation of this transcription factor via the 26S proteasome (Jain et al., 2005). According to this model, upon exposure of Keap1 to oxidants and electrophillic stress agents, Nrf2 dissociates from Keap1 and is able to localise to the nucleus owing to a nuclear localisation signal (NLS) located in its C-terminus which eventually leads to the coordinated activation of a number of antioxidant genes (Jain et al., 2005). The NLS domain of Nrf2 is thought to be critical in allowing its nuclear accumulation as deletion of this domain has been previously shown to result in a complete inhibition of Nrf2 nuclear accumulation and diminished transcriptional activity of genes downstream of Nrf2 (Jain et

al., 2005). In addition to this, a similar nuclear export signal (NES), also found on Nrf2 is thought to be important in the nuclear export and subsequent degradation of Nrf2 (Velichkova and Hasson, 2005).

In contrast to this, emerging evidence suggests that the relationship between Keap1/Nrf2 is more complicated than originally proposed. Watai and colleagues postulate that the Keap1/Nrf2 complex is localised in different locations in the cell. They suggest that although the majority of Keap1 resides in the cytoplasm there is also some localisation of this complex in the nucleus and in the endoplasmic reticulum (Watai et al., 2007). Interestingly, they also suggest that instead of being an inert protein in the cytoplasm, Keap1 is able to shuttle in and out of the nucleus in a NES-dependent manner (Watai et al., 2007). Velichkova and Hasson further explain this by suggesting that oxidative agents can directly oxidatively modify several cysteine residues in immediate proximity to the NES. Once oxidised, they can participate in the masking of the NES on Keap1, thereby allowing an increase in nuclear Nrf2 in the absence of Keap1–mediated export of Nrf2. Eventually this results in the transcription of various target genes via Nrf2/ARE, including HO-1 and NQO-1 (Velichkova and Hasson 2005). This is supported by findings in the fibroblast cell line, NIH3T3 that show that these cysteine residues are critical for the Keap1-mediated ubiquitination of Nrf2 (Zhang et al., 2003).

Further light was shed on the role of the Keap1/Nrf2 complex on ARE-mediated gene expression in HeLa cells. Keap1 was found to contain a NES domain, which allowed it to confer the nuclear-cytoplasmic shuttling of the Keap1/Nrf2 complex and mediate gene transcription (Karapetian et al., 2005). Perhaps the most controversial suggestion comes from Nguyen and colleagues who propose that the Keap1/Nrf2 interaction is a very brief one, whereby Keap1 transiently enters the nucleus and targets Nrf2 for ubiquitination in this compartment under non-stress conditions. However, they also go a step further by suggesting that even under stressed conditions, Keap1 is able to enter the nucleus and target Nrf2 for degradation once this transcription factor has activated its target genes (Nguyen et al., 2009). This supports findings from Jain et al., who found that treatment of cells with the stress agent, t-butylhydroquinone (t-BHQ), caused nuclear Nrf2 localisation but that this was abrogated between 1 – 4 h after treatment and gained normal localisation status 8 h after treatment (Jain et al., 2005).

Questions remain about the mechanism of Nrf2 nuclear localisation and activation by such diverse stimulants as TGF-β1 and SFN and it is likely that it involves cross-talk between several signalling pathways depending upon the stimulus, the cell and the tissue type. In the current study, Western blot analysis revealed several kinase pathways were activated in HAoAF in response to TGF-β1 and SFN, including PI3K/Akt, Erk and $p38^{MAPK}$ (see Chapter 3). Other studies have proposed the importance of kinases in the regulation of the ARE in an Nrf2-dependent manner; Erk has been reported as upregulating the ARE and $p38^{MAPK}$ has been shown to suppress this effect (Yu et al., 2000). Others have shown that the PI3K pathway results in Nrf2 nuclear translocation upon exposure to tBHQ-induced oxidative stress, an effect that is accompanied by actin rearrangement (Kang et al., 2002) whilst in HepG2 cells, phosphorylation of Ser40 on Nrf2 by protein kinase C was observed as being required for ARE-driven transcription (Huang et al., 2002).

More recently, Xu and colleagues have reported that treatment of a prostate carcinoma cell line with phenethyl isothiocyanate (PEITC) activated Erk and Jnk, which phosphorylated Nrf2, inducing its translocation to the nucleus and subsequent transcriptional activation of the ARE (Xu et al., 2006). In contrast, others have reported that phosphorylation of Nrf2 is not a requirement for its activation; Dinkova-Kostova and colleagues have proposed a model which suggests that oxidative insults that result in the induction of phase II enzymes directly modify cysteine residues on Keap1 allowing Nrf2 nuclear translocation (Dinkova-Kostova et al., 2002). This is further confirmed by work in mouse embryonic fibroblasts that proposed that Cys273 and 288 were the critical residues on Keap1 that were modified by electrophiles and required for Nrf2 release from Keap1 and its subsequent nuclear translocation (Wakabayashi et al., 2004). Although the current study did not investigate phosphorylation of Nrf2 following exposure to TGF-β1 or SFN, it has been shown previously that post-translational modification events, including acetylation of several lysine residues in the DNA binding domain of Nrf2 (Sun et al., 2009) are important in modulating the Nrf2-dependent antioxidant response (Sun et al., 2000) and therefore investigating such events would be important for future work in order to elucidate the mechanism by which TGF-β1 or SFN cause Nrf2 nuclear translocation, and in the case of SFN, its binding to the ARE of target genes in HAoAF.

6.2 Mechanisms of TGF-β1-mediated HAoAF migration

In this study, exposure of HAoAF to TGF-β1 caused changes in the Nrf2/ARE pathway, possibly affecting changes in the differentiation, migration and proliferation of these cells through altered redox status. These TGF-β1-mediated changes may contribute to adventitial activation, atherosclerotic disease progression, neointima formation and restenosis.

The differentiation and migration of adventitial fibroblasts in the setting of cardiovascular disease has recently been an area of intensive research. The role of the adventitia has only recently been given importance in the context of disease (Ruiz-Ortega et al., 2007) and the ability of adventitial fibroblasts to differentiate, proliferate and migrate into the intima and contribute to vascular remodelling in response to TGF-β1 can affect the outcome of disease progression (Ruiz-Ortega et al., 2007; Birukov, 2009).

TGF-β1 is a known activator of cell migration, with studies showing that this is due to its ability to activate integrins (Asnao et al., 2006). TGF-β1-modulated fibroblast migration and differentiation is a critical event during atherosclerotic disease progression and is likely to play a large role in the neointima formation and restenosis observed during advanced cardiovascular disease (Liu et al., 2010). The current study demonstrated a significant increase in migration of HAoAF treated with TGF-β1, an effect that was attenuated in the presence of the $O_2^{-\cdot}$ scavenger, superoxide dismutase (SOD; Fig 5.2). However, investigation of the levels of Nox2 or Nox4 mRNA in response to treatment with TGF-β1 revealed that there was no significant increase in the level of these NAD(P)H isoforms, though mRNA levels of these Nox isoforms are not indicative of their activity since assembly and activity of both Nox2 an Nox4 are subject to post-translational modification (Martin-Garrido et al., 2011; Fig. 5.5). The underlying mechanisms of this were not investigated here, however, changes in the actin cytoskeleton, to which the Nrf2 inhibitory protein, Keap1 is bound may result in the nuclear accumulation of Nrf2 and the consequent induction of cytoprotective antioxidant genes that may counteract ROS generation, and therefore attenuate migration of these cells.

Recent evidence from *in vitro* studies has implicated Nox1 and Nox4-derived ROS in cell migration as a reduction in the levels of these NAD(P)H oxidase catalytic subunits resulted in the abrogation of TGF-β1-mediated migration of VSMC, with an increase in migration

observed when these were overexpressed (Lyle et al., 2009; Schroder et al., 2007) and others have shown that ROS produced by Nox1 and Nox4 promotes matrix metalloproteinase (MMP) secretion from VSMC (Schroder et al., 2010). In particular, Rac1, which regulates cell growth, cellular transformation and especially cell migration, has been reported as being essential for the assembly of plasma membrane NAD(P)H oxidase (Dikalov et al., 2007). The mechanism by which Rac1 is able to facilitate these cellular processes is by controlling the production of ROS, which in turn can increase cell migration via the activation of stress-induced responses such as the activation of several MAPKs and subsequent downstream activation of several integrins and actin reorganization in order to drive the forward movement of the cell (Lassegue et al., 2010; see Fig 6.1). The initial assembly of these NAD(P)H oxidase isoforms may be in response to TGF-β1 production as a result of its autocrine and paracrine release and may explain the increase in the migration of HAoAF in response to this growth factor, an increase which was abrogated in the presence of SOD.

It is thought that the interaction between TGF-β1 receptors and integrins elicits a signalling cascade through FAK, subsequent integrin clustering and cellular migration (Margadant and Sonnenberg, 2010). Furthermore, TGF-β1 activation and its crosstalk with the integrin cluster, αvβ8 has been shown to induce the differentiation of airway fibroblasts into myofibroblasts and their subsequent migration (Araya et al., 2007). In the current study, TGF-β1 treatment resulted in an acute but significant increased protein expression of pAkt (Chapter 3, Fig 3.14) and other studies have purported that this may be a Smad-independent pathway by which TGF-β1 can stimulate migration via FAK; FAK has been proposed to bind the PI3K p85 regulatory subunit following TGF-β1 treatment in fibroblasts resulting in TGF-β1-mediated migration (Hong et al., 2011). Similarly, in monocytes, TGF-β1 has been reported as mediating motility in a Smad-independent manner as knockdown of these proteins did not affect migration, and it was observed that migration was dependent upon PI3K activation (Olieslagers et al., 2011). Results from the current study did not address the downstream functional effect of Akt phosphorylation in HAoAF upon treatment with TGF-β1, but as increased migration was observed in these cells, the phosphorylation of Akt may be a mechanism by which TGF-β1 modulates cell motility in HAoAF, however further work would need to be carried out to determine this.

Given the interactions reported between the Keap1/Nrf2 complex and focal adhesions (Velichkova and Hasson, 2005), which contain integrins and the proposed interaction between TGF-β1 and integrins as well as between TGF-β1 and Nrf2, further investigation of the mechanisms of signalling and activity between these various different pathways would need to be carried out in adventitial fibroblasts. One way in which this could be investigated in real-time is by using fluorescence resonance energy transfer (FRET) microscopy. Fluorescence resonance energy transfer (FRET) allows visualisation of individual proteins and allows the quantification of biochemical interactions (Worth and Parson, 2008). Based on the evidence that the Keap1/Nrf2 complex localises to the cytoskeleton at focal adhesions (Velichkova and Hasson, 2005), the use of FRET to assess Nrf2/Keap1 and adhesion complex interactions could clarify the crosstalk of migration machinery with this antioxidant pathway. Specific antibody/fluorophore probes would need to be generated to facilitate FRET imaging of possible TGF-β1-induced associations between Keap1/Nrf2 and focal adhesions.

A further method of determining cell motility behavior is to measure cell traction force (CTF), which is the force generated by actomyosin cross-bridges inside semi-sarcomeres containing actin-bundles (Wang and Lin, 2007). Adherent cells generate contractile forces by using the cell's contractile machinery, forces that are transmitted to the extracellular matrix (ECM) through focal adhesions which can be in the range of tens of nano-Newtons (Wang and Lin, 2007). CTF is essential for cell migration, cell shape maintenance and signal transduction and analysis of this force can offer critical understanding of physiological and pathophysiological events in cells and tissues. In the case of the current study, such information would provide an insight into the mechanisms by which TGF-β1 regulates cell migration in HAoAF.

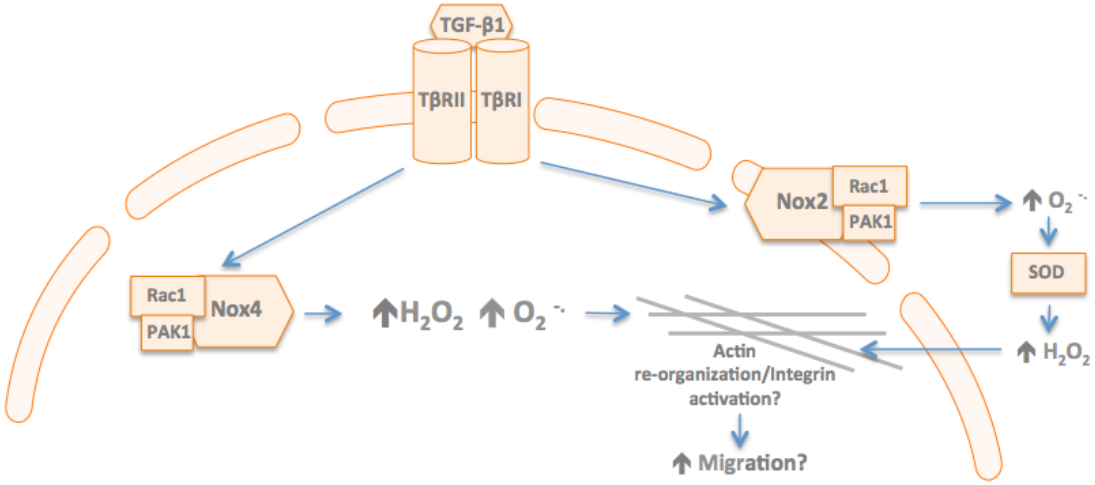


Figure 6.1 Possible mechanisms by which TGF-β1 may drive fibroblast migration in a ROS-dependent manner. In response to TGF-β1 Nox2/Nox4 produces reactive oxygen species that can cause the reorganization of the cytoskeleton and possible activation of integrins. This can lead to enhanced cell migration.

6.3 The Nrf2/ARE pathway as a mechanosensor

The localisation of the Nrf2-Keap1 complex is of particular relevance to the current study due to the reported association of Keap1 with the actin cytoskeleton, which may have further implications for cell phenotype, proliferation and migration.

It has been shown that Keap1 binds to the actin cytoskeleton and that the interaction between the two proteins contributes to its regulatory activity (Kang et al., 2004). In particular, the association between Keap1 and the actin cytoskeleton is thought to be through focal adhesions (see Fig 6.2). These sites of attachment of the cell membrane to the ECM and are formed by groups of cytoskeletal proteins clustered around a transmembrane integrin. Integrins consist of an α and a β subunit and constitute a family of transmembrane receptors which bind extracellularly to the ECM and intracellularly to the cytoskeleton to transduce signals between the extracellular and intracellular environment in order to regulate cell adhesion, spreading, proliferation and differentiation (Worth and Parsons, 2008). Actin remodelling, such as that seen during cell migration, is known to play a critical role in transforming a mechanical signal to a biological response (Velichkova and Hasson, 2003).

Previous work by Papaiahgari *et al.* has demonstrated the importance of the PI3k/Akt pathway in mediating ROS-dependent, hyperoxia-induced Nrf2 activation and similar work by Kang and colleagues has shown the importance of the PI3K/Akt pathway in regulating Nrf2 nuclear translocation via actin rearrangement, suggesting that actin plays a pivotal role in, and is closely related to the activation of the Nrf2/ARE pathway (Papaiahgari et al., 2006; Kang et al., 2002). More recently, immunoprecipitation and immmunocytochemical studies have found that disruption of the actin cytoskeleton promotes nuclear entry of Nrf2 suggesting a role for it in providing a scaffold essential for the function of Keap1 as a binding protein for Nrf2 as well as a sensor for oxidative and electrophilic stress (Kang et al., 2004). These findings were further confirmed in a study in epithelial and endothelial cells where it was found that blocking actin polymerization with the use of a pharmacological agent blocked cyclic-stretch induced ARE-mediated gene transcription in these cells (Papaiahgari et al., 2007). As a result of these studies, it has been postulated that actin plays an important role in enabling the Nrf2/ARE pathway to act as a

‘mechanosensor’ for oxidative stress and increased actin-polymerisation may result in a higher proportion of Keap1 within the cytoplasm where it also sequesters Nrf2, thereby preventing Nrf2 nuclear accumulation and subsequent ARE-mediated gene transcription.

Studies carried out in endothelial cells have suggested a ‘mechanosensing’ role for the Nrf2/ARE pathway, allowing this pathway to differentially regulate the redox status of cells. Investigation of the effect of laminar (atheroprotective) shear stress in human umbilical vein endothelial cells (HUVEC) dramatically increased intracellular ROS generation whilst also activating the Nrf2/ARE pathway and consequently the coordinated induction of downstream antioxidant enzymes such as HO-1 and NQO1 (Dai et al., 2007). Similarly, a separate study, also in HUVEC, found that laminar shear stress activated the Nrf2/ARE pathway and protected cells from external insults, however in cells exposed to oscillatory shear stress, Nrf2-regulated atheroprotective gene expression was suppressed, predisposing these areas of the blood vessel to the development of atherosclerosis (Hosoya et al., 2005). These studies highlight the importance of mechanical stress as a mediator of redox signalling in cardiovascular disease where biomechanical forces play a pivotal role during disease progression.

Further investigation of mechanical stress on the activation of Nrf2 in HAoAF is of particular relevance as the adventitial layer is exposed to cyclic stretch during normal vessel contraction and relaxation, which is enhanced during cardiovascular disease, particularly during hypertension (Stenmark et al., 2012; Li et al., 2011). Previous studies have reported increases in TGF-β1-dependent activation of NAD(P)H oxidase and consequently enhanced generation of ROS during cyclic stretch (Mata-Greenwood et al., 2005), a possible mechanism by which the Nrf2/ARE pathway may be activated during mechanical stress.

The expression pattern of Nrf2 in HAoAF was investigated in the present study, however the cellular localisation of Keap1 was not. Future work pertaining to the cellular localisation of Keap1 would help to elucidate the mechanism underlying the possible TGF-β1 or SFN-induced association between Nrf2/Keap1, focal adhesions and the cytoskeleton and may clarify whether this antioxidant pathway is responsible for ‘mechanosensing’ during exposure to mechanical stress in the adventitia during hypertension or adverse vascular remodelling.

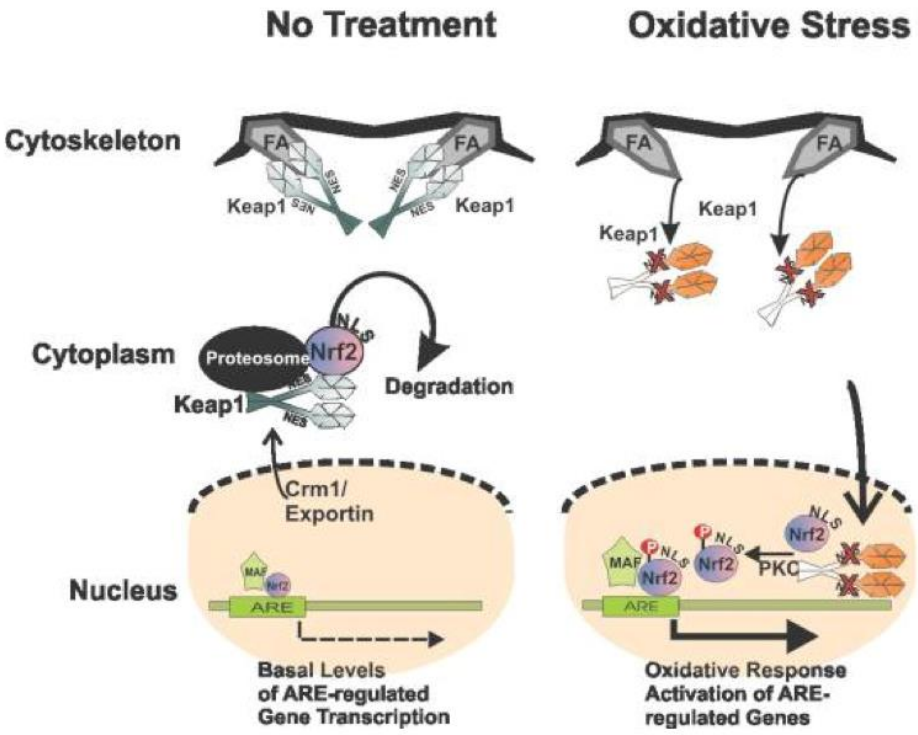


Figure 6.2 Model for the proposed association of Keap1 with the cytoskeleton and its regulation of Nrf2. Under basal conditions, Keap1 is observed in the cytoplasm, at focal adhesions and is involved in the targeted, ubiquitin-mediated degradation of Nrf2 via the 26S proteasome. Small amounts of the Keap1/Nrf2 complex are shuttled to the nucleus for basal levels of ARE-regulated gene expression. Chemical/mechanical stress modifies cysteine residues found within Keap1, resulting in the release of Keap1 from the cytoskeleton and from the degradation machinery. Pools of Keap1/Nrf2 accumulate within the nucleus which results in the Nrf2 mediated activation of ARE-genes. Adapted from Velichkova and Hasson., 2005.

The mechanisms of ROS generation and subsequent effect on activating 'mechanosensing' pathways remains to be fully elucidated, however it is thought that the binding of growth factors and chemoattractants to their cell surface receptors causes NAD(P)H oxidases to generate ROS (Hurd et al., 2012). The subsequent generation of ROS following ligand binding is thought to be mediated by p21-activated kinase-1 (PAK1) and the small GTPase Rac1 and an assortment of NAD(P)H regulatory subunits (Hurd et al., 2012). The generation of ROS can then direct cellular changes for migration by modifying the activity of several proteins, including Keap1 and actin, thereby setting in motion a series of signalling cascades, including the activation of cellular defense pathways such as the Nrf2/ARE pathway (see Fig 6.1).

6.4 Mechanisms of TGF-β1-mediated ROS generation in the adventitia

In the current study, the effect of TGF-β1 on ROS generation was investigated in HAoAF using the luminescent probe L-O12 and it was observed that TGF-β1 was able to elicit an increase in ROS production in HAoAF. Use of a NAD(P)H oxidase inhibitor revealed that the ROS was partially NAD(P)H oxidase-derived. Results from a recent study in rat kidney fibroblasts showed that TGF-β1 elicited an increase in ROS generation by increasing the activity of NAD(P)H oxidase and the expression of Nox2 and Nox4, whilst also causing an increase in the expression of α-smooth muscle actin, a marker of fibroblast differentiation. Knockdown of Nox4 was seen to markedly inhibit the TGF-β1-induced stimulation of NAD(P)H oxidase activity and reduced α-smooth muscle actin expression (Bondi et al., 2010). These findings demonstrate that TGF-β1 not only elicits ROS production in fibroblasts but in doing so, it also causes activation and differentiation of fibroblasts to myofibroblasts. In another study, TGF-β1 caused an increase in the levels of superoxide in aortic tissue from ApoE -/- mice, which was produced via NAD(P)H oxidase and which was inhibited in the presence of SOD (Buday et al., 2010). TGF-β1-mediated ROS production also caused endothelial dysfunction and accelerated atherosclerosis and hypertension (Buday et al., 2010). Similarly, TGF-β1 increased cellular ROS levels in fibroblasts in an ovarian cancer cell-fibroblast co-culture model, which caused an increase in the expression of α-smooth muscle actin and led to fibroblast transdifferentiation to myofibroblasts (Yao et al., 2009). Alveolar-epithelial cells also respond to TGF-β1 in a

similar way; exposure of these cells to TGF-β1 in the presence of hypoxia led to enhanced ROS production which was abrogated by the inhibition of a type I receptor kinase (Zhou et al., 2009), suggesting that in this cell type, TGF-β1 plays a pivotal role in ROS generation. Interestingly, TGF-β1 has been implicated in eliciting the generation of ROS via various sources within the cell, including the mitochondria, where it is thought to generate ROS through complex III, ROS, which in-turn, affect TGF-β1-mediated gene expression in lung fibroblasts (Jain et al., 2013). It should be noted that during mechanical stress, such as that seen during hypertension, TGF-β1 may mediate ROS production, particularly as it is seen in high concentration at the site of injury during the pathogenesis of atherosclerosis (Wang et al., 2007). Release of TGF-β1 from fibroblasts, SMC and endothelial cells may mediate the generation of ROS in the vessel wall, during mechanical stress in a similar way to that seen in the current study (see Table 1.1).

'Leakage' of immune cells from the vasa vasorum can also contribute to excessive local ROS production in the adventitial and medial layers of a blood vessel (Csanyi et al., 2009). One of the hallmarks of the 'outside-in' hypothesis is increased vasa vasorum angiogenesis and the coupling of this with enhanced ROS production in this outer layer of the blood vessel results in an increase in the delivery of inflammatory cells and further ROS production (Csanyi et al., 2009). The increased density of the vasa vasorum has been postulated as being due to decreased oxygen at the core of the neointima and hyperplastic media and consequent increase in the expression of transcription factors including hypoxia-inducible factor (HIF-1α, Kuwuhara et al., 2002). In addition to the increase in adhesion molecule expression along the endothelial lining of the vasa vasorum, this results in a positive feed back loop leading to the accumulation of leukocytes in the adventitia and consequent inflammatory cell-derived ROS, further compounding local ROS production, medial hypertrophy and neointima formation (Csanyi et al., 2009). Enhanced ROS at the endothelial layer has been reported to upregulate NFκB, which in turn leads to the release of proinflammatory cytokines such as IL-1, IL-6 and TNFα and the increased expression of endothelial adhesion molecules (Park et al., 2006). Cross-talk between leukocyte-derived and adventitia-derived ROS has also been implicated in further driving NFκB activation and increased adhesion molecule expression in the vasa vasorum (Stenmark et al., 2006). This positive association between adventitial ROS and adhesion molecule expression in the vasa vasorum has been demonstrated in the rat aorta where AngII infusion for 1 week

increased NAD(P)H oxidase-derived ROS and was accompanied by enhanced ICAM-1 expression and adventitial macrophage infiltration (Liu et al., 2003). In addition, the same study showed that adventitial ROS served as a direct chemotactic signal for leukocytes which were able to penetrate the vessel wall, thereby resulting in increased inflammation in the adventitia (Liu et al., 2003).

Use of an NAD(P)H oxidase inhibitor in the current study revealed that NAD(P)H oxidase was a partial source of ROS in HAoAF treated with TGF-β1. However, although the inhibitor used is specific for Nox (Altenhofer et al., 2012), there is currently no available pharmacological inhibitor that is able to inhibit specific Nox isoforms, and therefore it was not determined whether the source of ROS in HAoAF was Nox2 or Nox4-derived, both of which have been reported to be expressed by adventitial fibroblasts (Haurani and Pagano, 2007). Due to the lack of specific, freely available and validated antibodies for Nox4/Nox2, the protein expression of these isoforms was not determined in HAoAF. siRNA knockdown of either of these isoforms in order to observe differences in ROS production in TGF-β1-treated cells would also be important for future work, however due to lack of validated siRNAs in addition to lack of available antibodies to confirm knock-down, this approach would be technically difficult (Altenhofer et al., 2012).

6.5 Future Work

6.5.1 Investigation of miRNA regulation of TGF-β1 signalling

Interest in the regulation of gene expression by microRNA (miRNAs) in the context of cardiovascular disease (CVD) is currently a highly topical area of research. miRNAs are strands of short non-coding RNAs that regulate gene expression by modulating the translation and/or stability of target mRNAs (Boyd, 2008; see Introduction section 1.3.5a).

Preliminary data from the current study investigated the effect of TGF-β1 and two common inflammatory mediators observed in atherosclerosis, the lipid peroxidation product, 4-Hydroxynonenol (HNE; Abiko et al., 2011), and the H_2O_2 generator, glucose oxidase (GOx; Churchman et al., 2009), on the expression of several miRNAs in HAoAF.

Studies have found that TGF-β1-mediated expression of contractile smooth muscle genes is

in part mediated by miRNAs miR-21, miR-26a, miR-143 and miR-145. As one of the objectives of this study was to determine the effect of TGF-β1 on HAoAF differentiation, the expression of these mRNAs in response to treatment with this growth factor was investigated (Fig 6.3). It is widely known that in VSMC, TGF-β1 induces the expression of contractile proteins, however, potential mechanisms by which this occurs have not been fully elucidated. Recent studies have reported that several miRNAs, regulated by TGF-β1, may be involved in the differentiation process. In one study, miR-21 has been shown to mediate the expression of programmed cell death protein-4 (PDCD4) which is rapidly induced by TGF-β1 in VSMC and results in the expression of contractile proteins (Hata and Davis, 2009). The same study found that TGF-β1 could increase the expression of pre and mature miR-21 by facilitating the Drosha step (Hata and Davis, 2009). In addition, miR-21 has also been implicated in regulating the Erk-MAPK pathway in cardiac fibroblasts and the *in vivo* silencing of miR-21 by a specific antagomir in a mouse pressure-overload-induced disease model has been shown to inhibit interstitial fibrosis and attenuate cardiac dysfunction (Thum et al., 2008). In the current study, increased activation of the Erk-MAPK pathway by TGF-β1, though not statistically significant, was observed (see Chapter 3, Fig. 3.15) and investigation of miR-21 revealed that its expression was decreased in TGF-β1-treated cells. These two observations may be linked and it is possible that TGF-β1 regulation of the Erk-MAPK pathway is via miR-21 in HAoAF, but further work would be needed in order to determine this. Interestingly, the pro-inflammatory compounds, HNE and GOx were both shown to increase the expression of miR-21 in HAoAF, however this increase was non-significant (Fig 6.3, A). This observation may suggest a possible regulatory mechanism between miR-21 and HNE and GOx, though a link between these remains to be elucidated.

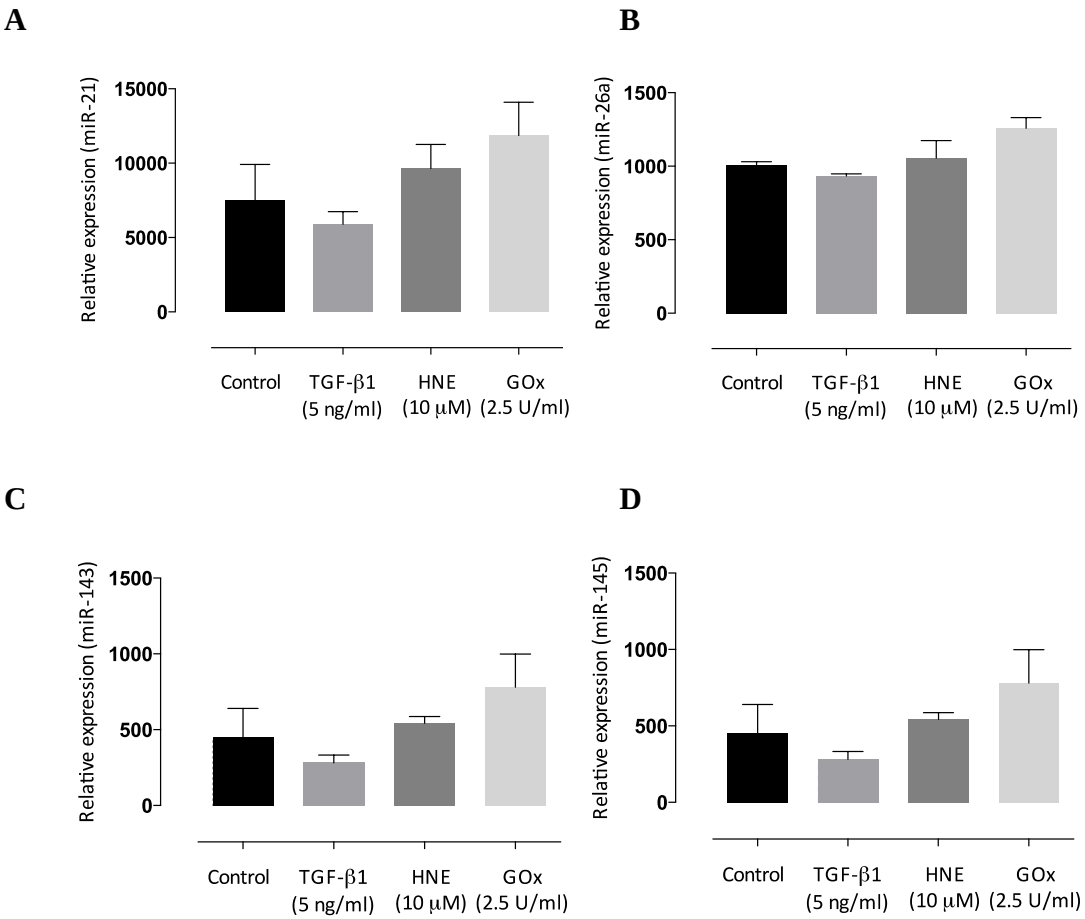


Figure 6.3 The effect of TGF-β1 on microRNA levels in HAoAF. Confluent HAoAF were equilibrated in phenol red-free DMEM supplemented with 1% FCS and subsequently treated with TGF-β1 (5 ng/ml), HNE (10 μM) or glucose oxidase (GOx; 2.5 U/ml) for 6 h. Micro-RNA levels in the samples were quantified by qRT-PCR and expressed relative to 3 housekeeping genes. Data was analysed using geNorm software and Graphpad Prism 5. Values denote means ± SEM. n = 3 independent experiments.

Similarly, in VSMC and fibroblasts, miR-143 and 145 have been reported as being direct transcriptional targets of serum response factor (SRF) and its co-factor myocardin and in response to these factors, both miRNAs were downregulated in atherosclerotic vessels resulting in a less differentiated cell population (Cordes et al., 2009). Another study in mesenchymal stem cells has reported that TGF-β1 led to a rapid and sustained up-regulation of miR-145, an effect that resulted in a decrease in Disabled-2 (Dab2), a protein that has a critical role in stem cell specification. After acute myocardial infarction, Dab2 levels were rapidly upregulated in cardiac myocytes in the border zone leading to fibrotic remodelling with a concomitant decrease in myocardial pri-miR-145 expression (Mayorga et al., 2012). These findings are in contrast to preliminary data in the current study that suggests that TGF- β1 downregulates the expression of both miR-143 and 145 in HAoAF after 6 h and conversely, may decrease the expression of characteristic markers of differentiation (Fig 6.3, C and D). Further work looking at the effect of long-term TGF-β1 treatment on the expression of these miRNAs would provide better insight into their regulation by TGF-β1 and the possible effects of this on HAoAF differentiation.

The role of Smad proteins, which are involved in facilitating TGF-β1 signalling, in the Drosha complex has also been studied; study of the nuclear factor Smad nuclear interacting protein1 (SNIP1), a nuclear protein partner of Smads, is found in complex with Drosha (Yu et al., 2008) suggesting a Smad-dependent regulation of miRNA biosynthesis could be modulated independently of TGF-β1 (Hata and Davis, 2009, Fig 6.4) and thereby be involved in many signalling pathways and as a result, a number of physiological processes.

Investigating the possible differential regulation of the Nrf2/ARE pathway by TGF-β1 would also be an interesting avenue of study. There is emerging evidence that has implicated several miRNAs in the regulation of Nrf2, particularly post-transcriptionally/translationally. One study has identified a miRNA targeting seed sequence within Nrf2 3' UTR in neuronal SH-SY5Y cells which has been postulated as being a possible target of miR153/miR27a/miR142-5p/miR144 (Narasimhan et al., 2012). Ectopic expression of these miRNAs resulted in diminished transactivation of Nrf2 with diminished GCLC and GSR expression, suggesting that alteration in the level of these miRNAs affects levels of Nrf2 and could be important in its regulation in disease (Narasimhan et al., 2012). As well as the direct regulation of Nrf2, regulation of Keap1 can also influence

transcriptional activity of Nrf2 and in breast cancer cells. miR200a has been reported as negatively regulating Keap1 via mRNA destabilization, thereby leading to an increase in Nrf2 levels and the phase II enzyme, NQO1 (Eades et al., 2011; Loignes et al., 2009). Although these miRNAs were not investigated in the current study, investigation into the overlap between TGF-β1 or SFN and miRNAs that regulate Nrf2/ARE signalling would be of interest, particularly because Nrf2 is known to be post-translationally/transcriptionally regulated, at least by SFN, (Negi et al., 2011; Zhang et al., 2003) and the potential mechanism by which this occurs may be via the alteration in the levels of several miRNAs.

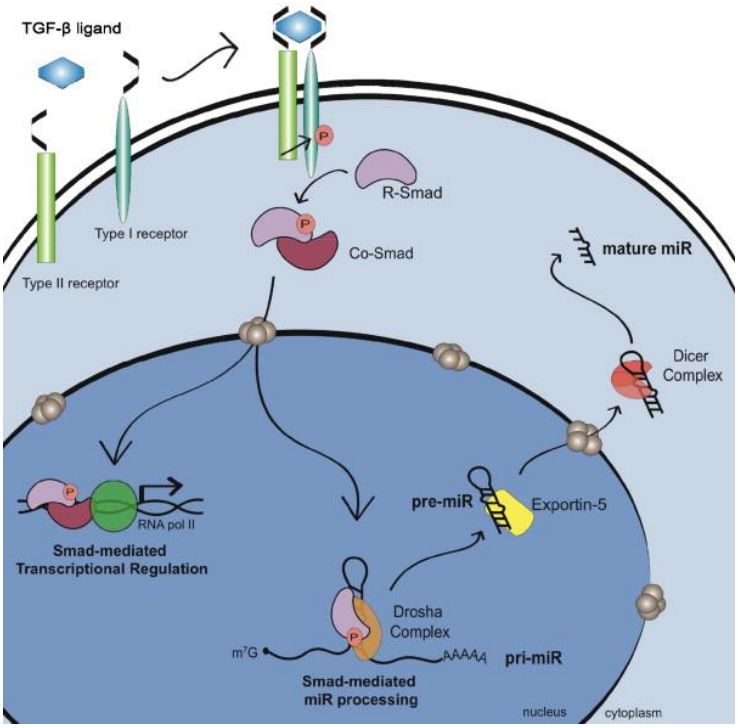


Figure 6.4 Regulation of miRNA maturation by TGF-β superfamily signalling. TGF-β signalling promotes the nuclear localisation of R-Smad proteins which stimulates the Drosha-mediated production of pre-miRNAs. Thus Smads regulate gene expression in two distinct manners (i) transcriptional regulation by DNA binding and (ii) regulation of miRNA maturation by associating with the Drosha complex. Taken from Hata and Davis, 2009.

6.5.2 The TGF-β1 signalling pathway: A therapeutic target?

As a result of the increasing evidence demonstrating the role of TGF-β1 in remodelling during vascular disease, it is not surprising that this signalling pathway has been a target for therapeutic intervention and a number of strategies have been employed in order to antagonise TGF-β1 signalling in the hope that it would confer clinical benefit in disease progression. Initial studies employed neutralizing anti- TGF-β1 antibodies to inhibit TGF-β1-mediated signalling. An *in vivo* study in a rat carotid artery injury model demonstrated that administration of neutralizing anti-TGF-β1 antibodies significantly reduced the size of the intimal lesions that developed after balloon injury when compared to untreated animals as well as an observed decrease in the extracellular matrix components, EDA and fibronectin (Wolf et al., 1994). This study not only confirmed that TGF-β1 was upregulated in the media of injured arteries, but was also one of the first to suggest that it was causally involved in the development of intimal hyperplasia (Wolf et al., 1994).

Similarly, Smith and colleagues also found that inhibiting the action of TGF-β1 by administering a recombinant TβR-II, which competes with cell-surface receptors for the binding of active TGF-β1, markedly attenuated adventitial myofibroblast migration and adverse remodelling in a rat model of balloon catheter carotid denudation (Smith et al., 1999). Intimal thickening was also reduced by up to 65% when compared with control animals (Smith et al., 1999). Ryan and colleagues showed that inhibition of TGF-β1 by antagonizing signalling also using a recombinant TβR-II prevented injury-induced reduction in lumen area by promoting vessel enlargement (Ryan et al., 2003). These studies demonstrate the potential therapeutic benefits of antagonizing TGF-β1 signalling either prophylactically or following vascular injury.

Use of ribozymes, which catalyse the cleavage of mRNA and therefore inhibit gene expression, is a technique that has been used to target TGF-β1 mRNA expression in vascular SMC from spontaneously hypertensive rats and have been found to inhibit cellular proliferation in this model (Teng et al., 2000). Transfection of FITC-labeled ribozymes *in vivo* to target TGF-β1 signalling has also shown similar results and as a consequence, prevented characteristic neointima formation in animals after balloon injury when the transfected vessel was examined 2 weeks after transfection (Yamamoto et al., 2000). Alternatively, inhibition of downstream TGF-β1 signalling using adenoviral over-

expression of Smad7, an inhibitor of Smad2 phosphorylation, has been shown to attenuate adventitial cell migration and remodelling in a rat model of balloon injury whilst also abrogating adventitial fibroblast contribution to neointimal formation (Mallawaarachichi et al., 2005).

Although these methods have proven efficacious in animal models and offer the possibility of preventing in-stent restenosis, further work in human studies would be essential in order to determine whether inhibiting TGF-β1 locally, perhaps using a drug-eluting stent at sites of angioplasty, would be clinically beneficial (Kotsar et al., 2010). One of the major factors that may influence the potential beneficial effects of antagonizing TGF-β1 signalling is the timepoint of administration. Several studies carried out in mice have reported that disruption of TGF-β1 signalling causes the formation of unstable plaques by abrogating the profibrogenic ability of TGF-β1 (Gojova et al., 2003; Mallat et al., 2001). In addition, inhibiting TGF-β1 has also been shown to result in an increase in the number of pro-inflammatory T-cells that populate atherosclerotic lesions (Tse and Ley, 2012; Ovchinnikova et al., 2009; Robertson et al., 2003; Lutgens et al., 2002). One study in which in-stent restenosis was investigated has also reported that adenoviral transfection of TβR-II to the coronary arterial segment of a pig, although decreased ECM accumulation, did not reduce stent-induced neointima formation and in fact induced vascular inflammation and aggravated lesion progression (Chung et al., 2010). Due to the diverse range of physiological process in which TGF-β1 is involved, the complete inhibition of this signalling pathway may potentially be detrimental in long-term recovery from vascular injury. Maximal therapeutic benefit may only be attained if the correct timepoint and mechanism of intervention are elucidated, both of which would need further investigation.

An alternative strategy by which TGF-β1 signalling could be modulated to confer protective effects would be via its ability to target and activate the Nrf2 pathway. In the current study it was observed that TGF-β1 increases the nuclear translocation of Nrf2 as well as causing the induction of HO-1. In human aortic SMC, Churchman and colleagues have also reported Nrf2 as being a target of TGF-β1, findings which may explain the 'protective cytokine' hypothesis of TGF-β1 (Churchman et al., 2009). Crosstalk between the TGF-β1 and Nrf2/ARE pathways has been demonstrated in the same study, where antagonism of TGF-β1 signalling using adenoviral overexpression of Smad7 resulted in a

decrease in the protein expression of HO-1 in response to electrophilic stress (Churchman et al., 2009). Although the underlying mechanisms of this remain unclear, it has been postulated that this interaction may be as a result of crosstalk between Smad and MAPK signalling pathways that contribute to Nrf2/ARE activation. Therefore, augmenting the activation of the Nrf2 pathway by TGF-β1, may be anti-atherogenic and a possible method by which neointima formation and restenosis can be attenuated.

6.5.3 Sulforaphane as a therapeutic agent in cardiovascular disease

Previous studies investigating the therapeutic potential of antioxidant supplementation have delivered inconsistent results in clinical trials (Kris-Etherton et al., 2004; Biondo-Zoccai; 2002; Steinberg, 2000) and focus has now shifted towards employing methods that activate endogenous antioxidant pathways to confer protection against oxidative stress during disease, such as the use of novel compounds like SFN that activate the cells own antioxidant defense systems against electrophilic stress.

There has been much interest in the prophylactic capacity of SFN and whether it can indeed be considered as a therapeutic option for the prevention of CVD. Pharmacokinetic studies have revealed that following the consumption broccoli (200g), volunteers displayed a peak plasma concentration of SFN of around 100 nM 1.5 h following ingestion of raw brocolli and 6 h following ingestion of cooked broccoli (Hanlon et al., 2009; Vermeulen et al., 2008). Bioavailabity of SFN based on the processing of glucoraphanin by myrosinase in broccoli has been reported as being between 3.4-10% and 32-37% for raw and cooked respectively (Vermeulen et al., 2008; Conaway et al., 2000). The absorption of SFN was more efficient for raw than cooked broccoli and plasma clearance was also reported as being faster for raw than cooked broccoli (Vermeulen et al., 2008; Conaway et al., 2000). Concentrations of SFN used in the current study (2.5 – 5 μM) were significantly higher than those observed in pharmacokinetic studies and therefore its metabolism, bioavailability and excretion would be expected to be different than that reported in those studies, especially since it is being used in its pure form as opposed to its precursor, glucaphoranin and within a cell culture model. These concentrations were able to activate the Nrf2/ARE pathway in HAoAF, however the amount available from the consumption of cruciferous vegetables in pharmacokinetic studies would not be sufficient enough to reach

plasma levels of 2.5 – 5 μM SFN and therefore further work investigating the effects of lower concentrations of SFN in HAoAF would need to be carried out to determine whether they would also be sufficient enough to elicit a similar activation of the Nrf2/ARE pathway *in vivo*.

Epidemiological evidence has highlighted the chemopreventative effectiveness of SFN in various cancers. One study has examined the effect of broccoli consumption on gene expression in human prostate biopsy tissue before, during and after a 12-month broccoli-rich diet and a 12-month pea-rich diet (Traka et al., 2008). Individuals with glutathione S-transferase mu 1 (GSTM1) positive and null genotypes were randomly assigned to either the broccoli or pea-rich diet and comparison of biopsies obtained pre- and post-intervention revealed more changes in gene expression had occurred in individuals on the broccoli-rich diet. The authors of the study suggest that this indicates the interaction of SFN with the GSTM1 genotype to result in complex changes in signalling pathways associated with inflammation and carcinogenesis in the prostate, including TGF-β1, epidermal growth factor and insulin signalling pathways, with direct chemical interaction of isothiocyanates with these signalling peptides (Traka et al., 2008).

The ability of SFN to acts as a tumor suppressor is dependent upon its activity as a histone deacetylase (HDAC) inhibitor; in mice a single dose of SFN (10 μM) resulted in a significant inhibition of HDAC activity in the colonic mucosa with a concomitant increase in acetylated histones and suppression of tumour development in the mucosa of these mice (Kaminski et al., 2010; Myazak et al., 2006). Further work in humans revealed that a single dose of broccoli (68 g) inhibited HDAC activity significantly in peripheral blood mononuclear cells (Myazak et al., 2007). The same study reported suppression of human PC-3 prostate cancer cells by 40% in male nude mice with a significant decrease in HDAC activity in xenografts (Myazak et al., 2007). These studies demonstrate the potential of SFN to be used as a preventative measure for carcinogenesis and as therapy during the disease process as a tumour suppressor.

The therapeutic benefits of SFN have also been highlighted in the context of cardiovascular disease models. Spontaneously hypertensive stroke-prone rats were fed dried broccoli sprouts (200 mg/day; 5 days/week for 24 weeks), after 14 weeks, animals on the broccoli-rich diet had significantly decreased oxidative stress levels in cardiovascular and kidney

tissues as demonstrated by increased GSH content and reduced oxidized GSH which correlated with improved endothelial-dependent relaxation of the aorta and significantly lower blood pressure (Wu et al., 2004). In this study, the beneficial effects of SFN were attributed to its ability to induce phase II antioxidant enzymes (Wu et al., 2004). Although this study did not clarify plasma levels of SFN following consumption of broccoli, they proposed that broccoli extracts contained the equivalent of 27.3 μM SFN, a concentration that is far above the physiological level of SFN in plasma following broccoli consumption.

Interestingly local delivery of SFN in a carotid artery injury model has been shown to inhibit the mRNA and protein expression of VCAM-1 by blocking the expression of the transcription factor GATA6 and preventing its binding to the promoter region of VCAM-1 whilst significantly reducing neointima formation 14 days after injury (Kwon et al., 2012). As well as this, SFN was also able to inhibit the proliferation and migration of VSMC highlighting its use as a potential therapeutic agent for the prevention of restenosis after vascular injury (Kwon et al., 2012). Currently there is a paucity of evidence about the beneficial effects of SFN in the adventitial layer, however results from this study suggest that it may be clinically beneficial during atherosclerotic disease progression as it is able to activate the Nrf2/ARE antioxidant defense pathway, and thereby induce downstream antioxidant gene expression.

The bioavailability of SFN and the promise it has shown as a beneficial therapeutic agent in cardiovascular disease and as a chemopreventative agent offers this dietary isothiocyanate as an ideal mode of prevention during these disease processes. However, due to its interaction and effect on diverse signalling pathways, the underlying mechanisms of SFN's beneficial effects will need to be fully elucidated in order to harness its true potential and to prevent any adverse off target effects.

6.5.4 Interactions between SFN and the TGF-β1/Smad signaling pathway

Recent evidence suggests that SFN is able to interact with the TGF-β1/Smad signalling pathway with varying effects on TGF-β1-mediated gene expression and activity. The first study to report an interaction was carried out in a heterogenous human epithelial colorectal adenocarcinoma cell line, Caco-2 cells where exposure of cells to different concentrations

of SFN (0 – 25.5 μM) resulted in a perturbation of TGF-β1 signalling; analysis of pSmad2 protein expression revealed that TGF-β1 increased the expression of pSmad2, however treatment of cells with broccoli extracts containing SFN resulted in a decrease in pSmad2 expression (Furniss et al., 2008). Interestingly, the same study found that treatment of cells with broccoli extract containing 25.5 μM SFN resulted in enhanced pSmad2 expression, although to a lesser extent than in cells treated with TGF-β1 alone (Furniss et al., 2008). The perturbation of TGF-β1 signalling, which is known to be involved in cell proliferation and cancer metastasis (Padua and Massague, 2009) may go some way in explaining the ability of SFN to act as a chemopreventative agent.

A study in NIH3T3 cells, quantified TGF-β1-induced Smad-mediated transcription by using a CAGA-luciferase plasmid in which luciferase activity can be measured upon activation of Smad proteins, found that exposure of cells to TGF-β1 that had been pre-incubated with SFN (2 μM) for 30 min resulted in an increase in Smad-mediated transcription compared to exposure of cells to TGF-β1 alone (Traka et al., 2008). The authors proposed that SFN is able to directly bind to and modify TGF-β1, thereby altering TGF-β1-mediated signalling (Traka et al., 2008). Other studies have reported an increase in TGF-β1 levels in response to SFN treatment. In colorectal cancer cells, incubation with SFN for 1 and 3 h resulted in an increase in the levels of TGF-β1 mRNA as well as mRNA of TβRI and TβRII suggesting a possible transcriptional regulation of the components of this signalling pathway by SFN, postulated as being as a result of SFN's ability to inhibit histone deacetylases (HDAC; Kaminski et al., 2010). Histone deacetylase inhibitors have been reported as being able to induce the expression of several tumour suppressive genes as well as the expression of TGF-β1 and its receptors (Kaminski et al., 2010).

Recently there has been some evidence to suggest that SFN is able to attenuate TGF-β1-mediated fibrosis via the Nrf2/ARE pathway. A study examining hepatic fibrosis in a HepG2 cell culture model has reported the ability of SFN to suppress TGF- β1-mediated fibrosis. Suppression of TGF-β1/Smad signalling by SFN is thought to be indirect and via its activation of Nrf2, which itself is thought to physically interact with Smad3 proteins (Oh et al., 2012). The authors postulate that this physical interaction may impede phosphorylation of Smad proteins either by masking their phosphorylation sites or by rendering them resistant to phosphorylation, preventing their nuclear accumulation and the

downstream transcription of Smad-regulated genes, which include plasminogen activator inhibitor-1 (PAI-1), α-smooth muscle actin and fibronectin, all of which are involved in tissue fibrosis (Oh et al., 2012). The ability of SFN to suppress hepatic stellate cell activation and fibrogenic gene expression in this model may provide some therapeutic benefit to individuals suffering from fibrotic conditions by simply incorporating SFN-rich foods into their diet, however further work, including pharamcokinetc studies, would need to be carried out in order to determine exact dietary amount required in order to reach a therapeutically beneficial concentration of SFN in plasma in these patients.

In the current study, the expression of fibrogenic proteins expressed by HAoAF in response to TGF-β1 was examined however cells were not treated with TGF-β1 and SFN together. It would be interesting to assess the effect of SFN pre-treatment on the expression of fibrogenic proteins including α-smooth muscle actin and vimentin in the presence of TGF-β1 and before they have become fully differentiated into myofibroblasts in order to assess its ability to attenuate the expression of these proteins in HAoAF. The interaction between SFN and TGF-β1/Smad signalling and the Nrf2 pathway is quite likely to be more complex than has been reported in the literature. Interaction of SFN with TGF-β1 signalling, which is involved in a diverse range of cellular processes is likely to have many targeted effects including the activation of other antioxidant pathways, including NFκB and AP-1 (Greco et al., 2011). In particular, a very recent study carried out in pulmonary fibroblasts has revealed that SFN reduces myofibroblastic dedifferentiation and inhibits the pro-fibrotic effects of TGF-β1 in this cell type, an inhibition which is abolished following Nrf2 knockdown suggesting that SFN mediated these effects via the Nrf2 pathway (Artaud-Macari et al, 2013). Therefore further work to fully elucidate the various pathways SFN may activate and the mechanism of its interaction with the TGF-β1 signalling pathway is important in order to take full advantage of its therapeutic benefit in relation to the disease processes that arise during the perturbation of TGF-β1 signalling and during impaired activation of antioxidant defense systems.

Effect in HAoAF	TGF-β1	SFN
Differentiation	There was basal expression of α-smooth muscle actin and vimentin. TGF-β1 treatment had no effect on the expression of these proteins in HAoAF.	-
Mitochondrial dehydrogenase activity	No effect.	No effect.
Smad phosphorylation	Increased.	-
Proliferation	Increased.	-
Effect on Smad-independent pathways	Significant increase in Akt phosphorylation at 1, 2 and 4 h. No effect on Erk or p38MAPK.	Significant increase in Akt phosphorylation at 1, 2 and 4 h. No effect on Erk or p38MAPK.
Nrf2 mRNA and protein	No effect on mRNA levels or total Nrf2 protein expression.	No effect on mRNA levels or total Nrf2 protein expression.
HO-1 mRNA and protein	No effect on mRNA levels. Significant increase in HO-1 protein at 8 h.	No effect on mRNA levels. Significant increase in HO-1 protein at 8 h.
NQO1 mRNA and protein	No effect on mRNA levels or total NQO1 protein expression.	No effect on mRNA levels or total NQO1 protein expression.
GSH levels	No effect.	GSH depletion.
Nrf2 nuclear translocation	Nuclear translocation observed at 2 h.	Nuclear translocation observed at 2 h.
Nrf2-ARE binding activity	No effect.	Significant increase.
Effect of Nrf2 siRNA on HO-1 protein expression	Significant decrease.	Significant decrease.
Migration	Significant increase in migration and velocity of HAoAF.	-
Nox2 and Nox4 mRNA	No effect	-
ROS generation	Significant increase.	Significant increase

Table 6.1 A summary of the results obtained in HAoAF treated with TGF-β1 and SFN. (-) indicates that these experiments were not performed with SFN.

6.6 Conclusions

Taken together, results from the current study are the first to suggest that TGF-β1 is able to induce the expression of HO-1 protein expression in HAoAF, however the underlying mechanism of this increase may be independent of the Nrf2/ARE pathway. In addition, this study has demonstrated for the first time that TGF-β1-driven migration of HAoAF is a ROS-dependent mechanism as abrogation of ROS results in the attenuation of migration in this cell type. Investigation into TGF-β1-driven ROS generation found that it was partially NAD(P)H oxidase-derived. The current study has also investigated the effect of the isothiocyanate, SFN on redox signalling in HAoAF. It has been found that SFN is able to activate the Nrf2/ARE pathway and as a consequence, induce HO-1 protein expression in this cell type. This study has provided novel data on the effects of TGF-β1 on the Nrf2/ARE pathway in HAoAF, its effect on potential modulation of HAoAF phenotype and migration and its effects on ROS-generation, all of which may provide an insight into the role of this growth factor on the adventitia during the progression of atherosclerotic disease. The modulation of endogenous antioxidant defense genes by SFN via the activation of the Nrf2/ARE pathway may offer potential therapeutic benefit against the progression of atherosclerotic disease. Future work to modulate TGF-β1 signalling and ROS generation and the relationship between TGF-β1 and SFN may help to establish clinically beneficial and possibly, adventitia-targeted interventions against the progression of atherosclerosis.

References

References

Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L & Wrana JL. (1997). TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *The Journal of biological chemistry* **272,** 27678-27685.

Abe J, Zhou W, Taguchi J, Takuwa N, Miki K, Okazaki H, Kurokawa K, Kumada M & Takuwa Y. (1994). Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery. *Biochemical and biophysical research communications* **198,** 16-24.

Abiko Y, Miura T, Phuc BH, Shinkai Y & Kumagai Y. (2011). Participation of covalent modification of Keap1 in the activation of Nrf2 by tert-butylbenzoquinone, an electrophilic metabolite of butylated hydroxyanisole. *Toxicology and applied pharmacology* **255,** 32-39.

Acharya PS, Majumdar S, Jacob M, Hayden J, Mrass P, Weninger W, Assoian RK & Pure E. (2008). Fibroblast migration is mediated by CD44-dependent TGF beta activation. *Journal of cell science* **121,** 1393-1402.

Afonyushkin T, Oskolkova OV, Philippova M, Resink TJ, Erne P, Binder BR & Bochkov VN. (2010). Oxidized phospholipids regulate expression of ATF4 and VEGF in endothelial cells via NRF2-dependent mechanism: novel point of convergence between electrophilic and unfolded protein stress pathways. *Arteriosclerosis, thrombosis, and vascular biology* **30,** 1007-1013.

Agrotis A, Kalinina N & Bobik A. (2005). Transforming growth factor beta, cell signaling and cardiovascular disorders. *Curr Vasc Pharmacol* **3,** 55-61.

Akamatsu Y, Haga M, Tyagi S, Yamashita K, Graca-Souza AV, Ollinger R, Czismadia E, May GA, Ifedigbo E, Otterbein LE, Bach FH & Soares MP. (2004). Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18,** 771-772.

Akerboom TP, Bilzer M & Sies H. (1982). Competition between transport of glutathione disulfide (GSSG) and glutathione S-conjugates from perfused rat liver into bile. *FEBS letters* **140,** 73-76.

Akhtar MJ, Ahamed M, Fareed M, Alrokayan SA & Kumar S. (2012). Protective effect of sulphoraphane against oxidative stress mediated toxicity induced by CuO nanoparticles in mouse embryonic fibroblasts BALB 3T3. *The Journal of toxicological sciences* **37,** 139-148.

Akhurst RJ. (2002). TGF-beta antagonists: why suppress a tumor suppressor? *The Journal of clinical investigation* **109,** 1533-1536.

Akin E, Clower B, Tibbs R, Tang J & Zhang J. (2002). Bilirubin produces apoptosis in cultured bovine brain endothelial cells. *Brain research* **931,** 168-175.

Akool el S, Doller A, Babelova A, Tsalastra W, Moreth K, Schaefer L, Pfeilschifter J & Eberhardt W. (2008). Molecular mechanisms of TGF beta receptor-triggered signaling cascades rapidly induced by the calcineurin inhibitors cyclosporin A and FK506. *J Immunol* **181,** 2831-2845.

Al Janobi AA, Mithen RF, Gasper AV, Shaw PN, Middleton RJ, Ortori CA & Barrett DA. (2006). Quantitative measurement of sulforaphane, iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography-tandem electrospray ionisation mass spectrometry. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* **844,** 223-234.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM & Cook JL. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *The Journal of biological chemistry* **274,** 26071-26078.

Aldieri E, Riganti C, Polimeni M, Gazzano E, Lussiana C, Campia I & Ghigo D. (2008). Classical inhibitors of NOX NAD(P)H oxidases are not specific. *Current drug metabolism* **9,** 686-696.
Alexandrova AY, Kopnin PB, Vasiliev JM & Kopnin BP. (2006). ROS up-regulation mediates Ras-induced changes of cell morphology and motility. *Experimental cell research* **312,** 2066-2073.

Alfieri A, Srivastava S, Siow RC, Modo M, Fraser PA & Mann GE. (2011). Targeting the Nrf2-Keap1 antioxidant defence pathway for neurovascular protection in stroke. *The Journal of physiology* **589,** 4125-4136.

Ali MH, Pearlstein DP, Mathieu CE and Schumacker PT (2004) Mitochondrial requirement for endothelial responses to cyclic strain: implications for mechanotransduction *Am J Physiol Lung Cell Mol Physiol* **3,** L486-96.

Ali MH, Schumacker PT (2002) Endothelial responses to mechanical stress: where is the mechanosensor? *Crit Care Med* **30,** S198-206.

Allioux-Guerin M, Icard-Arcizet D, Durieux C, Henon S, Gallet F, Mevel JC, Masse MJ, Tramier M & Coppey-Moisan M. (2009). Spatiotemporal analysis of cell response to a rigidity gradient: a quantitative study using multiple optical tweezers. *Biophysical journal* **96,** 238-247.

Altenhofer S, Kleikers PW, Radermacher KA, Scheurer P, Rob Hermans JJ, Schiffers P, Ho H, Wingler K & Schmidt HH. (2012). The NOX toolbox: validating the role of NADPH oxidases in physiology and disease. *Cellular and molecular life sciences : CMLS* **69,** 2327-2343.

Amma H, Naruse K, Ishiguro N and Sokabe M (2005) Involvement of reactive oxygen species in cyclic stretch-induced NF-kappaB activation in human fibroblast cells. *Br J Pharmacol.* **3,** 364-73.

Andreadi CK, Howells LM, Atherfold PA & Manson MM. (2006). Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols. *Molecular pharmacology* **69,** 1033-1040.

Andrew N & Insall RH. (2007). Chemotaxis in shallow gradients is mediated independently of PtdIns 3-kinase by biased choices between random protrusions. *Nature Cell Biol.* **9**, 193–200.

Anilkumar N, Weber R, Zhang M, Brewer A & Shah AM. (2008). Nox4 and nox2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation. *Arteriosclerosis, thrombosis, and vascular biology* **28,** 1347-1354.

Annabi B, Rojas-Sutterlin S, Laroche M, Lachambre MP, Moumdjian R & Beliveau R. (2008). The diet-derived sulforaphane inhibits matrix metalloproteinase-9-activated human brain microvascular endothelial cell migration and tubulogenesis. *Molecular nutrition & food research* **52,** 692-700.

Annes JP, Munger JS and Rifkin DB (2003) Making sense of latent TGFbeta activation. *J Cell Sci.* **116,** 217-24.

Antosiewicz J, Ziolkowski W, Kar S, Powolny AA & Singh SV. (2008). Role of reactive oxygen intermediates in cellular responses to dietary cancer chemopreventive agents. *Planta medica* **74,** 1570-1579.

Anwar AA, Li FY, Leake DS, Ishii T, Mann GE and Siow RC (2005) Induction of heme oxygenase 1 by moderately oxidized low-density lipoproteins in human vascular smooth muscle cells: role of mitogen-activated protein kinases and Nrf2. *Free Radic Biol Med* **39,** 227-36.

Apopa PL, He X & Ma Q. (2008). Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. *Journal of biochemical and molecular toxicology* **22,** 63-76.

Araujo AS, Fernandes T, Ribeiro MF, Khaper N & Bello-Klein A. (2010). Redox regulation of myocardial ERK 1/2 phosphorylation in experimental hyperthyroidism: role of thioredoxin-peroxiredoxin system. *Journal of cardiovascular pharmacology* **56,** 513-517.

Araya J, Cambier S, Morris A, Finkbeiner W and Nishimura SL (2006) Integrin-mediated transforming growth factor-beta activation regulates homeostasis of the pulmonary epithelial-mesenchymal trophic unit. *Am J Pathol.* **2,** 405-15.

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O & Caroni P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393,** 805-809.

Arsalane K, Dubois CM, Muanza T, Begin R, Boudreau F, Asselin C & Cantin AM. (1997). Transforming growth factor-beta1 is a potent inhibitor of glutathione synthesis in the lung epithelial cell line A549: transcriptional effect on the GSH rate-limiting enzyme gamma-glutamylcysteine synthetase. *American journal of respiratory cell and molecular biology* **17,** 599-607.

Artaud-Macari E, Goven D, Brayer S, Hamimi A, Besnard V, Marchal-Somme J, Ali ZE, Crestani B, Kerdine-Romer S, Boutten A & Bonay M. (2013). Nuclear factor erythroid 2-related factor 2 nuclear translocation induces myofibroblastic dedifferentiation in idiopathic pulmonary fibrosis. *Antioxidants & redox signaling* **18,** 66-79.

Asakage M, Tsuno NH, Kitayama J, Tsuchiya T, Yoneyama S, Yamada J, Okaji Y, Kaisaki S, Osada T, Takahashi K & Nagawa H. (2006). Sulforaphane induces inhibition of human umbilical vein endothelial cells proliferation by apoptosis. *Angiogenesis* **9,** 83-91.

Asano Y, Ihn H, Jinnin M, Mimura Y and Tamaki K (2006) Involvement of alphavbeta5 integrin in the establishment of autocrine TGF-beta signaling in dermal fibroblasts derived from localized scleroderma. *J Invest Dermatol.* **8,** 1761-9

auf dem Keller U, Huber M, Beyer TA, Kumin A, Siemes C, Braun S, Bugnon P, Mitropoulos V, Johnson DA, Johnson JA, Hohl D & Werner S. (2006). Nrf transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing. *Molecular and cellular biology* **26,** 3773-3784.

Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H & Yokoyama M. (2002). Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arteriosclerosis, thrombosis, and vascular biology* **22,** 1838-1844.

Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H & Yokoyama M. (1999). Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* **100,** 1494-1498.

Baird A & Durkin T. (1986). Inhibition of endothelial cell proliferation by type beta-transforming growth factor: interactions with acidic and basic fibroblast growth factors. *Biochemical and biophysical research communications* **138,** 476-482.

Bakin AV, Rinehart C, Tomlinson AK & Arteaga CL. (2002). p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. *Journal of cell science* **115,** 3193-3206.

Bakin AV, Stourman NV, Sekhar KR, Rinehart C, Yan X, Meredith MJ, Arteaga CL & Freeman ML. (2005). Smad3-ATF3 signaling mediates TGF-beta suppression of genes encoding Phase II detoxifying proteins. *Free radical biology & medicine* **38,** 375-387.

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL & Arteaga CL. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *The Journal of biological chemistry* **275,** 36803-36810.

Balestrini JL, Chaudhry S, Sarrazy V, Koehler A & Hinz B. (2012). The mechanical memory of lung myofibroblasts. *Integrative biology : quantitative biosciences from nano to macro* **4,** 410-421.

Ballinger SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, Reuf J, Horaist C, Lebovitz R, Hunter GC, McIntyre K and Runge MS (2002) Mitochondrial integrity and function in atherogenesis. *Circulation* **5,** 544-9.

Barbacanne MA, Souchard JP, Darblade B, Iliou, Nepveu F, Pipy B, Bayard F and Arnal JF (2000) Detection of superoxide anion released extracellularly by endothelial cells using cytochrome C reduction , ESR, flluorescence and lucigenin enhanced chemiluminescence techniques *Free Radical Biology and Medicine* **5,** 388-396.

Bar-Sagi D & Hall A. (2000). Ras and Rho GTPases: a family reunion. *Cell* **103,** 227-238.

Barcellos-Hoff MH, Derynck R, Tsang M L and Weatherbee J A (1994). Transforming growth factor-beta activation in irradiated murine mammary gland. *J. Clin. Invest.* **93,** 892 -899.

Barcellos-Hoff MH & Dix TA. (1996). Redox-mediated activation of latent transforming growth factor-beta 1. *Mol Endocrinol* **10,** 1077-1083.

Barnes JL & Gorin Y. (2011). Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. *Kidney international* **79,** 944-956.

Batut J, Howell M & Hill CS. (2007). Kinesin-mediated transport of Smad2 is required for signaling in response to TGF-beta ligands. *Developmental cell* **12,** 261-274.

Beck GR, Jr., Zerler B & Moran E. (2001). Gene array analysis of osteoblast differentiation. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **12,** 61-83.

Beeh KM, Beier J, Haas IC, Kornmann O, Micke P & Buhl R. (2002). Glutathione deficiency of the lower respiratory tract in patients with idiopathic pulmonary fibrosis. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* **19,** 1119-1123.

Benedict AL, Mountney A, Hurtado A, Bryan KE, Schnaar RL, Dinkova-Kostova AT & Talalay P. (2012). Neuroprotective Effects of Sulforaphane after Contusive Spinal Cord Injury. *Journal of neurotrauma*.

Bhatt A, Kaverina I, Otey C & Huttenlocher A. (2002). Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *Journal of cell science* **115,** 3415-3425.

Bhattacharyya S, Ishida W, Wu M, Wilkes M, Mori Y, Hinchcliff M, Leof E & Varga J. (2009). A non-Smad mechanism of fibroblast activation by transforming growth factor-beta via c-Abl and Egr-1: selective modulation by imatinib mesylate. *Oncogene* **28,** 1285-1297.

Bheemreddy RM & Jeffery EH. (2007). The metabolic fate of purified glucoraphanin in F344 rats. *Journal of agricultural and food chemistry* **55,** 2861-2866.

Bhowmick NA, Ghiassi M, Aakre M, Brown K, Singh V & Moses HL. (2003). TGF-beta-induced RhoA and p160ROCK activation is involved in the inhibition of Cdc25A with resultant cell-cycle arrest. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 15548-15553.

Bhowmick NA, Zent R, Ghiassi M, McDonnell M & Moses HL. (2001). Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *The Journal of biological chemistry* **276,** 46707-46713.

Bianchi A, Gervasi ME & Bakin A. (2010). Role of beta5-integrin in epithelial-mesenchymal transition in response to TGF-beta. *Cell Cycle* **9,** 1647-1659.

Bierie B and Moses HL (2006) Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer.* **7,** 506-20.

Biondi-Zoccai GG, Abbate A & Agostoni P. (2002). Antioxidant vitamins and coronary disease. *The New England journal of medicine* **346,** 1092-1093.

Birukova AA, Birukov KG, Gorshkov B, Liu F, Garcia JG & Verin AD. (2005). MAP kinases in lung endothelial permeability induced by microtubule disassembly. *American journal of physiology Lung cellular and molecular physiology* **289,** L75-84.

Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M & Bottinger EP. (2000). A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. *Genes & development* **14,** 187-197.

Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG & Gluud C. (2008). Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev***,** CD007176.

Bjorkerud B & Bjorkerud S. (1996). Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. *Arteriosclerosis, thrombosis, and vascular biology* **16,** 416-424.

Bjorkerud S. (1991). Effects of transforming growth factor-beta 1 on human arterial smooth muscle cells in vitro. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association* **11,** 892-902.

Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, Smieja M, Cambien F, Meyer J, Lackner KJ and AtheroGene Investigators (2003) Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *N Engl J Med.* **349,** 1605-13.

Bloom DA & Jaiswal AK. (2003). Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *The Journal of biological chemistry* **278,** 44675-44682.

Blumbach K, Zweers MC, Brunner G, Peters AS, Schmitz M, Schulz JN, Schild A, Denton CP, Sakai T, Fassler R, Krieg T & Eckes B. (2010). Defective granulation tissue formation in mice with specific ablation of integrin-linked kinase in fibroblasts - role of TGFbeta1 levels and RhoA activity. *Journal of cell science* **123,** 3872-3883.

Bobik A. (2006). Transforming growth factor-betas and vascular disorders. *Arteriosclerosis, thrombosis, and vascular biology* **26,** 1712-1720.

Bobik A, Agrotis A, Kanellakis P, Dilley R, Krushinsky A, Smirnov V, Tararak E, Condron M & Kostolias G. (1999). Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. *Circulation* **99,** 2883-2891.

Bobik A, Agrotis A, Kanellakis P, Dilley R, Krushinsky A, Smirnov V, Tararak E, Condron M and Kostolias G (1999) Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. *Circulation.* **22,** 2883-91.

Boddupalli S, Mein JR, Lakkanna S & James DR. (2012). Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins a, C, and e. *Frontiers in genetics* **3,** 7.

Boland S, Boisvieux-Ulrich E, Houcine O, Baeza-Squiban A, Pouchelet M, Schoevaert D & Marano F. (1996). TGF beta 1 promotes actin cytoskeleton reorganization and migratory phenotype in epithelial tracheal cells in primary culture. *Journal of cell science* **109 (Pt 9),** 2207-2219.

Bondi CD, Manickam N, Lee DY, Block K, Gorin Y, Abboud HE and Barnes JL (2010) NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. *J Am Soc Nephrol.* **1,** 93-102.

Borg TK, Rubin K, Carver W, Samarel A & Terracio L. (1996). The cell biology of the cardiac interstitium. *Trends in cardiovascular medicine* **6,** 65-70.

Borén J, Olin K, Lee I, Chait A, Wight TN and Innerarity TL (1998) Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J. Clin. Invest.* **101**, 2658–2664.

Borkowski P, Robinson MJ, Kusiak JW, Borkowski A, Brathwaite C and Mergner WJ (1995) Studies on TGF-beta 1 gene expression in the intima of the human aorta in regions with high and low probability of developing atherosclerotic lesions. *Mod Pathol* **5,** 478-82.

Boyd SD (2008) Everything you wanted to know about small RNA but were afraid to ask. *Lab Invest* **6,** 569-78.

Boyd JN, Babish JG & Stoewsand GS. (1982). Modification of beet and cabbage diets of aflatoxin B1-induced rat plasma alpha-foetoprotein elevation, hepatic tumorigenesis, and mutagenicity of urine. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **20,** 47-52.

Brandes RP, Takac I & Schroder K. (2011). No superoxide--no stress?: Nox4, the good NADPH oxidase! *Arteriosclerosis, thrombosis, and vascular biology* **31,** 1255-1257.

Brandes RP, Weissmann N, Schröder K (2010) NADPH oxidases in cardiovascular disease *Free Radical Biology and Medicine* **40,** 1-16.

Brasen JH, Leppanen O, Inkala M, Heikura T, Levin M, Ahrens F, Rutanen J, Pietsch H, Bergqvist D, Levonen AL, Basu S, Zeller T, Kloppel G, Laukkanen MO & Yla-Herttuala S. (2007). Extracellular superoxide dismutase accelerates endothelial recovery and inhibits in-stent restenosis in stented atherosclerotic Watanabe heritable hyperlipidemic rabbit aorta. *Journal of the American College of Cardiology* **50,** 2249-2253.

Brewer AC and Shah AM (2009) Redox signalling and miRNA function in cardiomyocytes. *J Mol Cell Cardiol* **1,** 2-4.

Brigelius-Flohe R & Banning A. (2006). Part of the series: from dietary antioxidants to regulators in cellular signaling and gene regulation. Sulforaphane and selenium, partners in adaptive response and prevention of cancer. *Free radical research* **40,** 775-787.

Bringuier AF, Seebold-Choqueux C, Moricard Y, Simmons DJ, Milhaud G & Labat ML. (1992). T-lymphocyte control of HLA-DR blood monocyte differentiation into neo-fibroblasts. Further evidence of pluripotential secreting functions of HLA-DR monocytes, involving not only collagen but also uromodulin, amyloid-beta peptide, alpha-fetoprotein and carcinoembryonic antigen. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **46,** 91-108.

Brose RD, Shin G, McGuinness MC, Schneidereith T, Purvis S, Dong GX, Keefer J, Spencer F & Smith KD. (2012). Activation of the stress proteome as a mechanism for small molecule therapeutics. *Human molecular genetics* **21,** 4237-4252.

Brouard S, Berberat PO, Tobiasch E, Seldon MP, Bach FH & Soares MP. (2002). Heme oxygenase-1-derived carbon monoxide requires the activation of transcription factor NF-kappa B to protect endothelial cells from tumor necrosis factor-alpha-mediated apoptosis. *The Journal of biological chemistry* **277,** 17950-

17961.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM & Soares MP. (2000). Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *The Journal of experimental medicine* **192,** 1015-1026.

Brown BG, Cheung MC, Lee AC, Zhao XQ & Chait A. (2002). Antioxidant vitamins and lipid therapy: end of a long romance? *Arteriosclerosis, thrombosis, and vascular biology* **22,** 1535-1546.

Brown BG, Zhao XQ, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, Marino EK, Bolson EL, Alaupovic P, Frohlich J & Albers JJ. (2001). Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *The New England journal of medicine* **345,** 1583-1592.

Brown KA, Ham AJ, Clark CN, Meller N, Law BK, Chytil A, Cheng N, Pietenpol JA & Moses HL. (2008). Identification of novel Smad2 and Smad3 associated proteins in response to TGF-beta1. *Journal of cellular biochemistry* **105,** 596-611.

Brown KA, Pietenpol JA & Moses HL. (2007). A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. *Journal of cellular biochemistry* **101,** 9-33.

Buday A, Orsy P, Godo M, Mozes M, Kokeny G, Lacza Z, Koller A, Ungvari Z, Gross ML, Benyo Z & Hamar P. (2010). Elevated systemic TGF-beta impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling, and increased plaque formation in apoE(-/-) mice. *American journal of physiology Heart and circulatory physiology* **299,** H386-395.

Bugyi B & Carlier MF. (2010). Control of actin filament treadmilling in cell motility. *Annual review of biophysics* **39,** 449-470.

Bujak M, Ren G, Kweon HJ, Dobaczewski M, Reddy A, Taffet G, Wang XF & Frangogiannis NG. (2007). Essential role of Smad3 in infarct healing and in the pathogenesis of cardiac remodeling. *Circulation* **116,** 2127-2138.

Burnette DT, Manley S, Sengupta P, Sougrat R, Davidson MW, Kachar B & Lippincott-Schwartz J. (2011). A role for actin arcs in the leading-edge advance of migrating cells. *Nature cell biology* **13,** 371-381.

Bustin SA. (2010). Why the need for qPCR publication guidelines?--The case for MIQE. *Methods* **50,** 217-226.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J & Wittwer CT. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry* **55,** 611-622.

Callaway EC, Zhang Y, Chew W & Chow HH. (2004). Cellular accumulation of dietary anticarcinogenic isothiocyanates is followed by transporter-mediated export as dithiocarbamates. *Cancer letters* **204,** 23-31.

Calo L, Giacon B, Davis PA, Pagnin E, Piccin A, Riegler P, Huber W, Antonello A & Semplicini A. (2002). Oxidative stress and TGFbeta in kidney-transplanted patients with cyclosporin-induced hypertension. Effect of carvedilol and nifedipine. *Clinical nephrology* **58,** 103-110.

Camara NO & Soares MP. (2005). Heme oxygenase-1 (HO-1), a protective gene that prevents chronic graft dysfunction. *Free radical biology & medicine* **38,** 426-435.

Carnesecchi S, Deffert C, Donati Y, Basset O, Hinz B, Preynat-Seauve O, Guichard C, Arbiser JL, Banfi B, Pache JC, Barazzone-Argiroffo C & Krause KH. (2011). A key role for NOX4 in epithelial cell death during development of lung fibrosis. *Antioxidants & redox signaling* **15,** 607-619.

Cascino T, Csanyi G, Al Ghouleh I, Montezano AC, Touyz RM, Haurani MJ & Pagano PJ. (2011). Adventitia-derived hydrogen peroxide impairs relaxation of the rat carotid artery via smooth muscle cell p38 mitogen-activated protein kinase. *Antioxidants & redox signaling* **15,** 1507-1515.

Chamseddine AH and Miller FJ Jr (2003) Gp91phox contributes to NADPH oxidase activity in aortic fibroblasts but not smooth muscle cells. *Am J Physiol Heart Circ Physiol* **285,** H2284-9.

Chan C, Lin HJ & Lin J. (2008). Stress-associated hormone, norepinephrine, increases proliferation and IL-6 levels of human pancreatic duct epithelial cells and can be inhibited by the dietary agent, sulforaphane. *International journal of oncology* **33,** 415-419.

Chan EC, Datla SR, Dilley R, Hickey H, Drummond GR & Dusting GJ. (2007). Adventitial application of the NADPH oxidase inhibitor apocynin in vivo reduces neointima formation and endothelial dysfunction in rabbits. *Cardiovascular research* **75,** 710-718.

Chan JY & Kwong M. (2000). Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. *Biochimica et biophysica acta* **1517,** 19-26.

Chan K, Han XD & Kan YW. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 4611-4616.

Chan K, Lu R, Chang JC & Kan YW. (1996). NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 13943-13948.

Chan KH, Ng MK & Stocker R. (2011). Haem oxygenase-1 and cardiovascular disease: mechanisms and therapeutic potential. *Clin Sci (Lond)* **120,** 493-504.

Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, Yamamoto M & Hayes JD. (2002). Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *The Biochemical journal* **365,** 405-416.

Chang G, Guo Y, Jia Y, Duan W, Li B, Yu J & Li C. (2010). Protective effect of combination of sulforaphane and riluzole on glutamate-mediated excitotoxicity. *Biological & pharmaceutical bulletin* **33,** 1477-1483.

Chang MW, Barr E, Lu MM, Barton K & Leiden JM. (1995). Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *The Journal of clinical investigation* **96,** 2260-2268.

Chapple SJ, Siow RC & Mann GE. (2012). Crosstalk between Nrf2 and the proteasome: therapeutic potential of Nrf2 inducers in vascular disease and aging. *The international journal of biochemistry & cell biology* **44,** 1315-1320.

Chauveau C, Bouchet D, Roussel JC, Mathieu P, Braudeau C, Renaudin K, Tesson L, Soulillou JP, Iyer S, Buelow R & Anegon I. (2002). Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **2,** 581-592.

Chen CS, Tseng YT, Hsu YY & Lo YC. (2012). Nrf2-Keap1 Antioxidant Defense and Cell Survival signaling are up-regulated by 17beta-Estradiol in Homocysteine-treated Dopaminergic SH-SY5Y Cells. *Neuroendocrinology*.

Chen G, Fang Q, Zhang J, Zhou D & Wang Z. (2011). Role of the Nrf2-ARE pathway in early brain injury after experimental subarachnoid hemorrhage. *Journal of neuroscience research* **89,** 515-523.

Chen G & Khalil N. (2006). TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respiratory research* **7,** 2.
Chen HB, Rud JG, Lin K & Xu L. (2005). Nuclear targeting of transforming growth factor-beta-activated Smad complexes. *The Journal of biological chemistry* **280,** 21329-21336.

Chen LX, Yang K, Sun M, Chen Q, Wang ZH, Hu GY & Tao LJ. (2012). Fluorofenidone inhibits transforming growth factor-beta1-induced cardiac myofibroblast differentiation. *Die Pharmazie* **67,** 452-456.

Chen XL, Dodd G & Kunsch C. (2009). Sulforaphane inhibits TNF-alpha-induced activation of p38 MAP kinase and VCAM-1 and MCP-1 expression in endothelial cells. *Inflammation research : official journal of the European Histamine Research Society [et al]* **58,** 513-521.

Chen Y, Inoyama D, Kong AN, Beamer LJ & Hu L. (2011). Kinetic analyses of Keap1-Nrf2 interaction and determination of the minimal Nrf2 peptide sequence required for Keap1 binding using surface plasmon resonance. *Chemical biology & drug design* **78,** 1014-1021.

Chen YG. (2009). Endocytic regulation of TGF-beta signaling. *Cell research* **19,** 58-70.

Chen YG, Hata A, Lo RS, Wotton D, Shi Y, Pavletich N & Massague J. (1998). Determinants of specificity in TGF-beta signal transduction. *Genes & development* **12,** 2144-2152.

Chen YT, Shi D, Yang D & Yan B. (2012). Antioxidant sulforaphane and sensitizer trinitrobenzene sulfonate induce carboxylesterase-1 through a novel element transactivated by nuclear factor-E2 related factor-2. *Biochemical pharmacology* **84,** 864-871.

Chen Z, Li DQ, Tong L, Stewart P, Chu C & Pflugfelder SC. (2006). Targeted inhibition of p57 and p15 blocks transforming growth factor beta-inhibited proliferation of primary cultured human limbal epithelial cells. *Molecular vision* **12,** 983-994.

Chen Z, Siu B, Ho YS, Vincent R, Chua CC, Hamdy RC, Chua BH (1998) Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J Mol Cell Cardiol* **11,** 2281-9.

Chen ZH, Yoshida Y, Saito Y, Sekine A, Noguchi N & Niki E. (2006). Induction of adaptive response and enhancement of PC12 cell tolerance by 7-hydroxycholesterol and 15-deoxy-delta(12,14)-prostaglandin J2 through up-regulation of cellular glutathione via different mechanisms. *The Journal of biological chemistry* **281,** 14440-14445.

Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, Arteaga CL, Neilson EG, Hayward SW & Moses HL. (2005). Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* **24,** 5053-5068.

Cheung KL & Kong AN. (2010). Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. *The AAPS journal* **12,** 87-97.

Chin YT, Liao YW, Fu MM, Tu HP, Shen EC, Nieh S, Shih KC & Fu E. (2011). Nrf-2 regulates cyclosporine-stimulated HO-1 expression in gingiva. *Journal of dental research* **90,** 995-1000.

Chiou YS, Ma NJ, Sang S, Ho CT, Wang YJ & Pan MH. (2012). Peracetylated (-)-epigallocatechin-3-gallate (AcEGCG) potently suppresses dextran sulfate sodium-induced colitis and colon tumorigenesis in mice. *Journal of agricultural and food chemistry* **60,** 3441-3451.

Chiou TJ, Chu ST and Tzeng WF (2003) Protection of cells from menadione-induced apoptosis by inhibition of lipid peroxidation. *Toxicology* **2-3,** 77-88.

Cho JS, Moon YM, Park IH, Um JY, Moon JH, Park SJ, Lee SH, Kang HJ & Lee HM. (2012). Epigenetic regulation of myofibroblast differentiation and extracellular matrix production in nasal polyp-derived fibroblasts. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **42,** 872-882.
Cho HY, Reddy SP and Kleeberger SR (2006) Nrf2 defends the lung from oxidative stress. *Antioxid Redox Signal* **8,** 76-87.

Choi KM, Lee YS, Sin DM, Lee S, Lee MK, Lee YM, Hong JT, Yun YP & Yoo HS. (2012). Sulforaphane inhibits mitotic clonal expansion during adipogenesis through cell cycle arrest. *Obesity (Silver Spring)* **20,** 1365-1371.

Choi ME, Ding Y & Kim SI. (2012). TGF-beta Signaling via TAK1 Pathway: Role in Kidney Fibrosis. *Seminars in nephrology* **32,** 244-252.

Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG & Kang SW. (2005). Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* **435,** 347-353.

Choi WY, Choi BT, Lee WH & Choi YH. (2008). Sulforaphane generates reactive oxygen species leading to mitochondrial perturbation for apoptosis in human leukemia U937 cells. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **62,** 637-644.

Chuang WY, Kung PH, Kuo CY & Wu CC. (2013). Sulforaphane prevents human platelet aggregation through inhibiting the phosphatidylinositol 3-kinase/Akt pathway. *Thrombosis and haemostasis* **109**.

Chung AC, Huang XR, Meng X and Lan HY (2010) miR-192 mediates TGF-{beta}/Smad3-driven renal fibrosis *J Am Soc Nephrol* **8,** 1317-1325.

Chung IM, Kim J, Pak YK, Jang Y, Yang WI, Han I, Park SJ, Park SW, Huh J, Wight TN & Ueno H. (2010). Blockade of TGF-beta by catheter-based local intravascular gene delivery does not alter the in-stent neointimal response, but enhances inflammation in pig coronary arteries. *International journal of cardiology* **145,** 468-475.

Chung SW, Kwon MY, Kang YH, Chung HT, Lee SJ, Kim HP & Perrella MA. (2012). Transforming growth factor-beta1 suppression of endotoxin-induced heme oxygenase-1 in macrophages involves activation of Smad2 and downregulation of Ets-2. *Journal of cellular physiology* **227,** 351-360.

Cifuentes ME, Rey FE, Carretero OA & Pagano PJ. (2000). Upregulation of p67(phox) and gp91(phox) in aortas from angiotensin II-infused mice. *American journal of physiology Heart and circulatory physiology* **279,** H2234-2240.

Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ and Motterlini R (2000) Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol* **278,** H643-51.

Clements CM, McNally RS, Conti BJ, Mak TW & Ting JP. (2006). DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 15091-15096.

Clerigues V, Guillen MI, Gomar F & Alcaraz MJ. (2012). Haem oxygenase-1 counteracts the effects of interleukin-1beta on inflammatory and senescence markers in cartilage-subchondral bone explants from osteoarthritic patients. *Clin Sci (Lond)* **122,** 239-250.

Coen M, Gabbiani G & Bochaton-Piallat ML. (2011). Myofibroblast-mediated adventitial remodeling: an underestimated player in arterial pathology. *Arteriosclerosis, thrombosis, and vascular biology* **31,** 2391-2396.

Collins AR, Lyon CJ, Xia X, Liu JZ, Tangirala RK, Yin F, Boyadjian R, Bikineyeva A, Pratico D, Harrison DG & Hsueh WA. (2009). Age-accelerated atherosclerosis correlates with failure to upregulate antioxidant genes. *Circulation research* **104,** e42-54.

Conaway CC, Getahun SM, Liebes LL, Pusateri DJ, Topham DK, Botero-Omary M & Chung FL. (2000). Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutrition and cancer* **38,** 168-178.

Conley BA, Koleva R, Smith JD, Kacer D, Zhang D, Bernabeu C & Vary CP. (2004). Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. *The Journal of biological chemistry* **279,** 27440-27449.

Copple IM. (2012). The Keap1-Nrf2 cell defense pathway--a promising therapeutic target? *Adv Pharmacol* **63,** 43-79.

Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN & Srivastava D. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* **460,** 705-710.

Cornelis MC, El-Sohemy A & Campos H. (2007). GSTT1 genotype modifies the association between cruciferous vegetable intake and the risk of myocardial infarction. *The American journal of clinical nutrition* **86,** 752-758.

Correa F, Mallard C, Nilsson M & Sandberg M. (2011). Activated microglia decrease histone acetylation and Nrf2-inducible anti-oxidant defence in astrocytes: restoring effects of inhibitors of HDACs, p38 MAPK and GSK3beta. *Neurobiology of disease* **44,** 142-151.

Correa-Costa M, Semedo P, Monteiro AP, Silva RC, Pereira RL, Goncalves GM, Marques GD, Cenedeze MA, Faleiros AC, Keller AC, Shimizu MH, Seguro AC, Reis MA, Pacheco-Silva A & Camara NO. (2010). Induction of heme oxygenase-1 can halt and even reverse renal tubule-interstitial fibrosis. *PloS one* **5,** e14298.

Coulson-Thomas VJ, Gesteira TF, Coulson-Thomas YM, Vicente CM, Tersariol IL, Nader HB & Toma L. (2010). Fibroblast and prostate tumor cell cross-talk: fibroblast differentiation, TGF-beta, and extracellular matrix down-regulation. *Experimental cell research* **316,** 3207-3226.

Coumailleau F, Furthauer M, Knoblich JA & Gonzalez-Gaitan M. (2009). Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell division. *Nature* **458,** 1051-1055.

Cowan DB, Jones M, Garcia LM, Noria S, del Nido PJ and McGowan FX Jr (2003) Hypoxia and stretch regulate intercellular communication in vascular smooth muscle cells through reactive oxygen species formation. *Arterioscler Thromb Vasc Biol* **10,** 1754-60.

Crawford DW and Kramsch DM (1988) The oxygen environment of the arterial media in early rabbit hypertension *Exp Mol Pathol* **49,** 215–233.

Crider BJ, Risinger GM, Jr., Haaksma CJ, Howard EW & Tomasek JJ. (2011). Myocardin-related transcription factors A and B are key regulators of TGF-beta1-induced fibroblast to myofibroblast differentiation. *The Journal of investigative dermatology* **131,** 2378-2385.

Csanyi G, Taylor WR & Pagano PJ. (2009). NOX and inflammation in the vascular adventitia. *Free radical biology & medicine* **47,** 1254-1266.

Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S & Sorescu D. (2005). NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circulation research* **97,** 900-907.

Cudmore MJ, Ahmad S, Sissaoui S, Ramma W, Ma B, Fujisawa T, Al-Ani B, Wang K, Cai M, Crispi F,

Hewett PW, Gratacos E, Egginton S & Ahmed A. (2012). Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: identification of inter-dependency between Akt-1 and heme oxygenase-1. *European heart journal* **33,** 1150-1158.

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ & Diehl JA. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Molecular and cellular biology* **23,** 7198-7209.

D'Autreaux B & Toledano MB. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews Molecular cell biology* **8,** 813-824.

Dabiri G, Campaner A, Morgan JR & Van De Water L. (2006). A TGF-beta1-dependent autocrine loop regulates the structure of focal adhesions in hypertrophic scar fibroblasts. *The Journal of investigative dermatology* **126,** 963-970.

Dai G, Vaughn S, Zhang Y, Wang ET, Garcia-Cardena G & Gimbrone MA, Jr. (2007). Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2. *Circulation research* **101,** 723-733.

Daiber A, Oelze M, August M, Wendt M, Sydow K, Wieboldt H, Kleschyov AL & Munzel T. (2004). Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. *Free radical research* **38,** 259-269.

Daniels CE, Wilkes MC, Edens M, Kottom TJ, Murphy SJ, Limper AH & Leof EB. (2004). Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis. *The Journal of clinical investigation* **114,** 1308-1316.

Das SK, White AC & Fanburg BL. (1992). Modulation of transforming growth factor-beta 1 antiproliferative effects on endothelial cells by cysteine, cystine, and N-acetylcysteine. *The Journal of clinical investigation* **90,** 1649-1656.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y & Wang XF. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 5545-5549.

Davies MR, Lund RJ & Hruska KA. (2003). BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. *Journal of the American Society of Nephrology : JASN* **14,** 1559-1567.

Davies PF (1995) Flow-mediated endothelial mechanotransduction. *Physiol Rev.* **3,** 519-60.

de Matos AC, Camara NO, Tonato EJ, Durao Junior Mde S, Franco MF, Moura LA & Pacheco-Silva A. (2010). Vimentin expression and myofibroblast infiltration are early markers of renal dysfunction in kidney transplantation: an early stage of chronic allograft dysfunction? *Transplantation proceedings* **42,** 3482-3488.

Deaton RA, Su C, Valencia TG & Grant SR. (2005). Transforming growth factor-beta1-induced expression of smooth muscle marker genes involves activation of PKN and p38 MAPK. *The Journal of biological chemistry* **280,** 31172-31181.

Deheuninck J & Luo K. (2009). Ski and SnoN, potent negative regulators of TGF-beta signaling. *Cell research* **19,** 47-57.

Deng C, Tao R, Yu SZ & Jin H. (2012). Sulforaphane protects against 6-hydroxydopamine-induced cytotoxicity by increasing expression of heme oxygenase-1 in a PI3K/Akt-dependent manner. *Molecular medicine reports* **5,** 847-851.

Deng YM, Wu BJ, Witting PK & Stocker R. (2004). Probucol protects against smooth muscle cell

proliferation by upregulating heme oxygenase-1. *Circulation* **110,** 1855-1860.

Denizot F & Lang R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of immunological methods* **89,** 271-277.

Derrett-Smith EC, Dooley A, Khan K, Shi-wen X, Abraham D & Denton CP. (2010). Systemic vasculopathy with altered vasoreactivity in a transgenic mouse model of scleroderma. *Arthritis research & therapy* **12,** R69.

Derynck R, Akhurst RJ & Balmain A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nature genetics* **29,** 117-129.

Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massagué J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF(1996) Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* 18, 173.

Desbuards N, Hyvelin JM, Machet MC, Eder V, Garrigue MA, Halimi JM & Antier D. (2009). Heme oxygenase-1 inducer hemin attenuates the progression of remnant kidney model. *Nephron Experimental nephrology* **113,** e35-44.

Desmouliere A. (1995). Factors influencing myofibroblast differentiation during wound healing and fibrosis. *Cell biology international* **19,** 471-476.

Desmouliere A, Chaponnier C & Gabbiani G. (2005). Tissue repair, contraction, and the myofibroblast. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **13,** 7-12.

Desmouliere A, Geinoz A, Gabbiani F & Gabbiani G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *The Journal of cell biology* **122,** 103-111.

Diaz MN, Frei B, Vita JA and Keaney JF (1997) Antioxidants and atherosclerotic heart disease *The New England Journal of Medicine* **337,** 408-416.

Di Guglielmo GM, Le Roy C, Goodfellow AF & Wrana JL. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nature cell biology* **5,** 410-421.

Di Wang H, Hope S, Du Y, Quinn MT, Cayatte A, Pagano PJ & Cohen RA. (1999). Paracrine role of adventitial superoxide anion in mediating spontaneous tone of the isolated rat aorta in angiotensin II-induced hypertension. *Hypertension* **33,** 1225-1232.

Di Wang H, Ratsep MT, Chapman A & Boyd R. (2010). Adventitial fibroblasts in vascular structure and function: the role of oxidative stress and beyond. *Canadian journal of physiology and pharmacology* **88,** 177-186.

Ding Q, Gladson CL, Wu H, Hayasaka H and Olman MA (2008) Focal adhesion kinase (FAK)-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. *J Biol Chem* **40,** 26839-49.

Dickinson BC, Huynh C & Chang CJ. (2010). A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *Journal of the American Chemical Society* **132,** 5906-5915.

Dickinson RB, Caro L & Purich DL. (2004). Force generation by cytoskeletal filament end-tracking proteins. *Biophysical journal* **87,** 2838-2854.

Didion SP, Kinzenbaw DA, Schrader LI & Faraci FM. (2006). Heterozygous CuZn superoxide dismutase deficiency produces a vascular phenotype with aging. *Hypertension* **48,** 1072-1079.

Didion SP, Ryan MJ, Didion LA, Fegan PE, Sigmund CD & Faraci FM. (2002). Increased superoxide and vascular dysfunction in CuZnSOD-deficient mice. *Circulation research* **91,** 938-944.

Dikalov S, Griendling KK & Harrison DG. (2007). Measurement of reactive oxygen species in cardiovascular studies. *Hypertension* **49,** 717-727.

Dikalov SI, Li W, Mehranpour P, Wang SS & Zafari AM. (2007). Production of extracellular superoxide by human lymphoblast cell lines: comparison of electron spin resonance techniques and cytochrome C reduction assay. *Biochemical pharmacology* **73,** 972-980.

Dimmeler S & Nicotera P. (2013). MicroRNAs in age-related diseases. *EMBO molecular medicine* **5,** 180-190.

Dinkova-Kostova AT, Fahey JW, Wade KL, Jenkins SN, Shapiro TA, Fuchs EJ, Kerns ML & Talalay P. (2007). Induction of the phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **16,** 847-851.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M & Talalay P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 11908-11913.

Dinkova-Kostova AT & Talalay P. (2010). NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Archives of biochemistry and biophysics* **501,** 116-123.

Discher DE, Janmey P & Wang YL. (2005). Tissue cells feel and respond to the stiffness of their substrate. *Science* **310,** 1139-1143.

Dobaczewski M, Bujak M, Li N, Gonzalez-Quesada C, Mendoza LH, Wang XF & Frangogiannis NG. (2010). Smad3 signaling critically regulates fibroblast phenotype and function in healing myocardial infarction. *Circulation research* **107,** 418-428.

Dong SM, Pang JC, Poon WS, Hu J, To KF, Chang AR & Ng HK. (2001). Concurrent hypermethylation of multiple genes is associated with grade of oligodendroglial tumors. *Journal of neuropathology and experimental neurology* **60,** 808-816.

Doudican NA, Bowling B & Orlow SJ. (2010). Enhancement of arsenic trioxide cytotoxicity by dietary isothiocyanates in human leukemic cells via a reactive oxygen species-dependent mechanism. *Leukemia research* **34,** 229-234.

Dourron HM, Jacobson GM, Park JL, Liu J, Reddy DJ, Scheel ML & Pagano PJ. (2005). Perivascular gene transfer of NADPH oxidase inhibitor suppresses angioplasty-induced neointimal proliferation of rat carotid artery. *American journal of physiology Heart and circulatory physiology* **288,** H946-953.

Doyle A, Marganski W & Lee J. (2004). Calcium transients induce spatially coordinated increases in traction force during the movement of fish keratocytes. *Journal of cell science* **117,** 2203-2214.

Dranoff JA & Wells RG. (2010). Portal fibroblasts: Underappreciated mediators of biliary fibrosis. *Hepatology* **51,** 1438-1444.

Droge W (2002) Free radicals in the physiological control of cell function *Physiology Reviews* **82,** 47-95.

Dugina V, Fontao L, Chaponnier C, Vasiliev J & Gabbiani G. (2001). Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *Journal of cell science* **114,** 3285-3296.

Dumont N, Bakin AV & Arteaga CL. (2003). Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *The Journal of biological chemistry* **278,** 3275-3285.

Durand E, Al Haj Zen A, Addad F, Brasselet C, Caligiuri G, Vinchon F, Lemarchand P, Desnos M, Bruneval P & Lafont A. (2005). Adenovirus-mediated gene transfer of superoxide dismutase and catalase decreases restenosis after balloon angioplasty. *Journal of vascular research* **42,** 255-265.

Dynoodt P, Speeckaert R, De Wever O, Chevolet I, Brochez L, Lambert J & Van Gele M. (2013). miR-145 overexpression suppresses the migration and invasion of metastatic melanoma cells. *International journal of oncology* **42,** 1443-1451.

Dziadzio M, Smith RE, Abraham DJ, Black CM & Denton CP. (2005). Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology (Oxford)* **44,** 1518-1524.

Eades G, Yang M, Yao Y, Zhang Y & Zhou Q. (2011). miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *The Journal of biological chemistry* **286,** 40725-40733.

Ebisawa T, Fukuchi M, Murakami G (2001) Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* **276,** 12477-12480.

Ebrahimian TG, Heymes C, You D, Blanc-Brude O, Mees OB, Waeckel L, Duriez M, Vilar J, Brandes RP, Levy BI, Shah A. and Silvestre JS, (2006) NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol* **169**, 719–728.

Edlund S, Bu S, Schuster N, Aspenstrom P, Heuchel R, Heldin NE, ten Dijke P, Heldin CH & Landstrom M. (2003). Transforming growth factor-beta1 (TGF-beta)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF-beta-activated kinase 1 and mitogen-activated protein kinase kinase 3. *Molecular biology of the cell* **14,** 529-544.

Eggler AL, Liu G, Pezzuto JM, van Breemen RB & Mesecar AD. (2005). Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 10070-10075.

Ehler E, Babiychuk E and Draeger A (1996) Human foetal lung (IMR-90) cells: Myofibroblasts with smooth muscle-like contractile properties *Cell Motility and the Cytoskeleton* **34,** 288-298.

Emmert SW, Desai D, Amin S & Richie JP, Jr. (2010). Enhanced Nrf2-dependent induction of glutathione in mouse embryonic fibroblasts by isoselenocyanate analog of sulforaphane. *Bioorganic & medicinal chemistry letters* **20,** 2675-2679.

Engel L & Ryan U. (1997). TGF-beta 1 reverses PDGF-stimulated migration of human aortic smooth muscle cells in vitro. *In vitro cellular & developmental biology Animal* **33,** 443-451.

Engelmann J, Volk J, Leyhausen G & Geurtsen W. (2005). ROS formation and glutathione levels in human oral fibroblasts exposed to TEGDMA and camphorquinone. *Journal of biomedical materials research Part B, Applied biomaterials* **75,** 272-276.

Engler A, Bacakova L, Newman C, Hategan A, Griffin M & Discher D. (2004). Substrate compliance versus ligand density in cell on gel responses. *Biophysical journal* **86,** 617-628.

Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL & Discher DE. (2004). Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff

microenvironments. *The Journal of cell biology* **166,** 877-887.

Enzerink A & Vaheri A. (2011). Fibroblast activation in vascular inflammation. *Journal of thrombosis and haemostasis : JTH* **9,** 619-626.

Ernst IM, Palani K, Esatbeyoglu T, Schwarz K & Rimbach G. (2013). Synthesis and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-inducing activity of the isothiocyanates iberverin, iberin and cheirolin. *Pharmacological research : the official journal of the Italian Pharmacological Society*.

Ernst IM, Palani K, Esatbeyoglu T, Schwarz K & Rimbach G. (2013). Synthesis and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-inducing activity of the isothiocyanates iberverin, iberin and cheirolin. *Pharmacological research : the official journal of the Italian Pharmacological Society*.

Evans PC. (2011). The influence of sulforaphane on vascular health and its relevance to nutritional approaches to prevent cardiovascular disease. *The EPMA journal* **2,** 9-14.

Eyden B. (2001). The myofibroblast: an assessment of controversial issues and a definition useful in diagnosis and research. *Ultrastructural pathology* **25,** 39-50.

Eyden B. (2004). Fibroblast phenotype plasticity: relevance for understanding heterogeneity in "fibroblastic" tumors. *Ultrastructural pathology* **28,** 307-319.

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P & Lozniewski A. (2002). Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 7610-7615.

Fahey JW, Zhang Y & Talalay P. (1997). Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 10367-10372.

Faraonio R, Vergara P, Di Marzo D, Pierantoni MG, Napolitano M, Russo T & Cimino F. (2006). p53 suppresses the Nrf2-dependent transcription of antioxidant response genes. *The Journal of biological chemistry* **281,** 39776-39784.

Faulkner K & Fridovich I. (1993). Luminol and lucigenin as detectors for O2. *Free radical biology & medicine* **15,** 447-451.

Feinberg MW & Jain MK. (2005). Role of transforming growth factor-beta1/Smads in regulating vascular inflammation and atherogenesis. *Panminerva medica* **47,** 169-186.

Feinberg MW, Watanabe M, Lebedeva MA, Depina AS, Hanai J, Mammoto T, Frederick JP, Wang XF, Sukhatme VP & Jain MK. (2004). Transforming growth factor-beta1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. *The Journal of biological chemistry* **279,** 16388-16393.

Feng XH & Derynck R. (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annual review of cell and developmental biology* **21,** 659-693.

Fenwick GR, Heaney RK & Mullin WJ. (1983). Glucosinolates and their breakdown products in food and food plants. *Critical reviews in food science and nutrition* **18,** 123-201.

Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD & Snyder SH. (1999). Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nature cell biology* **1,** 152-157.

Filomeni G, Piccirillo S, Rotilio G & Ciriolo MR. (2012). p38(MAPK) and ERK1/2 dictate cell death/survival response to different pro-oxidant stimuli via p53 and Nrf2 in neuroblastoma cells SH-SY5Y.

Biochemical pharmacology **83,** 1349-1357.

Fimognari C, Lenzi M, Sestili P, Turrini E, Ferruzzi L, Hrelia P & Cantelli-Forti G. (2012). Sulforaphane potentiates RNA damage induced by different xenobiotics. *PloS one* **7,** e35267.

Firth AL, Yao W, Ogawa A, Madani MM, Lin GY & Yuan JX. (2010). Multipotent mesenchymal progenitor cells are present in endarterectomized tissues from patients with chronic thromboembolic pulmonary hypertension. *American journal of physiology Cell physiology* **298,** C1217-1225.

Fleige S & Pfaffl MW. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular aspects of medicine* **27,** 126-139.

Fleige S, Walf V, Huch S, Prgomet C, Sehm J & Pfaffl MW. (2006). Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology letters* **28,** 1601-1613.

Fletcher NM, Jiang ZL, Diamond MP, Abu-Soud HM and Saed GM (2008) Hypoxia-generated superoxide induces the development of the adhesion phenotype. *Free Radic Biol Med* **4,** 530-6.

Floreani M., Petrone M, Debetto P, Palatini P (1997). A comparison between different methods for the determination of reduced and oxidized glutathione in mammalian tissues. *Free Radic Res* **26**(5): 449-455.

Fong YC, Hsu SF, Wu CL, Li TM, Kao ST, Tsai FJ, Chen WC, Liu SC, Wu CM & Tang CH. (2009). Transforming growth factor-beta1 increases cell migration and beta1 integrin up-regulation in human lung cancer cells. *Lung Cancer* **64,** 13-21.

Fornerod M, Ohno M, Yoshida M & Mattaj IW. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90,** 1051-1060.

Forstermann U. (2008). Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nature clinical practice Cardiovascular medicine* **5,** 338-349.

Fotakis G & Timbrell JA. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology letters* **160,** 171-177.

Franklin CC, Rosenfeld-Franklin ME, White C, Kavanagh TJ & Fausto N. (2003). TGFbeta1-induced suppression of glutathione antioxidant defenses in hepatocytes: caspase-dependent post-translational and caspase-independent transcriptional regulatory mechanisms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17,** 1535-1537.

Frederick KB, Sept D & De La Cruz EM. (2008). Effects of solution crowding on actin polymerization reveal the energetic basis for nucleotide-dependent filament stability. *Journal of molecular biology* **378,** 540-550.

Frey MT & Wang YL. (2009). A photo-modulatable material for probing cellular responses to substrate rigidity. *Soft matter* **5,** 1918-1924.

Frey RS & Mulder KM. (1997). Involvement of extracellular signal-regulated kinase 2 and stress-activated protein kinase/Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells. *Cancer research* **57,** 628-633.

Froh M, Conzelmann L, Walbrun P, Netter S, Wiest R, Wheeler MD, Lehnert M, Uesugi T, Scholmerich J & Thurman RG. (2007). Heme oxygenase-1 overexpression increases liver injury after bile duct ligation in rats. *World journal of gastroenterology : WJG* **13,** 3478-3486.

Frolik CA, Dart LL, Meyers CA, Smith DM & Sporn MB. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proceedings of the National Academy of Sciences of the United States of America* **80,** 3676-3680.

Frutkin AD, Otsuka G, Stempien-Otero A, Sesti C, Du L, Jaffe M, Dichek HL, Pennington CJ, Edwards DR, Nieves-Cintrón M, Minter D, Preusch M, Hu JH, Marie JC and Dichek DA (2009) TGF-[beta]1 limits plaque growth, stabilizes plaque structure, and prevents aortic dilation in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* **9,** 1251-7.

Fu J, Zhang Q, Woods CG, Zheng H, Yang B, Qu W, Andersen ME & Pi J. (2013). Divergent Effects of Sulforaphane on Basal and Glucose-Stimulated Insulin Secretion in beta-Cells: Role of Reactive Oxygen Species and Induction of Endogenous Antioxidants. *Pharmaceutical research*.

Fu Y, Zheng S, Lu SC & Chen A. (2008). Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis. *Molecular pharmacology* **73,** 1465-1473.

Fukai T, Folz RJ, Landmesser U & Harrison DG. (2002). Extracellular superoxide dismutase and cardiovascular disease. *Cardiovascular research* **55,** 239-249.

Fukai T, Galis ZS, Meng XP, Parthasarathy S & Harrison DG. (1998). Vascular expression of extracellular superoxide dismutase in atherosclerosis. *The Journal of clinical investigation* **101,** 2101-2111.

Fukai T & Ushio-Fukai M. (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxidants & redox signaling* **15,** 1583-1606.

Fukui T, Ishizaka N, Rajagopalan S, Laursen JB, Capers Qt, Taylor WR, Harrison DG, de Leon H, Wilcox JN & Griendling KK. (1997). p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circulation research* **80,** 45-51.

Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L & Roth MR. (2001). Proteoglycan expression during transforming growth factor beta -induced keratocyte-myofibroblast transdifferentiation. *The Journal of biological chemistry* **276,** 44173-44178.

Furman C, Rundlof AK, Larigauderie G, Jaye M, Bricca G, Copin C, Kandoussi AM, Fruchart JC, Arner ES & Rouis M. (2004). Thioredoxin reductase 1 is upregulated in atherosclerotic plaques: specific induction of the promoter in human macrophages by oxidized low-density lipoproteins. *Free radical biology & medicine* **37,** 71-85.

Furniss CS, Bennett RN, Bacon JR, LeGall G & Mithen RF. (2008). Polyamine metabolism and transforming growth factor-beta signaling are affected in Caco-2 cells by differentially cooked broccoli extracts. *The Journal of nutrition* **138,** 1840-1845.

Gabbiani G, Ryan GB & Majne G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* **27,** 549-550.

Galicia-Moreno M, Favari L & Muriel P. (2012). Antifibrotic and antioxidant effects of N-acetylcysteine in an experimental cholestatic model. *European journal of gastroenterology & hepatology* **24,** 179-185.

Gamet-Payrastre L. (2006). Signaling pathways and intracellular targets of sulforaphane mediating cell cycle arrest and apoptosis. *Current cancer drug targets* **6,** 135-145.

Gan L, Johnson DA & Johnson JA. (2010). Keap1-Nrf2 activation in the presence and absence of DJ-1. *The European journal of neuroscience* **31,** 967-977.

Garofalo M, Romano G, Di Leva G, Nuovo G, Jeon YJ, Ngankeu A, Sun J, Lovat F, Alder H, Condorelli G, Engelman JA, Ono M, Rho JK, Cascione L, Volinia S, Nephew KP & Croce CM. (2012). EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers. *Nature medicine* **18,** 74-82.

Gasper AV, Al-Janobi A, Smith JA, Bacon JR, Fortun P, Atherton C, Taylor MA, Hawkey CJ, Barrett DA & Mithen RF. (2005). Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli. *The American journal of clinical nutrition* **82,** 1283-1291.

Genkinger JM, Platz EA, Hoffman SC, Comstock GW & Helzlsouer KJ. (2004). Fruit, vegetable, and antioxidant intake and all-cause, cancer, and cardiovascular disease mortality in a community-dwelling population in Washington County, Maryland. *American journal of epidemiology* **160,** 1223-1233.

Ghosh AK, Wei J, Wu M & Varga J. (2008). Constitutive Smad signaling and Smad-dependent collagen gene expression in mouse embryonic fibroblasts lacking peroxisome proliferator-activated receptor-gamma. *Biochemical and biophysical research communications* **374,** 231-236.

Giannone G, Dubin-Thaler BJ, Rossier O, Cai Y, Chaga O, Jiang G, Beaver W, Dobereiner HG, Freund Y, Borisy G & Sheetz MP. (2007). Lamellipodial actin mechanically links myosin activity with adhesion-site formation. *Cell* **128,** 561-575.

Gimbrone, M. A. Jr (1999) Vascular endothelium, hemodynamic forces, and atherogenesis. *Am. J. Pathol.***155**, 1–5.

Giudice A & Montella M. (2006). Activation of the Nrf2-ARE signaling pathway: a promising strategy in cancer prevention. *BioEssays : news and reviews in molecular, cellular and developmental biology* **28,** 169-181.

Glagov S, Weisenberg E, Zarins CK, Stankunavicius R and Kolettis GJ (1987) Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med.* **2,** 1371-5.

Goffin JM, Pittet P, Csucs G, Lussi JW, Meister JJ & Hinz B. (2006). Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *The Journal of cell biology* **172,** 259-268.

Gojova A, Brun V, Esposito B, Cottrez F, Gourdy P, Ardouin P, Tedgui A, Mallat Z & Groux H. (2003). Specific abrogation of transforming growth factor-beta signaling in T cells alters atherosclerotic lesion size and composition in mice. *Blood* **102,** 4052-4058.

Goldstein JL, Ho YK, Basu SK and Brown MS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition *Proc Natl Acad Sci* **79,** 333-337.

Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A & Massague J. (2006). A FoxO-Smad synexpression group in human keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 12747-12752.

Gong D, Shi W, Yi SJ, Chen H, Groffen J & Heisterkamp N (2012) TGFbeta signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol* **13**: 31.

Gotlieb AI and Langille BL. (1996) The role of rheology in atherosclerotic coronary artery disease. Fuster V, Ross R, Topol EJ, eds. Atherosclerosis and coronary artery disease. Vol. 1. Philadelphia, Lippincott-Raven, 595-606.

Goto D, Nakajima H, Mori Y, Kurasawa K, Kitamura N & Iwamoto I. (2001). Interaction between Smad anchor for receptor activation and Smad3 is not essential for TGF-beta/Smad3-mediated signaling. *Biochemical and biophysical research communications* **281,** 1100-1105.

Goumans MJ, Liu Z & ten Dijke P. (2009). TGF-beta signaling in vascular biology and dysfunction. *Cell research* **19,** 116-127.

Grainger DJ (2007) TGF-β and atherosclerosis in man. *Cardiovasc Res* **74,** 213-222.

Grainger DJ, Kemp PR, Witchell CM, Weissberg PL & Metcalfe JC. (1994). Transforming growth factor beta decreases the rate of proliferation of rat vascular smooth muscle cells by extending the G2 phase of the cell cycle and delays the rise in cyclic AMP before entry into M phase. *The Biochemical journal* **299 (Pt**

1), 227-235.

Grainger, DJ, Kemp PR, Metcalf JC, Liu AC, Lawn RM, Williams NR, Grace AA, Schofield PM and Chauhan A (1995) The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis. *Nat Med* **1**: 74-79.

Grainger DJ, Metcalfe JC, Grace AA, Mosedale DE (1998) Transforming growth factor-beta dynamically regulates vascular smooth muscle differentiation in vivo. *J Cell ScI* **111,** 2977-88.

Grainger DJ, Mosedale DE, Metcalfe JC and Böttinger EP (2000) Dietary fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. *J Cell Sci* **113,** 2355-61.

Grattagliano I, Lauterburg BH, Portincasa P, Caruso ML, Vendemiale G, Valentini AM, Palmieri VO & Palasciano G. (2003). Mitochondrial glutathione content determines the rate of liver regeneration after partial hepatectomy in eu- and hypothyroid rats. *Journal of hepatology* **39,** 571-579.

Greco T, Shafer J & Fiskum G. (2011). Sulforaphane inhibits mitochondrial permeability transition and oxidative stress. *Free radical biology & medicine* **51,** 2164-2171.

Griendling KK & FitzGerald GA. (2003). Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* **108,** 1912-1916.

Grochot-Przeczek A, Dulak J & Jozkowicz A. (2012). Haem oxygenase-1: non-canonical roles in physiology and pathology. *Clin Sci (Lond)* **122,** 93-103.

Gruden G, Araf S, Zonca S, Burt D, Thomas S, Gnudi L and Viberti GC (2003) IGF-I induces vascular endothelial growth factor in human mesangial cells via a Src-dependent mechanism. *Kidney International* **63,** 1249–1255.

Gu L, Zhu YJ, Yang X, Guo ZJ, Xu WB & Tian XL. (2007). Effect of TGF-beta/Smad signaling pathway on lung myofibroblast differentiation. *Acta pharmacologica Sinica* **28,** 382-391.

Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C & Gutierrez L. (2009). Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of experimental botany* **60,** 487-493.

Guerrero-Beltran CE, Calderon-Oliver M, Pedraza-Chaverri J & Chirino YI. (2012). Protective effect of sulforaphane against oxidative stress: recent advances. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie* **64,** 503-508.

Guglielmo GM, Le Roy C, Goodfellow AF and Wrana JL (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* **5,** 410-21.

Gunaratne A, Benchabane H & Di Guglielmo GM. (2012). Regulation of TGFbeta receptor trafficking and signaling by atypical protein kinase C. *Cellular signalling* **24,** 119-130.

Guo X & Wang XF. (2009). Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research* **19,** 71-88.

Guyton JR & Klemp KF. (1996). Development of the lipid-rich core in human atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **16,** 4-11.

Guzik TJ & Harrison DG. (2006). Vascular NADPH oxidases as drug targets for novel antioxidant strategies. *Drug discovery today* **11,** 524-533.

Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, Korbut R & Channon KM. (2004). Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. *Arteriosclerosis, thrombosis, and vascular biology* **24,** 1614-1620.

Haines DD, Lekli I, Teissier P, Bak I & Tosaki A. (2012). Role of haeme oxygenase-1 in resolution of oxidative stress-related pathologies: focus on cardiovascular, lung, neurological and kidney disorders. *Acta Physiol (Oxf)* **204,** 487-501.

Halliwell B (1995) Antioxidant characterization. *Methodol Mech Biochem Pharmacol* **49,** 1341-1348.

Halloran BG, Prorok GD, So BJ & Baxter BT. (1995). Transforming growth factor-beta 1 inhibits human arterial smooth-muscle cell proliferation in a growth-rate-dependent manner. *American journal of surgery* **170,** 193-197.

Hamidi A, von Bulow V, Hamidi R, Winssinger N, Barluenga S, Heldin CH & Landstrom M. (2012). Polyubiquitination of transforming growth factor beta (TGFbeta)-associated kinase 1 mediates nuclear factor-kappaB activation in response to different inflammatory stimuli. *The Journal of biological chemistry* **287,** 123-133.

Han L & Gotlieb AI. (2011). Fibroblast growth factor-2 promotes in vitro mitral valve interstitial cell repair through transforming growth factor-beta/Smad signaling. *The American journal of pathology* **178,** 119-127.

Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K & Nishida E. (1999). Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *The Journal of biological chemistry* **274,** 27161-27167.

Hancock WW, Buelow R, Sayegh MH & Turka LA. (1998). Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nature medicine* **4,** 1392-1396.

Hanlon N, Coldham N, Gielbert A, Sauer MJ & Ioannides C. (2009). Repeated intake of broccoli does not lead to higher plasma levels of sulforaphane in human volunteers. *Cancer letters* **284,** 15-20.

Hannon GJ & Beach D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371,** 257-261.

Hannon, GJ & Rossi JJ (2004). Unlocking the potential of the human genome with RNA interference. *Nature* **431**(7006): 371-378

Hansson GK. (2005). Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine* **352,** 1685-1695.

Harfouche R, Malak NA, Brandes RP, Karsan A, Irani K & Hussain SN. (2005). Roles of reactive oxygen species in angiopoietin-1/tie-2 receptor signaling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19,** 1728-1730.

Hargrove JL and Schmidt FH. (1989). The role of mRNA and protein stability in gene expression. *The FASEB Journal* **12,** 2360-2370.

Harland B, Walcott S & Sun SX. (2011). Adhesion dynamics and durotaxis in migrating cells. *Physical biology* **8,** 015011.

Harman D (1956) Aging: A theory based on free radical and radiation chemistry *J Gerontol* 11,298-300.

Hartung R, Parapuram SK, Ganti R, Hunt DM, Chalam KV & Hunt RC. (2007). Vitreous induces heme oxygenase-1 expression mediated by transforming growth factor-beta and reactive oxygen species generation in human retinal pigment epithelial cells. *Molecular vision* **13,** 66-78.

Hashimoto Y, Parsons M & Adams JC. (2007). Dual actin-bundling and protein kinase C-binding activities of fascin regulate carcinoma cell migration downstream of Rac and contribute to metastasis. *Molecular biology of the cell* **18,** 4591-4602.

Hata A, Lo RS, Wotton D, Lagna G & Massague J. (1997). Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* **388,** 82-87.

Hata A and Davis BN (2009) Control of microRNA biogenesis by TGFbeta signaling pathway-A novel role of Smads in the nucleus. *Cytokine Growth Factor Rev* **5-6,** 517-21.

Hatic H, Kane MJ, Saykally JN & Citron BA. (2012). Modulation of transcription factor Nrf2 in an in vitro model of traumatic brain injury. *Journal of neurotrauma* **29,** 1188-1196.

Haurani MJ, Cifuentes ME, Shepard AD & Pagano PJ. (2008). Nox4 oxidase overexpression specifically decreases endogenous Nox4 mRNA and inhibits angiotensin II-induced adventitial myofibroblast migration. *Hypertension* **52,** 143-149.

Haurani MJ & Pagano PJ. (2007). Adventitial fibroblast reactive oxygen species as autacrine and paracrine mediators of remodeling: bellwether for vascular disease? *Cardiovascular research* **75,** 679-689.

Havelka GE & Kibbe MR. (2011). The vascular adventitia: its role in the arterial injury response. *Vascular and endovascular surgery* **45,** 381-390.

Hayashida T, Jones JC, Lee CK & Schnaper HW. (2010). Loss of beta1-integrin enhances TGF-beta1-induced collagen expression in epithelial cells via increased alphavbeta3-integrin and Rac1 activity. *The Journal of biological chemistry* **285,** 30741-30751.

Hayes JD & Ashford ML. (2012). Nrf2 orchestrates fuel partitioning for cell proliferation. *Cell metabolism* **16,** 139-141.

Hayes JD, Kelleher MO & Eggleston IM. (2008). The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *European journal of nutrition* **47 Suppl 2,** 73-88.

Hayes JD & McMahon M. (2006). The double-edged sword of Nrf2: subversion of redox homeostasis during the evolution of cancer. *Molecular cell* **21,** 732-734.

Hayes JD & McMahon M. (2009). NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends in biochemical sciences* **34,** 176-188.

Hayes JD, McMahon M, Chowdhry S & Dinkova-Kostova AT. (2010). Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. *Antioxidants & redox signaling* **13,** 1713-1748.

He M, Siow RC, Sugden D, Gao L, Cheng X & Mann GE. (2011). Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **21,** 277-285.

He X & Ma Q. (2009). NRF2 cysteine residues are critical for oxidant/electrophile-sensing, Kelch-like ECH-associated protein-1-dependent ubiquitination-proteasomal degradation, and transcription activation. *Molecular pharmacology* **76,** 1265-1278.

Hegazy SK, El-Bedewy M & Yagi A. (2012). Antifibrotic effect of aloe vera in viral infection-induced hepatic periportal fibrosis. *World journal of gastroenterology : WJG* **18,** 2026-2034.

Heldin CH, Miyazono K & ten Dijke P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390,** 465-471.

Higashi Y, Sasaki S, Nakagawa K, Fukuda Y, Matsuura H, Oshima T & Chayama K. (2002). Tetrahydrobiopterin enhances forearm vascular response to acetylcholine in both normotensive and hypertensive individuals. *American journal of hypertension* **15,** 326-332.

Higashi Y, Sasaki S, Nakagawa K, Kimura M, Noma K, Hara K, Jitsuiki D, Goto C, Oshima T, Chayama K & Yoshizumi M. (2006). Tetrahydrobiopterin improves aging-related impairment of endothelium-dependent vasodilation through increase in nitric oxide production. *Atherosclerosis* **186,** 390-395.

Higgins LG & Hayes JD. (2011). The cap'n'collar transcription factor Nrf2 mediates both intrinsic resistance to environmental stressors and an adaptive response elicited by chemopreventive agents that determines susceptibility to electrophilic xenobiotics. *Chemico-biological interactions* **192,** 37-45.

Higgins LG & Hayes JD. (2011). Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. *Drug metabolism reviews* **43,** 92-137.

Higgins LG, Kelleher MO, Eggleston IM, Itoh K, Yamamoto M & Hayes JD. (2009). Transcription factor Nrf2 mediates an adaptive response to sulforaphane that protects fibroblasts in vitro against the cytotoxic effects of electrophiles, peroxides and redox-cycling agents. *Toxicology and applied pharmacology* **237,** 267-280.

Hilenski LL, Clempus RE, Quinn MT, Lambeth JD & Griendling KK. (2004). Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* **24,** 677-683.

Hill CS. (2009). Nucleocytoplasmic shuttling of Smad proteins. *Cell research* **19,** 36-46.

Hill-Kapturczak N, Truong L, Thamilselvan V, Visner GA, Nick HS & Agarwal A. (2000). Smad7-dependent regulation of heme oxygenase-1 by transforming growth factor-beta in human renal epithelial cells. *The Journal of biological chemistry* **275,** 40904-40909.

Hinoi E, Fujimori S, Wang L, Hojo H, Uno K & Yoneda Y. (2006). Nrf2 negatively regulates osteoblast differentiation via interfering with Runx2-dependent transcriptional activation. *The Journal of biological chemistry* **281,** 18015-18024.

Hinz B. (2006). Masters and servants of the force: the role of matrix adhesions in myofibroblast force perception and transmission. *European journal of cell biology* **85,** 175-181.

Hinz B. (2007). Formation and function of the myofibroblast during tissue repair. *The Journal of investigative dermatology* **127,** 526-537.

Hinz B. (2009). Tissue stiffness, latent TGF-beta1 activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. *Current rheumatology reports* **11,** 120-126.

Hinz B. (2010). The myofibroblast: paradigm for a mechanically active cell. *Journal of biomechanics* **43,** 146-155.

Hinz B. (2012). Mechanical aspects of lung fibrosis: a spotlight on the myofibroblast. *Proceedings of the American Thoracic Society* **9,** 137-147.

Hinz B, Celetta G, Tomasek JJ, Gabbiani G & Chaponnier C. (2001). Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Molecular biology of the cell* **12,** 2730-2741.

Hinz B, Dugina V, Ballestrem C, Wehrle-Haller B & Chaponnier C. (2003). Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. *Molecular biology of the cell* **14,** 2508-2519.

Hinz B & Gabbiani G. (2003). Mechanisms of force generation and transmission by myofibroblasts. *Current opinion in biotechnology* **14,** 538-546.

Hinz B & Gabbiani G. (2010). Fibrosis: recent advances in myofibroblast biology and new therapeutic

perspectives. *F1000 biology reports* **2,** 78.

Hinz B, Gabbiani G & Chaponnier C. (2002). The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. *The Journal of cell biology* **157,** 657-663.

Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML & Gabbiani G. (2007). The myofibroblast: one function, multiple origins. *The American journal of pathology* **170,** 1807-1816.

Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmouliere A, Varga J, De Wever O, Mareel M & Gabbiani G. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *The American journal of pathology* **180,** 1340-1355.

Hinz B, Pittet P, Smith-Clerc J, Chaponnier C & Meister JJ. (2004). Myofibroblast development is characterized by specific cell-cell adherens junctions. *Molecular biology of the cell* **15,** 4310-4320.

Hissin PJ & Hilf R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical biochemistry* **74,** 214-226.

Ho E, Clarke JD & Dashwood RH. (2009). Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. *The Journal of nutrition* **139,** 2393-2396.

Hocevar BA, Brown TL & Howe PH. (1999). TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *The EMBO journal* **18,** 1345-1356.

Hoekstra KA, Godin DV and Cheng KM (2004) Protective role of heme oxygenase in the blood vessel wall during atherogenesis. *Biochem Cell Biol* **82,** 351-9.

Holmgren A. (1981). Regulation of ribonucleotide reductase. *Current topics in cellular regulation* **19,** 47-76.

Hong F, Freeman ML & Liebler DC. (2005). Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chemical research in toxicology* **18,** 1917-1926.

Hong M, Wilkes MC, Penheiter SG, Gupta SK, Edens M & Leof EB. (2011). Non-Smad transforming growth factor-beta signaling regulated by focal adhesion kinase binding the p85 subunit of phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **286,** 17841-17850.

Hopkins PN, Wu LL, Hunt SC, James BC, Vincent GM and Williams RR (1996) Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arterioscler Thromb Vasc Biol* **16,** 250–5.

Horbelt D, Denkis A & Knaus P. (2012). A portrait of Transforming Growth Factor beta superfamily signalling: Background matters. *The international journal of biochemistry & cell biology* **44,** 469-474.

Hori YS, Kuno A, Hosoda R, Tanno M, Miura T, Shimamoto K & Horio Y. (2011). Resveratrol ameliorates muscular pathology in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy. *The Journal of pharmacology and experimental therapeutics* **338,** 784-794.

Horwitz AR & Parsons JT. (1999). Cell migration--movin' on. *Science* **286,** 1102-1103.

Hoshino A, Chiba H, Nagai K, Ishii G & Ochiai A. (2008). Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. *Biochemical and biophysical research communications* **368,** 305-310.

Hosomi N, Noma T, Ohyama H, Takahashi T & Kohno M. (2002). Vascular proliferation and transforming growth factor-beta expression in pre- and early stage of diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats. *Atherosclerosis* **162,** 69-76.

Hosoya T, Maruyama A, Kang MI, Kawatani Y, Shibata T, Uchida K, Warabi E, Noguchi N, Itoh K & Yamamoto M. (2005). Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells. *The Journal of biological chemistry* **280,** 27244-27250.

Hou Y, Xue P, Bai Y, Liu D, Woods CG, Yarborough K, Fu J, Zhang Q, Sun G, Collins S, Chan JY, Yamamoto M, Andersen ME & Pi J. (2012). Nuclear factor erythroid-derived factor 2-related factor 2 regulates transcription of CCAAT/enhancer-binding protein beta during adipogenesis. *Free radical biology & medicine* **52,** 462-472.

Hough C, Radu M & Dore JJ. (2012). Tgf-beta induced Erk phosphorylation of smad linker region regulates smad signaling. *PloS one* **7,** e42513.

Hu B, Wu Z & Phan SH. (2003). Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *American journal of respiratory cell and molecular biology* **29,** 397-404.

Hu C, Eggler AL, Mesecar AD & van Breemen RB. (2011). Modification of keap1 cysteine residues by sulforaphane. *Chemical research in toxicology* **24,** 515-521.

Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, Lin W, Reddy B, Chan JY & Kong AN. (2006). Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice. *Cancer letters* **243,** 170-192.

Hu T, Ramachandrarao SP, Siva S, Valancius C, Zhu Y, Mahadev K, Toh I, Goldstein BJ, Woolkalis M & Sharma K. (2005). Reactive oxygen species production via NADPH oxidase mediates TGF-beta-induced cytoskeletal alterations in endothelial cells. *American journal of physiology Renal physiology* **289,** F816-825.

Hu Y & Xu Q. (2011). Adventitial biology: differentiation and function. *Arteriosclerosis, thrombosis, and vascular biology* **31,** 1523-1529.

Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B & Xu Q. (2004). Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *The Journal of clinical investigation* **113,** 1258-1265.

Huang HC, Nguyen T & Pickett CB. (2000). Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 12475-12480.

Huang HC, Nguyen T & Pickett CB. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *The Journal of biological chemistry* **277,** 42769-42774.

Huang TY, Chang WC, Wang MY, Yang YR & Hsu YC. (2012). Effect of sulforaphane on growth inhibition in human brain malignant glioma GBM 8401 cells by means of mitochondrial- and MEK/ERK-mediated apoptosis pathway. *Cell biochemistry and biophysics* **63,** 247-259.

Huang Y, Fang W, Wang Y, Yang W & Xiong B. (2012). Transforming growth factor-beta1 induces glutathione peroxidase-1 and protects from H2O2-induced cell death in colon cancer cells via the Smad2/ERK1/2/HIF-1alpha pathway. *International journal of molecular medicine* **29,** 906-912.

Huang ZZ, Chen C, Zeng Z, Yang H, Oh J, Chen L & Lu SC. (2001). Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **15,** 19-21.

Huang ZZ, Li H, Cai J, Kuhlenkamp J, Kaplowitz N & Lu SC. (1998). Changes in glutathione homeostasis during liver regeneration in the rat. *Hepatology* **27,** 147-153.

Hubmacher D and Apte SS (2013). The biology of the extracellular matrix: novel insights. *Curr Opin*

Rheumatol **25**(1): 65-70.

Hung HC, Joshipura KJ, Jiang R, Hu FB, Hunter D, Smith-Warner SA, Colditz GA, Rosner B, Spiegelman D & Willett WC. (2004). Fruit and vegetable intake and risk of major chronic disease. *Journal of the National Cancer Institute* **96,** 1577-1584.

Hurd TR, DeGennaro M & Lehmann R. (2012). Redox regulation of cell migration and adhesion. *Trends in cell biology* **22,** 107-115.

Huse, M., Chen, Y. G., Massagué, J. and Kuriyan, J (1999) Crystal structure of the cytoplasmic domain of the type I TGF-β receptor in complex with FKBP12. *Cell* **96**, 425–436.

Huttenlocher A, Palecek SP, Lu Q, Zhang W, Mellgren RL, Lauffenburger DA, Ginsberg MH & Horwitz AF. (1997). Regulation of cell migration by the calcium-dependent protease calpain. *The Journal of biological chemistry* **272,** 32719-32722.

Hwang C, Sinskey AJ & Lodish HF. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257,** 1496-1502.

Iglesias-De La Cruz MC, Ruiz-Torres P, Alcamí J, Díez-Marqués L, Ortega-Velázquez R, Chen S, Rodríguez-Puyol M, Ziyadeh FNand, Rodríguez-Puyol D (2001) Hydrogen peroxide increases extracellular matrix mRNA through TGF-beta in human mesangial cells. *Kidney Int* **1**, 87-95.

Ignotz RA, Endo T & Massague J. (1987). Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. *The Journal of biological chemistry* **262,** 6443-6446.

Iida S, Chu Y, Weiss RM, Kang YM, Faraci FM and Heistad DD (2006) Vascular effects of a common gene variant of extracellular superoxide dismutase in heart failure. *Am J Physiol Heart Circ Physiol* **291,** H914-20.

Ikeda H, Nishi S & Sakai M. (2004). Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis. *The Biochemical journal* **380,** 515-521.

Ikeda S, Yamaoka-Tojo M, Hilenski L, Patrushev NA, Anwar GM, Quinn MT & Ushio-Fukai M. (2005). IQGAP1 regulates reactive oxygen species-dependent endothelial cell migration through interacting with Nox2. *Arteriosclerosis, thrombosis, and vascular biology* **25,** 2295-2300.

Im JY, Lee KW, Woo JM, Junn E & Mouradian MM. (2012). DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway. *Human molecular genetics* **21,** 3013-3024.

Inman GJ, Nicolas FJ & Hill CS. (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Molecular cell* **10,** 283-294.

Ishikawa A, Jinno S, Suzuki T, Hayashi T, Kawai T, Mizuno T, Mori T & Hattori M. (2006). Global gene expression analyses of mouse fibroblast L929 cells exposed to IC50 MMA by DNA microarray and confirmation of four detoxification genes' expression by real-time PCR. *Dental materials journal* **25,** 205-213.

Ishizaka N, Saito K, Noiri E, Sata M, Ikeda H, Ohno A, Ando J, Mori I, Ohno M & Nagai R. (2005). Administration of ANG II induces iron deposition and upregulation of TGF-beta1 mRNA in the rat liver. *American journal of physiology Regulatory, integrative and comparative physiology* **288,** R1063-1070.

Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M and Mann GE (2004) Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages, activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ Res* **94,** 609-16.

Itoh F, Divecha N, Brocks L, Oomen L, Janssen H, Calafat J, Itoh S and Dijke (2002) The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF- -Smad signalling. *Genes Cells* **7**, 321–331.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M and Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **2,** 313-22.

Itoh K, Mimura J & Yamamoto M. (2010). Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxidants & redox signaling* **13,** 1665-1678.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD & Yamamoto M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & development* **13,** 76-86.

Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin CH & ten Dijke P. (2003). Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. *The Journal of biological chemistry* **278,** 3751-3761.

Iwata S, Hori T, Sato N, Ueda-Taniguchi Y, Yamabe T, Nakamura H, Masutani H & Yodoi J. (1994). Thiol-mediated redox regulation of lymphocyte proliferation. Possible involvement of adult T cell leukemia-derived factor and glutathione in transferrin receptor expression. *J Immunol* **152,** 5633-5642.

Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T & Pagano PJ. (2003). Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery. *Circulation research* **92,** 637-643.

Jadhav A, Torlakovic E & Ndisang JF. (2008). Interaction among heme oxygenase, nuclear factor-kappaB, and transcription activating factors in cardiac hypertrophy in hypertension. *Hypertension* **52,** 910-917.

Jain AK, Bloom DA & Jaiswal AK. (2005). Nuclear import and export signals in control of Nrf2. *The Journal of biological chemistry* **280,** 29158-29168.

Jain AK & Jaiswal AK. (2006). Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *The Journal of biological chemistry* **281,** 12132-12142.

Jain AK & Jaiswal AK. (2007). GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *The Journal of biological chemistry* **282,** 16502-16510.

Jain AK, Mahajan S & Jaiswal AK. (2008). Phosphorylation and dephosphorylation of tyrosine 141 regulate stability and degradation of INrf2: a novel mechanism in Nrf2 activation. *The Journal of biological chemistry* **283,** 17712-17720.

Jain M, Rivera S, Monclus EA, Synenki L, Zirk A, Eisenbart J, Feghali-Bostwick C, Mutlu GM, Budinger GR & Chandel NS. (2013). Mitochondrial Reactive Oxygen Species Regulate Transforming Growth Factor-beta Signaling. *The Journal of biological chemistry* **288,** 770-777.

Jaiswal AK (2004) Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biology and Medicine* **10,** 1199-1207.

Jaiswal AK. (2000). Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free radical biology & medicine* **29,** 254-262.
Jardine H, MacNee W, Donaldson K & Rahman I. (2002). Molecular mechanism of transforming growth factor (TGF)-beta1-induced glutathione depletion in alveolar epithelial cells. Involvement of AP-1/ARE and Fra-1. *The Journal of biological chemistry* **277,** 21158-21166.

Jardine H, MacNee W, Donaldson K & Rahman I. (2002). Molecular mechanism of transforming growth

factor (TGF)-beta1-induced glutathione depletion in alveolar epithelial cells. Involvement of AP-1/ARE and Fra-1. *The Journal of biological chemistry* **277,** 21158-21166.

Jeffery TK & Morrell NW. (2002). Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. *Progress in cardiovascular diseases* **45,** 173-202.

Jenkins JK, Huang H, Ndebele K & Salahudeen AK. (2001). Vitamin E inhibits renal mRNA expression of COX II, HO I, TGFbeta, and osteopontin in the rat model of cyclosporine nephrotoxicity. *Transplantation* **71,** 331-334.

Jeong WS, Jun M & Kong AN. (2006). Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. *Antioxidants & redox signaling* **8,** 99-106.

Ji J, Xu F, Li L, Chen R, Wang J & Hu WC. (2010). Activation of adventitial fibroblasts in the early stage of the aortic transplant vasculopathy in rat. *Transplantation* **89,** 945-953.

Jiang YB, Zhang XL, Tang YL, Ma GS, Shen CX, Wei Q, Zhu Q, Yao YY & Liu NF. (2011). Effects of heme oxygenase-1 gene modulated mesenchymal stem cells on vasculogenesis in ischemic swine hearts. *Chinese medical journal* **124,** 401-407.

Jiang F, Roberts SJ, Datla SR and Dusting GJ *(*2006) NO modulates NADPH oxidase function via Heme Oxygenase-1 in human endothelial cells. *Hypertension* **48,** 950-7.

Jiang X, Zeng H, Guo Y, Zhou ZB, Tang B and LI F (2004) The expression of matrix metalloproteinases-9, transforming growth factor-β1 and transforming growth factor-β receptor in human atherosclerotic plaque and their relationship with plaque stability. *Chinese Medical Journal* **117,** 1825-18.

Jiang Z, Yu P, Tao M, Fernandez C, Ifantides C, Moloye O, Schultz GS, Ozaki CK and Berceli SA (2007) TGF-B and CTGF mediated fibroblast recruitment influences early outward vein graft remodelling *Am J Physiol Heart Circ* **293,** H482-H488.

Jones CB, Sane DC & Herrington DM. (2003). Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. *Cardiovascular research* **59,** 812-823.
Joshi G & Johnson JA. (2012). The Nrf2-ARE Pathway: A Valuable Therapeutic Target for the Treatment of Neurodegenerative Diseases. *Recent patents on CNS drug discovery*.

Juan SH, Lee TS, Tseng KW, Liou JY, Shyue SK, Wu KK & Chau LY. (2001). Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **104,** 1519-1525.

Juge N, Mithen RF & Traka M. (2007). Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cellular and molecular life sciences : CMLS* **64,** 1105-1127.

Jung KA & Kwak MK. (2010). The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules* **15,** 7266-7291.

Junn E, Lee KN, Ju HR, Han SH, Im JY, Kang HS, Lee TH, Bae YS, Ha KS, Lee ZW, Rhee SG & Choi I. (2000). Requirement of hydrogen peroxide generation in TGF-beta 1 signal transduction in human lung fibroblast cells: involvement of hydrogen peroxide and Ca2+ in TGF-beta 1-induced IL-6 expression. *J Immunol* **165,** 2190-2197.

Juurlink BH. (2012). Dietary Nrf2 activators inhibit atherogenic processes. *Atherosclerosis* **225,** 29-33.

Kacem K & Sercombe R. (2008). Similar pathological effects of sympathectomy and hypercholesterolemia on arterial smooth muscle cells and fibroblasts. *Acta histochemica* **110,** 302-313.

Kadam SD, Gucek M, Cole RN, Watkins PA & Comi AM. (2012). Cell proliferation and oxidative stress pathways are modified in fibroblasts from Sturge-Weber syndrome patients. *Archives of dermatological*

research **304,** 229-235.

Kallifatidis G, Labsch S, Rausch V, Mattern J, Gladkich J, Moldenhauer G, Buchler MW, Salnikov AV & Herr I. (2011). Sulforaphane increases drug-mediated cytotoxicity toward cancer stem-like cells of pancreas and prostate. *Molecular therapy : the journal of the American Society of Gene Therapy* **19,** 188-195.

Kallifatidis G, Rausch V, Baumann B, Apel A, Beckermann BM, Groth A, Mattern J, Li Z, Kolb A, Moldenhauer G, Altevogt P, Wirth T, Werner J, Schemmer P, Buchler MW, Salnikov AV & Herr I. (2009). Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. *Gut* **58,** 949-963.

Kalra M & Miller VM. (2000). Early remodeling of saphenous vein grafts: proliferation, migration and apoptosis of adventitial and medial cells occur simultaneously with changes in graft diameter and blood flow. *Journal of vascular research* **37,** 576-584.

Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ, 2nd & Ischiropoulos H. (2012). Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free radical biology & medicine* **52,** 1-6.

Kamii H, Kato I, Kinouchi H, Chan PH, Epstein CJ, Akabane A, Okamoto H & Yoshimoto T. (1999). Amelioration of vasospasm after subarachnoid hemorrhage in transgenic mice overexpressing CuZn-superoxide dismutase. *Stroke; a journal of cerebral circulation* **30,** 867-871; discussion 872.

Kaminski BM, Loitsch SM, Ochs MJ, Reuter KC, Steinhilber D, Stein J & Ulrich S. (2010). Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-beta/Smad signaling pathway. *Molecular nutrition & food research* **54,** 1486-1496.

Kanellakis P, Nestel P & Bobik A. (2004). Angioplasty-induced superoxide anions and neointimal hyperplasia in the rabbit carotid artery: suppression by the isoflavone trans-tetrahydrodaidzein. *Atherosclerosis* **176,** 63-72.

Kang JS, Liu C & Derynck R. (2009). New regulatory mechanisms of TGF-beta receptor function. *Trends in cell biology* **19,** 385-394.

Kang KW, Lee SJ, Park JW & Kim SG. (2002). Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. *Molecular pharmacology* **62,** 1001-1010.

Kang MI, Kobayashi A, Wakabayashi N, Kim SG & Yamamoto M. (2004). Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 2046-2051.

Kang P & Svoboda KK. (2002). PI-3 kinase activity is required for epithelial-mesenchymal transformation during palate fusion. *Developmental dynamics : an official publication of the American Association of Anatomists* **225,** 316-321.

Kang SJ, You A & Kwak MK. (2011). Suppression of Nrf2 signaling by angiotensin II in murine renal epithelial cells. *Archives of pharmacal research* **34,** 829-836.

Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. (2013) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biology* **1,** 45-49.

Kaplowitz N, Aw TY & Ookhtens M. (1985). The regulation of hepatic glutathione. *Annual review of pharmacology and toxicology* **25,** 715-744.

Kaspar JW, Niture SK & Jaiswal AK. (2009). Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free radical biology & medicine* **47,** 1304-1309.

Kaspar JW and Jaiswal AK (2010) Antioxidant-induced phosphorylation of tyrosine 486 leads to rapid nuclear export of Bach1 that allows Nrf2 to bind to the antioxidant response element and activate defensive gene expression. *J Biol Chem* **1,** 153-62.

Kato S, Ueda S, Tamaki K, Fujii M, Miyazono K, ten Dijke P, Morimatsu M & Okuda S. (2001). Ectopic expression of Smad7 inhibits transforming growth factor-beta responses in vascular smooth muscle cells. *Life sciences* **69,** 2641-2652.

Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A & Yamamoto M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes to cells : devoted to molecular & cellular mechanisms* **6,** 857-868.

Kaur P, Kali S and Bansal MP (2006) Effects of diethyl maleate induced oxidative stress on male reproductive activity I nmice: Redox active enzymes and transcription factors expression. *Mol and Cell Biochem* **291,** 55-61.

Kavsak P, Rasmussen RK and Causing CG (2000) et al Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. *Mol Cell* **6,** 1365-1375

Kawai Y, Garduno L, Theodore M, Yang J & Arinze IJ. (2011). Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *The Journal of biological chemistry* **286,** 7629-7640.

Kawashima A, Oda Y, Yachie A, Koizumi S & Nakanishi I. (2002). Heme oxygenase-1 deficiency: the first autopsy case. *Human pathology* **33,** 125-130.

Kensler TW, Egner PA, Agyeman AS, Visvanathan K, Groopman JD, Chen JG, Chen TY, Fahey JW & Talalay P. (2012). Keap1-Nrf2 Signaling: A Target for Cancer Prevention by Sulforaphane. *Topics in current chemistry*.

Kensler TW & Wakabayashi N. (2010). Nrf2: friend or foe for chemoprevention? *Carcinogenesis* **31,** 90-99.

Keum YS. (2011). Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications. *Annals of the New York Academy of Sciences* **1229,** 184-189.

Keum YS, Han YH, Liew C, Kim JH, Xu C, Yuan X, Shakarjian MP, Chong S & Kong AN. (2006). Induction of heme oxygenase-1 (HO-1) and NAD[P]H: quinone oxidoreductase 1 (NQO1) by a phenolic antioxidant, butylated hydroxyanisole (BHA) and its metabolite, tert-butylhydroquinone (tBHQ) in primary-cultured human and rat hepatocytes. *Pharmaceutical research* **23,** 2586-2594.

Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A & Kong AN. (2006). Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. *Cancer research* **66,** 8804-8813.

Khan R, Agrotis A & Bobik A. (2007). Understanding the role of transforming growth factor-beta1 in intimal thickening after vascular injury. *Cardiovascular research* **74,** 223-234.

Khurana VG and Meyer FB. (2003) Translational paradigms in cerebrovascular gene transfer *J Cereb Blood Flow Metab.* **11,** 1251-62.

Ki SH, Cho IJ, Choi DW & Kim SG. (2005). Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. *Molecular and cellular biology* **25,** 4150-4165.

Kie JH, Kapturczak MH, Traylor A, Agarwal A & Hill-Kapturczak N. (2008). Heme oxygenase-1 deficiency promotes epithelial-mesenchymal transition and renal fibrosis. *Journal of the American Society of Nephrology : JASN* **19,** 1681-1691.

Kim JY, Park HJ, Um SH, Sohn EH, Kim BO, Moon EY, Rhee DK & Pyo S. (2012). Sulforaphane suppresses vascular adhesion molecule-1 expression in TNF-alpha-stimulated mouse vascular smooth muscle cells: involvement of the MAPK, NF-kappaB and AP-1 signaling pathways. *Vascular pharmacology* **56,** 131-141.

Kim MS, Ahn SM & Moon A. (2002). In vitro bioassay for transforming growth factor-beta using XTT method. *Archives of pharmacal research* **25,** 903-909.

Kim S, Lee Y, Seo JE, Cho KH & Chung JH. (2008). Caveolin-1 increases basal and TGF-beta1-induced expression of type I procollagen through PI-3 kinase/Akt/mTOR pathway in human dermal fibroblasts. *Cellular signalling* **20,** 1313-1319.

Kim SG & Kim SO. (2004). PKC downstream of Pl3-kinase regulates peroxynitrite formation for Nrf2-mediated GSTA2 induction. *Archives of pharmacal research* **27,** 757-762.

Kim YS, Lee HL, Lee KB, Park JH, Chung WY, Lee KS, Sheen SS, Park KJ & Hwang SC. (2011). Nuclear factor E2-related factor 2 dependent overexpression of sulfiredoxin and peroxiredoxin III in human lung cancer. *The Korean journal of internal medicine* **26,** 304-313.

Kingston PA, Sinha S, David A, Castro MG, Lowenstein PR & Heagerty AM. (2001). Adenovirus-mediated gene transfer of a secreted transforming growth factor-beta type II receptor inhibits luminal loss and constrictive remodeling after coronary angioplasty and enhances adventitial collagen deposition. *Circulation* **104,** 2595-2601.

Kirschenlohr HL, Metcalfe JC, Weissberg PL & Grainger DJ. (1995). Proliferation of human aortic vascular smooth muscle cells in culture is modulated by active TGF beta. *Cardiovascular research* **29,** 848-855.

Kitisin K, Saha T, Blake T, Golestaneh N, Deng M, Kim C, Tang Y, Shetty K, Mishra B & Mishra L. (2007). Tgf-Beta signaling in development. *Science's STKE : signal transduction knowledge environment* **2007,** cm1.

Klingberg F, Hinz B & White ES. (2012). The myofibroblast matrix:implications for tissue repair and fibrosis. *The Journal of pathology* **34**, 231-234.

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K & Yamamoto M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and cellular biology* **24,** 7130-7139.

Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K & Yamamoto M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Molecular and cellular biology* **26,** 221-229.

Kobayashi A, Kang M, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K and Masayuki Yamamoto (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-Based E3 ligase to regulate proteasomal degradation of Nrf2 *Mol Cell Biol.* **16,** 7130–7139.

Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y, Eguchi M, Wada Y, Kumagai Y & Yamamoto M. (2009). The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Molecular and cellular biology* **29,** 493-502.

Kobayashi M & Yamamoto M. (2005). Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & redox signaling* **7,** 385-394.

Kobayashi M & Yamamoto M. (2006). Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Advances in enzyme regulation* **46,** 113-140.

Kole TP, Tseng Y, Jiang I, Katz JL & Wirtz D. (2005). Intracellular mechanics of migrating fibroblasts. *Molecular biology of the cell* **16,** 328-338.

Koli K, Myllarniemi M, Keski-Oja J & Kinnula VL. (2008). Transforming growth factor-beta activation in the lung: focus on fibrosis and reactive oxygen species. *Antioxidants & redox signaling* **10,** 333-342.

Kominsky SL, Doucet M, Brady K & Weber KL. (2007). TGF-beta promotes the establishment of renal cell carcinoma bone metastasis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **22,** 37-44.

Kong AN, Yu R, Chen C, Mandlekar S & Primiano T. (2000). Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. *Archives of pharmacal research* **23,** 1-16.

Kong X, Thimmulappa R, Craciun F, Harvey C, Singh A, Kombairaju P, Reddy SP, Remick D & Biswal S. (2011). Enhancing Nrf2 pathway by disruption of Keap1 in myeloid leukocytes protects against sepsis. *American journal of respiratory and critical care medicine* **184,** 928-938.

Kortlever RM, Nijwening JH & Bernards R. (2008). Transforming growth factor-beta requires its target plasminogen activator inhibitor-1 for cytostatic activity. *The Journal of biological chemistry* **283,** 24308-24313.

Kotsar A, Niemenen R, Isotalo T, Mikkonen J Uurto I, Kellomaki M, Taija M, Moilanen E & Tammela TL (2010). Biocompatibility of new drug-elutinh biodegradable urethral stent materials. *Urology* **75:** 229-234.

Kratschmar DV, Calabrese D, Walsh J, Lister A, Birk J, Appenzeller-Herzog C, Moulin P, Goldring CE & Odermatt A. (2012). Suppression of the Nrf2-dependent antioxidant response by glucocorticoids and 11beta-HSD1-mediated glucocorticoid activation in hepatic cells. *PloS one* **7,** e36774.

Krendel M, Sgourdas G & Bonder EM. (1998). Disassembly of actin filaments leads to increased rate and frequency of mitochondrial movement along microtubules. *Cell motility and the cytoskeleton* **40,** 368-378.

Kris-Etherton PM, Lichtenstein AH, Howard BV, Steinberg D & Witztum JL. (2004). Antioxidant vitamin supplements and cardiovascular disease. *Circulation* **110,** 637-641.

Kundi R, Hollenbeck ST, Yamanouchi D, Herman BC, Edlin R, Ryer EJ, Wang C, Tsai S, Liu B & Kent KC. (2009). Arterial gene transfer of the TGF-beta signalling protein Smad3 induces adaptive remodelling following angioplasty: a role for CTGF. *Cardiovascular research* **84,** 326-335.

Kunsch C & Medford RM. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circulation research* **85,** 753-766.

Kusano Y, Horie S, Shibata T, Satsu H, Shimizu M, Hitomi E, Nishida M, Kurose H, Itoh K, Kobayashi A, Yamamoto M & Uchida K. (2008). Keap1 regulates the constitutive expression of GST A1 during differentiation of Caco-2 cells. *Biochemistry* **47,** 6169-6177.

Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ & Wiggert B. (1994). Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-beta. *Journal of cellular physiology* **159,** 371-378.

Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K & Imaizumi T. (2002). Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. *Circulation* **106,** 130-135.

Kuwahara F, Kai H, Tokuda K, Shibata R, Kusaba K, Tahara N, Niiyama H, Nagata T & Imaizumi T. (2002). Hypoxia-inducible factor-1alpha/vascular endothelial growth factor pathway for adventitial vasa vasorum formation in hypertensive rat aorta. *Hypertension* **39,** 46-50.

Kwak MK, Itoh K, Yamamoto M & Kensler TW. (2002). Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Molecular and cellular biology* **22,** 2883-2892.

Kwon JS, Joung H, Kim YS, Shim YS, Ahn Y, Jeong MH & Kee HJ. (2012). Sulforaphane inhibits restenosis by suppressing inflammation and the proliferation of vascular smooth muscle cells. *Atherosclerosis* **225,** 41-49.

Laakso J, Mervaala E, Himberg JJ, Teräväinen TL, Karppanen H, Vapaatalo H and Lapatto R (1998) Increased kidney xanthine oxidoreductase activity in salt-induced experimental hypertension. *Hypertension.* **5,** 902-6.

Labat ML, Bringuier AF, Seebold C, Moricard Y, Meyer-Mula C, Laporte P, Talmage RV, Grubb SA, Simmons DJ & Milhaud G. (1991). Monocytic origin of fibroblasts: spontaneous transformation of blood monocytes into neo-fibroblastic structures in osteomyelosclerosis and Engelmann's disease. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **45,** 289-299.

Ladoux B & Nicolas A. (2012). Physically based principles of cell adhesion mechanosensitivity in tissues. *Rep Prog Phys* **75,** 116601.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.
LaFramboise WA, Scalise D, Stoodley P, Graner SR, Guthrie RD, Magovern JA & Becich MJ. (2007). Cardiac fibroblasts influence cardiomyocyte phenotype in vitro. *American journal of physiology Cell physiology* **292,** C1799-1808.

Lakkisto P, Palojoki E, Backlund T, Saraste A, Tikkanen I, Voipio-Pulkki LM & Pulkki K. (2002). Expression of heme oxygenase-1 in response to myocardial infarction in rats. *Journal of molecular and cellular cardiology* **34,** 1357-1365.

Lakkisto P, Siren JM, Kyto V, Forsten H, Laine M, Pulkki K & Tikkanen I. (2011). Heme oxygenase-1 induction protects the heart and modulates cellular and extracellular remodelling after myocardial infarction in rats. *Exp Biol Med (Maywood)* **236,** 1437-1448.

Lal H, Verma SK, Golden HB, Foster DM, Smith M and Dostal DE (2008) Stretch-induced regulation of angiotensinogen gene expression in cardiac myocytes and fibroblasts: opposing roles of JNK1/2 and p38alpha MAP kinases. *J Mol Cell Cardiol.* **6**, 770-8.

Lamouille S & Derynck R. (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *The Journal of cell biology* **178,** 437-451.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE and Harrison DG (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest.* **8,** 1201-9.

Lassegue B & Clempus RE. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *American journal of physiology Regulatory, integrative and comparative physiology* **285,** R277-297.

Lassegue B & Griendling KK. (2010). NADPH oxidases: functions and pathologies in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology* **30,** 653-661.

Latte KP, Appel KE & Lampen A. (2011). Health benefits and possible risks of broccoli - an overview.

Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association **49,** 3287-3309.

Lau A, Tian W, Whitman SA & Zhang DD. (2012). The Predicted Molecular Weight of Nrf2: It Is What It Is Not. *Antioxidants & redox signaling*.

Lauffenburger DA & Horwitz AF. (1996). Cell migration: a physically integrated molecular process. *Cell* **84,** 359-369.

Laukkanen MO, Kivela A, Rissanen T, Rutanen J, Karkkainen MK, Leppanen O, Brasen JH & Yla-Herttuala S. (2002). Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. *Circulation* **106,** 1999-2003.

Laukkanen MO, Kivela A, Rissanen T, Rutanen J, Karkkainen MK, Leppanen O, Brasen JH & Yla-Herttuala S. (2002). Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. *Circulation* **106,** 1999-2003.

Le Roy C and Wrana JL (2005) Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat Rev Mol Cell Biol* **2,** 112-26.

Lee DY, Lee CI, Lin TE, Lim SH, Zhou J, Tseng YC, Chien S & Chiu JJ. (2012). Role of histone deacetylases in transcription factor regulation and cell cycle modulation in endothelial cells in response to disturbed flow. *Proceedings of the National Academy of Sciences of the United States of America* **109,** 1967-1972.

Lee JH, Moon MH, Jeong JK, Park YG, Lee YJ, Seol JW & Park SY. (2012). Sulforaphane induced adipolysis via hormone sensitive lipase activation, regulated by AMPK signaling pathway. *Biochemical and biophysical research communications* **426,** 492-497.

Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ & Adcock IM. (2006). NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. *J Immunol* **176,** 603-615.

Lee MY, San Martin A, Mehta PK, Dikalova AE, Garrido AM, Datla RS, Lyons E, Krause KH, Banfi B, Lambeth JD, Lassegue B and Griendling KK (2009) Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler, Thromb Vasc Biol* **29,** 480-487**.**

Lee SS, Gao W, Mazzola S, Thomas MN, Csizmadia E, Otterbein LE, Bach FH & Wang H. (2007). Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21,** 3450-3457.

Lee YJ, Jeong HY, Kim YB, Lee YJ, Won SY, Shim JH, Cho MK, Nam HS & Lee SH. (2012). Reactive oxygen species and PI3K/Akt signaling play key roles in the induction of Nrf2-driven heme oxygenase-1 expression in sulforaphane-treated human mesothelioma MSTO-211H cells. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **50,** 116-123.

Lee YJ & Lee SH. (2011). Sulforaphane induces antioxidative and antiproliferative responses by generating reactive oxygen species in human bronchial epithelial BEAS-2B cells. *Journal of Korean medical science* **26,** 1474-1482.

Leite PF, Danilovic A, Moriel P, Dantas K, Marklund S, Dantas AP & Laurindo FR. (2003). Sustained decrease in superoxide dismutase activity underlies constrictive remodeling after balloon injury in rabbits. *Arteriosclerosis, thrombosis, and vascular biology* **23,** 2197-2202.

Lenaerts I, Braeckman F, Matthissjens F and Vanfleteren JR (2002) A high-throughput microtiter plate assay for superoxide dismutase based in lucigenin chemiluminescence *Analytical Biochemistry* **311,** 9092.

Levitzki A. (2005). PDGF receptor kinase inhibitors for the treatment of restenosis. *Cardiovascular research* **65,** 581-586.

Levonen AL, Inkala M, Heikura T, Jauhiainen S, Jyrkkänen HK, Kansanen E, Määttä K, Romppanen E, Turunen P, Rutanen J and Ylä-Herttuala S (2007) Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo. *Arterioscler Thromb Vasc Biol.* **27,** 741-7.

Levy AS, Chung JC, Kroetsch JT & Rush JW. (2009). Nitric oxide and coronary vascular endothelium adaptations in hypertension. *Vascular health and risk management* **5,** 1075-1087.

Li F, Fan C, Cheng T, Jiang C & Zeng B. (2009). Efficient inhibition of fibroblast proliferation and collagen expression by ERK2 siRNAs. *Biochemical and biophysical research communications* **382,** 259-263.

Li F, Liu S, Ouyang Y, Fan C, Wang T, Zhang C, Zeng B, Chai Y & Wang X. (2012). Effect of celecoxib on proliferation, collagen expression, ERK1/2 and SMAD2/3 phosphorylation in NIH/3T3 fibroblasts. *European journal of pharmacology* **678,** 1-5.

Li G, Chen SJ, Oparil S, Chen YF & Thompson JA. (2000). Direct in vivo evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. *Circulation* **101,** 1362-1365.

Li G, Chen YF, Greene GL, Oparil S & Thompson JA. (1999). Estrogen inhibits vascular smooth muscle cell-dependent adventitial fibroblast migration in vitro. *Circulation* **100,** 1639-1645.

Li G, Chen YF, Kelpke SS, Oparil S & Thompson JA. (2000). Estrogen attenuates integrin-beta(3)-dependent adventitial fibroblast migration after inhibition of osteopontin production in vascular smooth muscle cells. *Circulation* **101,** 2949-2955.

Li H, Wang F, Zhang L, Cao Y, Liu W, Hao J, Liu Q & Duan H. (2011). Modulation of Nrf2 expression alters high glucose-induced oxidative stress and antioxidant gene expression in mouse mesangial cells. *Cellular signalling* **23,** 1625-1632.

Li J, Johnson D, Calkins M, Wright L, Svendsen C & Johnson J. (2005). Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicological sciences : an official journal of the Society of Toxicology* **83,** 313-328.

Li JM & Shah AM. (2004). Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *American journal of physiology Regulatory, integrative and comparative physiology* **287,** R1014-1030.

Li L, Zhu DL, Shen WL & Gao PJ. (2006). Increased migration of vascular adventitial fibroblasts from spontaneously hypertensive rats. *Hypertension research : official journal of the Japanese Society of Hypertension* **29,** 95-103.

Li M, Riddle SR, Frid MG, El Kasmi KC, McKinsey TA, Sokol RJ, Strassheim D, Meyrick B, Yeager ME, Flockton AR, McKeon BA, Lemon DD, Horn TR, Anwar A, Barajas C & Stenmark KR. (2011). Emergence of fibroblasts with a proinflammatory epigenetically altered phenotype in severe hypoxic pulmonary hypertension. *J Immunol* **187,** 2711-2722.

Li S, Tabar SS, Malec V, Eul BG, Klepetko W, Weissmann N, Grimminger F, Seeger W, Rose F & Hanze J. (2008). NOX4 regulates ROS levels under normoxic and hypoxic conditions, triggers proliferation, and inhibits apoptosis in pulmonary artery adventitial fibroblasts. *Antioxidants & redox signaling* **10,** 1687-

1698.

Li S, Li X, Zheng H, Xie B, Bidasee KR and Rozanski GJ (2008) Pro-oxidant effect of transforming growth factor- beta1 mediates contractile dysfunction in rat ventricular myocytes. *Cardiovasc Res* **1,**107-17.

Li W, Jain MR, Chen C, Yue X, Hebbar V, Zhou R & Kong AN. (2005). Nrf2 Possesses a redox-insensitive nuclear export signal overlapping with the leucine zipper motif. *The Journal of biological chemistry* **280,** 28430-28438.

Li W & Kong AN. (2009). Molecular mechanisms of Nrf2-mediated antioxidant response. *Molecular carcinogenesis* **48,** 91-104.

Li X, Zhang D, Hannink M & Beamer LJ. (2004). Crystal structure of the Kelch domain of human Keap1. *The Journal of biological chemistry* **279,** 54750-54758.

Li Y, Paonessa JD & Zhang Y. (2012). Mechanism of chemical activation of Nrf2. *PloS one* **7,** e35122.

Li Z, Dranoff JA, Chan EP, Uemura M, Sevigny J & Wells RG. (2007). Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology* **46,** 1246-1256.

Li ZD, Bork JP, Krueger B, Patsenker E, Schulze-Krebs A, Hahn EG & Schuppan D. (2005). VEGF induces proliferation, migration, and TGF-beta1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Biochemical and biophysical research communications* **334,** 1049-1060.

Liang YY, Brunicardi CF and Lin X (2008) Smad3 mediates immediate early induction of Id1 by TGF-β. *Cell Res* **19,** 140-148.

Libby P. (1998). The interface of atherosclerosis and thrombosis: basic mechanisms. *Vasc Med* **3,** 225-229.

Libby P, Ridker PM & Maseri A. (2002). Inflammation and atherosclerosis. *Circulation* **105,** 1135-1143.
Lin CC, Chiang LL, Lin CH, Shih CH, Liao YT, Hsu MJ & Chen BC. (2007). Transforming growth factor-beta1 stimulates heme oxygenase-1 expression via the PI3K/Akt and NF-kappaB pathways in human lung epithelial cells. *European journal of pharmacology* **560,** 101-109.

Lin Y, Soares MP, Sato K, Takigami K, Csizmadia E, Smith N & Bach FH. (1999). Accommodated xenografts survive in the presence of anti-donor antibodies and complement that precipitate rejection of naive xenografts. *J Immunol* **163,** 2850-2857.

Lindskog H, Athley E, Larsson E, Lundin S, Hellstrom M & Lindahl P. (2006). New insights to vascular smooth muscle cell and pericyte differentiation of mouse embryonic stem cells in vitro. *Arteriosclerosis, thrombosis, and vascular biology* **26,** 1457-1464.

Liu C, Nath KA, Katusic ZS & Caplice NM. (2004). Smooth muscle progenitor cells in vascular disease. *Trends in cardiovascular medicine* **14,** 288-293.

Liu H, Wang H, Shenvi S, Hagen TM & Liu RM. (2004). Glutathione metabolism during aging and in Alzheimer disease. *Annals of the New York Academy of Sciences* **1019,** 346-349.

Liu J, Ormsby A, Oja-Tebbe N & Pagano PJ. (2004). Gene transfer of NAD(P)H oxidase inhibitor to the vascular adventitia attenuates medial smooth muscle hypertrophy. *Circulation research* **95,** 587-594.

Liu J, Yang F, Yang XP, Jankowski M & Pagano PJ. (2003). NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. *Arteriosclerosis, thrombosis, and vascular biology* **23,** 776-782.

Liu JF, Hou SM, Tsai CH, Huang CY, Yang WH & Tang CH. (2012). Thrombin induces heme oxygenase-

1 expression in human synovial fibroblasts through protease-activated receptor signaling pathways. *Arthritis research & therapy* **14,** R91.

Liu L, Rodriguez-Belmonte EM, Mazloum N, Xie B & Lee MY. (2003). Identification of a novel protein, PDIP38, that interacts with the p50 subunit of DNA polymerase delta and proliferating cell nuclear antigen. *The Journal of biological chemistry* **278,** 10041-10047.

Liu P, Zhang C, Feng JB, Zhao YX, Wang XP, Yang JM, Zhang MX, Wang XL & Zhang Y. (2008). Cross talk among Smad, MAPK, and integrin signaling pathways enhances adventitial fibroblast functions activated by transforming growth factor-beta1 and inhibited by Gax. *Arteriosclerosis, thrombosis, and vascular biology* **28,** 725-731.

Liu P, Zhang C, Zhao YX, Feng JB, Liu CX, Chen WQ, Yao GH, Zhang M, Wang XL & Zhang Y. (2010). Gax gene transfer inhibits vascular remodeling induced by adventitial inflammation in rabbits. *Atherosclerosis* **212,** 398-405.

Liu RM & Gaston Pravia KA. (2010). Oxidative stress and glutathione in TGF-beta-mediated fibrogenesis. *Free radical biology & medicine* **48,** 1-15.

Liu RM, Liu Y, Forman HJ, Olman M & Tarpey MM. (2004). Glutathione regulates transforming growth factor-beta-stimulated collagen production in fibroblasts. *American journal of physiology Lung cellular and molecular physiology* **286,** L121-128.

Liu RM, Vayalil PK, Ballinger C, Dickinson DA, Huang WT, Wang S, Kavanagh TJ, Matthews QL & Postlethwait EM. (2012). Transforming growth factor beta suppresses glutamate-cysteine ligase gene expression and induces oxidative stress in a lung fibrosis model. *Free radical biology & medicine* **53,** 554-563.

Liu YC, Hsieh CW, Weng YC, Chuang SH, Hsieh CY & Wung BS. (2008). Sulforaphane inhibition of monocyte adhesion via the suppression of ICAM-1 and NF-kappaB is dependent upon glutathione depletion in endothelial cells. *Vascular pharmacology* **48,** 54-61.

Liu XM, Chapman GB, Peyton KJ, Schafer AI and Durant W (2002) Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. *Cardiovasc Res* **55,** 396–405.

Liu ZY & Kong W. (2010). [The role of adventitia in atherosclerosis]. *Sheng li ke xue jin zhan [Progress in physiology]* **41,** 177-182.

Lo SC, Li X, Henzl MT, Beamer LJ & Hannink M. (2006). Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *The EMBO journal* **25,** 3605-3617.

Loignon M, Miao W, Hu L, Bier A, Bismar TA, Scrivens PJ, Mann K, Basik M, Bouchard A, Fiset PO, Batist Z & Batist G. (2009). Cul3 overexpression depletes Nrf2 in breast cancer and is associated with sensitivity to carcinogens, to oxidative stress, and to chemotherapy. *Molecular cancer therapeutics* **8,** 2432-2440.

Lonn P, Moren A, Raja E, Dahl M & Moustakas A. (2009). Regulating the stability of TGFbeta receptors and Smads. *Cell research* **19,** 21-35.

Looi YH, Grieve DJ, Siva A, Walker SJ, Anilkumar N, Cave AC, Marber M, Monaghan MJ and Shah AM (2008) Involvement of Nox2 NADPH oxidase in adverse cardiac remodeling after myocardial infarction. *Hypertension* **51**, 319–325.

Lovett JK and Rothwell PM (2003) Site of carotid plaque ulceration in relation to direction of blood flow, an angiographic and pathological study. *Cerebrovasc Dis.* **4,** 369-75.

Lu H, Lu F, Liu G & Gao J. (2012). [Effect of human serum extracted by two different methods on human dermal fibroblast growth in vitro]. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University* **32,** 61-65.

Lu SC. (2009). Regulation of glutathione synthesis. *Molecular aspects of medicine* **30,** 42-59.

Lu Z, Murray JT, Luo W, Li H, Wu X, Xu H, Backer JM & Chen YG. (2002). Transforming growth factor beta activates Smad2 in the absence of receptor endocytosis. *The Journal of biological chemistry* **277,** 29363-29368.

Luga V, McLean S, Le Roy C, O'Connor-McCourt M, Wrana JL & Di Guglielmo GM. (2009). The extracellular domain of the TGFbeta type II receptor regulates membrane raft partitioning. *The Biochemical journal* **421,** 119-131.

Luoma JS, Stralin P, Marklund SL, Hiltunen TP, Sarkioja T & Yla-Herttuala S. (1998). Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions: colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arteriosclerosis, thrombosis, and vascular biology* **18,** 157-167.

Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE & Daemen MJ. (2002). Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arteriosclerosis, thrombosis, and vascular biology* **22,** 975-982.

Lynch SM, Frei B, Morrow JD, Roberts LJ, Xu AI, Jackson T, Reyna R, Klevay LM, Vita JA and Keaney JF (1997) Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17,** 2975-2981.

Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, Papaharalambus C, Lassegue B & Griendling KK. (2009). Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circulation research* **105,** 249-259.

Madamanchi NR, Moon SK, Hakim ZS, Clark S, Mehrizi A, Patterson C & Runge MS. (2005). Differential activation of mitogenic signaling pathways in aortic smooth muscle cells deficient in superoxide dismutase isoforms. *Arteriosclerosis, thrombosis, and vascular biology* **25,** 950-956.

Madamanchi NR & Runge MS. (2007). Mitochondrial dysfunction in atherosclerosis. *Circulation research* **100,** 460-473.

Maeda M, Johnson KR & Wheelock MJ. (2005). Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *Journal of cell science* **118,** 873-887.

Mahadevan VS, Campbell M, McKeown PP & Bayraktutan U. (2006). Internal mammary artery smooth muscle cells resist migration and possess high antioxidant capacity. *Cardiovascular research* **72,** 60-68.

Maiellaro K and Taylor WR (2007) The role of the adventitia in vascular inflammation. *Cardiovascular Research* **75:** 640-648.

Maines MD. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annual review of pharmacology and toxicology* **37,** 517-554.
Majesky MW, Dong XR, Hoglund V, Daum G & Mahoney WM, Jr. (2012). The adventitia: a progenitor cell niche for the vessel wall. *Cells, tissues, organs* **195,** 73-81.

Majesky MW, Lindner V, Twardzik DR, Schwartz SM & Reidy MA. (1991). Production of transforming growth factor beta 1 during repair of arterial injury. *The Journal of clinical investigation* **88,** 904-910.

Majkut SF & Discher DE. (2012). Cardiomyocytes from late embryos and neonates do optimal work and striate best on substrates with tissue-level elasticity: metrics and mathematics. *Biomechanics and modeling in mechanobiology* **11,** 1219-1225.

Malhotra D, Portales-Casamar E, Singh A, Srivastava S, Arenillas D, Happel C, Shyr C, Wakabayashi N, Kensler TW, Wasserman WW & Biswal S. (2010). Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic acids research* **38,** 5718-5734.

Malhotra D, Thimmulappa R, Navas-Acien A, Sandford A, Elliott M, Singh A, Chen L, Zhuang X, Hogg J, Pare P, Tuder RM & Biswal S. (2008). Decline in NRF2-regulated antioxidants in chronic obstructive pulmonary disease lungs due to loss of its positive regulator, DJ-1. *American journal of respiratory and critical care medicine* **178,** 592-604.

Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R, Fradelizi D & Tedgui A. (2001). Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circulation research* **89,** 930-934.

Mallawaarachchi CM, Weissberg PL & Siow RC. (2005). Smad7 gene transfer attenuates adventitial cell migration and vascular remodeling after balloon injury. *Arteriosclerosis, thrombosis, and vascular biology* **25,** 1383-1387.

Mann GE, Bonacasa B, Ishii T & Siow RC. (2009). Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. *Current opinion in pharmacology* **9,** 139-145.

Mann GE, Niehueser-Saran J, Watson A, Gao L, Ishii T, de Winter P & Siow RC. (2007). Nrf2/ARE regulated antioxidant gene expression in endothelial and smooth muscle cells in oxidative stress: implications for atherosclerosis and preeclampsia. *Sheng li xue bao : [Acta physiologica Sinica]* **59,** 117-127.

Manning G, Whyte DB, Martinez R, Hunter T and Sudarsanam S (2002) The protein kinase complement of the human genome *Science* **298,** 1215-1224.

Margadant C & Sonnenberg A. (2010). Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO reports* **11,** 97-105.

Mari M, Morales A, Colell A, Garcia-Ruiz C, Kaplowitz N & Fernandez-Checa JC. (2012). Mitochondrial glutathione: Features, regulation and role in disease. *Biochimica et biophysica acta*.

Mark A, Hock T, Kapturczak MH, Agarwal A & Hill-Kapturczak N. (2005). Induction of heme oxygenase-1 modulates the profibrotic effects of transforming growth factor-beta in human renal tubular epithelial cells. *Cell Mol Biol (Noisy-le-grand)* **51,** 357-362.

Marklund SL. (1984). Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *The Biochemical journal* **222,** 649-655.

Markovic J, Mora NJ, Broseta AM, Gimeno A, de-la-Concepción N, Viña J and Pallardó FV (2009) The depletion of nuclear glutathione impairs cell proliferation in 3t3 fibroblasts. *PLoS One* **7,** e6413.

Marrot L, Jones C, Perez P & Meunier JR. (2008). The significance of Nrf2 pathway in (photo)-oxidative stress response in melanocytes and keratinocytes of the human epidermis. *Pigment cell & melanoma research* **21,** 79-88.

Martin-Garrido A, Brown DI, Lyle AN, Dikalova A, Seidel-Rogol B, Lassegue B, San Martin A & Griendling KK. (2011). NADPH oxidase 4 mediates TGF-beta-induced smooth muscle alpha-actin via p38MAPK and serum response factor. *Free radical biology & medicine* **50,** 354-362.

Martinez EF, Araujo VC, Sousa SO & Arana-Chavez VE. (2007). TGF-beta1 enhances the expression of alpha-smooth muscle actin in cultured human pulpal fibroblasts: immunochemical and ultrastructural

analyses. *Journal of endodontics* **33,** 1313-1318.

Martino F, Loffredo L, Carnevale R, Sanguigni V, Martino E, Catasca E, Zanoni C, Pignatelli P and Violi F (2008) Oxidative stress is associated with arterial dysfunction and enhanced intima-media thickness in children with hypercholesterolemia: the potential role of nicotinamide-adenine dinucleotide phosphate oxidase, *Pediatrics* **122**, e648–e655.

Maruoka H, Sasaya H, Sugihara K, Shimoke K & Ikeuchi T. (2011). Low-molecular-weight compounds having neurotrophic activity in cultured PC12 cells and neurons. *Journal of biochemistry* **150,** 473-475.

Massague J. (2012). TGFbeta signalling in context. *Nature reviews Molecular cell biology* **13,** 616-630.

Massague J and Chen YG (2000) Controlling TGF-β signalling. *Genes & Dev.* **14,** 627-644.

Massague J & Wotton D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. *The EMBO journal* **19,** 1745-1754.

Masur SK, Dewal HS, Dinh TT, Erenburg I & Petridou S. (1996). Myofibroblasts differentiate from fibroblasts when plated at low density. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 4219-4223.

Mata-Greenwood E, Grobe A, Kumar S, Noskina Y & Black SM. (2005). Cyclic stretch increases VEGF expression in pulmonary arterial smooth muscle cells via TGF-beta1 and reactive oxygen species: a requirement for NAD(P)H oxidase. *American journal of physiology Lung cellular and molecular physiology* **289,** L288-289.

Mata-Greenwood E, Meyrick B, Steinhorn RH, Fineman JR & Black SM. (2003). Alterations in TGF-beta1 expression in lambs with increased pulmonary blood flow and pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology* **285,** L209-221.

Matsumura F, Ono S, Yamakita Y, Totsukawa G & Yamashiro S. (1998). Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. *The Journal of cell biology* **140,** 119-129.

Matsuno K, Yamada H, IwataDenan Jin K, Katsuyama M, Matsuki M, Takai S, Yamanishi K, Miyazaki M, Matsubara H and Yabe-Nishimura C (2005) Nox 1 is involved in angiotensin II mediated hypertension a study in nox1 deficient mice *Circulation* **112,** 2677-2685.

Matsuno H, Kozawa O, Niwa M, Abe A, Takiguchi Y & Uematsu T. (2001). Characterization of simple and reproducible vascular stenosis model in hypercholesterolemic hamsters. *Lipids* **36,** 453-460.

MacMillan DE. (1985) Blood flow and the localization of atherosclerotic plaques. *Stroke* **16,** 582-7.

McNally JS, Davis ME, Giddens DP, Saha A, Hwang J, Dikalov S, Jo H and Harrison DG (2003) Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. *Am J Physiol Heart Circ Physiol* **6,** H2290-7.

Mavrakis KJ, Andrew RL, Lee KL, Petropoulou C, Dixon JE, Navaratnam N, Norris DP & Episkopou V. (2007). Arkadia enhances Nodal/TGF-beta signaling by coupling phospho-Smad2/3 activity and turnover. *PLoS biology* **5,** e67.

Mayorga ME & Penn MS. (2012). miR-145 is differentially regulated by TGF-beta1 and ischaemia and targets Disabled-2 expression and wnt/beta-catenin activity. *Journal of cellular and molecular medicine* **16,** 1106-1113.

McCaffrey TA. (2000). TGF-betas and TGF-beta receptors in atherosclerosis. *Cytokine & growth factor reviews* **11,** 103-114.

McLean S & Di Guglielmo GM. (2010). TGF beta (transforming growth factor beta) receptor type III directs clathrin-mediated endocytosis of TGF beta receptor types I and II. *The Biochemical journal* **429,** 137-145.

McMahon M, Itoh K, Yamamoto M & Hayes JD. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *The Journal of biological chemistry* **278,** 21592-21600.

McMahon M, Thomas N, Itoh K, Yamamoto M & Hayes JD. (2004). Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *The Journal of biological chemistry* **279,** 31556-31567.

Mebratu Y & Tesfaigzi Y. (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* **8,** 1168-1175.

Meister A. (1983). Selective modification of glutathione metabolism. *Science* **220,** 472-477.
Meister A. (1988). Glutathione metabolism and its selective modification. *The Journal of biological chemistry* **263,** 17205-17208.

Meister A. (1988). On the discovery of glutathione. *Trends in biochemical sciences* **13,** 185-188.

Meister A & Anderson ME. (1983). Glutathione. *Annual review of biochemistry* **52,** 711-760.

Meister, G. and T. Tuschl (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**(7006): 343-349.

Menè P (1996) Physiology and pathophysiology of the mesangial cell. *Nefrologica.***16,** 3.

Mercado N, Thimmulappa R, Thomas CM, Fenwick PS, Chana KK, Donnelly LE, Biswal S, Ito K & Barnes PJ. (2011). Decreased histone deacetylase 2 impairs Nrf2 activation by oxidative stress. *Biochemical and biophysical research communications* **406,** 292-298.

Meredith MJ & Reed DJ. (1982). Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *The Journal of biological chemistry* **257,** 3747-3753.

Messina JP & Lawrence DA. (1989). Cell cycle progression of glutathione-depleted human peripheral blood mononuclear cells is inhibited at S phase. *J Immunol* **143,** 1974-1981.

Meyer-ter-Vehn T, Sieprath S, Katzenberger B, Gebhardt S, Grehn F & Schlunck G. (2006). Contractility as a prerequisite for TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Investigative ophthalmology & visual science* **47,** 4895-4904.

Mi L & Chung FL. (2008). Binding to protein by isothiocyanates: a potential mechanism for apoptosis induction in human non small lung cancer cells. *Nutrition and cancer* **60 Suppl 1,** 12-20.

Mi L, Sirajuddin P, Gan N & Wang X. (2010). A cautionary note on using N-acetylcysteine as an antagonist to assess isothiocyanate-induced reactive oxygen species-mediated apoptosis. *Analytical biochemistry* **405,** 269-271.

Miano JM, Long X and Fujiwara K (2007) Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol* **1,** C70-81.

Michaeloudes C, Chang PJ, Petrou M & Chung KF. (2011). Transforming growth factor-beta and nuclear factor E2-related factor 2 regulate antioxidant responses in airway smooth muscle cells: role in asthma. *American journal of respiratory and critical care medicine* **184,** 894-903.
Mii S, Ware JA & Kent KC. (1993). Transforming growth factor-beta inhibits human vascular smooth muscle cell growth and migration. *Surgery* **114,** 464-470.

Miller CJ, Gounder SS, Kannan S, Goutam K, Muthusamy VR, Firpo MA, Symons JD, Paine R, 3rd, Hoidal JR & Rajasekaran NS. (2012). Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle. *Biochimica et biophysica acta* **1822,** 1038-1050.

Miller ER, 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ & Guallar E. (2005). Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Annals of internal medicine* **142,** 37-46.

Miller FJ, Jr. (2009). NADPH oxidase 4: walking the walk with Poldip2. *Circulation research* **105,** 209-210.

Mimoto T, Miyazaki K, Morimoto N, Kurata T, Satoh K, Ikeda Y & Abe K. (2012). Impaired antioxydative Keap1/Nrf2 system and the downstream stress protein responses in the motor neuron of ALS model mice. *Brain research* **1446,** 109-118.

Mishra L, Derynck R & Mishra B. (2005). Transforming growth factor-beta signaling in stem cells and cancer. *Science* **310,** 68-71.

Miyazono K. (2009). Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. *Proc Jpn Acad Ser B Phys Biol Sci* **85,** 314-323.

Mo MS, Li HB, Wang BY, Wang SL, Zhu ZL & Yu XR. (2012). PI3K/Akt and NF-kappaB activation following intravitreal administration of 17beta-estradiol: Neuroprotection of the rat retina from light-induced apoptosis. *Neuroscience* **228C,** 1-12.

Modarressi A, Pietramaggiori G, Godbout C, Vigato E, Pittet B & Hinz B. (2010). Hypoxia impairs skin myofibroblast differentiation and function. *The Journal of investigative dermatology* **130,** 2818-2827.

Moi P, Chan K, Asunis I, Cao A & Kan YW. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 9926-9930.

Moinova HR & Mulcahy RT. (1998). An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gamma-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *The Journal of biological chemistry* **273,** 14683-14689.

Moon DO, Kang SH, Kim KC, Kim MO, Choi YH & Kim GY. (2010). Sulforaphane decreases viability and telomerase activity in hepatocellular carcinoma Hep3B cells through the reactive oxygen species-dependent pathway. *Cancer letters* **295,** 260-266.

Mori K, Shibanuma M & Nose K. (2004). Invasive potential induced under long-term oxidative stress in mammary epithelial cells. *Cancer research* **64,** 7464-7472.

Morita T. (2005). Heme oxygenase and atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **25,** 1786-1795.

Morita T and Kourembanas S (1995) Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. *J Clin Invest* **96,** 2676–82.

Moses HL. (1992). TGF-beta regulation of epithelial cell proliferation. *Molecular reproduction and development* **32,** 179-184.

Moses HL, Arteaga CL, Alexandrow MG, Dagnino L, Kawabata M, Pierce DF, Jr. & Serra R. (1994). TGF

beta regulation of cell proliferation. *Princess Takamatsu symposia* **24,** 250-263.

Moses HL, Coffey RJ, Jr., Leof EB, Lyons RM & Keski-Oja J. (1987). Transforming growth factor beta regulation of cell proliferation. *Journal of cellular physiology Supplement* **Suppl 5,** 1-7.

Motohashi H, Katsuoka F, Engel JD & Yamamoto M. (2004). Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 6379-6384.

Motohashi H & Yamamoto M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends in molecular medicine* **10,** 549-557.

Motterlini R, Foresti R, Intaglietta M & Winslow RM. (1996). NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *The American journal of physiology* **270,** H107-114.

Moustakas A & Heldin CH. (2005). Non-Smad TGF-beta signals. *Journal of cell science* **118,** 3573-3584.

Mu Y, Gudey SK & Landstrom M. (2012). Non-Smad signaling pathways. *Cell and tissue research* **347,** 11-20.

Muchaneta-Kubara EC & el Nahas AM. (1997). Myofibroblast phenotypes expression in experimental renal scarring. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **12,** 904-915.

Mucsi I, Skorecki KL & Goldberg HJ. (1996). Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-beta1 on gene expression. *The Journal of biological chemistry* **271,** 16567-16572.

Mukherjee S, Gangopadhyay H & Das DK. (2008). Broccoli: a unique vegetable that protects mammalian hearts through the redox cycling of the thioredoxin superfamily. *Journal of agricultural and food chemistry* **56,** 609-617.

Mulcahy RT & Gipp JJ. (1995). Identification of a putative antioxidant response element in the 5'-flanking region of the human gamma-glutamylcysteine synthetase heavy subunit gene. *Biochemical and biophysical research communications* **209,** 227-233.

Mulcahy RT & Gipp JJ. (1995). Identification of a putative antioxidant response element in the 5'-flanking region of the human gamma-glutamylcysteine synthetase heavy subunit gene. *Biochemical and biophysical research communications* **209,** 227-233.

Muller T & Hengstermann A. (2012). Nrf2: friend and foe in preventing cigarette smoking-dependent lung disease. *Chemical research in toxicology* **25,** 1805-1824.

Munday R, Mhawech-Fauceglia P, Munday CM, Paonessa JD, Tang L, Munday JS, Lister C, Wilson P, Fahey JW, Davis W & Zhang Y. (2008). Inhibition of urinary bladder carcinogenesis by broccoli sprouts. *Cancer research* **68,** 1593-1600.

Muscarella LA, Parrella P, D'Alessandro V, la Torre A, Barbano R, Fontana A, Tancredi A, Guarnieri V, Balsamo T, Coco M, Copetti M, Pellegrini F, De Bonis P, Bisceglia M, Scaramuzzi G, Maiello E, Valori VM, Merla G, Vendemiale G & Fazio VM. (2011). Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer. *Epigenetics : official journal of the DNA Methylation Society* **6,** 710-719.

Myzak MC & Dashwood RH. (2006). Chemoprotection by sulforaphane: keep one eye beyond Keap1. *Cancer letters* **233,** 208-218.

Myzak MC, Dashwood WM, Orner GA, Ho E & Dashwood RH. (2006). Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice. *FASEB journal : official publication*

of the Federation of American Societies for Experimental Biology **20,** 506-508.

Myzak MC, Tong P, Dashwood WM, Dashwood RH & Ho E. (2007). Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Exp Biol Med (Maywood)* **232,** 227-234.

Nabel EG, Shum L, Pompili VJ, Yang ZY, San H, Shu HB, Liptay S, Gold L, Gordon D, Derynck R & et al. (1993). Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 10759-10763.

Nabel EG, Shum L, Pompili VJ, Yang ZY, San H, Shu HB, Liptay S, Gold L, Gordon D, Derynck R & et al. (1993). Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 10759-10763.

Nagaraj NS & Datta PK. (2010). Targeting the transforming growth factor-beta signaling pathway in human cancer. *Expert opinion on investigational drugs* **19,** 77-91.

Nakamura T, Matsushima M, Hayashi Y, Shibasaki M, Imaizumi K, Hashimoto N, Shimokata K, Hasegawa Y & Kawabe T. (2011). Attenuation of transforming growth factor-beta-stimulated collagen production in fibroblasts by quercetin-induced heme oxygenase-1. *American journal of respiratory cell and molecular biology* **44,** 614-620.

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH & ten Dijke P. (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* **389,** 631-635.

Nakao A, Otterbein LE, Overhaus M, Sarady JK, Tsung A, Kimizuka K, Nalesnik MA, Kaizu T, Uchiyama T, Liu F, Murase N, Bauer AJ & Bach FH. (2004). Biliverdin protects the functional integrity of a transplanted syngeneic small bowel. *Gastroenterology* **127,** 595-606.

Nakashima Y, Raines EW, Plump AS, Breslow JL and Ross R (1998) Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* **18,** 842-51.

Nakatsuji S, Yamate J & Sakuma S. (1998). Relationship between vimentin expressing renal tubules and interstitial fibrosis in chronic progressive nephropathy in aged rats. *Virchows Archiv : an international journal of pathology* **433,** 359-367.

Nam HJ, Park YY, Yoon G, Cho H & Lee JH. (2010). Co-treatment with hepatocyte growth factor and TGF-beta1 enhances migration of HaCaT cells through NADPH oxidase-dependent ROS generation. *Experimental & molecular medicine* **42,** 270-279.

Narasimhan M, Patel D, Vedpathak D, Rathinam M, Henderson G & Mahimainathan L. (2012). Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells. *PloS one* **7,** e51111.

Nath KA, Croatt AJ, Warner GM & Grande JP. (2011). Genetic deficiency of Smad3 protects against murine ischemic acute kidney injury. *American journal of physiology Renal physiology* **301,** F436-442.

Natsch A. (2010). The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers--functional relevance and a hypothesis on innate reactions to skin sensitizers. *Toxicological sciences : an official journal of the Society of Toxicology* **113,** 284-292.

Navab M, Berliner JA and Watson AD (1996) The yin and yang of oxidation in the development of the fatty streak: a review based on the 1994 George Lyman Duff Memorial lecture *Arterioscler Thromb Vasc Biol* **16,** 831-842.

Negi G, Kumar A & Sharma SS. (2011). Nrf2 and NF-kappaB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes. *Current neurovascular research* **8,** 294-304.

Neuzil J and Stocker R (1994) Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. *Mol. Endocrinol.* **269,** 16712-9.

Nguyen AD, Itoh S, Jeney V, Yanagisawa H, Fujimoto M, Ushio-Fukai M & Fukai T. (2004). Fibulin-5 is a novel binding protein for extracellular superoxide dismutase. *Circulation research* **95,** 1067-1074.

Nguyen T, Nioi P & Pickett CB. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *The Journal of biological chemistry* **284,** 13291-13295.

Nguyen T, Sherratt PJ, Huang HC, Yang CS & Pickett CB. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *The Journal of biological chemistry* **278,** 4536-4541.

Nguyen T, Sherratt PJ, Nioi P, Yang CS & Pickett CB. (2005). Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *The Journal of biological chemistry* **280,** 32485-32492.

Nguyen T, Yang CS & Pickett CB. (2004). The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free radical biology & medicine* **37,** 433-441.

Nicolas FJ, De Bosscher K, Schmierer B & Hill CS. (2004). Analysis of Smad nucleocytoplasmic shuttling in living cells. *Journal of cell science* **117,** 4113-4125.

Niethammer P, Grabher C, Look AT & Mitchison TJ. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* **459,** 996-999.

Ning W, Song R, Li C, Park E, Mohsenin A, Choi AM & Choi ME. (2002). TGF-beta1 stimulates HO-1 via the p38 mitogen-activated protein kinase in A549 pulmonary epithelial cells. *American journal of physiology Lung cellular and molecular physiology* **283,** L1094-1102.

Nioi P & Hayes JD. (2004). Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. *Mutation research* **555,** 149-171.

Nioi P, Nguyen T, Sherratt PJ & Pickett CB. (2005). The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. *Molecular and cellular biology* **25,** 10895-10906.

Nishikawa T, Tsuno NH, Okaji Y, Sunami E, Shuno Y, Sasaki K, Hongo K, Kaneko M, Hiyoshi M, Kawai K, Kitayama J, Takahashi K & Nagawa H. (2010). The inhibition of autophagy potentiates anti-angiogenic effects of sulforaphane by inducing apoptosis. *Angiogenesis* **13,** 227-238.

Nishinaka Y, Aramaki Y, Yoshida H, Masuya H, Sugawara T & Ichimori Y. (1993). A new sensitive chemiluminescence probe, L-012, for measuring the production of superoxide anion by cells. *Biochemical and biophysical research communications* **193,** 554-559.

Niso-Santano M, Gonzalez-Polo RA, Bravo-San Pedro JM, Gomez-Sanchez R, Lastres-Becker I, Ortiz-Ortiz MA, Soler G, Moran JM, Cuadrado A & Fuentes JM. (2010). Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis. *Free radical biology & medicine* **48,** 1370-1381.

Niture SK & Jaiswal AK. (2011). Inhibitor of Nrf2 (INrf2 or Keap1) protein degrades Bcl-xL via

phosphoglycerate mutase 5 and controls cellular apoptosis. *The Journal of biological chemistry* **286,** 44542-44556.

Nobes CD & Hall A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *The Journal of cell biology* **144,** 1235-1244.

Noyan-Ashraf MH, Wu L, Wang R & Juurlink BH. (2006). Dietary approaches to positively influence fetal determinants of adult health. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20,** 371-373.

Numazawa S, Ishikawa M, Yoshida A, Tanaka S & Yoshida T. (2003). Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *American journal of physiology Cell physiology* **285,** C334-342.

Nussler AK, Hao L, Knobeloch D, Yao P, Nussler NC, Wang Z, Liu L & Ehnert S. (2010). Protective role of HO-1 for alcohol-dependent liver damage. *Dig Dis* **28,** 792-798.

O'Brien KD, McDonald TO, Chait A, Allen MD & Alpers CE. (1996). Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* **93,** 672-682.

Ogunjimi AA, Briant DJ, Pece-Barbara N, Le Roy C, Di Guglielmo GM, Kavsak P, Rasmussen RK, Seet BT, Sicheri F & Wrana JL. (2005). Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Molecular cell* **19,** 297-308.

Oh CJ, Kim JY, Choi YK, Kim HJ, Jeong JY, Bae KH, Park KG & Lee IK. (2012). Dimethylfumarate Attenuates Renal Fibrosis via NF-E2-Related Factor 2-Mediated Inhibition of Transforming Growth Factor-beta/Smad Signaling. *PloS one* **7,** e45870.

Oh CJ, Kim JY, Min AK, Park KG, Harris RA, Kim HJ & Lee IK. (2012). Sulforaphane attenuates hepatic fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-beta/Smad signaling. *Free radical biology & medicine* **52,** 671-682.

Ohara Y, Peterson TE and Harrison DG (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* **6,** 2546-51.

Ohnuma T, Nakayama S, Anan E, Nishiyama T, Ogura K & Hiratsuka A. (2010). Activation of the Nrf2/ARE pathway via S-alkylation of cysteine 151 in the chemopreventive agent-sensor Keap1 protein by falcarindiol, a conjugated diacetylene compound. *Toxicology and applied pharmacology* **244,** 27-36.

Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M & Hirohashi S. (2008). Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer research* **68,** 1303-1309.

Okouchi M, Okayama N, Alexander JS & Aw TY. (2006). NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia- induced brain endothelial cell apoptosis. *Current neurovascular research* **3,** 249-261.

Olieslagers S, Pardali E, Tchaikovski V, ten Dijke P & Waltenberger J. (2011). TGF-beta1/ALK5-induced monocyte migration involves PI3K and p38 pathways and is not negatively affected by diabetes mellitus. *Cardiovascular research* **91,** 510-518.

Olieslagers S, Pardali E, Tchaikovski V, ten Dijke P & Waltenberger J. (2011). TGF-beta1/ALK5-induced monocyte migration involves PI3K and p38 pathways and is not negatively affected by diabetes mellitus. *Cardiovascular research* **91,** 510-518.

Olsson N, Piek E, Sundstrom M, ten Dijke P & Nilsson G. (2001). Transforming growth factor-beta-mediated mast cell migration depends on mitogen-activated protein kinase activity. *Cellular signalling* **13,**

483-490.

Osburn WO & Kensler TW. (2008). Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutation research* **659,** 31-39.

Ossola JO, Kristoff G and Tomaro ML (2000) Heme oxygenase induction by menadione bisulfite adduct-generated oxidative stress in rat liver. *Comp. Biochem. Phys* **127,** 91–99.

Otranto M, Sarrazy V, Bonte F, Hinz B, Gabbiani G & Desmouliere A. (2012). The role of the myofibroblast in tumor stroma remodeling. *Cell adhesion & migration* **6,** 203-219.

Otterbein LE, Soares MP, Yamashita K & Bach FH. (2003). Heme oxygenase-1: unleashing the protective properties of heme. *Trends in immunology* **24,** 449-455.

Ottesen JJ, Huse M, Sekedat MD & Muir TW. (2004). Semisynthesis of phosphovariants of Smad2 reveals a substrate preference of the activated T beta RI kinase. *Biochemistry* **43,** 5698-5706.

Ovchinnikova O, Robertson AK, Wagsater D, Folco EJ, Hyry M, Myllyharju J, Eriksson P, Libby P & Hansson GK. (2009). T-cell activation leads to reduced collagen maturation in atherosclerotic plaques of Apoe(-/-) mice. *The American journal of pathology* **174,** 693-700.

Owens GK. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiological reviews* **75,** 487-517.

Ozumi K, Tasaki H, Takatsu H, Nakata S, Morishita T, Koide S, Yamashita K, Tsutsui M, Okazaki M, Sasaguri Y, Adachi T & Nakashima Y. (2005). Extracellular superoxide dismutase overexpression reduces cuff-induced arterial neointimal formation. *Atherosclerosis* **181,** 55-62.

Padua D & Massague J. (2009). Roles of TGFbeta in metastasis. *Cell research* **19,** 89-102.

Pagan R, Martin I, Llobera M & Vilaro S. (1997). Growth and differentiation factors inhibit the migratory phenotype of cultured neonatal rat hepatocytes induced by HGF/SF. *Experimental cell research* **235,** 170-179.

Pagano PJ, Chanock SJ, Siwik DA, Colucci WS & Clark JK. (1998). Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. *Hypertension* **32,** 331-337.

Pagano PJ, Chanock SJ, Siwik DA, Colucci WS & Clark JK. (1998). Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. *Hypertension* **32,** 331-337.

Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM & Quinn MT. (1997). Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 14483-14488.

Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI & Cohen RA. (1995). An NADPH oxidase superoxide-generating system in the rabbit aorta. *The American journal of physiology* **268,** H2274-2280.

Palecek SP, Horwitz AF & Lauffenburger DA. (1999). Kinetic model for integrin-mediated adhesion release during cell migration. *Annals of biomedical engineering* **27,** 219-235.

Palecek SP, Huttenlocher A, Horwitz AF & Lauffenburger DA. (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *Journal of cell science* **111 (Pt 7),** 929-940.

Palecek SP, Schmidt CE, Lauffenburger DA & Horwitz AF. (1996). Integrin dynamics on the tail region of migrating fibroblasts. *Journal of cell science* **109 (Pt 5),** 941-952.

Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, Portugal S, Soares MP & Mota MM. (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nature medicine* **13,** 703-710.

Ossola JO, Kristoff G and Tomaro ML (2000) Heme oxygenase induction by menadione bisulfite adduct-generated oxidative stress in rat liver. *Comp. Biochem. Phys* **127,** 91–99.

Papaharalambus CA & Griendling KK. (2007). Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends in cardiovascular medicine* **17,** 48-54.

Papaiahgari S, Kleeberger SR, Cho HY, Kalvakolanu DV & Reddy SP. (2004). NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. *The Journal of biological chemistry* **279,** 42302-42312.

Papaiahgari S, Yerrapureddy A, Hassoun PM, Garcia JG, Birukov KG & Reddy SP. (2007). EGFR-activated signaling and actin remodeling regulate cyclic stretch-induced NRF2-ARE activation. *American journal of respiratory cell and molecular biology* **36,** 304-312.

Papaiahgari S, Zhang Q, Kleeberger SR, Cho HY & Reddy SP. (2006). Hyperoxia stimulates an Nrf2-ARE transcriptional response via ROS-EGFR-PI3K-Akt/ERK MAP kinase signaling in pulmonary epithelial cells. *Antioxidants & redox signaling* **8,** 43-52.

Paravicini TM, Gulluyan LM, Dusting GJ & Drummond GR. (2002). Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circulation research* **91,** 54-61.

Paravicini TM & Touyz RM. (2006). Redox signaling in hypertension. *Cardiovascular research* **71,** 247-258.

Pardali K and Moustakas A (2007) Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta.* **1,** 21-62.

Park HS, Chun JN, Jung HY, Choi C & Bae YS. (2006). Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells. *Cardiovascular research* **72,** 447-455.

Park SA, Park IH, Cho JS, Moon YM, Lee SH, Kim TH, Lee SH & Lee HM. (2012). Effect of [6]-gingerol on myofibroblast differentiation in transforming growth factor beta 1-induced nasal polyp-derived fibroblasts. *American journal of rhinology & allergy* **26,** 97-103.

Park SH. (2005). Fine tuning and cross-talking of TGF-beta signal by inhibitory Smads. *J Biochem Mol Biol* **38,** 9-16.

Parsons M & Adams JC. (2008). Rac regulates the interaction of fascin with protein kinase C in cell migration. *Journal of cell science* **121,** 2805-2813.

Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL & Majesky MW. (2008). A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 9349-9354.

Patil AS, Sable RB & Kothari RM. (2011). An update on transforming growth factor-beta (TGF-beta): sources, types, functions and clinical applicability for cartilage/bone healing. *Journal of cellular physiology* **226,** 3094-3103.

Paul R, Heil P, Spatz JP & Schwarz US. (2008). Propagation of mechanical stress through the actin cytoskeleton toward focal adhesions: model and experiment. *Biophysical journal* **94,** 1470-1482.

Pauly K, Fritz K, Furey A & Lobner D. (2011). Insulin-like growth factor 1 and transforming growth factor-beta stimulate cystine/glutamate exchange activity in dental pulp cells. *Journal of endodontics* **37,** 943-947.

Pavlov D, Muhlrad A, Cooper J, Wear M & Reisler E. (2007). Actin filament severing by cofilin. *Journal of molecular biology* **365,** 1350-1358.

Payen L, Courtois A, Loewert M, Guillouzo A & Fardel O. (2001). Reactive oxygen species-related induction of multidrug resistance-associated protein 2 expression in primary hepatocytes exposed to sulforaphane. *Biochemical and biophysical research communications* **282,** 257-263.

Pedruzzi LM, Stockler-Pinto MB, Leite M, Jr. & Mafra D. (2012). Nrf2-keap1 system versus NF-kappaB: The good and the evil in chronic kidney disease? *Biochimie*.

Pelham RJ, Jr. & Wang Y. (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 13661-13665.

Pellacani A, Wiesel P, Sharma A, Foster LC, Huggins GS, Yet SF & Perrella MA. (1998). Induction of heme oxygenase-1 during endotoxemia is downregulated by transforming growth factor-beta1. *Circulation research* **83,** 396-403.

Pels K, Labinaz M, Hoffert C & O'Brien ER. (1999). Adventitial angiogenesis early after coronary angioplasty : correlation with arterial remodeling. *Arteriosclerosis, thrombosis, and vascular biology* **19,** 229-238.

Pendyala S, Moitra J, Kalari S, Kleeberger SR, Zhao Y, Reddy SP, Garcia JG & Natarajan V. (2011). Nrf2 regulates hyperoxia-induced Nox4 expression in human lung endothelium: identification of functional antioxidant response elements on the Nox4 promoter. *Free radical biology & medicine* **50,** 1749-1759.

Penheiter SG, Mitchell N, Garamszegi N, Edens M, Doré JJE and Leof EB (2002) Internalization-dependent and independent requirements for transforming growth factor ß receptor signaling via the Smad pathway. *Molecular and Cellular Biology* **22,** 4750-4759.

Penttinen RP, Kobayashi S & Bornstein P. (1988). Transforming growth factor beta increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 1105-1108.

Petersen SV, Oury TD, Ostergaard L, Valnickova Z, Wegrzyn J, Thogersen IB, Jacobsen C, Bowler RP, Fattman CL, Crapo JD & Enghild JJ. (2004). Extracellular superoxide dismutase (EC-SOD) binds to type i collagen and protects against oxidative fragmentation. *The Journal of biological chemistry* **279,** 13705-13710.

Petrache I, Otterbein LE, Alam J, Wiegand GW & Choi AM. (2000). Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *American journal of physiology Lung cellular and molecular physiology* **278,** L312-319.

Petri N, Tannergren C, Holst B, Mellon FA, Bao Y, Plumb GW, Bacon J, O'Leary KA, Kroon PA, Knutson L, Forsell P, Eriksson T, Lennernas H & Williamson G. (2003). Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. *Drug metabolism and disposition: the biological fate of chemicals* **31,** 805-813.

Petri S, Korner S & Kiaei M. (2012). Nrf2/ARE Signaling Pathway: Key Mediator in Oxidative Stress and

Potential Therapeutic Target in ALS. *Neurology research international* **2012,** 878030.

Petrie R, Doyle AD & Yamada KM. (2009). Random versus directionally persistent cell migration. *Nature Reviews Molecular Cell Biology* **10,** 538:549.

Petrone WF, English DK, Wong K & McCord JM. (1980). Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. *Proceedings of the National Academy of Sciences of the United States of America* **77,** 1159-1163.

Pettazzoni P, Ciamporcero E, Medana C, Pizzimenti S, Dal Bello F, Minero VG, Toaldo C, Minelli R, Uchida K, Dianzani MU, Pili R & Barrera G. (2011). Nuclear factor erythroid 2-related factor-2 activity controls 4-hydroxynonenal metabolism and activity in prostate cancer cells. *Free radical biology & medicine* **51,** 1610-1618.

Pi J, Bai Y, Reece JM, Williams J, Liu D, Freeman ML, Fahl WE, Shugar D, Liu J, Qu W, Collins S & Waalkes MP. (2007). Molecular mechanism of human Nrf2 activation and degradation: role of sequential phosphorylation by protein kinase CK2. *Free radical biology & medicine* **42,** 1797-1806.

Piao MS, Park JJ, Choi JY, Lee DH, Yun SJ, Lee JB & Lee SC. (2012). Nrf2-dependent and Nrf2-independent induction of phase 2 detoxifying and antioxidant enzymes during keratinocyte differentiation. *Archives of dermatological research* **304,** 387-395.

Pierreux CE, Nicolas FJ & Hill CS. (2000). Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Molecular and cellular biology* **20,** 9041-9054.

Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM & Moses HL. (1990). TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61,** 777-785.

Pilkington MF, Sims SM & Dixon SJ. (2001). Transforming growth factor-beta induces osteoclast ruffling and chemotaxis: potential role in osteoclast recruitment. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **16,** 1237-1247.

Poerschke RL, Franklin MR, Bild AH & Moos PJ. (2012). Major differences among chemopreventive organoselenocompounds in the sustained elevation of cytoprotective genes. *Journal of biochemical and molecular toxicology* **26,** 344-353.

Pollard TD & Borisy GG. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112,** 453-465.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM & Koff A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & development* **8,** 9-22.

Pompella A, Visvikis A, Paolicchi A, De Tata V & Casini AF. (2003). The changing faces of glutathione, a cellular protagonist. *Biochemical pharmacology* **66,** 1499-1503.

Ponti A, Machacek M, Gupton SL, Waterman-Storer CM & Danuser G. (2004). Two distinct actin networks drive the protrusion of migrating cells. *Science* **305,** 1782-1786.

Pool-Zobel B, Veeriah S & Bohmer FD. (2005). Modulation of xenobiotic metabolising enzymes by anticarcinogens -- focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutation research* **591,** 74-92.

Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH. (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta *Journal of Experimental Medicine* **1,** 251-256.

Popova EN, Pletjushkina OY, Dugina VB, Domnina LV, Ivanova OY, Izyumov DS, Skulachev VP & Chernyak BV. (2010). Scavenging of reactive oxygen species in mitochondria induces myofibroblast differentiation. *Antioxidants & redox signaling* **13,** 1297-1307.

Powell DW (2000) Myofibroblasts: paracrine cells important in health and disease. *Trans Am Clin Climatol Assoc* **111,** 271–293.

Purdom-Dickinson SE, Sheveleva EV, Sun H & Chen QM. (2007). Translational control of nrf2 protein in activation of antioxidant response by oxidants. *Molecular pharmacology* **72,** 1074-1081.

Qi H, Han Y & Rong J. (2012). Potential roles of PI3K/Akt and Nrf2-Keap1 pathways in regulating hormesis of Z-ligustilide in PC12 cells against oxygen and glucose deprivation. *Neuropharmacology* **62,** 1659-1670.

Qi S, den Hartog GJ & Bast A. (2009). Superoxide radicals increase transforming growth factor-beta1 and collagen release from human lung fibroblasts via cellular influx through chloride channels. *Toxicology and applied pharmacology* **237,** 111-118.

Quinn MT, Parthasarathy S, Fong LG and Steinberg D (1987) Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocytes/macrophages during atherogenesis*Proc Natl Acad Sci* **84,** 2995-2998.

Quinn TP, Schlueter M, Soifer SJ, Gutierrez JA (2002) Cyclic mechanical stretch induces VEGF and FGF-2 expression in pulmonary vascular smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* **5,** L897-903.

Radjendirane V, Joseph P, Lee YH, Kimura S, Klein-Szanto AJ, Gonzalez FJ, Jaiswal AK (1998) Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. *J Biol Chem* **13,**7382-9.

Rachakonda G, Sekhar KR, Jowhar D, Samson PC, Wikswo JP, Beauchamp RD, Datta PK & Freeman ML. (2010). Increased cell migration and plasticity in Nrf2-deficient cancer cell lines. *Oncogene* **29,** 3703-3714.

Rada P, Rojo AI, Chowdhry S, McMahon M, Hayes JD & Cuadrado A. (2011). SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Molecular and cellular biology* **31,** 1121-1133.

Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z & Bissell MJ. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* **436,** 123-127.

Radpour R, Sikora M, Grussenmeyer T, Kohler C, Barekati Z, Holzgreve W, Lefkovits I & Zhong XY. (2009). Simultaneous isolation of DNA, RNA, and proteins for genetic, epigenetic, transcriptomic, and proteomic analysis. *Journal of proteome research* **8,** 5264-5274.

Rahimi RA & Leof EB. (2007). TGF-beta signaling: a tale of two responses. *Journal of cellular biochemistry* **102,** 593-608.

Rahman I, Biswas SK & Kirkham PA. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. *Biochemical pharmacology* **72,** 1439-1452.

Rahman I, Skwarska E, Henry M, Davis M, O'Connor CM, FitzGerald MX, Greening A & MacNee W. (1999). Systemic and pulmonary oxidative stress in idiopathic pulmonary fibrosis. *Free radical biology & medicine* **27,** 60-68.

Raines EW and Ross R (1993) Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis *British Heart Journal* **69,** S30-S37.

Rajasekaran NS, Varadharaj S, Khanderao GD, Davidson CJ, Kannan S, Firpo MA, Zweier JL & Benjamin IJ. (2011). Sustained activation of nuclear erythroid 2-related factor 2/antioxidant response element signaling promotes reductive stress in the human mutant protein aggregation cardiomyopathy in mice. *Antioxidants & redox signaling* **14,** 957-971.

Ramachandran A, Levonen AL, Brookes PS, Ceaser E, Shiva S, Barone MC and Darley-Usmar V (2002) Mitochondria, nitric oxide, and cardiovascular dysfunction. *Free Radic Biol Med* **11,** 1465-74.

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P & Kensler TW. (2001). Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 3410-3415.

Rao RK, Basuroy S, Rao VU, Karnaky Jr KJ & Gupta A. (2002). Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *The Biochemical journal* **368,** 471-481.

Reckless J, Rubin EM, Verstuyft JB, Metcalfe JC and Grainger DJ (2001) A common phenotype associated with atherogenesis in diverse mouse models of vascular lipid lesions. *J Vasc Res.* **3,** 256-65.

Reichard JF & Petersen DR. (2004). Hepatic stellate cells lack AP-1 responsiveness to electrophiles and phorbol 12-myristate-13-acetate. *Biochemical and biophysical research communications* **322,** 842-853.

Reisdorf P, Lawrence DA, Sivan V, Klising E & Martin MT. (2001). Alteration of transforming growth factor-beta1 response involves down-regulation of Smad3 signaling in myofibroblasts from skin fibrosis. *The American journal of pathology* **159,** 263-272.

Ren M, Wang B, Zhang J, Liu P, Lv Y, Liu G, Jiang H & Liu F. (2011). Smad2 and Smad3 as mediators of the response of adventitial fibroblasts induced by transforming growth factor beta1. *Molecular medicine reports* **4,** 561-567.

Rey FE, Cifuentes ME, Kiarash A, Quinn MT & Pagano PJ. (2001). Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O(2)(-) and systolic blood pressure in mice. *Circulation research* **89,** 408-414.

Rhee SG. (2006). Cell signaling. H2O2, a necessary evil for cell signaling. *Science* **312,** 1882-1883.

Ridley AJ. (1994). Signal transduction through the GTP-binding proteins Rac and Rho. *Journal of cell science Supplement* **18,** 127-131.

Ridley AJ. (1994). Membrane ruffling and signal transduction. *BioEssays : news and reviews in molecular, cellular and developmental biology* **16,** 321-327.

Ridley AJ. (1994). Signal transduction through the GTP-binding proteins Rac and Rho. *Journal of cell science Supplement* **18,** 127-131.

Ridley AJ. (2001). Rho GTPases and cell migration. *Journal of cell science* **114,** 2713-2722.

Ridley AJ, Comoglio PM & Hall A. (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Molecular and cellular biology* **15,** 1110-1122.

Riedl MA, Saxon A & Diaz-Sanchez D. (2009). Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. *Clin Immunol* **130,** 244-251.

Riedy MC, Brown MC, Molloy CJ & Turner CE. (1999). Activin A and TGF-beta stimulate phosphorylation of focal adhesion proteins and cytoskeletal reorganization in rat aortic smooth muscle cells. *Experimental cell research* **251,** 194-202.

Joseph-Silverstein, J. and. Rifkin DB (1987) Endothelial cell growth factors and the vessel wall. *Semin Thromb Hemost* **13**: 504-513.

Roberts AB & Sporn MB. (1989). Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-beta. *The American review of respiratory disease* **140,** 1126-1128.

Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA & Hansson GK. (2003). Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *The Journal of clinical investigation* **112,** 1342-1350.

Rocic P & Lucchesi PA. (2005). NAD(P)H oxidases and TGF-beta-induced cardiac fibroblast differentiation: Nox-4 gets Smad. *Circulation research* **97,** 850-852.

Roelofs M, Faggian L, Pampinella F, Paulon T, Franch R, Chiavegato A & Sartore S. (1998). Transforming growth factor beta1 involvement in the conversion of fibroblasts to smooth muscle cells in the rabbit bladder serosa. *The Histochemical journal* **30,** 393-404.

Rojo AI, Medina-Campos ON, Rada P, Zuniga-Toala A, Lopez-Gazcon A, Espada S, Pedraza-Chaverri J & Cuadrado A. (2012). Signaling pathways activated by the phytochemical nordihydroguaiaretic acid contribute to a Keap1-independent regulation of Nrf2 stability: Role of glycogen synthase kinase-3. *Free radical biology & medicine* **52,** 473-487.

Rojo AI, Rada P, Egea J, Rosa AO, Lopez MG & Cuadrado A. (2008). Functional interference between glycogen synthase kinase-3 beta and the transcription factor Nrf2 in protection against kainate-induced hippocampal cell death. *Molecular and cellular neurosciences* **39,** 125-132.

Ross D (2004) Quinone reductases multitasking in the metabolic world. *Drug Metab Rev* **3-4**, 639-54.

Ross R. (1999). Atherosclerosis--an inflammatory disease. *The New England journal of medicine* **340,** 115-126.

Rost M, Karge E & Klinger W. (1998). What do we measure with luminol-, lucigenin- and penicillin-amplified chemiluminescence? 1. Investigations with hydrogen peroxide and sodium hypochlorite. *Journal of bioluminescence and chemiluminescence* **13,** 355-363.

Roum JH, Buhl R, McElvaney NG, Borok Z & Crystal RG. (1993). Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol* **75,** 2419-2424.

Rousar, T., O. Kucera, et al. (2012). Assessment of reduced glutathione: comparison of an optimized fluorometric assay with enzymatic recycling method."*Anal Biochem* **423**(2): 236-240.

Ruiz-Ortega, M., J. Rodriguez-Vita, et al. (2007). TGF-beta signaling in vascular fibrosis. *Cardiovasc Res* **74**(2): 196-206

Ryan ST, Koteliansky VE, Gotwals PJ & Lindner V. (2003). Transforming growth factor-beta-dependent events in vascular remodeling following arterial injury. *Journal of vascular research* **40,** 37-46.

Safdar A, deBeer J & Tarnopolsky MA. (2010). Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old. *Free radical biology & medicine* **49,** 1487-1493.

Sakao K & Singh SV. (2012). D,L-sulforaphane-induced apoptosis in human breast cancer cells is regulated by the adapter protein p66Shc. *Journal of cellular biochemistry* **113,** 599-610.

Sampson N, Koziel R, Zenzmaier C, Bubendorf L, Plas E, Jansen-Durr P & Berger P. (2011). ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. *Mol Endocrinol* **25,** 503-515.

Sandbo N, Kregel S, Taurin S, Bhorade S and Dulin NO (2009) Critical role of serum response factor in pulmonary myofibroblast differentiation induced by TGF-beta. *Am J Respir Cell Mol Biol* **3,** 332-8.

Sanchez A, Alvarez AM, Benito M & Fabregat I. (1997). Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor beta in fetal rat hepatocytes in primary culture. *Hepatology* **26,** 935-943.

Sander EE, ten Klooster JP, van Delft S, van der Kammen RA & Collard JG. (1999). Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *The Journal of cell biology* **147,** 1009-1022.

Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T & Ishii S. (1999). ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. *The Journal of biological chemistry* **274,** 8949-8957.

Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, Bedosky KM, Freed DH, Kardami E & Dixon IM. (2010). Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Developmental dynamics : an official publication of the American Association of Anatomists* **239,** 1573-1584.

Sarada S, Himadri P, Mishra C, Geetali P, Ram MS and Ilavazhagan G (2008) Role of oxidative stress and NFkB in hypoxia-induced pulmonary edema. *Exp Biol Med* **9,** 1088-98.

Sarsour EH, Venkataraman S, Kalen AL, Oberley LW & Goswami PC. (2008). Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. *Aging cell* **7,** 405-417.

Sartore S, Chiavegato A, Faggin E, Franch R, Puato M, Ausoni S & Pauletto P. (2001). Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circulation research* **89,** 1111-1121.

Sarzani R, Brecher P & Chobanian AV. (1989). Growth factor expression in aorta of normotensive and hypertensive rats. *The Journal of clinical investigation* **83,** 1404-1408.

Sass G, Soares MC, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, Kaczmarek E, Ritter T, Volk HD & Tiegs G. (2003). Heme oxygenase-1 and its reaction product, carbon monoxide, prevent inflammation-related apoptotic liver damage in mice. *Hepatology* **38,** 909-918.

Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH & Soares MP. (2001). Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol* **166,** 4185-4194.

Sato M, Muragaki Y, Saika S, Roberts AB & Ooshima A. (2003). Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *The Journal of clinical investigation* **112,** 1486-1494.

Satoh T, Okamoto SI, Cui J, Watanabe Y, Furuta K, Suzuki M, Tohyama K & Lipton SA. (2006). Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophilic [correction of electrophillic] phase II inducers. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 768-773.

Schaller V, Weber C, Semmrich C, Frey E & Bausch AR. (2010). Polar patterns of driven filaments. *Nature* **467,** 73-77.

Schmierer B & Hill CS. (2005). Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. *Molecular and cellular biology* **25,** 9845-9858.

Schmitt-Graff A, Desmouliere A & Gabbiani G. (1994). Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. *Virchows Archiv : an international journal of pathology* **425,** 3-24.

Schroder K. (2010). Isoform specific functions of Nox protein-derived reactive oxygen species in the vasculature. *Current opinion in pharmacology* **10,** 122-126.

Schroder K, Helmcke I, Palfi K, Krause KH, Busse R & Brandes RP. (2007). Nox1 mediates basic fibroblast growth factor-induced migration of vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* **27,** 1736-1743.

Schurch W, Seemayer TA, Lagace R & Gabbiani G. (1984). The intermediate filament cytoskeleton of myofibroblasts: an immunofluorescence and ultrastructural study. *Virchows Archiv A, Pathological anatomy and histopathology* **403,** 323-336.

Schwartz SM, deBlois D & O'Brien ER. (1995). The intima. Soil for atherosclerosis and restenosis. *Circulation research* **77,** 445-465.

Scott NA, Cipolla GD, Ross CE, Dunn B, Martin FH, Simonet L and Wilcox JN (1996) Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries. *Circulation* **12,** 2178-87.

Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH and Gelbart WM (1995) Genetic characterisation and cloning of mothers against dpp, a gene required for decapentaplegic function in drosophila melanogaster *Genetics* **139,** 1347-1358.

Sekhar KR, Crooks PA, Sonar VN, Friedman DB, Chan JY, Meredith MJ, Starnes JH, Kelton KR, Summar SR, Sasi S & Freeman ML. (2003). NADPH oxidase activity is essential for Keap1/Nrf2-mediated induction of GCLC in response to 2-indol-3-yl-methylenequinuclidin-3-ols. *Cancer research* **63,** 5636-5645.

Selmeci L, Antal M, Horkay F, Merkely B, Szokodi I, Bíró L, Székely M, Jobbágy J, Szépvölgyi J and Tóth M (2000) Enhanced accumulation of pericardial fluid ferritin in patients with coronary artery disease. *Coron Artery Dis.* **1,** 53-6.

Senanayake GV, Banigesh A, Wu L, Lee P & Juurlink BH. (2012). The dietary phase 2 protein inducer sulforaphane can normalize the kidney epigenome and improve blood pressure in hypertensive rats. *American journal of hypertension* **25,** 229-235.

Sentman ML, Brannstrom T, Westerlund S, Laukkanen MO, Yla-Herttuala S, Basu S & Marklund SL. (2001). Extracellular superoxide dismutase deficiency and atherosclerosis in mice. *Arteriosclerosis, thrombosis, and vascular biology* **21,** 1477-1482.

Serini G & Gabbiani G. (1999). Mechanisms of myofibroblast activity and phenotypic modulation. *Experimental cell research* **250,** 273-283.

Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Forro L, Schlegel W & Krause KH. (2007). NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *The Biochemical journal* **406,** 105-114.

Serviddio G, Pereda J, Pallardo FV, Carretero J, Borras C, Cutrin J, Vendemiale G, Poli G, Vina J & Sastre J. (2004). Ursodeoxycholic acid protects against secondary biliary cirrhosis in rats by preventing mitochondrial oxidative stress. *Hepatology* **39,** 711-720.

Sestili P, Paolillo M, Lenzi M, Colombo E, Vallorani L, Casadei L, Martinelli C & Fimognari C. (2010). Sulforaphane induces DNA single strand breaks in cultured human cells. *Mutation research* **689,** 65-73.

Seth S, Johns R & Templin MV. (2012). Delivery and biodistribution of siRNA for cancer therapy:

challenges and future prospects. *Therapeutic delivery* **3,** 245-261.

Shah AM and Ray R (2005) NADPH oxidase and endothelial cell function *Clinical Science* 109, 217-226.

Shan Y, Lambrecht RW, Donohue SE & Bonkovsky HL. (2006). Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20,** 2651-2653.

Sharma A, Rodier JT, Tandon A, Klibanov AM & Mohan RR. (2012). Attenuation of corneal myofibroblast development through nanoparticle-mediated soluble transforming growth factor-beta type II receptor (sTGFbetaRII) gene transfer. *Molecular vision* **18,** 2598-2607.

Shaw JP & Chou IN. (1986). Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. *Journal of cellular physiology* **129,** 193-198.

Shephard P, Hinz B, Smola-Hess S, Meister JJ, Krieg T & Smola H. (2004). Dissecting the roles of endothelin, TGF-beta and GM-CSF on myofibroblast differentiation by keratinocytes. *Thrombosis and haemostasis* **92,** 262-274.

Shephard P, Martin G, Smola-Hess S, Brunner G, Krieg T & Smola H. (2004). Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. *The American journal of pathology* **164,** 2055-2066.

Shi Y & Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113,** 685-700.

Shi Y, Niculescu R, Wang D, Ormont M, Magno M, San Antonio JD, Williams KJ & Zalewski A. (2000). Myofibroblast involvement in glycosaminoglycan synthesis and lipid retention during coronary repair. *Journal of vascular research* **37,** 399-407.

Shi Y, Niculescu R, Wang D, Patel S, Davenpeck KL & Zalewski A. (2001). Increased NAD(P)H oxidase and reactive oxygen species in coronary arteries after balloon injury. *Arteriosclerosis, thrombosis, and vascular biology* **21,** 739-745.

Shi Y, O'Brien JE, Jr., Ala-Kokko L, Chung W, Mannion JD & Zalewski A. (1997). Origin of extracellular matrix synthesis during coronary repair. *Circulation* **95,** 997-1006.

Shi Y, O'Brien JE, Fard A, Mannion JD, Wang D & Zalewski A. (1996). Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. *Circulation* **94,** 1655-1664.
Shi Y, O'Brien JE, Jr., Fard A & Zalewski A. (1996). Transforming growth factor-beta 1 expression and myofibroblast formation during arterial repair. *Arteriosclerosis, thrombosis, and vascular biology* **16,** 1298-1305.

Shi Y, Pieniek M, Fard A, O'Brien J, Mannion JD & Zalewski A. (1996). Adventitial remodeling after coronary arterial injury. *Circulation* **93,** 340-348.

Shi W, Sun C and He B (2004) GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. *J Cell Biol* 2004 **164,** 291-300.

Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K & Ghosh S. (2005). TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes & development* **19,** 2668-2681.

Shin DH, Park HM, Jung KA, Choi HG, Kim JA, Kim DD, Kim SG, Kang KW, Ku SK, Kensler TW & Kwak MK. (2010). The NRF2-heme oxygenase-1 system modulates cyclosporin A-induced epithelial-mesenchymal transition and renal fibrosis. *Free radical biology & medicine* **48,** 1051-1063.

Shin I, Bakin AV, Rodeck U, Brunet A & Arteaga CL. (2001). Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. *Molecular biology of the cell* **12,** 3328-3339.

Siegel PM & Massague J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature reviews Cancer* **3,** 807-821.

Silva M, Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana F and Jeffrey L (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFb receptor. *Cell* **95,** 779–791.

Simeone DM, Zhang L, Graziano K, Nicke B, Pham T, Schaefer C & Logsdon CD. (2001). Smad4 mediates activation of mitogen-activated protein kinases by TGF-beta in pancreatic acinar cells. *American journal of physiology Cell physiology* **281,** C311-319.

Simeone DM, Zhang L, Graziano K, Nicke B, Pham T, Schaefer C & Logsdon CD. (2001). Smad4 mediates activation of mitogen-activated protein kinases by TGF-beta in pancreatic acinar cells. *American journal of physiology Cell physiology* **281,** C311-319.

Sin S, Bonin F, Petit V, Meseure D, Lallemand F, Bieche I, Bellahcene A, Castronovo V, de Wever O, Gespach C, Lidereau R & Driouch K. (2011). Role of the focal adhesion protein kindlin-1 in breast cancer growth and lung metastasis. *Journal of the National Cancer Institute* **103,** 1323-1337.

Singh S, Vrishni S, Singh BK, Rahman I & Kakkar P. (2010). Nrf2-ARE stress response mechanism: a control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases. *Free radical research* **44,** 1267-1288.

Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, Zeng Y, Watkins SC, Johnson CS, Trump DL, Lee YJ, Xiao H & Herman-Antosiewicz A. (2005). Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. *The Journal of biological chemistry* **280,** 19911-19924.

Sinha S, Heagerty AM, Shuttleworth CA & Kielty CM. (2002). Expression of latent TGF-beta binding proteins and association with TGF-beta 1 and fibrillin-1 following arterial injury. *Cardiovascular Res.* **53,** 971-983.

Siow RC & Churchman AT. (2007). Adventitial growth factor signalling and vascular remodelling: potential of perivascular gene transfer from the outside-in. *Cardiovascular research* **75,** 659-668.

Siow RC, Mallawaarachchi CM & Weissberg PL. (2003). Migration of adventitial myofibroblasts following vascular balloon injury: insights from in vivo gene transfer to rat carotid arteries. *Cardiovascular research* **59,** 212-221.

Siow RC & Mann GE. (2010). Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? *Molecular aspects of medicine* **31,** 468-477.

Skutek M, van Griensven M, Zeichen J, Brauer N and Bosch U (2001) Cyclic mechanical stretching modulates secretion pattern of growth factors in human tendon fibroblasts. *Eur J Appl Physiol* **1,** 48-52.

Sivakumar P, Czirok A, Rongish BJ, Divakara VP, Wang YP & Dallas SL. (2006). New insights into extracellular matrix assembly and reorganization from dynamic imaging of extracellular matrix proteins in living osteoblasts. *Journal of cell science* **119,** 1350-1360.

Smith JD, Bryant SR, Couper LL, Vary CP, Gotwals PJ, Koteliansky VE & Lindner V. (1999). Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circulation research* **84,** 1212-1222.

Smith JD, Bryant SR, Couper LL, Vary CP, Gotwals PJ, Koteliansky VE & Lindner V. (1999). Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circulation research* **84,** 1212-1222.

Smith PC, Caceres M & Martinez J. (2006). Induction of the myofibroblastic phenotype in human gingival fibroblasts by transforming growth factor-beta1: role of RhoA-ROCK and c-Jun N-terminal kinase signaling pathways. *Journal of periodontal research* **41,** 418-425.

Smithies O & Maeda N (1995)Gene targeting approaches to complex diseases: atherosclerosis andessential hypertension. *Proc Natl Acad Sci USA* **92,** 5266–5272.

So HS, Kim HJ, Lee JH, Lee JH, Park SY, Park C, Kim YH, Kim JK, Lee KM, Kim KS, Chung SY, Jang WC, Moon SK, Chung HT & Park RK. (2006). Flunarizine induces Nrf2-mediated transcriptional activation of heme oxygenase-1 in protection of auditory cells from cisplatin. *Cell death and differentiation* **13,** 1763-1775.

Song H, Guo B, Zhang J & Song C. (2010). Transforming growth factor-beta suppressed Id-1 Expression in a smad3-dependent manner in LoVo cells. *Anat Rec (Hoboken)* **293,** 42-47.

Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR & Griendling KK. (2002). Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* **105,** 1429-1435.

Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH & Landstrom M. (2008). The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nature cell biology* **10,** 1199-1207.

Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P & Heldin CH. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *The Journal of biological chemistry* **272,** 28107-28115.

Spagnoli LG, Bonanno E, Sangiorgi G & Mauriello A. (2007). Role of inflammation in atherosclerosis. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **48,** 1800-1815.

Sprague AH & Khalil RA. (2009). Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochemical pharmacology* **78,** 539-552.

Stanyon CA & Bernard O. (1999). LIM-kinase1. *The international journal of biochemistry & cell biology* **31,** 389-394.

Steinberg D. (1993). Antioxidant vitamins and coronary heart disease. *The New England journal of medicine* **328,** 1487-1489.

Steinberg D. (2000). Is there a potential therapeutic role for vitamin E or other antioxidants in atherosclerosis? *Current opinion in lipidology* **11,** 603-607.
Steinhubl SR. (2008). Why have antioxidants failed in clinical trials? *The American journal of cardiology* **101,** 14D-19D.

Stenmark KR, Davie N, Frid M, Gerasimovskaya E & Das M. (2006). Role of the adventitia in pulmonary vascular remodeling. *Physiology (Bethesda)* **21,** 134-145.

Stenmark KR, Frid MG, Yeager M, Li M, Riddle S, McKinsey T & El Kasmi KC. (2012). Targeting the adventitial microenvironment in pulmonary hypertension: A potential approach to therapy that considers epigenetic change. *Pulmonary circulation* **2,** 3-14.

Stenmark KR, Yeager ME, El Kasmi KC, Nozik-Grayck E, Gerasimovskaya EV, Li M, Riddle SR & Frid MG. (2013). The adventitia: essential regulator of vascular wall structure and function. *Annual review of physiology* **75,** 23-47.

Stephens P, Genever PG, Wood EJ & Raxworthy MJ. (1997). Integrin receptor involvement in actin cable formation in an in vitro model of events associated with wound contraction. *The international journal of biochemistry & cell biology* **29,** 121-128.

Stewart D, Killeen E, Naquin R, Alam S & Alam J. (2003). Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *The Journal of biological chemistry* **278,** 2396-2402.

Stockwell BR & Schreiber SL. (1998). Probing the role of homomeric and heteromeric receptor interactions in TGF-beta signaling using small molecule dimerizers. *Current biology : CB* **8,** 761-770.

Stocker R and Perrella M (2006) Heme Oxygenase-1: A novel drug target for atherosclerotic diseases? *Circulation* **114,** 2178-89.

Strachan GD, Ostrow LA & Jordan-Sciutto KL. (2005). Expression of the fetal Alz-50 clone 1 protein induces apoptotic cell death. *Biochemical and biophysical research communications* **336,** 490-495.

Stralin P, Karlsson K, Johansson BO & Marklund SL. (1995). The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arteriosclerosis, thrombosis, and vascular biology* **15,** 2032-2036.

Strauss BH & Rabinovitch M. (2000). Adventitial fibroblasts: defining a role in vessel wall remodeling. *American journal of respiratory cell and molecular biology* **22,** 1-3.

Strutz F, Zeisberg M, Renziehausen A, Raschke B, Becker V, van Kooten C & Muller G. (2001). TGF-beta 1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2). *Kidney international* **59,** 579-592.

Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, Kennedy TP & Hoidal JR. (2006). Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *American journal of physiology Lung cellular and molecular physiology* **290,** L661-L673.

Su JZ, Fukuda N, Hu WY & Kanmatsuse K. (2000). Ribozyme to human TGF-beta1 mRNA inhibits the proliferation of human vascular smooth muscle cells. *Biochemical and biophysical research communications* **278,** 401-407.

Sugiura H, Ichikawa T, Liu X, Kobayashi T, Wang XQ, Kawasaki S, Togo S, Kamio K, Mao L, Ann Y, Ichinose M & Rennard SI. (2009). N-acetyl-L-cysteine inhibits TGF-beta1-induced profibrotic responses in fibroblasts. *Pulmonary pharmacology & therapeutics* **22,** 487-491.

Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM & Hagen TM. (2004). Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 3381-3386.

Sun AJ, Gao PJ, Liu JJ, Ji KD & Zhu DL. (2004). [Osteopontin enhances migratory ability of cultured aortic adventitial fibroblasts from spontaneously hypertensive rats]. *Sheng li xue bao : [Acta physiologica Sinica]* **56,** 21-24.

Sun DX, Liu Z, Tan XD, Cui DX, Wang BS & Dai XW. (2012). Nanoparticle-mediated local delivery of an antisense TGF-beta1 construct inhibits intimal hyperplasia in autogenous vein grafts in rats. *PloS one* **7,** e41857.

Sun J, Brand M, Zenke Y, Tashiro S, Groudine M & Igarashi K. (2004). Heme regulates the dynamic

exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 1461-1466.

Sun L, Shi T, Qiao H, Jiang X, Jiang H, Krissansen GW & Sun X. (2011). Hepatic overexpression of heme oxygenase-1 improves liver allograft survival by expanding T regulatory cells. *The Journal of surgical research* **166,** e187-194.

Sun Z, Chin YE & Zhang DD. (2009). Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Molecular and cellular biology* **29,** 2658-2672.

Sun Z, Huang Z & Zhang DD. (2009). Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. *PloS one* **4,** e6588.

Sundaresan M, Yu ZX, Ferrans VJ, Irani K & Finkel T. (1995). Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science* **270,** 296-299.

Suraneni P, Rubinstein B, Unruh JR, Durnin M, Hanein D & Li R. (2012). The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *The Journal of cell biology* **197,** 239-251.

Suzuki C, Murakami G, Fukuchi M, Shimanuki T, Shikauchi Y, Imamura T, Miyazono K (2002) Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem.* **42,** 39919-25.

Suzuki H, DeLano FA, Parks DA, Jamshidi N, Granger DN, Ishii H, Suematsu M, Zweifach BW and Schmid-Schönbein GW (1998) Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proc Natl Acad Sci* **8,** 4754-9.

Suzuki K, Wilkes MC, Garamszegi N, Edens M & Leof EB. (2007). Transforming growth factor beta signaling via Ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses. *Cancer research* **67,** 3673-3682.

Szocs K, Lassegue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD & Griendling KK. (2002). Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arteriosclerosis, thrombosis, and vascular biology* **22,** 21-27.

Takac I, Schroder K & Brandes RP. (2012). The Nox family of NADPH oxidases: friend or foe of the vascular system? *Current hypertension reports* **14,** 70-78.

Takac I, Schroder K, Zhang L, Lardy B, Anilkumar N, Lambeth JD, Shah AM, Morel F & Brandes RP. (2011). The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. *The Journal of biological chemistry* **286,** 13304-13313.

Takaya K, Suzuki T, Motohashi H, Onodera K, Satomi S, Kensler TW & Yamamoto M. (2012). Validation of the multiple sensor mechanism of the Keap1-Nrf2 system. *Free radical biology & medicine* **53,** 817-827.

Takehara K, LeRoy EC & Grotendorst GR. (1987). TGF-beta inhibition of endothelial cell proliferation: alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell* **49,** 415-422.

Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K & Saito H. (2002). Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *The EMBO journal* **21,** 6473-6482.

Tanaka N, Ikeda Y, Ohta Y, Deguchi K, Tian F, Shang J, Matsuura T & Abe K. (2011). Expression of Keap1-Nrf2 system and antioxidative proteins in mouse brain after transient middle cerebral artery occlusion. *Brain research* **1370,** 246-253.

Tapia E, Soto V, Ortiz-Vega KM, Zarco-Marquez G, Molina-Jijon E, Cristobal-Garcia M, Santamaria J, Garcia-Nino WR, Correa F, Zazueta C & Pedraza-Chaverri J. (2012). Curcumin induces Nrf2 nuclear translocation and prevents glomerular hypertension, hyperfiltration, oxidant stress, and the decrease in antioxidant enzymes in 5/6 nephrectomized rats. *Oxidative medicine and cellular longevity* **2012,** 269039.

Tarpey M and Fridovich I (2001) Methods of detection of vascular reactive oxygen species: Nitric oxide, Superoxide, hydrogen peroxide and peroxynitrite *Circulation Res* **89,** 224-236.

Taylor S, Wakem M, Dijkman G, Alsarraj M & Nguyen M. (2010). A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods* **50,** S1-5.

ten Dijke P, Goumans MJ & Pardali E. (2008). Endoglin in angiogenesis and vascular diseases. *Angiogenesis* **11,** 79-89.

Teng J, Fukuda N, Hu WY, Nakayama M, Kishioka H & Kanmatsuse K. (2000). DNA-RNA chimeric hammerhead ribozyme to transforming growth factor-beta(1) mRNA inhibits the exaggerated growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Cardiovascular research* **48,** 138-147.

Tew KD. (2011). NrF2 Keap1 as gatekeepers of redox homeostasis - do they prevent or cause cancer? *Pigment cell & melanoma research* **24,** 1078-1079.

Thacher T, da Silva RF and Stergiopulos N (2009) Differential effects of reduced cyclic stretch and perturbed shear stress within the arterial wall and on smooth muscle function. *American Journal of Hypertension* **2,** 1250–1257.

Thakur N, Sorrentino A, Heldin CH & Landstrom M. (2009). TGF-beta uses the E3-ligase TRAF6 to turn on the kinase TAK1 to kill prostate cancer cells. *Future Oncol* **5,** 1-3.

Thannickal VJ, Aldweib KD & Fanburg BL. (1998). Tyrosine phosphorylation regulates H2O2 production in lung fibroblasts stimulated by transforming growth factor beta1. *The Journal of biological chemistry* **273,** 23611-23615.

Thannickal VJ & Fanburg BL. (1995). Activation of an H2O2-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *The Journal of biological chemistry* **270,** 30334-30338.

Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM & Thomas PE. (2003). Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *The Journal of biological chemistry* **278,** 12384-12389.

Thejass P & Kuttan G. (2006). Antimetastatic activity of Sulforaphane. *Life sciences* **78,** 3043-3050.

Theodore M, Kawai Y, Yang J, Kleshchenko Y, Reddy SP, Villalta F & Arinze IJ. (2008). Multiple nuclear localization signals function in the nuclear import of the transcription factor Nrf2. *The Journal of biological chemistry* **283,** 8984-8994.

Thews O, Lambert C, Kelleher DK, Biesalski HK, Vaupel P & Frank J. (2009). Impact of reactive oxygen species on the expression of adhesion molecules in vivo. *Advances in experimental medicine and biology* **645,** 95-100.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M & Biswal S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer research* **62,** 5196-5203.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M & Biswal S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer research* **62,** 5196-5203.

Thom SR, Fisher D, Xu YA, Notarfrancesco K & Ischiropoulos H. (2000). Adaptive responses and

apoptosis in endothelial cells exposed to carbon monoxide. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 1305-1310.

Thomas M, Gavrila D, McCormick ML, Miller FJ Jr, Daugherty A, Cassis LA, Dellsperger KC and Weintraub NL (2006) Deletion of p47phox attenuates angiotensin II-induced abdominal aortic aneurysm formation in apolipoprotein E-deficient mice. *Circulation* **114,** 404–413.

Thorpe GH and Kricka LJ (1986) Enhanced chemiluminescent reactions catalyzed by horseradish peroxidase. *Methods Enzymol* **133,** 331-53.

Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J & Engelhardt S. (2008). MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature* **456,** 980-984.

Tian H, Zhang B, Di J, Jiang G, Chen F, Li H, Li L, Pei D & Zheng J. (2012). Keap1: one stone kills three birds Nrf2, IKKbeta and Bcl-2/Bcl-xL. *Cancer letters* **325,** 26-34.

Tigges U, Komatsu M & Stallcup WB. (2013). Adventitial pericyte progenitor/mesenchymal stem cells participate in the restenotic response to arterial injury. *Journal of vascular research* **50,** 134-144.

Tobar N, Guerrero J, Smith PC & Martinez J. (2010). NOX4-dependent ROS production by stromal mammary cells modulates epithelial MCF-7 cell migration. *British journal of cancer* **103,** 1040-1047.

Toda S, Matsumura S, Fujitani N, Nishimura T, Yonemitsu N & Sugihara H. (1997). Transforming growth factor-beta1 induces a mesenchyme-like cell shape without epithelial polarization in thyrocytes and inhibits thyroid folliculogenesis in collagen gel culture. *Endocrinology* **138,** 5561-5575.

Tomaro ML and Batlle AM (2002) Bilirubin: its role in cytoprotection against oxidative stress. *The Int J of Biochem & Cell Biol* **34,** 216-20.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C & Brown RA. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature reviews Molecular cell biology* **3,** 349-363.

Tomczyk J & Olejnik A. (2010). [Sulforaphane--a possible agent in prevention and therapy of cancer]. *Postepy Hig Med Dosw (Online)* **64,** 590-603.

Tong KI, Katoh Y, Kusunoki H, Itoh K, Tanaka T and Yamamoto M (2006) Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Mol Cell Bio* **8,** 2887-900 (a).

Tong KI, Kobayashi A, Katsuoka F and Yamamoto M (2006) Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol Chem* **10-11,** 1311-20 (b).

Tong KI, Padmanabhan B, Kobayashi A, Shang C, Hirotsu Y, Yokoyama S and Yamamoto M (2007) Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol.* **21,** 7511-21.

Tong X & Schroder K. (2009). NADPH oxidases are responsible for the failure of nitric oxide to inhibit migration of smooth muscle cells exposed to high glucose. *Free radical biology & medicine* **47,** 1578-1583.

Torchinsky MB, Garaude J, Martin AP & Blander JM. (2009). Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature* **458,** 78-82.

Torzewski M, Ochsenhirt V, Kleschyov AL, Oelze M, Daiber A, Li H, Rossmann H, Tsimikas S, Reifenberg K, Cheng F, Lehr HA, Blankenberg S, Förstermann U and Münzel T (2007) Deficiency of

glutathione peroxidase-1 accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **27,** 850-7.

Touhami A, Di Pascuale MA, Kawatika T, Del Valle M, Rosa RH, Jr., Dubovy S & Tseng SC. (2005). Characterisation of myofibroblasts in fibrovascular tissues of primary and recurrent pterygia. *The British journal of ophthalmology* **89,** 269-274.

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* **9,** 4350-4.

Traka M, Gasper AV, Melchini A, Bacon JR, Needs PW, Frost V, Chantry A, Jones AM, Ortori CA, Barrett DA, Ball RY, Mills RD & Mithen RF. (2008). Broccoli consumption interacts with GSTM1 to perturb oncogenic signalling pathways in the prostate. *PloS one* **3,** e2568.

Tsai S, Hollenbeck ST, Yamanouchi D, Herman BC, Edlin R, Ryer EJ, Wang C, Tsain S, Liu B & Kent KC. (2009). Arterial gene transfer of the TGF-beta signaling protein Smad3 induces adaptive remodeling following angioplasty: a role for CTGF. *Cardiovasc Res* **2,** 326-35.

Tse K & Ley K. (2012). Transforming growth factor-beta: transforming plaque to stability. *European heart journal*.

Tsuruda T, Kato J, Hatakeyama K, Masuyama H, Cao YN, Imamura T, Kitamura K, Asada Y & Eto T. (2005). Antifibrotic effect of adrenomedullin on coronary adventitia in angiotensin II-induced hypertensive rats. *Cardiovascular research* **65,** 921-929.

Tulis DA, Durante W, Liu X, Evans AJ, Peyton KJ & Schafer AI. (2001). Adenovirus-mediated heme oxygenase-1 gene delivery inhibits injury-induced vascular neointima formation. *Circulation* **104,** 2710-2715.

Tulis DA, Keswani AN, Peyton KJ, Wang H, Schafer AI & Durante W. (2005). Local administration of carbon monoxide inhibits neointima formation in balloon injured rat carotid arteries. *Cell Mol Biol (Noisy-le-grand)* **51,** 441-446.

Ungefroren H, Groth S, Sebens S, Lehnert H, Gieseler F & Fandrich F. (2011). Differential roles of Smad2 and Smad3 in the regulation of TGF-beta1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: control by Rac1. *Molecular cancer* **10,** 67.

Ungvari Z, Bailey-Downs L, Gautam T, Jimenez R, Losonczy G, Zhang C, Ballabh P, Recchia FA, Wilkerson DC, Sonntag WE, Pearson K, de Cabo R & Csiszar A. (2011). Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. *American journal of physiology Heart and circulatory physiology* **300,** H1133-1140.

Uruno A & Motohashi H. (2011). The Keap1-Nrf2 system as an in vivo sensor for electrophiles. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **25,** 153-160.

Ushio-Fukai M. (2006). Localizing NADPH oxidase-derived ROS. *Science's STKE : signal transduction knowledge environment* **2006,** re8.

Ushio-Fukai M. (2007). VEGF signaling through NADPH oxidase-derived ROS. *Antioxidants & redox signaling* **9,** 731-739.

Usuki J, Matsuda K, Azuma A, Kudoh S & Gemma A. (2012). Sequential analysis of myofibroblast differentiation and transforming growth factor-beta1/Smad pathway activation in murine pulmonary fibrosis. *Journal of Nippon Medical School = Nippon Ika Daigaku zasshi* **79,** 46-59.

Valster A, Tran NL, Nakada M, Berens ME, Chan AY & Symons M. (2005). Cell migration and invasion assays. *Methods* **37,** 208-215.

van den Borne SWM, Diez J, Blankesteijn W, Verjans J, Hofstra L and Narula J (2010) Myocardial remodeling after infarction: the role of myofibroblasts. *Nature Reviews Cardiology* **7,** 30-37.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3,** RESEARCH0034.

Vargas MR, Pehar M, Cassina P, Martinez-Palma L, Thompson JA, Beckman JS & Barbeito L. (2005). Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival. *The Journal of biological chemistry* **280,** 25571-25579.

Velichkova M & Hasson T. (2003). Keap1 in adhesion complexes. *Cell motility and the cytoskeleton* **56,** 109-119.

Velichkova M & Hasson T. (2005). Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Molecular and cellular biology* **25,** 4501-4513.

Venugopal R & Jaiswal AK. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 14960-14965.

Vermeulen M, Klopping-Ketelaars IW, van den Berg R & Vaes WH. (2008). Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. *Journal of agricultural and food chemistry* **56,** 10505-10509.

Verschueren K & Huylebroeck D. (1999). Remarkable versatility of Smad proteins in the nucleus of transforming growth factor-beta activated cells. *Cytokine & growth factor reviews* **10,** 187-199.

Villeneuve NF, Lau A & Zhang DD. (2010). Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. *Antioxidants & redox signaling* **13,** 1699-1712.

Vinals F & Pouyssegur J. (2001). Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling. *Molecular and cellular biology* **21,** 7218-7230.

Vogelmann R, Nguyen-Tat MD, Giehl K, Adler G, Wedlich D & Menke A. (2005). TGFbeta-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN. *Journal of cell science* **118,** 4901-4912.

Vomhof-Dekrey EE & Picklo MJ, Sr. (2012). The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism. *The Journal of nutritional biochemistry* **23,** 1201-1206.

Vomhof-DeKrey EE & Picklo MJ. (2012). NAD(P)H:quinone oxidoreductase 1 activity reduces hypertrophy in 3T3-L1 adipocytes. *Free radical biology & medicine* **53,** 690-700.

Vozenin-Brotons MC, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, Lefaix JL & Martin M. (2001). Antifibrotic action of Cu/Zn SOD is mediated by TGF-beta1 repression and phenotypic reversion of myofibroblasts. *Free radical biology & medicine* **30,** 30-42.
Wach F, Hein R, Adelmann-Grill BC & Krieg T. (1987). Inhibition of fibroblast chemotaxis by superoxide dismutase. *European journal of cell biology* **44,** 124-127.

Waghray M, Cui Z, Horowitz JC, Subramanian IM, Martinez FJ, Toews GB and Thannickal VJ (2005) Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. *FASEB J* **7,** 854-6.

Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, Kensler TW & Talalay P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 2040-2045.

Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, Harada T, Engel JD & Yamamoto M. (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nature genetics* **35,** 238-245.

Wakabayashi N, Shin S, Slocum SL, Agoston ES, Wakabayashi J, Kwak MK, Misra V, Biswal S, Yamamoto M & Kensler TW. (2010). Regulation of notch1 signaling by nrf2: implications for tissue regeneration. *Science signaling* **3,** ra52.

Wakabayashi N, Slocum SL, Skoko JJ, Shin S & Kensler TW. (2010). When NRF2 talks, who's listening? *Antioxidants & redox signaling* **13,** 1649-1663.

Walsh JE & Young MR. (2011). TGF-beta regulation of focal adhesion proteins and motility of premalignant oral lesions via protein phosphatase 1. *Anticancer research* **31,** 3159-3164.

Walsh MF, Ampasala DR, Hatfield J, Vander Heide R, Suer S, Rishi AK & Basson MD. (2008). Transforming growth factor-beta stimulates intestinal epithelial focal adhesion kinase synthesis via Smad- and p38-dependent mechanisms. *The American journal of pathology* **173,** 385-399.

Wang B, Zhu X, Kim Y, Li J, Huang S, Saleem S, Li RC, Xu Y, Dore S & Cao W. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free radical biology & medicine* **52,** 928-936.

Wang F, Tian F, Whitman SA, Zhang DD, Nishinaka T, Zhang N & Jiang T. (2012). Regulation of transforming growth factor beta1-dependent aldose reductase expression by the Nrf2 signal pathway in human mesangial cells. *European journal of cell biology* **91,** 774-781.

Wang H, Radjendirane V, Wary KK & Chakrabarty S. (2004). Transforming growth factor beta regulates cell-cell adhesion through extracellular matrix remodeling and activation of focal adhesion kinase in human colon carcinoma Moser cells. *Oncogene* **23,** 5558-5561.

Wang HD, Xu S, Johns DG, Du Y, Quinn MT, Cayatte AJ & Cohen RA. (2001). Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circulation research* **88,** 947-953.

Wang JH & Lin JS. (2007). Cell traction force and measurement methods. *Biomechanics and modeling in mechanobiology* **6,** 361-371.

Wang JN, Shi N & Chen SY. (2012). Manganese superoxide dismutase inhibits neointima formation through attenuation of migration and proliferation of vascular smooth muscle cells. *Free radical biology & medicine* **52,** 173-181.

Wang L, Chen Y, Sternberg P & Cai J. (2008). Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Investigative ophthalmology & visual science* **49,** 1671-1678.

Wang L, Lee JY, Kwak JH, He Y, Kim SI & Choi ME. (2008). Protective effects of low-dose carbon monoxide against renal fibrosis induced by unilateral ureteral obstruction. *American journal of physiology Renal physiology* **294,** F508-517.

Wang Q, Li J, Yang X, Sun H, Gao S, Zhu H, Wu J & Jin W. (2013). Nrf2 is associated with the regulation of basal transcription activity of the BRCA1 gene. *Acta biochimica et biophysica Sinica*.

Wang XL, Liu SX & Wilcken DE. (1997). Circulating transforming growth factor beta-1 and coronary artery disease. *Cardiovasc Res* **34,** 404–410.

Wang S, Wilkes MC, Leof EB & Hirschberg R. (2005). Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis in vivo. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19,** 1-11.
Wang Z, Castresana MR & Newman WH. (2001). Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells. *Biochemical and biophysical research communications* **285,** 669-674.

Warabi E, Takabe W, Minami T, Inoue K, Itoh K, Yamamoto M, Ishii T, Kodama T and Noguchi N (2007) Shear stress stabilizes NF-E2-related factor 2 and induces antioxidant genes in endothelial cells: role of reactive oxygen/nitrogen species. *Free Radic Biol Med.* **42,** 260-9.

Ward MR, Agrotis A, Kanellakis P, Dilley R, Jennings G & Bobik A. (1997). Inhibition of protein tyrosine kinases attenuates increases in expression of transforming growth factor-beta isoforms and their receptors following arterial injury. *Arteriosclerosis, thrombosis, and vascular biology* **17,** 2461-2470.

Ward MR, Pasterkamp G, Yeung AC & Borst C. (2000). Arterial remodeling. Mechanisms and clinical implications. *Circulation* **102,** 1186-1191.

Watabe T & Miyazono K. (2009). Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell research* **19,** 103-115.

Watai Y, Kobayashi A, Nagase H, Mizukami M, McEvoy J, Singer JD, Itoh K and Yamamoto M (2007) Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes Cells.* **10,**1163-78.

Watanabe M, Masuyama N, Fukuda M & Nishida E. (2000). Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO reports* **1,** 176-182.

Watkins SJ, Borthwick GM, Oakenfull R, Robson A & Arthur HM. (2012). Angiotensin II-induced cardiomyocyte hypertrophy in vitro is TAK1-dependent and Smad2/3-independent. *Hypertension research : official journal of the Japanese Society of Hypertension* **35,** 393-398.

Wattenberg LW. (1983). Inhibition of neoplasia by minor dietary constituents. *Cancer research* **43,** 2448s-2453s.

Weber DS, Taniyama Y, Rocic P, Seshiah PN, Dechert MA, Gerthoffer WT, Griendling KK (2004) Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. *Circ Res* **94**, 1219–1226.

Wen H, Blume PA and Sumpio BE (2009) Role of integrins and focal adhesion kinase in the orientation of dermal fibroblasts exposed to cyclic strain. *Int Wound J* **6,** 149-58.

Wenner CE & Yan S. (2003). Biphasic role of TGF-beta1 in signal transduction and crosstalk. *Journal of cellular physiology* **196,** 42-50.

Werner S & Grose R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiological reviews* **83,** 835-870.
Werth D, Grassi G, Konjer N, Dapas B, Farra R, Giansante C, Kandolf R, Guarnieri G, Nordheim A and Heidenreich O (2010) Proliferation of human primary vascular smooth muscle cells depends on serum response factor. *Eur J Cell Biol* **2-3,** 216-24.

Weston CR & Davis RJ. (2007). The JNK signal transduction pathway. *Current opinion in cell biology* **19,** 142-149.

Weyhe D, Hoffmann P, Belyaev O, Mros K, Muller C, Uhl W & Schmitz F. (2007). The role of TGF-beta1 as a determinant of foreign body reaction to alloplastic materials in rat fibroblast cultures: comparison of different commercially available polypropylene meshes for hernia repair. *Regulatory peptides* **138,** 10-14.

White AC, Das SK & Fanburg BL. (1992). Reduction of glutathione is associated with growth restriction and enlargement of bovine pulmonary artery endothelial cells produced by transforming growth factor-beta 1. *American journal of respiratory cell and molecular biology* **6,** 364-368.

White CR, Darley-Usmar V, Berrington WR, McAdams M, Gore JZ, Thompson JA, Parks DA, Tarpey MM and Freeman BA (1996) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc Natl Acad Sci* **16,** 8745-9.

Wilcox JN, Waksman R, King SB and Scott NA. (1996) The role of the adventitia in the arterial response to angioplasty: the effect of intravascular radiation. *Int J Radiat Oncol Biol Phys.* **4,** 789-96.

Wild AC, Moinova HR & Mulcahy RT. (1999). Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *The Journal of biological chemistry* **274,** 33627-33636.

Wilkes MC, Mitchell H, Penheiter SG, Dore JJ, Suzuki K, Edens M, Sharma DK, Pagano RE & Leof EB. (2005). Transforming growth factor-beta activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer research* **65,** 10431-10440.

Wilkinson FL, Liu Y, Rucka AK, Jeziorska M, Hoyland JA, Heagerty AM, Canfield AE & Alexander MY. (2007). Contribution of VCAF-positive cells to neovascularization and calcification in atherosclerotic plaque development. *The Journal of pathology* **211,** 362-369.

Williamson TP, Johnson DA & Johnson JA. (2012). Activation of the Nrf2-ARE pathway by siRNA knockdown of Keap1 reduces oxidative stress and provides partial protection from MPTP-mediated neurotoxicity. *Neurotoxicology* **33,** 272-279.

Willis Al Pierre-Paul D, Sumpio BE and Gahtan V (2004) Vascular smooth muscle cell migration: current research and clinical implications. *Vasc Endovascular Surg* **1,** 11-23.

Wipff PJ & Hinz B. (2009). Myofibroblasts work best under stress. *Journal of bodywork and movement therapies* **13,** 121-127.

Wipff PJ, Rifkin DB, Meister JJ & Hinz B. (2007). Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *The Journal of cell biology* **179,** 1311-1323.

Wirohadidjojo YW, Radiono S, Budiyanto A & Soebono H. (2011). Cellular viability, collagen deposition, and transforming growth factor beta1 production among ultraviolet B-irradiated keloid fibroblasts. *Aesthetic plastic surgery* **35,** 1050-1055.

Wolf YG, Rasmussen LM & Ruoslahti E. (1994). Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. *The Journal of clinical investigation* **93,** 1172-1178.

Worth DC and Parsons M (2008) Adhesion dynamics: mechanisms and measurements. *Int Journal of Biochem & Cell Biol* **40,** 2397–2409.

Wotton D, Lo RS, Lee S & Massague J. (1999). A Smad transcriptional corepressor. *Cell* **97,** 29-39.

Wotton D, Lo RS, Swaby LA & Massague J. (1999). Multiple modes of repression by the Smad transcriptional corepressor TGIF. *The Journal of biological chemistry* **274,** 37105-37110.

Wotton D & Massague J. (2001). Smad transcriptional corepressors in TGF beta family signaling. *Current topics in microbiology and immunology* **254,** 145-164.

Wrighton KH, Lin X & Feng XH. (2009). Phospho-control of TGF-beta superfamily signaling. *Cell research* **19,** 8-20.

Wu CS, Wu PH, Fang AH & Lan CC. (2012). FK506 inhibits the enhancing effects of transforming growth factor (TGF)-beta1 on collagen expression and TGF-beta/Smad signalling in keloid fibroblasts: implication for new therapeutic approach. *The British journal of dermatology* **167,** 532-541.

Wu G, Chen YG, Ozdamar B, Gyuricza CA, Chong PA, Wrana JL, Massague J & Shi Y. (2000). Structural basis of Smad2 recognition by the Smad anchor for receptor activation. *Science* **287,** 92-97.

Wu JW, Hu M, Chai J, Seoane J, Huse M, Li C, Rigotti DJ, Kyin S, Muir TW, Fairman R, Massagué J, Shi Y (2001) Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signalling. *Mol Cell.* **6,** 1277-89.

Wu L & Juurlink BH. (2001). The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats. *Journal of hypertension* **19,** 1819-1825.

Wu L, Noyan Ashraf MH, Facci M, Wang R, Paterson PG, Ferrie A & Juurlink BH. (2004). Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 7094-7099.

Wu ML, Ho YC & Yet SF. (2011). A central role of heme oxygenase-1 in cardiovascular protection. *Antioxidants & redox signaling* **15,** 1835-1846.

Wu RF, Xu YC, Ma Z, Nwariaku FE, Sarosi GA, Jr. & Terada LS. (2005). Subcellular targeting of oxidants during endothelial cell migration. *The Journal of cell biology* **171,** 893-904.

Wu WS, Wu JR & Hu CT. (2008). Signal cross talks for sustained MAPK activation and cell migration: the potential role of reactive oxygen species. *Cancer metastasis reviews* **27,** 303-314.

Wu XB, Li Y, Schneider A, Yu W, Rajendren G, Iqbal J, Yamamoto M, Alam M, Brunet LJ, Blair HC, Zaidi M & Abe E. (2003). Impaired osteoblastic differentiation, reduced bone formation, and severe osteoporosis in noggin-overexpressing mice. *The Journal of clinical investigation* **112,** 924-934.

Wu XG, Peng SB & Huang Q. (2012). [Transcriptional regulation of breast cancer resistance protein]. *Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji* **34,** 1529-1536.

Xiao J, Xiang Q, Xiao YC, Su ZJ, Huang ZF, Zhang QH, Tan Y, Li XK & Huang YD. (2010). The effect of transforming growth factor-beta1 on nasopharyngeal carcinoma cells: insensitive to cell growth but functional to TGF-beta/Smad pathway. *Journal of experimental & clinical cancer research : CR* **29,** 35.

Xiao L, Du Y, Shen Y, He Y, Zhao H & Li Z. (2012). TGF-beta 1 induced fibroblast proliferation is mediated by the FGF-2/ERK pathway. *Frontiers in bioscience : a journal and virtual library* **17,** 2667-2674.

Xiao Q, Luo Z, Pepe AE, Margariti A, Zeng L & Xu Q. (2009). Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H2O2. *American journal of physiology Cell physiology* **296,** C711-723.

Xie B, Li H, Wang Q, Xie S, Rahmeh A, Dai W & Lee MY. (2005). Further characterization of human DNA polymerase delta interacting protein 38. *The Journal of biological chemistry* **280,** 22375-22384.

Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH & Kong AN. (2006). Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer research* **66,** 8293-8296.

Xu C, Yuan X, Pan Z, Shen G, Kim JH, Yu S, Khor TO, Li W, Ma J & Kong AN. (2006). Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. *Molecular cancer therapeutics* **5,** 1918-1926.

Xu L, Chen YG & Massague J. (2000). The nuclear import function of Smad2 is masked by SARA and unmasked by TGFbeta-dependent phosphorylation. *Nature cell biology* **2,** 559-562.

Xu L, Kang Y, Col S & Massague J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Molecular cell* **10,** 271-282.

Xue M, Qian Q, Adaikalakoteswari A, Rabbani N, Babaei-Jadidi R & Thornalley PJ. (2008). Activation of NF-E2-related factor-2 reverses biochemical dysfunction of endothelial cells induced by hyperglycemia linked to vascular disease. *Diabetes* **57,** 2809-2817.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y & Koizumi S. (1999). Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *The Journal of clinical investigation* **103,** 129-135.

Yamada SD, Baldwin RL & Karlan BY. (1999). Ovarian carcinoma cell cultures are resistant to TGF-beta1-mediated growth inhibition despite expression of functional receptors. *Gynecologic oncology* **75,** 72-77.

Yamamoto K, Morishita R, Tomita N, Shimozato T, Nakagami H, Kikuchi A, Aoki M, Higaki J, Kaneda Y & Ogihara T. (2000). Ribozyme oligonucleotides against transforming growth factor-beta inhibited neointimal formation after vascular injury in rat model: potential application of ribozyme strategy to treat cardiovascular disease. *Circulation* **102,** 1308-1314.

Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H & Yamamoto M. (2008). Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. *Molecular and cellular biology* **28,** 2758-2770.

Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H & Yamamoto M. (2008). Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. *Molecular and cellular biology* **28,** 2758-2770.

Yamashita K, Ollinger R, McDaid J, Sakahama H, Wang H, Tyagi S, Csizmadia E, Smith NR, Soares MP & Bach FH. (2006). Heme oxygenase-1 is essential for and promotes tolerance to transplanted organs. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20,** 776-778.

Yamashita M, Fatyol K, Jin C, Wang X, Liu Z & Zhang YE. (2008). TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Molecular cell* **31,** 918-924.

Yanaka A. (2011). Sulforaphane enhances protection and repair of gastric mucosa against oxidative stress in vitro, and demonstrates anti-inflammatory effects on Helicobacter pylori-infected gastric mucosae in mice and human subjects. *Current pharmaceutical design* **17,** 1532-1540.

Yang H, Magilnick N, Lee C, Kalmaz D, Ou X, Chan JY & Lu SC. (2005). Nrf1 and Nrf2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via NF-kappaB and AP-1. *Molecular and cellular biology* **25,** 5933-5946.

Yang H, Magilnick N, Ou X & Lu SC. (2005). Tumour necrosis factor alpha induces co-ordinated activation of rat GSH synthetic enzymes via nuclear factor kappaB and activator protein-1. *The Biochemical journal* **391,** 399-408.

Yang HC & Pon LA. (2002). Actin cable dynamics in budding yeast. *Proceedings of the National Academy*

of Sciences of the United States of America **99,** 751-756.

Yang L, Li W, Wang S, Wang L, Li Y, Yang X & Peng R. (2012). Smad4 disruption accelerates keratinocyte reepithelialization in murine cutaneous wound repair. *Histochemistry and cell biology* **138,** 573-582.

Yang H, Roberts LJ, Shi MJ, Zhou LC, Ballard BR, Richardson A and Guo ZM (2004) Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. *Circ. Res.* **95,** 1075–1081.

Yang L, Qu M, Wang Y, Duan H, Chen P, Wang Y, Shi W, Danielson P & Zhou Q. (2013). Trichostatin A Inihibits TGF-beta-induced Reactive Oxygen Species Accumulation and Myofibroblast Differentiation Via Enhanced Nrf2-ARE Signaling. *Molecular pharmacology*.

Yao H, Wang H, Zhang Z, Jiang BH, Luo J & Shi X. (2008). Sulforaphane inhibited expression of hypoxia-inducible factor-1alpha in human tongue squamous cancer cells and prostate cancer cells. *International journal of cancer Journal international du cancer* **123,** 1255-1261.

Yao Q, Qu X, Yang Q, Wei M and Kong B. (2009) CLIC4 mediates TGF-beta1-induced fibroblast-to-myofibroblast transdifferentiation in ovarian cancer. *Oncol Rep* **3,** 541-8.

Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA & Talalay P. (2002). Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clinica chimica acta; international journal of clinical chemistry* **316,** 43-53.

Ye L, Zhang HY, Yang GH, Bu H & Guo H. (2005). [Effects of transforming growth factor-beta/Smad signaling on the growth and apoptosis of human rhabdomyosarcoma cell line RD]. *Zhonghua bing li xue za zhi Chinese journal of pathology* **34,** 407-412.

Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE & Perrella MA. (2003). Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17,** 1759-1761.

Yi JY, Shin I & Arteaga CL. (2005). Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **280,** 10870-10876.

Yijing L, Liu H, Yuan C, Wang B, Ren M, Yan L, Wang X & Zhang J. (2013). The effects of qindan-capsule-containing serum on the TGF-beta1/ERK signaling pathway, matrix metalloproteinase synthesis and cell function in adventitial fibroblasts. *Pharmaceutical biology* **51,** 712-721.

Yochum L, Kushi LH, Meyer K & Folsom AR. (1999). Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. *American journal of epidemiology* **149,** 943-949.

Yokoyama M, Inoue N & Kawashima S. (2000). Role of the vascular NADH/NADPH oxidase system in atherosclerosis. *Annals of the New York Academy of Sciences* **902,** 241-247; discussion 247-248.

Yoshida T, Watanabe M, Engelman DT, Engelman RM, Schley JA, Maulik N, Ho YS, Oberley TD and Das DK (1996) Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. *J Mol Cell Cardiol.* **8,** 1759-67.

Yoshida T, Maulik N, Engelman RM, Ho YS, Magnenat JL, Rousou JA, Flack JE, Deaton D and Das DK (1997) Glutathione peroxidase knockout mice are susceptible to myocardial ischemia reperfusion injury. *Circulation* **96,** 216-20.

Yu B, Bi L, Zheng B, Ji L, Chevalier D, Agarwal M, Ramachandran V, Li W, Lagrange T, Walker JC and Chen X (2008) The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. *Proc Natl Acad Sci* **29,** 10073-8.

Yu L, Hebert MC & Zhang YE. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-

independent TGF-beta responses. *The EMBO journal* **21,** 3749-3759.

Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J & Kong AN. (1999). Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *The Journal of biological chemistry* **274,** 27545-27552.

Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH & Kong AN. (2000). p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. *The Journal of biological chemistry* **275,** 2322-2327.

Yuan SM, Wang YQ, Shen Y & Jing H. (2011). Transforming growth factor-beta in graft vessels: histology and immunohistochemistry. *Clinics (Sao Paulo)* **66,** 895-901.

Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR and Griendling KK (1998) Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy *Hypertension* **3,** 488-495.

Zakkar M, Van der Heiden K, Luong le A, Chaudhury H, Cuhlmann S, Hamdulay SS, Krams R, Edirisinghe I, Rahman I, Carlsen H, Haskard DO, Mason JC & Evans PC. (2009). Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state. *Arteriosclerosis, thrombosis, and vascular biology* **29,** 1851-1857.

Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etayo JC & Diez J. (2000). Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* **35,** 1055-1061.

Zalewski A & Shi Y. (1997). Vascular myofibroblasts. Lessons from coronary repair and remodeling. *Arteriosclerosis, thrombosis, and vascular biology* **17,** 417-422.

Zanetti M, Sato J, Katusic ZS & O'Brien T. (2001). Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. *American journal of physiology Heart and circulatory physiology* **280,** H2516-2523.

Zanichelli F, Capasso S, Cipollaro M, Pagnotta E, Carteni M, Casale F, Iori R & Galderisi U. (2012). Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect. *Age (Dordr)* **34,** 281-293.

Zargham R (2008) Preventing restenosis after angioplasty: a multistage approach. *Clin Sci* **4,** 257-64.

Zarifis JH. (2005). Atherosclerosis, thrombosis, and inflammatory risk factors, from history and the laboratory to real life. *European heart journal* **26,** 317-318.

Zarjou A & Agarwal A. (2012). Heme oxygenase-1 as a target for TGF-beta in kidney disease. *Seminars in nephrology* **32,** 277-286.

Zhang DD. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug metabolism reviews* **38,** 769-789.
Zhang DD & Hannink M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Molecular and cellular biology* **23,** 8137-8151.

Zhang DD, Lo SC, Cross JV, Templeton DJ & Hannink M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Molecular and cellular biology* **24,** 10941-10953.

Zhang H & Forman HJ. (2009). Signaling pathways involved in phase II gene induction by alpha, beta-unsaturated aldehydes. *Toxicology and industrial health* **25,** 269-278.

Zhang J, Dinh TN, Kappeler K, Tsaprailis G & Chen QM. (2012). La autoantigen mediates oxidant induced de novo Nrf2 protein translation. *Molecular & cellular proteomics : MCP* **11,** M111 015032.

Zhang L, Zalewski A, Liu Y, Mazurek T, Cowan S, Martin JL, Hofmann SM, Vlassara H & Shi Y. (2003). Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries. *Circulation* **108,** 472-478.

Zhang M, Fraser D & Phillips A. (2006). ERK, p38, and Smad signaling pathways differentially regulate transforming growth factor-beta1 autoinduction in proximal tubular epithelial cells. *The American journal of pathology* **169,** 1282-1293.

Zhang R, Ruan D & Zhang C. (2006). Effects of TGF-beta1 and IGF-1 on proliferation of human nucleus pulposus cells in medium with different serum concentrations. *Journal of orthopaedic surgery and research* **1,** 9.

Zhang SS, Gong ZJ, Li WH, Wang X & Ling TY. (2012). Antifibrotic effect of curcumin in TGF-beta 1-induced myofibroblasts from human oral mucosa. *Asian Pacific journal of cancer prevention : APJCP* **13,** 289-294.

Zhang X, Chen X, Song H, Chen HZ & Rovin BH. (2005). Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. *European journal of immunology* **35,** 3258-3267.

Zhang X, Su Y, Zhang M & Sun Z. (2012). Opposite effects of arsenic trioxide on the Nrf2 pathway in oral squamous cell carcinoma in vitro and in vivo. *Cancer letters* **318,** 93-98.

Zhang Y. (2000). Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis* **21,** 1175-1182.

Zhang Y. (2001). Molecular mechanism of rapid cellular accumulation of anticarcinogenic isothiocyanates. *Carcinogenesis* **22,** 425-431.

Zhang Y & Callaway EC. (2002). High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. *The Biochemical journal* **364,** 301-307.

Zhang Y, Kensler TW, Cho CG, Posner GH & Talalay P. (1994). Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 3147-3150.

Zhang Y & Talalay P. (1998). Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer research* **58,** 4632-4639.

Zhang Y, Talalay P, Cho CG & Posner GH. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 2399-2403.

Zhang Y & Tang L. (2007). Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. *Acta pharmacologica Sinica* **28,** 1343-1354.
Zhang YE. (2009). Non-Smad pathways in TGF-beta signaling. *Cell research* **19,** 128-139.

Zhang R, Wu W, Luo S (2011) Different behaviors of glutathione in aqueous and DMSO solutions: Molecular dynamics simulation and NMR experimental study. *Journal of Solution Chemistry* **10:** 1784-1795.

Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J & Kalyanaraman B. (2005). Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 5727-5732.

Zhao J, Shi W, Wang YL, Chen H, Bringas P, Jr., Datto MB, Frederick JP, Wang XF & Warburton D. (2002). Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *American journal of physiology Lung cellular and molecular physiology* **282,** L585-593.

Zheng W, Seftor EA, Meininger CJ, Hendrix MJ and Tomanek RJ (2001) Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-beta. *Am J Physiol Heart Circ Physiol* **2,** H909-17.

Zheng H, Whitman SA, Wu W, Wondrak GT, Wong PK, Fang D & Zhang DD. (2011). Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes* **60,** 3055-3066.

Zhong C, Kinch MS & Burridge K. (1997). Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. *Molecular biology of the cell* **8,** 2329-2344.

Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, Varga J and Sznajder JI (2009) Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. *Am J Physiol Lung Cell Mol Physiol* **6,** L1120-30.

Zhu H, Itoh K, Yamamoto M, Zweier JL & Li Y. (2005). Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS letters* **579,** 3029-3036.

Zhu H, Jia Z, Strobl JS, Ehrich M, Misra HP & Li Y. (2008). Potent induction of total cellular and mitochondrial antioxidants and phase 2 enzymes by cruciferous sulforaphane in rat aortic smooth muscle cells: cytoprotection against oxidative and electrophilic stress. *Cardiovascular toxicology* **8,** 115-125.

Zhu H & Li Y. (2012). NAD(P)H: quinone oxidoreductase 1 and its potential protective role in cardiovascular diseases and related conditions. *Cardiovascular toxicology* **12,** 39-45.

Zipper LM & Mulcahy RT. (2000). Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochemical and biophysical research communications* **278,** 484-492.

Zipper LM & Mulcahy RT. (2003). Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. *Toxicological sciences : an official journal of the Society of Toxicology* **73,** 124-134.

Zou X, Feng Z, Li Y, Wang Y, Wertz K, Weber P, Fu Y & Liu J. (2012). Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways. *The Journal of nutritional biochemistry* **23,** 994-1006.

Zucker B, Hanusch J & Bauer G. (1997). Glutathione depletion in fibroblasts is the basis for apoptosis-induction by endogenous reactive oxygen species. *Cell death and differentiation* **4,** 388-395.

Zuo W & Chen YG. (2009). Specific activation of mitogen-activated protein kinase by transforming growth factor-beta receptors in lipid rafts is required for epithelial cell plasticity. *Molecular biology of the cell* **20,** 1020-1029.

Appendices

Appendices

Appendix I – Media composition

Table AI.1 Dulbecco's Phosphate Buffered Saline-10 x (D1408)

Inorganic salts	g/L	Molecular weight	mM
$CaCl_2 \bullet 2H_2O$	—		
$MgCl \bullet 6H_20$	—		
KCl	2.0	74.55	26.83
KH_2PO_4	2.0	136.07	14.70
NaCl	80	58.44	368.9
Na_2HPO_4	11.5	142	81.4

Other	g/L	Molecular weight	mM
D-Glucose	—		
Kanamyein Sulfate	—		
Penicillin G (sulfate)	—		
Pyruvic Acid•Na	—		
Streptomycin Sulfate	—		

REFERENCE

1. Dulbecco, R. And Vogt, M, (1957). Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exp.Med 106, 167-169.

Table AI.2 Dulbecco's Modified Eagle's Medium – low glucose (D5921)

Inorganic salts	g/L	Molecular weight	mM
$CaCl_2 \bullet 2H_20$	0.265	147	1.803
$Fe(NO_3)_3 \bullet 9H_2O$	0.0001	403.8	0.00025
$MgSO_2$	0.09767	120.4	0.8112
KCl	0.4	74.55	5.3655
$NaHCO_3$	3.7	84	44.048
NaCl	6.4	58.44	109.51
NaH_2PO_4	0.109	120	0.9083
Succinic Acid	—		
Sodium Succinate	—		

Amino acids	g/L	Molecular weight	mM
L-Arginine•HCl	0.084	210.7	0.3986
L-Cystine•2HCl	0.0626	313.2	0.1998
L-Glutamine	—		
Glycine	0.030	75.05	0.3997
L-Histidine•HCl•H_20	0.042	209.6	0.2004
L-Isoleucine	0.105	131.2	0.8003
L-Leucine	0.105	131.2	0.8003
L-Lysine •HCl	0.146	182.6	0.7996
L-Methionine	0.030	149.2	0.2011
L-Phenylalamine	0.066	165.2	0.3995
L-Serine	0.042	105.1	0.3996
L-Threonine	0.095	119.1	0.7976
L-Tryptophan	0.016	204.2	0.0784
L-Tyrosine (free base	—		
L-Tyrosine•2Na•$2H_20$	0.10379	261.2	0.3973
L-Valine	0.094	117.1	0.8027

Vitamins	g/L	Molecular weight	mM
Choline Bitartrate	—		
Choline Chloride	0.004	139.6	0.0287
Folic Acid	0.004	441.4	0.00907
myo-Inositol	0.0072	180.2	0.03996
Niacinamide	0.004	122.1	0.03276
D-Pantothenic Acid•½ Ca	0.004		
Pyridoxal•HCl	—		
Pyridoxine•HCl	0.004	205.6	0.01946
Riboflavin	0.0004	376.4	0.00106
Thiamine•HCl	0.004	337.3	0.01186

Other	g/L	Molecular weight	mM
D-Glucose	1.0	180.16	5.5506
HEPES	—		
Phenol Red•Na	—		
Pyruvic Acid•Na	—		

Add	g/L	Molecular weight	mM
Glucose	—		
L-Glutamine	0.584	146.15	3.996
L-Cystine•2HCl	—		
L-Leucine	—		
L-Lysine•HCl	—		
L-Methionine	—		
$NaHC0_2$	—		
NaH_2P0_4	—		
Phenol Red•Na	—		
Pyruvic Acid•Na	—		

Appendix II – Composition of SDS lysis buffer

Components (Sigma Aldrich, UK)	Concentration (%)
Tris base (pH 6.8)	50 mM
Glycerol	10%
SDS	2%
Protease Inhibitor Cocktails	1:1000 dilution

Appendix III – Composition of tank buffer for SDS-PAGE

Components (Sigma Aldrich, UK)	Conconcentration (%)
Tris base	0.3
Glycine	1.44
SDS	0.1

Appendix IV – Composition of transfer buffer for Western blotting

Components	Concentration
Tris base	25mM
Glycine	192mM
Methanol	20%

Appendix V - Composition of Resolving Gel for SDS-PAGE

Components	Concentration
4 x Tris-SDS (pH 8.8)	1 X (375 mM Tris-base 0.1% SDS)
Acrylamide	8, 10 or 12%
ddH_20	—
Ammonium persulfate (APS)	0.04%
Tetramethylethylenediamine (TEMED)	0.08%

Appendix VI - Composition of Stacking Gel for SDS-PAGE

Components	Concentration
4 x Tris-SDS (pH 6.8)	1 x (125mM Tris-base 0.1% SDS)
Acrylamide	4%
ddH_20	—
Ammonium persulfate (APS)	0.05%
Tetramethylethylenediamine (TEMED)	0.06%

Appendix VII - Source of antibodies

Primary Antibody	Origin	Source
HO-1	Mouse monoclonal	Transduction Laboratories
NQO1	Goat polyclonal	Santa Cruz
CD36	Mouse monoclonal	Santa Cruz
Nrf2	Rabbit polyclonal	Spring Biosciences
pan-Akt	Rabbit polyclonal	Cell Signalling
Phospho-Akt	Rabbit polyclonal	Cell Signlling
$p38^{MAPK}$	Rabbit polyclonal	Cell Signalling
pan-Erk 1/2	Rabbit polyclonal	Cell Signalling
Phospho-Erk	Rabbit polyclonal	Cell Signalling
pan-JNK	Rabbit polyclonal	Cell Signaling
Phospho-JNK	Rabbit polyclonal	Cell Signalling
Phospho-smad2	Rabbit monoclonal	Millipore
α-Tubulin	Rat polyclonal	Chemicon
Lamin A/C	Goat polyclonal	Santa Cruz
Vimentin	Rabbit monoclonal	Abcam
α-Smooth muscle actin	Mouse monoclonal	Santa Cruz

Secondary Antibody	Origin	Source
Goat anti-mouse-HRP	Goat	Santa Cruz
Donkey anti-mouse-HRP	Donkey	Santa Cruz
Goat anti-rabbit-HRP	Goat	Santa Cruz
Goat anti-rat-HRP	Goat	Santa Cruz
Alexa Flour 488 anti-rabbit	Goat	Molecular Probes
Alexa Flour 488 anti-mouse	Goat	Molecular Probes
Alexa Flour 468 anti-rabbit	Goat	Molecular Probes

Appendix VIII – Composition of Krebs buffer

Components (Sigma Aldrich, UK)	Concentration (mM)
NaCl	136
KCl	2.6
Na_2HPO_4	3.2
KH_2PO_4	1.46
HEPES	5
D-Glucose	5

Components (Sigma Aldrich, UK)	Concentration (mM)
Adjust to pH 7.4	
$CaCl_2$	2.5
$MgCl_2$	1
L-arginine	0.1

Appendix IX – Addresses of suppliers

Agar Scientific Ltd
66A Cambridge Road
Stanstead, Essex
CM24 8DA
UK

Alexis Biochemicals
San Diego
California
USA

Amersham Life Sciences
GE Healthcare UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA
UK

Biomol International, L.P.
Palatine House
Matford Court
Exeter EX2 8NL
UK

Cell Signalling Technology
Inc. 3 Trask LaneDanvers
MA 01923
UK

Chemicon Europe Ltd
The Sciences Centre
Eagle Close
Chandlers Close
Hampshire
S053 4NF
UK

Molecular Probes
Invitrogen Ltd
3 Fountain Drive
Inchiman Business Park
Paisley
PA4 9RF
UK

National Diagnostics Ltd
Unit 4 Fleet Business Park
Itlings Lane
Hessle
East Riding of Yorkshire
Hull
HUI39LX
UK

Pierce Perbio
USA

Promega
Delta House
Southampton Science Park
Southampton
SO16 7NS
UK

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH12 4QH

Santa Cruz Biotechnology Inc.
2145 Delaware Avenue
Santa Cruz CA, 95060
USA

Signal Transduction Laboratories
21 Between Towns Road
Cowley
Oxford
OX4 3LY
UK

List of Publications

Published Abstracts

T. Mughal, GE. Mann and RC Siow (2010) Transforming growth factor- β1enhances expression of oxidative stress proteins in human aortic adventitial fibroblasts. *Proc Physiol Soc*. **19,** PC188.

T. Mughal, J. Wong, GE. Mann, M. Parsons & RC. Siow (2011) Sulforaphane modulates antioxidant redox signalling in human aortic adventitial fibroblasts *Proc Physiol Soc*. **38,** PC75.

T Mughal, GE. Mann, M. Parsons & RC. Siow (2011) Transforming growth factor-β1 induces antioxidant defense pathways via activation of Nrf2 and Akt pathways. *Society for Free Radical Research Europe* **12,** 650.

T Mughal, M. Parsons & RC. Siow (2012) Transforming growth factor- β1 mediates redox signalling via activation of the Nrf2 and Akt pathways and mediates adventitial fibroblast migration in human aortic adventitial fibroblasts. *British Microcirlculation Society* **565P,** C73.

T Mughal, M. Parsons & RC. Siow (2012*)* Transforming growth factor- β1 mediates adventitial fibroblast migration and redox signalling in human aortic adventitial fibroblasts. *Society for Free Radical Research International.*