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Endothelial Lineage Differentiation from iPS Cells is Regulated by miRNA-21/AKT and TGF- β 2 Pathways

Di Bernardini, Elisabetta

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Endothelial Lineage Differentiation from iPS Cells is Regulated by miRNA-21/AKT and TGF- β 2 Pathways

A thesis submitted for the degree of Doctor of
Philosophy for the King's College London

Elisabetta Di Bernardini

July

2013

Cardiovascular Division
King's College London, School of Medicine

Dedicated to my Family

ABSTRACT

Endothelial death/dysfunction is a critical process in the development of cardiovascular diseases. Finding a source of endothelial cells (ECs) for regenerative medicine is a challenging yet fundamental issue. Induced pluripotent stem cells (iPSCs) constitute an attractive source of cells for transplantation because of their high proliferation and differentiation potential.

We established a protocol using collagen and VEGF to drive the functional differentiation of iPSCs into ECs. After 7 days, the cells strongly expressed EC markers (VE-cadherin, Flk1, vWF and eNOS) and formed tubes on Matrigel. Next, we compared the miRNA signature of undifferentiated and differentiated iPSCs with VEGF. Amongst the validated miRNAs, we focused on miR-21 which was previously shown to be involved in angiogenesis. Overexpression of miR-21 (Pre-21) in pre-differentiated iPSCs induced EC marker upregulation and *in vitro* and *in vivo* capillary formation; accordingly, inhibition of miR-21 (LNA-21) produced the opposite effects. Interestingly, miR-21 overexpression increased TGF- β 2 mRNA and secreted protein level, consistent with the strong upregulation of TGF- β 2 during iPSC differentiation. In addition, treatment of iPSCs with TGF- β 2 induced EC marker expression and *in vitro* tube formation, through induction of VEGF secretion. Inhibition of SMAD3, a downstream effector of TGF- β 2, strongly decreased VE-cadherin expression. Furthermore, TGF- β 2 neutralization inhibited miR-21 induced EC marker upregulation, indicating that TGF- β 2/SMAD3 pathway is required during iPSC differentiation into ECs. We then confirmed the PTEN/AKT pathway as a direct target of miR-21 and we showed that PTEN shutdown during the differentiation of iPSCs increased EC marker expression.

We demonstrated that miR-21 directly targets the PTEN/AKT pathway, which also involves TGF- β 2 pathway regulation by miR-21. Moreover the PTEN/AKT pathway is required in the VEGF-induced EC differentiation of iPSCs. Thus, the molecular mechanisms elucidated in this work might provide the basic information for stem cell therapy for vascular disease, e.g. tissue engineering and endothelial repair in damaged vessels.

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I finally would like to thank Dr Claire Potter for her help in reading the final version of my thesis, and all the other members of our group for their kind cooperation and friendship.

DECLARATION

I, Elisabetta Di Bernardini, confirm that the work presented in this thesis is my own and I have been involved in the design, planning and conduct of all the experiments and the thesis writing. This includes the differentiation of iPSCs to ECs, the lentiviral particle production, standard molecular biology methods and analysis of the results.

Expert assistance was provided in some aspects of the project by the following colleagues from the Cardiovascular Division of King's College.

Dr Anna Zampetaki performed the microRNA arrays in the Proteomics laboratory directed by Professor Manuel Mayr.

Dr. Yanhua Hu injected the cell samples in C57BL/6 mice, for the *in vivo* Matrigel assay.

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ABBREVIATIONS

±SEM	standard error of mean
AKT	protein kinase B (PKB)
AP-1	activator protein 1
bFGF	basic fibroblast growth factor
BMPs	bone morphogenetic proteins
CD133	cluster of differentiation 133
CD144	cluster of differentiation 144/VE-cadherin
CD31	cluster of differentiation 31/PECAM-1
CD34	cluster of differentiation 34
CD45	cluster of differentiation 45
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
cKit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
cMyc	similar to v-MYC (myelocytomatosis viral oncogene)
DAPI	4',6-diamidino-2-phenylindole
ECs	endothelial cells
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial–mesenchymal transition
EndMT	endothelial to mesenchymal transition
eNOS	endothelial nitric oxide
EPCs	endothelial progenitor cells
ERK	extracellular-signal-regulated kinase
ESCs	embryonic stem cells
EtBr	ethidium Bromide
FGF	fibroblast growth factor
FACS	fluorescence activating cell sorting
FITC	fluorescein isothiocyanate
Flk1	vascular endothelial growth factor receptor 2/KDR
Flt1	vascular endothelial growth factor receptor 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
h	hours
HDACs	histone deacetylases
HE	Hematoxylin
HIF-1 α	hypoxia-inducible factor 1-alpha
HRP	horse radish peroxidase
HUVECs	human umbilical vein endothelial cells
IL-6	interleukin 6

iPSCs	induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
KDR	kinase insert domain receptor/Flk1
KLF4	krüppel-like family of transcription factor 4
LDL	low density lipoprotein
LiCl	lithium chloride
LIF	leukaemia inhibitory factor
LNA-Ctrl	control of miRNA inhibitor
LNA-21	miR-21 inhibitor
MAPKs	mitogen-activated protein kinases
MCP-1	monocyte chemotactic protein-1
MEFs	mouse embryonic fibroblasts
min	minutes
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NO	nitric oxide
NOS	nitric oxide synthase
OCT4	octamer-binding transcription factor 4
OSS	oscillatory shear stress
PDCD4	programmed cell death 4
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI3K	phosphatidylinositide 3-kinases
Pre-Ctrl	control of miRNA precursor
Pre-21	miR-21 precursor
PTEN	phosphatase and tensin homolog
Q-PCR	quantitative real time polymerase chain reaction
RhoB	ras homolog gene family, member B
ROS	reactive oxygen species
rpm	rounds per minute
s	seconds
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulphate
shRNA	short hairpin ribonucleic acid
SM22	smooth muscle protein 22-alpha or transgelin
SMA	smooth muscle actin
SMCs	smooth muscle cells
SMMHC	smooth muscle myosin heavy chain
SOX2	SRY (Sex determining region Y)-box2
STAT3	signal transducer and activator of transcription factor 3
TGF- β	transforming growth factor β
TGF- β I	transforming growth factor β -induced

TNF	tumour necrosis factor
TRITC	tetramethyl Rhodamine Iso-Thiocyanate
VCAM-1	vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial-cadherin/CD144
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vWF	von Willebrand factor
µg	Micrograms
µl	Microliters

CHAPTER 1. INTRODUCTION

1.1 THE VASCULAR SYSTEM

The cardiovascular system is a complex organ that passes blood around the body, bringing nutrients and oxygen to the cells and removing waste products and carbon dioxide.

The main mechanisms leading to blood vessel formation are two, namely vasculogenesis and angiogenesis. Vasculogenesis is the formation of new vessels from progenitor cells and was historically believed to occur in embryo development, during the formation of the primitive vascular network from angioblasts, which occurs in several stages. First, hemangioblasts, the common putative precursors of endothelial and hematopoietic cells in the embryo, differentiate from the mesoderm. These differentiated cells then aggregate to form blood islands. The inner cells of the blood islands become hematopoietic stem cells, or blood-forming cells and the outer cells become angioblasts, which give rise to the blood vessels (Gilbert 2000). Angiogenesis is the formation of new blood vessels from pre-existing ones and occurs both during embryonic development and during physiological and pathological conditions in adult life. Angiogenesis can be initiated by several stimuli including hypoxia and is pivotal to the development of many diseases, such as cancer (Carmeliet 2003). More recently, the discovery of vasculogenic bone marrow and tissue resident progenitor cells in the adult has challenged the definition of vasculogenesis and extended it into adult life (Garin, Mathews et al. 2005). Finally, another form of *de novo* vascular development is arteriogenesis, which is caused by mechanical forces like shear stress and occurs through the remodelling of pre-existing vasculature and expansion of collateral vessels (Carmeliet 2000, Heil, Eitenmuller et al. 2006).

Large blood vessels are composed of three layers: the innermost is the tunica intima, which is composed of a single continuous layer of endothelial cells (ECs) and mediates the exchange of nutrients and cells with the circulation. Surrounding the intima is the thick layer of smooth muscle cells (SMCs) composing the tunica media, which is responsible for the maintenance of the vessel tone and elasticity. Finally, adventitia is the external layer mainly composed of fibroblasts and connective tissue and incorporating the vasa vasorum, the small network of vessels that provides oxygen and nutrients to the cells in the vessel wall (Figure 1). Vessels within the

microvascular system (i.e. capillaries and venules) are formed by contractile cells called pericytes that wrap around the endothelial cell layer (Carmeliet 2000).

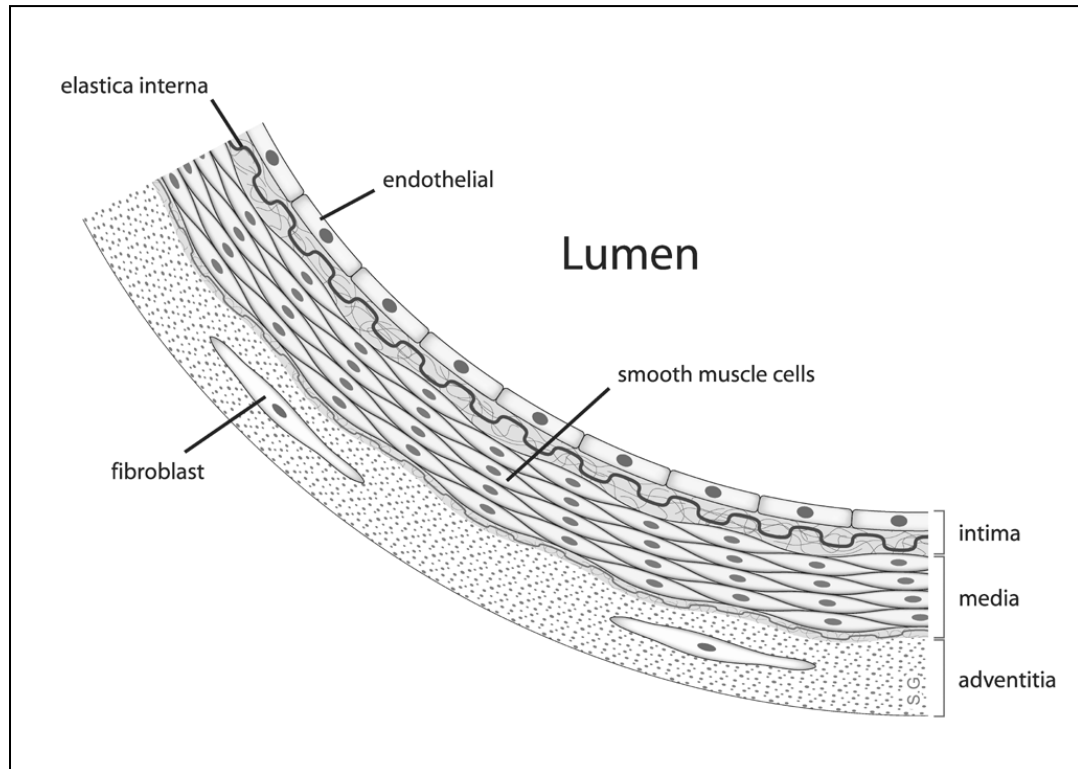


Figure 1 Anatomy of artery

In this schematic representation of the arterial wall the three layers of a blood vessel (intima, media and adventitia) are shown together with the cells that compose each layer (endothelial cells, smooth muscle cells and fibroblast). http://en.wikipedia.org/wiki/File:Anatomy_artery.png

1.1.1. ENDOTHELIAL CELL CHARACTERIZATION AND FUNCTION

Endothelial cells line the blood vessels of the entire circulatory system, from the aorta to capillaries, and represent the barrier between circulating blood and the rest of the vessel wall. The endothelium is a dynamic and heterogeneous organ with secretory, metabolic, synthetic and immunological functions (Fishman 1982).

Endothelial cells cover a surface in an adult human of approximately 1 to 7 m², which is composed of approximately 1 to 6 x 10¹³ cells and weighs approximately 1 kg (Augustin, Kozian et al. 1994). ECs, which line the vessels of all organs, regulate

the flow of nutrients, biologically active molecules and blood cells through the action of membrane-bound receptors, lipid transporting particles, metabolites and hormones and specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions (Cines, Pollak et al. 1998).

A unique characteristic of ECs is that, despite their many common functional and morphological features, they display a great heterogeneity in different organs or in the same organ in the endothelium of large and small vessels, veins and arteries. For instance the kidney contains different types of ECs: fenestrated in the peritubular capillaries, discontinuous in glomerular capillaries and continuous in other regions (Risau 1995).

As mentioned before, vascular and hematopoietic tissues develop together after implantation, with the formation of blood islands within the primitive yolk sac. During embryonic development, ECs differentiate from a common precursor called angioblast and acquire organ-specific properties. The angioblasts form the outer layer of ECs in the blood island, whereas the hematopoietic stem cells compose the inner cluster, which gives rise to the first embryonic blood cells (Risau and Flamme 1995). Angioblasts are primarily found in embryonic mesoderm, arising from the lateral mesodermal plate and cardiac crescent and some cells migrate into the forming brain or into the endocardium of the early heart tube (Palis, McGrath et al. 1995). Other angioblasts differentiate into the ECs of the vitelline vessels, which allow the blood cells from the yolk sac to circulate within the body of the embryo (Risau and Flamme 1995). Moreover, ECs directly differentiated from the surrounding mesenchyme of invading vessels compose the vasculature of the viscera (Risau and Flamme 1995). Endodermally-derived cells of the branching airway have been shown to induce formation of angioblasts that become part of the pulmonary vasculature (Buck, Edelman et al. 1996).

One of the determinants of EC specific pathways of differentiation is the local environment in which the cells differentiate and their interaction with surrounding cells, which may occur through the release of soluble mediators, cell-to-cell adhesion and the synthesis and assembly of matrix proteins on which the endothelium develop (Garlanda and Dejana 1997). Importantly, many recent studies showed that vascular development is mediated by the action of two important factors, basic fibroblast

growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Beck and D'Amore 1997).

ECs have been shown to express specific markers which are used to identify these cells *in vivo* and *in vitro*. In the majority of cases these molecules have been discovered using monoclonal antibodies directed to ECs. The most identified markers for ECs are von Willebrand factor (vWF), platelet–endothelial cell adhesion molecule (PECAM-1/CD31), angiotensin-converting enzyme, type I scavenger receptor, vascular endothelial cadherin (VE-cadherin), CD34, CD102/ICAM-2, CD105/engoglin, CD36 and thrombomodulin (Garlanda and Dejana 1997).

Detailed studies of endothelial function started in the 1970s with the development of techniques to culture ECs *in vitro* (Jaffe, Nachman et al. 1973, Lewis, Hoak et al. 1973).

Endothelium is a semi-permeable barrier that regulates the conduction of blood and the passage of fluids and solutes between the blood and the interstitial space; the permeability of this barrier and cell adhesion is tightly regulated by intracellular junction. The most important intracellular junctions that have been characterized as the cell–cell adhesive barrier structures in the microvascular endothelium are the adherens junction and the tight junctions; the structural and functional integrity of these junctions is a major determinant of paracellular permeability (Michel and Curry 1999). Adherens junctions are found in nearly all types of vascular beds, especially in the peripheral microvasculature and are impermeable to albumin (69 kDa) and other large proteins, indeed representing the major responsible of endothelial barrier to macromolecules in many organs and tissues (Mehta and Malik 2006). Amongst the adherens junctions, VE-cadherin, also known as cadherin-5, is believed to be the most important protein in forming the molecular basis and regulating the function of adherens junctions. VE–cadherin is a transmembrane receptor, whose extracellular domain binds to the extracellular domain of another VE–cadherin expressed in the membrane of an adjacent endothelial cell; it is found almost exclusively on ECs and promotes cell-cell adhesion by a calcium-dependent homotypic mechanism (Dejana, Orsenigo et al. 2008). Intracellularly, VE–cadherin is connected to the actin cytoskeleton through binding to β -catenin and γ -catenin, which in turn are connected to actin via binding to α -catenin (Mehta and Malik

2006). The catenins indeed not only represent a structural linkage between VE-cadherin and the cytoskeleton, but they can also transduce biochemical signals for cell-cell communications; the maintenance of endothelial barrier function is indeed ensured by the stability of the VE-cadherin-catenin-cytoskeleton complex (Vincent, Xiao et al. 2004). Adherens junctions are also involved in many signalling events regulating gene expression (Bazzoni and Dejana 2004).

There are many other proteins at cell-cell contacts which can interact with adherens junctions, such as E-cadherin, junctional adhesion molecules, and PECAM-1, a member of the Ig superfamily. Within the vascular compartment, PECAM-1 is expressed on leukocytes, platelets, and on ECs mostly at junctions between adjacent cells. PECAM-1 is known to bind to integrins on leukocytes to facilitate their transmigration across the microvascular endothelium; in addition to its adhesive properties, it plays a role in signal transduction, angiogenesis, platelet function, thrombosis and endothelial mechanosensing of fluid shear stress (Woodfin, Voisin et al. 2007).

Tight junctions are less common than adherens junctions in the peripheral microvasculature, and are mainly found in the microvascular endothelium of some specialized tissues, such as the blood-brain or blood-retinal barriers (Hawkins and Davis 2005). Tight junctions contribute to endothelial barrier function by impeding the passage of much smaller molecules (<1 kDa), such as small inorganic ions (e.g., Na⁺). Endothelial tight junctions are based on the interactions of the tight junction proteins occludin, claudins (3/5), which are integral membrane proteins, and junctional adhesion molecule-A, a member of the immunoglobulin superfamily of proteins. Occludin, claudins and junctional adhesion molecules-A are connected to the actin cytoskeleton through binding to zona occludens proteins (ZO-1, ZO-2) and α -catenin (Schneeberger and Lynch 2004, Hawkins and Davis 2005, Abbott, Patabendige et al. 2010).

Additionally, links between the endothelial basolateral membrane and the surrounding extracellular matrix of the microvascular wall are maintained by focal adhesions (Wu 2005). The main components of focal adhesions are the integrins, transmembrane receptors which belong to a family of glycoproteins. Intracellularly integrins interact with the cytoskeleton through the linker proteins paxillin, talin,

vinculin, or α -actinin, whereas extracellularly they bind to the matrix proteins fibronectin, collagen, vitronectin, fibrinogen and laminin (Petit and Thiery 2000, Hodivala-Dilke, Reynolds et al. 2003).

ECs also show an anti-thrombotic function, preventing the progression of the thrombotic response after endothelial damage. The release of tissue factor inhibitor prevents coagulation. Furthermore, healthy ECs respond to a number of stimuli, such as serotonin from aggregating platelets and thrombin, by releasing nitric oxide (NO), which relaxes the underlying vascular SMCs (Kolluru, Siamwala et al. 2010). In ECs NO production is mediated by the enzyme nitric oxide synthase (NOS), of which there are three isoforms: inducible, neuronal and endothelial (eNOS); eNOS activity regulates blood vessel dilatation (van Hinsbergh 2001). Increasing concentration of Ca^{2+} by the action of eNOS agonists such as bradykinin, acetylcholine, ATP, ADP, substance P and thrombin, induces the association between Ca^{2+} and calmodulin, which binds to and activates eNOS. eNOS activity is also increased by phosphorylation and acetylation (Butt, Bernhardt et al. 2000, Jung, Kim et al. 2010). eNOS is expressed in human endothelial progenitor cells (EPCs) (Qiao, Niu et al. 2010) and during the differentiation of bone marrow stem cells into ECs (Liu, Jiang et al. 2007).

NO, together with prostacyclin and prostaglandin- E_2 , inhibits platelet aggregation, preventing abnormal constriction (vasospasm) of the coronary arteries and inhibiting the expression of endothelial adhesion molecules and thus preventing the adhesion and penetration of macrophages.

The endothelium is also responsible for regulating vascular tone, which is maintained by a balance between the production of vasodilatation and vasoconstriction factors. Synthesis of plasminogen activators stimulates fibrinolysis to lyse a developing thrombus before it causes damage (van Hinsbergh 2001). NO diffusion from the EC into adjacent vascular SMCs leads to reduced SMC contraction.

Endothelium-derived NO also prevents the proliferation of vascular SMCs and limits the formation of oxidised low density lipoprotein (LDL), reducing the risk of atherogenesis (Michel and Vanhoutte 2010).

Finally vWF, a blood glycoprotein involved in homeostasis, is considered a specific EC marker and is produced by ECs and megakaryocytes only; it is heterogeneously distributed throughout the vasculature and differently expressed by ECs (Zanetta, Marcus et al. 2000). It has been shown that increased plasma levels of vWF are involved in a large number of cardiovascular, neoplastic and connective tissue diseases, and may contribute to an increased risk of thrombosis (Budde and Schneppenheim 2001).

In vivo, ECs are in contact on their basal surface with the basement membrane, a highly specialized extracellular matrix. This matrix covers the ECs and maintains the tube-like structures of the blood vessels (Kalluri 2003). Because endothelial cell tube formation on basement membrane replicates many steps of angiogenesis, it has been established as a method to screen for angiogenic and antiangiogenic factors (Auerbach, Lewis et al. 2003). In the past 20 years it has been demonstrated that ECs rapidly form capillary-like structures *in vitro* when plated on a reconstituted basement membrane extracellular matrix, such as Matrigel (Grant, Kinsella et al. 1995). The formation of the capillary-like tubes is indeed specific to ECs and it is used as an *in vitro* angiogenesis assay to confirm the functionality of the ECs. These capillary-like tube structures take up acetylated-low density lipoprotein (Ac-LDL), which is a marker of differentiation for these cells (Arnaoutova 2009). Ac-LDL is taken up by macrophages and ECs via the "scavenger cell pathway" of LDL metabolism; the increased metabolism of Ac-LDL in ECs is used to identify these cells, using Ac-LDL labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) (Voyta, Via et al. 1984).

1.1.2 ENDOTHELIAL CELL DYSFUNCTION

Environmental risk factors (LDL levels, smoking, diabetes, hypertension, infection and hemodynamic forces), which accelerate cardiovascular diseases result in a reduced release of NO, an increase in superoxide, cytokine, prostaglandin-D₂ and adhesion molecule production and an acceleration of the apoptotic process in the endothelium. This process increases inflammation and thrombosis, thus leading to progression of the disease.

In cases of endothelial damage, growth factors and chemokines are released in order to increase EC proliferation and motility and to reduce neointima formation, caused by matrix deposition. The apoptotic cells are removed by the blood stream and can be replaced by proliferating neighbouring cells and/or circulating EPCs which circulate postnatally in peripheral blood. EPCs may be able to target and repair damaged endothelial regions (Xu, Zhang et al. 2003, Xu 2008). In a process named postnatal vasculogenesis EPCs may be recruited from the bone marrow and target sites of active neovascularisation in ischemic hindlimbs or myocardium, tumour vasculature and damaged corneas (Asahara, Masuda et al. 1999).

However, the proliferative capacity of the endothelium is thought to be low, due to the presence of senescent cells. Moreover these cells are thought to be unable to produce the required amounts of NO, which together with low proliferative capacity facilitates the inflammatory response, ultimately leading to the formation of atherosclerotic plaques (de Nigris, Lerman et al. 2003).

Endothelial dysfunction is considered the initial stage of many cardiovascular diseases, such as atherosclerosis.

1.1.3 ENDOTHELIAL CELLS AND ATHEROSCLEROSIS

Cardiovascular disease is the major cause of death worldwide, and current therapies can only delay progression of the disease (Segers and Lee 2008). The main forms of cardiovascular related mortality develop following atherogenesis, which is thought to be initiated by endothelial cell dysfunction.

Cardiovascular diseases include arteriosclerosis, aneurysms, cerebrovascular disease, heart failure, coronary heart disease, myocardial infarction, hypertension and stroke. The most common form of arteriosclerosis is atherosclerosis, which is the primary cause of cerebrovascular disease and coronary heart disease. Atherosclerosis mainly occurs in elastic arteries causing vessel wall thickening and narrowing of the blood vessel lumen and loss of elasticity, followed by the ischemia of connected tissue.

Atherosclerosis is a chronic inflammatory disease, started by endothelial dysfunction (Sima, Stancu et al. 2009, Sandoo, van Zanten et al. 2010). ECs dysfunction is a key

early event in the formation of the atherosclerotic plaque. Atherosclerosis develops in specific regions of the vasculature following interactions between modified lipoproteins, endogenous SMCs and ECs, macrophages and immune cells and it is the result of multiple risk factors.

In these atheroprone regions, ECs show increased permeability, cytokine production and expression of cell adhesion molecules, transcytosis of lipoproteins, secretory function and hyperplasia of the basement membrane (Sima, Stancu et al. 2009). Furthermore, in atheroprone regions ECs are sensitive to changes in blood flow, such as disturbed flow, which modify their morphology and signalling cascade activation (World, Garin et al. 2006).

The pathogenesis of atherosclerosis can be classified into several stages, which were initially described by Ross in 1976. Once ECs become damaged, the activation of an inflammatory response induces the exposure of adhesion molecules on the apical surface of the endothelium; this promotes the adhesion and migration of monocytes which can penetrate into the subendothelial space (Figure 2 A). The penetration and accumulation of LDL contributes to this process leading to the formation of a fatty-streak in the vessel. With the progress of the lesion, SMC migration, T-cell activation and platelet adhesion and aggregation further contribute to the process. The excessive uptake of oxidised LDL by macrophages, through recognition by scavenger receptors, results in a morphological change into a foam cell (Figure 2 B).

Foam cells have high intracellular levels of cholesterol, which induces apoptosis, releasing the fatty contents into the subendothelial space and driving the formation of the atherosclerotic lesion. Finally, the lesion progresses with the formation of a fibrous cap formed from SMC-produced extracellular matrix proteins. The continued expansion of the lipid-filled interior leads to the formation of a necrotic core (Figure 2 C) (Ross and Glomset 1976).

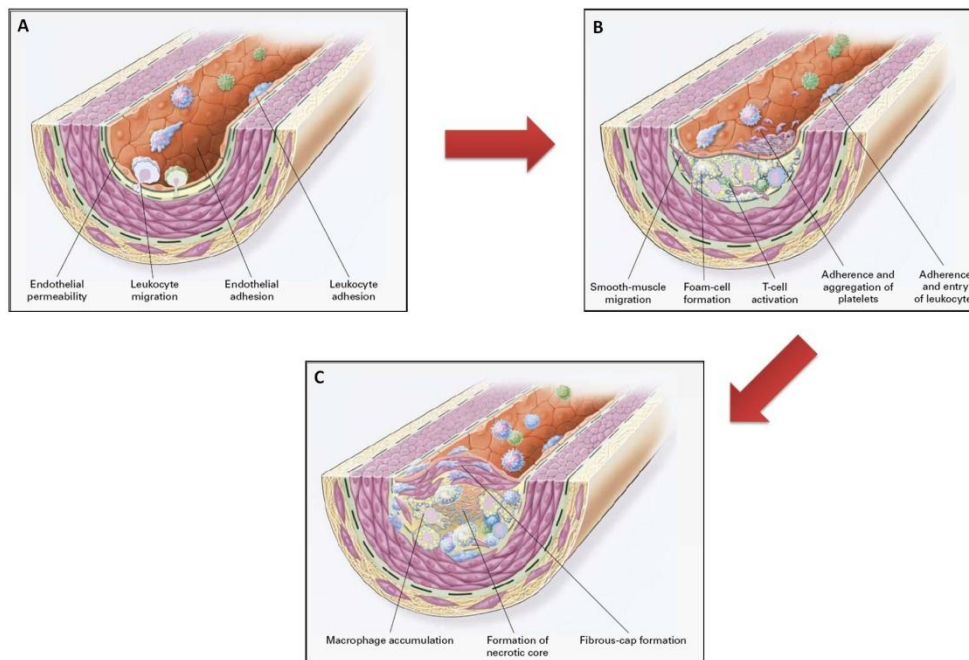


Figure 2 Atherosclerotic development

Adhesion and migration of leukocytes follow endothelial cell dysfunction (A). The initial development of atherosclerosis is the formation of a fatty streak upon the lumen surface, following platelet and leukocyte adhesion, T-cell activation and smooth muscle cell migration. Moreover, macrophage engulfment of oxidised LDL leads to foam cell formation (B). As atherosclerosis progresses, the lesion forms a fibrous cap, which narrows the lumen and a necrotic core forms (C) (Ross 1999).

There are two types of atherosclerotic plaques: stable and unstable, due to their position in the vessel and composition. Stable plaques can grow until the lumen is totally occluded; unstable plaques are prone to rupture following proteolytic degradation at the edges of the fibrous cap. This results in the formation of a thrombus which occludes the lumen at the site of rupture, causing ischemia and damage of tissue, such as myocardial infarction.

Balloon angioplasty and stenting are routinely used in clinical practice to treat patients with angina pectoris or myocardial infarction. However, one of the major issues of stenting is restenosis, leading to the recurrence of symptoms; balloon inflation at high pressure and use of metallic stent struts cause EC loss with subsequent SMC proliferation and matrix deposition, thus originating luminal narrowing (Xiao, Zeng et al. 2006). It has been reported that after vascular injury restenosis development can be prevented through accelerated re-endothelialization

by mature ECs, which inhibits SMC migration, proliferation and neointima formation (Nowak, Karrar et al. 2004).

In the past several years, accumulating evidence suggests that embryonic stem cells (ESCs), a promising source of pluripotent stem cells with unlimited growth and self-renewal abilities, are able to differentiate into ECs *in vitro* and *in vivo* (Levenberg, Golub et al. 2002). Understanding of the molecular mechanisms of stem cell differentiation into ECs might lead to definition of new methods to produce large number of ECs with high purity from ESCs: ESC-derived ECs can be used to treat damaged vessels and to avoid restenosis. However, so far still little is known about the EC differentiation mechanisms and the therapeutic potential of ESC-derived ECs in cardiovascular diseases.

1.1.4 ANGIOGENESIS IN INFARCTED TISSUE

When myocardium is deprived of blood, ischemia, infarction and myocardium remodelling are initiated. Following epicardial coronary artery obstruction, DNA synthesis in ECs and SMCs is strongly induced, indicating active neovascularisation (White, Carroll et al. 1992). Formation of new blood vessels is fundamental for oxygen and nutrient supply to the infarcted myocardium in order to sustain metabolism. Neovascularisation is indeed essential to develop collateral blood vessels able to compensate for the compromised vascular function. The infarct size, the amount of viable myocardium and the prognosis in patients with acute myocardial infarction are determined by the amount of coronary collateral blood vessels (Sabia, Powers et al. 1992). Recurrent myocardial ischaemia may play an active role in inducing EC proliferation essential to expand the myocardial vascular network (Banai, Shweiki et al. 1994).

Angiogenesis in the infarcted tissue occurs through a complex link between extracellular matrix, ECs and pericytes, in response to an imbalance of angiogenic factors compared to angiostatic factors in the local environment (Ferrara and Alitalo 1999, Frangiannis, Smith et al. 2002).

Early release of angiogenic factors in the injured areas has been associated with myocardial infarction. Many studies have shown that the angiogenic factors VEGF, interleukin 8 (IL-8) and bFGF are rapidly induced in the ischemic myocardium and may induce infarct neovascularisation (Kukielka, Smith et al. 1995, Lee, Wolf et al. 2000).

VEGF is a secreted growth factor that is angiogenic *in vivo* and specifically targets vascular ECs (Houck, Ferrara et al. 1991). VEGF expression has been shown to be increased *in vitro* in myocardial cells in hypoxia and by myocardial ischemia *in vivo*; indeed VEGF is a likely mediator in the myocardial neovascularisation induced by ischemia (Banai, Shweiki et al. 1994).

Use of angiogenic growth factors to promote new collateral vessel formation in the ischemic tissues is now a very exciting frontier of cardiovascular medicine (Ferrara and Alitalo 1999, Frangogiannis, Smith et al. 2002). One of the initial attempts in this direction was made through the delivery of VEGF recombinant protein in patients with coronary artery disease. However, this procedure was not really effective due to half-life of VEGF-A *in vivo*, insufficient myocardial uptake after coronary infusion and de-sensitization of chronically ischemic tissues to VEGF treatment. Therefore, the next step was trying to deliver VEGF-A gene directly to the ischemic tissues. Although the outcome of the clinical trials showed an indication of functional improvement in myocardial perfusion and cardiac function, in particular at early time points after treatment, the efficiency of naked DNA uptake by muscle and cardiac cells was still very low (Giacca and Zacchigna 2012).

Indeed, there are still many issues related to local delivery of growth factors in the infarcted tissues to induce neovascularisation; the aim of this study is to develop a differentiation protocol based on the use of the angiogenic growth factor VEGF, to induce the differentiation of pluripotent cells into ECs able to heal the damaged ischemic tissues when locally delivered.

1.1.5 TISSUE ENGINEERING AND VASCULAR GRAFTS

Atherosclerosis and stenosis are phenomena that lead to the narrowing of a blood vessel and are the cause of several cardiovascular diseases, including myocardial infarction. One approach for the treatment of arterial stenoses is the application of a vascular bypass performed using the patient's own saphenous vein or mammary artery. When natural vessels are not available, due to concomitant vascular complications or earlier procedures, alternative grafts need to be developed. The challenge of creating tissue engineered graft (artificial or from decellularized natural vessels) is to obtain a result with the mechanical and anti-thrombotic properties of the natural vessel, in particular in the case of small diameter vessel (<6mm).

The concept of tissue engineering is based on three essential components: cells, which can either be seeded *in vitro* or mobilized *in vivo*; scaffolds, onto which the extracellular matrix is organized and signals, which can be classified as humoral and mechanical (Bell 1991) (Figure 3).

An ideal cell source for vascular tissue engineering should be easy to isolate and expand in culture; it should be able to differentiate into functional vascular cells and be non-immunogenic for recipients. Recent studies showed that adult stem cells, ESCs and induced pluripotent stem cells (iPSCs) could be used as a source for vascular tissue engineering since they show all the above mentioned characteristics (detailed description follows).

However, there are still limitations in the use of synthetic vascular grafts, such as thrombogenicity, risk of infection and lack of growth potential. The first clinical trial using tissue engineered vascular grafts by seeding autologous bone marrow-derived mononuclear cells onto biodegradable tubular scaffolds has been recently performed; approximately 16% of grafts within the first seven years after implantation of this tissue engineered vascular grafts showed stenosis, an abnormal narrowing in the blood vessel (Naito, Shinoka et al. 2011).

Despite the numerous studies conducted on ESC differentiation into vascular lineages, limited studies have used vascular cells derived from ESC as a source for vascular grafts *in vitro*. In 2003, Shen and his colleagues implanted small diameter

tissue engineered vascular grafts (3mm) subcutaneously into nude mice by seeding SMCs from rabbit arteries onto a biodegradable polymer scaffold wrapped around a silicon tube. After 8 weeks from implantation the graft was retrieved and silicon tube removed. Then, ECs derived from mouse ESCs were seeded onto the lumen of the graft to form an intimal layer, and the graft was re-implanted subcutaneously for a further 5 days. Histological and immunohistochemical staining of this tissue engineered graft showed ESC-derived ECs lining the intimal surface and the presence of SMCs and collagen in the wall, resembling a vascular structure. Indeed, ECs differentiated from mouse ESCs can be used as seed cells for endothelium lining in tissue engineered blood vessels (Shen, Tsung et al. 2003). However, this tissue engineered vascular graft has not been tested yet *in vivo* by implanting it into the circulatory system; therefore, the mechanical and functional properties of the graft still remain to be elucidated.

In one of the most recent studies, sheets created from iPSC-derived vascular cells were used as an alternative and attractive source for tissue engineered vascular grafts (Hibino, Duncan et al. 2012).

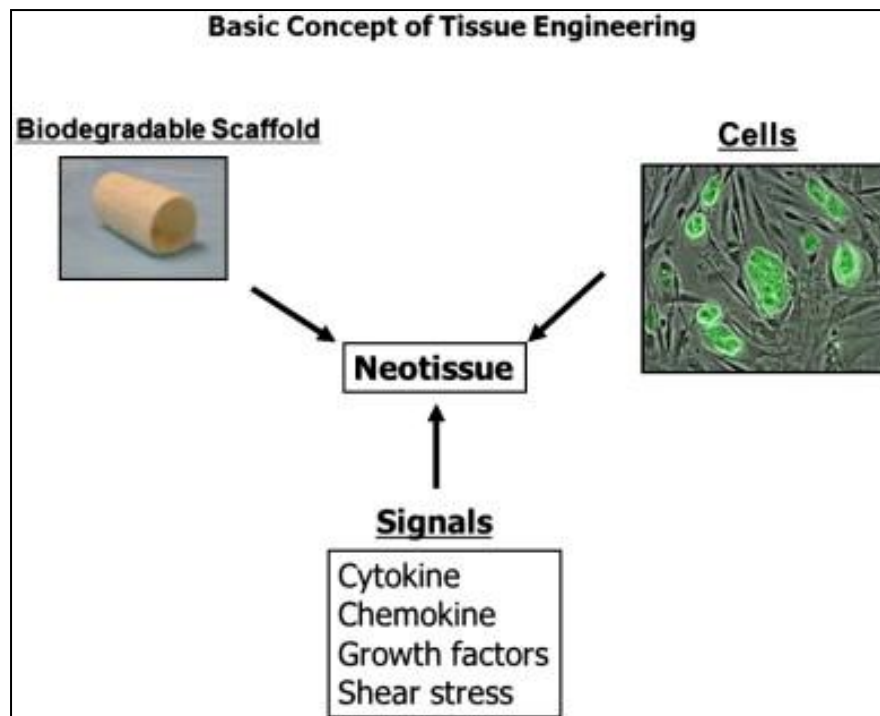


Figure 3 The basic concept of tissue engineering

Three essential components are required for the construction of tissue engineering grafts: cells, biodegradable scaffold onto which the extracellular matrix is organized to support the neotissue and signals such as cytokines, chemokines, growth factors and mechanical forces such as shear stress, required for the formation of an organized tissue (Naito, Shinoka et al. 2011).

Following the initial tissue engineering techniques developed, recent studies have been trying to improve re-endothelialisation of vascular scaffolds using autogenous cells in order to create a tissue engineered vessel with autogenous ECs and SMCs. However, the possibility of isolating adequate numbers of functional ECs or SMCs from patients with vascular disease is often not possible.

Developing tissue engineered vascular grafts is now a major challenge for the future of medicine. Clinical research studies and innovation of tissue engineering techniques, combined with the emerging iPSC technology may represent the future of regenerative medicine.

1.2 SOURCES OF ENDOTHELIAL CELLS

Recently, the mechanisms involved in the maintenance of endothelial layer integrity have become an important focus of research due to their importance in the pathophysiology of the vessels. Furthermore, new strategies have been developed to allow the isolation, culture and differentiation of cells that can contribute to vascular repair (Kirton and Xu 2010). However, current cell transplantation therapies devised for the treatment of cardiovascular diseases are hampered by the lack of a viable source of committed cells. Indeed, fully mature ECs isolated from patients' blood vessels have limited proliferation and expansion capabilities. Hence, alternatives such as the use of ESCs or iPSCs, which have an unlimited self-renewal capability, are increasingly sought after for tissue regeneration in the damaged areas.

In order to fulfil the potential of ESCs or iPSCs as a platform for treating cardiovascular diseases, an understanding of their roles and functional biology in vasculogenesis is pivotal. As such, the following sections will discuss current studies focusing on ESCs and in particular on iPSCs as model systems, and the relevant mechanisms that are involved in their differentiation.

1.2.1 EMBRYONIC STEM CELLS (ESCs)

Embryonic stem cells (ESCs) are derived from the inner mass of the embryonic blastocysts and can be cultured *in vitro* under the appropriate conditions in order to maintain their original pluripotency. This pluripotent ability has attracted the interest of numerous researchers, both to expand the fundamental understanding of developmental biology and for their potential application in regenerative medicine. Isolation of ESCs was firstly reported in 1981 from the inner cell mass of mouse E3.5 blastocysts (Evans and Kaufman 1981). In *in vitro* culture, these cells were shown to maintain their proliferative and undifferentiated state, growing on a feeder layer of mouse embryonic fibroblasts (MEF) or in medium containing leukaemia inhibitory factor (LIF) (Conner 2001).

Pluripotency is the ability of a cell to differentiate into all the cell types that make up an individual. Pluripotency can be tested *in vitro*, with the capacity of forming embryoid bodies, three-dimensional aggregates that are able to recapitulate the three germ layers. *In vivo*, cells can be injected into a blastocyst to assess whether they will be able to contribute to the development of a chimeric mouse and be transmitted through the germ line. Furthermore, proof of pluripotency can be obtained by subcutaneously injecting the cells in immunocompromised mice and monitoring for the formation of teratoma, a tumour composed by all three germ layers (Figure 4).

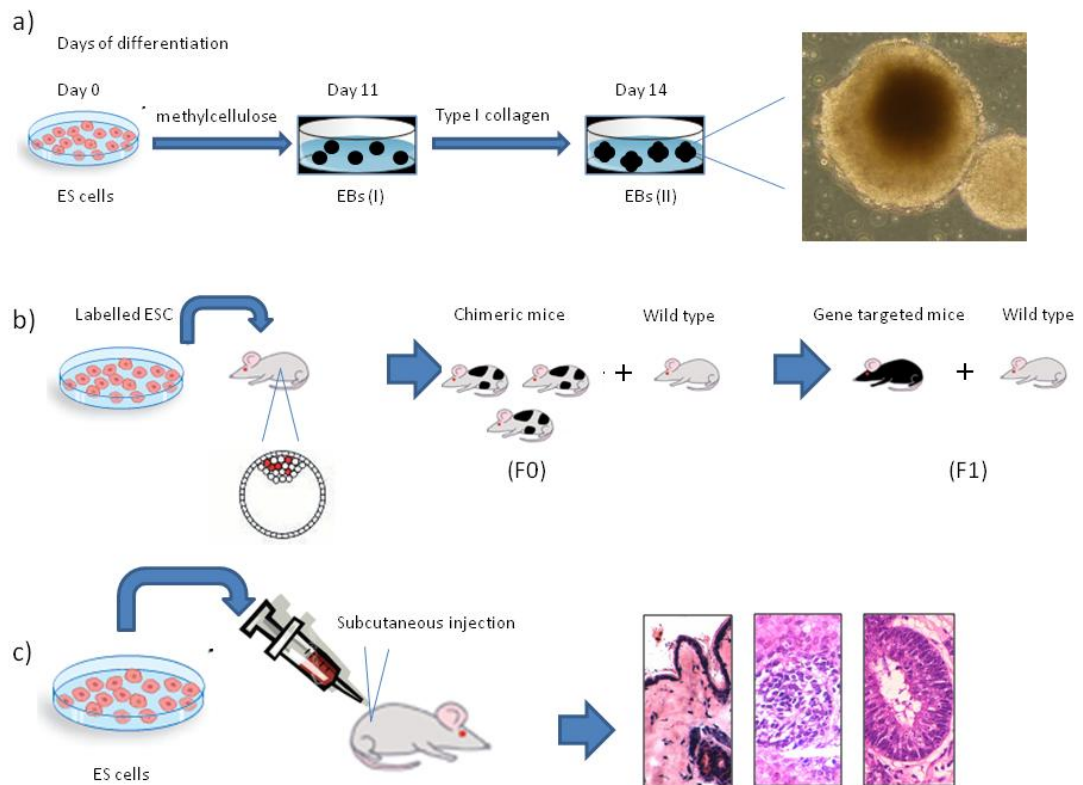


Figure 4 *In vitro* and *in vivo* tests for pluripotency

a) Embryoid body formation. Embryonic stem cells (ESCs) are isolated from the inner cell mass of a blastocyst and cultured in suspension in semisolid media without Leukemia inhibitory factor. After eleven days, the cells spontaneously differentiate to form three-dimensional multicellular aggregates called embryoid bodies (EBs), which comprise the three embryonic germ layers. b) Production of chimeric mice. Labeled or mutated ESCs are injected into the diploid blastocyst of a wild type mouse. F0 generation chimeric mice are only partially derived from the modified ESCs and are bred to obtain F1 generation in which some of the mice are derived from the labelled ESCs c) Teratoma formation. ESCs subcutaneously injected into immunodeficient mice spontaneously originate tumours comprised of all three germ layers, called teratoma.

1.2.1.1 ESC SPECIFIC MOLECULAR NETWORKS

As mentioned above, pluripotency is the key characteristic of stem cells in general and ESCs in particular. Understanding the network of factors that regulates this process has been the crucial focus of a large number of recent papers. In ESCs, pluripotency is maintained by a transcription factor network, which activates pluripotency-associated genes, including themselves, while repressing the developmentally regulated genes (Yeo and Ng 2013).

LIF, a member of the IL-6 family, is a supplement that is necessary to maintain ESCs in an undifferentiated state in the absence of a feeder layer. On the cell surface, LIF binds to a two-part receptor complex that consists of the LIF receptor and the gp130 receptor. This binding leads to the phosphorylation of a signal transducer and to the activation of the latent signal transducer and activator of transcription 3 (STAT3). This event is necessary for the maintenance of a proliferative state in mouse ESCs. Activated STAT3 translocates to the nucleus to activate a variety of downstream genes like Krüppel-like factor 4 (KLF4), which is involved in the regulation of numerous processes including proliferation and differentiation, and cMyc, a potent oncogene known to strongly promote proliferation (Figure 5a).

Src homology-2 domain (SH2)-containing tyrosine phosphatases (SHP-2) and extracellular-signal-regulated kinase (ERK) are both components of a signal-transduction pathway that counteracts the proliferative effects of STAT3 activation, influencing the self-renewal of mouse ESCs. SHP-2 is a tyrosine phosphatase that interacts with the intracellular domain of the gp130 receptor, and ERK is one of several enzymes activated when the gp130 receptor and other cell-surface receptors are stimulated. If ERK and SHP-2 are active, they inhibit ESC self-renewal promoting cell differentiation (Figure 5b).

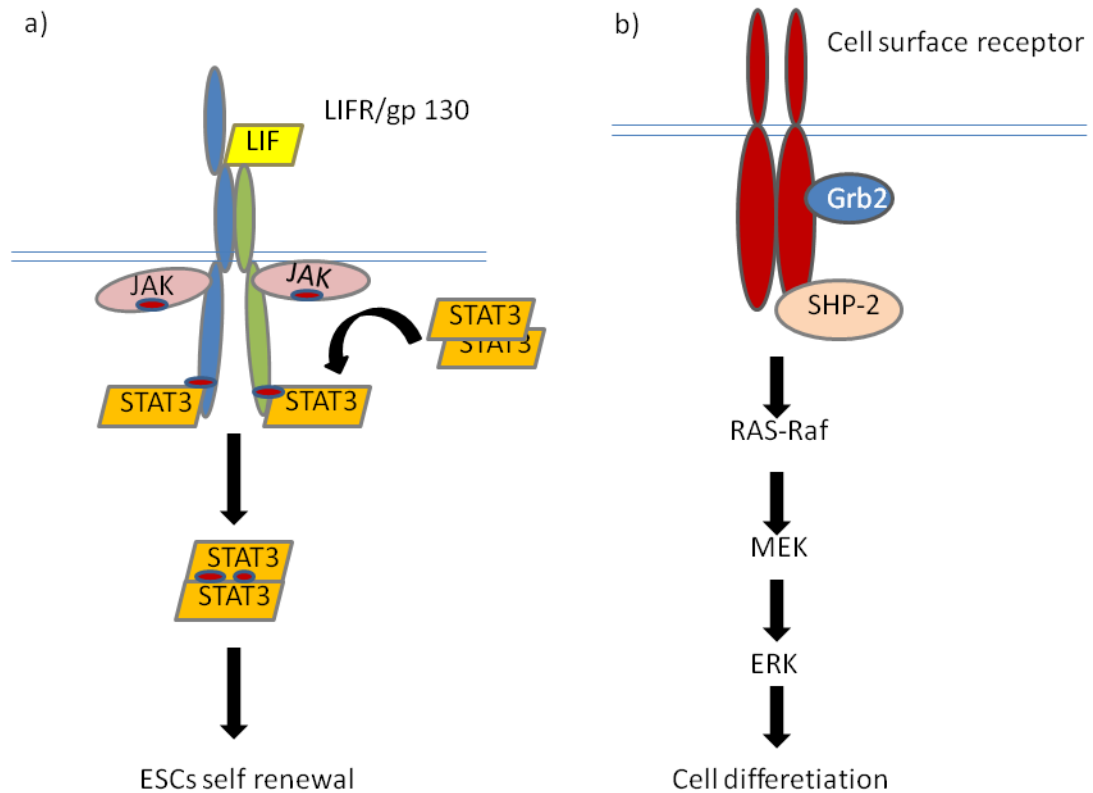


Figure 5 Mechanisms of ESC self renewal and differentiation

a) Promotion of ESC self-renewal via LIF-dependent activation of STAT3. Leukemia inhibitory factor (LIF) stabilizes the binding between Leukemia inhibitory factor receptor (LIFR) and glycoprotein 130 (gp130) cytokine receptor resulting in the activation of receptor-associated Janus-associated (JAK) tyrosine kinases. This causes the recruitment, phosphorylation and dimerization of the transcription factor signal transducer and activator of transcription 3 (STAT3). When the dimers translocate to the nucleus they can regulate the expression of self-renewal genes. b) Ras/MEK/ERK signalling pathway induces cell differentiation. The recruitment on cell-surface receptors, such as gp130 of a complex containing the growth factor receptor-bound protein 2 (Grb2) adaptor and sos guanine-nucleotide-exchange factor, is mediated by src homology-2 domain (SH2)-containing tyrosine phosphatase (SHP-2). Subsequent activation of rat sarcoma (RAS) initiates a cascade of transphosphorylations involving Raf and MAPK kinase (MEK) kinases with the final activation of and extracellular-signal-regulated kinase (ERK). Active ERK promotes ESC differentiation after phosphorylating cytoplasmic targets and undergoing nuclear translocation.

Other important transcription factors involved in the maintenance of pluripotency are: sex determining region (SRY) Y-box 2 (Sox2), homeobox protein Nanog (Nanog) and octamer-binding transcription factor 4 (Oct4).

Sox2 gene, a member of the SOX gene family, encodes for a transcription factor, which is initially expressed in the blastocyst inner cell mass and, in later stages of development, in the germ cells and in the ectoderm. After gastrulation, Sox2 expression is detectable in the developing neural tube throughout the early neuroepithelium (Wood and Episkopou 1999) and is probably induced by the activity of some regionally restricted enhancer elements (Zappone, Galli et al. 2000). *In vitro*, Sox2 is essential for maintaining pluripotency and it has been shown that a reduction of its expression induces mouse ESCs to differentiate into cells of the trophoectoderm lineage (Masui, Nakatake et al. 2007).

Nanog is a homeobox-containing transcription factor expressed in the inner cells of the morula, prior to blastocyst formation and in the inner cell mass of the blastocyst up until the implantation stage (Chambers, Colby et al. 2003). Nanog expression is still detectable in the proximal epiblast at embryonic day 6 and in the epiblast as the development progresses (Hart, Hartley et al. 2004). *In vitro*, overexpression of Nanog in differentiation-inducing conditions prevents ESCs from exiting their pluripotent and self-renewing stage (Chambers, Colby et al. 2003).

Oct4 is expressed in the mouse zygote, and is required throughout blastocyst development to establish and maintain the pluripotency of the inner cell mass and the epiblast. Oct4 is also expressed in the primordial and mature germ cells of mice (Schöler, Dressler et al. 1990). Oct4 expression *in vitro* is required to maintain the pluripotent, undifferentiated state of ESCs and its expression level is very important in the developmental program of mouse ESCs, making the protein a candidate "master regulator" of ESCs pluripotency. Indeed, Oct4 regulates gene expression by acting in synergy with Sox2 and binding to the Oct-Sox enhancer. This results in activation of pluripotency genes and silencing of differentiation-associated genes (Rodda, Chew et al. 2005). Furthermore, the binding of Oct4/Sox2 to the proximal region of Nanog promoter enhances its expression. LIF, with its downstream effectors STAT3 may be also involved in the regulation of Nanog gene expression (Pan and Thomson 2007) (Figure 6).

Therefore, the pluripotency of ESCs is externally regulated through several molecules such as LIF, whose signalling pathway activates transcription factors in the nucleus. The transcriptional activity of Klf4, cMyc and Nanog, along with Oct4

and Sox2, maintains the unlimited proliferative capacity and differentiation potential of the cells.

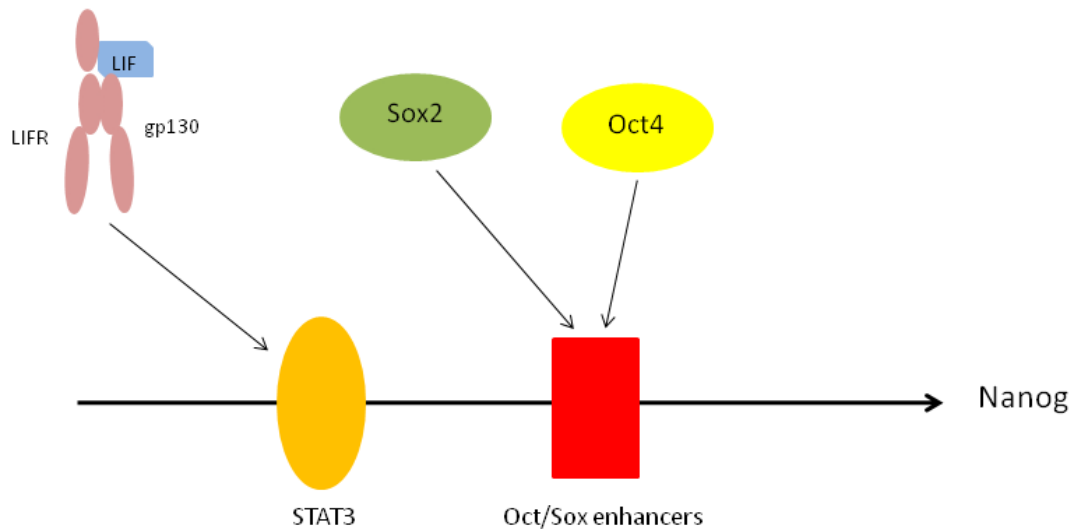


Figure 6 Regulation of Nanog expression in the ESC transcriptional network of pluripotency

Octamer-binding transcription factor 4 (Oct4) regulates gene expression by acting in synergy with sex determining region (SRY) Y-box 2 (Sox2) and binding to the Oct-Sox enhancer; the binding of Oct4/Sox2 to the proximal region of homeobox protein Nanog (NANOG) promoter enhances its expression. Leukemia inhibitory factor (LIF), with its downstream effector signal transducer and activator of transcription 3 (STAT3), may be also involved in the regulation of Nanog gene expression.

1.2.1.2 ESCs DIFFERENTIATION INTO MESODERMAL LINEAGE

Recently ESCs have been successfully differentiated into mesoderm-derived lineages using specific stimulations with different substrates and growth factors. For instance, mouse ESCs under optimised culture conditions and following serum induction have been shown to differentiate into hematopoietic cells (Keller 1995). Moreover, mouse ESCs plated on matrigel originated embryonic bodies and differentiated into blood vessels spontaneously (Nakagami, Nakagawa et al. 2006). Furthermore, ESCs have been differentiated recently into ECs and SMCs (Levenberg, Golub et al. 2002, Xiao, Zeng et al. 2007), vascular progenitors (Yamashita, Itoh et al. 2000) and cardiomyocytes (Kehat, Kenyagin-Karsenti et al. 2001, Yamashita, Takano et al.

2005), offering a wide range of cell lineages for clinical transplantation and therapeutic applications, which will be discussed in one of the following sections (1.4).

It has also been shown that CD34⁺ cells isolated from differentiated hESCs act as vascular progenitor cells capable of originating both ECs and SMCs (Hill, Obrtlíkova et al. 2010).

Generation of vascular ECs, SMCs and cardiomyocytes from ESCs offers a prospective source for cardiovascular tissue repair (Gepstein 2002).

Differentiation potential of stem cells into mesodermal lineages will be better discussed in some of the following sections, with a particular focus on iPSCs differentiation and the different stimuli inducing stem cell differentiation.

1.2.2 ADULT STEM CELLS

Adult stem cells, also known as somatic stem cells, are multipotent stem cells, found throughout the body after development in specific niches, that multiply by cell division to replenish dying cells and regenerate damaged tissues (Yin and Li 2006). Scientific interest in adult stem cells is due to their self-renewability and to their potential to differentiate into one or more cell types of the organ from which they originate. Unlike embryonic stem cells, the use of adult stem cells for therapeutic application does not raise ethical issues, since these cells are derived from adult tissue samples without the need for destroying human embryos.

Adult stem cells can be divided into three categories: bone marrow, circulating and tissue-resident stem cells. The first application of adult stem cell therapy was discovered in the 1950s, with the first bone marrow transplantation study. Stem cells present in the bone marrow can be used to replenish the depleted tissue and are able to produce all the derived populations. Subsequent studies isolated two main stem cell populations in the bone marrow: the hematopoietic stem cells, which form all the types of blood cells in the body, and the bone marrow stromal stem cells (also called mesenchymal stem cells), which can generate bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue (Nirmalanandhan and

Sittampalam 2009). They have been used for the therapy of blood disorders, such as leukemia, multiple myeloma and lymphoma, and disorders with defective genes such as severe combined immune deficiency (Papewalis, Topolar et al. 2013). Beside the reconstitution of host bone marrow, many other regenerative medicine applications of these cells have been progressively discovered. Mesenchymal stem cells have been differentiated *in vitro* into functional cardiomyocytes offering a great potential for therapeutic applications in myocardial infarction (Xu, Zhang et al. 2004). Moreover, mesenchymal stem cells have been used in the effective cell therapy of the infarcted heart (Pittenger and Martin 2004). Furthermore, mesenchymal stem cells have been transplanted in a model of limb ischemia, after artery occlusion and their angiogenic potential has been tested, compared to a control cell population composed of bone marrow-derived mononuclear cells. Blood flow recovery was improved by both cell types and mesenchymal stem cells were able to differentiate into ECs and SMCs and to contribute to the sprouting of collateral vessels (Iwase, Nagaya et al. 2005).

Gradually, it has become evident that adult stem cells play an important role in the maintenance and repair of tissues and organs during the life span of the individual (Nirmalanandhan and Sittampalam 2009, Teo and Vallier 2010).

Progenitor cells are found in many organs and are unipotent or multipotent with limited plasticity. Even though their self-renewability is highly limited, progenitor cells are able to repair injury in tissues where they reside. There are many types of progenitor cells so far identified, such as osteoblasts and chondrocytes, satellite cells in muscles, angioblasts, bone marrow, cardiac, stromal cells and the intermediate progenitor cells in the subventricular zone of the brain (Nirmalanandhan and Sittampalam 2009).

Bone marrow cells are recruited to the site of injury and participate in the healing of several organs. During the reparative process, progenitor cells are mobilized from the bone marrow into the circulation by directed migration along growth factor/cytokine gradients, to differentiate into a more mature phenotype and integrate in the nascent vasculature. The most widely studied subpopulation of circulating progenitor cells so far is represented by the EPCs, which have been shown to offer a great potential for angiogenesis applications and atherosclerosis treatment.

EPCs are derived from the bone marrow and appear to be implicated in the repair and maintenance of the vasculature through re-endothelialisation and neovascularisation (Shi, Rafii et al. 1998). It has been shown that the number and function of circulating EPCs decrease in response to risk factors associated with coronary artery disease (CAD), such as age, diabetes and high levels of blood cholesterol, potentially contributing to increased cardiac risk, reduced angiogenic capacity, and impaired cardiac repair effectiveness; however, the mechanisms underlying this process are not yet fully understood (Vasa, Fichtlscherer et al. 2001). Putative circulating EPCs were initially described as CD133⁺CD34⁺VEGFR2⁺ or CD34⁺VEGFR2⁺ (Asahara, Murohara et al. 1997). EPCs were originally characterized as a circulating population carrying the marker CD34 and coexpressing hematopoietic antigens (such as CD45). During culture/expansion, EPCs stop expressing CD45 (leukocyte common antigen) and start expressing EC markers, such as Factor VIII, CD31, UEA-1 (ulex europeaeus agglutinin-1), eNOS, E-selectin and become able to incorporate Dil-labeled acLDL. EPCs injected in a non-immunocompetent mice model of limb tumour were shown to successfully integrate into nascent capillaries (Asahara, Murohara et al. 1997). The therapeutic potential of EPC transplantation has been exploited by many studies conducted in mouse and rabbit hind limb ischemia models (Takahashi, Kalka et al. 1999, Kalka, Masuda et al. 2000). Moreover, EPCs expanded *ex vivo* have been reported to incorporate into foci of myocardial neovascularization (Kawamoto, Gwon et al. 2001).

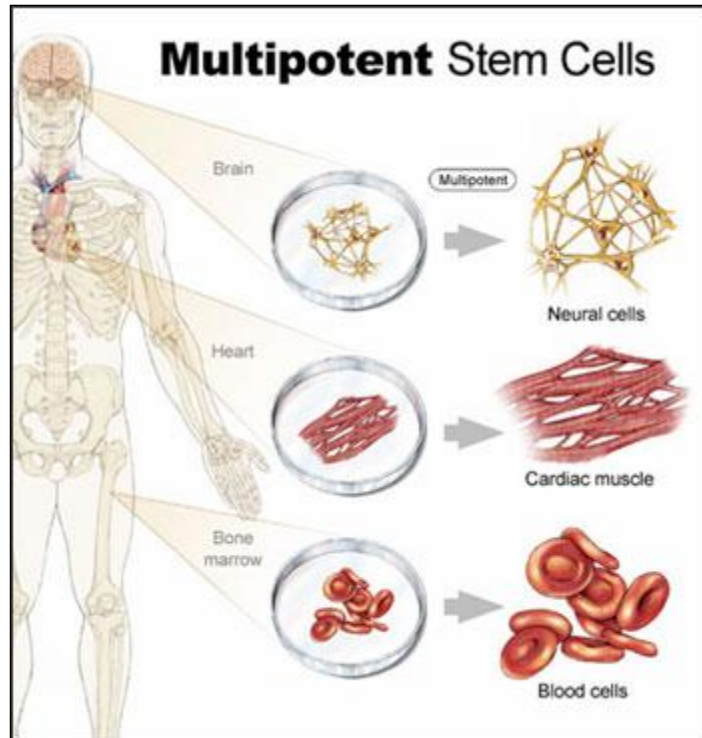


Figure 7 Niches of adult stem cells

Adult stem cells have been identified in specific niches in the human body, such as in the bone marrow, the heart and the brain. These cells show self-renewability and the potential to differentiate into all the cell types of the organ from which they originate. http://www.kokhucredernegi.org.tr/eng/kok_hucre/eriskin.html

Adult stem cells have been shown to be present in many different adult tissues. For instance, vascular progenitor cells such as Sca-1⁺ have been shown to be abundant in the adventitia (Hu, Zhang et al. 2004) and perivascular progenitor cells have been identified in the human adult vena saphena (Campagnolo, Cesselli et al. 2010); adult stem cells can be also found in the skin (Bickenbach and Grinnell 2004), the liver (Kung and Forbes 2009), the muscle (Martin, Russell et al. 2006), the lung (Neuringer and Randell 2006) and the heart (Beltrami, Barlucchi et al. 2003).

Despite the success in the use of stem cells from the bone marrow, adult stem cells clinical use is still restricted because of the limited differentiation potential of these cells. Furthermore, the identification of adult stem cells in the human body and indeed their characterization is still challenging (Teo and Vallier 2010). In addition, the therapeutic potential of somatic stem cells might be reduced in pathological conditions, since they might be impaired by the disease itself. Finally, using

endogenous stem cells in genetic diseases is not likely to be beneficial, since the stem cells would still produce progeny carrying the same phenotype (Teo and Vallier 2010).

Mobilization of endogenous somatic stem cells represents the ultimate non-invasive objective of regenerative medicine. It has been recently demonstrated that stimulation with granulocyte colony-stimulating factor (G-CSF) is able to mobilize hematopoietic progenitor cells from the bone marrow (Levesque, Hendy et al. 2003). Moreover, populations of progenitor cells can be selectively mobilized from the bone marrow as a response to different factors in various pathologies (Pitchford, Furze et al. 2009).

A better environment should be created in order to facilitate a therapeutic use of adult stem cells, for instance through the activation and mobilization of resident progenitor cells from their niches. Cytokines may be used to mimic the physiological situation in which BM cells are mobilized into the circulation, after stimulation from damaged tissues. Moreover, in a the study conducted on adult epicardium-derived progenitor cells, it has been shown that the actin monomer-binding protein thymosin beta4 (T β 4) is able to induce and revert these resident progenitor cells to their embryonic phenotype, giving rise to endothelial cells and vascular smooth muscle cells *ex vivo*. Indeed, through an “endogenous repair,” T β 4 promotes cardiac neovascularization to sustain the myocardium after ischemic damage (Smart, Risebro et al. 2010).

1.2.3 INDUCED PLURIPOTENT STEM CELLS (iPSCs)

The knowledge of the complex network of transcription factors cooperating to maintain the pluripotent state of ESCs has recently been exploited in order to induce somatic cell reprogramming. In the past, techniques such as somatic cell nuclear transfer have proved successful in inducing cell de-differentiation; however, there are technical and ethical issues in applying this type of approach to human cells, for example oocyte donor-associated risk of transmissible diseases (Yamanaka 2007).

In a landmark study, Yamanaka and colleagues showed the generation of induced pluripotent stem cells (iPSCs), following retroviral overexpression of four

transcription factors, Oct4, Sox2, Klf4, and c-Myc (OSKM) in mouse fibroblasts (Takahashi and Yamanaka 2006).

However, iPSCs could not produce chimeric mice and there were major differences between their global gene expression pattern and that of ESCs. Indeed, in 2007 a new method to generate mouse iPSCs was described; resultant cells showed phenotypes more closely related to ESCs and were able to generate chimeric mice and be transmitted through the germline (Meissner, Wernig et al. 2007, Okita, Ichisaka et al. 2007, Wernig, Meissner et al. 2007).

Hence, reprogrammed cells, like ESCs, exhibited unlimited proliferation and satisfied all the pluripotency standard assays, such as *in vitro* differentiation into the three germ layers, teratoma formation, contribution to chimeric mice, germline transmission (Maherali and Hochedlinger 2008), and tetraploid complementation (Woltjen, Michael et al. 2009).

Moreover, as iPSCs may be obtained from somatic cells, they offer the potential of generating patient-specific cell lines and consequently will overcome the immune rejection response. Furthermore, being generated from adult cells, iPSCs are not subjected to the ethical considerations related to ESCs.

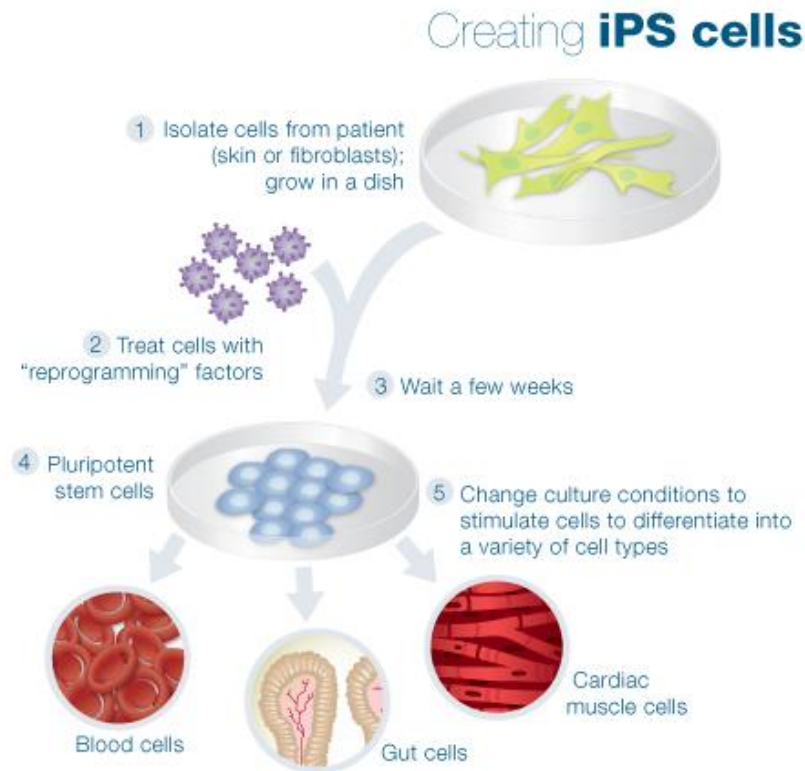


Figure 8 Generation of iPSCs

iPSCs are generated from adult fibroblasts by introducing a cocktail of reprogramming factors. Reprogrammed cells are pluripotent and if cultured with different stimuli can originate cells from the three germ layers, such as blood cells, gut cells or cardiac cells. <http://learn.genetics.utah.edu/content/tech/stemcells/quickref/>

Following the publication of the protocol for iPSC generation, a number of research groups focused on demonstrating that iPSCs can originate from different types of somatic cells derived from all three germ layers: neuronal progenitor cells and keratinocytes from the ectoderm (Aasen, Raya et al. 2008, Shi, Tae Do et al. 2008), progenitor B cells from the mesoderm (Hanna, Markoulaki et al. 2008) and stomach cells and hepatocytes from the endoderm (Aoi, Yae et al. 2008). Furthermore, iPSCs were derived from human cells using either the OSKM factors (Takahashi, Tanabe et al. 2007, Park, Zhao et al. 2008) or Oct4, Sox2, Nanog and lin-28 (Yu, Vodyanik et al. 2007).

The availability of pluripotent stem cell populations and the understanding of the mechanisms that maintain the undifferentiated state, provide a powerful tool to drive stem cell differentiation into therapeutically interesting cell types, such as ECs. In order to design an efficient protocol of differentiation, it is fundamental to

understand the physiological stimuli involved in EC maturation and proliferation during development and adulthood.

1.2.3.1 iPSC DIFFERENTIATION INTO MESODERMAL LINEAGES

As mentioned before, iPSCs are able to differentiate towards cells derived from all the three germ layers, such as pancreatic beta-cells from the endoderm (Zhang, Jiang et al. 2009), and several neuronal cell types from the ectoderm (Wernig, Zhao et al. 2008). In particular, in this work we will focus on iPSC differentiation towards mesodermal lineages and specifically endothelial cell lineage.

iPSCs are believed to possess the same characteristics and differentiation potential of ESCs. Studies to confirm this statement are of extreme importance and will help to elucidate more clearly the degree of similarity between those two cell types. In regards to the process of endothelial differentiation, the protocols applied and the results obtained so far seem to be comparable. A recent study elucidated the features of the directed differentiation of human iPSCs into vascular ECs and mural cells, revealing that the properties of human iPSC differentiation into vascular cells are nearly identical to those observed in human ESCs (Taura, Sone et al. 2009).

To initiate the differentiation process, iPSCs are cultured on collagen IV, an extracellular matrix protein, which has been reported to direct ESC differentiation to mesodermal lineages, including SMCs, ECs, and hematopoietic cells, in both mouse (Nishikawa, Nishikawa et al. 1998, Schenke-Layland, Angelis et al. 2007) and human cultures (Gerecht-Nir, Ziskind et al. 2003). Fluorescence-activated cell sorter (FACS) analysis of collagen IV differentiated iPSCs showed the presence of Flk1⁺ progenitor cells which, when isolated and cultured in differentiation promoting conditions, differentiated into functional SMCs, ECs and spontaneously beating cardiomyocytes, with concomitant decrease of stem and progenitor cell gene expression (Figure 9) (Narazaki, Uosaki et al. 2008, Schenke-Layland, Rhodes et al. 2008).

Additionally iPSCs monolayers have been shown to differentiate into functional SMCs, after treatment with retinoic acid for 8 days (Xie et al., 2009).

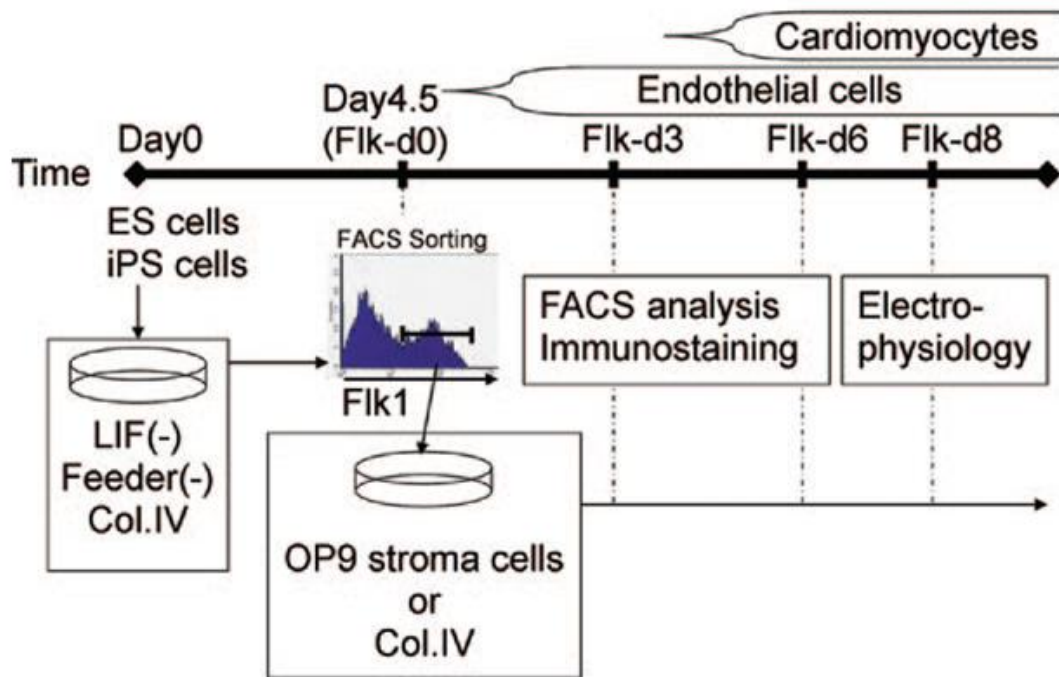


Figure 9 Procedure and time course of mESC and iPSC differentiation into ECs and cardiomyocytes

When undifferentiated induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) were cultured for 4.5 days on collagen IV-coated dishes in differentiation medium, without leukemia inhibitory factor (LIF), a mixed population of cells containing vascular progenitor cells arose. Vascular endothelial receptor 2 (Flk1⁺) putative vascular progenitor cells were sorted by flow cytometry and plated onto collagen IV dish or OP9 stroma cells. Flk1⁺ cells differentiated for one day (Flk-d1) or four and five days (Flk-d4-5) give rise respectively to a mixed population of endothelial cells (ECs) and cardiomyocytes. Fluorescence-activated cell sorter (FACS) analysis and immunostaining were performed at Flk-d3 and Flk-d5 to evaluate the differentiation of ECs and cardiomyocytes; electrophysiological studies were carried out on cardiomyocytes at Flk-d8. Adapted from (Narazaki, Uosaki et al. 2008).

In one particular study, cells derived from Flk1⁺ progenitors were thoroughly characterized. Amongst the populations obtained, the authors identified mural cells (vascular SMCs and pericytes), lymphatic, vascular ECs (arterial and venous ECs) and a novel population of cardiac progenitor cells. From cardiac progenitor cells, a population of self-beating cardiomyocytes arose as a mix of atrial, ventricular, pacemaker and conduction system cells of the heart (Figure 10) (Narazaki, Uosaki et al. 2008).

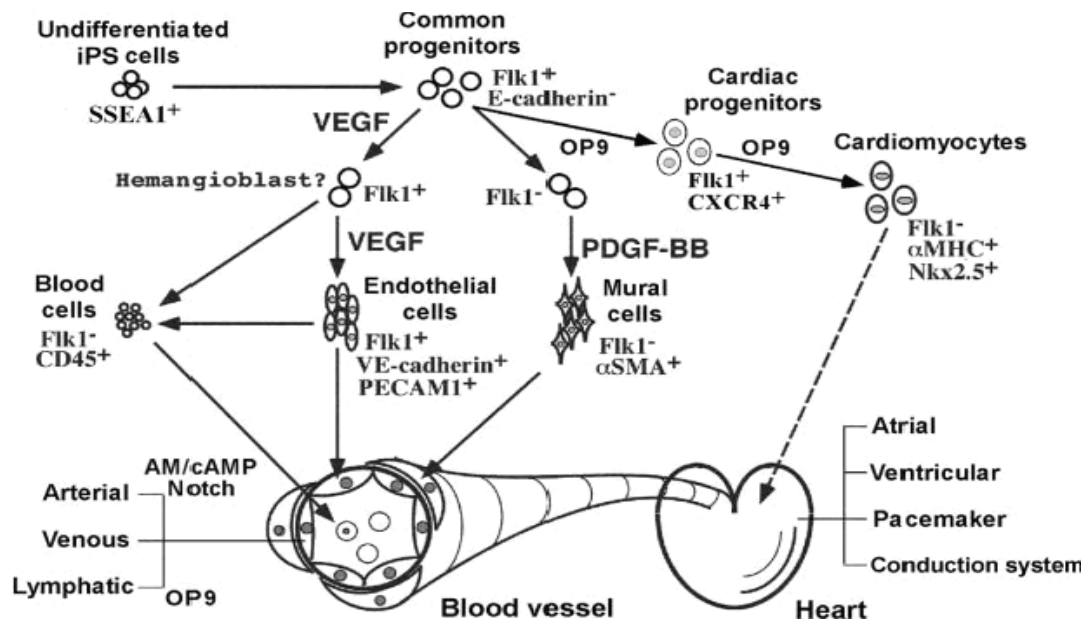


Figure 10 Systematic induction of different cardiovascular cell types from common progenitor Flk1⁺ of mouse iPSCs

A population of common progenitor cells positive for Flk1⁺ and endothelial cadherin (E-cadherin) is selected from pre-differentiated mouse induced pluripotent stem cells (iPSCs), initially positive for stage specific embryonic antigen (SSEA1⁺). Flk1⁺ cells cultured in presence of vascular endothelial growth factor (VEGF) give rise to hemangioblasts-like precursors able to give rise to Flk1⁺ and VE-cadherin⁺ ECs and blood cells (Flk1⁻ and CD45⁺). Furthermore, Flk1⁺ cells give rise to smooth muscle actin positive (αSMA⁺) mural cells in presence of platelet-derived growth factor BB (PDGF-BB). On the other hand, Flk1⁺ cells cultured on OP9 stroma cells differentiated into a cardiac progenitor cells population, positive for Flk1 and chemokine receptor type 4 (CXCR4⁺), that can then be further differentiated into cardiomyocytes, which are negative for Flk1 and positive for α-myosin heavy chain (αMHC⁺) and homeobox protein Nkx2.5. Adapted from (Narazaki, Uosaki et al. 2008).

In a similar study, using collagen IV culture or EB formation, Flk1⁺ progenitor cell-derived mouse iPSCs were differentiated into mesodermal lineages, including cardiovascular and hematopoietic lineages (Schenke-Layland, Rhodes et al. 2008).

Finally, mouse iPSCs have been shown to differentiate *in vitro* into functional cardiomyocytes, which have been characterized for the expression of functional cardiac ion channels and hormonal regulation (Kuzmenkin, Liang et al. 2009).

1.3 STIMULI REGULATING ENDOTHELIAL DIFFERENTIATION

Initial methods to generate vascular progenitors from ESCs tried to mimic the *in vivo* pattern of blood vessel formation. In terms of differentiation both cytokines and mechanical forces are able to initiate a signal cascade leading to progenitor cell acquisition of phenotypic features typical of ECs.

1.3.1 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Both vasculogenesis and angiogenesis are regulated by the actions of a series of growth factors, such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) and, in the adult, platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF- β).

VEGF is an EC-specific mitogen that plays an important role in many of the events necessary for angiogenesis (Connolly 1991) and other EC functions, such as permeability, vascular tone and production of vasoactive molecules (Zachary 1998). The VEGF gene is located on the short arm of chromosome 6 and it is composed of eight exons and seven introns (Tischer, Mitchell et al. 1991, Vincenti, Cassano et al. 1996). Hypoxia-mediated control of gene transcription and alternative splicing regulate the transcription of VEGF gene and the production of differing isoforms (Giles 2001). VEGF transcript is subjected to alternative splicing from exons 5 to 8, leading to the production of different isoforms which show variable diffusibility depending on their length: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ represent the main forms (Tischer, Mitchell et al. 1991). Exon 6, which is not present in VEGF₁₂₁ and VEGF₁₆₅, together with exon 7 provide heparin-binding affinity; exon 8, which is present in all the active isoforms, is required to stimulate mitosis (Ferrara 2004). The smaller isoform VEGF₁₂₁, which lacks exons 6 and 7, is freely diffusible, whereas the longer isoforms are highly basic and remain cell-associated (Houck, Ferrara et al. 1991). VEGF₁₆₅, which lacks only exon 6, shows intermediary properties: it is largely soluble, with a distinct cell-associated fraction (Houck, Ferrara et al. 1991). VEGF₁₆₅ is the prevailing isoform, and is most biologically active in the physiological state (Ferrara 2004). VEGF regulates key cellular events during

vasculogenesis, supporting EC proliferation and motility. In particular, low doses of VEGF induce EC proliferation while high doses enhance EC motility. This dose-dependent effect can also explain the abnormal blood vessel formation and lethality of embryos lacking only one single VEGF allele (heterozygous VEGF^{+/-}). As expected, the development of blood vessels is further impaired in homozygous VEGF-deficient embryos (VEGF^{-/-}) (Carmeliet, Ferreira et al. 1996, Ferrara, Carver-Moore et al. 1996).

VEGF exerts its action binding to three different receptors: VEGFR-1, also known as fms-related tyrosine kinase (Flt1), VEGFR-2 or kinase insert domain receptor (KDR), the human homolog of fetal liver kinase (Flk1), and VEGFR-3 (Flt4) (Neufeld, Cohen et al. 1999, Ellis, Takahashi et al. 2000). Both VEGFR-1 and 2 consist of an extracellular domain composed of seven immunoglobulin (Ig)-like domains, a transmembrane domain, a juxtamembrane domain, a long kinase domain insert in the middle of the tyrosine kinase domain and a C-terminal tail (Takahashi, Yamaguchi et al. 2001). VEGFR-1 can be activated by VEGF-A or placenta growth factor-1 in arteriogenesis. Wound healing processes and their signalling pathways have been characterized in human monocytes. As a result of VEGFR-1 activation there is an induction of chemotactic responses, with the expression of tissue factors, cytokines and chemokines (Tchaikovski, Fellbrich et al. 2008). VEGFR-2 is expressed in monocytes and ECs and mediates the effect of VEGF on cell motility (Gille, Kowalski et al. 2001), vascular permeability and proliferation (Clauss, Weich et al. 1996). VEGFR-2 is a major progenitor cell marker for hematopoietic and endothelial lineage, expressed from the hemangioblast to mature ECs (Schatteman and Awad 2004). VEGFR-3 is involved in the lymphoangiogenic process and is homologous with the neuropilin-1 receptor (Ferrara 2000, Yancopoulos, Davis et al. 2000). Study of knock-out animals demonstrated that not only VEGF, but also its receptors 1 and 2 are fundamental in the development of the fetal vasculature. As it appeared from the knock-out animal's phenotype, these two receptors mediate different responses: Flk-1 null mutant mice showed an impaired endothelial and hematopoietic cell development (Shalaby, Rossant et al. 1995) and Flt1 knock-out resulted in an overgrowth of ECs and disorganization of blood vessels (Fong, Rossant et al. 1995).

1.3.1.1 ROLE OF VEGF IN EC DIFFERENTIATION

VEGF is a potent stimulus, able to drive endothelial differentiation in a large number of progenitor cell populations, leading to the creation of a reservoir of cells that could be therapeutically used to repair the endothelial monolayer and to improve vascular function following injury [reviewed in (Zampetaki, Kirton et al. 2008)].

Indeed VEGF has been shown so far to be one of the most potent growth factors able to stimulate progenitor cell differentiation into ECs. In particular, treatment of Flk1⁺ cells derived from ESCs with VEGF was able to induce EC differentiation (Yamashita, Itoh et al. 2000). Moreover, ESC-derived Flk1⁺ cells could differentiate into both endothelial and mural cells, after stimulation with VEGF and platelet-derived growth factor BB (PDGF-BB) respectively, and reproduce the vascular organization process. To confirm this concept, Yamashita and his co-workers engineered mouse Flk1⁺ cells to express LacZ, and injected them into the developing hearts of stage 16–17 chick embryos. The β -gal⁺ mouse cells populated blood vessels in the chicks' head, yolk sac, heart, and regions between the somites, showing differentiation towards ECs and mural cell lineages (Yamashita, Itoh et al. 2000). Indeed, Flk1⁺ cells can act as “vascular progenitor cells” in the formation of mature vessels offering big potential for tissue engineering of the vascular system.

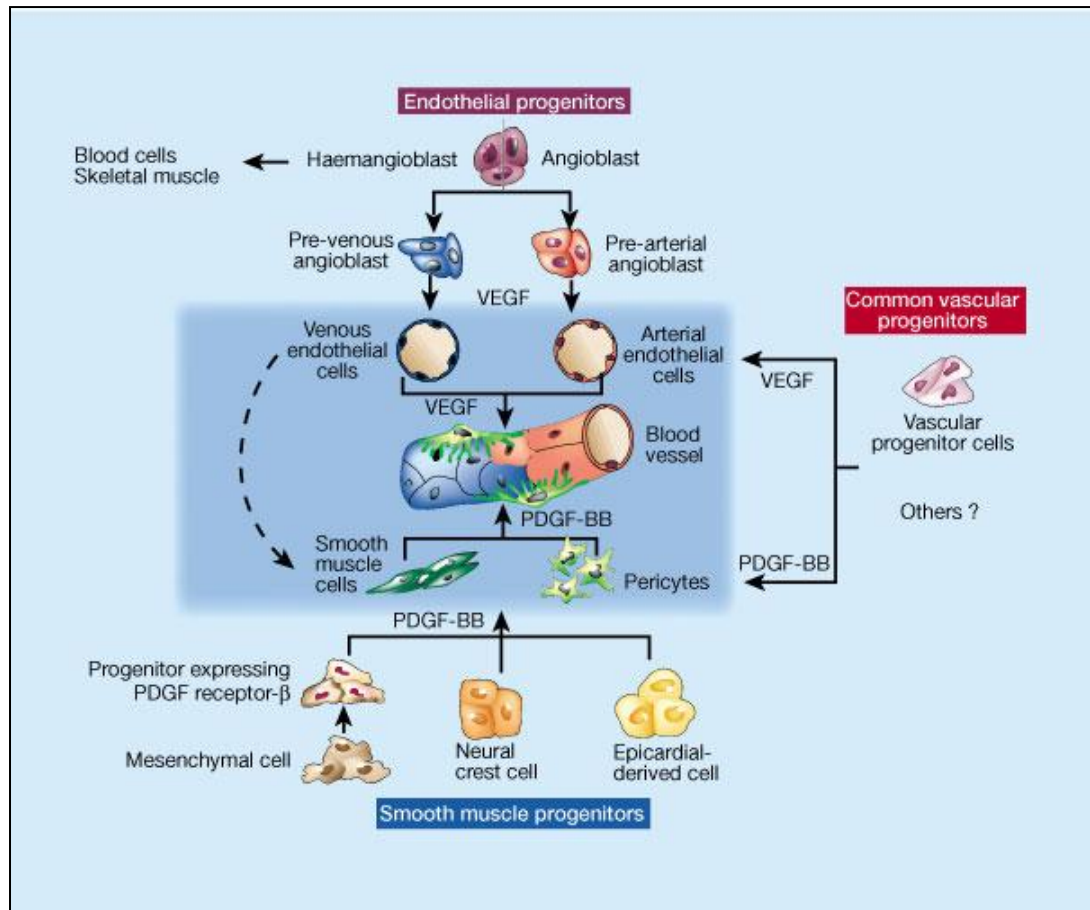


Figure 11 The two types of blood-vessel cells arise from a common progenitor

Blood vessels are generally composed of two cell types: while endothelial cells line the inside and form channels that conduct blood, smooth muscle cells cover the outside, protecting the channels from rupture and controlling blood flow. These two cell types have been considered to arise from separate precursors: endothelial cells from the angioblasts or haemangioblasts in the embryo, or from circulating endothelial progenitors in the adult (not shown) and smooth muscle cells and pericytes from a variety of progenitors. However recently it has been shown that the two types of cells that make up blood vessels, endothelial cells and smooth muscle cells, can develop from common vascular progenitors upon stimulation with vascular endothelial growth factor (VEGF) or platelet-derived growth factor BB (PDGF-BB) respectively (Carmeliet 2000).

Several years later, Flk1⁺ cells isolated from human embryonic stem cells (hESCs) were used to differentiate into ECs and SMCs, which not only expressed phenotypic makers, but were also able to contract when treated with retinoic acid and/or dibutyryl-cyclic adenosine monophosphate (db-cAMP) (Drab, Haller et al. 1997, Huang, Zhao et al. 2006).

Moreover ECs generated from Flk1⁺ precursors were shown to organize into vessel-like structures when grown in collagen gel suspensions or transplanted *in vivo*. Interestingly, immunofluorescence analysis showed that the tube-like structures obtained mimicked the organization of blood islands in the early embryo, being composed of endothelial (PECAM-1⁺) and mural (SMA⁺) cells and containing blood cells (CD45⁺ and Ter119⁺) (Yurugi-Kobayashi, Itoh et al. 2003).

Additionally, in a study conducted in our group, stem cell antigen-1-positive (Sca1⁺) progenitor cells, isolated from predifferentiated ESCs were cultured in medium containing VEGF which led to their differentiation into a pure and functional population of ECs, via VEGF-induced activation of histone deacetylase 3 (HDAC3) (Xiao, Zeng et al. 2006). When cultured with VEGF for 21 days Sca1⁺ progenitors displayed *cobblestone* morphology and the majority of the cells expressed high levels of endothelial markers (CD31, CD106, CD144, Flk1, Flt1 and vWF). These ESC-derived ECs were also shown to form vascular structures when mixed with Matrigel and subcutaneously injected in mice (Xiao, Zeng et al. 2006). Moreover, when Sca1⁺ cells were injected in a mouse model of femoral artery denudation injury, the progenitor-derived ECs were able to decrease the process of neointimal formation (Xiao, Zeng et al. 2006).

In another work, hESCs isolated from 10 to 15 day old human embryoid bodies were dissociated and labeled with anti-human CD34 antibodies. CD34⁺ cells were then isolated from the cell mixture using magnetic-activated cell separation and cultured in endothelial growth medium supplemented with VEGF. These specific differentiation conditions gave rise to an homogenous endothelial-like population (Levenberg, Ferreira et al. 2010). Similarly, undifferentiated ESCs were cultured on collagen IV-coated dishes, in a medium containing fetal calf serum but no LIF. This induced the generation of cells of the mesodermal lineage, including Flk1⁺ cells that were then purified by flow cytometry sorting. When these cells were cultured with medium containing VEGF, sheets of ECs expressing typical markers, such as PECAM-1, arose (Hirashima, Kataoka et al. 1999, Yamashita, Itoh et al. 2000).

The growing number of publications showing the centrality of the role of VEGF in the process of endothelial differentiation of progenitor cells prompted us to apply it to our differentiation system.

1.3.2 SHEAR STRESS

Apart from VEGF, another potent stimulus used to induce EC differentiation is the mechanical force of shear stress.

Endothelial shear stress is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is expressed in units of force/unit area (N/m^2 or Pascal [Pa] or dyne/cm^2 ; $1\text{N/m}^2 = 1\text{ Pa} = 10\text{ dyne/cm}^2$). Endothelial shear stress is proportional to the product of the blood viscosity (μ) and the spatial gradient of blood velocity at the wall (endothelial shear stress = $\mu \times dv/dy$).

Abnormal responses of ECs to shear stress lead to impaired vascular functions and contribute to many vascular diseases, such as hypertension, thrombosis, and atherosclerosis. Previous studies showed that shear stress could lead to morphological changes of ECs via cytoskeleton reorganization with actin filaments becoming rearranged into stress fibers aligned in the direction of the shear stress (Ando and Yamamoto 2009).

Laminar shear stress occurs naturally *in vivo* in straight regions of the vasculature but becomes turbulent in areas of disturbed flow, such as the inside wall of curved regions and lateral branching points, where a higher incidence of atherosclerosis is observed (Ku, Giddens et al. 1985). In healthy regions of the human vasculature where the laminar blood flow is unidirectional and pulsatile, the shear stress sensed by the endothelium is between 15 and 70 dynes/cm^2 . High shear stress induces an atheroprotective and anticoagulant endothelial phenotype (Boon and Horrevoets 2009). On the other hand, regions of the vasculature predisposed to atherosclerotic lesion formation still sense pulsatile flow, though this is highly turbulent and bidirectional. The oscillatory shear stress sensed by the endothelium in these regions is between 0 and 10 dynes/cm^2 . Application of turbulent shear stress through use of an *in vivo* cuff model determines a higher probability of development of atherosclerotic lesions (VanderLaan, Reardon et al. 2004).

Moreover, occluded sections of atherosclerotic lesions which are subjected to elevated laminar shear stress showed an 85% decrease in EC apoptosis (Tricot, Mallat et al. 2000). Furthermore, it has been shown that ECs exposed to 24h of steady laminar shear flow at 12 dyn/cm^2 , which reproduces approximately the

hemodynamic force in straight parts of arteries, undergo anti-inflammatory (Wang, Miao et al. 2006) and anti-proliferative (Lin, Hsu et al. 2000) modifications. In contrast, exposure of ECs to disturbed flow, which mimics the hemodynamic force at branch points, leads to opposite responses (Hsiai, Cho et al. 2003)

Cyclin-dependent-kinase (CDK) inhibitors (e.g., p21cip, p27kip) and tumour suppressor p53 expression and retinoblastoma (Rb) hypophosphorylation have been found to be involved in laminar shear-induced EC growth arrest (Akimoto, Mitsumata et al. 2000, Lin, Hsu et al. 2000).

Different types of flow, such as steady, pulsatile, low and oscillatory, can initiate signalling cascades, leading to up or downregulation of a variety of genes and to cytoskeleton organization. Steady laminar shear stress activates mechanosensitive, atheroprotective signalling pathways, limiting oxidative stress, inflammation and apoptosis and maintaining EC integrity (Chatzizisis, Coskun et al. 2007). In contrast, in regions exposed to low shear stress, or high oscillatory (disturbed) flow, nitric oxide, reactive oxygen species (ROS) scavengers and prostacyclin production is attenuated with a consequent reduction of the vasodilatory, atheroprotective role of these molecules. Disturbed flow is also probably involved in neovascularisation, calcification and thrombogenicity of the atherosclerotic plaque, enhancing inflammation and leading to excessive expansive remodelling. All these factors can transform an early fibroatheroma into a high-risk plaque (Chatzizisis, Coskun et al. 2007).

1.3.2.1 ROLE OF SHEAR STRESS IN EC DIFFERENTIATION

Beside the action of chemokines and growth factors, such as VEGF, endothelial differentiation can also be induced by mechanical stimuli. In particular shear stress has been studied in both *in vitro* and *in vivo* settings.

Many studies have demonstrated that shear stress plays a critical role in promoting the differentiation of ESC-derived progenitor cells into ECs.

In particular, Yamamoto *et al.* showed that when Flk1⁺ progenitors are exposed to shear stress (1.5 to 10 dynes/cm²), EC marker expression, such as Flk1, Flt1, PECAM-1 and VE-cadherin, is significantly increased at both protein and mRNA

levels as it is increased their tube formation capacity (Yamamoto, Sokabe et al. 2005).

In 2005 Wang *et al.* demonstrated for the first time that mesenchymal progenitors differentiate into ECs after exposure to shear stress (Wang, Riha et al. 2005). One year later the same investigators showed that shear stress may promote EC transdifferentiation from SMCs (Wang, Yan et al. 2006).

Additionally, it has been demonstrated that murine ESC derived-ECs, when exposed to laminar flow, undergo cytoskeletal rearrangements and show increased vasculogenic and angiogenic potential *in vitro*, as compared to cells cultured in static conditions (McCloskey, Smith et al. 2006). Also hESC-derived ECs are capable of functionally responding to changes in fluid shear stress by modulating cell morphology and gene expression (Metallo, Vodyanik et al. 2008)

Furthermore, data from our group showed that exposing Sca1⁺ progenitor cells to laminar shear stress (12 dynes/cm²) increased their proliferation and differentiation. Sheared Sca1⁺ cells displayed increased expression levels of Flk1, eNOS and VCAM-1 and showed improved tube-like structure formation on Matrigel (Xiao, Zeng et al. 2006).

In another work, recently conducted by our research group, ckit⁺/Sca⁻ progenitor cells, when exposed to short-term shear stress using an orbital shaker, showed an increased EC marker expression (in revision, Campagnolo *et al.*).

Shear stress has been also shown to induce differentiation of EPCs towards arterial ECs by increasing the expression of ephrinB2 in the progenitor cells through specific protein 1 (Sp1) activation (Obi, Yamamoto et al. 2009).

In another study, two days application of fluid-based shear stress at levels comparable to physiological during the first stages of mouse ESC differentiation induced an increase in cell proliferation and in the expression of the endothelial markers Flk1, VE-cadherin and CD31. Shear stress also induced the number of Flk1⁺ cells from 1% to 40%, inducing the ability of the cells to form vessel-like structures *in vitro* (Ahsan T, Nerem RM. 2010).

Finally, in a work published this year laminar shear stress has been applied to ESCs using a 2D adherent parallel plate configuration to study in a systematic way the

effects of mechanical parameters involved. Shear stress in the range of 1.5 to 15 dyne/cm² promoted endothelial and hematopoietic differentiation of cells seeded on collagen, fibronectin or laminin substrates. Prolonged duration of the treatment consistently induced an endothelial response, while application of shear at later stages of differentiation impaired hematopoietic differentiation (Wolfe and Ahsan 2013).

Thus, shear stress may be used to direct differentiation of ESCs and EPCs toward an endothelial-like phenotype, helping to find new cell sources in tissue engineering and cardiovascular regenerative medicine.

1.3.3 MicroRNA (miRNA)

Amongst the factors able to drive EC differentiation and to modify their proliferation/migration pathway are microRNAs (miRNAs), which have been recently discovered and are now the topic of cutting edge research.

MiRNAs are single-stranded, noncoding molecules of RNA, 20-25 nucleotides long, able to regulate a wide range of cellular processes by binding to non-coding regions of messenger RNA (mRNA) (Daubman 2010). The first miRNAs, *lin-4* and *let-7*, were discovered in *Caenorhabditis elegans* in 1993 (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993, Reinhart, Slack et al. 2000). Extensive research has shown that miRNAs are expressed by most eukaryotic cells and regulate several cell functions (Ambros 2008). The exact mechanism of this regulation remains unclear, however miRNAs seem to bind to 3' untranslated regions (3' UTRs) of target mRNAs by traditional base-pairing; in this way miRNA can promote mRNA degradation or modify its translational levels, therefore regulating the corresponding protein expression (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993, Reinhart, Slack et al. 2000); (Lewis, Burge et al. 2005).

MiRNA genes are mostly transcribed by RNA polymerase II (Pol II), resulting in a primary miRNA (pri-miRNA) which can be spliced (Lee, Jeon et al. 2002, Bracht 2004, Cai, Hagedorn et al. 2004). Pri-miRNA are processed in the nucleus by the RNase III Droscha to produce a ~70-nt precursor miRNA (pre-miRNA) (Lee, Ahn et al. 2003) that is then transported in the cytoplasm by Exportin-5 (Bohnsack,

Czaplinski et al. 2004). In the cytoplasm, another RNase III, Dicer, cleaves the pre-miRNA in a ~22-nt miRNA duplex (Bernstein, Caudy et al. 2001, Chendrimada, Gregory et al. 2005). At this point, the strand of miRNA duplex with lower stability of base-pairing at its 5' is incorporated into the RNA induced silencing complex (RISC), whereas the other strand is degraded. RISC is a trimeric complex composed of Dicer and the Argonaute protein Ago, which is recruited by the TAR RNA binding *protein* (TRBP) in human cells (Gregory, Chendrimada et al. 2005, Maniataki and Mourelatos 2005) (Figure 12). MiRNAs included in the RISC can target the mRNAs by base-pairing. If the base-pairing interaction is complementary, mRNAs are cleaved and actively degraded (Hutvagner and Zamore 2002, Martinez and Tuschl 2004).

Theoretically, each miRNA should be able to regulate more than 100 mRNAs, potentially controlling the activity of 30% of all genes at the post-transcriptional level (Xie, Lu et al. 2005, Filipowicz, Bhattacharyya et al. 2008, Cordes, Sheehy et al. 2009)

In the human genome, more than 1000 miRNAs have been identified and there is strong evidence that these small molecules are involved in a wide range of physiological and pathological processes such as cell proliferation and differentiation, angiogenesis and oncogenesis (Lu, Getz et al. 2005, Suarez and Sessa 2009). Recent studies showed that miRNAs play an important role in vascular development and homeostasis (Suárez, Fernández-Hernando et al. 2008, Bonauer, Carmona et al. 2009).

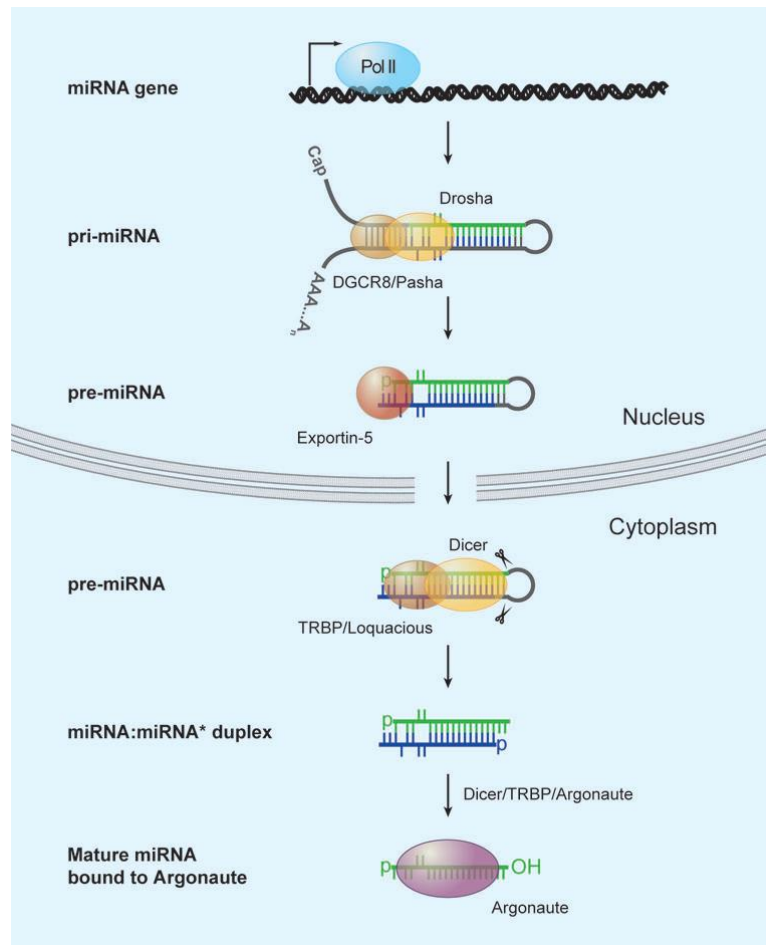


Figure 12 miRNA production in cells

RNA polymerase II (Pol II) transcribes miRNA genes and originates the primary miRNA (pri-miRNA). In the nucleus the pri-miRNA is cleaved by the RNase III endonuclease Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8/Pasha, originating a ~70-nt precursor miRNA (pre-miRNA). The pre-miRNA is transported from the nucleus to the cytoplasm by the protein Exportin-5. In the cytoplasm the pre-miRNA is cleaved by Dicer, another RNase III endonuclease, together with the TAR RNA binding protein (TRBP) /Loquacious, in a ~22-nt miRNA duplex*. The RNA induced silencing complex (RISC), a trimeric complex also composed by Dicer and the Argonaute protein Ago, incorporates the miRNA strand, whereas the miRNA* strand is degraded. Adapted from (Bushati and Cohen 2007).

MiRNA expression is generally tissue specific and dysregulation can cause a cellular dysfunction, thus leading to development of diseases such as cancer (Iorio and Croce 2012), metabolic diseases (Fernández-Hernando, Ramírez et al. 2013) or cardiovascular diseases (Ono, Kuwabara et al. 2011).

1.3.3.1 ROLE OF miRNAs IN ANGIOGENESIS AND EC DIFFERENTIATION AND FUNCTION

Several miRNAs have been shown to be involved in regulating function, proliferation and growth of vascular ECs (Jakob and Landmesser 2012). The first evidence of a possible involvement of miRNAs in ESC differentiation was found in experiments conducted on Dicer or Drosha deficient ESCs, which are not able to produce mature miRNAs and therefore lack differentiation capacity (Murchison, Partridge et al. 2005, Wang, Medvid et al. 2007).

Recent studies focused on angiogenesis-associated miRNAs which are involved in ESC differentiation towards ECs (Wu, Yang et al. 2009). In particular, miRNA-126 has been shown to act as a key regulator of vascular integrity and angiogenesis in mouse and zebrafish. Indeed, miRNA-126 is enriched in human ECs and in developing mouse embryos and has been found to regulate vascular development, regeneration, and integrity (Fish, Santoro et al. 2008, Van Solingen, Seghers et al. 2009). Knockdown of miRNA-126 in zebrafish caused hemorrhaging and loss of vascular integrity in the embryo (Fish, Santoro et al. 2008). In a similar way, endothelial specific deletion of miR-126 in mouse embryos is lethal in ~40% of cases and in the surviving miR-126 null mice, lack of tight cell-cell interactions between ECs cause loss of vascular integrity (Wang, Aurora et al. 2008).

Importantly, predicted targets of miRNA-126 include negative regulators of the VEGF pathway, such as Sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta). These results were confirmed by showing that overexpression of SPRED1 and inhibition of the VEGF pathway in zebrafish, led to results similar to those observed after miRNA-126 knockdown (Fish, Santoro et al. 2008). Finally, overexpression of miRNA-126 in pluripotent stem cells induced differentiation to endothelial lineage, corroborating its leading role in angiogenesis (Fish, Santoro et al. 2008). MiR-126 also regulates the expression of VCAM-1, a critical adhesion molecule, which promotes adherence between ECs and leukocytes (Harris, Yamakuchi et al. 2008).

The family of pro-angiogenic miRNAs includes the miRNA-17-92 cluster, which is expressed in ECs and plays a role in tumour vascularization (Otsuka, Zheng et al. 2008), the miRNA-92a, which has been shown to control angiogenesis *in vivo* and *in*

vitro and to induce functional recovery of mice ischemic tissues (Bonauer, Carmona et al. 2009), and the miRNAs Let-7b and f, which regulate sprout formation (Kuehbacher, Urbich et al. 2007). Moreover, the miRNA-130a controls the angiogenic phenotype of ECs and is considered to promote angiogenesis in ECs by inhibiting the expression of GAX, a homeodomain gene, an angiogenesis inhibitor in vascular ECs (Chen and Gorski 2008). The miRNA-210 is involved in EC migration and capillary-like structure formation (Fasanaro, D'Alessandra et al. 2008), the miRNA-378 promotes tumour angiogenesis (Lee, Deng et al. 2007) and the miRNA-296 regulates EC migration and tube formation, and tumour angiogenesis *in vivo* and it has been shown to target hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), thus leading to a reduction of HGS-mediated degradation of VEGFR2 and PDGFR β (Würdinger, Tannous et al. 2008). Finally miR-10 promotes the VEGFR2-mediated signalling to control EC proliferation, adhesion and migration (Hassel, Cheng et al. 2012). Therefore all the above mentioned pro-angiogenic miRNAs can be used as new therapeutic targets in the selective modulation of angiogenesis, for the treatment of cardiovascular diseases or tumours.

On the other hand, the group of the anti-angiogenic miRNAs comprises for instance the miRNA-221 and 222 which inhibit EC migration and proliferation and have been shown to target the angiogenic ability of c-kit, the receptor for stem cell factor (SCF), thus modulating the capacity of ECs to form new capillaries (Poliseno, Tuccoli et al. 2006, Suarez, Fernandez-Hernando et al. 2007). Moreover, the miRNA-328 reduces formation of capillary structure (Wang, Lee et al. 2008), the miRNA-15b and 16 induce cell apoptosis (Guo, Pan et al. 2009) and together with the miRNA-20a and 20b, have been found to be downregulated in hypoxic conditions and to directly decrease VEGF expression in carcinoma cell lines (Hua, Lv et al. 2006). miR-92a inhibition has been shown to induce proliferation and migration in rat aortic ECs *in vitro* and to promote re-endothelialisation in injured rat carotid arteries *in vivo* (Poliseno, Tuccoli et al. 2006). Finally, the miRNA-100 shows an anti-angiogenic function and represses mammalian target of rapamycin (mTOR) signalling in endothelial and vascular smooth muscle cells (Grundmann, Hans et al. 2011).

Study of miRNA expression in feeder and serum-free directed EC differentiation protocol in hESCs showed upregulation of angiogenesis-related miRNA let7b, 7f, miRNA-126, 130a, 133a and b, 210 and 296 (Wu, Yang et al. 2009) and downregulation of anti-angiogenic miRNA-20a, 20b, 221, and 222 (Wu, Yang et al. 2009), concomitant with an increase in angiogenesis-associated proteins (Kane, Meloni et al. 2010).

Additionally, in a recent work, Kane *et al.* demonstrated that miR-99b, 181a, and 181b take part in the endothelial-miRNA signature and are able to enhance EC differentiation from pluripotent hESCs and to improve hESC-EC-induced therapeutic neovascularization *in vivo* (Kane, Howard et al. 2012).

Finally, even though the miR-17-92 cluster plays a role in regulating vascular integrity and angiogenesis and EC function (Bonauer and Dimmeler 2009), it has been reported that knockdown of the miRNAs in this cluster using antagomirs had no effect on ESC differentiation into ECs (Tréguer 2012).

Further investigations are required to explore miRNA involvement in the early development of the vascular system and EC fate commitment. In particular, analysis and confirmation of miRNA targets will help to elucidate the complicated network of proteins involved and the mechanisms underlying. A better understanding of miRNA regulation of cell commitment to vascular endothelial lineages, and the elucidation of their role in mature endothelial cells, may help in developing new vascular regeneration strategies, to repair damaged tissues after ischemic injury.

1.3.3.2 ROLE OF MiRNA-21 IN ANGIOGENESIS AND ECs

MiR-21 is one of the most well characterized miRNAs and it is overexpressed in many solid tumours (Volinia, Calin et al. 2006). Recent studies suggested that miR-21 plays an important role in tumour growth and metastasis, showing that miR-21 targets several tumour suppressors such as phosphatase and tensin homolog (PTEN) in human hepatocellular cancer (Meng, Henson et al. 2007), programmed cell death 4 (PDCD4) in breast cancer cells (Frankel, Christoffersen et al. 2008), tumour suppressor gene tropomyosin (Zhu, Si et al. 2007) and matrix metalloproteinases inhibitors RECK and TIMP3 in promoting glioma invasion (Gabriely, Wurdinger et

al. 2008). Moreover, miR-21 inhibitor has been shown to suppress cell growth of breast cancer *in vitro* and tumour growth in a xenograft mouse model (Si, Zhu et al. 2006). The role of miR-21 in tumour growth and metastasis led to the hypothesis that it might be involved in the development of tumour angiogenesis.

Indeed, it has been reported that miR-21 overexpression in human prostate cancer cells increased hypoxia-inducible factor-1 α (HIF-1 α) and VEGF expression, thereby inducing tumour angiogenesis. The molecular mechanism involved AKT and ERK 1/2 activation by miR-21. On the other hand, miR-21 inhibition using the antagomir blocked this process. MiR-21 directly targeted PTEN, whose inhibition activated AKT and ERK and increased HIF-1 and VEGF expression, thus inducing tumour angiogenesis. Moreover, miR-21-induced tumour angiogenesis was abolished after inhibiting AKT and ERK using the inhibitors LY294002 and U0126 respectively. The same effect was observed by HIF-1 α inhibition, underlying the importance of HIF-1 α in this process (Liu, Li et al. 2011).

In another work conducted in primary bovine retinal microvascular endothelial cells, which represent a well-characterized *in vitro* system to study angiogenesis, RNA extracted from the cells was used to create RNA library for deep sequencing. Amongst the 250 known microRNAs mapped, the most highly expressed was miR-21. Inhibition of miR-21 using a LNA inhibitor was found to decrease proliferation, migration and tube-formation ability of RMECs, suggesting that miR-21 is involved in the regulation of angiogenesis in the retinal microvasculature (Guduric-Fuchs, O'Connor et al. 2012).

However, miR-21 has been also reported to exhibit anti-angiogenic functions. For instance, in a study conducted in human umbilical vein endothelial cells (HUVECs), miR-21 expression has been confirmed to be negatively regulated by the pro-angiogenic factors serum and bFGF. Furthermore, *in vitro* angiogenic assays showed that miR-21 overexpression led to decreased proliferation, migration and tube formation capacity of ECs, whereas miR-21 inhibition with LNA-21 exerted the opposite action. The decrease in cell migration can probably be explained with a reduction in the organization of actin filaments into stress fibers by miR-21. In this process miR-21 targeted Ras homolog gene family, member B (RhoB), whose inhibition impaired EC migration and tubulogenesis; indeed the mechanism of miR-

miR-21 inhibition of angiogenesis is probably mediated by RhoB repression (Sabatel, Malvaux et al. 2011). Finally, in a mouse model of choroidal neovascularisation, miR-21 has been shown to act as a potential therapeutic inhibitor of angiogenesis (Sabatel, Malvaux et al. 2011).

MiR-21 has also been implicated in shear stress-mediated endothelial differentiation. Mechanical forces associated with blood flow play in fact a relevant role in the regulation of vascular signalling and gene expression in ECs. In the work of Weber *et al.* the miRNA expression profile in human ECs subjected to unidirectional shear stress has been determined and the role of miR-21 in shear stress-induced changes in EC function has been elucidated. In HUVECs exposed to prolonged unidirectional shear stress (24h, 15 dynes/cm²) the miRNA that showed the greatest change was miR-21, which showed an upregulation of 5.2-fold, as compared to untreated cells. Protein expression of PTEN was also downregulated in HUVECs exposed to unidirectional shear stress or transfected with pre-miR-21. Interestingly, overexpression of miR-21 in HUVECs led to reduced apoptosis and increased eNOS phosphorylation and NO production. In conclusion, this study demonstrated that miR-21 expression is regulated by shear stress forces in ECs and these mechanisms are involved in the control of vascular homeostasis (Weber, Baker et al. 2010).

While unidirectional shear stress is a known differentiation stimulus for endothelial differentiation, oscillatory shear stress is typically associated with the vascular inflammation processes leading to atherosclerosis. Oscillatory shear stress has been shown to induce transcription factor activator protein-1 (AP-1)-dependent miR-21 expression in HUVECs. Moreover, miR-21 directly targeted PPAR α , thus inducing the expression of VCAM-1 and monocyte chemoattractant protein-1 (MCP-1) and the consequential adhesion of monocytes to ECs. In conclusion, the induction of miR-21 by oscillatory shear stress contributes to proinflammatory responses of vascular endothelium (Zhou, Wang et al. 2011).

MiRNAs have been shown to regulate many different biological processes, and miR-21 has been reported to play a role not only in EC, but also in EPC function. In particular, a recent study described the miR-21 regulation of EPC senescence, although the mechanisms underlying this process in EPCs are still unknown. In particular, microRNA profiling and microarray analysis have been performed in

lineage-negative bone marrow cells from young and aged wild-type and apolipoprotein E-deficient mice, in order to map the microRNA/gene expression signatures of EPC senescence. From this analysis, miR-10A and miR-21, together with their common target gene *Hmga2* have been identified as critical regulators of EPC senescence. Overexpression of miR-10A and miR-21 in young EPCs led to upregulation of senescence-associated β -galactosidase, decreased self-renewal potential, increased p16(Ink4a)/p19(Arf) expression and impaired EPC angiogenesis *in vitro* and *in vivo*, causing EPC senescence. On the other hand, inhibition of miR-10A* and miR-21 in aged EPCs, decreased senescence-associated β -galactosidase expression, increased self-renewal potential, decreased p16(Ink4a)/p19(Arf) expression and improved EPC angiogenesis *in vitro* and *in vivo*, thereby rejuvenating EPCs. In conclusion, miR-10A and miR-21 have been reported to regulate EPC senescence via inhibiting *Hmga2* expression. Importantly, modulation of senescence-associated microRNAs may offer new therapeutic applications to improve EPC-mediated angiogenesis and vascular repair (Zhu, Deng et al. 2013).

In summary, from the above reported studies, a contradictory role for miR-21 in angiogenesis is emerging and the molecular mechanisms involved in this regulation remain to be clarified. Furthermore, no studies have demonstrated a link between miR-21 and endothelial differentiation from stem cells. Indeed, in the experiments conducted in this thesis, we will try to investigate the role of miR-21 in promoting iPSC differentiation into functional ECs, also elucidating the underlying molecular mechanisms.

1.3.4 TRANSFORMING GROWTH FACTOR BETA (TGF- β) FAMILY

The transforming growth factor β (TGF- β) family is a superfamily of growth factors, which includes 2 families: the TGF- β /activin/Nodal family and the bone morphogenetic proteins (BMPs)/growth and differentiation factor (GDF)/Mullerian inhibiting substance (MIS) family.

The TGF- β family regulates many different biological processes, such as differentiation, angiogenesis, cell growth, apoptosis, migration, extracellular matrix

production, tumour metastasis and invasion and embryonic development (Rahimi and Leof 2007, Zhang 2009).

TGF- β is the most potent inducer of endothelial-to-mesenchymal transition (EndMT) and one of the best promoters of fibrosis in many organs (Hartsough and Mulder 1995, Yu, Hebert et al. 2002).

Epithelial-to-mesenchymal transition (EMT) is an essential process occurring during development, when epithelial cells lose their epithelial markers and start to express fibroblast markers, such as fibroblast-specific protein-1 (FSP-1). EMT occurs in many situations during normal development and also in pathologic conditions such as the progression of malignant epithelial tumours (Engel, McDonnell et al. 1999) and organ fibrosis (Wilkes, Mitchell et al. 2005). In a similar way, EndMT participates to fibroblast formation in fibrotic diseases of the heart (Lee, Hempel et al. 2010), lung (Kurpinski, Lam et al. 2010), kidney (Chen and Lechleider 2004), liver (Kennard, Liu et al. 2008) and carcinoma-associated fibrosis (Pardali and Ten Dijke 2009).

In a recent study it has been shown that miR-21, which is highly expressed in fibroblasts and rapidly inducible by TGF- β , plays an important role in partially mediating EndMT via targeting the PTEN/AKT pathway (Myhre and Blobel 2009).

Five distinct isoforms of TGF- β have been so far described, each approximately 65–85% homologous, and arising after proteolytic cleavage of longer precursors. The mammalian TGF- β isoforms (TGF- β -1, -2 and -3) are secreted as latent precursors and mediate signal transduction through binding to multiple cell surface receptors (Clark and Coker 1998). The biological activities of the mature isoforms are not species-specific. The various TGF- β isotypes share many biological activities and their action on cells are qualitatively similar in most cases, apart from few examples of distinct functions. The biggest differences in the TGF- β isoforms are related to the spatial and temporal expression of their mRNAs and proteins in developing, regenerating and pathologic tissues (Roberts 1992).

The TGF- β family exerts its action on the cells via binding of TGF- β 1, 2 and 3 ligands to specific type I and type II serine/threonine kinase receptors and

intracellular SMAD transcription factors. The human and mouse genomes encode for eight SMAD proteins, which are classified into three groups. The first group, receptor-regulated SMAD (R-SMAD), comprises SMAD1, SMAD5, and SMAD8 which are activated by the BMP-specific type I receptors, and SMAD2 and SMAD3 which are activated by the TGF- β -specific type I receptors; the second group is represented by a common mediator SMAD (Co-SMAD), such as SMAD4; the third SMAD group involves inhibitory SMADs (I-SMADs), such as SMAD6 and SMAD7 (Massagué, Seoane et al. 2005). Activated R-SMADs form complexes with the Co-SMAD, which translocate into the nucleus, where they regulate transcription of target genes (Song, Estrada et al. 2009). It is well-known that TGF- β exerts multiple biological effects via SMAD (Massagué, Seoane et al. 2005, Moustakas and Heldin 2005) and non-SMAD (Moustakas and Heldin 2005) pathways. In the SMAD pathway, two type I and two type II receptors (T β R-I and II) form a tetrameric complex through ligand binding to type II receptors on the cell surface. Both TGF- β 1 and TGF- β 3 show a high affinity for TGF- β RII. In contrast, TGF- β 2 has a low affinity for TGF- β RII and requires an accessory receptor, TGF- β RIII (also known as β -glycan), for high-affinity interaction with the heteromeric-signalling complex. After ligand binding to the complex, the type II receptor kinase activates the type I receptor kinase, which then transduces the signal through phosphorylation of receptor-activated SMADs (R-SMADs) (Goumans, Liu et al. 2009). In addition to the signalling via the canonical SMAD pathway described above, TGF- β can also activate other signalling molecules such as mitogen-activated protein kinases (MAPKs) in a cell-type dependent manner (Mu, Gudey et al. 2012). Examples of such MAPKs are ERK (Hartsough and Mulder 1995), c-Jun N-terminal kinase (JNK) (Engel, McDonnell et al. 1999), p38 kinase (Yu, Hebert et al. 2002), phosphatidylinositol-3-kinase (PI3K)/AKT (Wilkes, Mitchell et al. 2005), or Rho-like GTPase (Bhowmick, Ghiassi et al. 2001) signalling pathways (Figure 13).

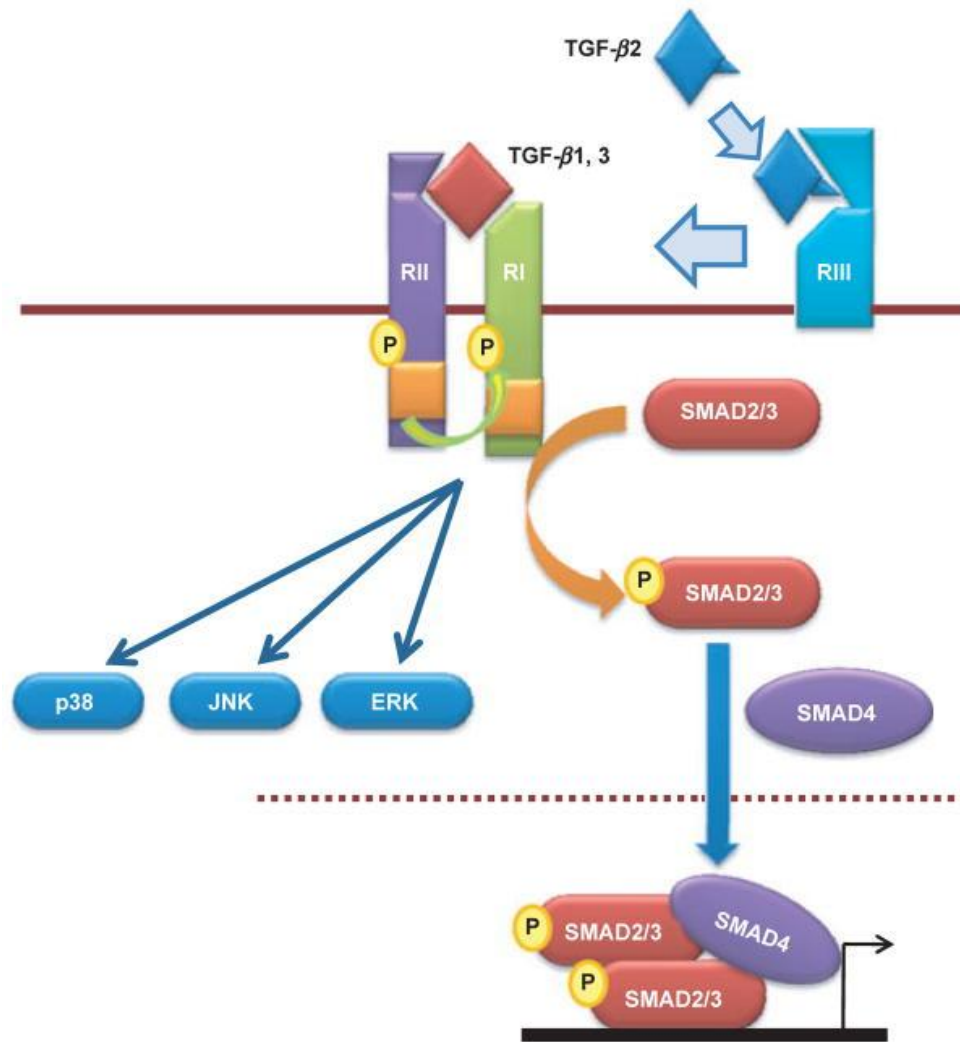


Figure 13 Mechanisms of TGF- β signalling

TGF- β signalling is initiated by assembling receptor complexes that activate SMAD transcription factors. Firstly, TGF- β 1, - β 2, or - β 3 isoform binds to the TGF- β type II receptor (T β R_{II}), which becomes phosphorylated and activated. While TGF- β 1 and TGF- β 3 show a high affinity for TGF- β R_{II}, TGF- β 2 has a low affinity for TGF- β R_{II} and TGF- β R_{III} is required for its binding. Secondly, TGF- β R_{II}-mediated phosphorylation recruits TGF- β R_I into the complex and activates it. Activated TGF- β R_I recruits into the complex and phosphorylates the receptor-associated SMADs (R-SMAD), SMAD2 and SMAD3. This phosphorylation event causes R-SMAD dissociation from the activated receptor complex, association with the SMAD4, translocation into the nucleus and subsequent transcriptional activation or repression. In some physiological and pathological conditions TGF- β can also activate SMAD-independent signalling cascades, including the ERK, JNK, and p38 MAPK kinase pathways (Iwata, Parada et al. 2011).

1.3.4.1 ROLE OF TGF- β IN ANGIOGENESIS, VASCULOGENESIS AND EC DIFFERENTIATION AND FUNCTION

The role of TGF- β signalling in the vascular development has been studied mainly in the context of SMC differentiation. The role of TGF- β in EC differentiation has not been investigated in detail and in particular the specific role of the different isoforms is still in need of clarification, since limited research has been done to date to distinguish the function of the main isoforms in the different biological situations.

The essential roles of TGF- β in angiogenesis have been clarified by genetic studies in human and mouse models. Deletions of many members of the TGF- β family, such as TGF- β 1, TGF- β RII, activin receptor-like kinase 1(ALK1) and TGF- β RI (ALK5), endoglin, SMAD1, 4 and 5, cause vascular remodelling defects and absence of mural cell formation, thus leading to embryonic lethality (Pardali and Ten Dijke 2009). For instance, deletion of TGF- β 1 in the mouse results in embryonic lethality because of defective yolk sac vasculogenesis and EC deletion of TGF- β RI and II results in embryonic lethality at embryonic day 10.5 due to vascular defects. However, TGF- β 1 deletion leads to vascular abnormalities only in a specific genetic background, suggesting that other factors may be involved in the development of vascular abnormalities caused by defects in TGF- β signalling (Pardali, Goumans et al. 2010). Moreover, mutations in the components of the TGF- β signalling pathways, such as endoglin, ALK1 and SMAD4 are responsible for the most clinical cases of Hereditary Hemorrhagic Telangiectasia (Pardali and Ten Dijke 2009).

TGF- β plays a critical role in stem cells differentiation into SMCs by modulating and interacting with other pathways such as the Notch signalling pathway. In a recent work conducted on human mesenchymal stem cells, it has been shown that TGF- β induced the expression of Notch ligand Jagged 1 (JAG1) and SMC markers, including smooth muscle alpha-actin (ACTA2), calponin 1 (CNN1), and myocardin (MYOCD), via SMAD3 and Rho kinase-dependent activation. Furthermore, Notch signalling mediated TGF- β regulation of MSC differentiation and induced the differentiation of mesenchymal stem cells and hESCs into SMCs (Kurpinski, Lam et al. 2010). In another study, it was demonstrated that TGF- β -activated SMAD2 and SMAD3 were necessary for the induction of the SMC marker α -SMA, and that SMAD2 and SMAD3 may cooperate to induce a smooth muscle phenotype in neural crest stem cell line Monc-1 (Chen and Lechleider 2004).

In endothelial cells, TGF- β has been shown to bind to and signal through two distinct types of receptors: TGF- β R1 (ALK5) and ACVRL1 (ALK1); this results in activation of SMAD2/3 and SMAD1/5/8, respectively (Goumans, Valdimarsdottir et al. 2002, Goumans, Valdimarsdottir et al. 2003). The role of TGF signalling in ECs is still unclear and studies have led to opposite conclusions.

TGF- β /ALK1 signalling has been reported to promote proliferation and migration of ECs whereas TGF- β /ALK5 has been shown to do the opposite (Figure 3.4) (Goumans, Valdimarsdottir et al. 2002, Goumans, Valdimarsdottir et al. 2003). Indeed, the balance between TGF- β /ALK1 and TGF- β /ALK5 signalling may determine the pro- or the anti-angiogenic effects of TGF- β .

Furthermore, TGF- β 1 negatively impacts on hESC-derived EC proliferation and commitment, through the inhibition of inhibitor of DNA binding protein-1 (Id1) expression (James, Nam et al. 2010). It has been also demonstrated that shear stress can induce endothelial differentiation from mouse embryonic mesenchymal progenitor cells, by suppressing TGF- β 1 functions through down-regulation of TGF- β 1, TGF- β R1, TGF- β R2, SMAD2, SMAD3 and SMAD4 and up-regulation of SMAD7 (Wang, Li et al. 2008)

Additionally, different effects of TGF- β on angiogenesis are usually dose- and cellular context- dependent; for instance the role of TGF- β 1 in tumour angiogenesis is context-dependent, since it has been shown that TGF- β 1 prevents tumour growth and angiogenesis in early phases of tumour development, whereas it promotes it in late-stages of tumour progression (Goumans, Valdimarsdottir et al. 2002, Serrati, Margheri et al. 2009, Pardali, Goumans et al. 2010). Moreover, low concentrations of TGF- β were shown to promote EC proliferation and migration and to enhance the angiogenic effects of bFGF or VEGF in a 3D fibrin or collagen assay, while high concentrations led to the opposite effect (Pepper 1997, Pardali and Ten Dijke 2009).

In another study, it has been reported that treating bovine capillary endothelial (BCE) cells with TGF- β initially leads to apoptosis by inducing VEGF expression through conversion of VEGF/VEGFR2-activated p38 (MAPK) into a pro-apoptotic signal by TGF- β signalling (Ferrari, Pintucci et al. 2006). Prolonged TGF- β treatment resulted instead in EC remodelling and formation of cord-like structures (Ferrari, Cook et al. 2009).

In addition, the role of the different receptors TGF- β type I and II receptors on EC function is still unclear because of the remarkable diversity and context-dependent effects of TGF- β family members on the complex process of blood vessel formation. Recent studies suggested that the effects of TGF- β RII or TGF- β RI loss on angiogenesis are caused by defects in SMC function and not by their role on ECs (Harris, Yamakuchi et al. 2008).

Some studies also remarked on a crosstalk between VEGF and ALK1/endoglin signalling in angiogenesis, even though the exact underlying molecular mechanisms remain to be elucidated. In particular, inhibition of ALK1/endoglin signalling using the soluble chimeric proteins ALK1-fragment crystallizable region (Fc) and endoglin-Fc impaired VEGF-induced EC sprouting *in vitro*. Moreover, ALK1-Fc has been shown to inhibit VEGF/bFGF-induced angiogenesis in an *in vivo* matrigel plug assay (Cunha, Pardali et al. 2010). In addition, treatment with ALK5 kinase inhibitor enhanced VEGF/bFGF-induced angiogenesis in a matrigel-plug assay *in vivo* (Liu, Kobayashi et al. 2009).

The regulation of angiogenesis by TGF- β is further complicated by the role of several other key factors, such as SMAD and BMP, as well as the co-receptor endoglin. The activation of different classes of SMADs by the same ligand, such as TGF- β , may activate the formation of diverse receptor complexes, thus inducing opposite effects. The exact role of BMP9-induced SMAD1 or SMAD2 phosphorylation in angiogenesis is still not fully clear. Although BMP9 and BMP10 were considered to have an anti-angiogenic action, BMP9 in combination with TGF- β was shown to enhance VEGF-induced proliferation of ECs *in vitro* and VEGF/bFGF-induced angiogenesis *in vivo* (Cunha, Pardali et al. 2010).

In conclusion, TGF- β and BMP signalling appears to play crucial roles in EC function and angiogenesis; however the molecular mechanisms by which these molecules regulate vascular system still remain to be elucidated. TGF- β signalling exerts apparently contradictory actions during the different stages of angiogenesis which are dependent on the distinct cellular context, local concentration of the ligands, receptors, coreceptors, antagonists and their interactions. Although there have been new insights into the role of TGF- β signalling in vascular development and function, the exact mechanisms remain to be elucidated. A better understanding

of the molecular mechanisms underlying the different effects of TGF- β signalling on angiogenesis will help in developing new therapeutic solutions to pathological vascular malformations and tumour growth and angiogenesis (Pardali, Goumans et al. 2010).

1.3.5 SIGNALLING PATHWAYS INVOLVED IN EC DIFFERENTIATION AND ANGIOGENESIS

1.3.5.1 VEGF AND SHEAR STRESS SIGNALLING INTERACTION

External and environmental stimuli such as VEGF and shear stress have been demonstrated to play a pivotal role in the induction of endothelial differentiation. Both the binding of VEGF to its receptors and the stimulation of mechanoreceptors by shear stress activate a signal cascade inside the cells, which results in the promotion of endothelial-specific proteins. Interestingly, some of these pathways are common to multiple stimuli. The mechanisms of EC differentiation induced by VEGF have been thoroughly investigated. The effect of VEGF in ECs and their precursors is strongly dependent on its binding to VEGFR-2. For instance, it is well known that VEGFR-2 is fundamental in the recruitment and differentiation of endothelial precursors from mouse ESCs (Yamashita, Itoh et al. 2000, Schmidt, Brixius et al. 2007). Lack of this gene (Flk1^{-/-}) in a mouse model arrests vascular and haematopoietic development; the reintroduction of VEGFR-2 through lentiviral transduction of ESC cultures differentiating *in vitro* as embryoid bodies is sufficient to rescue the phenotype (Li, Edholm et al. 2007).

The action of VEGF, through its binding to VEGFR-2, is mediated by the activation of phospholipase C (PLC)/calcium and phosphokinase C (PKC), PI3K/AKT, focal adhesion kinase (FAK) and the RAS/RAF/MEK/ERK pathways (Figure 14) (Giles 2001, Gélinas, Bernatchez et al. 2002, Zeng, Xiao et al. 2006, McCubrey, Steelman et al. 2007).

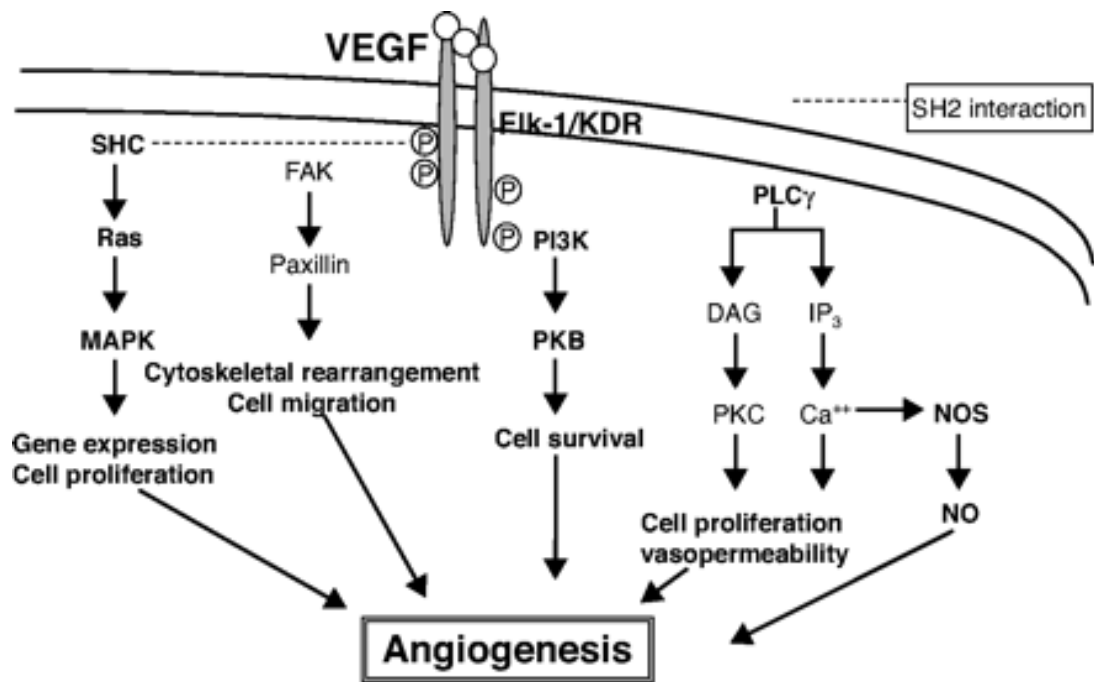


Figure 14 VEGF activates different signalling pathways leading to angiogenesis

When vascular endothelial growth factor (VEGF) binds to Flk1/KDR, this tyrosine kinase receptor is activated by phosphorylation and activates different signalling cascades, which all together lead to angiogenesis. The activation of phospholipase C gamma (PLC γ) leads to cell proliferation and vasopermeability via the inositol triphosphate and calcium (IP $_3$ /Ca $^{++}$) or diacylglycerol and phosphokinase C (DAG/PKC) pathways. VEGF induces immediate synthesis of nitric oxide (NO), also involved in promoting angiogenesis, through the PLC/Ca $^{++}$ pathway. Activation of phosphoinositide 3-kinase (PI3K) promotes cell survival via the phosphokinase B (PKB) pathway. When SHC, an intermediate in the activation of the rat sarcoma (Ras) pathway, is phosphorylated by VEGF receptor, it activates the Ras/mitogen-activated protein kinase (MAPK) pathway, which promotes gene expression and cell proliferation. Finally, cytoskeletal rearrangement and cell migration are also involved in angiogenesis through the signalling cascade of focal adhesion kinase (FAK) and paxillin, two focal adhesion-associated proteins. Adapted from (Giles 2001).

Recent evidence has revealed shear stress as an important key regulator for EPC differentiation. However, the specific mechanisms of mechanotransduction that contribute to the shear stress-induced EPC differentiation have still to be elucidated.

Recently, in EPCs isolated from rat bone marrow and stimulated by shear stress, an increased expression of the EC markers vWF and CD31 was observed, which was found to be related to the levels of integrin β 1 and β 3. These integrins have indeed been demonstrated to play important roles in regulating the shear stress-induced

endothelial cell marker expression in late EPCs. Further study of these integrins may provide novel insights into the mechanisms of mechanotransduction in late EPC differentiation mediated by shear stress (Cui, Zhang et al. 2012).

In addition the mechanosensor heparan sulphate proteoglycan has been found to mediate shear stress-induced expression of vWF, VE-cadherin, tight junction protein gene ZO-1 and vasodilatory genes eNOS and COX-2 in ESC-derived ECs (Nikmanesh, Shi et al. 2012).

VEGF signalling is also deeply involved in the effect of shear stress on endothelial differentiation. In particular, in Flk1⁺ progenitor cells derived from mouse ESCs exposed to shear stress, the mRNA levels of venous endothelial marker Ephrin B4 decreased, whereas mRNA of the arterial EC marker Ephrin B2 levels increased dose-dependently. The increased Ephrin B2 expression after exposure to shear stress is mediated by VEGF-Notch signalling pathway, in which VEGF receptor phosphorylation leads to the activation of Notch (Masumura, Yamamoto et al. 2009).

In a very recent study showing that endothelial and hematopoietic differentiation are both stimulated by shear stress application to ESCs, inhibition of Flk1 neutralized this effect. This result indicates that the membrane protein is a critical mediator of both endothelial and hematopoietic differentiation by applied shear stress to ESCs (Wolfe and Ahsan 2013).

Moreover VEGF-R2 and the PI3K/AKT/mTOR signal pathway have been shown to be activated by shear stress and to induce differentiation, migration, adhesion, proliferation, prevent apoptosis and ultimately to increase the vasculogenesis potential of circulating EPCs (Obi, Masuda et al. 2012)

Furthermore, in the study by Ye *et al.*, activation of AKT has been shown to be essential in the shear stress-induced differentiation of EPCs. Both shear stress and VSMCs co-culture were shown to induce the differentiation of EPCs, increasing the expression of the endothelial markers CD31 and simultaneously decreasing the progenitor markers CD133 and CD34. This effect was blunted by the presence of AKT inhibitor, indicating the essential role of AKT activation in shear stress and/or VSMC-induced EPC differentiation, which provide a new insight to clinical application on the regeneration of the vascular endothelium (Ye, Bai et al. 2008).

Lee *et al.* studied the role of HDACs in EC differentiation, showing that ESCs undergo gene-specific and chromatin remodelling which results in the inhibition of HDACs and prevents differentiation (Lee, Hart et al. 2004). In particular, data from our group demonstrated that the Flk1–PI3K–AKT–HDAC3–p53–p21 pathway is crucial in the shear stress-induced endothelial differentiation of progenitor cells (Sca1⁺) derived from ESCs, and also that VEGF induces EC differentiation through a similar pathway. Specifically, it has been shown that that laminar shear stress (12 dyne/cm²) is able to activate HDAC3 through activation of Flk1 and its downstream PI3K-AKT cascade. Activated HDAC3 in turn deacetylates p53 and activates p21, resulting in EC differentiation and survival (Xiao, Zeng et al. 2006).

1.3.5.2 miRNA-21 INTERACTION WITH THE VEGF AND SHEAR STRESS SIGNALLING

MiR-21 function in angiogenesis is still unclear, since some studies show that it promotes the angiogenic process, while others show the opposite.

Its role in tumour angiogenesis and the mechanisms involved in this process were described in a work by Liu *et al.* miR-21 was found to induce tumour angiogenesis in human prostate cancer by targeting PTEN, leading to the activation of AKT and ERK1/2 signalling pathways, which enhanced HIF-1 α and VEGF downstream expression. On the other hand, overexpression of PTEN inhibited tumour angiogenesis by partially inactivating AKT and ERK and decreasing the expression of HIF-1 and VEGF. Moreover, using AKT and ERK inhibitors, HIF-1 α and VEGF expression and angiogenesis were suppressed. Finally, inhibition of HIF-1 α abolished miR-21-inducing tumour angiogenesis, indicating that this gene is a key downstream target of miR-21 in the regulation of tumour angiogenesis (Liu, Li et al. 2011).

Moreover, in human pancreatic cells miR-21 expression has been found to be increased by hypoxia, together with VEGF and IL6 expression, leading to increase in cancer stem cell marker expression, cell migration/invasion and angiogenesis (Bao, Ali et al. 2012).

In contrast, miR-21 has been shown to negatively regulate angiogenesis in HUVECS, where its expression is negatively regulated by serum and bFGF, two pro-angiogenic factors. In *in vitro* angiogenic assays, miR-21 overexpression reduced endothelial cell proliferation, migration and the ability of the cells to form tube-like structures, whereas miR-21 inhibition led to opposite results. After miR-21 overexpression, the organization of actin into stress fibers was also reduced, explaining the decrease in cell migration. This negative regulation of angiogenesis by miR-21 occurs through directly targeting RhoB expression (Sabatel, Malvaux et al. 2011).

In a recently published study, the drug rapamycin, which is eluted by some coronary artery stents, was found to suppress endothelial proliferation and migration in HUVECs, through overexpression of miR-21. RhoB is directly targeted by miR-21 in this rapamycin-mediated mechanism. Accordingly, miR-21 inhibition abolished the negative effects of rapamycin on endothelial cell growth and mobility (Jin, Zhao et al. 2013).

Furthermore it has been shown that shear stress can regulate the expression of some miRNAs. In particular, in a study conducted by Weber *et al.* in 2010 in HUVECs subjected to prolonged unidirectional shear stress (24h, 15 dynes/cm²), the expression of miR-21 was increased approximately 5 fold, as compared to static control cells. Overexpression of miR-21 in the cells influenced endothelial biology by decreasing apoptosis and activating the NO pathway. This demonstrates that the positive effect of shear stress is at least partially mediated by miR-21 (Weber, Baker et al. 2010).

1.3.5.3 TGF- β INTERACTION WITH THE VEGF AND SHEAR STRESS SIGNALLING

Both VEGF and TGF- β 1 have been shown to induce angiogenesis, but with opposite effects on ECs. In fact, in ECs VEGF promotes survival, while TGF- β 1 induces apoptosis through a VEGF/Flk1 mediated signalling. Genetic deficiency of VEGF abolishes TGF- β 1 induction of EC differentiation and formation of vascular structures in embryoid bodies. Furthermore, inhibition of VEGF blocks TGF- β 1 induction of both apoptosis and angiogenesis. TGF- β 1 induces endothelial cell

apoptosis via up-regulation of VEGF expression and activation of VEGF/Flk1 signalling, which is a rapid and transient process required for TGF- β 1-induced angiogenesis. In conclusion, in this study a new role of VEGF in mediating EC apoptosis has been elucidated, which might represent a target to control TGF- β 1-induced-angiogenic process (Ferrari, Cook et al. 2009).

VEGF induction of cell survival also occurs via activation of PI3K/AKT pathway through the formation of a multi-protein complex that involves Flk1 and the adherens junction proteins VE-cadherin and beta-catenin. In another work conducted by Cook and Ferrari on the TGF- β 1-VEGF interaction in ECs, it has been reported that TGF- β 1 induces changes in the adherens junction structure by separating Flk1 from VE-cadherin and promoting association of beta-catenin with Flk1 and VE-cadherin. These rearrangements are mediated by VEGF/Flk1 signalling. In conclusion, the adherens junction plays an important role in the TGF- β 1-VEGF interaction in ECs. (Cook, Ferrari et al. 2008)

Finally, in mouse embryonic mesenchymal progenitor cells, fluid shear stress has been shown to promote EC differentiation. Shear stress application also abolished TGF- β function through inhibition of TGF- β 1, TGF-R1 and 2 and positive signalling molecules SMAD2, SMAD3 and SMAD4 and induction of negative signalling molecule SMAD7. In conclusion, this study suggests that shear stress-induced EC differentiation in mesenchymal progenitor cells might involve a negative regulation of TGF-1 (Wang, Li et al. 2008).

In synthesis, in this paragraph we have clustered the most important mechanisms and signalling pathways involved in EC differentiation and angiogenesis. In particular, we focused on the interactions amongst the pathways activated by VEGF, shear stress, miR-21 and TGF- β stimuli. Indeed, in this work, the mechanisms involving these four stimuli during the iPSC differentiation process into ECs have been investigated.

1.4 USE OF STEM CELLS IN CARDIOVASCULAR THERAPY

The study of the differentiation potential of stem cells and the associated mechanisms presents the opportunity of a new and exciting prospect of therapy for many illnesses, including cardiovascular diseases. The possibility of using cell-based therapies to improve endothelial and cardiac function after injury is causing a great interest and has prompted a large number of studies. In the recent years, a wide variety of cell types have been considered as candidates for therapeutic delivery in patients with cardiovascular problems, in particular in the context of myocardial infarction (Figure 15) (Segers and Lee 2008). Although the ideal cell type has not been found yet, many studies have been carried out to compare the efficacy of different stem cell populations (Wollert and Drexler 2005).

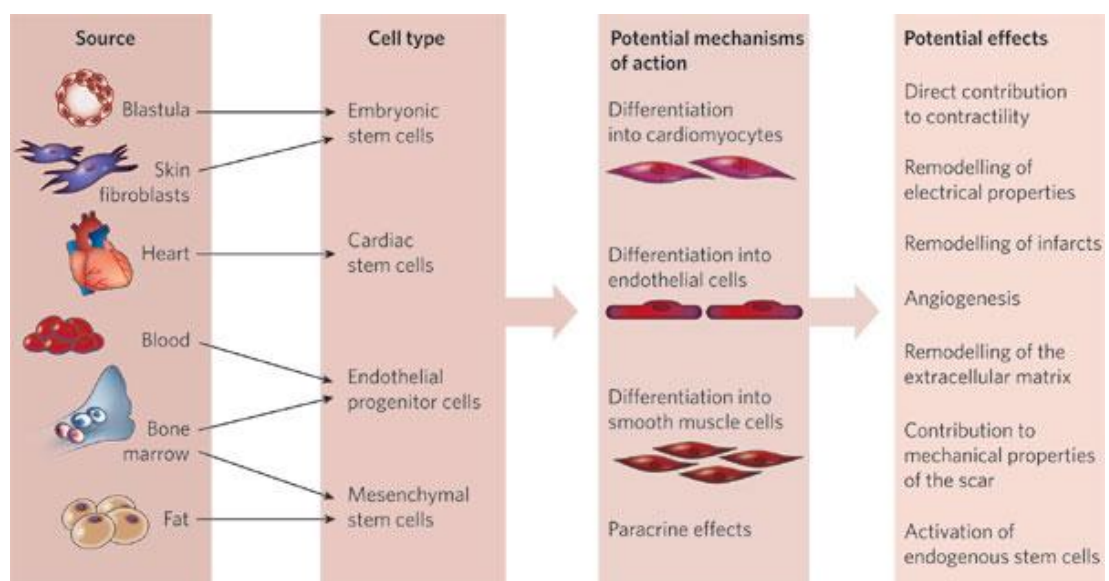


Figure 15 Potential cell types and mechanism involved in cardiac therapy

Many types of stem or progenitor cell populations can be isolated from different autologous or allogeneic organs or tissues: stem cells from blastula or induced from skin fibroblasts, cardiac stem cells from the heart, endothelial progenitor cells from blood and bone marrow and mesenchymal stem cells from bone marrow or adipose tissue. Most of those cells are able to differentiate into cardiomyocytes, endothelial cells, smooth muscle cells and/or to release pro-angiogenic and pro-mitotic paracrine factors, potentially leading to remodelling, angiogenesis or activation of exogenous stem cells in the infarcted heart. Adapted from (Segers and Lee 2008).

Ever since the first therapeutic bone marrow transplantation was performed in dogs in the early fifties by E. Donnall Thomas, the possibility of using adult stem cells in autologous or heterologous cell therapy has been deeply exploited. Adult stem cells are therapeutically attractive because they can potentially be derived from the patient in need of transplantation and therefore they do not present immunological or ethical issues. Furthermore, they possess limited proliferation capacity and differentiation potential, reducing the risk of tumourigenesis.

On the other hand, adult stem cells use is hampered by their limited availability and accessibility and their low *ex-vivo* proliferation, which reduce their application. More importantly, while proof of efficient differentiation of adult stem cells in *in vivo* settings is still debated (Hombach-Klonisch, Panigrahi et al. 2008), clinical trials involving the use of autologous stem cells have shown limited improvement in the functional outcome (Dill, Schächinger et al. 2009, Zhang, Sun et al. 2009, Perez Simon, Lopez-Villar et al. 2011). The prospective of stimulation of resident stem cells within the organs through the use of specific drugs or by the delivery of pro-angiogenic and pro-survival progenitor cells is of major interest, although it is not yet clear if the resident stem cell number would be sufficient after a severe injury (Chavakis, Koyanagi et al. 2010). Additionally, it has recently been shown that an epicardial progenitor population is able to give rise to *de novo* cardiomyocytes in the case of myocardial infarction through stimulation by peptide thymosin β 4; this suggests a regenerative mechanism within the mammalian adult heart (Smart, Bollini et al. 2011).

The use of allogenic cells for transplantation presents the same immunological problems associated with any other tissue or organ transplantation. Within all the cell types studied, ESCs have the advantage of an immunoprivileged phenotype, as shown in pre-clinical studies (Deb and Sarda 2008). This, together with their reported high proliferation and differentiation capacity, made ESCs the focus of numerous *in vivo* studies aiming to investigate their clinical potential. During the past few years, ESCs have become a major focus of translational medicine, regenerative medicine (Ehnert, Glanemann et al. 2009) and functional tissue engineering (Guilak, Butler et al. 2001), due to their potential clinical applications in the treatment of degenerative diseases such as metabolic diseases, brain and myelin disorders, heart disease and many other diseases (Teo and Vallier 2010).

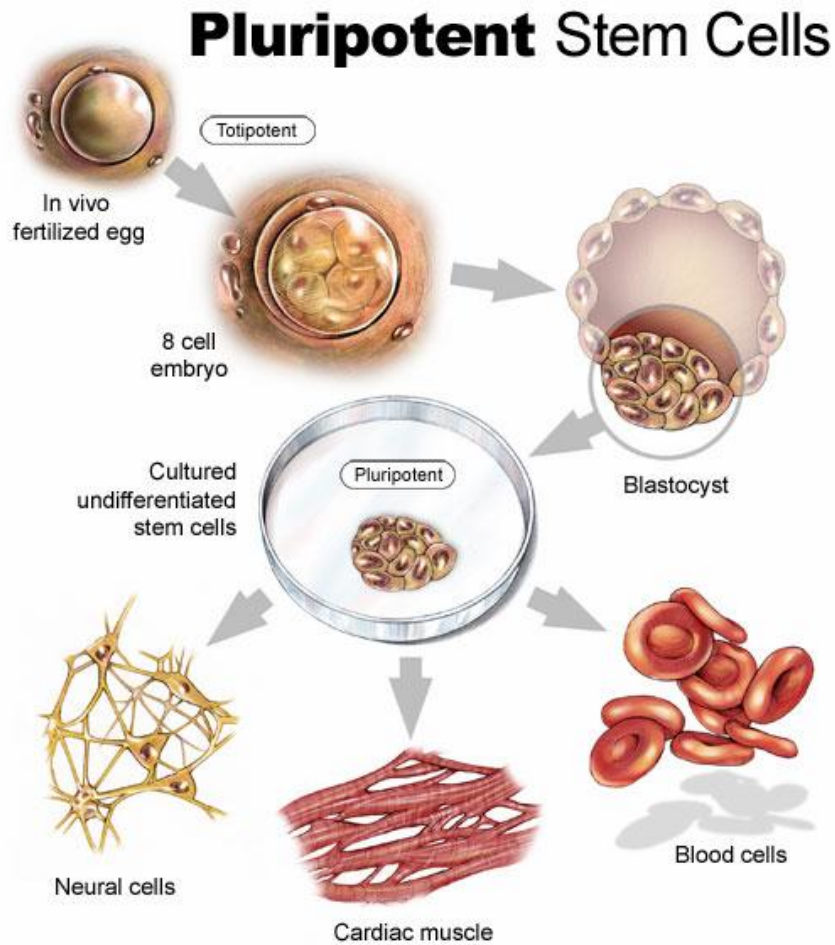


Figure 16 Isolation, culture and differentiation of embryonic stem cells

Embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst and then cultured and differentiated *in vitro* towards different cell types using established differentiation protocols. <http://www.csa.com/discoveryguides/stemcell/overview.php>.

Recent reports showed that ESCs delivered into a mouse infarcted myocardium were able to originate cardiomyocytes, which integrated in the host tissue. Treatment resulted in a stable and beneficial outcome observed over 12 weeks of follow-up and normalization of the ventricular architecture, decrease of the entity of scars and of myocardial necrosis (Hodgson, Behfar et al. 2004). ESC transplantation also reduced cardiac fibrosis, cardiomyocyte hypertrophy and apoptosis resulting in the inhibition of adverse cardiac remodelling (Singla, Lyons et al. 2007).

Beside their ability to give rise to functional cardiomyocytes, ESCs were also shown to be able to release pro-angiogenic and pro-survival factors contributing to their therapeutic effect. A recent study investigated the effect of embryonic and adult stem

cells treatments in a mouse model of myocardial infarction. ESC-treated hearts showed a greater post-ischemic recovery in function, increased cardioprotection and levels of VEGF and IL-10, relative to adult bone marrow cells (BMC)-treated hearts or medium-injected controls. A marked decrease of pro-inflammatory cytokines was also observed after ESC treatment (Crisostomo, Abarbanell et al. 2008).

Before therapeutic applications can be fully realized it has to be taken into consideration that ESC-based therapy has raised many important problems. Firstly ethical issues arise from the derivation of human ESCs from *in vitro* fertilized blastocysts and the isolation of human ESCs requires the destruction of the embryo. Furthermore there are technical issues in the directed differentiation into somatic cell populations, which is still inefficient and generates heterogeneous cell populations. Moreover transplanted ESCs might form teratoma, which is caused by the accumulation of undifferentiated cells in a non-cancerous tumour, and could be rejected immunologically due to the different genetic makeup between the patient and donor. Therefore the use of anti-rejection drugs with serious side effects would be required to avoid immune rejection problems. The risk of tumour formation is due to the innate pluripotency and high proliferative capacity of ESCs (Laflamme and Murry 2005, Swijnenburg, Sheikh et al. 2007).

To reduce this possibility, recent research has focused on the implantation of predifferentiated ESC-derived endothelial or cardiomyocytes (Laflamme, Chen et al. 2007, Tomescot, Leschik et al. 2007). For instance, ESCs pre-differentiation into cardiomyocytes can be promoted with the addition of tumour necrosis factor-alpha (TNF- α). Differentiated ESCs can be implanted in the infarcted myocardium in presence of a “cocktail” of prosurvival factors such as Matrigel, cyclosporine A, insulin-like growth factor-1 (IGF-1) and the caspase inhibitor ZVAD-fmk. Downstream analysis demonstrated the survival of the transplanted cells and their ability to improve myocardial function and recovery (Laflamme, Chen et al. 2007). In another work, ESCs-derived ECs transplanted in a mouse model of myocardial infarction, were shown to improve ventricular function and to promote neoangiogenesis without inducing teratoma formation, as reported in the control animals treated with ESCs (Li, Wu et al. 2007).

When ESC-derived ECs were injected intramuscularly into ischemic mouse limbs, increased limb salvage and blood perfusion were observed (Cho, Moon et al. 2007). Similarly, implantation of hESC-ECs in a hindlimb ischemia model of immunodeficient mice has demonstrated a therapeutic improvement of blood perfusion and limb salvage: transplanted hESC-derived ECs were successfully incorporated into the host circulation and significantly accelerated improvement of local blood flow (Sone, Itoh et al. 2007).

Additionally, in a recent study, hESCs have been directly differentiated into ECs using a feeder- and serum-free protocol. Differentiated cells showed rapid loss of pluripotency markers and progressive induction of vascular markers, such as CD31 and VE-cadherin and angiogenic growth factors, like VEGF; the cells also showed an increased expression of angiogenesis-associated microRNAs, including miR-126 and miR-210, and EC morphology. *In vitro*, differentiated cells were able to produce NO, to migrate across a wound and to form tubular structures in presence or absence of Matrigel. *In vivo*, implantation of the cells previously differentiated for 10 days induced therapeutic neovascularisation, and hESC-derived ECs were incorporated into the blood-perfused vasculature of recipient mice (Kane, Meloni et al. 2010).

Furthermore, mouse ESCs pretreated with retinoic acid to promote neuronal differentiation, were transplanted into an ischemic brain of an adult rat. After 1-8 weeks, transplanted ESCs originated cells able to fill the lesion cavity and to express markers typical of neurons, oligodendrocytes, astrocytes and ECs. ESC-transplanted animals showed an increased recovery in neuronal function and behaviour as compared with the controls rats injected with adult mouse cortical cells or vehicle (Wei, Cui et al. 2005).

Although the tumourigenesis and immunological issues connected with the use of ESCs have been partially addressed, the ethical debate associated with the use of human embryos is still ongoing. Furthermore, the number of available human ESC lines may also be insufficient for their therapeutic potential (Wobus and Boheler 2005).

In order to find a solution to all the issues involving the use of ESCs, scientists are searching for alternative sources of pluripotent cells, such as the generation of iPSCs. The ethical and immunological problems associated with the use of ESCs are

bypassed using iPSCs as they can be derived from somatic cell population isolated from the patient.

iPSCs are able to differentiate into many cell types and, like ESCs, are considered a powerful cell source for regenerative medicine, such as vascular tissue engineering. These cells also show a huge potential for drug screening and for the construction of disease models by obtaining somatic cells from patients with specific diseases.

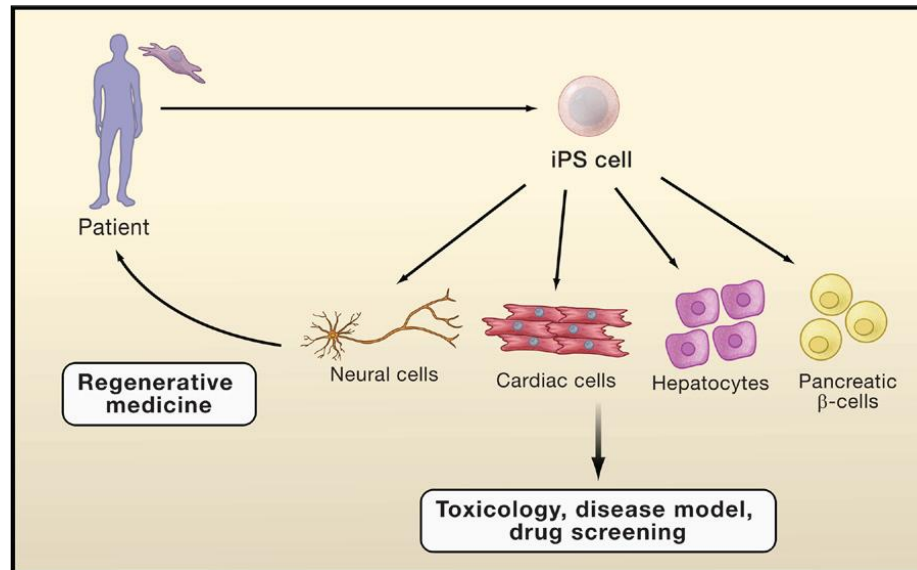


Figure 17 iPSC technology for regenerative medicine and drug screening

iPSCs generated from patient adult cells can differentiate into many different somatic cell types, which could be used for regenerative medicine applications, to screen new and safe drugs and to construct models of disease (Yamanaka 2009).

Some research groups have already shown applications of iPSCs to treat disease states. For instance, it has been demonstrated that iPSCs can treat sickle cell anemia *in vivo* in mice. iPSCs reprogrammed from fibroblasts of mice affected by sickle cell anemia, have been differentiated into hematopoietic progenitor cells, after correction of the human sickle hemoglobin allele by gene-specific targeting. Mice can be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPS cells. These results prove that genetically modified iPSCs can be applied to treat diseases in mice.

In addition, skin fibroblasts from an 82 year-old woman with a familial form of amyotrophic lateral sclerosis, have been reprogrammed to iPSCs and differentiated into motor neurons, the cells affected by the disease (Dimos, Rodolfa et al. 2008).

Similarly to what has been found for ESCs, iPSCs can be predifferentiated and transplanted into a mice model of hind-limb ischemia promoting vascular and muscle regeneration via direct *de novo* differentiation and via paracrine mechanisms. Furthermore, their effect was stronger than that obtained with the control population, adult bone marrow mesenchymal stem cells (Lian, Zhang et al. 2010). In another study, iPSC-derived Flk1⁺ cells were transplanted in a mouse model of hind limb ischemia and were shown to improve recovery and angiogenesis (Suzuki, Shibata et al. 2010). Furthermore, iPSCs directly injected into damaged areas after induction of ischemic stroke in the rat cortex were able to reduce infarct size, improve the motor function and attenuate the inflammation response (Chen, Chang et al. 2010).

The use of patient specific iPSCs derived from adult tissues has opened the possibility of autologous regenerative medicine, but there are many limitations to the iPSC application in the clinical setting. These limitations are mainly due to the delivery of reprogramming factors that introduces risks of permanent transgene integration into the genome and to the oncogenicity of the reprogramming factors. Reprogramming factors such as the known oncogene c-Myc, although mostly silenced in iPSCs, can be reactivated and lead to tumourigenesis (Okita, Ichisaka et al. 2007). Teratoma formation is indeed one of the most severe and common issues concerning the use of iPSCs and even small numbers of undifferentiated cells can originate teratomas (Yamanaka 2009). Moreover, most of the protocols used so far showed reprogramming efficiencies as low as 0.01% suggesting that the methods used to generate iPSCs need to be improved and new strategies will have to be developed (Hochedlinger and Plath 2009).

However, new methods have been developed to reduce the risk of stable integration of the reprogramming factors in the genome of iPSCs during the reprogramming procedure [reviewed in (Muller, Daley et al. 2009)].

Reprogramming can in fact be obtained using plasmids containing the four factors or by direct delivery of reprogramming proteins (Okita, Nakagawa et al. 2008, Kim, Kim et al. 2009), avoiding the risk of using retroviruses and lentiviruses which can

be integrated into the host genome disrupting the normal gene expression. A number of studies are also focusing on enhancing the efficiency of the process, in particular by using specific chemicals, such as the TGF- β inhibitor Alk5i, which increases reprogramming in the absence of c-Myc (Lin, Ambasudhan et al. 2009), or small molecules that promote reprogramming (Xu, Shi et al. 2008). Further research into this field could thus produce a highly efficient method of reprogramming that does not involve genetic modification of the initial somatic cell.

Another recently raised issue concerning the therapeutic application of iPSCs is about their immunogenicity. Although iPSCs might not show alloreactivity, if the iPSC harbors a genetic abnormality which is corrected before transplantation into the iPSC donor, then an immune response can occur (Fairchild 2010). It has been, in fact, demonstrated that abnormal expression in some cells derived from iPSCs can induce T-cell-dependent immune response in syngenic recipients (Zhao, Zhang et al. 2011). Indeed the immunogenicity of the patient-specific iPSCs should be seriously evaluated before their clinical application.

There is also a big concern about the consistency of the current methods used to assess pluripotency of the iPSCs lines. In a recent study it has been shown that amongst 122 published iPSC lines, only 21% of the researchers used the teratoma formation assay, one of the most important tests for pluripotency, to characterize these cell lines (Smith, Luong et al. 2009). Furthermore, recent microarray analyses showed that there are still differences in the gene expression profiles of iPSC and ESCs.

In conclusion, the recent innovative approaches in generating iPSCs have significantly advanced stem cell research. However, several technical issues still remain to be addressed, in order to exploit the full potential of iPSCs.

However, despite success in pre-clinical settings, iPSC technology is not yet ready for transplanting cells into patients, because iPSCs, like ESCs, tend to form teratomas, and the current differentiation protocols are not designed to efficiently eliminate residual undifferentiated cells (Wernig, Zhao et al. 2008).

Furthermore, the potential therapeutic use of iPSCs is also limited by the relatively little knowledge of their molecular and functional equivalence to ESCs. Indeed a

careful analysis of the genomic and epigenomic integrity of iPSCs as well the development of optimized differentiation protocols will be required to evaluate the functionality of iPSCs-derived specialized cells.

1.5 HYPOTHESIS AND AIMS OF THE STUDY

The hypothesis in this work is that the VEGF-induced iPSC differentiation into an endothelial lineage is regulated by miRNAs and their specific signalling pathways.

This project aimed to identify the mechanisms regulating iPSC differentiation into ECs, with a particular focus on miRNAs and their targets.

Initially, we aimed to optimise a protocol to provide an efficient differentiation of iPSCs towards an EC lineage, through the use of collagen IV, VEGF and shear stress in different combinations.

After establishing the optimal differentiation protocol, microRNA array technique was performed in collaboration with Dr. Anna Zampetaki, to identify changes in miRNA expression during the initial days of differentiation; this allowed us to investigate the crucial early changes in miRNA expression that drive the downstream differentiation process. In particular, we aimed to study the function of miRNAs selected from the array, able to drive the iPSC specific differentiation towards ECs, via activation of downstream targets.

Next, we intended to elucidate the molecular targets involved in this process and the regulating mechanisms occurring between those, in order to clarify the miRNA-regulated EC differentiation process. In an effort to find the miRNA targets, we performed bioinformatic *in silico* analysis and we then confirmed the results by evaluating target gene expression after miRNA overexpression or anti-miRNA-mediated silencing. Furthermore, we aimed to study the direct effect of miRNA on specific 3'-UTR of its target using a luciferase reporter assay. Finally, our understanding of the miRNA-targets networks was furthered through modification of their expression during the iPSC differentiation process.

Ultimately, iPSCs might represent an ethically and technically suitable source of cells for transplantation. iPSC derived-ECs could become useful for the treatment of cardiovascular diseases such as myocardial infarction, ischemia and atherosclerosis, in which EC death and dysfunction represent a critical initiation process.

Furthermore, an understanding of the molecular mechanisms that involve miRNA regulation during differentiation may accelerate the translation of this basic science to a clinical setting. The identification of molecular targets might translate into new treatments for patients affected by cardiovascular diseases; it might also offer potential applications for stem cell therapy, e.g. tissue engineering and endothelial repair in damaged vessels.

CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS

Cell culture media, serum and cell culture supplements were purchased from ATCC, Millipore, Gibco and PAA. Mouse recombinant VEGF₁₆₅ and human recombinant TGF- β 2 were purchased from Peprotech, aliquoted into stock solutions of 0.1mg/ml in BSA and stored at -20°C. Human TGF β -2 quantikine ELISA kit was purchased from R&D systems. Mouse VEGF ELISA kit was purchased from Invitrogen. Mouse VEGF₁₆₄ and TGF β -2 neutralizing antibodies were purchased from R&D systems, aliquoted into stock solutions of 0.2mg/ml and 1mg/ml respectively in sterile PBS and stored at -20°C (normal IgG was used as control). SMAD3 inhibitor SIS3 was purchased from Calbiochem, aliquoted into stock solutions of 25mM in DMSO and stored at -20°C. PTEN inhibitor PTP bpV (phen) was purchased from Enzo Life Sciences, aliquoted into stock solutions of 25mM in DMSO and stored at -20°C. Total RNAs were extracted with the RNeasy and miRNeasy kits purchased from Qiagen. Taqman microRNA Reverse Transcription kit, rodent Taqman microRNA assay stem loop primers and rodent preamplification primers were purchased from Applied Biosystems. Resulting cDNAs were used for quantitative real-time PCR using Taqman microRNA assay and Taqman universal PCR master mix reagents from Applied Biosystems. Rodent Taqman miRNA Arrays A and B were purchased from Applied Biosystems. MiR-20b and miR-21 precursors (Pre-20b and Pre-21) and the precursor control (Pre-Ctrl) 5nmol were purchased from Ambion AB and resuspended in 100 μ l sterile water to have a stock concentration of 50 μ M. 1:10 dilution was applied to Pre-20b, Pre-21 and Pre-Ctrl for the transfection experiments. MiR-20b and miR-21 inhibitors (LNA-20b and LNA-21) and the inhibitor control (LNA-Ctrl) 5 nmol were purchased from Exiqon and resuspended in 100 μ l sterile water to have a stock concentration of 50 μ M. LipofectamineTM RNAiMAX for miRNA transfection was purchased from Invitrogen. The Luciferase Assay System and Renilla were purchased from Promega. DNA & siRNA cotransfection reagent jetPRIME[®] was purchased from Polyplus-transfection SA. Mission[®] Sh-RNA for PTEN was purchased from SIGMA-ALDRICH. Polybrene for lentiviral infection was purchased from Millipore. Reporter plasmids pGL3-control-PTEN-3'UTR-wild type and -mutant were created by Joshua Mendell

laboratory and distributed by Addgene (Addgene plasmid 21326 and 21327 respectively). Matrigel for the *in vitro* angiogenesis assay was purchased from BD Biosciences. Purchased antibodies used for immunoblotting and their recommended dilutions are shown in Table 8. Primary antibodies used for immunoblotting were diluted in PBS containing 5% milk, 0.05 Tween[®] 20 (Sigma-Aldrich, Cat No P2287) and 0.02% sodium azide and stored at -20°C. Buffers made for the methods herein are shown in Table 7.

2.2 METHODS

2.2.1 CELL CULTURE OF mESC AND iPSCs

mESCs (ES-D3, from ATCC) and iPSCs were cultured on gelatine-coated flasks (PBS containing 0.04% of gelatine from bovine skin, Sigma) in DMEM (ATCC) supplemented with 10% Fetal Bovine Serum ES cell qualified (Embriomax, Millipore), 100IU/ml penicillin and 100µg/ml streptomycin (Gibco), non essential aminoacids (NEAA) 100X (Gibco) (for mESC culture only), 10ng/ml recombinant human leukemia inhibitory factor (LIF, Millipore) and 0.1mM 2-mercaptoethanol (Gibco) in a humidified incubator supplemented with 5% CO₂. The cells were passaged every 2 days at a ratio of 1:4 to 1:6.

2.2.2 GENERATION OF MOUSE iPSCs

iPSCs were generated in our laboratory starting from MEF isolated as stated in (Takahashi, Okita et al. 2007), using a similar method stated in (Kaji, Norrby et al. 2009). Briefly, 2 x10⁶ MEF were nucleofected with 2µg of linerised pCAG2LMKOSimO (Addgene) using the MEF Nucleofector[®] Kit 2 and nucleofector programme T-020 (Amaxa) and re-plated on gelatine-coated tissue culture plastic with standard MEF media. The media was then changed to ESC media on day 1. Cells were then re-nucleofected on day 4 and maintained in ESC media. iPS-like colonies were picked between day 18-22 and expanded as described before (Takahashi, Okita et al. 2007). Colonies were expanded, characterised for ESC markers and mOrange expression, indicating exogenous gene expression. iPSC

colonies were transfected using Fugene with pCre-GFP (Addgene) according to the manufactures instructions, in order to excise the remaining transgene plasmid. Two days post transfection, GFP positive cells were selected using a cell sorter and clonally re-seeded and expanded. Successful Cre-excision of exogenous DNA was confirmed by genomic DNA PCR, as previously demonstrated in (Kaji, Norrby et al. 2009), and immunoflorescence to show lack of mOrange expression.

2.2.3 DIFFERENTIATION

Mouse ESCs were seeded ($7-10 \times 10^3/\text{cm}^2$) on type IV mouse collagen ($5\mu\text{g}/\text{ml}$, VWR International) coated flasks and maintained for four days in differentiation medium (DM) containing alpha MEM (Gibco) supplemented with 10% FBS (Gibco), 0.2mM 2-mercaptoethanol and $100\text{u}/\text{ml}$ penicillin and $100\mu\text{g}/\text{ml}$ streptomycin. Subsequently, $c\text{-kit}^+$ progenitor cells were isolated with magnetic-activated cell separation magnetic beads as described below, and seeded on collagen IV coated wells and maintained in differentiation medium as mentioned before. After 3 days of differentiation, shear stress was applied using an orbital shaking platform (POS-330 Grant-Bio), at an orbital speed of 120 rpm which correspond to $8\text{ dynes}/\text{cm}^2$ as previously described (Dardik, Chen et al. 2005), for 24h and 48h in presence or absence in the media of $50\text{ng}/\text{ml}$ vascular endothelial growth factor (VEGF, Peprotech). The cells in the central area of the plate, which are subjected to oscillatory stress as compared to the directional and laminar flow in the periphery, were removed by scraping before proceeding to further analysis (Chakraborty, Chakraborty et al. 2012). Differentiation of iPSCs was obtained by seeding them on type IV mouse collagen ($5\mu\text{g}/\text{ml}$) coated dishes in differentiation medium (DM) that contains alpha-MEM supplemented with 10% FBS (Gibco), 0.05mM 2-mercaptoethanol and $100\text{u}/\text{ml}$ penicillin and $100\mu\text{g}/\text{ml}$ streptomycin. The medium was supplemented with $50\text{ng}/\text{ml}$ VEGF and the cells were maintained under these conditions for 3, 5 and 7 days when samples were harvested. The number of iPSCs seeded on collagen IV for the differentiation was $1.3 \times 10^4/\text{cm}^2$ (d3), $6.6 \times 10^3/\text{cm}^2$ (d5) and $3.3 \times 10^3/\text{cm}^2$ (d7). Shear stress studies were performed on iPSCs differentiated for three days, shear stress was applied for 48h in presence or absence in the media of $50\text{ng}/\text{ml}$ VEGF.

2.2.4 CELL SORTING

Pre-differentiated mESCs were trypsinized and centrifuged at 1000rpm for 5min. The cell pellet was resuspended in 60µl of magnetic-activated cell separation buffer (2mM EDTA, 0.5% bovine serum albumin, BSA) to which 40µl of CD117 MicroBeads (Miltenyi) were added. Cell suspension was incubated for 15min at 4-8°C and then washed in 2.5ml of buffer and centrifuged at 1000rpm for 5min. Meanwhile, a MS magnetic-activated cell separation column (Miltenyi) was placed in the magnetic field of a magnetic-activated cell separator and washed with 500µl of buffer. The cell pellet was resuspended in 500µl of buffer and applied onto the column. The unlabeled cells that do not bind to the column pass through and are discarded. The column was washed further three times by adding 500µl of buffer each time. The column was then removed from the separator and placed in a suitable collection tube. Finally, 1ml of buffer was pipetted onto the column and the magnetically labelled cells were flushed out by pushing the plunger into the column.

2.2.5 HARVESTING CELLS

The supernatant medium was removed or collected from the flasks or dishes and cells were washed with cold PBS (4°C). An additional volume (5-10ml) of cold PBS was added and cells were scraped off, decanted into 15ml tubes and centrifuged at 1000rpm for 5min. Supernatant PBS was discarded and cells were resuspended in 1ml of which 750µl and 250µl were transferred to micro centrifuge tubes and centrifuged shortly at 4°C (high speed) for protein and RNA extraction, respectively.

2.2.6 RNA EXTRACTION

RNA extraction was performed using RNeasy Mini Kit for isolation of total RNA from animal cells (Qiagen), according to the manufacturer's protocol at room temperature with a centrifugal force of 8000 g unless otherwise stated. The cell pellet was resuspended in 350µl of RLT lysis buffer that disrupt the cell membrane and organelles to release total RNA contained in the sample. The lysate was then transferred into a QIAshredder spin column and centrifuged for 2min to create a homogenous lysate by shearing high-molecular-weight genomic DNA and other cellular components. An equal volume of 70% ethanol was added to the

homogenized lysate to provide appropriate conditions for the RNA to bind to the RNeasy spin column membrane. At this point the mixture was applied to the RNeasy mini column placed in a 2ml collection tube and centrifuged for 30s and the flow through obtained was discarded. 350µl of RW1 washing buffer were added to the column followed by centrifugation for 15s, and the flow through was once again discarded. On-column DNase digestion was then performed mixing gently 10µl of DNase I stock solution to 70µl of buffer RDD and adding the DNase I incubation mix directly to the RNase spin column membrane (RNase-Free DNase Set, Qiagen). After placing the reaction for 15min at room temperature, 350µl of RW1 washing buffer were added to the column followed by centrifugation for 15s. The flow through was discarded and 2 more washes were carried out with 500µl of RPE buffer. The washing buffers are applied to remove all contaminants. The flow through was discarded and an additional centrifugation of 1min took place to remove all remaining solution that could reduce the purity of the RNA extract. To elute the RNA, the RNeasy column was transferred to a clean 1.5ml autoclaved micro centrifuge collection tube and 30µl of RNase-free water was added to the RNeasy column membrane followed by spinning for 1min. The RNA concentration was measured using a Nanodrop Spectrophotometer.

2.2.7 REVERSE TRANSCRIPTION

cDNA was synthesised from 1µg total RNA. Reverse transcription was performed using the Improm-II reverse transcription system (Promega) in a 25µl reaction as outlined below. Mix A was heated at 70°C for 5min to allow denaturation of cDNA. After adding Mix B, the samples were kept at 4°C for 5min and 25°C for further 5min and then at 42°C for 90min for the extension step to take place. Finally a temperature of 72°C was applied for 5min in order to deactivate the enzyme (Table 1). The cDNA obtained was diluted with 75µl of DEPC-treated water to obtain a final concentration of 10ng/µl.

Table 1 Reverse Transcription reaction

	Component	Volume (μ l)	Final concentration
Mix A	Random primers	0.2	0.1
	Improm II 5x reaction Buffer	5	1X
	25mM MgCl ₂	3	3mM
	25mM dNTPs	1	1mM
	RNA	10	1 μ g
	DEPC-treated water	to 19,2 μ l	-
Mix B	40IU/ μ l RNasin ribonuclease inhibitor	0.625	25IU
	Improm II reverse transcription	1	1IU
	DEPC-treated water	to 5,8 μ l	-

2.2.8 POLYMERASE CHAIN REACTION

50ng of cDNA was used to perform polymerase chain reaction using 2x PCR Master Mix (containing 50units/ml Taq DNA polymerase, 400 μ M each: dATP, dGTP, dCTP, dTTP, and 3mM MgCl₂) from Promega. The reaction mix for 25 μ l reaction volume and primer parameters are given in the table below (Table 2 and 3).

Table 2 PCR reaction

Component	Volume (μ l)	Final concentration
cDNA	5	50ng
PCR Master Mix, 2X	12.5	1x
20 μ M Forward Primer	1	0.8 μ M
20 μ M Reverse Primer	1	0.8 μ M
DEPC-treated water	to 25 μ l	-

Primers specific to the PCR template were designed from human mRNA sequences using the Primer-BLAST tool (www.ncbi.nlm.gov.uk/tools/primer-blast). All primers were ordered from Sigma-Aldrich, resuspended in DEPC water and the stocks (100mM) were stored at -80 °C. Working solutions of 20mM were kept at -20 °C.

Table 3 Primer parameters and their sequence

Gene Target	Sequence (5' → 3')	Start Position	End Position	Annealing Temperature (°C)	Cycles	Product Size (bp)
βactin Forward βactin Reverse	AGCCATGTACGTAGCCATCC CTCTCAGCTGTGGTGGTGAA	469 696	488 677	55	30	227
CD31 Forward CD31 Reverse	ATGACCCAGCAACATTCACA CACAGAGCACCGAAGTACCA	1572 1771	1591 1752	55	30	199
VE-cad Forward VE-cad Reverse	GACCAGTGACAGAGGCCAAT CGGAGGGTTGTCATTCTCAT	1283 1481	1302 1462	55	35	198
vWF Forward vWF Reverse	TTCATCCGGGACTTTGAGAC AGCCTTGGCAAACCTCTCA	5185 5385	5204 5366	55	35	200
Flk1 Forward Flk1 Reverse	GGCGGTGGTGACAGTATCTT GTCAGTACAGAGGCGATGA	498 659	517 640	55	30	161
eNOS Forward eNOS Reverse	GACCCTCACCGCTACAACAT GCTCATTTTCCAGGTGCTTC	1121 1319	1140 1300	55	35	198

The PCR protocols were optimized: Initial denaturation, 95°C for 5min; 30-35 cycles of 94 °C for 30s, 58 °C for 30s and 72 °C for 1min; Final extension 72 °C for 10min.

2.2.9 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (Q-PCR)

Total RNA was isolated as previously described. Relative gene expression was detected by Q-PCR using the Eppendorf Mastercycler® ep realplex. Each real-time PCR reaction was performed in duplicate. Each real-time reaction contained 20ng cDNA, 6.5µl DEPC-treated water and 0.75µl of each 20mM forward and reverse primers and 10µl of sybr green reaction mix (Applied Biosystems). The reaction mixture was placed in each well of a 96-well plate (Eppendorf, twin.tec real-time PCR plates) and placed into a sequence detection system 7000 (Eppendorf). The Q-PCR conditions were 5min at 95°C and then 40 cycles of 95°C for 15s and 60°C for 30s followed by 10min of 95°C to establish the melting curve of the primers. The threshold cycle (Ct) values were automatically obtained in excel format and the 18s RNA Ct values served as the internal endogenous control. For every sample, Q-PCR was performed in duplicate. The primers used for Q-PCR were designed using a software provided by DNA Integrated Technologies (<http://eu.idtdna.com/scitools/Applications/RealTimePCR/>) and are shown below (table 4).

Table 4 Real Time PCR primer sequences

Gene symbol	Gene name	Sequence (5'→3')
VE-cad	Vascular endothelial cadherin 5	AAGAAACCGCTGATCGGCA TCGGAAGAATTGGCCTCTGTC
CD31	PECAM-1	CAAACAGAAACCCGTGGAGAT ACCGTAATGGCTGTGGCTTC
Flk1	VEGFR-2	TGAAATTGAGCTATCTGCCGG TTTGAAGGTGGAGAGTGCCAG
vWF	Von Willebrand Factor	GGCTGTGCGGTGATTTTAACAT CGTTTACACCGCTGTTCTCA
eNOS	Endothelia nitric oxide synthase	GGCTGGGTTTAGGGCTGTG CTGAGGGTGTCGTAGGTGATG
18s	18s ribosomal RNA	CCCAGTAAGTGCCGGGCATAA CCGAGGGCCTCACTAAACC
SMA	Smooth Muscle Actin	TCCTGACGCTGAAGTATCCGAT GGCCACACGAAGCTCGTTATAG
SM22	Smooth muscle 22	GATATGGCAGCAGTGCAGAG AGTTGGCTGTCTGTGAAGTC
CNN1	Calponin1	GGTCCTGCCTACGGCTTGTC TCGCAAAGAATGATCCCGTC
SmMHC	Smooth muscle Myosin Heavy Chain	AAGCAGCCAGCATCAAGGAG AGCTCTGCCATGTCCTCCAC
GATA4	GATA Binding Protein 4	TCCAGTGCTGTCTGCTCTAAGC TGGCCTGCGATGTCTGAGT
MEF2C	Myocyte Enhancer Factor 2C	AAGCCAAATCTCCTCCCCCTAT TGATTCACTGATGGCATCGTGT
Nkx 6.1	NK6 Homeobox 1	GCCTGTACCCCCATCAAG ACGTGGGTCTGGTGTGTTTTTC
AFP	alpha-fetoprotein	ACCCGCTTCCCTCATCCT AAACTCATTTTCGTGCAATGCTT
TUBB3	Tubulin beta3	TCCGTTTCGCTCAGGTCCTT CCCAGACTGACCGAAAACGA
Krt 2-5	Keratin 2-5	CCCTCTGAACCTGCAAATCG TGATCTGCTCCCTCTCCTCAGT
TGF-β2	Transforming Growth Factor beta 2	CTTCGACGTGACAGACGCT GCAGGGGCAGTGTAACCTTATT

PITX2	Paired-like Homeodomain Transcription Factor 2	TGGACCAACCTTACGGAAGC GACAGAGACGTTGACGTGAGG
TGFβI	Transforming Growth Factor beta 1	CAGCACGGCCCCAATGTAT GGGACCTTTTCATATCCAGGAA
TGF-βRIII	Transforming Growth Factor beta Receptor III	GGTGTGAACTGTCACCGATCA GTTTAGGATGTGAACCTCCCTTG
HIF-1α	Hypoxia Inducible Factor 1, alpha subunit	ACCTTCATCGGAAACTCCAAAG CTGTTAGGCTGGGAAAAGTTAGG
VEGFA	Vascular Endothelial Growth Factor A	CTGCCGTCCGATTGAGACC CCCCTCCTGTACCACTGTC
TCF4	Transcription Factor 4	AAAGTCCGAAAAGTTCCTCCG CTCCATAGCCCGGCTGATT
WNT1	Wingless-related MMTV Integration Syte 1	GGTTTCTACTACGTTGCTACTGG GGAATCCGTCAACAGGTTCTGT
RhoB	Ras Homolog Family Member B	GTGCCTGCTGATCGTGTCA CCGAGAAGCACATAAGGATGAC
Spry1	Sprouty Homolog 1	ATGGATTCCCCAAGTCAGCAT CCTGTCATAGTCTAACCTCTGCC
SRY	Sex Determing Region 1	CAAGATGCTGGGTAAGTGCG CCTTTCCGAACAGCGTTGTC
Vcl	Vinculin	TGGACGGCAAAGCCATTC GCTGGTGGCATATCTCTTTCAG
Pcd4	Programmed Cell Death 4	CCACTGACCCTGACAATTTAAGC TTTTCCGCAGTCGCTTTTTGG
Timp3	TIMP Metallopeptidase Inhibitor 3	CTTCTGCAACTCCGACATCGT GGGGCATCTTACTGAAGCCTC
PTEN	Phosphatase and Tensin Homolog	ACAATTCCAGTCAGAGGCG ACTGAGGATTGCAAGTTCCG
REGFX1	Regulatory Factor X1	GGCAGCCAGAAGCAGTATGT GCACCTTCCGATACGGTGA
TNF	Tumor Necrosis Factor	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
ASPN	Asporin	AAGGAGTATGTGATGCTACTGCT ACATTGGCACCCAAATGGACA
BMRII	Bone Morphogenetic Protein Receptor, type II	TTGGGATAGGTGAGAGTCAAT

		TGTTTCACAAGATTGATGTCCCC
Pax3	Paired Box 3	TTTCACCTCAGGTAATGGGACT GAACGTCCAAGGCTTACTTTGT
CCND1	Cyclin D1	AGACCTGTGCGCCCTCCGTA GGCCAGCGGGAAGACCTCCT
LEF1	Lymphoid Enhancer Binding Factor 1	AGAGAAAGGAGCAGGAGCCAAA ACACTCAGCAACGACATTCGC
ID2	Inhibitor of DNA Binding2	GGACCTGCAGATCGCCCTGG TGGACGCCTGGTTCTGTCCA
TCF1	HNF1 Homeobox 1	TGGCCCTCCTCGATGTCCC GGAGGCACCATCCAACGGGC
END1	Endothelin 1	GCACCGGAGCTGAGAATGG GTGGCAGAAGTAGACACACTC
AXIN2	Axin2	TGACTCTCCTCCAGATCCCA TGCCACACTAGGCTGACA
Ctnb1	Catenin, beta1	TGCAGCTTCTGGGTTCCGATGATA AGATGGCAGGCTCAGTGATGTCTT
GSK3A	Glycogen Synthase Kinase 3 alpha	AGCCCAATGTGTCTACATCTGCT AGTAGCTCAGCAAGTACACAGCCA
GSK3B	Glycogen Synthase Kinase 3 beta	TCCGAGGAGAGCCCAATGTTTCAT TGGACGTGTAATCAGTGGCTCAA

2.2.10 AGAROSE GEL ELECTROPHORESIS

A 1.5% Agarose (Sigma) gel, was made with Tris-Acetate EDTA buffer and 0.01 % safeview (NBS), and placed in the electrophoresis apparatus immersed in 1x Tris-Acetate EDTA buffer. Samples were loaded after the addition of 6x DNA Loading dye (30% glycerol, 0.3% bromophenol blue). The electrophoresis was performed at 180V for ~25min and visualised under ultraviolet light.

2.2.11 MiRNA EXTRACTION

Extraction of total RNA including miRNA was performed using miRNeasy Mini Kit (Qiagen). It was performed according to the manufacturer's protocol at room temperature with a centrifugal force of 8000 x g unless otherwise stated. After harvesting the cells as described above, the cell pellet was disrupted by adding 700µl of QIAzol Lysis Reagent to up to 2×10^6 cells and pipetting to mix well. Disruption of plasma membranes of cells and organelles is required to release all the RNA contained in the sample. The tube containing the homogenate was then placed on the benchtop for 5min. 140µl of chloroform were added to the tube shaking it vigorously for 15s and the tube was left at room temperature for further 2-3min. At this point the homogenate was centrifugated for 15min at 12000 x g at 4°C. After centrifugation the sample separated in three phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The volume of the aqueous phase, approximately 350µl, was then transferred to a new collection tube and 1.5 volumes (usually 525µl) of 100% ethanol were added mixing thoroughly by pipetting several times. Up to 700µl of the sample were pipetted into an RNeasy mini spin column in a 2ml collection tube and the sample was centrifuged at 8000 x g for 15s and the flow through obtained was discarded. 700µl of RWT washing buffer provided by the kit were then added to the column and another centrifugation at 8000 x g for 15s was performed; the flow through was once again discarded. Two more washes were carried out with 500µl of RPE buffer. The washing buffers are applied to remove all contaminants. The flow through was discarded and an additional centrifugation of 1min took place to remove all remaining solution that could reduce the purity of the RNA extract. To elute the RNA, the RNeasy column was transferred to a clean 1.5ml autoclaved micro centrifuge collection tube and 30µl of RNase-free water was added to the RNeasy column membrane followed by spinning for 1min. The RNA concentration was measured using a Nanodrop Spectrophotometer.

2.2.12 REVERSE TRANSCRIPTION AND PREAMPLIFICATION

A fixed volume of 2µl of RNA solution from the 30µl eluate was used as input in each reverse transcription reaction. miRNAs were reverse transcribed using the Megaplex reverse transcription Primers which is a set of two predefined pools

(Rodent Pools A and B v3.0) from Applied Biosystem. Reverse transcription reaction was performed according to the company's recommendations combining 2µl of RNA with 0.8µl of Pooled Primers 0.2µl of 100mmol/L dNTPs with dTTP, 0.8µl of 10x Reverse-Transcription Buffer, 0.9µl of MgCl₂ (25mmol/L), 1.5µl of Multiscribe Reverse-Transcriptase, 0.1µl of RNAsin (20U/µl) to a final volume of 7.5µl. The reverse transcription-PCR reaction was set as follows: 16°C for 2min, 42°C for 1min and 50°C for 1s for 40 cycles and then incubation at 85°C for 5min using a Veriti thermocycler (Applied Biosystems). The reverse transcription reaction products were further amplified using the Megaplex PreAmp Primers (Rodent Primers A and B v3.0). The PreAmp Primers significantly enhance the ability to detect low-expression miRNAs, enabling the generation of a comprehensive expression profile using as little as 1ng of input total RNA. A 1µl aliquot of the reverse transcription product was combined with 5µl of Pre-amplification Mastermix (2x), 1µl of Megaplex PreAmp Primers (10x) to a final volume of 10µl. The pre-amplification reaction was performed by heating the samples at 95°C for 10min, followed by 12 cycles of 95°C for 15s and 60°C for 4min. Finally, samples were heated at 95°C for 10min to ensure enzyme inactivation. Pre-amplification reaction products were diluted to a final volume of 40µl and stored at -20°C.

2.2.13 TAQMAN miRNA ARRAY

The expression profile of miRNAs in the samples was determined using the Rodent Taqman miRNA Arrays A and B (Applied Biosystems), which is a set of two 384-well microfluidic cards (Array A and Array B v3.0). The arrays enable quantification of gene expression levels of 375 miRNAs per pool and are specific to human, mouse or rat. Five endogenous controls and a negative control were included in each array for data normalization. PCR reactions were performed using 450µl of the Taqman Universal PCR Master Mix No AmpErase UNG (2x) and 9µl of the diluted pre-amplification product to a final volume of 900µl. 100µl of the PCR mix were loaded to each port of the Taqman miRNA Array. The fluidic card was then centrifuged and mechanically sealed. Real-Time PCR was carried out on an Applied Biosystems 7900HT thermocycler using the manufacturer's recommended programme.

2.2.14 TAQMAN qPCR ASSAY

Taqman miRNA assays were used to assess the expression of individual miRNAs. 0.5µl of the diluted pre-amplification product were combined with 0.25µl of Taqman miRNA Assay (20x) (Applied Biosystems) and 2.5µl of the Taqman Universal PCR Master Mix No AmpErase UNG (2x) to a final volume of 5µl. QPCR was performed on an Applied Biosystems 7900HT thermocycler at 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. All samples were run in duplicates and standardized to miR-202, 135 and RNU6b using SDS2.2 (Applied Biosystems) software.

2.2.15 MiRNA TRANSIENT TRANSFECTION

To alter miR-20b and miR-21 levels in iPSCs differentiated in presence of VEGF for 3 days, cells were cultured to 60–70% confluence, and transfected with the pre-20b and pre-21, the negative control precursor miRNA (Ambion), miR-20b and miR-21 inhibitor, and the negative control of miRNA inhibitor (Exiqon) using Lipofectamine™ RNAiMAX in serum-free alpha MEM medium. The final concentration of the oligomers was 50µM for LNA-20b, LNA-21 and the LNA-Ctrl and 5µM (1:10 dilution) for pre-20b, pre-21 and the pre-Ctrl. Briefly, for each petri dish we removed complete DM and we added 5ml of serum-free alpha MEM medium. In sterile tubes we prepared mastermix A containing 394µl of serum-free alpha MEM and 6µl of precursors/inhibitors/controls; then we prepared mastermix B containing 400µl of serum-free alpha MEM and 25µl of Lipofectamine™ RNAiMAX. We indeed combined mastemix A and B mixing gently and incubating for 10min at room temperature. Next, we added the precursor/inhibitor/controls – Lipofectamine complexes to each petri dish, mixing gently. After 5h, an equal volume of fresh alpha MEM containing 20% FBS, but without GP, was added to the transfection medium. 24h after transfection, the media was refreshed with complete DM until the next day, when cells were harvested and RNA and protein expression was analyzed by Q-PCR and Western blot, respectively. The above mentioned protocol has been scaled down to accommodate different cell numbers.

2.2.16 PROTEIN EXTRACTION

The cell pellet was resuspended in 30-50µl of a protein lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1mM EDTA pH 8.0). The lysate was then sonicated with the Branson Sonifier 150 at the lowest setting for 12s at 4°C and incubated on ice for at least 20min. The lysate was then centrifuged at full speed for 2min at 4°C. The supernatant was transferred to a new tube and the protein level was detected using a Biorad Protein Assay. Briefly, 2µl of the protein lysate was mixed with 998µl of the Bio-Rad Reagent (diluted 1:5 in water) and incubated at room temperature for 5min. Duplicates were measured using the Bio-Rad Spectrophotometer 3000. Lysis buffer was used as the blank measurement.

2.2.17 WESTERN BLOT

30-50µg of protein was mixed with 1X SDS loading buffer and heated at 94°C for 10min before loading onto NuPage®, 6-8% Bis-Tris gel immersed in NuPage® MOPS SDS running buffer in a XCell *SureLock*[™] Mini-Cell (Life technologies (Novex®)). Protein ladder (Precision Plus Protein Ladder, Bio-Rad) was loaded simultaneously and the samples were run at 160V. The gel was then transferred onto a PVDF membrane (Amersham, hypond-P) with the XCell[™] Blot Module (Invitrogen) at 30V for 2h immersed in transfer buffer. The membrane was then blocked with 5% milk in PBS-Tween and then incubated overnight at 4°C in the primary antibody solution. The primary antibodies used are shown below (Table 5).

Table 5 Primary antibodies used in Western Blotting

Primary Antibody	Dilution (in 5% milk in PBS-Tween)	Company of origin
Rabbit anti-VE-cad	1:1000	Abcam
Rabbit anti-CD31	1:750	ABBiotech
Goat anti-vWF	1:1000	Santa Cruz
Rabbit anti-Flk1	1:1000	Abcam
Rabbit anti-eNOS	1:1000	Abcam
Goat anti-Akt 1/2	1:200	Santa Cruz
Rabbit anti-PSer473 AKT	1 :200	Santa Cruz
Rabbit anti-PTEN	1:1000	New England Biolabs
Mouse anti-GAPDH	1:5000	Abcam

The membranes were incubated in the primary antibody (diluted in PBS-Tween containing 5% milk) overnight at 4°C. Secondary antibodies diluted in 5% milk PBS-Tween (1:3000) were incubated in room temperature for 1h after washing with PBS-Tween (10min x3). Further washing was then carried out before addition of ECL detection solutions (GE healthcare, 5min). Exposure of films (Amersham, Kodak) was carried out using the Compact X4 (Xograph Imaging System).

2.2.18 INDIRECT IMMUNOFLUORESCENCE ASSAY

Cells were trypsinised and resuspended in medium and 4×10^3 and 8×10^3 cells (for 3 or 5 days of differentiation respectively) were seeded on each well of an 8-chamber slide coated with gelatine (for undifferentiated iPSCs that were used as negative control) or collagen IV (for iPSCs cultured in DM with VEGF). Three to five days after seeding, medium was discarded and cells were washed twice with warm PBS. The cells were then fixed with 4% paraformaldehyde in PBS for 20min in room temperature. They were then washed x3 with PBS and permeabilised with 0.1% Triton-X-100 in PBS for 10min in room temperature. An additional wash with PBS took place at room temperature for 5min.

Frozen sections of *in vivo* matrigel plaque were instead fixed in 100% acetone for 10min and then washed x3 with PBS. All samples were then blocked with 5% normal swine serum in PBS and incubated for 30min at room temperature. The primary antibodies used and the dilutions in which they were used are shown in Table 6 and the incubation was carried out overnight at 4°C. The samples were once again washed with PBS (10min x3) and secondary antibodies were applied in the concentrations shown in Table 10, for 30min at 37°C. Samples were washed 3 x 5min before counterstaining with DAPI (1:1000 in PBS) for 2min at room temperature. They were then mounted with fluorescent mounting media (Dako) and images were taken with the Axio Imager M2 microscope and AxioVision Digital Imaging System (Carl Zeiss Ltd) or SP5 confocal microscopy.

Table 6 Primary and secondary antibodies used in immunofluorescence staining

Primary Antibody	Dilution (in swine serum)	Company of origin	Secondary Antibody conjugated to Fluorophore	Dilution (in PBS)
Rabbit anti-CD144	1:100	Abcam	Anti-rabbit conjugated to FITCI (Alexa488)	1:1000
Goat anti-CD-31	1:50	Santa Cruz	Anti-goat conjugated to FITCI (Alexa488)	1:1000
Goat anti-vWF	1:50	Santa Cruz	Anti-goat conjugated to FITCI (Alexa488)	1:1000
Rabbit anti-eNOS	1:100	Abcam	Anti-rabbit conjugated to FITCI (Alexa488)	1:1000

2.2.19 IN VITRO AND IN VIVO TUBE FORMATION ASSAY

iPSCs were differentiated in presence of VEGF for 4 days and then transfected with Pre-21, Pre-ctrl, LNA-21 and LNA-ctrl. *In vitro* and *in vivo* angiogenesis assays were performed after further 48h. For the *in vitro* assay, 100µl of complete Matrigel (BD) was layered in each well of an 8 well chamber slide and let to solidify. Cells were detached and counted and 4×10^4 cells were plated in each well; quadruplicates were performed for each condition. Representative images were acquired 7-8h later

with Axioplan 2 imaging and total tube length was quantified with ImageJ image processing program.

In vivo angiogenesis was performed by mixing 5×10^5 cells with 200 μ l Matrigel and injecting it subcutaneously in mice (C57BL/6), where it rapidly solidifies, forming a plug; triplicates were performed per each condition. After 7 days, mice were euthanized, and the skin of the mouse was pulled back to expose the Matrigel. Plugs were then removed and fixed in liquid nitrogen and cryosections were prepared. Some of the cryosections were stained with hematoxylin, which colours nuclei of cells blue and eosin, which colours other structures in various shades of red. Some of the cryosections were stained with CD31 and VE-cadherin antibodies. Immunostaining was assessed by confocal imaging and capillary density was calculated as the number of capillary number per mm^2 .

In vitro angiogenesis assay was performed using the protocol described above also after 7 days of differentiation in presence of VEGF or TGF- β 2 treatment.

2.2.20 BACTERIA CULTURE FROM STAB AND PLASMID PURIFICATION

Using a sterile pipette tip the bacteria was touched within the punctured area of the stab culture and streaked on Ampicilin LB Agar sterile plates (50 μ g/ml) and incubated overnight at 37°C. Single colonies were picked and amplified in LB containing Ampicilin (100 μ g/ml) overnight. Plasmid purification was then performed using a QIAprep Spin Miniprep kit (Qiagen) as instructed by the manufacturer.

Verification of plasmid size was performed by running the plasmidic DNA on a 1% agarose gel.

2.2.21 LENTIVIRAL PARTICLE PRODUCTION

Lentiviral particles were produced by transfecting HEK 293T with shPTEN pLKO.1 plasmid (Sigma Mission) together with the packaging plasmids. Non-targeting pLKO.1 plasmid was used to obtain non-targeting lentiviral particles. Cells were plated at 60% confluency, the next day cells were transfected using a mix of Fugene (Promega) and 4.5 μ g shRNA-PTEN (or NT), 3.6 μ g pCMV-dR8.2 packaging

plasmid and 0.9µg pCMV-VSV-G envelope plasmid (Addgene) at a ratio of 3:1 (µl of Fugene:µg of plasmids). During the overnight transfection period, cells were kept in DMEM containing 2% FBS without antibiotics. The media was changed the next morning to complete medium and the supernatants containing the lentivirus particles were harvested 48h and 96h after transfection, pooled and then filtered through a 0.45 µm filter. The transduction unit (TU) was calculated as previously described (Margariti, Zampetaki et al. 2010).

2.2.22 shRNA LENTIVIRAL INFECTION

Knockdown of PTEN was achieved by infecting iPSCs differentiated for 3 days in DM containing VEGF 50ng/ml with lentiviruses expressing with short hairpin RNA (shRNA). Lentiviruses were generated as described before from plasmids encoding for shRNA specific for PTEN or non-coding control. Cells were infected with shPTEN or the non targeting control (10^7 TU/ml) complete growth medium supplemented with 10µg/ml of polybrene for 16-24h. The viruses were then removed and the cells were transfected with LNA-21 and LNA-Ctrl, as described before. Cells were harvested for further analysis after 48h.

2.2.23 LUCIFERASE REPORTER ASSAY

For the Luciferase Reporter Assays, 3×10^4 iPSCs cells were seeded in each collagen-coated well of a 12-well plate in DM VEGF. 72h later, cells were transfected with the plasmid expressing Luciferase under the control of the PTEN 3'UTR (pGL3-control-PTEN-3'UTR-wild type) and the miR-21 inhibitor and its control. As a control the same experiment was performed with a plasmid expressing luciferase under the control of a mutated and inactive PTEN 3'UTR. Briefly, 0.33µg/well of the reporter plasmids (AddGene, Joshua Mendell laboratory (O'Donnell, Wentzel et al. 2005)) were cotransfected with the miR-21 precursor and inhibitor and their negative controls, (5µM and 50µM respectively) using jetPRIME® (2µl/well) (Polyplus-transfection SA), according to the protocol provided. pGL3-Luc-Renilla (0.1µg/well) (AddGene) was also included in all transfections as a control of the transfection efficiency. Cells were lysated 48h later using Reporter lysis 5x buffer (Promega). The Luciferase (Luciferase Assay System, Promega) and Renilla (Coelenterazine,

Promega) enzymatic activities were detected 48h after transfection using the Lumat LB 9507 illuminometer. Relative luciferase unit was defined as the ratio of luciferase activity to Renilla activity and setting the control value to 1.0.

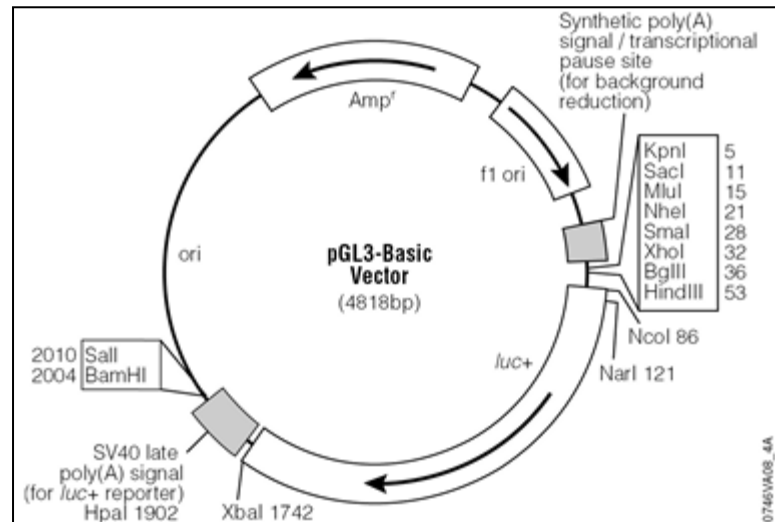


Table 7 Map of the pGL3-Luciferase Basic vector.

Backbone of pGL3; PTEN-3'UTR-wt and -mut have been inserted at the XBAI restriction side.

2.2.24 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Supernatant was collected from the cells, aliquoted and stored at -80°C until used. The concentration of the VEGF and TGF-β2 released glycoprotein in the supernatant was detected by a VEGF and TGF-β2 ELISA kit (Invitrogen and R&D respectively). For the TGF-β2 ELISA kit, before starting the assay, latent TGF-β2 had to be activated to the immunoreactive form according to the manufacturer's protocol. A monoclonal antibody specific for TGF-β2 was pre-coated onto a 96-well microplate. 100µl of Assay Diluent was added to each well. The microplate was subsequently coated with 100µl per well of the standards, control or activated sample and then sealed and incubated for 2h at room temperature (Standards comprised human recombinant TGF-β2 in 7 2-fold serial dilutions with 250pg/mL peak and were used for the generation of a standard curve). The solution was then removed and the wells were washed three times with Wash Buffer (0.05% Tween® 20 in PBS). Each well was then incubated with 200µl of TGF-β2 conjugated (polyclonal antibody against TGF-β2 conjugated to horseradish peroxidase) and the plate was sealed and maintained in room temperature for a further 2h. The wells were once again washed

three times with Wash Buffer. 200µl of the Substrate Solution were added per well (1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine)). The plate was incubated at room temperature for 25min avoiding direct exposure to light. Finally, the reaction was stopped using 50µl of Stop Solution (2N H₂SO₄). The optical density of each well was determined immediately using a Tecan microplate reader set to 450nm. Wavelength correction was set to 540nm and was used for normalisation of the readings. A standard curve was generated and the concentration of released TGF-β₂ of each sample was calculated in pg/ml.

Before starting the ELISA to detect VEGF secretion, cells were serum-deprived overnight. The supernatants were then harvested from an equal cell number (4x10⁵ cells) and were 7.7-fold concentrated after centrifuging for 1h with centrifugal filter units (ultracel YM-3, Millipore). A purified antibody specific for VEGF was pre-coated onto a 96-well microplate. The microplate was subsequently coated with 100µl per well of the standards, standard diluent buffer to the blank standard wells or sample of concentrated supernatants and then sealed and incubated for 1h at room temperature (Standards comprised mouse recombinant VEGF in 7 2-fold serial dilutions with 250pg/mL peak and were used for the generation of a standard curve). The solution was then removed and the wells were washed three times with Wash Buffer (0.05% Tween® 20 in PBS). Each well was then incubated with 100µl of biotinylated mouse VEGF biotin conjugate solution into each well without the chromogen blank and the plate was sealed and maintained in room temperature for further 1h. The wells were once again washed three times with Wash Buffer. 100µl of streptavidin-HRP working solution (1X) were added per well without the chromogen blank. The plate was incubated at room temperature for 30min. After washing once again, the plate was incubated with 100µl of stabilized chromogen for 25min at room temperature in the dark. Finally, the reaction was stopped using 100µl of Stop Solution (2N H₂SO₄) to each well. The optical density of each well was determined immediately using a Tecan microplate reader set to 450nm. Wavelength correction was set to 540nm and was used for normalisation of the readings. A standard curve was generated and the concentration of released VEGF of each sample was calculated in pg/ml.

2.2.25 PHARMACOLOGICAL INHIBITION OF SIGNALLING PATHWAYS

To chemically inhibit PTEN iPSCs cells were seeded on Collagen IV, in DM containing VEGF 50ng/ml up to day 5 when the medium was removed and refreshed with serum free alpha MEM for 4h. Then it was replaced with fresh serum free alpha MEM containing 5 μ M PTEN inhibitor PTP bpV (phen) (Enzo Life Sciences) for 24h, 2h and 30min, when the cells were harvested. DMSO was added to the cells for 2h as a control. Protein expression of P-AKT, tot AKT and PTEN was analyzed.

For SMAD3 inhibition iPSCs cells were cultured from day 1 in DM containing 5 μ M SMAD3 inhibitor SIS3 (Calbiochem) or DMSO as a control, in presence or absence of TGF- β 2. At day seven the cells were harvested and EC marker expression was analyzed at protein and RNA level.

In order to neutralize TGF β -2 secretion, iPSCs were transfected with Pre-21 and Pre-Ctrl as described before. 5h after transfection to the medium was added fresh alpha MEM containing FBS serum and either 1 μ g/ml TGF β -2 neutralizing antibody or IgG control (R&D System). 24h after transfection the media was refreshed with complete DM containing TGF β -2 neutralizing antibody or IgG control for further 24h. The gene expression of the EC markers was analyzed with Q-PCR.

In order to neutralize VEGF secretion, iPSCs were seeded on collagen IV, in DM containing TGF- β 2 for seven days. From day 1 the cells were treated with either 0.1 μ g/ml VEGF neutralizing antibody or IgG as a control (R&D System). The protein expression of the EC markers was analyzed with Western Blot.

2.2.26 STATISTICAL ANALYSIS OF DATA

Statistical analysis of data was performed using ABI Graphpad Prism 5 software. Data were analyzed using a student's unpaired T-test to compare two data sets for statistical differences and 1way ANOVA for multiple comparisons. A value of $p < 0.05$ was considered significant.

Table 8 Buffers and their composition

Buffer	Composition
5X SDS Loading Buffer	10% sodium dodecyl sulphate, 50% glycerol, 0.05% bromophenol blue, 10mM β -mercaptoethanol in 500mM Tris-hydrochloride (pH 6.8)
6x DNA Loading Buffer	30% glycerol, 0.3% bromophenol blue in distilled water
Phosphate Buffered Saline	137 mM NaCl, 2.7mM KCl, 8.1mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 1.76mM KH_2PO_4 to pH 7.4
Protein Lysis Buffer	1mM EDTA, 50mM tris-hydrochloride (pH7.4), 150mM NaCl, 1% Triton X, cocktail of protease inhibitors (Roche;1 tablet in 50ml)
Transfer Buffer	25mM Tris, 192mM glycine, 10% methanol in ddH ₂ O.

CHAPTER 3. RESULTS

3.1 ROLE OF VEGF AND SHEAR STRESS IN STEM CELL DIFFERENTIATION INTO ECs

Previous work from our laboratory identified a protocol for ESC differentiation towards ECs involving the use of collagen IV, VEGF and shear stress (Zeng, Xiao et al. 2006). In order to adapt this protocol to the differentiation of mouse iPSCs, several protocols involving different VEGF concentrations, and time points were tested. A protocol combining VEGF and shear stress was also tested, firstly on ESCs and then on iPSCs.

3.1.1 VEGF induces functional differentiation of iPSC towards EC lineage

Firstly, in order to identify the optimal concentration of VEGF able to induce the strongest level of endothelial cell differentiation in iPSCs, cells were cultured in differentiation medium (DM) containing 0, 10, 20, 50 and 100ng/ml of VEGF for 5 days. Quantitative gene expression analysis showed a consistent upregulation of the endothelial markers VE-cadherin, Flk1 and vWF, in particular when the cells were stimulated with 20 and 50ng/ml of VEGF, as compared to untreated cells cultured in differentiation (Figure 18).

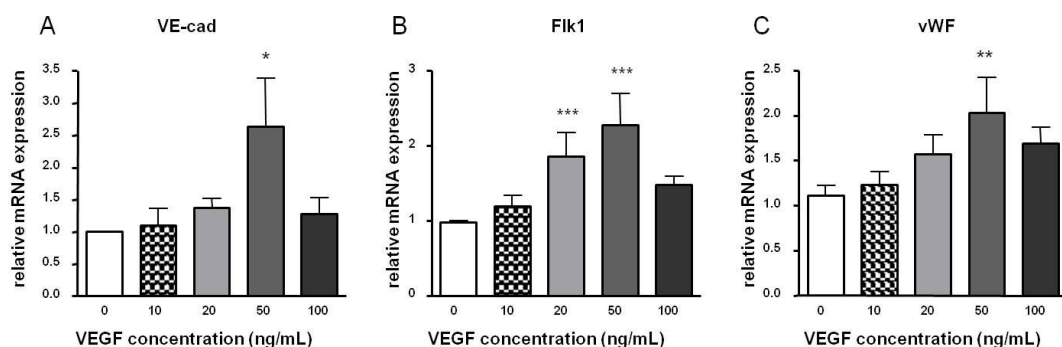


Figure 18 Dose-dependent response of iPSCs to VEGF

iPSCs were differentiated for 5 days on collagen IV and in the presence of differentiation medium containing different concentrations of VEGF (0, 10, 20, 50 and 100 ng/ml). Gene expression level was assessed by real-time PCR for the endothelial markers VE-cadherin (VE-cad, A), Flk1 (B), and

vWF (C). Statistical analysis performed is 1-way ANOVA for multiple comparisons. Data are shown as mean \pm SEM and are representative of 4 individual experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. 0ng/ml. Controls (0ng/ml) represent iPSCs cultured in differentiation medium containing 0ng/mL of VEGF.

On the basis of results obtained from the VEGF concentration curve, the next series of experiments were designed to elucidate the timing of the progression towards endothelial differentiation. The concentrations of 20 and 50ng/ml of VEGF were chosen because they induced the greatest upregulation of endothelial marker expression. The effects of these two conditions were tested after 3, 5 and 7 days of stimulation.

Cells cultured in the presence of 20ng/ml of VEGF showed an increase in endothelial marker expression starting at day 5. Strong and significant upregulation of VE-cadherin, Flk1 and vWF was observed at day 7 (Figure 19).

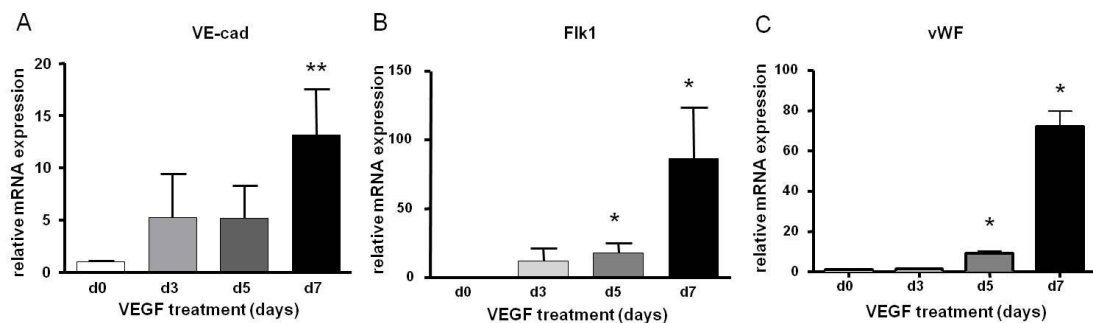


Figure 19 Time course study of EC markers expression in iPSCs differentiated with 20ng/ml VEGF

iPSCs were seeded on collagen IV and cultured in differentiation medium with 20ng/ml VEGF. Gene expression analysis was performed by real-time PCR after 3, 5, and 7 days of differentiation. Results are shown for VE-cadherin (VE-cad, A), Flk1 (B) and vWF (C). The statistical analysis used is 1-way ANOVA for multiple comparisons. Data are shown as mean \pm SEM and are representative of 4 individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. d0 (d0 represents iPSCs seeded on gelatin and cultured in undifferentiated conditions).

Since the concentration curve analysis showed that the addition of 50ng/ml of VEGF was able to induce a stronger endothelial differentiation than 20ng/ml, we tested the progression of differentiation over a 7 days period adding 50ng/ml of VEGF to the medium. Results showed a strong upregulation of the endothelial marker expression

at day 7 (Figure 20). As compared to the results obtained with 20ng/ml of VEGF, the higher dose of the growth factor induced a stronger expression of some differentiation markers, in particular VE-cadherin and an earlier upregulation of the mature endothelial cell marker vWF, starting from day 3.

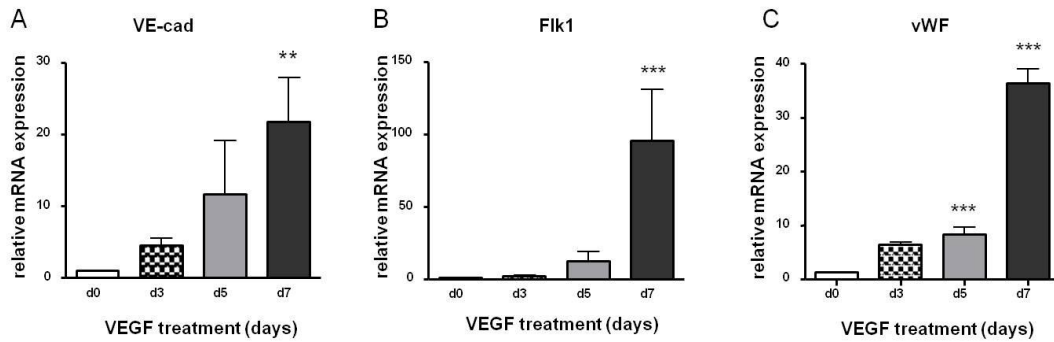


Figure 20 Time course study of EC marker expression in iPSCs differentiated with 50ng/ml VEGF

iPSCs were cultured on collagen IV and in differentiation medium supplemented with 50ng/ml VEGF. Real-time PCR was performed after 3, 5, and 7 days of differentiation to analyze the gene expression of VE-cadherin (Ve-cad, A), Flk1 (B) and vWF (C). The statistical analysis used is 1 way ANOVA for multiple comparisons. Data are shown as mean \pm SEM and are representative of 4 individual experiments. $**P < 0.01$ and $***P < 0.001$ vs. d0 (d0 represents iPSCs seeded on gelatin and cultured in undifferentiated conditions).

To confirm the expression of the differentiation markers, Western blot analysis was performed on cell lysate derived from iPSCs differentiated at different time points. Protein analysis confirmed the differentiation pattern observed at gene expression level. In particular, cells showed an increased level of the endothelial cell markers CD31 and Flk1 starting at day 3, further increasing at day 5 and day 7. A similar pattern was observed for the late endothelial cell marker VE-cadherin, which was upregulated from day 5 (Figure 21).

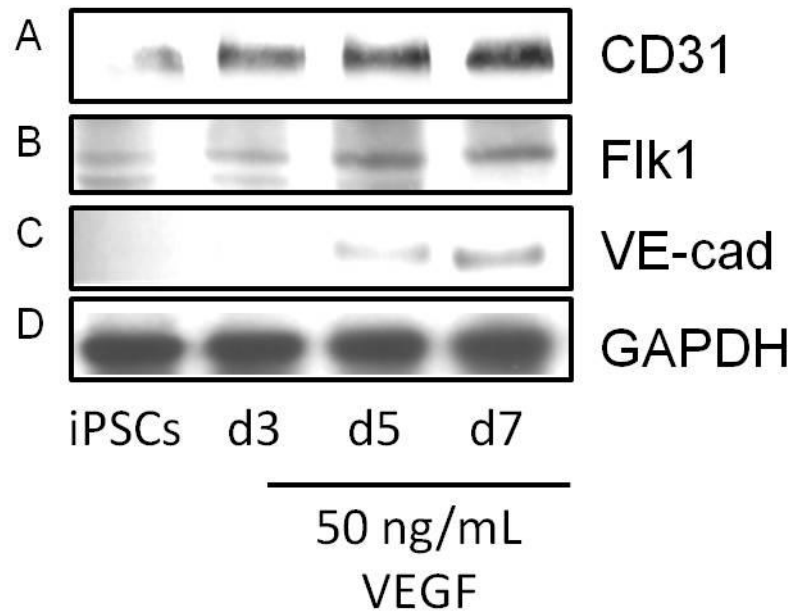


Figure 21 EC marker protein expression in iPSCs differentiated with 50ng/ml VEGF at different time points

iPSCs were seeded on collagen IV, in differentiation medium containing 50ng/ml VEGF and the samples were harvested after 3, 5 and 7 days of differentiation. Protein expression level was assessed by Western Blot analysis of early endothelial markers CD31 (A) and Flk1 (B), and the late marker VE-cadherin (VE-cad, C). As a control iPSCs were seeded on gelatin and cultured in undifferentiating conditions. GAPDH (D) was used as loading control.

We also observed a different morphology of the cells cultured on collagen IV, in DM containing VEGF 50ng/ml, as compared to the undifferentiated cells in gelatin.

While the undifferentiated iPSCs appeared clustered in round three-dimensional colonies, the differentiated cells assumed a flat adherent phenotype, becoming increasingly elongated (Figure 22).

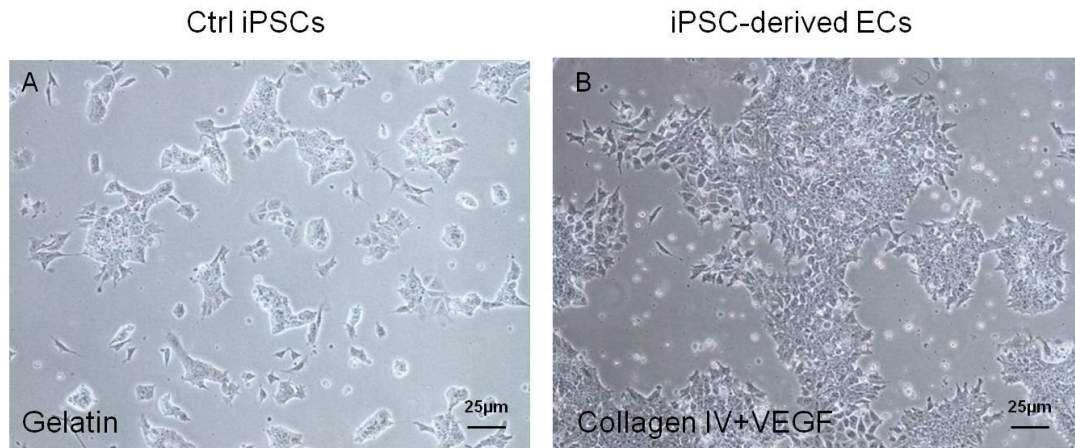


Figure 22 Morphology of iPSC-derived ECs and undifferentiated iPSCs

iPSCs were differentiated for 5 days on collagen IV and cultured in differentiation medium containing 50ng/ml VEGF (B) or cultured in undifferentiated conditions on gelatin (A). Light microscopy images were taken with the Nikon Eclipse TS100 and are representative of at least 3 experiments. Scale bar, 25 µm.

Furthermore, immunofluorescence analysis of the endothelial cell markers VE-cadherin, eNOS and vWF was performed to confirm expression and assess the correct localization of the proteins in the cells. Cells were seeded on collagen in the presence of 50ng/ml of VEGF for 5 days. Confocal imaging showed a clear expression pattern of VE-cadherin at cell junction level, and of eNOS and vWF on the cytoplasm/membrane of the cells (Figure 23).

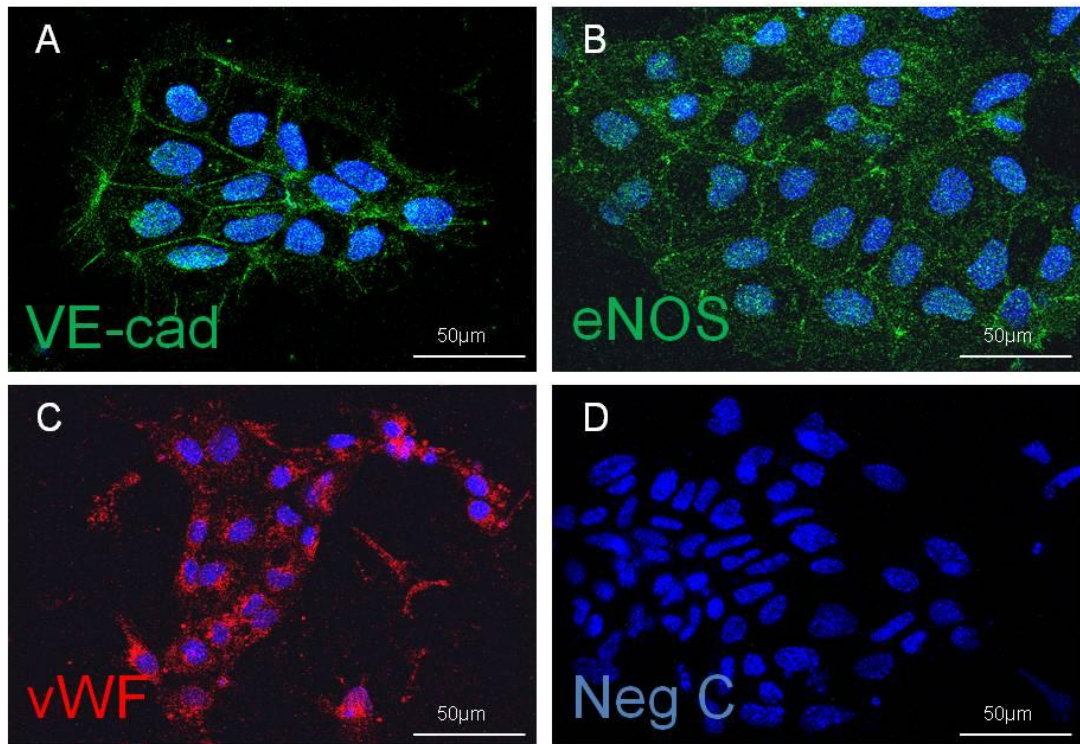


Figure 23 Staining of iPSC-derived ECs treated with 50ng/ml VEGF

iPSCs were differentiated on collagen IV and in differentiation medium containing 50ng/ml VEGF for 5 days. Leica SP5 inverted Confocal microscope images showed positivity for the endothelial cell markers VE-cadherin (VE-cad, A) and eNOS (B), and vWF (C). Negative controls consisted of cells incubated with IgG followed by secondary antibody (D). Scale bar, 50 µm.

In addition, expression of VE-cadherin after 7 days was confirmed by immunocytochemistry. Cells cultured for 7 days on collagen IV in DM containing 50ng/ml VEGF, showed a clear pattern of VE-cadherin expression at the cell-cell junctions (Figure 24). Comparison with the results of the previous experiment (performed after five days with 50ng/ml VEGF) demonstrated that an increased culture time led to a stronger VE-cadherin positivity.

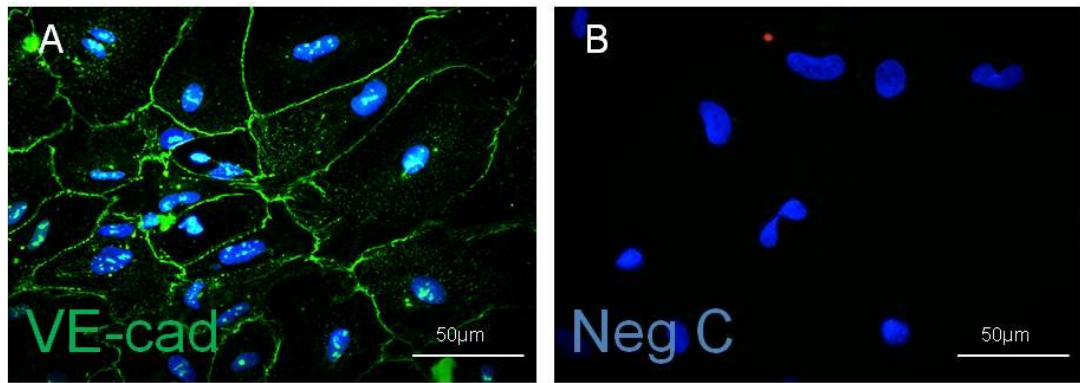


Figure 24 Staining of iPSC-derived ECs treated with 50ng/ml VEGF

iPSCs were differentiated for 7 days on collagen IV and cultured in differentiation medium containing 50ng/ml VEGF. Leica SP5 inverted confocal imaging showed a strong positivity of the cells for the endothelial cell marker VE-cadherin (VE-cad, A). Negative controls consisted of cells incubated with IgG followed by secondary antibody (Neg C, B). Scale bar, 50 µm.

Finally, to test the functionality of iPSCs after VEGF treatment we performed an *in vitro* angiogenesis assay. Cells treated for 7 days with VEGF (Figure 25, B) showed an increased tube formation ability, as compared to the cells grown in absence of VEGF (Figure 25, A). The results of the assay were confirmed by total tube length quantification, which showed a 4-fold increase in tube-like structure formation capacity in cells differentiated with VEGF (Figure 25, C). These findings showed that the endothelial lineage differentiation of iPSCs has given rise to functional, mature endothelial cells.

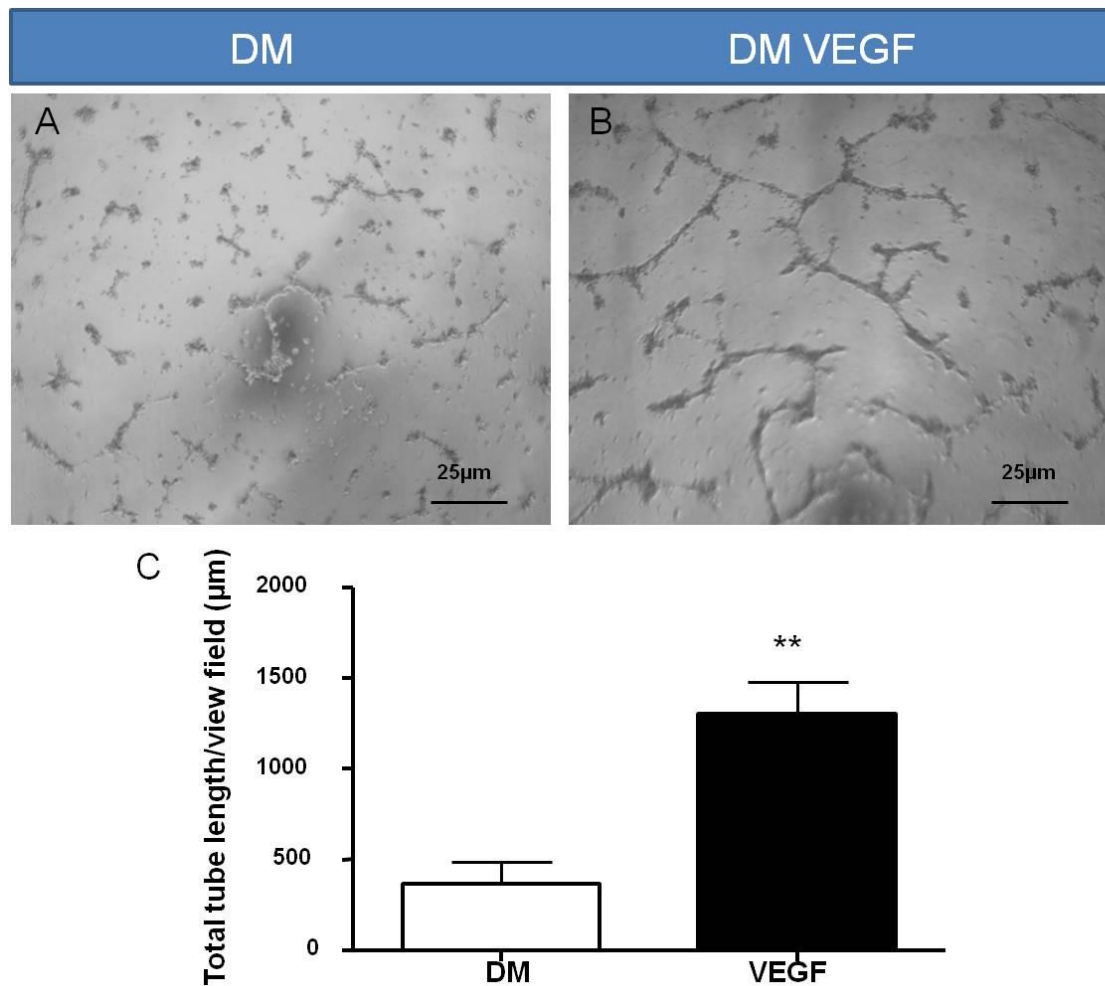


Figure 25 In vitro angiogenesis assay in iPSCs differentiated with VEGF

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) with or without VEGF. After 7 days, cells were seeded on Matrigel and incubated for 7-8h to test their angiogenesis potential. Representative images show tube formation in the cells treated with VEGF (B) and untreated in DM (A). Total tube length was measured to quantify the effect of differentiation on tube-like formation capacity (C). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM and are representative of 3 individual experiments. *** $P < 0.01$ vs. DM (DM represents iPSCs seeded on collagen IV and cultured in differentiation medium). Scale bars, 25 μ m.

3.1.2 Role of shear stress in stem cell differentiation

Previous studies conducted in our group revealed the importance of shear stress in the differentiation of stem and progenitor cells. In particular, application of shear stress on Sca1⁺ progenitor cells increased their proliferation and induced EC marker upregulation and this effect is mediated by the VEGF pathway (Xiao, Zeng et al. 2006).

In order to further improve the differentiation protocol described in the first part of the work, we included a set of experiments performed on ESCs and then replicated with iPSCs, combining VEGF and shear stress. In particular, we chose to study a ckit⁺ population that is being characterized in our laboratory and has been demonstrated to represent a novel vascular progenitor cell population.

ESCs were seeded on collagen IV and cultured in differentiation medium for 4 days. Subsequently, ckit⁺ progenitor cells were isolated using immunomagnetic beads and seeded on collagen IV coated wells in the presence of differentiation medium. After three days of culture, shear stress was applied for either 24h or 48h and then cells were collected and analyzed for their expression of endothelial markers. Quantitative gene expression analysis on cells subjected to 24h of shear stress showed some degree of upregulation of the endothelial markers CD31 and VE-cadherin, although consistent results were obtained only for CD31 after 24h. Flk1 and eNOS were upregulated approximately 2-fold, as compared to cells cultured in static conditions. Treatment of the cells with shear stress for 48h did not induce a relevant increase in their differentiation (Figure 26).

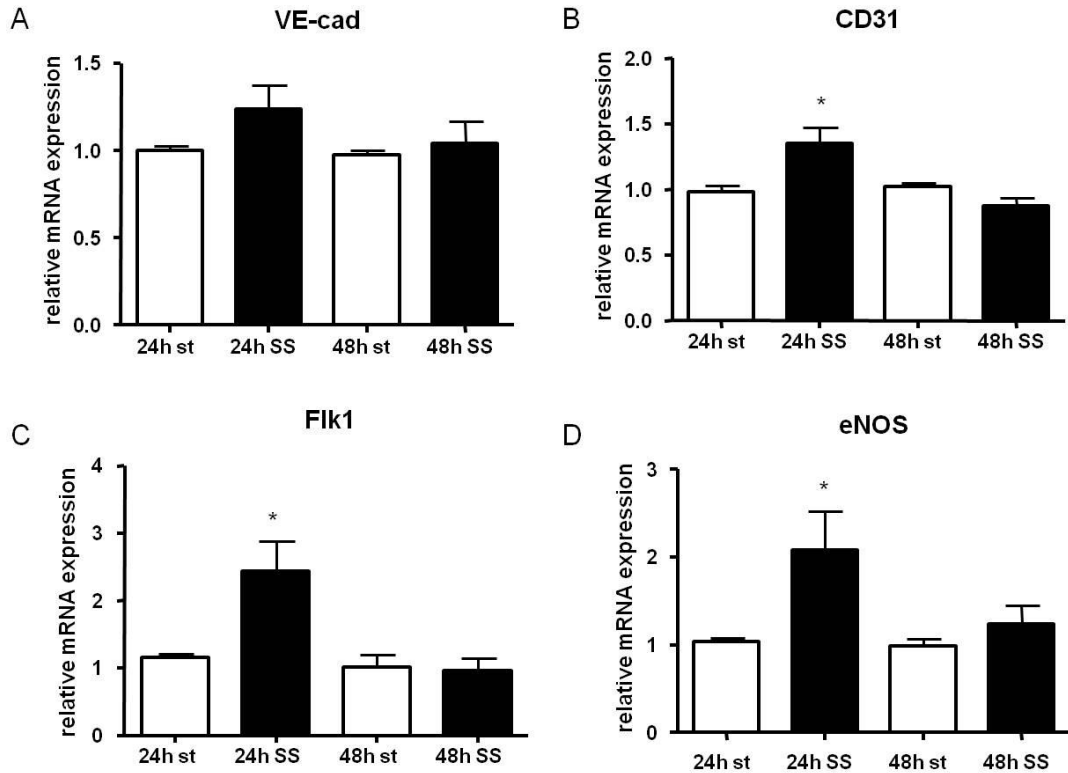


Figure 26 Effect of shear stress on ESC differentiation

$ckit^+$ progenitor cells isolated from mESCs pre-differentiated for 4 days on collagen IV and in differentiation medium, were cultured in the same differentiation conditions for a further three days and subjected to shear stress for 24h and 48h. Real time PCR shows the effect on endothelial cell marker expression of the addition of shear stress (SS) to the static differentiation conditions (st). Results are shown for VE-cadherin (VE-cad, A), CD31 (B), Flk1 (C) and eNOS (D). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean \pm SEM of 3 individual experiments. * $P < 0.05$ vs. iPSCs differentiated for 24h in static conditions. The shear stress results were normalized to the relative time points.

3.1.3 Synergistic action of shear stress and VEGF in ESC differentiation into ECs

Previous results in this chapter showed that shear stress is able to induce some upregulation of the endothelial cell markers in mESC. Therefore, in order to increase the response we decided to combine the mechanical force of shear stress to the biochemical action of VEGF.

ESC-derived $ckit^+$ progenitor cells were obtained as described previously (Campagnolo *et al.*, submitted to Circulation) and differentiated for three days in

collagen IV before stimulation with 50ng/ml VEGF for 24 and 48h, with or without the application of shear stress. Quantitative gene expression analysis of the endothelial markers CD31, Flk1 and eNOS showed upregulation of approximately 2 fold in the cells treated for 24h with a combination of shear stress and VEGF, relative to the cells treated with VEGF alone. eNOS and the late EC marker VE-cadherin showed an increase of 2 and 2.5 fold respectively after 48h of combined treatment with VEGF and shear stress, compared to static cells (Figure 27).

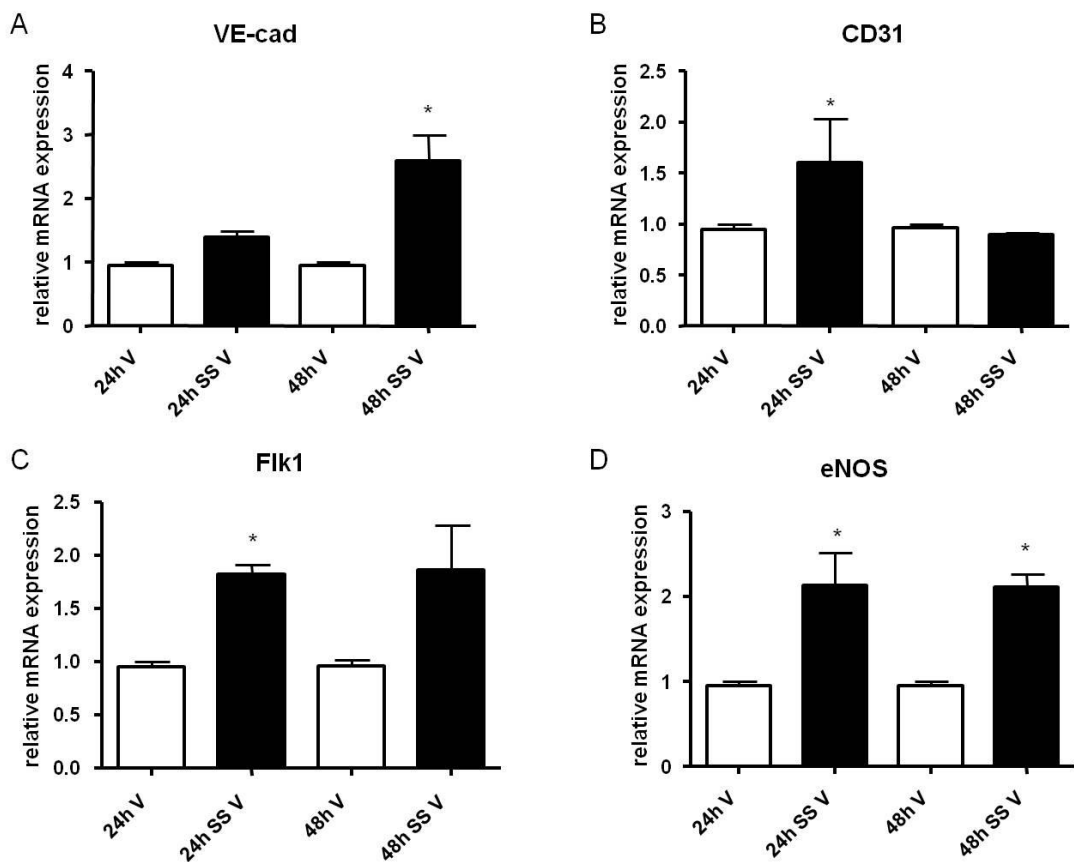


Figure 27 Combined application of shear stress and VEGF improves EC differentiation of ckit+ cells

ckit⁺ progenitor cells were differentiated for a further three days in DM on collagen and then stimulated with VEGF 50ng/ml for 24h and 48h, in presence or absence of shear stress stimulation. Real time PCR shows the synergistic effect on the endothelial marker expression of the combination of shear stress (SS) and VEGF (V). Results are reported for VE-cadherin (VE-cad, A), CD31 (B), Flk1 (C) and eNOS (D). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are shown as mean \pm SEM and are representative of 3 individual experiments. * $P < 0.05$ vs. iPSCs cultured in VEGF only (V). Results of shear stress and VEGF combined treatment were normalized to the relative time points.

As in $ckit^+$ cells the combined action of shear stress and VEGF was shown to induce the expression of EC markers to a greater level, as compared to each stimulus applied alone, the logical next step was application of this protocol to iPSCs.

3.1.4 Synergistic action of shear stress and VEGF in iPSC differentiation into ECs

Once we had established the protocol in ESC-derived $ckit^+$ progenitors, we proceeded to apply it to iPSCs.

We used a mixed population of iPSCs and pre-differentiated them for three days. These cells were then treated with shear stress, VEGF or a combination of the two for 48h. After combined application of VEGF and shear stress, results showed a significant and stronger EC marker expression, as compared to each stimulus applied alone (Figure 28).

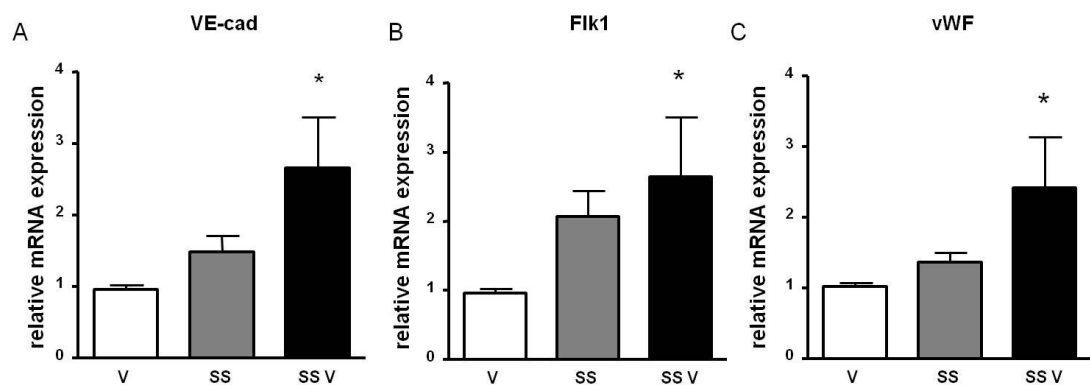


Figure 28 Combined application of shear stress and VEGF improves EC differentiation in iPSCs

iPSCs were differentiated for three days on collagen IV and in differentiation medium and then treated with shear stress, VEGF or a combination of the two stimuli for 48h. Real time PCR shows a synergistic effect on endothelial marker expression after addition of shear stress (SS) and VEGF (V) to the differentiation conditions, as compared to either stimulus alone. Results are shown for VE-cadherin (VE-cad, A), Flk1 (B) and vWF (C). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean \pm SEM of 3 individual experiments.* $P < 0.05$ vs. iPSC differentiated in presence of VEGF (V).

3.1.5 Conclusions

In conclusion, in this first part of the work we described the optimization of the differentiation protocols for ESCs and iPSCs.

Firstly, we tested several conditions and established that a greater level of endothelial differentiation could be obtained by culturing iPSCs with differentiation medium containing 50ng/ml VEGF for up to 7 days, as shown by EC marker expression results and *in vitro* angiogenesis assay.

Additionally, using ESCs as a model, we further optimized the previous protocol through the addition of shear stress.

We finally applied the combination of shear stress, a mechanical force, and VEGF, a chemical stimulation, to iPSCs. VEGF and shear stress together were able to increase the EC differentiation of the cells, as compared to each stimulus applied alone. However, a limitation of this protocol is represented by the poor reproducibility of the experiments performed in presence of shear stress, which will be explained in greater depth in the discussion.

3.2 CHARACTERIZATION OF miRNA PROFILE DURING VEGF- AND SHEAR STRESS- INDUCED iPSC DIFFERENTIATION: MiRNA-21 REGULATES VEGF-INDUCED iPSC DIFFERENTIATION INTO ECs

The optimization of the protocol described in the previous section (3.1) led to the design of a method to differentiate iPSCs into ECs, involving the use of DM with 50ng/ml of VEGF or a combination of VEGF and shear stress treatment. Using these protocols we now aimed to characterize the miRNA signature of the differentiating iPSCs in order to study the involvement of miRNAs in the relevant signalling pathways and to elucidate the underlying molecular mechanism.

3.2.1 MiRNA array analysis on iPSCs differentiated with VEGF and undifferentiated

Our results showed that the treatment of iPSCs with 50ng/ml of VEGF induced endothelial differentiation, starting at day 5, with a peak at day 7. Minor changes in marker expression were detected also at day 3, but were not consistent in all the markers.

Using the protocol described in Chapter 3 we then aimed to study the molecular mechanisms underlying iPSC differentiation.

For this purpose, we performed a miRNA array to study the miRNA involvement in the differentiation process. The array was performed in order to compare the expression of miRNAs in iPSCs undifferentiated and differentiated for three days in the presence of 50ng/ml VEGF.

The choice of such an early time point was made in order to investigate the initial changes in miRNA expression that might initiate the subsequent differentiation cascade.

Each experiment was performed in quadruplicates, and 2 controls (undifferentiated iPSCs) and 2 treated samples (3 days VEGF 50ng/ml) were loaded on two microfluidic cards each (pool A and B).

The two microfluidic cards used in the experiment contained probes for 750 target miRNAs. Card A focuses on more highly characterized miRNAs, while Card B contains many of the more recently discovered miRNAs along with the miR* sequences or passenger strands. Indeed, before assembling the active RNA induced silencing complex (RISC) to perform gene silencing, the double-stranded duplex is separated into the guide strand, functional and complementary to the mRNA target, and the passenger strand, which is subsequently degraded (Narazaki, Uosaki et al. 2008). The function of these passenger strands is still unclear, but well-conserved miRNA* strands may contribute to the regulation network (Park, Afrikanova et al. 2004).

Data obtained from the miRNA array were normalized to 3 different control miRNAs, differential expression was then considered for those miRNAs that obtained similar results in all three normalizations. Out of the 750 probes present in the cards, 123 in pool A and 88 in pool B reported expression of the relative miRNA in our system and therefore could be detected under the threshold of 30 cycles. Amongst the detected miRNAs, differential expression was observed in 25 (pool A) and 15 (pool B) miRNAs; only miRNAs consistently up or down regulated in both the experiments analyzed were considered. Finally, miRNAs which showed a consistent differential expression of at least 2 fold were picked. The final number of miRNAs selected was 24 for pool A (20 upregulated and 4 downregulated, Table 9) and 11 for pool B (8 upregulated and 3 downregulated, Table 10).

Some of the miRNAs identified in the array analysis are already known to be involved in stem cells maintenance, such as the miRNA-302-367 cluster (Taniyama and Griendling 2003), angiogenesis, such as the miRNA-20b and 21 (Cascio, D'Andrea et al. 2010, Sabatel, Malvaux et al. 2011), and smooth muscle cell differentiation, like the miRNA-145 (Willert, Brown et al. 2003).

Target name	snoRNA202	snoRNA135	MammU6
mmu-let-7c	6.7 ± 3.4	4.9 ± 3.2	6.8 ± 1.9
mmu-miR-129-3p	33.9 ± 15	22.6 ± 6.2	39.1 ± 25.9
mmu-miR-133a	7.2 ± 4.4	5.3 ± 3.9	7.2 ± 2.8
mmu-miR-139-5p	3.6 ± 2.5	2.3 ± 1.3	4.2 ± 3.7
mmu-miR-188-5p	3.3 ± 0.5	2.3 ± 0.7	3.5 ± 0.4
mmu-miR-20b	3.1 ± 1.6	2.1 ± 0.7	3.6 ± 2.6
mmu-miR-21	22.7 ± 27.3	17.5 ± 21.8	21.0 ± 23.4
mmu-miR-218	2.9 ± 0.5	2.1 ± 0.7	3.1 ± 0.3
mmu-miR-224	126.5 ± 170.7	98.5 ± 134.3	114.3 ± 151.0
mmu-miR-26b	23.0 ± 13.1	15.1 ± 6.3	26.9 ± 20.7
mmu-miR-29b	145.9 ± 189.9	113.2 ± 150.1	132.7 ± 166.7
mmu-miR-302a	88.1 ± 3.6	61.5 ± 13.4	95.7 ± 20.7
mmu-miR-302b	4432 ± 4226	2820 ± 2391	5436 ± 5862
mmu-miR-302c	27395 ± 34072	21156 ± 27080	25158 ± 29548
mmu-miR-340-5p	7.8 ± 2.0	5.6 ± 2.4	8.3 ± 0.0
mmu-miR-34b-3p	6.6 ± 1.1	4.7 ± 1.1	7.1 ± 0.6
mmu-miR-367	65.9 ± 16.9	44.8 ± 3.6	74.4 ± 37.0
mmu-miR-449a	19.8 ± 12.9	14.5 ± 11.4	19.8 ± 8.6
mmu-miR-685	2.9 ± 0.2	2.0 ± 0.2	3.2 ± 1.1
rno-miR-224	9.3 ± 5.1	6.1 ± 2.4	10.8 ± 8.2
mmu-miR-145	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
mmu-miR-467e	0.5 ± 0.3	0.4 ± 0.3	0.5 ± 0.2
mmu-miR-669a	0.5 ± 0.2	0.3 ± 0.2	0.5 ± 0.1
mmu-miR-409-3p	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.3

Table 9 Pool A miRNAs target. The miRNA array for pool A was performed on iPSCs differentiated for three days on collagen IV and in differentiation medium containing 50ng/ml of VEGF; undifferentiated cells were used as control. The results represent an average of the differential expression between two independent experiments. In the table are shown 20 (green panel) and 4 (red panel) miRNAs, respectively up and down regulated at least two fold relative to the undifferentiated iPSCs. Results were obtained after normalization with three different endogenous controls (mouse snoRNA202 and 135, and mammalian MammU6). These miRNAs were initially characterized in mouse or rat (mmu= *mus musculus*; rno= *rattus norvegicus*).

Target name	snoRNA135	snoRNA202	Mamm U6
hsa-miR-200b	2.0 ± 0.6	2.0 ± 0.3	2.5 ± 0.6
mmu-miR-212	4.4 ± 1.0	4.3 ± 0.5	5.6 ± 1.6
mmu-miR-2138	6.5 ± 4.5	6.2 ± 3.8	7.1 ± 1.8
mmu-miR-302a#	108.1 ± 46.1	104.7 ± 34.8	129.4 ± 9.8
mmu-miR-34c#	7.0 ± 2.5	6.8 ± 1.7	8.5 ± 1.3
mmu-miR-374-5p	8.9 ± 7.1	8.4 ± 6.1	9.5 ± 3.6
mmu-miR-449b	370.8 ± 512.4	341.1 ± 469.7	326.7 ± 440.3
mmu-miR-92a#	4.1 ± 2.1	3.9 ± 1.7	4.7 ± 0.1
mmu-miR-467a	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
mmu-miR-690	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
mmu-miR-706	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.0

Table 10 Pool B miRNAs target. miRNA array for pool B on iPSCs undifferentiated used as control, and differentiated for three days on collagen IV and in differentiation medium containing 50ng/ml of VEGF. The results represent an average of the differential expression between two independent experiments. In the table are shown 8 (green panel) and 3 (red panel) miRNAs, respectively up and down regulated at least two times relative to the control. MiR-302a#, 34c# and 92a# represent miR* passenger strands. Results were normalized with three different endogenous controls (mouse snoRNA202 and 135, and mammalian MammU6). These miRNAs were initially characterized in human or mouse (hsa= *homo sapiens*; mmu= *mus musculus*).

3.2.2 Real time PCR validation of selected miRNAs

In order to choose some relevant candidates and to study their involvement in the mechanisms of iPSC differentiation toward an endothelial lineage, we decided to validate some of the most consistently upregulated or downregulated miRNAs in the array and some of those previously shown to contribute to angiogenesis.

We chose to analyze the expression of five of the selected miRNAs in the cells treated with VEGF, compared to the untreated cells (Figure 29).

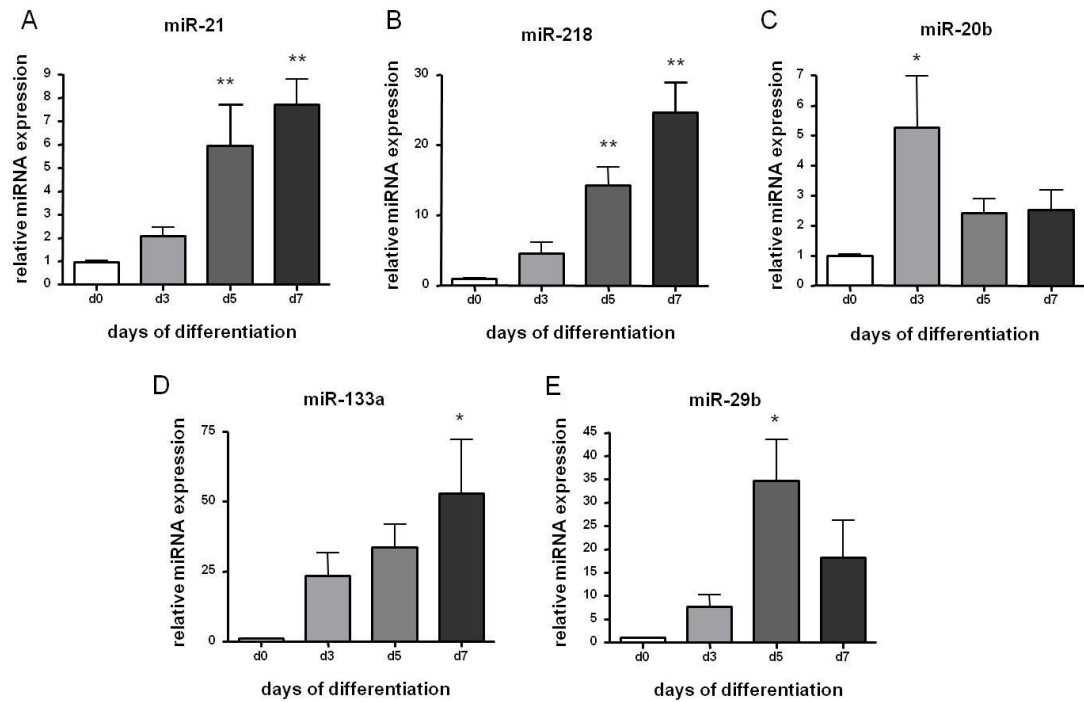


Figure 29 Analysis of the expression of selected miRNAs in iPSCs differentiated with 50ng/ml VEGF for up to 7 days

Five of the most consistently upregulated miRNAs were selected from the miRNA array and validated by real time PCR. Results show the expression levels of miRNAs 21 (A), 218 (B), miRNA 20b (C), 133a (D) and 29b (E) after 3, 5 and 7 days of VEGF treatment, as compared to the undifferentiated cells. The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean \pm SEM of 4 individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. d0 (d0 represents iPSCs seeded on gelatin and cultured in undifferentiated conditions).

3.2.3 MiRNA array analysis on iPSCs treated with shear stress for 48h and static

In section 3.1 we described a differentiation protocol for ESCs and iPSCs involving the application of shear stress and VEGF. In order to investigate possible common mechanisms in the iPSC differentiation process induced by the two stimuli, we aimed to investigate the miRNA signature of the cells treated with shear stress and untreated. For this purpose we performed a second miRNA array to compare the miRNA expression in iPSCs differentiated for three days on collagen IV, in DM and iPSCs differentiated in the same conditions and with 48h of shear stress treatment.

Each experiment was performed in quadruplicates, and 2 controls (3 days collagen IV and DM) and 2 treated samples (3 days collagen IV and DM, 48h of shear stress) were loaded on two microfluidic cards each (pool A and B).

As explained before, data obtained from the miRNA array were normalized to 3 different control miRNAs, differential expression was then considered for those miRNAs that obtained similar results in all three normalizations. Out of the 750 probes present in the cards, 138 in pool A and 94 in pool B reported expression of the relative miRNA in our system and therefore could be detected under the threshold of 30 cycles. Only differentially expressed miRNAs consistently up or down regulated of at least 2 fold in both the experiments analyzed were considered. The final number of miRNAs selected was 6 for pool A (4 upregulated and 2 downregulated, Table 11) and 8 for pool B (3 upregulated and 5 downregulated, Table 12).

Target name	snoRNA202	snoRNA135	MammU6
mmu-miR-218	1.9 ± 0.0	4.1 ± 4.2	2.3 ± 0.6
mmu-miR-369-5p	37.8 ± 49.1	42.0 ± 43.2	39.2 ± 49.1
mmu-miR-425	2.4 ± 0.3	5.6 ± 6.0	3.0 ± 1.1
mmu-let-7e	14.6 ± 8.9	21.6 ± 13.1	16.6 ± 6.6
mmu-miR-328	0.2 ± 0.0	0.3 ± 0.3	0.2 ± 0.0
mmu-miR-302b	0.4 ± 0.0	0.7 ± 0.7	0.4 ± 0.1

Table 11 Pool A miRNAs target. The miRNA array for pool A was performed on iPSCs differentiated for three days on collagen VI and in differentiation medium (DM), under shear stress stimuli for 48h. Differentiated cells on collagen IV and in DM were used as controls. The results represent an average of the differential expression between two independent experiments. In the table are shown 4 (green panel) and 2 (red panel) miRNAs, respectively up and down regulated at least two fold relative to the untreated iPSCs. Results were obtained after normalization with three different endogenous controls (mouse snoRNA202 and 135, and mammalian MammU6). These miRNAs were initially characterized in mouse or rat (mmu= *mus musculus*; rno= *rattus norvegicus*).

Target name	snoRNA202	snoRNA135	MammU6
hsa-miR-200b	1.2 ± 0.0	1.5 ± 0.2	1.6 ± 0.4
mmu-miR-31#	6.1 ± 2.9	7.2 ± 2.7	0.9 ± 0.3
rno-miR-7a#	128.4 ± 36.4	152.6 ± 28.5	0.8 ± 0.2
Y1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0
hsa-miR-15b#	0.5 ± 0.2	0.6 ± 0.3	0.6 ± 0.1
hsa-miR-378	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2
hsa-miR-99b#	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.4
mmu-miR-721	0.4 ± 0.1	0.5 ± 0.1	4.6 ± 3

Table 12 Pool B miRNAs target. MiRNA array for pool B on iPSCs differentiated for three days on collagen IV and in differentiation medium (DM), under shear stress stimuli for 48h. Differentiated cells on collagen IV and in DM were used as controls. The results represent an average of the differential expression between two independent experiments. In the table are shown 3 (green panel) and 5 (red panel) miRNAs, respectively up and down regulated at least two times relative to the untreated control iPSCs. MiR-31#, 7a#, 15b# and 99b# represent miR* passenger strands. Results were normalized to three different endogenous controls (mouse snoRNA202 and 135, and mammalian MammU6). These miRNAs were initially characterized in human or mouse (hsa= *homo sapiens*; mmu= *mus musculus*).

3.2.4 Analysis of differential expression of selected miRNAs in iPSCs treated with shear stress for 48h and untreated

We then aimed to validate observed changes to some of the most consistently upregulated and downregulated miRNAs from the array performed in cells after shear stress treatment, in order to study their involvement in the mechanisms of iPSC differentiation toward an endothelial lineage. We analyzed the differential expression of six selected miRNAs in the cells treated with shear stress for 48h, compared to the untreated cells on collagen IV and DM.

On analysis of the results of the Real time PCR we were unable to confirm any significant differential expression of the selected miRNAs in the cells treated with shear stress, compared to the static (Figure 30).

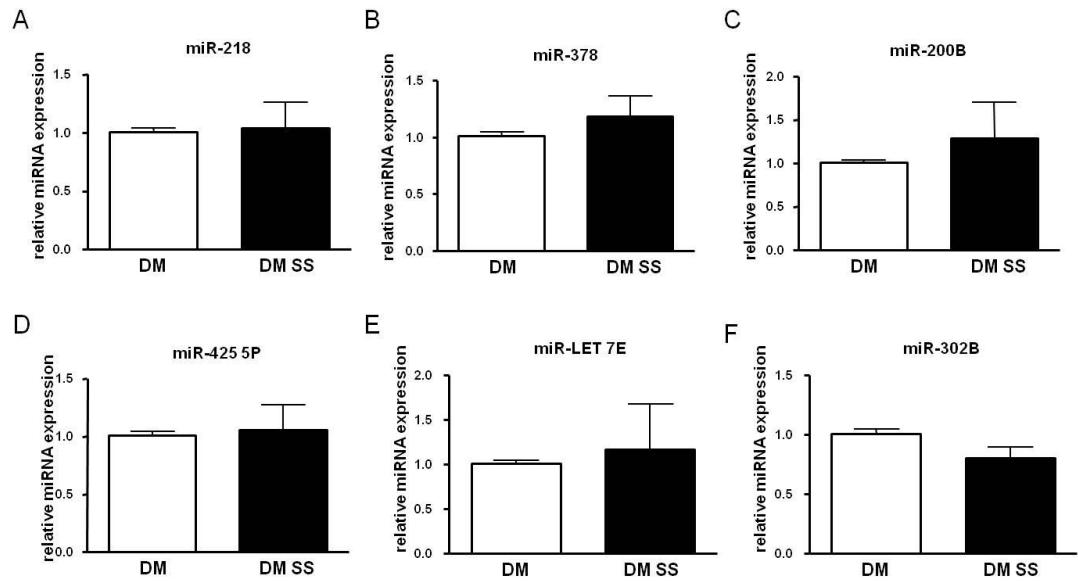


Figure 30 Analysis of the expression of miRNAs selected from the miRNA array performed in iPSCs treated with shear stress and static

Four of the most consistently upregulated miRNAs, miR-218 (A), miR-425 5p (D), miR-LET 7E (E) and miR-302B (F), and two of the most consistently downregulated miRNAs, miR-378 (B) and miR-202B (C), were selected from the array and validation experiments performed by real time PCR. Expression levels of these markers are shown in the static cells differentiated for 3 days on collagen IV, in differentiation medium (DM) and in cells differentiated in the same conditions plus 48h of shear stress treatment (DM SS). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean \pm SEM of 4 individual experiments. No results were significant ($*P < 0.05$).

A possible reason for these results is that, as mentioned in section 3.1, the experiments performed in the presence of shear stress showed a lot of variability, which does not allow the establishment of an efficient differentiation system. Explanation of this observed variability will be further explored in the discussion.

3.2.5 Study of the role of miRNAs in endothelial differentiation

Based on the data shown above, we decided to select one of the most suitable candidates amongst the validated miRNAs to investigate its role in iPSC differentiation towards an EC lineage. We decided to focus on miR-20b and miRNA-21, since their involvement in angiogenesis has already been documented.

3.2.5.1 MiR-20b expression does not affect iPSC endothelial differentiation

MiR-20b is considered one of the potential anti-angiogenic miRNAs, through targeting VEGF expression (Wang and Olson 2009); miR-20b has also been shown to be downregulated in hypoxic conditions and to directly decrease VEGF expression in carcinoma cell lines (Hua, Lv et al. 2006). Therefore, miR-20b appears to play a role in regulating angiogenesis, but its function in EC differentiation has not yet been clarified.

Aiming to investigate a potential involvement of miR-20b in the VEGF-induced iPSC differentiation towards ECs, we first altered the levels of this miRNA in iPSCs differentiated with VEGF for three days. In order to do so, we overexpressed miR-20b by transfecting iPSCs with the miR-20b precursor (Pre-20b). The mature miRNA expression was increased by approximately 300-fold in iPSCs transfected with Pre-20b, as compared to cells transfected with the precursor control (Pre-Ctrl) (Figure 4.3, A). We then inhibited miR-20b expression by transfecting iPSCs with an anti-miR LNA-20b, which is a chemically modified, single stranded nucleic acid designed to specifically bind to and inhibit endogenous miR-20b molecules. Results showed an approximately 13-fold decrease in the mature expression level of miR-20b in iPSCs transfected with LNA-20b, as compared to cells transfected with the inhibitor negative control (LNA-Ctrl) (Figure 31, B).

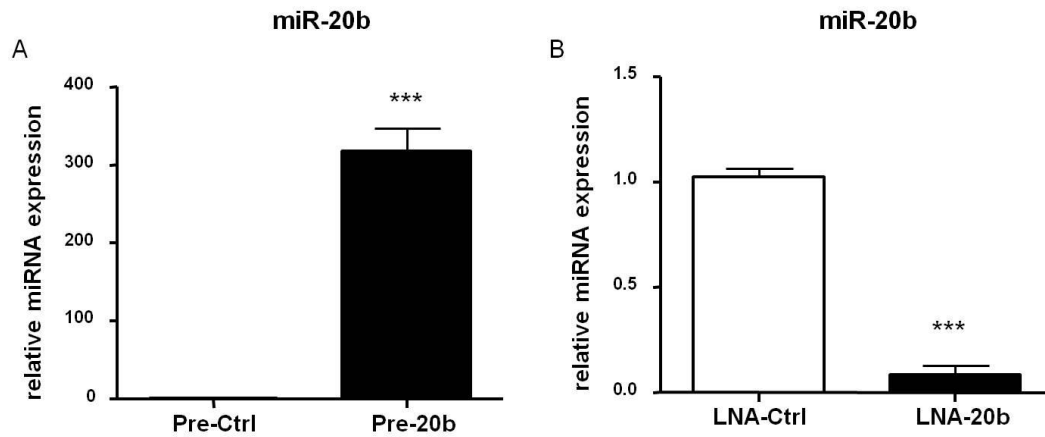


Figure 31 MiR-20b overexpression and inhibition in iPSCs differentiated with VEGF

iPSCs were differentiated for three days on collagen IV and in differentiation medium containing 50ng/ml VEGF. The levels of miR-20b were altered by transfecting the cells with precursor of miR-20b (Pre-20b), negative control precursor (Pre-Ctrl), inhibitor of miR-20b (LNA-20b) and negative control of miRNA inhibitor (LNA-Ctrl). Real time PCR analysis was performed after 48h to assess the expression level of the mature miR-20b. Results are shown for miR-20b expression after transfection with Pre-20b compared to Pre-Ctrl (A) and with LNA-20b, compared to LNA-Ctrl (B). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl and LNA-Ctrl.

Next, we aimed to analyze the effect of miR-20b on the expression of the EC markers VE-cadherin and Flk1 in iPSCs differentiated in presence of VEGF. However, Q-PCR results showed that miR-20b overexpression (Figure 32 A and C) and inhibition (Figure 32, B and D) did not affect the expression levels of these genes.

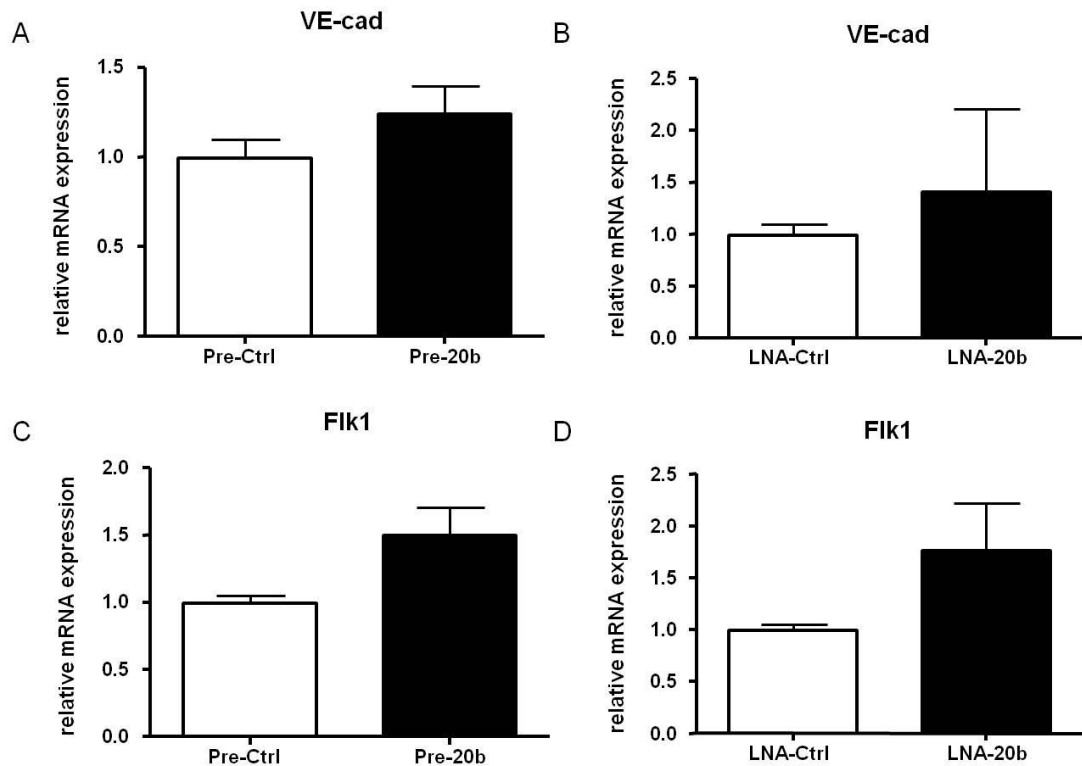


Figure 32 Analysis of EC marker expression in iPSCs differentiated with VEGF, after miR-20b overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-20b precursor (Pre-20b), inhibitor (LNA-20b) and the negative controls of the miR-20b precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis was performed 48h later. Results are shown for the EC markers VE-cadherin (VE-cad) (A and B) and Flk1 (C and D), after miR-20b overexpression and inhibition. The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

From the above shown results, we can indeed conclude that miR-20b does not regulate iPSC differentiation into ECs.

3.2.5.2 MiR-21 specifically regulates iPSC differentiation into ECs

MiR-21 has previously been shown to induce tumour angiogenesis through AKT and ERK pathway activation, increasing the expression of VEGF and HIF-1 α (Liu, Li et al. 2011). However, the role of miR-21 in regulating endothelial differentiation remains to be elucidated. Therefore we decided to investigate its involvement in the VEGF-induced iPSC differentiation into ECs by altering the levels of miR-21 in iPSCs differentiated in the presence of VEGF for three days.

Firstly we overexpressed miR-21 by transfecting with the miR-21 precursor (Pre-21). The level of miR-21 was increased by approximately 80 fold in the presence of the Pre-21, as compared to cells transfected with the precursor control (Pre-Ctrl) (Figure 33, A). We then inhibited miR-21 expression by transfecting iPSCs with an anti-miR LNA-21, which is a chemically modified, single stranded nucleic acid designed to specifically bind to and inhibit endogenous miR-21 molecules. Results showed an approximately 34-fold decrease in the mature miRNA expression after LNA-21 transfection, as compared to the cell transfected with the inhibitor negative control (LNA-Ctrl) (Figure 33, B).

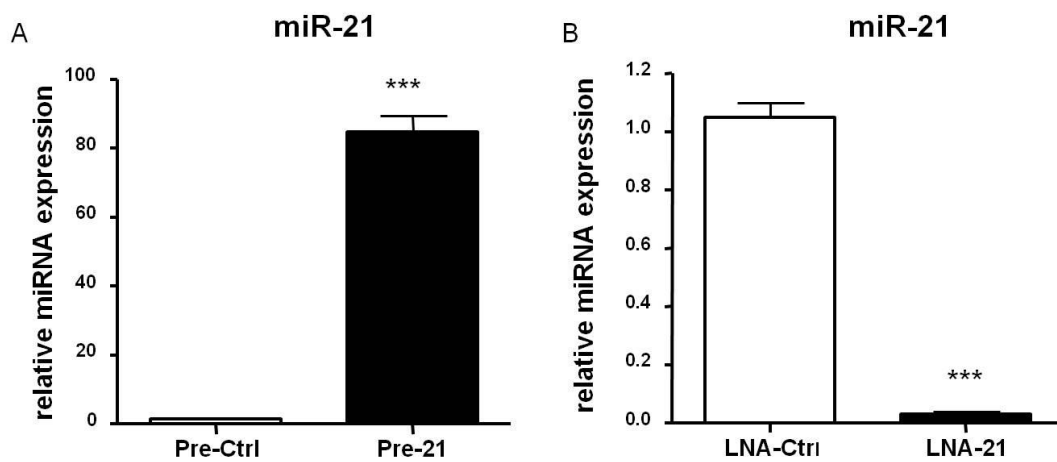


Figure 33 MiR-21 overexpression and inhibition in iPSCs differentiated with VEGF

iPSCs were differentiated for three days on collagen IV and in differentiation medium containing 50ng/ml VEGF. In order to alter the levels of miR-21, cells were transfected with precursor (Pre-21), negative control precursor (Pre-Ctrl), inhibitor of miR-21(LNA-21) and negative control of miRNA inhibitor (LNA-Ctrl). After 48h gene expression was assessed by Real time PCR analysis, results are shown for miR-21 expression after transfection with pre-21 compared to Pre-Ctrl (A) and with LNA-21, compared to LNA-Ctrl (B). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl and LNA-Ctrl.

We then analyzed the EC marker expression in iPSCs differentiated with VEGF for three days and then transfected with miR-21 precursor and inhibitor.

Transfection with Pre-21 led to an upregulation of approximately 5- and 2.5-fold in the expression of the EC markers VE-cadherin and Flk1, respectively (Figure 34, A

and C). Whereas, after transfecting the cells with LNA-21 we did not observe any significant change in the EC marker expression (Figure 34, B and D).

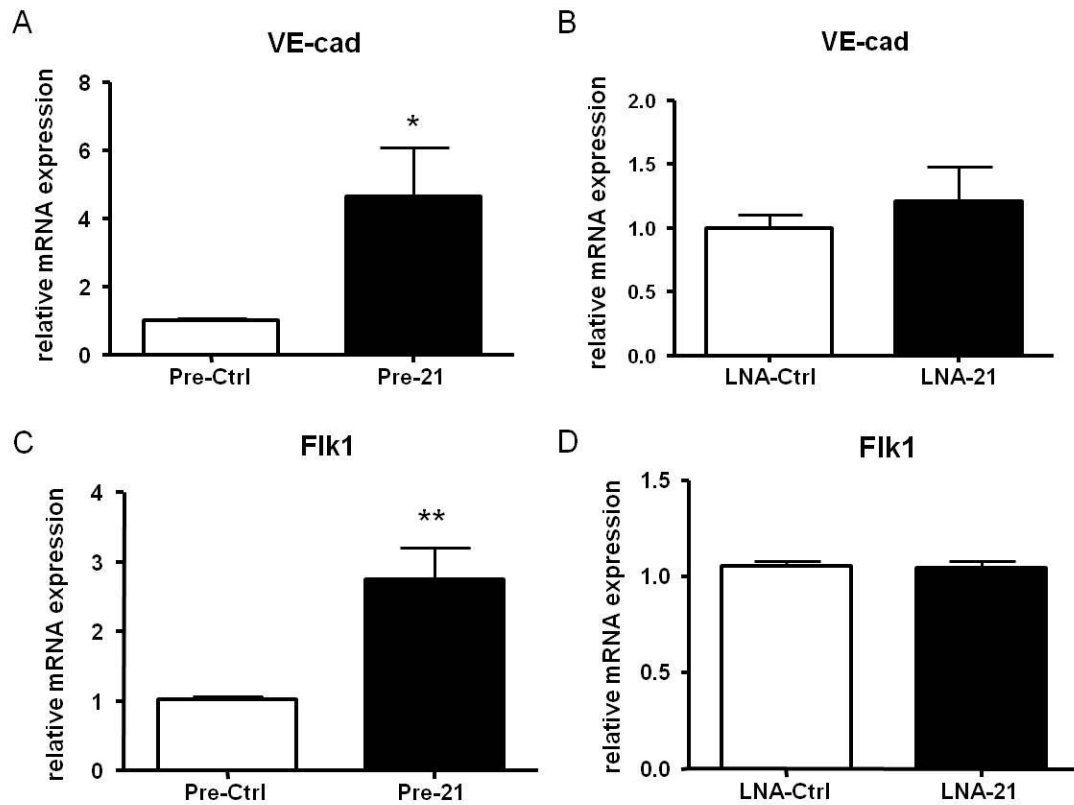


Figure 34 Analysis of EC marker expression in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis was performed 48h later. Results are shown for the EC markers VE-cadherin (VE-cad) (A and B) and Flk1 (C and D), after miR-21 overexpression and inhibition. The statistical analysis used is Student's unpaired T-test (the mean \pm SEM of 4 individual experiments is shown). * $P < 0.05$ and ** $P < 0.01$ vs. Pre-Ctrl.

In accordance with the Real time PCR results, protein expression analysis on iPSCs transfected with Pre-21 confirmed the increased expression of the EC markers VE-cadherin and CD31, compared to Pre-Ctrl (Figure 35, A and C). Surprisingly, despite no detectable changes in gene expression, transfection with LNA-21 led to a decrease in the protein levels of these markers (Figure 35, B and D).

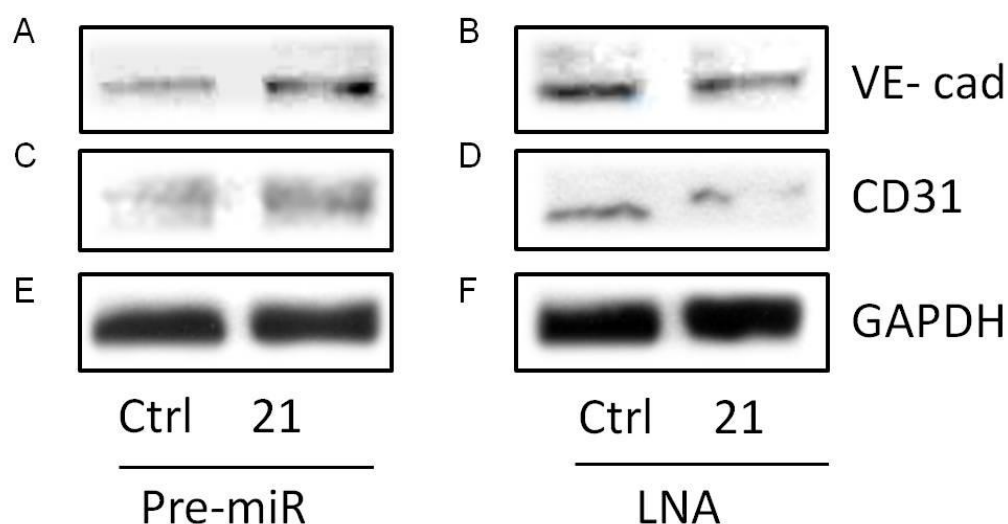


Figure 35 Protein analysis of EC markers in iPSCs after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) or the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Protein expression was assessed after 48h by Western Blot analysis of VE-cadherin (VE-cad) (A and B) and CD31(C and D) after miR-21 overexpression and inhibition. GAPDH was used as a loading control for cells transfected with Pre-miR (E) and LNA (F).

ECs, like SMCs which form the tunica media of all the blood vessels, and cardiac cells are derived from the embryonic mesoderm layer. Indeed, in order to exclude a non-specific effect of miR-21 on the differentiation of iPSCs towards the SMC or other mesoderm cell lineages, we investigated the expression of markers typical of these cell types after miR-21 overexpression and inhibition.

Real time PCR was performed after transfecting VEGF-differentiated iPSCs with Pre-21 and showed some upregulation in the expression of the SMC markers calponin and smooth muscle actin (SMA), but no statistical significance was detected (Figure 36, A and C). There were no changes in the expression levels of the SMC markers after transfection with miR-21 inhibitor (Figure 36, B and D).

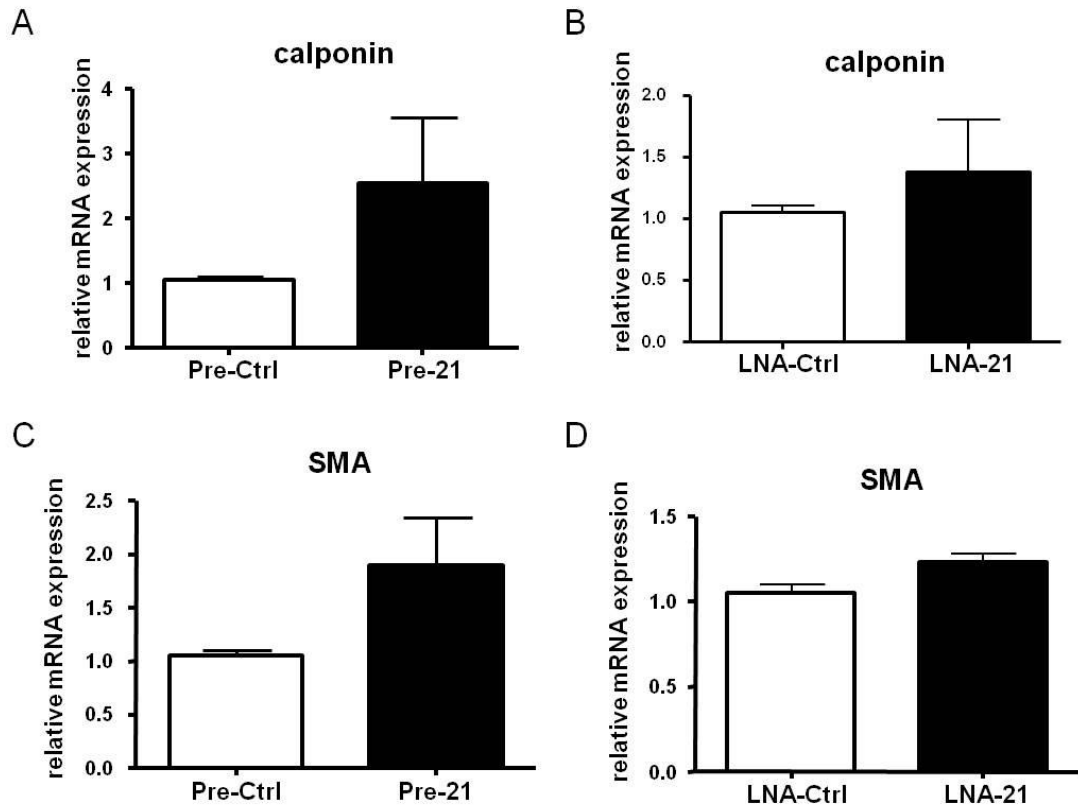


Figure 36 Analysis of SMC marker expression in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis was performed after 48h. Results are shown for Calponin (A and B) and Smooth muscle actin (SMA, C and D). The data are representative of three independent experiments. The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

Furthermore, we analyzed the expression of the cardiac markers GATA4 and MEF2C and could not detect any significant changes in their expression, even if a trend could be observed for GATA4 (Figure 37, A-D).

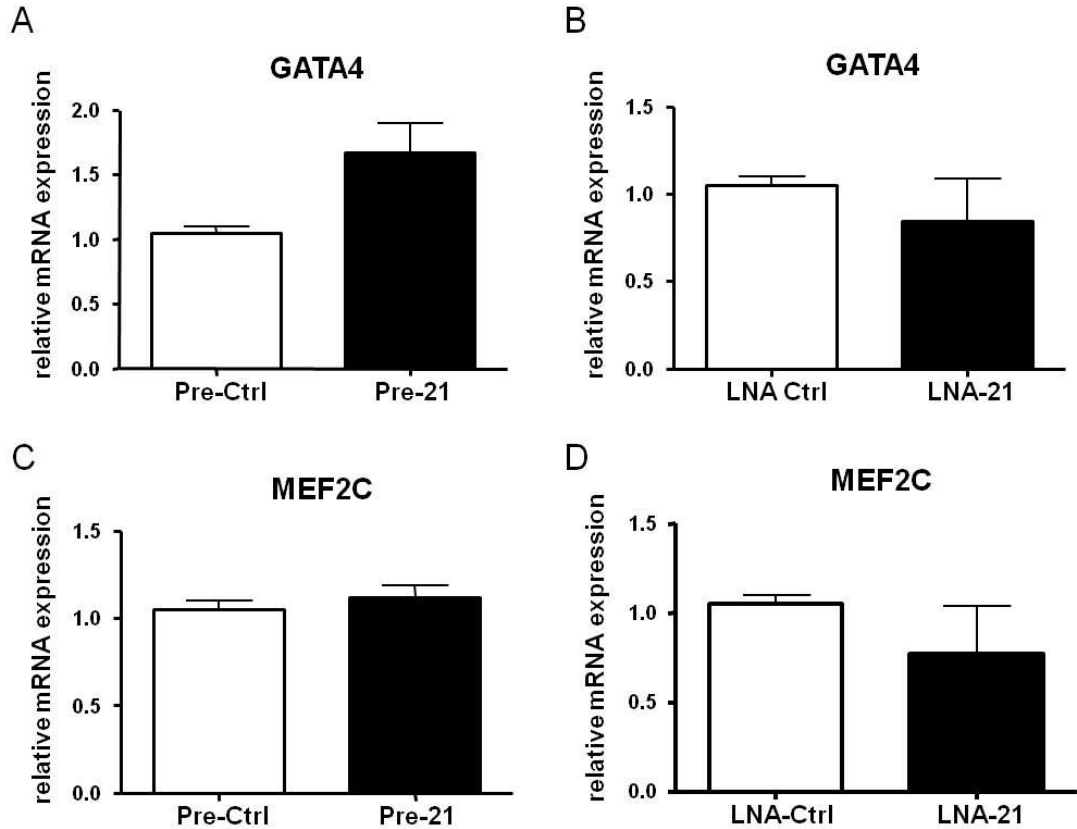


Figure 37 Analysis of SMC marker expression in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis was performed after 48h. Results are shown for GATA4 (A and B) and MEF2C (C and D). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

After having excluded an effect of miR-21 on any other cell type of the mesodermal lineage, we proceeded to test its effect on the endoderm and ectoderm.

After transfecting differentiated iPSCs with precursor or inhibitor of miR-21, no significant differences were observed in the expression levels of the pancreatic marker Nkx 6.1 (Figure 38, A and B) or the liver marker alpha-fetoprotein (AFP) (Figure 38, C and D), both derived from the endoderm.

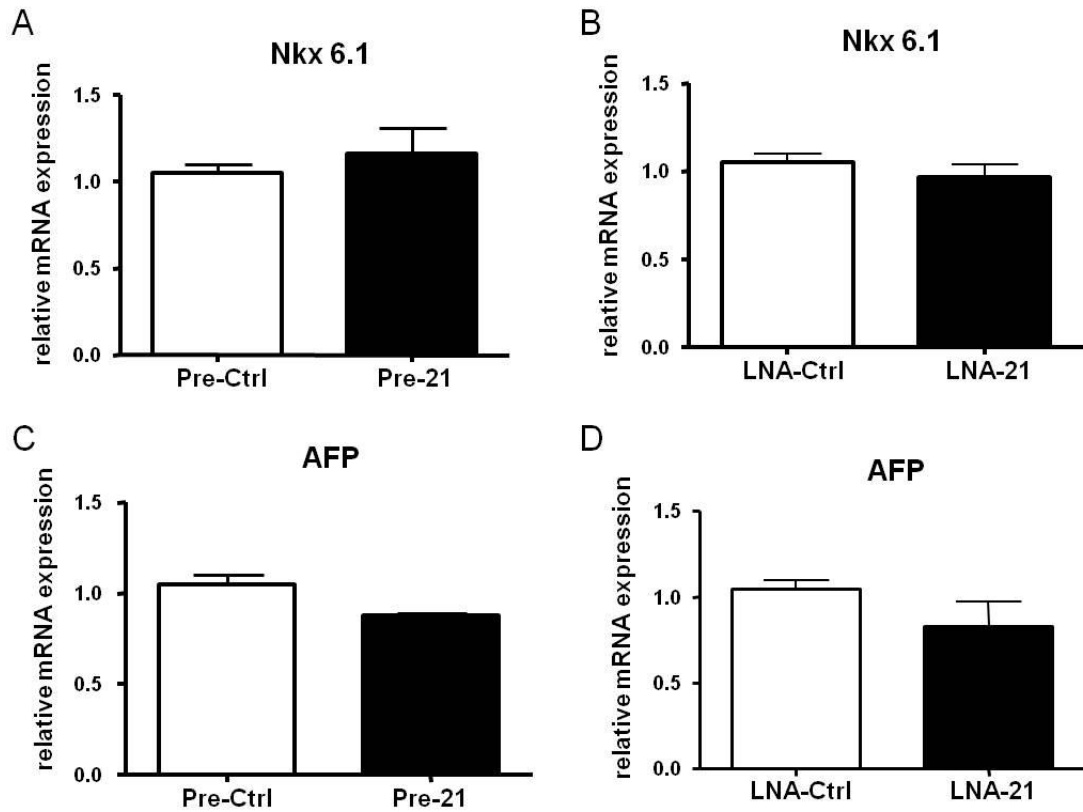


Figure 38 Analysis of pancreatic and liver marker expression in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis performed after 48h; results are shown for Nkx 6.1 (A and B) and alpha-fetoprotein (AFP, C and D). The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

Similarly, results obtained from the analysis of the ectoderm markers, showed no significant changes in the expression of neuronal or epidermal markers. Results after miR-21 overexpression (Figure 39, A and C) and inhibition (Figure 39, B and D) are shown for the neuronal marker beta3 tubulin ($\beta 3$ tubulin) and the epidermal marker Krt 2-5.

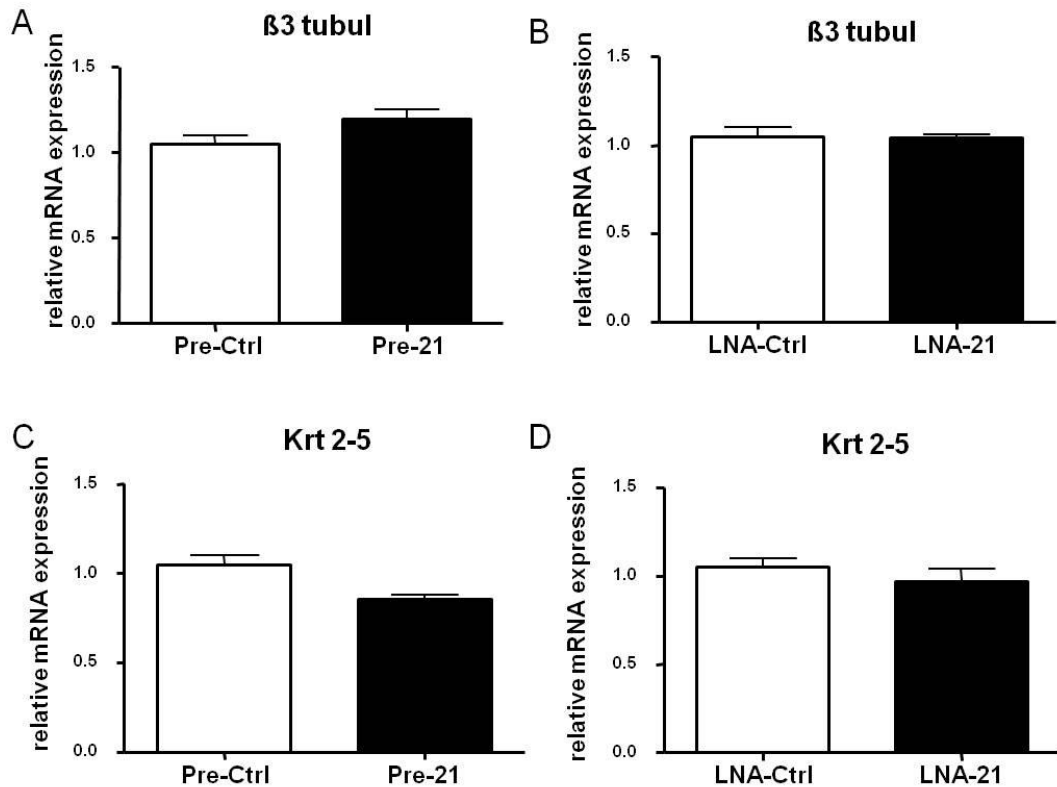


Figure 39 Analysis of neuronal and epidermal markers expression in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Real time PCR analysis was performed after 48h; results are reported for beta 3 tubulin ($\beta 3$ tubul, A and B) and Krt 2-5 (C and D). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

In conclusion, the expression levels of SMC, cardiac, endoderm and ectoderm markers were not affected by miR-21 overexpression and inhibition. We confirmed that miR-21 specifically regulates iPSC differentiation towards ECs and not towards any other cell type.

3.2.5.3 MiR-21 regulates the functional differentiation of iPSCs into ECs in *in vitro* angiogenesis assay

The next step to confirm the specific role of miR-21 in iPSC differentiation into ECs was to assess whether this miRNA is able to drive the functional differentiation of the cells, modulating the cell organization into tube-like structures.

Therefore, we performed an *in vitro* angiogenesis assay on iPSCs differentiated with VEGF for four days and then transfected with Pre-21, Pre-Ctrl, LNA-21 or LNA-Ctrl.

Results showed that cells transfected with miR-21 were endowed with increased capacity for tube-like structure formation in Matrigel (Figure 40, B), as compared to the cells transfected with Pre-Ctrl (Figure 40, A). Results of the *in vitro* angiogenesis assay were then quantified measuring the total tube length; transfection with Pre-21 increased the total tube length by approximately 4 fold (Figure 40, C).

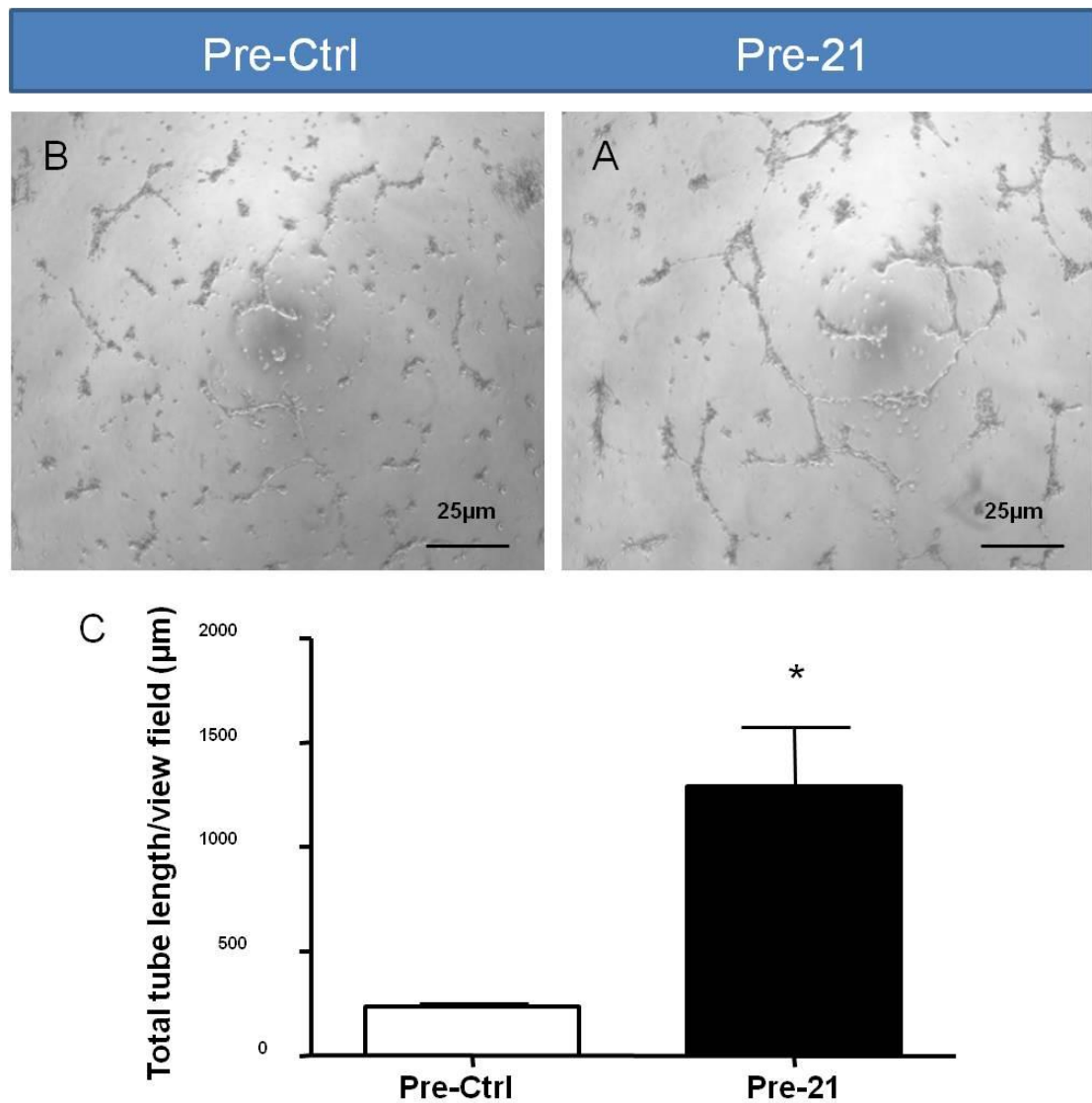


Figure 40 In vitro angiogenesis assay in iPSCs transfected with miR-21

iPSCs differentiated for four days on collagen IV in differentiation medium containing VEGF, were transfected with miR-21 precursor (Pre-21) or precursor negative control (Pre-Ctrl). After a further 72h of differentiation, cells were seeded on Matrigel and incubated for 7-8h to test their angiogenesis potential. Representative images show the increased tube formation capacity in the cells transfected with Pre-21 (B), as compared to precursor negative control (A). Results were confirmed by total tube length quantification in the cells transfected with Pre-21 and Pre-Ctrl (C). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. * $P < 0.05$ vs. Pre-Ctrl. Scale bar, 25 μ m.

On the other hand, inhibition of miR-21 in differentiating iPSCs seeded onto Matrigel *in vitro* decreased the capacity of the cells to form a tube network. Results showed that transfection with LNA-21 (Figure 41, B) impaired the ability of the cells to form tube-like structures, as compared to transfection with LNA-Ctrl (Figure 41,

A). Total tube length quantification showed that LNA-21 transfected iPSCs had total tube lengths decreased by approximately 50% (Figure 41, C).

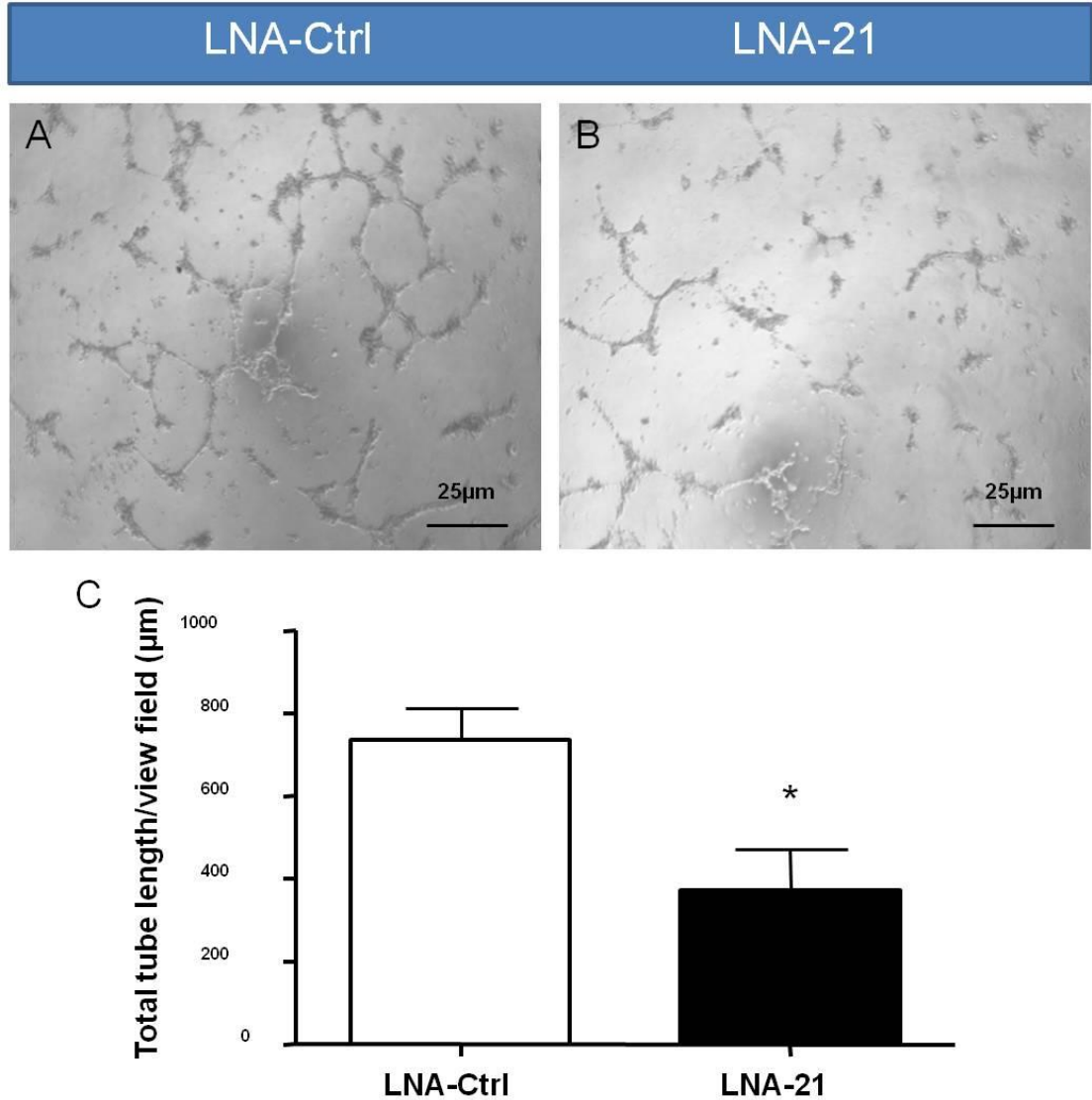


Figure 41 In vitro angiogenesis assay in iPSCs transfected with miR-21 inhibitor

iPSCs differentiated for four days on collagen IV in differentiation medium containing VEGF, were transfected with miR-21 inhibitor (LNA-21) and inhibitor negative control (LNA-Ctrl). After a further 72h of differentiation the cells were seeded on Matrigel and incubated for 7-8h to test their angiogenic potential. Representative images show tube formation capacity in the cells transfected with LNA-21 (B), as compared to LNA-Ctrl (A). Results were confirmed by total tube length quantification (C). The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. * $P < 0.05$ vs. LNA-Ctrl. Scale bar, 25 μ m.

Indeed, we showed that miR21 is able to drive the functional differentiation of iPSCs as shown by their increased capacity for organisation into tube-like structures in an *in vitro* angiogenesis assay. Accordingly, in response to miR-21 inhibition we observed a reduction in tube formation, indicating a reduction in functional differentiation.

3.2.5.4 MiR-21 regulates the functional differentiation of iPSCs into ECs in an *in vivo* angiogenesis assay

The results obtained so far indicated that miR-21 is able to induce iPSCs to differentiate towards the endothelial lineage and to form tube-like structures *in vitro*. Next, we aimed at assessing the effect of miR-21 in an *in vivo* angiogenesis assay. iPSCs were differentiated in the presence of VEGF for 4 days and then transfected with Pre-21, Pre-ctrl, LNA-21 and LNA-ctrl and then mixed with Matrigel and injected subcutaneously into nude C57BL/6 mice. After the injections, the Matrigel rapidly formed a subcutaneous plug that was collected after 7 days for capillary quantification. After 7 days, plugs were removed and cryosections were prepared. We stained the sections in hematoxylin and eosin or we performed immunohistochemistry for CD31 and VE-cadherin to quantify capillary density.

Immunohistochemical analysis of the Matrigel plugs showed a significant induction in the density of CD31+ capillaries in the plugs seeded with miR-21 transfected cells (Figure 42, B), as compared to the control cells (Figure 42, A). Results of the capillary density quantification showed a 2.7 fold induction in the number CD31-positive capillaries per mm² in miR-21 overexpressing cells (Figure 42, C).

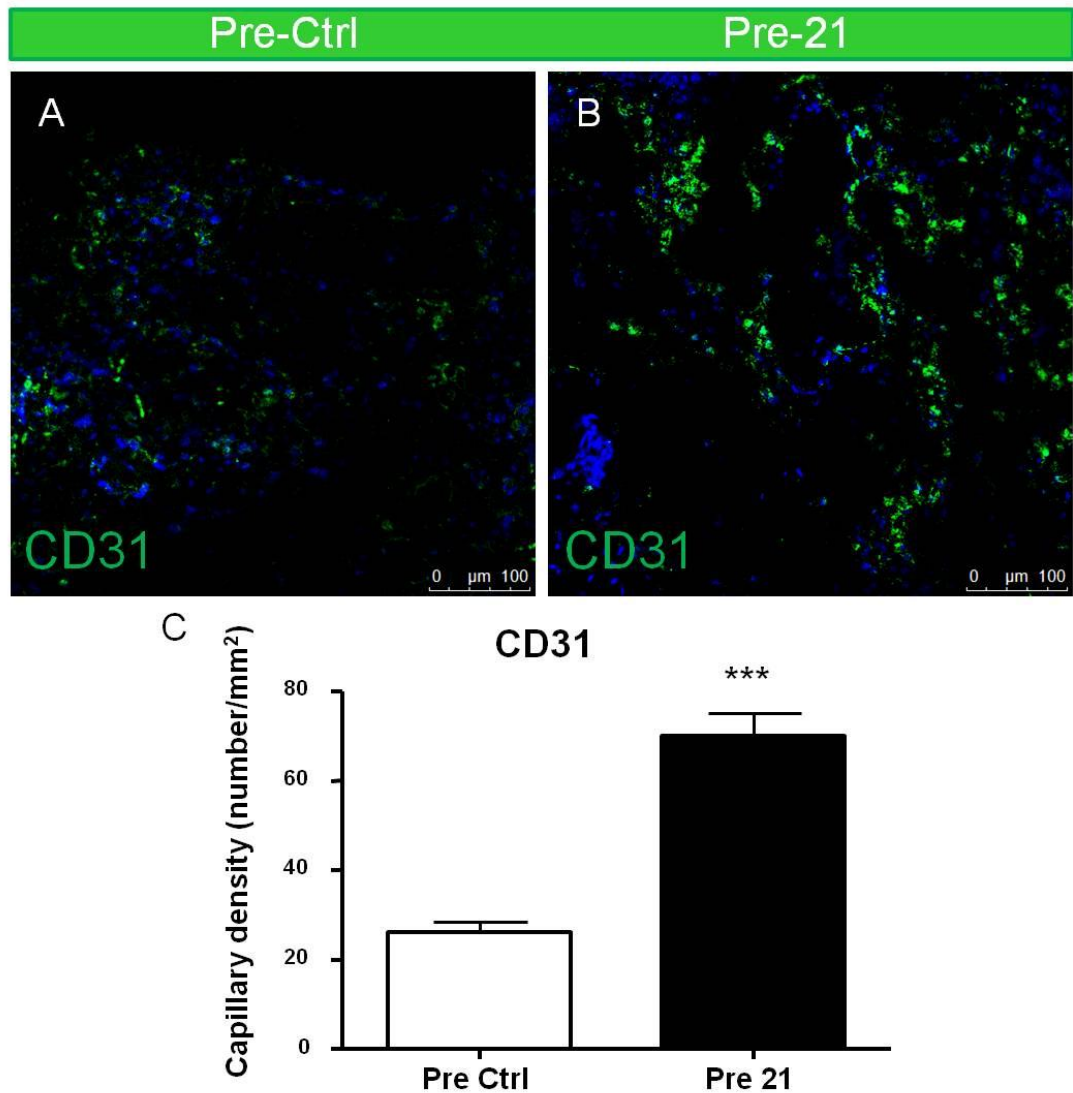


Figure 42 miR-21 overexpression increases the number of CD31-positive capillaries in in vivo Matrigel plugs

iPSCs differentiated for four days on collagen IV in differentiation medium containing VEGF, were transfected with miR-21 precursor (Pre-21) and precursor negative control (Pre-Ctrl). After further 72h of differentiation the cells were mixed with Matrigel and subcutaneously injected into mice for 1 week to test their angiogenic potential. Confocal microscopy images showed increased number of CD31⁺ capillaries in plugs containing the cells transfected with Pre-21 (B), as compared to Pre-Ctrl (A). Results were confirmed by capillary density quantification (C). The statistical analysis used is a Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl. Scale bar, 100 μ m.

Additionally, to confirm these results we quantified the number of VE-cadherin positive capillaries and found a similar trend, where the number of capillaries was induced by miR-21 overexpression (Figure 43, B), as compared to Pre-Ctrl (Figure

43, A). Quantification of VE-cadherin⁺ capillaries showed 2.3 folds of induction by miR-21(Figure 43, C).

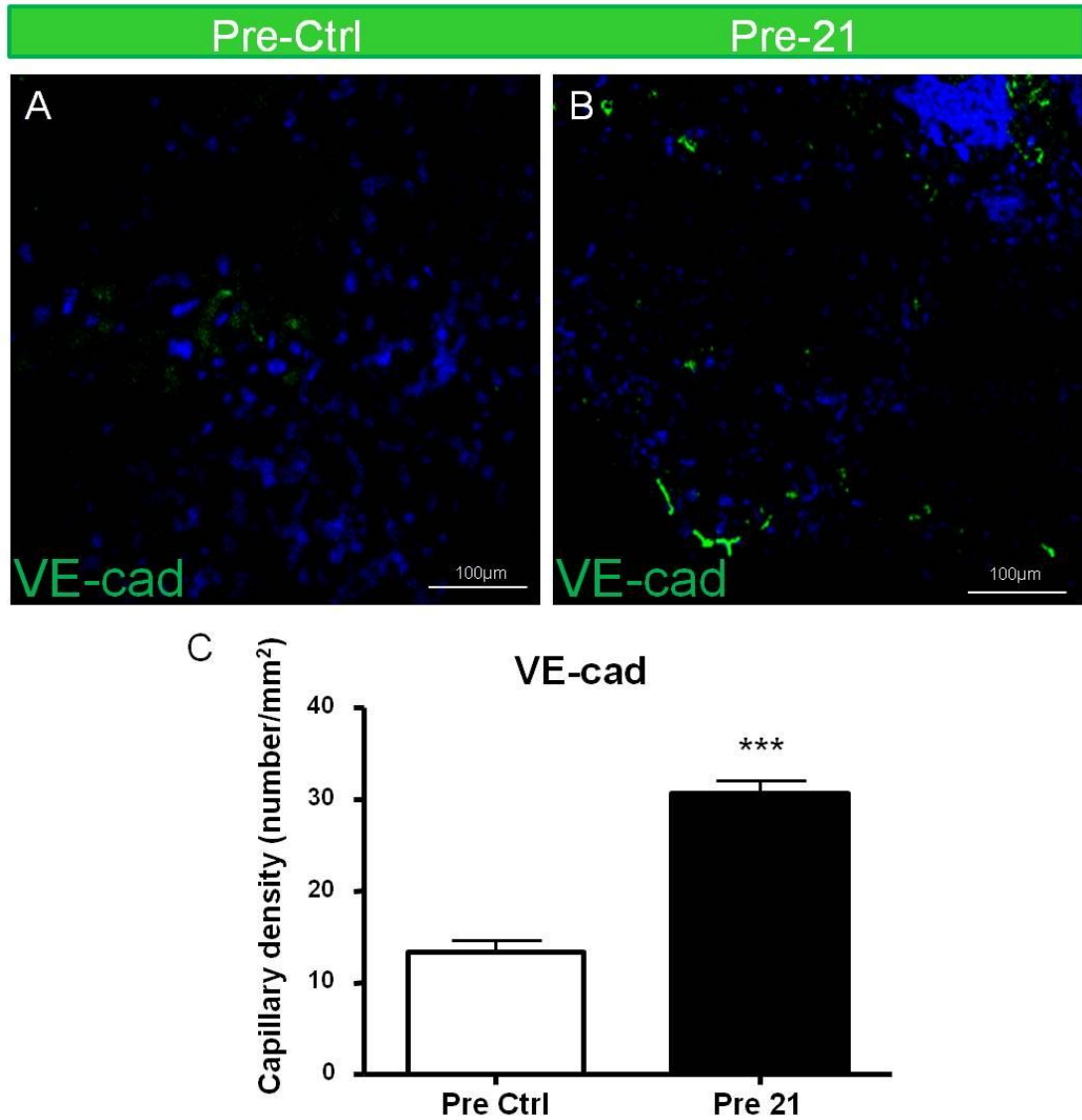


Figure 43 miR-21 overexpression induces the number of VE-cadherin-positive capillaries in matrigel plaques

iPSCs differentiated for four days on collagen IV in differentiation medium containing VEGF, were transfected with miR-21 precursor (Pre-21) and precursor negative control (Pre-Ctrl). After a further 72h of differentiation the cells were mixed with Matrigel and subcutaneously injected into the mice for 1 week to test the angiogenesis potential. Confocal microscopy imaging revealed a higher density of VE-cadherin (VE-cad)-positive capillaries in plugs seeded with Pre-21 transfected cells (B), as compared to Pre-Ctrl (A). Results were confirmed by capillary density quantification of the number of VE-cad⁺ capillaries per mm² (C). The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl. Scale bar, 100 μ m.

To confirm the results obtained with the cells transfected with Pre-21, we knocked down miR-21 expression by transfecting the cells with LNA-21 and used them in an *in vivo* Matrigel plug assay. Similarly to the results obtained *in vitro*, inhibition of miR-21 led to a decreased number of capillaries positive for the endothelial cell markers CD31 (Figure 44, B) and VE-cadherin (Figure 45, B), as compared to LNA-Ctrl (Figure 44 A and 45 A, respectively).

Quantification of capillary density indicated a significant reduction in the number of CD31-and VE-cadherin-positive capillaries per unit of area (Figure 44, C and 45, C, respectively).

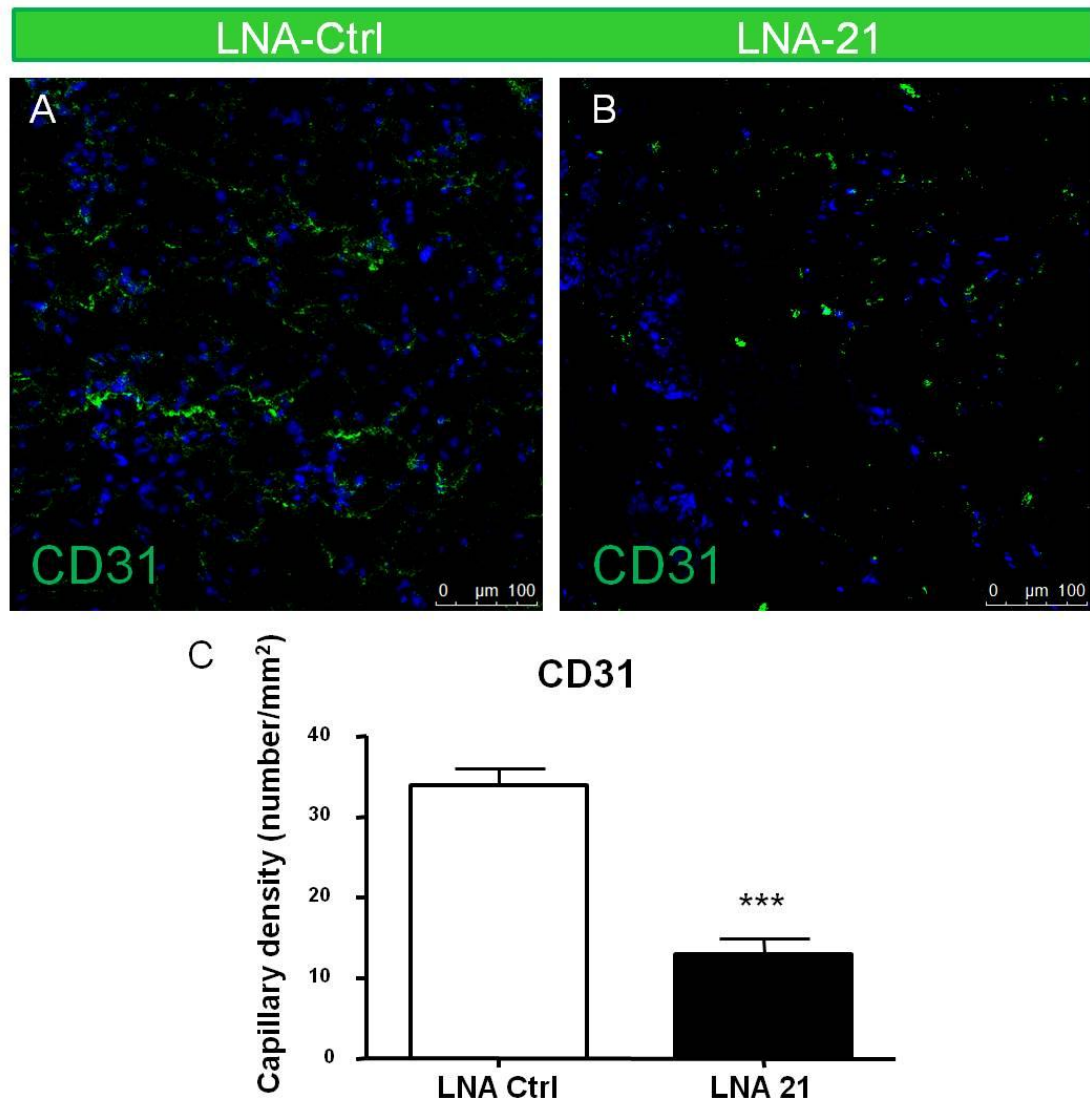


Figure 44 miR-21 inhibition decreases the number of CD31-positive capillaries in Matrigel plugs

iPSCs differentiated for four days on collagen IV in differentiation medium containing VEGF, were transfected with miR-21 inhibitor (LNA-21) and inhibitor negative control (LNA-Ctrl). After a further 72h of differentiation the cells were mixed with Matrigel and subcutaneously injected into the mice for 1 week to test their angiogenic potential. Confocal microscopy images of the Matrigel plugs show CD31-positive capillaries in the cells transfected with LNA-21 (B), as compared to LNA-Ctrl (A). Capillary density was calculated by quantification of the number of CD31-positive capillaries per unit of area (C). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. LNA-Ctrl. Scale bar, 100 μ m.

Similar results were obtained when the Matrigel plugs were stained and quantified for VE-cadherin⁺ capillaries (Figure 45).

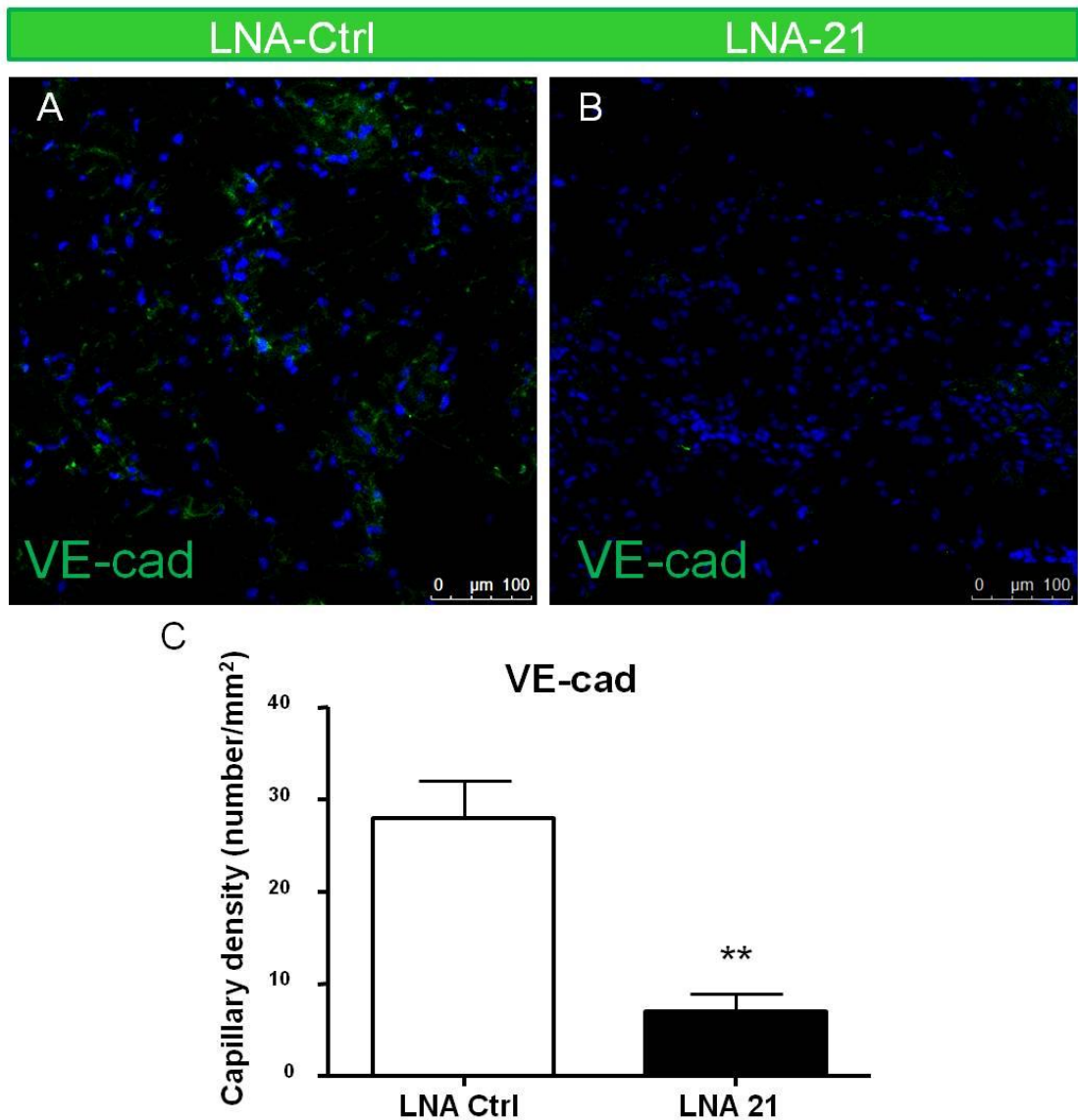


Figure 45 miR-21 inhibition decreases the number of VE-cadherin-positive capillaries in Matrigel plugs

iPSCs differentiated for four days on collagen IV and differentiation medium containing VEGF, were transfected with miR-21 inhibitor (LNA-21) and inhibitor negative control (LNA-Ctrl). After a further 72h of differentiation the cells were mixed with Matrigel and subcutaneously injected into the mice for 1 week to test their angiogenic potential. Confocal microscopy showed a decreased number of VE-cadherin (VE-cad)-positive capillaries in the cells transfected with LNA-21 (B), as compared to LNA-Ctrl (A). Results were confirmed by capillary density quantification of the number of VE-cad-positive capillaries per mm² (C). The statistical analysis used is Student's unpaired t-test. Data are presented as mean ±SEM of 3 individual experiments. *** $P < 0.001$ vs. LNA-Ctrl. Scale bar, 100 μm.

3.2.5.5 VEGF stimulation is required in miR-21-induced EC differentiation

In the first part of this work we showed that VEGF is required for the differentiation of iPSCs into ECs. We also reported that iPSCs differentiated in the presence of VEGF display a specific miRNA profile and express more miR-21 than undifferentiated iPSCs.

We showed that in iPSCs pre-differentiated with VEGF, miR-21 overexpression induces a functional differentiation of the cells into ECs. In contrast, inhibition of miR-21 impairs iPSC differentiation into ECs and reduces capillary formation *in vitro* and *in vivo*.

Next, we decided to examine the requirement for the VEGF stimulation in the process of miR-21-induced iPSC differentiation into ECs.

In order to do so, we pre-differentiated iPSCs in DM without VEGF for 3 days and then we altered the expression levels of miR-21 in the cells. As described previously, cells were transfected with precursor (Pre-21), negative control precursor (Pre-Ctrl) or inhibitor of miR-21 (LNA-21) and negative control of miRNA inhibitor (LNA-Ctrl) and then expression levels of mature miR-21 were assessed.

The expression of miR-21 increased by approximately 60 fold after Pre-21 transfection when compared to the negative control, indicating that transfection of Pre-21 increased mature miR-21 expression (Figure 46, A). We then overexpressed miR-21 inhibitor, and detected a 33-fold reduction in the mature miRNA expression as compared to the negative control (Figure 46, B).

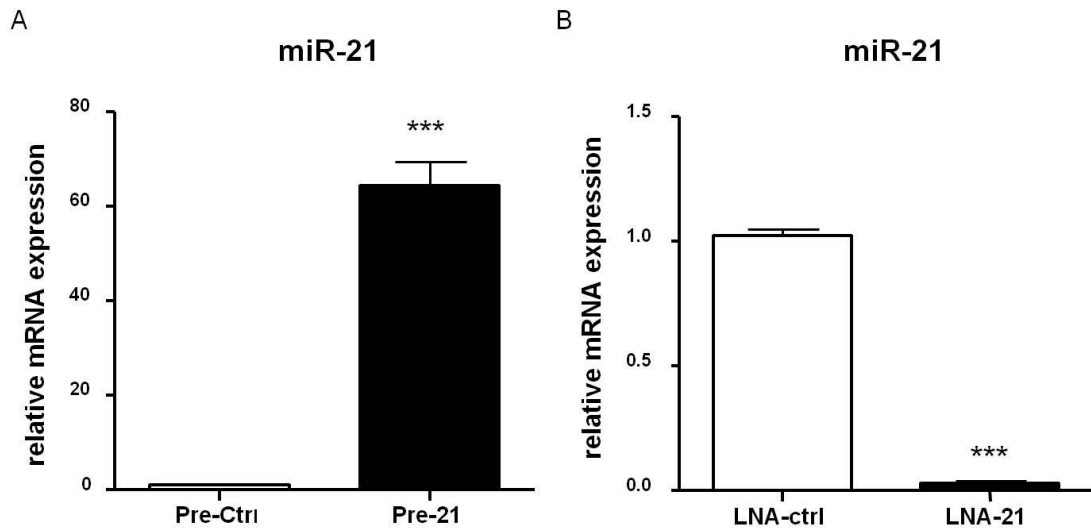


Figure 46 miR-21 overexpression and inhibition in iPSCs differentiated in absence of VEGF

iPSCs were seeded for three days on collagen IV and cultured in differentiation medium without VEGF. In order to alter the levels of miR-21, cells were transfected with precursor (Pre-21), negative control precursor (Pre-Ctrl), inhibitor of miR-21(LNA-21) and negative control of miRNA inhibitor (LNA-Ctrl), using lipofectamin RNAiMAX. After 48h gene expression was assessed by Real time PCR analysis. Results show miR-21 expression levels after transfection with Pre-21, compared to Pre-Ctrl (A) and after transfection with LNA-21, compared to LNA-Ctrl (B).). The statistical analysis used is Student's unpaired t-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl and LNA-Ctrl.

Subsequently, we then analyzed the EC marker expression in iPSCs pre-differentiated without VEGF, after miR-21 overexpression and inhibition. Transfection with Pre-21 did not alter the expression of VE-cadherin, as compared to cells transfected with Pre-Ctrl (Figure 47, A). Flk1 gene expression was increased by approximately two fold by Pre-21, but this result was not statistically significant (Figure 47, C).

After transfecting the cells with LNA-21, we did not observe any significant downregulation in the EC marker expression (Figure 47, B and D).

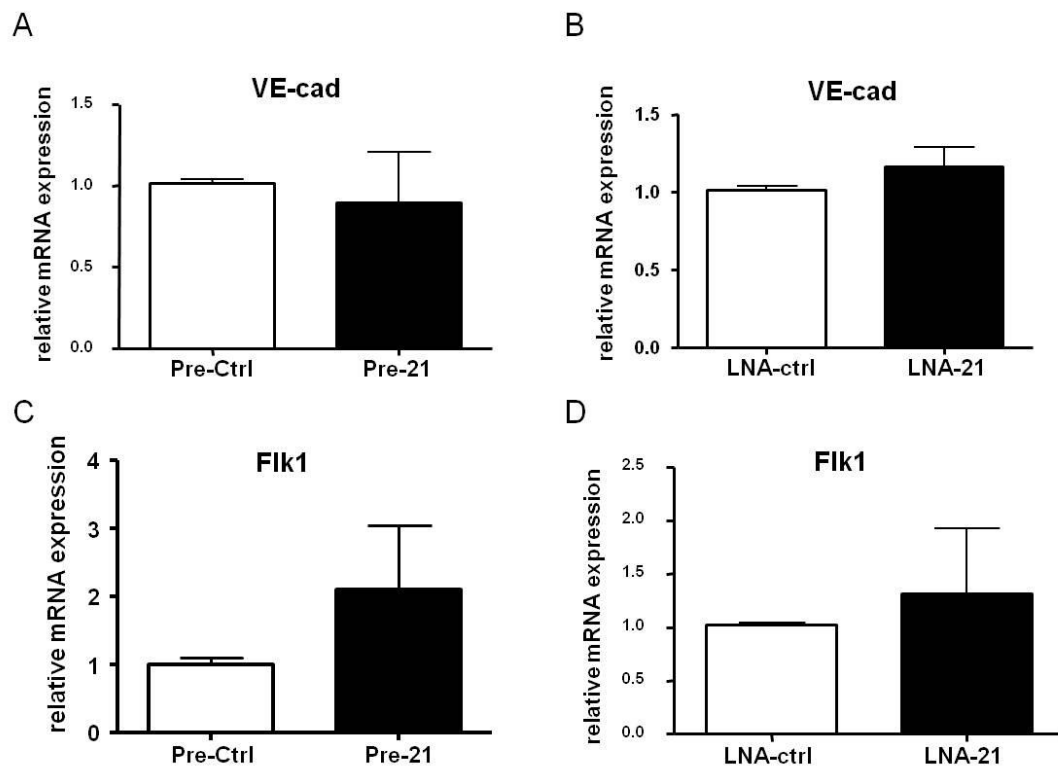


Figure 47 Analysis of the EC marker expression in iPSCs differentiated in the absence of VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium without VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Gene expression was analysed by Real time PCR in samples collected after 48h. Results are shown for VE-cadherin (VE-cad, A and B) and Flk1 (C and D) after miR-21 overexpression and inhibition. The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

In conclusion, without adding VEGF to the iPSCs differentiation system, miR-21 induction of the EC marker expression was abolished. These data indeed show that the action of this cytokine is required for miR-21 induced iPSC differentiation into ECs.

3.2.6 Conclusions

In conclusion, in this second part of our study we characterized the miRNA signature typical of iPSC during their differentiation towards an EC lineage.

We performed a miRNA array on iPSCs differentiated for 3 days in presence of VEGF and undifferentiated and we confirmed a differential expression of five miRNAs in the two groups of cells.

We also performed a miRNA array on iPSCs differentiated for 3 days on collagen IV, in static condition and compared them to iPSCs cultured in the same way, but subjected to 48h of shear stress. However, due to the lack of reproducibility of the experiments performed with shear stress application, we could not confirm differential expression of any miRNAs in the two groups of cells.

Amongst the five validated miRNAs obtained from the differentiated vs. undifferentiated array, we further investigated the role of miR-20b and of miR-21 in driving EC differentiation. Results showed no indication of EC differentiation in cells transfected with miR-20b, but significant upregulation of EC markers by miR-21.

Further studies, allowed us to establish that miR-21 was able to specifically induce EC marker expression exclusively in cells pre-differentiated in the presence of VEGF.

Furthermore, *in vitro* and *in vivo* tube formation assays revealed that miR-21 also induced functional differentiation of iPSCs, promoting the organization of the cells into tube-like structures on Matrigel.

3.3 IDENTIFICATION OF THE MOLECULAR TARGETS OF MiRNA-21: TGF- β 2 PATHWAY IS A DOWNSTREAM TARGET OF MiR-21 AND DRIVES iPSC DIFFERENTIATION INTO ECs

In the present study we so far optimized a differentiation protocol for iPSCs differentiation into ECs involving the use of VEGF. Based on this protocol we characterized a miRNA signature during the iPSC differentiation process and in particular we found that miR-21 plays a role in specifically inducing iPSC differentiation towards the EC lineage.

At this point we aimed to elucidate the molecular mechanisms which occur during the miR-21 induced differentiation of iPSCs. Indeed, we started by identifying the molecular targets of miR-21 in order to elucidate the signalling pathways involved in the differentiation process.

3.3.1 Screening for the potential target genes of miR-21

The next important step in this work is the identification of the molecular targets of miR-21, using bioinformatic tools and *in silico* target screenings.

As mentioned before, miRNAs induce mRNA degradation and post-transcriptional gene silencing by binding to conserved regions in the 3' untranslated region (UTR) of target mRNAs (Pillai, Bhattacharyya et al. 2007).

In order to identify putative target genes regulated by miR-21 and involved in the control of iPSC differentiation, we evaluated targets predicted by several of the available algorithms. In particular, we used a miRNA target database (miRGEN) based on the intersection of four prediction algorithms: DIANA-micro-T, miRanda, Pic Tar and TargetScanS. From the lists of *in silico* predicted targets, we focused on genes that may be involved in the angiogenic processes. Among those we analyzed genes regulating endothelial cell function and vessel growth, such as transforming growth factor receptor II (TGF- β RII), the regulator of ERK activation Sprouty 1 (SPRY1), the RhoGTPase RhoB and Sox7. From the same list we also searched for genes encoding for regulators of cell migration such as vinculin (Vcl).

Together with the predicted targets, we also considered other pathways that were implicated with miR-21 in several published papers. For instance, miR-21 is known to be involved in the hypoxia-inducible factor 1-alpha (HIF1 α) pathway, which induces tumor angiogenesis through stimulation of VEGF gene expression (Kong, Kong et al. 2012). Furthermore, we considered the Wnt signalling pathway, which has previously been found to be involved in EC proliferation and differentiation during development and healing and also in physiological and pathological angiogenesis (Logan and Nusse 2004, Dejana 2010). Furthermore the Wnt/ β -catenin signalling pathway has been reported to play a role in cardiomyocyte differentiation from human pluripotent stem cells (Lian, Zhang et al. 2013). Moreover, wnt5a has been shown to drive the EC differentiation of ESCs, through both Wnt/ β -catenin and Protein Kinase C α and is implicated in vascular development *in vivo* (Yang, Yoon et al. 2009).

Analysis of miR-21 regulation of the chosen genes from miRgen or part of the Wnt and the HIF1 α /VEGF signalling pathways is reported in Figure 48 and 49. Interestingly, we found that transfection with miR-21 inhibitor was able to reduce VEGF gene expression. These results highlighted the possibility of a mutual regulation between miR-21 and VEGF during iPSC differentiation, by showing that not only VEGF regulates miR-21 expression, but also miR-21 is important in the regulation of VEGF, since cells lacking miR-21 expressed reduced amount of VEGF mRNA (Figure 49).

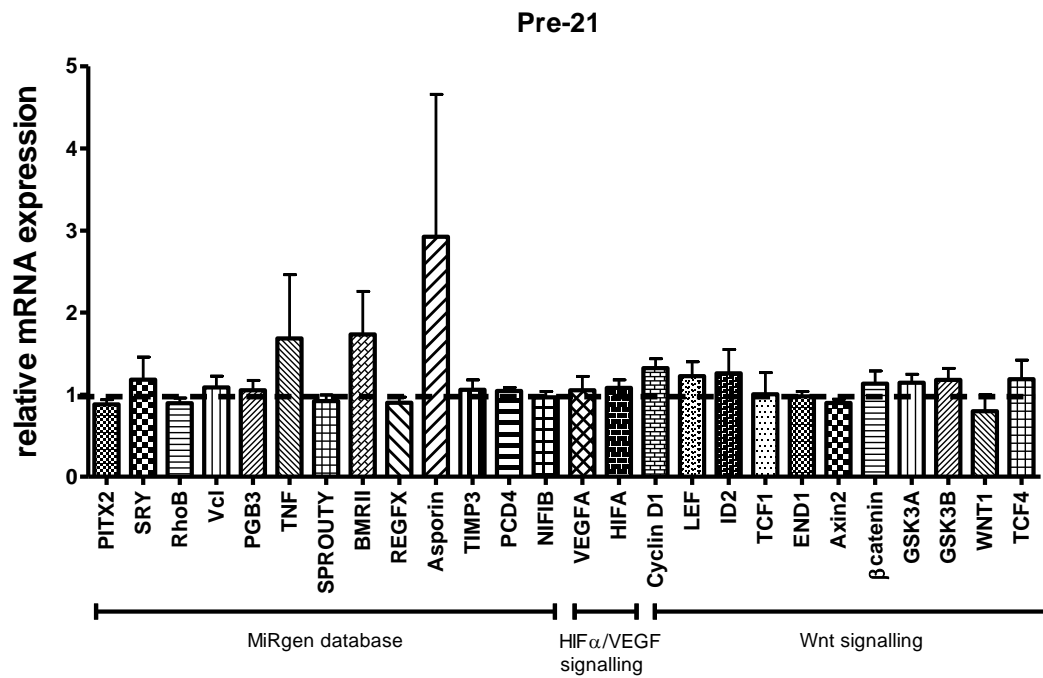


Figure 48 Analysis of the expression of potential targets of miR-21 after overexpression

iPSCs differentiated for 3 days on collagen IV and in DM containing VEGF were transfected with miR-21 precursor (Pre-21) or the negative control of the miR-21 precursor. Real time PCR analysis was performed after 48h. Results show the expression levels of some of the genes predicted by MiRgen database and some genes from the Wnt signalling pathway, after miR-21 overexpression. The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

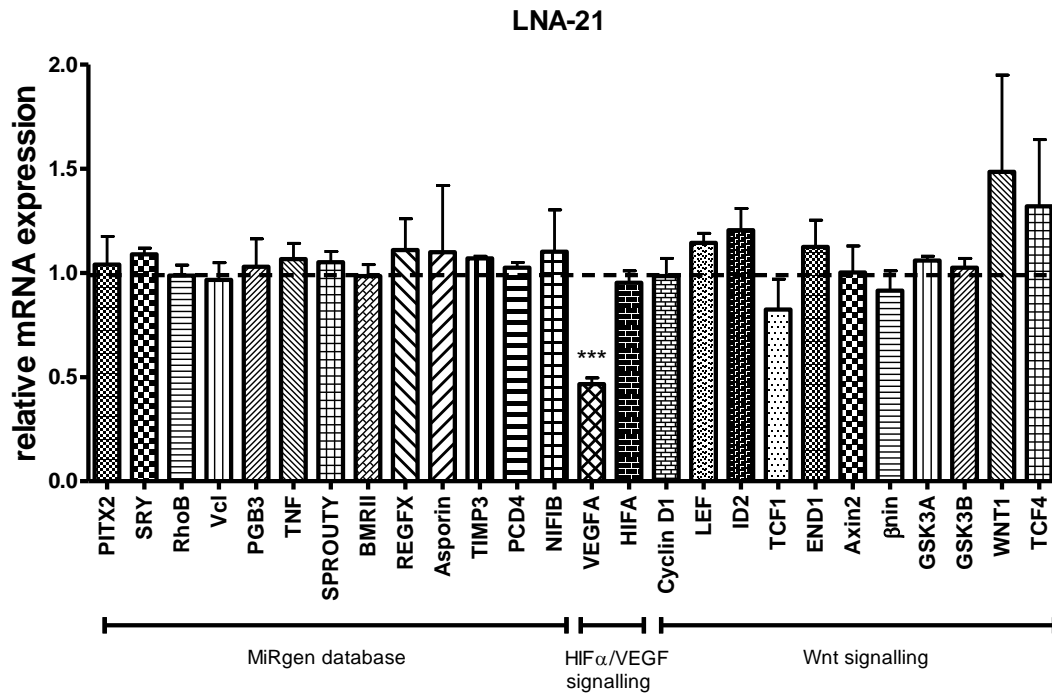


Figure 49 Analysis of the expression of potential targets of miR-21, after its inhibition

iPSCs differentiated for 3 days on collagen IV and in DM containing VEGF were transfected with miR-21 inhibitor (LNA-21) and the negative control of the miR-21 inhibitor. Real time PCR analysis was performed after 48h. Results show the expression levels of some of the genes predicted by the MiRgen database and some genes from the Wnt signalling pathway, after miR-21 inhibition. The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$), except for VEGFA expression ($***P < 0.001$ vs LNA-Ctrl).

3.3.2 TGF- β 2 is a downstream target of miR-21

Despite the lack of variation in the other genes identified through miRGEN, we decided to expand our investigation starting from other two predicted targets, TGF- β RII and transforming growth factor-beta-induced (TGF- β I), which are known components of the TGF- β signalling pathway.

TGF- β is a multifunctional cytokine which regulates proliferation, migration, differentiation and survival of many different cell types (Munger, Harpel et al. 1997); in mammals there are three known isoforms, TGF- β 1, TGF- β 2 and TGF- β 3, with distinct and shared functions. Interestingly, knockout studies for the different components of the TGF- β signalling pathway have shown the pivotal role of TGF- β

signalling in angiogenesis. Deletions of many members of the TGF- β family, such as TGF- β 1, TGF- β RII, TGF- β RI or ALK5, SMAD1, 4 and 5 cause vascular remodelling defects and the absence of mural cell formation, thus leading to embryonic lethality (Pardali and Ten Dijke 2009). Moreover, mutations in ALK1 and the accessory TGF- β receptor endoglin led to hereditary hemorrhagic telangiectasia, a severe vascular disorder (Bertolino, Deckers et al. 2005, Lebrin, Deckers et al. 2005).

Indeed, we decided to investigate the role of the TGF- β signalling pathway in miR-21-dependent iPSCs differentiation. We firstly analyzed the effect of miR-21 on the expression of TGF- β RII and TGF- β I. However, Q-PCR results showed that miR-21 overexpression (Figure 50, A and C) and inhibition (Figure 50, B and D) did not affect the expression levels of these genes.

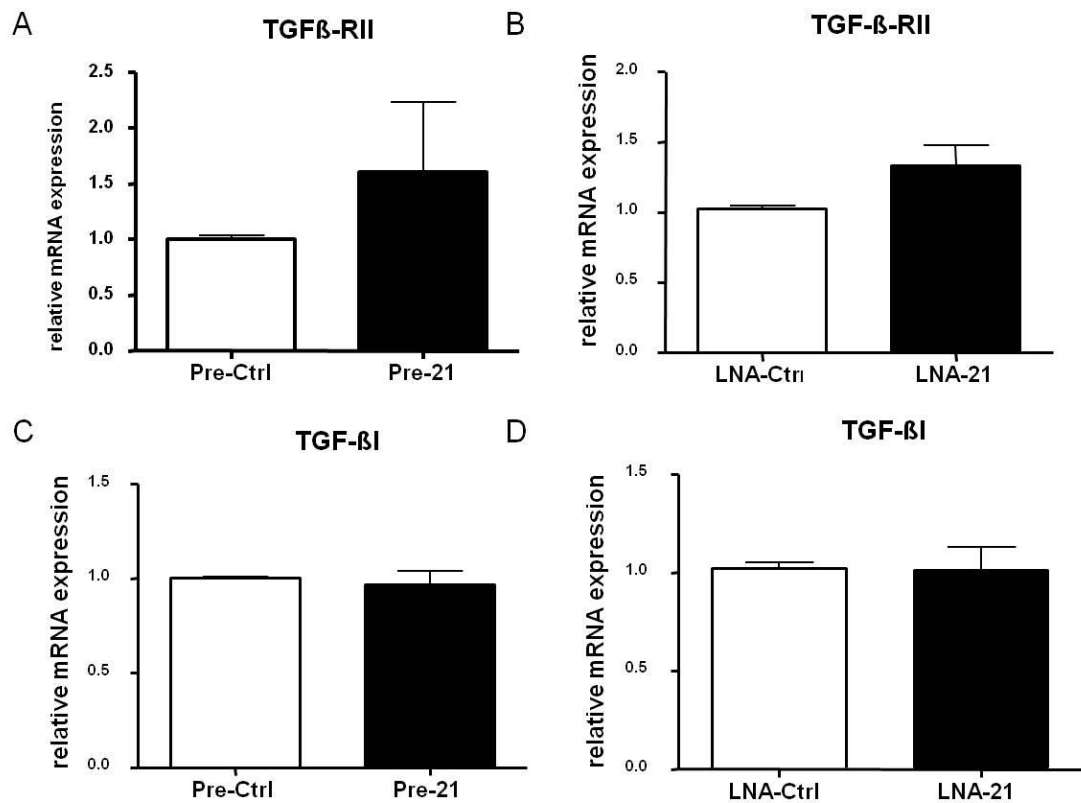


Figure 50 Analysis of TGF-βRII and TGF-βI expression after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in DM containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl) and samples for Real time PCR were collected after 48h. Results show the expression levels of transforming growth factor-beta receptor II (TGF-βRII, A and B) and transforming growth factor-beta-induced (TGF-βI, C and D) after miR-21 overexpression and inhibition. The statistical analysis used is Student's unpaired T-test. Data are presented as mean ±SEM of 3 individual experiments. No results were significant (* $P < 0.05$).

Additionally, we analysed the expression of other components of this pathway, such as TGF-βRI, TGF-β1 and TGF-β2. The analysis of the results showed that the expression of TGF-β1 and TGF-βRI was not affected by miR-21 overexpression (Figure 51, A and C) or inhibition (Figure 51 B and D).

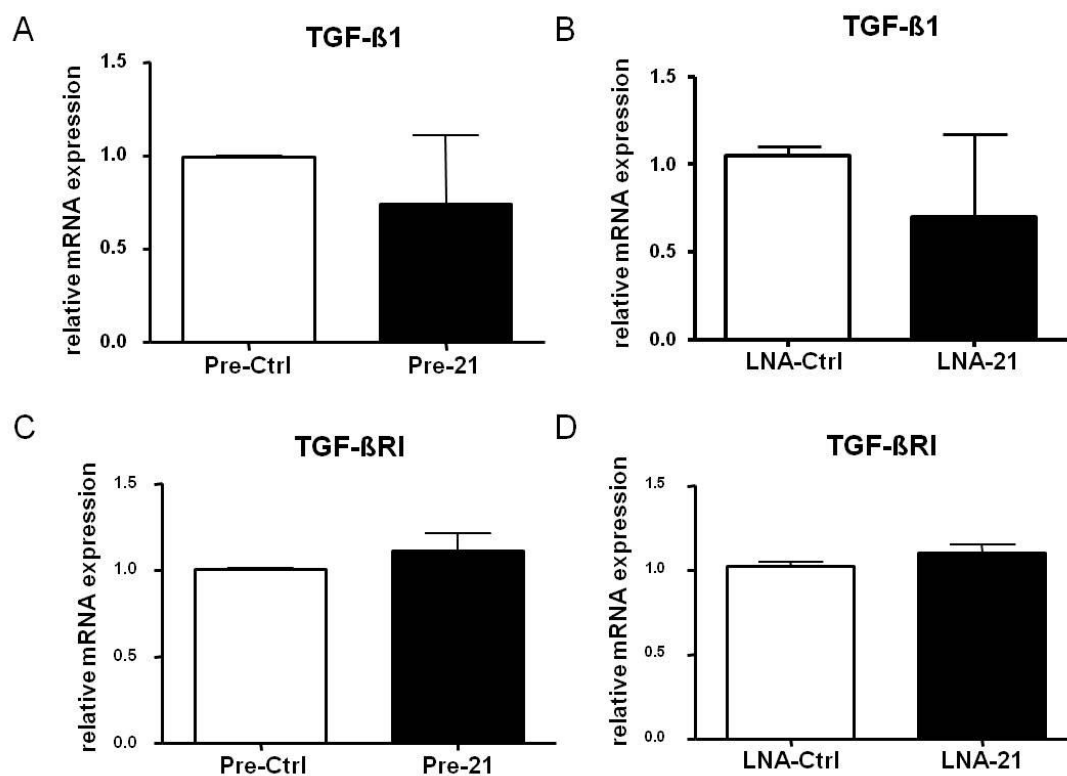


Figure 51 Analysis of the TGF-β1 and TGF-βRI expression in iPSCs differentiated with VEGF after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium with VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl) and samples for Real time PCR were collected after 48h. Results show the expression levels of transforming growth factor-beta1 (TGF-β1, A and B) and transforming growth factor-beta receptor I (TGF-βRI, C and D) after miR-21 overexpression and inhibition. The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. No results were significant ($*P < 0.05$).

Finally, analysis of TGF-β2 gene expression revealed approximately 2 fold induction upon miR-21 overexpression (Figure 52, A). On the other hand, after miR-21 inhibition the gene expression of TGF-β2 was slightly, but not significantly diminished (Figure 52, B).

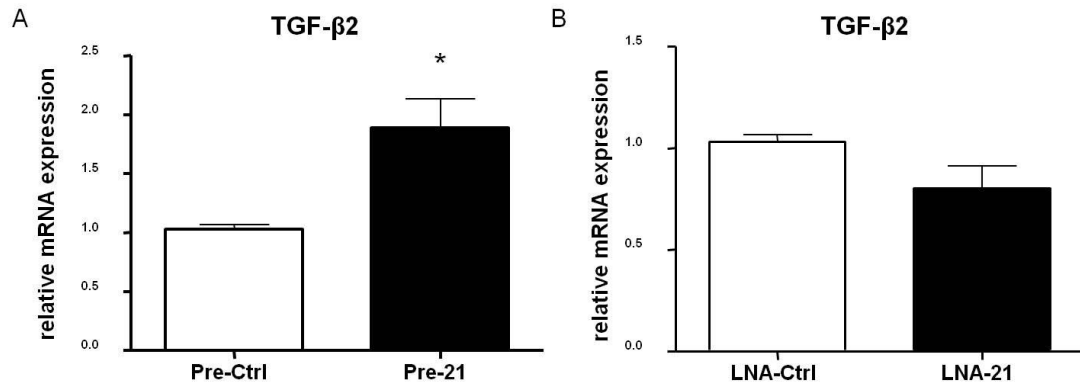


Figure 52 Analysis of TGF-β2 expression in iPSCs pre-differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in DM containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl) and samples for Real time PCR were collected after 48h. Results show TGF-β2 expression levels after transfection with Pre-21, compared to Pre-Ctrl (A) and after transfection with LNA-21, compared to LNA-Ctrl (B). The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. * $P < 0.05$ vs. Pre-Ctrl.

We performed ELISAs in order to confirm that the increase in TGF-β2 mRNA after miR-21 transfection corresponded to an increase in the secreted protein. An ELISA specific for TGF-β2 showed a 2-fold increase in the protein in the supernatants of cells transfected with Pre-21 (Figure 53, A). Interestingly, we also observed a significant decrease in the secreted protein after miR-21 inhibition (Figure 53, B).

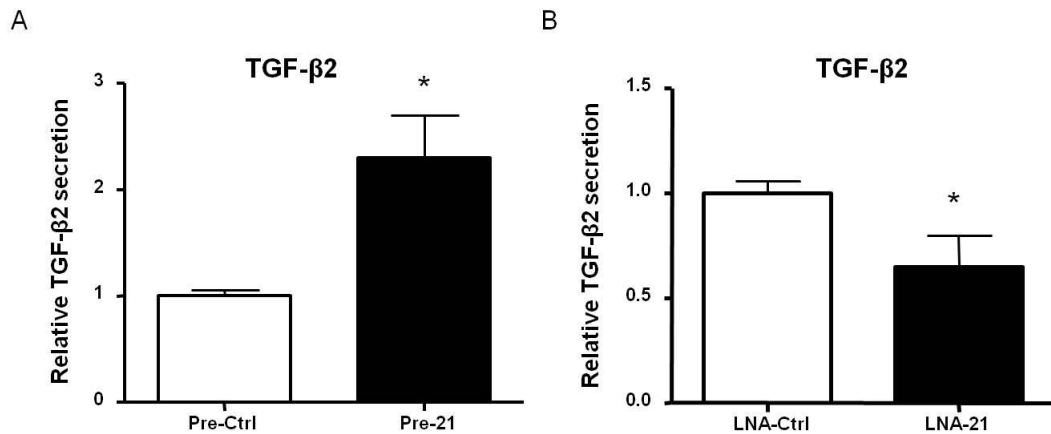


Figure 53 Analysis of TGF-β2 secretion after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl) and supernatants were collected for ELISA analysis after 48h. Results show the secretion level of TGF-β2 after transfection with Pre-21 and Pre-Ctrl (A) and after transfection with LNA-21, as compared to LNA-Ctrl (B). The statistical analysis used is Student's unpaired T-test. Data are shown as mean ±SEM of 3 individual experiments. * $P < 0.05$ vs. Pre-Ctrl and LNA-Ctrl.

From the data showed above we could conclude that miR-21 induced the expression and the secretion of TGF-β2, which indeed can be considered a downstream target of the miRNA.

3.3.3 TGF-β2 pathway is required in the miR-21-inducing iPSC differentiation into ECs

To further determine whether TGF-β2 is an essential downstream molecule mediating miR-21-inducing EC differentiation, iPSCs were differentiated with VEGF for 3 days and then transfected with pre-21 and its control. After 5h from transfection, the cells were treated with 1μg/ml TGFβ-2 neutralizing antibody or IgG as a control. 48h after transfection the cells were harvested and the protein expression was analyzed.

Real time PCR analysis showed that treating the cells with 1μg/ml TGFβ-2 neutralizing antibody inhibited miR-21-induced VE-cad upregulation, as compared to IgG control (Figure 54).

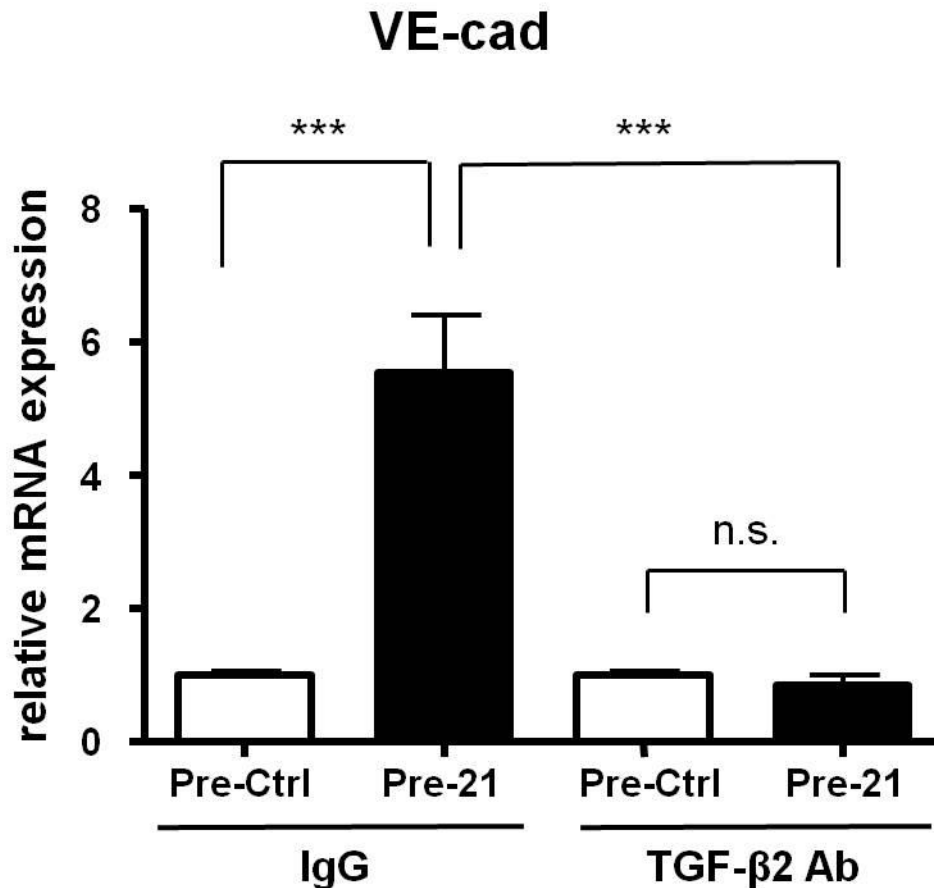


Figure 54 TGF-β2 is required in miR-21 induced iPSC differentiation into ECs

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21) and precursor control (Pre-Ctrl). After 5h from transfection, 1μg/ml TGF-β2 neutralizing antibody (TGF-β2 Ab) or IgG, as a control were added to the culture medium. Q-PCR performed 48h after transfection shows the expression levels of VE-cadherin (VE-cad). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean ±SEM of 3 individual experiments. *** $P < 0.001$ vs. Pre-Ctrl IgG and vs. Pre-21 IgG.

Western Blot analysis for CD31 expression level confirmed that TGF-β2 is required in miR-21 induced iPSC differentiation into ECs. In particular, miR-21-induced CD31 upregulation observed in the cells treated with IgG as a control was abolished by treatment with 1μg/ml TGFβ-2 neutralizing antibody (Figure 55).

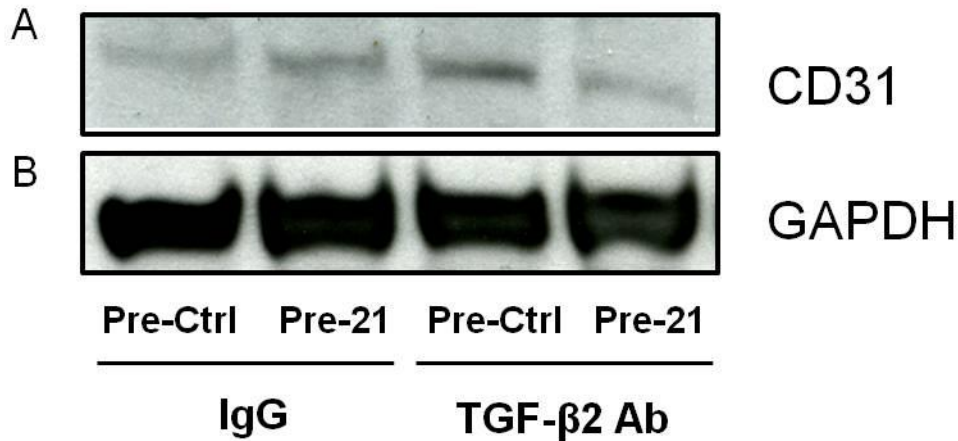


Figure 55 Analysis of CD-31 protein expression in iPSCs transfected with miR-21 precursor and treated with TGF-β2 antibody

iPSCs were transfected with miR-21 precursor (Pre-21) and precursor control (Pre-Ctrl) after 3 days of differentiation on collagen IV and in differentiation medium containing VEGF. After 5h from transfection, 1μg/ml TGF-β2 neutralizing antibody (TGF-β2 Ab) or IgG, as a control, was added to the culture medium. Protein expression was assessed after 48h by Western Blot analysis of CD31 (A). GAPDH was used as a loading control (B).

These data suggest that the induction of TGFβ-2 is necessary for miR-21 to drive iPSC differentiation into ECs.

3.3.4 TGF-β2 pathway regulates iPSC differentiation into ECs

At this point we decided to investigate the involvement of TGF-β2 miR-21-dependent iPSC differentiation into ECs. In order to do so, we first analyzed its expression during VEGF-induced EC differentiation.

3.3.4.1 TGF-β2 expression is increased during VEGF-induced iPSC differentiation

Data collected so far demonstrated that TGF-β2 plays a role in the differentiation of iPSCs towards the endothelial lineage. We therefore analyzed its expression during the first 7 days of differentiation with VEGF. As results showed, TGF-β2 expression was increased 50 and 200-fold, after 5 and 7 days respectively (Figure 56, A). In contrast, TGF-β1 was increased by approximately 5 and 10 fold after 5 and 7 days, and its expression was highly variable among experiments (Figure 56, B).

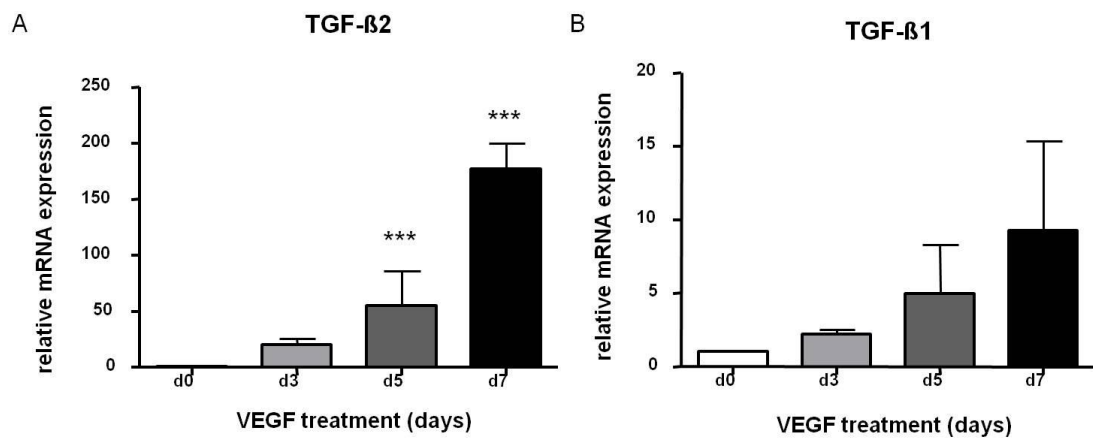


Figure 56 Analysis of TGF-β2 and TGF-β1 gene expression during VEGF induced iPSC differentiation

iPSCs were seeded on collagen IV and cultured in differentiation medium with 50ng/ml VEGF. Gene expression analysis was performed by real-time PCR after 3, 5, and 7 days of differentiation. Results are shown for transforming growth factor-β2 (TGF-β2, A) and transforming growth factor-β1 (TGF-β1, B). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. d0 (d0 represents iPSCs seeded on gelatin and cultured in undifferentiated conditions).

Next, we analyzed the expression of the receptors of TGF-β signalling during the VEGF-induced iPSC differentiation. Consistent with the expression of the ligands, we found a strong upregulation of TGF-βRII and III of approximately 20 and 200 fold after 7 days (Figure 57, B and C). Although a trend was visible, the TGF-βRI gene was upregulated to a lesser extent and not always consistently (Figure 57, A).

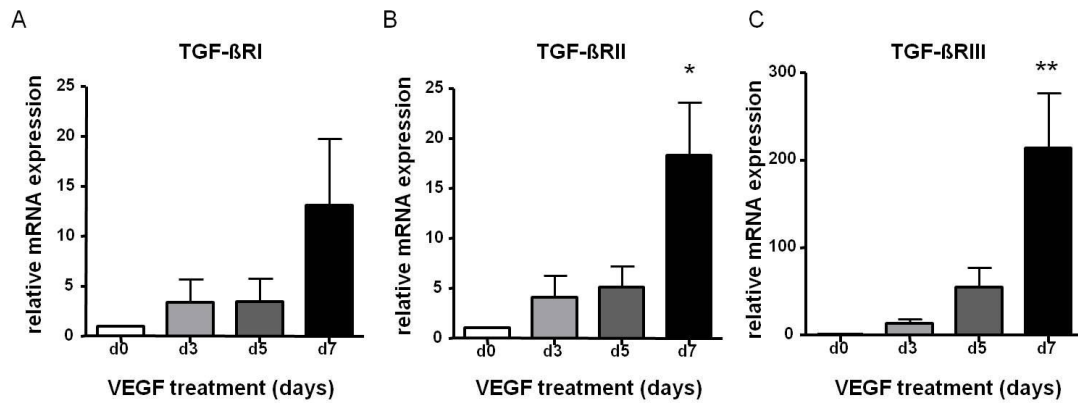


Figure 57 Analysis of TGF-βRIII, II and I expression during VEGF induced iPSC differentiation

iPSCs were seeded on collagen IV and cultured in differentiation medium with 50ng/ml VEGF. Gene expression analysis was performed by real-time PCR after 3, 5, and 7 days of differentiation. Results are shown for transforming growth factor-β receptor III (TGF-βRIII, A), transforming growth factor-β receptor II (TGF-βRII, B) and transforming growth factor-β receptor I (TGF-βRI, C). The statistical analysis used is 1way ANOVA for multiple comparisons. Data are shown as mean ±SEM of 3 individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. d0 (d0 represents iPSCs seeded on gelatin and cultured in undifferentiated conditions).

We showed that the expression of TGF-β2 and its receptors TGF-βRIII and II is increased during the VEGF-induced iPSC differentiation after 5 and 7 days. On the other hand, the expression of TGF-β1 and its receptor TGF-βRI was not significantly induced during the differentiation of iPSCs in presence of VEGF.

These data indicate that TGF-β2 has a specific role during the iPSC differentiation process induced by VEGF.

3.3.4.2 TGF-β2 treatment induces iPSC differentiation specifically towards EC lineage

At this point, in order to clarify the role of TGF-β2 in iPSC differentiation, we treated iPSCs for up to 7 days with TGF-β2 and analyzed the expression of the endothelial cell markers.

Accordingly, as with previous results, our findings showed an upregulation of the endothelial markers VE-cadherin (VE-cad, Figure 58, A) and Flk1 (Figure 58, B) after TGF- β 2 treatment.

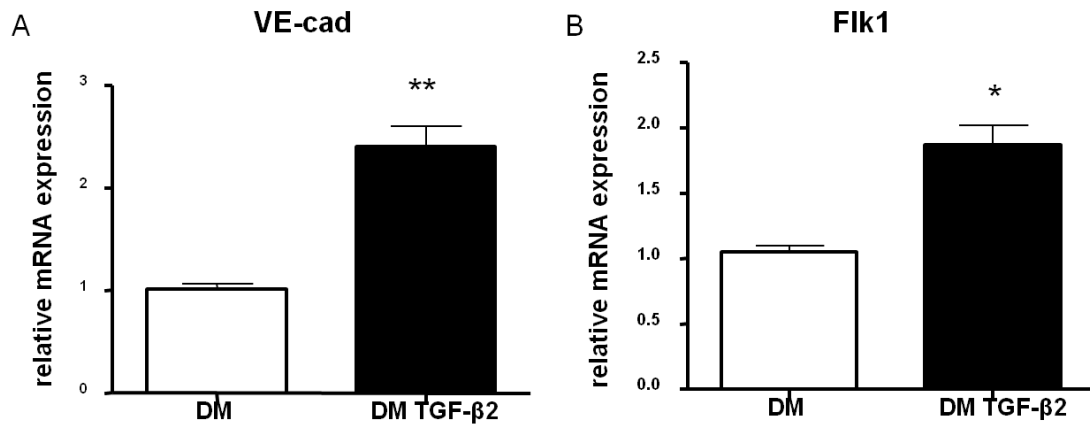


Figure 58 Analysis of EC marker expression in iPSCs treated with TGF- β 2

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) containing 3ng/ml TGF- β 2. Gene expression analysis performed by real-time PCR after 7 days. Results show the expression levels of VE-cadherin (VE-cad) and Flk1 (B). The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. iPSCs differentiated for 7 days on collagen IV and in DM (DM).

Protein analysis confirmed the upregulation of the EC markers observed at a gene expression level. Treatment of iPSCs with TGF- β 2 for 7 days induced the expression of VE-cadherin (VE-cad, Figure 59, A) and CD31 (Figure 59, B), compared to untreated iPSCs in DM.

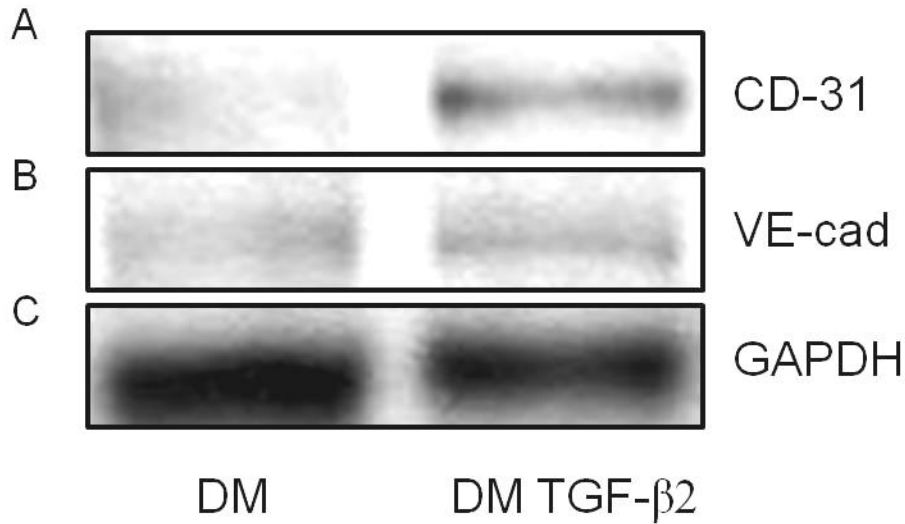


Figure 59 Protein expression analysis of EC markers in iPSCs treated with TGF-β2

iPSCs were seeded on collagen IV and cultured in differentiation medium containing 3ng/ml TGF-β2 (DM TGF-β2) or normal differentiation medium (DM). Protein expression was assessed by Western blot analysis after 7 days. Results are shown for VE-cadherin (Ve-cad, A) and CD31 (B). GAPDH was used as loading control (C).

In order to exclude any unspecific effect of TGF-β2 on iPSC differentiation towards other mesodermal lineages, we analyzed the expression of SMC markers after 7 days of TGF-β2 treatment. The expression of smooth muscle myosin heavy chain (SM-MHC), smooth muscle 22 (SM22) and calponin was not affected by treatment with TGF-β2 (Figure 60, A, B and C).

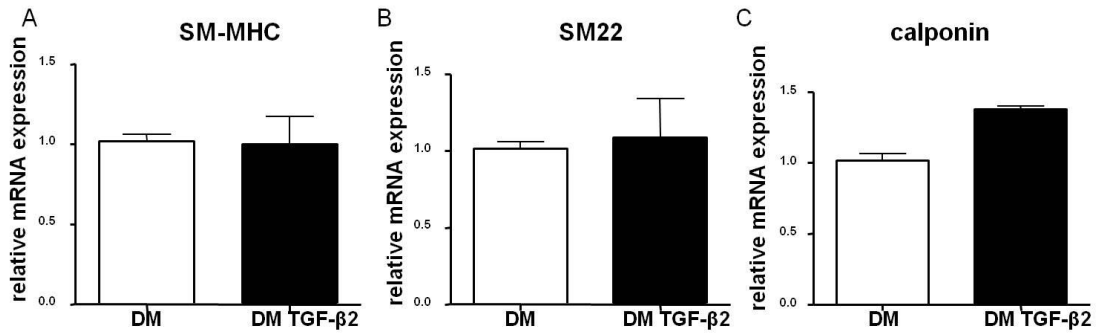


Figure 60 Analysis of SMC markers expression in iPSCs treated with TGF-β2

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) containing 3ng/ml TGF-β2. Gene expression analysis performed by real-time PCR after 7 days. Results are shown for smooth muscle myosin heavy chain (A, SM-MHC), smooth muscle 22 (B, SM22) and calponin (C). The statistical analysis used is Student's unpaired T-test. Data are shown as mean ±SEM of 3 individual experiments. No results were significant (* $P < 0.05$).

We could indeed confirm that TGF-β2 is able to drive iPSCs differentiation specifically towards the EC lineage.

3.3.4.3 TGF-β2 promotes the functional differentiation of iPSCs

To test the functionality of iPSCs after TGF-β2 treatment we performed *in vitro* angiogenesis assays. Cells treated for 7 days with TGF-β2 (Figure 61, B) showed increased tube formation ability, as compared to the untreated cells (Figure 61, A). The results of the assay were confirmed by total tube length quantification. TGF-β2 treatment increased iPSC total tube length 7-fold (Figure 61, C).

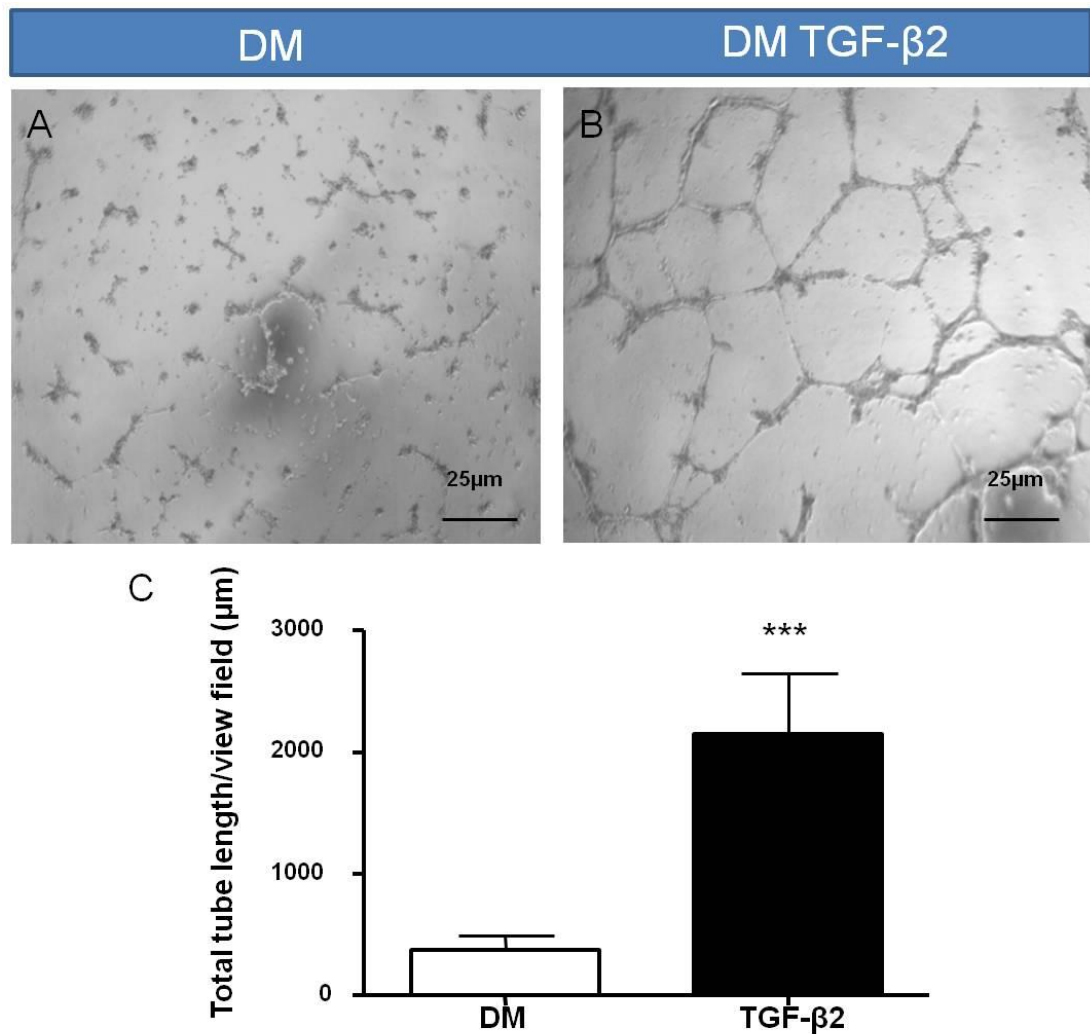


Figure 61 In vitro angiogenesis assay in iPSCs differentiated with TGF-β2

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) containing 3ng/ml TGF-β2. After 7 days cells were seeded on Matrigel and incubated for 7-8h to test the angiogenesis potential. Representative images show tube formation capacity in the cells treated with TGF-β2 (B) and untreated cell in DM (A). Results were confirmed by total tube length quantification (C). The statistical analysis used is Student's unpaired T-test. Data are presented as mean ±SEM of 3 individual experiments. *** $P < 0.001$ vs. DM (DM represents iPSCs seeded on collagen IV and cultured in differentiation medium). Scale bar, 25 μm.

In vitro angiogenesis assays showed that TGF-β2 treatment promotes the functional differentiation of iPSCs, increasing the formation of tube-like structures on Matrigel.

3.3.4.4 Inhibition of SMAD3 abolishes TGF- β 2 stimulation of VE-cadherin expression

As already mentioned in the introduction section (1.3.4), the TGF- β family exerts its action on the cells via specific type I and type II serine/threonine kinase receptors and intracellular SMAD transcription factors. SMAD3, together with SMAD1, SMAD5, and SMAD8 and SMAD2 are part of the R-SMAD, and like SMAD2, are activated by the TGF- β -specific type I receptors (Massagué, Seoane et al. 2005). In ECs TGF- β has been shown to bind to TGF- β RI, ALK1, which induces SMAD1/5 phosphorylation to potentiate angiogenic reactions; on the other hand, binding of TGF- β to ALK5, which is ubiquitously expressed in the majority of the cells, induces phosphorylation of SMAD2/3, thereby inhibiting proliferation, tube formation, and migration of ECs (Goumans, Valdimarsdottir et al. 2003). However, so far there are no studies reporting the role of SMAD3 in EC differentiation. Interestingly, in our system the inhibition of SMAD3 strongly reduced VE-cadherin expression, indicating the importance of the TGF β -2/ SMAD3 pathway in the differentiation process of iPSCs.

iPSCs were differentiated for 7 days on collagen IV and in DM with or without TGF- β 2. From day 1 the cells were treated with 5 μ M SMAD3 inhibitor (SIS3) or DMSO as a control. Q-PCR results showed approximately two fold upregulation of VE-cadherin induced by TGF- β 2 treatment. SMAD3 inhibition not only decreased the baseline expression level of VE-cadherin, but it also abolished the TGF- β 2 induction of the EC marker (Figure 62).

VE-cad

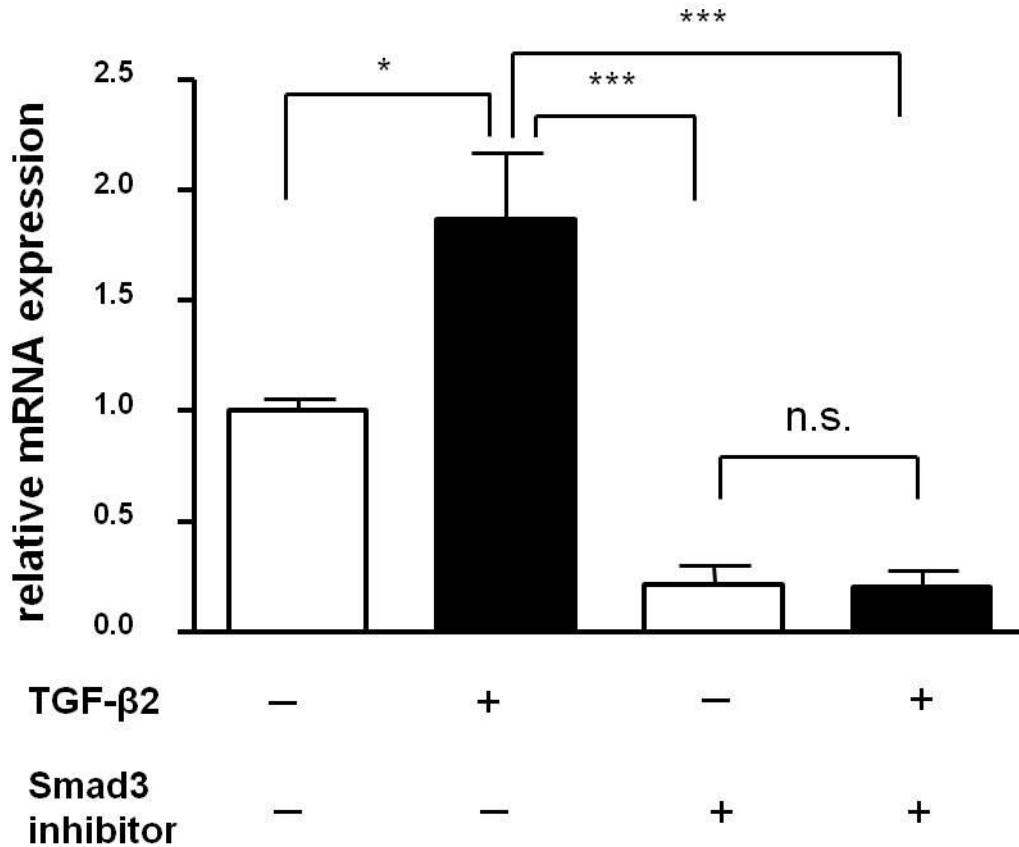


Figure 62 Inhibition of SMAD3 strongly reduces the baseline expression of VE-cadherin and abolished its induction by TGF-β2

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) containing 3ng/ml TGF-β2 or not. From day 1 the cells were treated with 5μM Smad3 inhibitor or DMSO as a control. Gene expression analysis was assessed by real-time PCR after 7 days. Results show the expression levels of VE-cadherin (VE-cad) in the presence or absence of TGF-β2 treatment and Smad3 inhibition. The statistical analysis used is 1way ANOVA for multiple comparisons. Data are presented as mean ±SEM of 3 individual experiments. * $P < 0.05$ vs. iPSCs differentiated for 7 days on collagen IV and in DM and *** $P < 0.001$ vs. iPSCs differentiated for 7 days on collagen IV and in DM containing 3ng/ml TGF-β2.

These data demonstrated that the effect of TGF-β2 on iPSC differentiation is mediated by Smad3 and highlighted the importance of the TGF-β2 pathway in the differentiation of iPSC into ECs.

3.3.4.5 TGF- β 2 induces EC differentiation of iPSCs through the secretion of VEGF

In order to elucidate how TGF- β 2 induces iPSC differentiation into ECs, we hypothesized an indirect mechanism of VEGF secretion.

We performed ELISAs in order to analyze the secretion of VEGF after 7 days of treatment with TGF- β 2. At this point the cells were serum-deprived overnight, and then the supernatants were concentrated and loaded into an ELISA microplate to analyze the secretion level of VEGF. Results showed a significant induction of 1.6 fold in the secretion of VEGF after TGF- β 2 treatment, as compared to untreated cells (Figure 63).

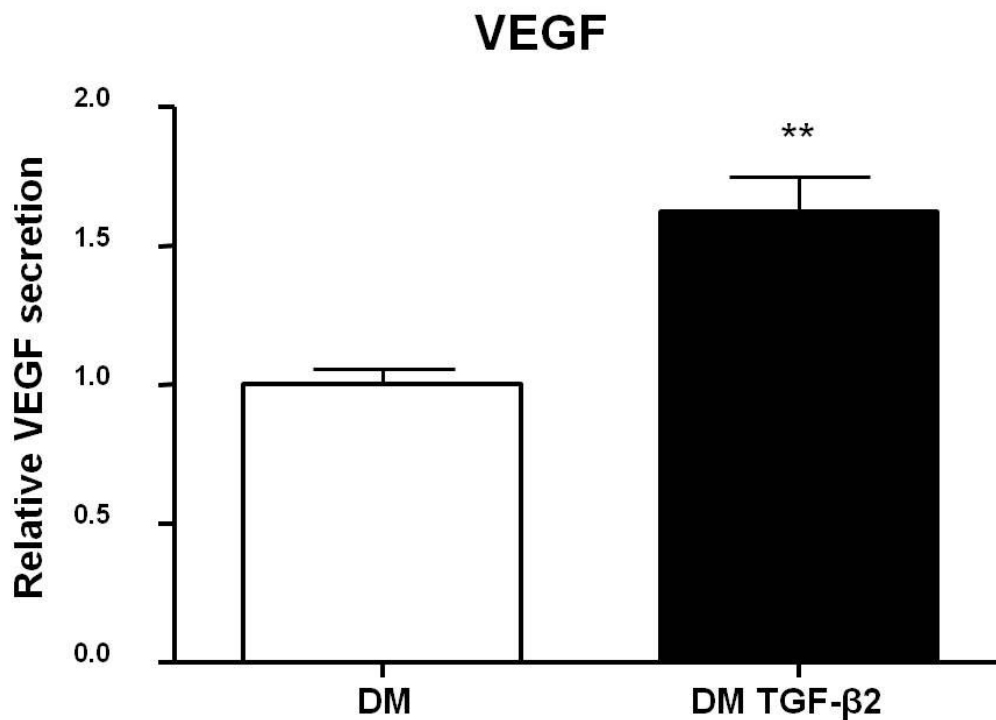


Figure 63 Analysis of VEGF secretion in iPSCs treated with TGF- β 2 for 7 days

iPSCs were seeded on collagen IV and cultured in differentiation medium (DM) containing 3ng/ml TGF- β 2. After 7 days, the medium was removed and refreshed with serum free alpha MEM overnight. Cell supernatants were then harvested and 7.7 fold-concentrated using centrifugal filter units. Samples of concentrated supernatants were used to perform ELISAs. Results show relative VEGF secretion after TGF- β 2 treatment, as compared to untreated cells in DM. The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. ** $P < 0.01$ vs. iPSCs differentiated for 7 days on collagen IV and in DM (DM).

These data indicate that TGF- β 2 promotes a specific and functional differentiation of iPSCs into ECs through induction of VEGF secretion. These results are in accordance with our previous data showing that miR-21 inhibition decreased VEGF gene expression, thus supporting the hypothesis of a link between miR-21, TGF- β 2 and VEGF pathways.

To further confirm that VEGF secretion is required for TGF- β 2 induced iPSC differentiation into ECs, we treated iPSCs for 7 days with TGF- β 2 in the presence of 0.1 μ g/ml VEGF neutralizing antibody or IgG as a control and analyzed the expression of the EC markers.

Results of Western Blots showed that treatment with VEGF blocking antibody reduced the TGF- β 2-dependent increase of VE-cadherin, as compared to control cells (Figure 64).

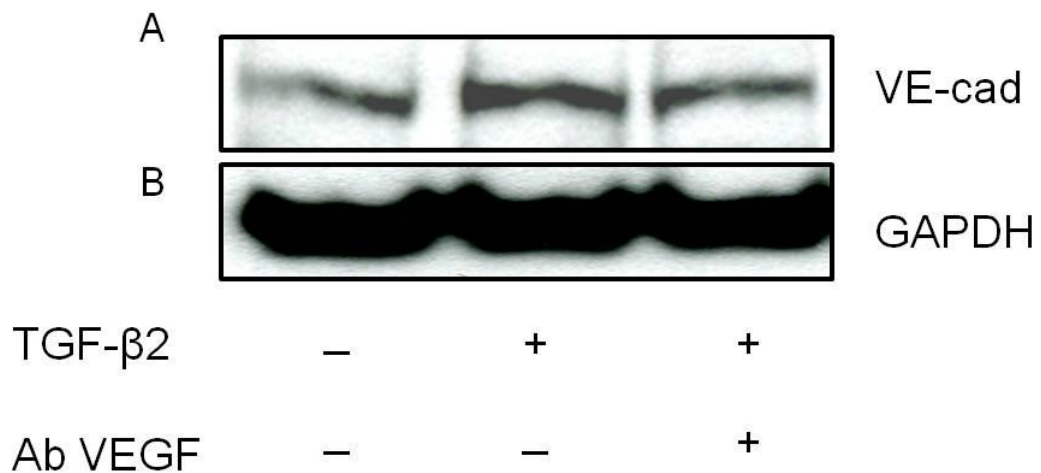


Figure 64 Protein expression analysis of VE-cadherin in iPSCs treated with TGF- β 2 and VEGF neutralizing antibody

iPSCs were seeded on collagen IV and cultured in differentiation medium containing 3ng/ml TGF- β 2 or not for 7 days. From day one either 0.1 μ g/ml VEGF neutralizing antibody or IgG was added to the cells as a control. Protein expression was assessed by Western blot analysis after 7 days. Results are shown for VE-cadherin (VE-cad, A). GAPDH was used as loading control (B).

These results show that VEGF secretion is necessary for the TGF- β 2 induced iPSC differentiation into ECs.

3.3.5 Conclusions

In this section of the work we presented a screening of the *in silico* predicted and other potential targets of miR-21.

MiR-21 has been found to induce the expression and the secretion of TGF- β 2. TGF- β 2 has been identified as a downstream, indirect target of miR-21.

Indeed, after neutralizing TGF- β 2 in our differentiation system, miR-21 induction of EC marker expression was abolished. This data indicates that the TGF- β 2 pathway is required for miR-21 inducing iPSC differentiation into ECs.

The expression of TGF- β 2 and its receptors TGF- β RIII and II has been shown to be strongly increased during VEGF induced iPSC differentiation into ECs, in particular after 5 and 7 days. These results demonstrate that TGF- β 2 plays a role in the iPSC differentiation process.

Furthermore, TGF- β 2 treatment of iPSCs for 7 days induced the expression of EC markers at a gene and protein expression level, whereas it did not affect SMC marker expression. This data indicates that TGF- β 2 regulation of iPSC differentiation acts specifically towards the EC lineage. In addition, treating iPSCs with TGF- β 2 for 7 days promoted the organization of the cells into tubular structures on Matrigel *in vitro*.

To further confirm the importance of the TGF- β 2 pathway in the EC differentiation of iPSCs, we inhibited SMAD3, a downstream effector of TGF- β 2 and we analyzed the EC marker expression. Inhibition of SMAD3 not only repressed the baseline level of VE-cadherin, but it also abolished the induction of this marker observed after TGF- β 2 treatment in the control cells.

Finally, ELISAs performed on iPSCs treated for 7 days with TGF- β 2 showed an induction of VEGF secretion from the cells. Furthermore, neutralization of VEGF secretion from the differentiation system repressed the TGF- β 2-dependent increase in EC marker expression. These data might indicate that TGF- β 2 indirectly regulates iPSC differentiation through stimulation of VEGF secretion.

3.4 IDENTIFICATION OF THE MOLECULAR TARGETS OF miRNA-21: MiR-21 TARGETS THE PTEN/AKT PATHWAY, WHICH REGULATES iPSC DIFFERENTIATION INTO ECs

3.4.1 MiR-21 targets the PTEN/AKT pathway

3.4.1.1 PTEN is a direct target of miR-21

After identifying TGF- β 2 as a necessary downstream functional target of miR-21 regulated iPSC differentiation into ECs, we aimed to identify the direct target of miR-21.

One of the *in silico* predicted targets for miR-21 is PTEN. Recent studies indicated that miR-21 inhibited the tumor suppressor PTEN by binding to its 3' UTR (Meng, Henson et al. 2007).

MiR-21 has recently been shown to induce cell proliferation, migration and invasion by modulating tumor suppressor gene PTEN in human hepatocellular cancer (Meng and Henson, Gastroenterology 2007). Furthermore, inhibition of PTEN by miR-21 has been shown to play a role in inducing tumor angiogenesis through AKT and ERK activation and HIF-1 α expression (Liu L Z et al). However, the role of PTEN in miR-21 inducing endothelial cell differentiation remains to be elucidated.

In iPSCs pre-differentiated in the presence of VEGF, overexpression of miR-21 decreased the protein level of PTEN, as shown by Western Blot analysis (Figure 65, A). By contrast, inhibition of miR-21 expression increased PTEN protein level (Figure 65, B).

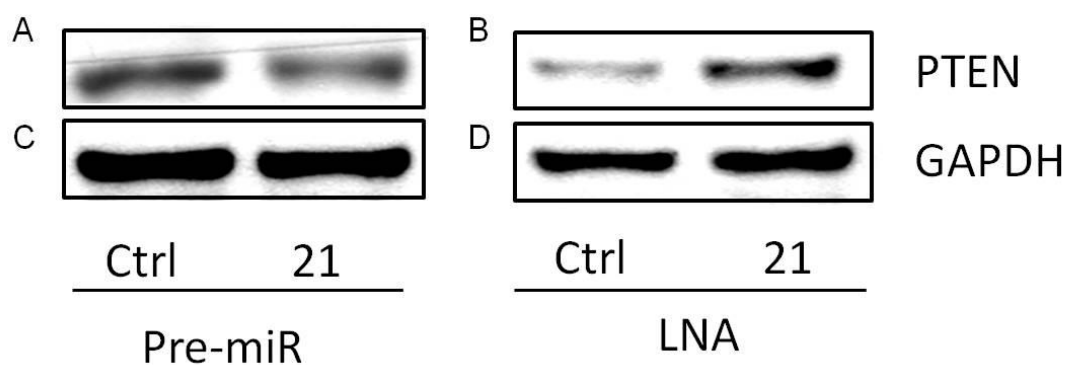


Figure 65 Protein expression analysis of PTEN in iPSCs differentiated with VEGF after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Protein expression level of PTEN (A and B) was assessed after 48h by Western Blot analysis. GAPDH was used as a loading control (C and D).

In order to confirm PTEN as a direct target of miR-21, we used a luciferase reporter vector encoding the complete 3'UTR of PTEN (WT PTEN 3'UTR) and a control vector containing mismatches in the predicted miR-21 binding site (Mut PTEN 3'UTR). Co-transfection of the WT PTEN 3'UTR plasmid and LNA-21 in iPSCs differentiating with VEGF resulted in an approximately 1.4 fold increase in luciferase activity (Figure 66, A). This data suggests that PTEN mRNA is a direct target of miR-21. Importantly, mutations in the sequence targeted by miR-21 in PTEN 3'UTR abolished the observed up-regulation of luciferase activity by miR-21 (Figure 66, B). This result confirmed PTEN as a direct target of miR-21 and validated its binding to the predicted binding site.

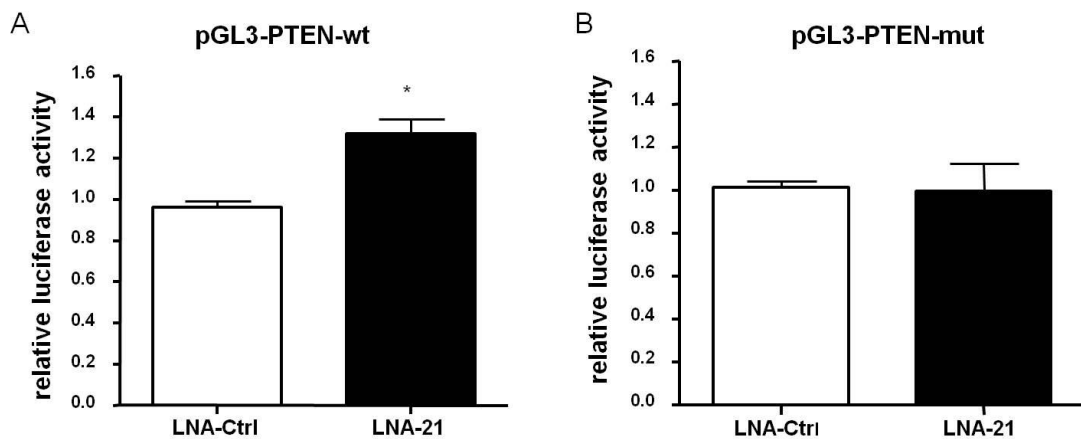


Figure 66 Luciferase reporter assay confirms PTEN as a direct target for miR-21

The wild type or mutated reporter plasmid pGL3 for PTEN 3'UTR (pGL3-PTEN-wt or pGL3-PTEN-mut) was co-transfected with miR-21 inhibitor (LNA-21) and its control (LNA-Ctrl) in iPSCs differentiated for 3 days on collagen IV, in DM containing VEGF. 48h after transfection the luciferase activity was quantified. Results of relative luciferase activity are shown for pGL3-PTEN-wt (A) and pGL3-PTEN-mut (B). Renilla luciferase activity was used as a normalization control for transfection efficiency. The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. * $P < 0.05$ vs. LNA-Ctrl.

3.4.1.2 Phosphorylation of AKT is induced by miR-21

PTEN is the antagonist of PI3K, which removes the 39 phosphate of Phosphatidylinositol 3-phosphate (PIP3), resulting in inhibition of the AKT signalling pathway (Jiang and Liu 2008). The PI3K/AKT signalling pathway plays a crucial role in many intracellular cascade events including tumor angiogenesis and tumor growth (Xia, Meng et al. 2006). Importantly, as already mentioned, the PI3K/AKT pathway has been shown to drive shear- and VEGF-induced stem cell differentiation into ECs (Zeng, Xiao et al. 2006).

AKT (also known as protein kinase B), together with extracellular-signal regulated kinase (ERK), is one of the major signalling pathways regulating cell proliferation, survival and migration (Zhong, Chiles et al. 2000).

Overexpression of miR-21 increased the phosphorylation of AKT at the Serine 473 site (P-AKT), as compared to the negative control of precursor miRNA (Figure 67, A). On the other hand, inhibition of miR-21 reduced AKT phosphorylation, as compared to the negative control of an miRNA inhibitor (Figure 67, B). The total

level of AKT (AKT tot) was not significantly altered by miR-21 overexpression (Figure 67, C) or inhibition (Figure 67, D).

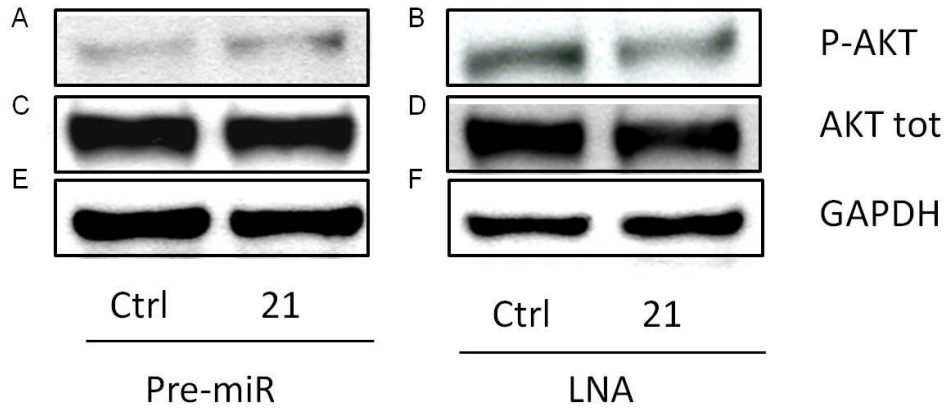


Figure 67 Protein expression analysis of P-AKT and total AKT in iPSCs differentiated with VEGF, after miR-21 overexpression and inhibition

iPSCs differentiated for 3 days on collagen IV and in differentiation medium containing VEGF were transfected with miR-21 precursor (Pre-21), inhibitor (LNA-21) and the negative controls of the miR-21 precursor (Pre-Ctrl) and inhibitor (LNA-Ctrl). Protein expression level was assessed after 48h by Western Blot analysis of phosphorylated AKT (P-AKT, A and B) and total AKT (AKT tot, C and D). GAPDH was used as a loading control for Pre-miR (E) and LNA (F).

Taken together, these data show that miR-21 inhibited PTEN expression, which in turn increased AKT phosphorylation.

3.4.2 The PTEN/AKT pathway regulates iPSC differentiation into ECs

3.4.2.1 PTEN inhibition leads to AKT activation in the VEGF-induced iPSC differentiation

We then aimed to assess whether PTEN inhibition is required for the activation of AKT during the VEGF-induced iPSC differentiation process.

PTEN shares the same active centre, the CX5R motif, with protein tyrosine phosphatases (PTPases) (Li, Ping et al. 2012); therefore, we inhibited PTEN using the chemical inhibitor bisperoxovanadium (bpV), a well-established PTPase inhibitor. BpV has been shown to target the phosphatidylinositol 3-phosphatase active site of PTEN (Schmid, Byrne et al. 2004); testing several different compounds *in vitro*, Schmid *et al.* showed that bpVs with polar N,O ligands had a strong preference towards the active site of PTEN, while bpVs with the neutral N,N ligands seemed to be more prone to target both PTPases and PTEN, although with distinct affinities. They concluded that all bpVs inhibit PTEN with 10- to 100-fold lower concentrations than PTPases (Bauters, Kumarswamy et al.). Although PTEN shows dual phosphatase activity, dephosphorylating both protein and lipid substrates, it has a higher specificity towards 3-phosphorylated phosphoinositides (PI) such as PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. In the above mentioned study of Schmid *et al.*, after treatment with bpVs, the loss of PTEN activity has been monitored with an increase of PtdIns (3,4,5)P₃ levels and therefore with a dose-dependent increase of Ser473 phosphorylation of AKT, a well characterised downstream target of PTEN-dependent signalling (Bauters, Kumarswamy et al.).

After 5 days of differentiation, iPSCs were starved for 4h and then treated with 5µM PTEN inhibitor for 30min, 2h and 24h and then harvested. Phosphorylation of AKT at the Ser-473 was increased after 30min and 24h of PTEN inhibition, as compared to the cells treated with DMSO as a control (Ctrl) (Figure 68, A). The protein level of total AKT (AKT tot) was not altered by PTEN inhibition (Figure 68, B).

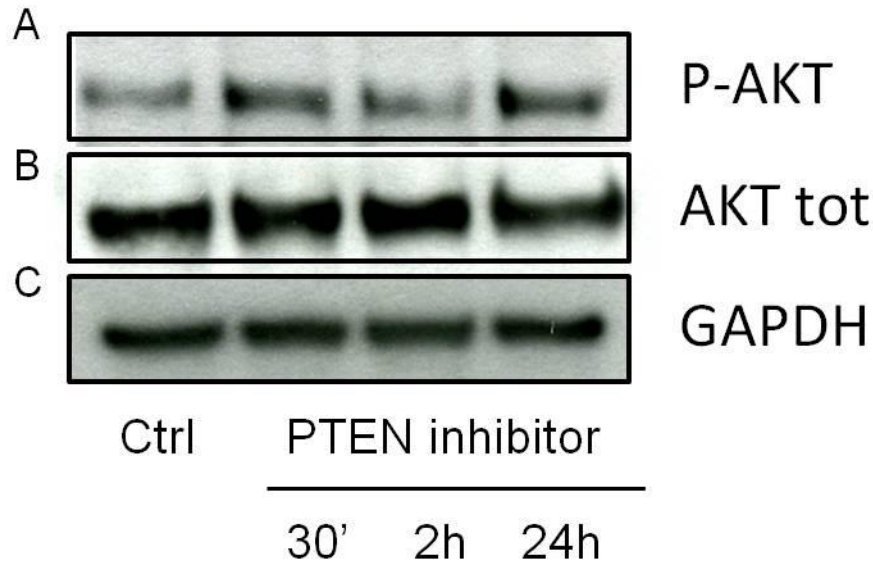


Figure 68 Protein expression analysis of P-AKT, total AKT and PTEN in iPSCs differentiated with VEGF, after PTEN chemical inhibition

iPSCs were seeded on Collagen IV, in DM containing VEGF 50ng/ml up to day 5 when the medium was removed and refreshed with serum free alpha MEM for 4h. 5µM PTEN inhibitor was then added for 24h, 2h and 30'(minutes), after which time the cells were harvested. Cells were treated with DMSO for 2h as a control (Ctrl). Protein expression of phospho-Ser-473 AKT (P-AKT, A) and total AKT (AKT tot, B) was analyzed by Western Blot. GAPDH was used as a loading control (C).

These data indicate that during the VEGF-induced iPSC differentiation process, inhibition of PTEN increases the AKT phosphorylation and its consequent activation.

3.4.2.2 PTEN downregulation increases the EC marker expression in differentiating iPSCs

After demonstrating that PTEN inhibition is required for AKT activation during the VEGF-induced iPSC differentiation, we aimed to clarify whether the PTEN/AKT pathway is essential for the EC differentiation of iPSCs.

Since we found that prolonged exposure of iPSCs to bpV strongly diminished cell survival and in order to establish a stable knockdown of PTEN, we used specific shRNAs to shutdown its expression. We then assessed the effect of the knockdown on EC marker expression.

iPSCs were differentiated for 3 days on collagen IV and in DM supplemented with 50ng/ml VEGF and then infected with lentiviruses expressing shRNA specific for PTEN (shPTEN) or non targeting shRNAs (shNT), as a control. 48h later, the cells were harvested to analyze the gene expression level of PTEN and of the EC markers.

Q-PCR results showed a significant and efficient repression of PTEN gene expression in iPSCs infected with shPTEN, as compared to cells infected with shNT (Figure 69).

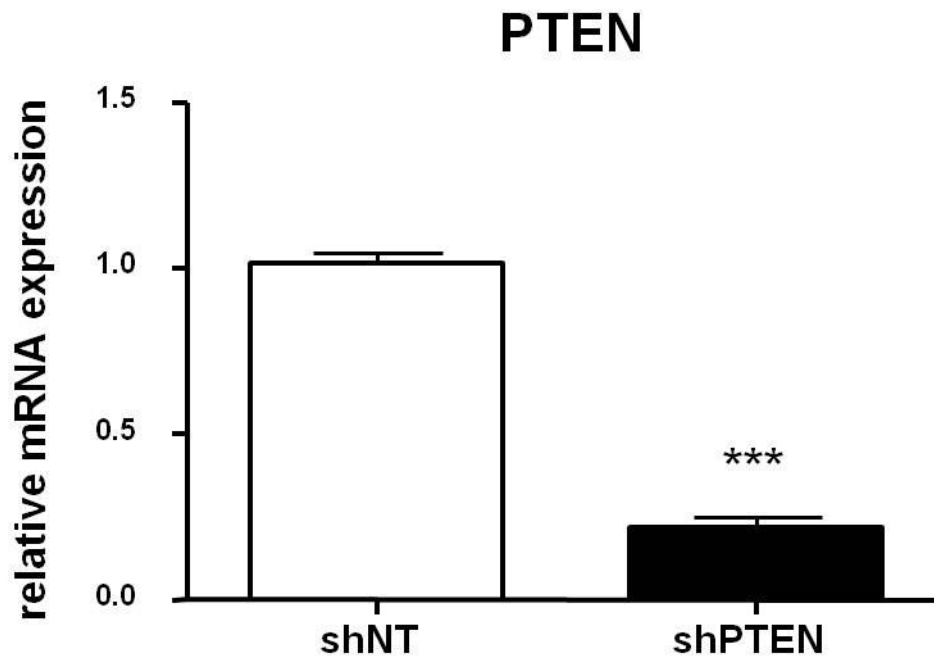


Figure 69 PTEN knockdown using lentiviral-mediated shRNA expression

After 3 days of differentiation on collagen IV and in DM containing 50ng/ml VEGF, iPSCs were infected with shRNA specific for PTEN (shPTEN) or non targeting shRNAs (shNT), as a control. The gene expression level of PTEN was assessed 48h later by real time-PCR. The statistical analysis used is Student's unpaired T-test. Data are presented as mean \pm SEM of 3 individual experiments. *** $P < 0.001$ vs. iPSCs infected with non targeting shRNA (shNT).

After confirming the efficiency of the shRNA knockdown of PTEN, we analyzed the expression of the EC markers after shPTEN infection. Real time-PCR analysis

showed a 1.5-fold increase in the expression of VE-cadherin (VE-cad, Figure 70, A) and Flk1 (Figure 70, B) after shPTEN infection, as compared to shNT infection.

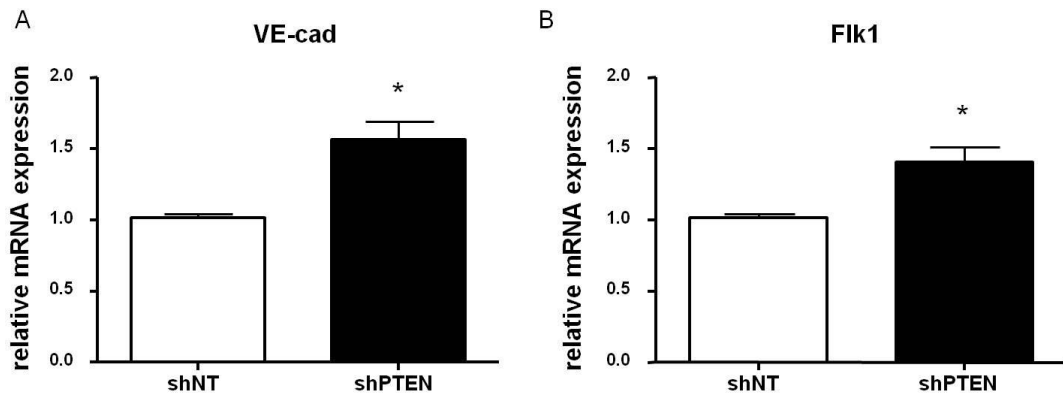


Figure 70 Knockdown of PTEN induces EC marker upregulation in differentiating iPSCs

iPSCs were differentiated for 3 days on collagen IV, in DM supplemented with 50ng/ml VEGF and then infected with shRNA specific for PTEN (shPTEN) or non targeting shRNAs (NT), as a control. Real time-PCR was performed 48h later to analyze the gene expression level of the EC markers VE-cad (A) and Flk1 (B). The statistical analysis used is Student's unpaired T-test. Data are shown as mean \pm SEM of 3 individual experiments. *** $P < 0.05$ vs. iPSCs infected with non targeting shRNA (shNT).

We can conclude that during the VEGF-induced iPSC differentiation, inhibition of PTEN with specific shRNAs is able to induce EC marker expression in differentiating iPSCs.

3.4.3 Conclusions

In summary, in the last part of the work we partially elucidated the signalling pathways through which miR-21 induces iPSC differentiation into ECs.

We firstly confirmed PTEN as a direct target of miR-21 and validated its binding to the predicted binding site.

Secondly, we showed that miR-21 regulates the AKT pathway by targeting PTEN and thus inducing AKT phosphorylation.

Finally, we assessed the requirement of the PTEN/AKT pathway in the VEGF-induced EC differentiation of iPSCs.

Indeed, during the differentiation of iPSCs, shutdown of PTEN with specific shRNAs increased EC marker expression, thereby indicating that PTEN inhibition is essential to induce iPSC differentiation towards EC lineage.

CHAPTER 4. DISCUSSION

4.1 ROLE OF VEGF AND SHEAR STRESS IN STEM CELL DIFFERENTIATION INTO ECs

The generation of an efficient, patient-specific vessel graft requires the identification of a suitable source of endothelial cells that can be derived from the patient in adequate number for clinical use. In this work we focused on the differentiation of iPSCs to ECs. Somatic cells from patients have the potential to be reprogrammed to iPSCs, which can be expanded in an undifferentiated state, or to be subjected to lineage specific differentiation in response to a stimulus.

It is very important to establish an efficient and reproducible protocol of differentiation. We started from a protocol recently published from our lab to induce ESC differentiation towards ECs based on the use of collagen IV, VEGF and shear stress (Zeng, Xiao et al. 2006). Aiming to adapt this protocol to iPSCs differentiation, we tested many combinations, using different VEGF concentrations and time points. We also tried a combination of VEGF and shear stress firstly on ESCs, and then on iPSCs. After plating iPSCs on collagen IV, in DM containing VEGF, we first observed a different morphology as compared to the undifferentiated iPSCs on gelatine: the differentiating cells adhered to the plate and became flat and elongated, while the undifferentiated iPSCs appeared to cluster in round three-dimensional colonies.

We then aimed to find an optimal dose of VEGF able to obtain the best response of ECs differentiation generated by iPSCs. Zeng *et al.* stimulated Sca-1⁺ vascular progenitor cells derived from ESC with medium containing 10ng/ml VEGF. On the other hand, in the work of Narazaki *et al.*, iPSC and ESC-derived Flk1⁺ progenitor cells were stimulated with 100ng/ml VEGF (Narazaki, Uosaki et al. 2008). In our study we aimed to test several concentrations ranging between these two extremes. Furthermore, we used we used undifferentiated iPSCs without pre-differentiating the cells or selecting for a specific population. The results of a dose-response curve experiment showed that concentrations of 20 and 50 ng/ml of VEGF were able to induce a robust upregulation of the EC markers VE-cadherin, Flk1 and vWF. Interestingly, immunostaining experiments of the differentiated cells revealed a homogeneous expression of the endothelial markers, showing a remarkably high

differentiation rate. Indeed, the cellular distribution of endothelial-specific proteins observed by immunostaining confirmed the presence of a clear pattern of VE-cadherin expression at the cell junctions and eNOS and vWF at the cytoplasm/membrane of the cells. Therefore we confirmed that we had devised a protocol that bypassed the need of selecting for a specific marker.

We also investigated the timeframe of differentiation by analysing the EC marker expression in cells treated with 20 and 50ng/ml of VEGF at different time points. Previously published protocols indicate that the peak of differentiation starts after 3-day culture of iPSC-derived Flk1⁺ cells using 100 ng/ml VEGF (Narazaki, Uosaki et al. 2008), or after 7-day culture of ESC-derived Sca-1⁺ cells using 10 ng/ml VEGF (Zeng, Xiao et al. 2006).

Our experiments showed that the highest upregulation of the EC markers (VE-cadherin, Flk1 and vWF) was after 7 days. However, using 50ng/ml of VEGF for 7 days, we observed an increased expression of the late EC marker VE-cad (20 fold), as compared to 20ng/ml VEGF (14 fold). Furthermore, VE-cad and vWF expression started at an early time, around day 3, as compared to the lower dose.

In summary, we established that the treatment of iPSCs with increasing concentrations of VEGF had an effect on endothelial differentiation up to 50ng/ml, while higher concentrations did not achieve a better result. Furthermore, 50ng/ml achieved a quicker pattern of differentiation as compared to 20ng/ml and was therefore chosen for further experiments. Importantly, compared to previously described differentiation protocols, our protocol shows a higher scale of EC marker induction, up to a 100 fold increase in Flk1 expression, thus indicating better differentiation efficiency than existing protocols. For instance, Narazaki *et al.* showed that, in Flk1⁺ progenitor cells, the effect of VEGF only led to a 20 fold increase in EC marker expression (Narazaki, Uosaki et al. 2008).

In conclusion, our protocol allows the direct and efficient EC differentiation from undifferentiated iPSCs in a relative short time and at a highest level, as compared to the previously published protocols.

In addition to phenotypical characterization, the functionality of the obtained endothelial cells was investigated using an *in vitro* angiogenesis assay. The

phenomenon that ECs rapidly form capillary-like structures *in vitro* when plated on a reconstituted basement membrane extracellular matrix, such as Matrigel, was demonstrated almost 20 years ago (Grant, Kinsella et al. 1995). This formation of capillary-like structures is specific to ECs and is used to assess EC functionality. Cells differentiated using our protocol showed the ability to organize into tube-like structures, confirming their endothelial nature.

In conclusion, using such an optimized differentiation protocol for iPSCs we characterized the differentiated cells and established that they were highly positive for main endothelial markers at gene and protein level and resemble endothelial cells in their morphology and their ability to organise into tubular structures *in vitro*.

A separate section of the work was dedicated to a set of experiments involving the use of shear stress. Shear stress has been shown by our group and others to play an important role in stem cell differentiation, in particular in the differentiation of the progenitor cell population Sca1⁺ (Zeng, Xiao et al. 2006). We first performed preliminary experiments on a better established population of progenitor cells, ckit⁺, which has been characterized in our laboratory and has been demonstrated to represent a novel vascular progenitor cell population.

In the study of Yamamoto *et al.*, ESC-derived Flk1⁺ progenitors exposed to shear stress (1.5 to 10 dynes/cm²) showed a significant induction in EC marker expression (Flk1, Flt-1, PECAM-1 and VE-cadherin) at both protein and mRNA levels, and had an increased tube formation capacity (Yamamoto, Sokabe et al. 2005).

Furthermore, data from our group showed that exposing Sca1⁺ progenitor cells to laminar shear stress (12 dynes/cm²) for 24h increased their proliferation and differentiation. Sheared Sca1⁺ cells displayed increased expression levels of Flk1, eNOS and VCAM-1, and showed improved tube-like structure formation on Matrigel (Xiao, Zeng et al. 2006). In the work of Xiao *et al.*, the predifferentiated cells were exposed to a laminar flow generated by a peristaltic pump; the shear stress obtained (12 dyne/cm²) was determined by the flow rate and the channel dimensions, and was comparable with the physiological range in human major arteries.

Additionally, in another work recently conducted by our group, ckit⁺/Sca⁻ progenitor cells were exposed to short-term shear stress using an orbital shaker, and showed an increased EC marker expression (in revision, Campagnolo *et al.*).

Following the protocol described in the study of Xiao *et al.*, we indeed differentiated ESCs on collagen IV and in DM for 4 days to obtain a mixed population of progenitor cells. Subsequently, we decided to isolate ckit⁺ progenitor cells, rather than Sca-1⁺, using immunomagnetic beads and to differentiate this progenitor population for a further 3 days in the same conditions. Shear stress was then applied, using an orbital shaker, for either 24h or 48h, after which cells were collected and endothelial marker expression analyzed. We could only observe some degree of upregulation of the EC markers after 24h of shear stress.

Interestingly, previous data from our group demonstrated that shear stress and VEGF share a similar activation pathway (the Flk1–PI3K–AKT–HDAC3–p53–p21 pathway) in the induction of EC differentiation of Sca1⁺ derived from ESCs (Xiao, Zeng et al. 2006). We decided to combine shear stress stimulation with VEGF treatment, in order to increase the rate of EC differentiation. Results showed an enhanced expression of EC markers such as CD31, VE-cad, Flk1 and eNOS after 24 or 48h (about 2 folds), as compared to each stimuli applied alone. However the results were often quite inconsistent and poorly reproducible.

After optimising the conditions for this differentiation protocol on ckit⁺ progenitor cells, we then applied the shear stress and VEGF treatment to iPSCs for 48h. Synergistic application of VEGF and shear stress to iPSCs has been never shown so far in the literature. We observed an approximately 3 fold induction in EC marker expression. Although it was possible to appreciate some effect of the synergistic action of shear stress and VEGF on the iPSC differentiation, we decided to focus only on the VEGF stimuli to study the mechanisms underlying iPSC differentiation. The shear stress application system, using an orbital shaker platform, presents too many limitations, which will be better explained in the following paragraph.

4.2 CHARACTERIZATION OF miRNA PROFILE DURING VEGF- AND SHEAR STRESS- INDUCED iPSC DIFFERENTIATION: MiRNA-21 REGULATES VEGF-INDUCED iPSC DIFFERENTIATION INTO ECs

The main aim in this thesis was to establish the molecular mechanisms driving the differentiation of iPSCs to ECs. . MiRNAs are small non-coding RNAs that have been shown the potential to regulate complex processes by targeting multiple proteins at the same time. For this reason we decided to investigate the miRNA signature of differentiated and undifferentiated iPSCs using a miRNA array.

Previous work on embryonic stem cells has shown that pluripotent cells and cells differentiated to vascular ECs express different pools of miRNAs. In particular, some of the miRNAs found to be highly overexpressed by ESC-derived EC were previously found to be involved in angiogenesis (Kane, Meloni et al. 2010).

To our knowledge, there are no studies investigating the miRNA profile during vascular differentiation of iPSCs though recent publications have characterized the miRNA profile of iPSCs during osteogenic differentiation (Okamoto, Matsumi et al. 2012). We performed a miRNA array on iPSCs differentiated for 3 days with VEGF and in undifferentiated iPSCs, in order to compare their miRNA profiles. We found a pool of differentially expressed miRNAs and selected some of the most consistently up or downregulated miRNAs to validate their expression over a 7 day-long differentiation toward the endothelial lineage.

We validated a total of 5 miRNAs, including miR-218, miR-133a, miR-29b, miR-20b and miR-21. Among the validated miRNAs, miR-218, showed an induction of up to 25 fold after 7 days of VEGF-induced differentiation. This miRNA has been mainly studied as a tumour suppressor in different types of cancer (Yamasaki, Seki et al. , Li, Ping et al. 2012). MiR-133a showed an upregulation of approximately 50 fold after 7 days of differentiation; miR-133a has been shown to be mainly involved in cardiac and muscle remodelling and is a known biomarker expressed after myocardial infarction (Bauters, Kumarswamy et al.). MiR-133a was also shown to

be involved in myocardial and liver fibrosis (Castoldi, di Gioia et al. 2012, Roderburg, Luedde et al. 2013) and to promote cardiogenic differentiation (Lee, Ham et al. 2013). Another miRNA showing a strong induction, of approximately 35 fold at day 5 of differentiation, was miR-29b. This miRNA plays a role in osteogenic differentiation (Trompeter, Dreesen et al. 2013) and acts as a tumour suppressor (Melo and Kalluri 2013), in particular repressing tumour angiogenesis, invasion and metastasis (Fang, Zhou et al. 2011). Additionally, miR-20b was found to be upregulated by 6 fold at day 3 of differentiation. Previous publications have shown that miR-20b has antiangiogenic properties (Wu, Yang et al. 2009) being downregulated in hypoxic conditions and decreasing the expression of VEGF in carcinoma cell lines (Hua, Lv et al. 2006). Finally miR-21 was found to be upregulated by approximately 6 and 8 fold after 5 and 7 days, respectively. MiR-21 has been shown to increase tumour progression in several types of tumours by targeting and inhibiting tumour suppression proteins such as phosphatase and tensin homolog (PTEN) (Meng, Henson et al. 2007). More relevant to our research, miR-21 has been previously shown to induce tumour angiogenesis through the AKT and ERK pathway activation, increasing the expression of VEGF and HIF-1 α (Liu, Li et al. 2011). Conversely, it has also been reported to exhibit antiangiogenic functions, by targeting RhoB in mature ECs (Sabatel, Malvaux et al. 2011). This double aspect of miR-21 in the context of angiogenesis led us to hypothesise a possible role in EC differentiation.

After validating the expression of the above mentioned miRNAs, we decided to select the two most interesting candidates, in order to study their involvement in the mechanisms underlying iPSC differentiation into ECs. Although miRNAs 133a, 29b and 218 were highly upregulated during iPSC differentiation, we decided to focus on miRNAs which had previously been shown to be related to angiogenic processes, such as miR-20b and miR-21. Despite some studies relating these two miRNAs with tumour angiogenesis and mature EC biology, no studies have investigated their involvement in the VEGF-induced differentiation of iPSCs.

Our initial aim was to establish a miRNA link between the differentiation pathway induced by VEGF and the pathway induced by shear stress. We therefore ran a parallel experiment of miRNA array to compare cells grown in static and shear stress conditions. Interestingly, miR-218, one of the most consistently overexpressed

miRNAs was also upregulated in the VEGF array, although at a lower level. We attempted to confirm this result by analysing its expression with real time PCR, but we could not validate it. We also attempted to validate other interesting miRNAs that emerged from the array, but the high variability and poor reproducibility of the conditions invalidated the results.

The shear stress model that we used is an orbital shaker platform; in this model, the shear stress cannot be uniformly applied to the cells across the plate and the entity of the shear is difficult to define mathematically. It has been shown that shear stress generated by an orbital shaker is low and oscillatory at the centre of the well, while at the periphery of the well it is directional and laminar as in atheroprotected physiological conditions (Chakraborty, Chakraborty et al. 2012). Therefore, in order to overcome this issue we systematically removed the cells in the centre of the well before collecting our samples. This expedient did not succeed in eliminating the problem. This may be due to variability within the area we defined as the periphery. In a study based on the analysis of computational fluid dynamics (CFD), time and location dependent wall shear stress (WSS) generated by an orbital shaker was determined. WSS was shown to be uniform (0–1 dyne/cm²) across the bottom of the dish, when low orbital speed (50 rpm) was applied; however, using higher orbital speeds (100 and 150 rpm), the WSS remained uniform near the centre, but varied significantly (0–9 dyne/cm²) near the side walls of the dish (Dardik, Chen et al. 2005). This variation can be thought of as similar to the pulsatile nature of shear stress experienced by native ECs.

We applied an orbital speed of 120 rpm, which corresponds to a peak shear of 8 dyne/cm² (Dardik, Chen et al. 2005), but this level of shear stress was not necessarily sensed in a homogeneous pattern by the cells across the surface. Differentiating iPSCs at day 3 do not form a uniform monolayer, in contrast to the appearance of mature ECs, and this might prevent the cells from transmitting shear sensing mechanical signals to adjacent cells.

These factors should be considered in the interpretation of the results of experiments performed with shear stress, which were not always consistent and reproducible. This may also explain why we were not able to validate the expression of the miRNAs found in the array performed with shear stress. For these reasons, we

decided to focus on the VEGF differentiation system, in order to study the involvement of miRNA candidates in the EC differentiation of iPSCs.

In conclusion, we performed the first miRNA array during iPSC differentiation towards an EC lineage. Our miRNA characterization may open future studies: for instance, the role of the other validated miRNAs. Study of these miRNAs was beyond the scope of this thesis but the role of these could be analyzed in future, thus elucidating new molecular pathways.

As mentioned previously, we chose to investigate the involvement of miR-20b and miR-21 in the VEGF-induced iPSC differentiation towards ECs. We first altered the levels of miR-20b and miR-21 in iPSCs differentiated with VEGF for three days. The efficiency of transfection with miRNA precursor (Pre20b/21) and inhibitor (LNA-20b/21) was significantly high, as showed by the relative increase and decrease in the mature miRNA expression. However, the gene expression of the EC markers was not affected by miR-20b overexpression and inhibition. On the other hand, when the cells were differentiated for 3 days in presence of VEGF and transfected with Pre-21, we could observe an induction of approximately 5 and 3 fold in the gene expression level of VE-cadherin and Flk1 respectively; however, miR-21 inhibition did not affect the gene expression of these markers. At a protein expression level, we could confirm an increase in EC markers after transfecting iPSCs with Pre-21; importantly, we could also observe a significant decrease in the protein expression of VE-cadherin and flk1 after inhibiting miR-21. The differences in the results of miR-21 inhibition observed at a gene and protein expression levels could be explained in two ways; firstly, the effect of the inhibitor may depend on the miRNA expression level at the time of transfection: if the miRNA is not expressed by the cells at a significant level, the LNA cannot efficiently bind to and inhibit the endogenous molecules of miRNA. Secondly, miRNA regulation is known to occur at a post-transcriptional level, and this may explain why we could see the miR-21 inhibition effect on the EC markers at a protein expression level only. In order to understand whether VEGF was required in this miR-21 regulation of EC marker expression, we cultured the cells in the absence of VEGF and we performed the same experiment. Importantly, miR-21 was not able to affect the expression of the EC markers at a gene expression level. These data indicate the requirement of VEGF stimulation for the miR-21 induced EC marker induction.

At this point, in order to verify that miR-21 regulation of iPSC differentiation occurred specifically towards the endothelial lineage, we analyzed the expression of SMC and cardiac markers, which, like ECs, are derived from the mesoderm and additionally, the expression of endoderm and ectoderm markers. Importantly, we confirmed that the expression of all these markers was not affected by miR-21 overexpression and inhibition. We therefore proved that miR-21 specifically regulates iPSC differentiation towards ECs and not towards any other cell lineage. The specificity of miR-21 regulation towards EC differentiation is a strong point in this work and will allow us to study the mechanisms involved in this process.

We then assessed the miR-21 regulation on iPSC differentiation into functional ECs, using an angiogenesis assay *in vitro*. Overexpression of miR-21 in iPSCs differentiated with VEGF for four days induced the formation of tube-like structures. Accordingly, miR-21 inhibition decreased the tube formation ability on Matigel; these results further confirmed the effect of miRNA inhibition at a post-transcriptional level.

We also aimed to study the effect of miR-21 regulation using an *in vivo* angiogenesis assay. The results revealed a significant induction in the density of CD31⁺ and VE-cadherin⁺ capillaries in the plugs seeded with miR-21 transfected cells, as compared to the control cells. Similarly to the results obtained *in vitro*, inhibition of miR-21 led to a decreased density of capillaries positive for the EC markers CD31 and VE-cad, as compared to LNA-Ctrl.

In vivo assays that mimic human angiogenesis has been previously reported, for instance in the work of Skovseth *et al.*, where native HUVECs are suspended in Matrigel, injected into immunodeficient mice, and develop into mature, functional vessels able to vascularize the Matrigel plug within 30 days (Skovseth, K uchler *et al.* 2007).

Moreover, in the study of Liu *et al.*, human prostate cancer cells transfected with Pre-21, LNA-21 and the relative negative controls were resuspended in serum-free medium, and mixed with Matrigel; aliquots of the different mixture were then applied onto the chicken chorioallantoic membrane of 9-day-old embryos to assess tumour angiogenesis. The number of branches of microvessels were increased by miR-21 overexpression and *viceversa* decreased by miR-21 inhibition, indicating a

pro-angiogenic function of miR-21 (Liu, Li et al. 2011). This study indeed shows a direct role of miR-21 in inducing angiogenesis, thus supporting our findings.

Our results of the *in vivo* angiogenesis assay suggested a potential use of miR-21 inhibitor in the development of new antiangiogenesis therapies; for instance this may be used for the treatment of diseases like cancer, where angiogenesis plays an important role.

Since in the *in vivo* angiogenesis assay we did not label the miR-21 transfected cells before the injection, we cannot exclude that the capillary structures observed in the Matrigel plugs were also composed by the host ECs, and not only by the miR-21-induced ECs. However, since the previously performed *in vitro* Matrigel assay showed that cells transfected with miR-21 were able to organize tube like structures, probably in the Matrigel plugs the same cells are able to promote the angiogenic process *in vivo*. There is a possibility that host cells are recruited by miR-21 transfected cells and integrate with them to form new capillaries, following the inflammation process induced by Matrigel plug injection. In both the cases, either if the effect on the capillary formation is due to the injected cells only or to their interaction with the host cells, *in vivo* angiogenesis is promoted by miR-21 transfected cells.

In conclusion, we showed for the first time that miR-21 specifically regulates the VEGF-induced differentiation of iPSCs into functional ECs and that VEGF stimulation is required in this process. Interestingly, a link between miR-21 and VEGF expression has already been established in the work of Liu *et al.*, where miR-21 has been reported to induce tumour angiogenesis through AKT and ERK pathway activation, increasing the expression of VEGF and hypoxia-inducible factor 1-alpha (HIF-1 α) (Liu, Li et al. 2011). The possibility of a mutual regulation between the VEGF and miR-21 pathways will be examined in the following section (4.3).

4.3 IDENTIFICATION OF THE MOLECULAR TARGETS OF MiRNA-21: TGF- β 2 PATHWAY IS A DOWNSTREAM TARGET OF MiR-21 AND DRIVES iPSC DIFFERENTIATION INTO ECs

At this point we aimed to elucidate the molecular mechanisms that occur during miR-21 induced differentiation of iPSCs. We started by identifying the molecular targets of miR-21 in order to elucidate the signalling pathways involved in the differentiation process. This was a difficult process as miR-21 has been shown to target multiple genes, which have not yet been shown to have a role in stem cell differentiation towards the EC lineage.

In order to start screening the molecular targets of miR-21 we used an miRNA target database (miRGEN), based on the intersection of four different prediction algorithms. From the lists of *in silico* predicted targets, we mainly focused on genes that may be involved in the angiogenic process. Among those, we focused on genes known to regulate EC functions and vessel growth, such as TGF- β R2, regulator of ERK activation Sprouty 1 (SPRY1), RhoGTPase RhoB and Sox7, and genes encoding for regulators of cell migration such as vinculin (Vcl).

We also decided to include genes of the Wnt and the HIF1 α /VEGF signalling pathways, which are known regulators of EC differentiation during embryogenesis and tumour growth (Logan and Nusse 2004, Dejana 2010). Furthermore, miR-21 is known to regulate HIF1 α , and induce tumour angiogenesis, through stimulation of VEGF gene expression (Kong, Kong et al. 2012). Interestingly, we found that transfection with miR-21 inhibitor was able to reduce VEGF gene expression. These results highlighted the connection between miR-21 and VEGF by showing that while miR-21 is regulated during VEGF-dependent differentiation, it is also important in the regulation of VEGF, since cells lacking miR-21 expressed a reduced amount of VEGF mRNA.

Despite the lack of variation in the other genes identified through miRGEN, we decided to expand our investigation stemming from the two predicted members of the TGF- β family, TGF- β R2 and transforming TGF- β I, to the rest of the components

of the pathway. TGF- β is a multifunctional cytokine which regulates proliferation, migration, differentiation and survival of many different cell types (Munger, Harpel et al. 1997). In mammals there are three known isoforms, TGF- β 1, TGF- β 2 and TGF- β 3, with distinct and shared functions. Deletions of different members of the TGF- β family, have been shown to cause vascular remodelling defect and absence of mural cell formation, leading to embryonic lethality (Pardali and Ten Dijke 2009), and mutations of other components of this family have been reported to lead to severe vascular disorders (Bertolino, Deckers et al. 2005, Lebrin, Deckers et al. 2005).

Interestingly, TGF- β 2 was found to be upregulated by miR-21 overexpression. Importantly, this observation was matched by increased levels of secreted protein, showing that miR-21 was not only able to influence TGF- β 2 at a transcriptional level, but also that this resulted in an increase of the functional protein. Furthermore, protein levels in the supernatant were reduced by the treatment with miR-21 inhibitor, despite no apparent change in RNA levels. This inhibitory effect on TGF- β 2 protein secretion only might be due to the fact that miRNAs, as stated earlier, are known to act at a posttranscriptional level.

To confirm TGF- β 2 as an essential downstream molecule mediating miR-21-inducing EC differentiation, Pre-21-transfected iPSCs were treated with TGF- β 2 neutralizing antibody. TGF- β 2 neutralization inhibited the miR-21-induced EC marker induction, as compared to IgG control, at both gene and protein expression level. This experiment was relevant to assess for the first time the existence of a link between miR-21 and TGF- β 2 during iPSCs differentiation, thus attesting that TGF- β 2 secretion is an essential step in the miR-21 induced EC differentiation.

We therefore aimed to analyze TGF- β 2 expression during the VEGF-induced iPSC differentiation. Our results showed a strong upregulation of TGF- β 2 gene expression, up to 200 fold after 7 days of differentiation, while the expression of TGF- β 1 was much lower, with a peak of 10 fold induction after 7 days. Interestingly and supporting our previous results, TGF- β 2 expression appeared to be concomitant with miR-21 expression, after 5 and 7 days of VEGF-induced iPSC differentiation; these data further support the connection between miR-21 and TGF- β 2 in the iPSC differentiation process. Next, we analyzed the expression of receptors of TGF- β

signalling during VEGF-induced iPSC differentiation. Consistently with the expression of the ligands, we found a strong upregulation of TGF- β RII and III. Although a trend was visible, TGF- β RI gene was upregulated to a lesser extent and not always consistently. TGF- β RIII was the most highly upregulated, reaching 200 fold induction after 7 days. This result may be explained by considering that TGF- β 2, which has a low affinity for TGF- β RII, requires the accessory receptor TGF- β RIII or β -glycan, for high-affinity interaction with the heteromeric-signalling receptor complex; binding of TGF- β 2 to TGF- β RII then leads to activation of TGF- β RI for the signal transduction (Goumans, Liu et al. 2009).

After indentifying TGF- β 2 as an important downstream effector of miR-21, and after showing its expression during VEGF-induced iPSC differentiation, we aimed to clarify the role of TGF- β 2 in this process. We therefore treated iPSCs for up to 7 days with TGF- β 2 to analyze the expression of EC markers. Results showed an upregulation of approximately 2.3 and 2 fold in the gene expression of VE-cadherin and Flk1 respectively. Importantly, after TGF- β 2 treatment, we could not see any effect on the SMC markers. In contrast, it has previously been demonstrated that TGF- β induces ESC differentiation into SMCs via Notch or SMAD2 and SMAD3 signalling (Yamashita, Takano et al. 2005, Kurpinski, Lam et al. 2010); additionally, in a neural crest stem cell line Chen *et al.* showed a TGF- β induction of SMC markers and phenotype (Chen and Lechleider 2004). Here, we report for the first time that TGF- β 2 treatment is able to specifically induce iPSC differentiation towards EC lineage, without affecting SMC marker expression.

From the ELISA results iPSCs secretion of TGF- β 2 appeared to be in the scale of pg/ml, indeed at a low concentration level; however, since iPSCs continuously secrete TGF- β 2, which probably exerts a paracrine action on neighbouring cells, that concentration was enough to be effective in iPSC differentiation. However, in order to study the response of the cells to TGF- β 2 treatment for a defined time of 7 days, we had to use a significantly higher dose of the cytokine; we chose 3ng/ml TGF- β 2 which has previously been shown to induce EMT in epithelial cells (Hatanaka, Koizumi et al. 2012).

Importantly, results of protein expression analysis confirmed the induction of the EC markers observed at a transcriptional level. Moreover, TGF- β 2 regulation of the

functional iPSC differentiation was attested by *in vitro* angiogenesis assay; TGF- β 2 treated cells showed a significantly increased tube formation capacity, as compared to untreated cells. Notably, so far there are no other studies reporting the role of TGF- β 2 on stem cell functional differentiation towards ECs.

It has previously been shown that the TGF- β family exerts its action on cells via specific intracellular transcription factors, such as RhoA/ROCK and SMAD. The ROCK pathway has been shown to play a negative role in stem cell differentiation, since its suppression promotes differentiation and expansion of ECs from ESC-derived Flk1⁺ cells (Yamasaki, Seki et al.). On the other hand, in ECs TGF- β has been shown to bind to TGF- β RI, and to induce phosphorylation of SMAD2/3, thereby inhibiting proliferation, tube formation, and migration of ECs (Goumans, Valdimarsdottir et al. 2003). However, so far there are no studies reporting the role of SMAD3 in stem cell differentiation into ECs. Indeed, in order to clarify the molecular mechanisms through which TGF- β 2 is able to drive iPSC differentiation into ECs, we decided to investigate the role of the TGF- β 2/SMAD3 pathway in this process. We therefore treated iPSCs differentiated for 7 days in the presence or absence of TGF- β 2, with a SMAD3 inhibitor. Q-PCR results showed that the SMAD3 inhibitor significantly abolished TGF- β 2 induction of EC markers, as compared to untreated cells. These data confirm the importance of the TGF- β 2/SMAD3 pathway in the iPSC differentiation process. Interestingly, our results also showed that inhibition of SMAD3 significantly reduced the baseline expression level of VE-cadherin, in the absence of TGF- β 2 stimulation; this result may be explained by considering that SMAD3 is at the centre of other molecular pathways and it is not only a TGF- β 2 downstream effector. For instance activin, a member of the TGF- β superfamily, has been shown to share some biological activities with TGF- β and to signal through SMAD proteins, such as SMAD3 (Massagué and Chen 2000). Moreover, it has been reported that SMAD2/3 is a downstream effector of Activin/Nodal signalling, which binds and directly controls the activity of the Nanog gene to maintain pluripotency in hESCs (Singh, Reynolds et al. 2012). Furthermore, studies conducted in EC-specific SMAD2/3 double KO (SMAD2/3KO) mice embryos, revealed hemorrhage leading to embryonic lethality around E12.5 and incomplete vascular maturation because of inadequate assembly of mural cells in the vasculature. In the vasculature of EC-SMAD2/3KO mice it is possible to observe

wide gaps between ECs and mural cells, because of the reduced expression of N-cadherin and sphingosine-1-phosphate receptor-1 (S1PR1) in ECs. Indeed the SMAD2/3 signalling has been reported to be essential in ECs to keep the vascular integrity through regulating N-cadherin, VE-cadherin, and S1PR1 expressions (Itoh, Itoh et al. 2012). This last study could also explain why the baseline level of VE-cadherin expression was affected after SMAD3 inhibition in our system.

In conclusion, our results demonstrated that SMAD3 mediates the effect of TGF- β 2 on iPSC differentiation and for the first time highlighted the importance of the TGF- β 2/SMAD3 pathway in the differentiation of iPSCs into ECs.

At this point, we aimed to elucidate the mechanisms behind the TGF- β 2 induction of iPSC differentiation into ECs. Our results showed so far that miR-21 inhibition reduces VEGF gene expression, and that VEGF stimulation is required in the miR-21 regulation of iPSC differentiation into ECs. We indeed hypothesized an indirect mechanism of VEGF secretion induced by TGF- β 2 treatment.

We therefore performed ELISAs in order to analyze the secretion of VEGF after 7 days of treatment with TGF- β 2. Our results showed a significant induction of 1.6 folds in the secretion of VEGF after TGF- β 2 treatment, as compared to untreated cells; this data indicates that TGF- β 2 promotes a specific and functional differentiation of iPSCs into ECs through induction of VEGF secretion. Other studies previously conducted in different cellular contexts showed that VEGF secretion can be induced by TGF- β 2, thus supporting our findings. For instance, VEGF secretion has been shown to play an important role in retinal and choroidal neovascularisation; induction of VEGF secretion by TGF- β 2 in human retinal pigment epithelium is mediated by MEK, p38, JNK, PI3K and NF-kappaB and by many other important signalling intermediates, such as PKC, PTK and ROS (Bian, Elnor et al. 2007). Moreover, in the work of Ma *et al.* it has been reported that TGF- β 2 stimulation increases VEGF mRNA expression and secretion; both VEGF and TGF- β 2 were in fact able to induce retinal pigment epithelium cell-mediated collagen gel contraction *in vitro* through upregulation of α -SMA expression (Ma, Zhang et al. 2012).

To further confirm our hypothesis that VEGF secretion is required for TGF- β 2 induced iPSC differentiation into ECs, we treated iPSCs for 7 days with TGF- β 2 in

the presence of VEGF neutralizing antibody or IgG as a control and we analyzed the expression of the EC markers. Western Blot results showed that treatment with VEGF blocking antibody reduced the TGF- β 2-dependent increase of VE-cadherin, as compared to control cells. These results indeed strongly support our data from the ELISAs and indicate for the first time that VEGF secretion is required for the TGF- β 2 induced iPSC differentiation into ECs. Indeed, our data so far demonstrated that TGF- β 2 expression and secretion were increased during the VEGF- and miR-21-induced iPSC differentiation and *vice versa* VEGF secretion was induced by TGF- β 2 treatment; after neutralizing VEGF secretion, there was a decrease in the TGF- β 2 induction of the EC markers. Indeed, we could conclude that there is an evident link between the miR-21, TGF- β 2 and VEGF pathways, which appears to be a mutual regulation.

4.4 IDENTIFICATION OF THE MOLECULAR TARGETS OF miRNA-21: MiR-21 TARGETS THE PTEN/AKT PATHWAY, WHICH REGULATES iPSC DIFFERENTIATION INTO ECs

In the last part of this work, after identifying TGF- β 2 as an essential downstream functional target of the miR-21 regulated iPSC differentiation into ECs, we focused on the research of the direct target of miR-21. We also aimed to elucidate the molecular pathway underlying the miR-21 regulation of iPSC differentiation.

One of the *in silico* predicted targets for miR-21 is PTEN. Recent studies indicated that miR-21 inhibited the tumour suppressor PTEN by binding to its 3' UTR (Meng, Henson et al. 2007). Modulation of the tumour suppressor gene PTEN by miR-21 in human hepatocellular cancer has recently been shown to induce cell proliferation, migration and invasion (Meng and Henson, Gastroenterology 2007), and inhibition of PTEN by miR-21 has been reported to induce tumour angiogenesis through AKT and ERK activation and HIF-1 α expression (Liu L Z et al). However, so far the role of PTEN in miR-21 induction of endothelial cell differentiation has not yet been elucidated. We aimed to clarify it.

We first confirmed PTEN as a direct target of miR-21 at a protein expression level. In iPSCs pre-differentiated in the presence of VEGF for 3 days, transfection with Pre-21 caused a significant reduction of PTEN protein expression, whereas transfection with LNA-21 led to a significant induction of PTEN.

To further confirm this result, we performed a luciferase assay, which has the consistency of a gene expression analysis, but is more specific since it assesses the binding of miRNA to mRNA, using wild type and mutated 3' UTR sequences. Co-transfection of the WT PTEN 3'UTR plasmid and LNA-21 in iPSCs differentiating with VEGF resulted in an approximately 1.4 fold increase in luciferase activity. Importantly, mutations in the sequence targeted by miR-21 in PTEN 3'UTR abolished the observed up-regulation of luciferase activity by miR-21. This result confirmed PTEN as a direct target of miR-21 and validated its specific binding to the predicted binding site.

At this point, since PTEN is known to be the antagonist of PI3K, which removes the 39 phosphate of PIP3, resulting in inhibition of the AKT signalling pathway (Jiang and Liu 2008), we aimed to assess whether PTEN inhibition is required for the activation of AKT during the VEGF-induced iPSC differentiation process. In order to do so, in iPSCs differentiated for 5 days with VEGF, we inhibited PTEN using the chemical inhibitor bisperoxovanadium (bpV) for 30min, 2h and 24h. BpV is a well-established PTPase inhibitor, which binds to the phosphatidylinositol 3-phosphatase active site of PTEN through competitive inhibition, with 10- to 100-fold lower concentrations than the other PTPase inhibitors (Bauters, Kumarswamy et al.). PTEN shows dual phosphatase activity, dephosphorylating both protein and lipid substrates, with a higher specificity towards 3-phosphorylated phosphoinositides (PI) such as PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3; indeed loss of PTEN activity can be monitored with an increase of PtdIns (3,4,5)P3 levels and therefore with a dose-dependent increase of Ser473 phosphorylation of AKT (Bauters, Kumarswamy et al.).

Our results showed that phosphorylation of AKT at the Ser-473 was increased after 30min and 24h of PTEN inhibition, as compared to the cells treated with DMSO as a control; the protein level of total AKT was instead not altered by PTEN inhibition. With this experiment we proved that during the VEGF-induced iPSC differentiation

process, inhibition of PTEN increases the AKT phosphorylation and its consequent activation.

Interestingly it has been shown that the PI3K/AKT signalling pathway plays a crucial role in many intracellular cascade events including tumour angiogenesis and tumour growth (Xia, Meng et al. 2006) and, in particular, the PI3K/AKT pathway has been reported to drive the shear- and VEGF-induced stem cell differentiation into ECs (Zeng, Xiao et al. 2006). We therefore decided to assess whether the PTEN/AKT pathway was essential in driving iPSC differentiation into EC. We indeed aimed to inhibit PTEN in iPSCs differentiating in presence of VEGF, in order to analyze the expression of the EC markers. Knockdown of genes using specific shRNAs is optimal for differentiation studies, since it has a long term and stable action on gene inhibition. Furthermore, we found that prolonged expositions of iPSCs to the PTEN chemical inhibitor bpV strongly diminished the cell survival, and it was indeed only possible to treat the cells with bpV for a short time. Therefore, in iPSCs differentiated with VEGF, in order to establish a stable knockdown of PTEN we used specific shRNAs to shutdown its expression and we then assessed the effect of the knockdown on EC marker expression. PTEN gene expression showed a significant repression in iPSCs infected with shPTEN, as compared to cells infected with shNT, confirming the efficiency of the shRNA knockdown of PTEN. Importantly, VE-cadherin and Flk1 showed a 1.5-fold increase in the gene expression after shPTEN infection, as compared to shNT infection.

Our results indeed highlight for the first time that PTEN inhibition is required to induce EC marker expression in iPSCs differentiated with VEGF.

To conclude our mechanistic studies, after linking the inhibition of PTEN to AKT activation and therefore to EC differentiation of iPSCs, we finally wanted to confirm it in the miR-21-induced iPSC differentiation system. We indeed showed that miR-21 overexpression increased the phosphorylation of AKT at the Serine 473 site, whereas inhibition of miR-21 reduced AKT phosphorylation, as compared to the relative negative controls. The total level of AKT was not significantly altered by miR-21 overexpression or inhibition. In conclusion, these data show that miR-21 inhibits PTEN expression, which in turn increases AKT phosphorylation.

All together our results show for the first time that miR-21 inhibits PTEN, therefore inducing AKT activation, and stimulates the secretion of TGF- β 2, in order to drive iPSC differentiation into ECs, through VEGF stimulation.

We established a new link between miR-21, TGF- β 2 and VEGF, which, as mentioned before, appears to act through a mutual regulation. Elucidation of the molecular pathway involved in the miR-21 induced iPSC differentiation into ECs might provide the basic information for stem cell therapy of vascular diseases, e.g. tissue engineering and endothelial repair in damaged vessels.

4.5 CONCLUSIONS AND FUTURE WORK

The work presented in this thesis was based on the hypothesis that specific miRNAs and their targets may be able to drive the VEGF-induced iPSC differentiation towards EC lineage.

Indeed, we have demonstrated that differentiation of iPSCs in presence of VEGF induces miR-21 expression and that miR-21 is able to induce TGF- β 2 expression and secretion, which in turn increases iPSC differentiation. Moreover TGF- β 2 is expressed during the VEGF-induced iPSC differentiation and iPSCs treatment with the cytokine induces functional endothelial differentiation. We have then proved that TGF- β 2 regulates EC differentiation of iPSCs through induction of VEGF secretion; moreover, miR-21 inhibition decreases VEGF expression in iPSCs. Indeed we have showed that the miR-21, TGF- β 2 and VEGF pathways are tightly linked by a mutual regulation during iPSC differentiation into ECs. Finally, we have partially elucidated an additional signalling pathway through which miR-21 induces iPSC differentiation by validating PTEN/AKT pathway as a direct target of miR-21 and demonstrating that the PTEN/AKT pathway is required in the VEGF-induced EC differentiation of iPSCs.

Evidences presented in this thesis suggest that endothelial lineage differentiation from iPSCs is regulated by miR-21/AKT and TGF- β 2 pathways.

Moreover, further elucidation of the molecular targets/pathways involved in the iPSC differentiation process regulated by VEGF, miR-21 and TGF- β 2, will be useful

to fulfil the therapeutic potential of these enhanced ECs. For instance, the downstream targets of TGF- β 2 need still to be elucidated in order to complete the mechanistic study of the miR-21/TGF- β 2-regulated differentiation of iPSC. Interestingly, in a study from Kato *et al.* it has been reported that TGF- β can increase FoxO3a phosphorylation and transcriptional inactivation via PI3K/AKT; this study suggests that AKT/FoxO pathway regulation by TGF- β may be a new mechanism in inducing mesangial cell survival and oxidant stress in diabetic kidney disease (Kato, Yuan et al. 2006). Even though this mechanism has been described in a different cell type, it might be worth investigating it in our differentiation, since the forkhead box (Fox) family of transcription factors plays an important role in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (Tuteja and Kaestner 2007).

Moreover, for possible future applications, iPSCs can be modified with the overexpression or knockdown of miR-21 and/or its targets, such as PTEN/AKT and TGF- β 2, to improve their endothelial differentiation potential in order to obtain more homogeneous EC populations.

Improvement in EC differentiation will be assessed in *in vivo* settings. In particular, enhanced ECs will be used for tissue engineering of decellularized vessels, in order to reduce the stenosis occurrence in vessel grafts. Furthermore, ECs obtained will be used for cell transplantation purposes to repair the damaged lumen in a model of wire-induced vessel de-endothelialization.

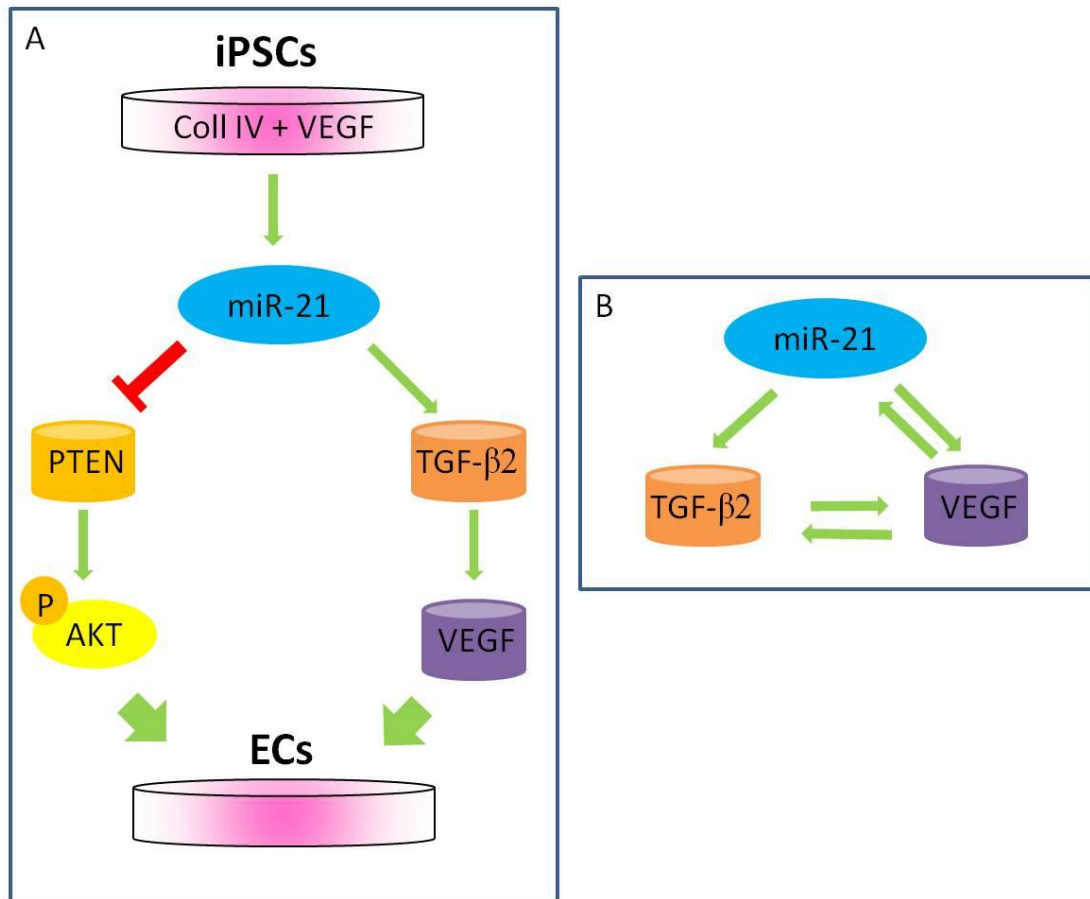


Figure 71 Endothelial lineage differentiation from iPSCs is regulated by miR-21/AKT and TGF-β2 pathways

A. Differentiation of iPSCs in presence of collagen IV and VEGF induces miR-21 expression. miR-21 induces iPSC differentiation into EC by inhibiting PTEN and therefore inducing AKT phosphorylation; moreover miR-21 is able to induce TGF-β2 expression and secretion, which in turn increases iPSC differentiation into ECs, through induction of VEGF secretion.

B. TGF-β2 expression is induced by VEGF during iPSC differentiation and iPSCs treatment with TGF-β2 induces functional endothelial differentiation through induction of VEGF secretion. Inhibition of miR-21, whose expression is induced by VEGF during iPSC differentiation, decreases VEGF expression in iPSCs. Moreover, miR-21 induces TGF-β2 expression and secretion. Indeed, miR-21, TGF-β2 and VEGF pathways are tightly linked by a mutual regulation during iPSC differentiation into ECs.

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CHAPTER 5. PUBLICATIONS AND AWARDS

JOURNAL ARTICLES

In peer Review

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Yanhua Hu, Qingbo Xu. **Endothelial lineage differentiation from iPS cells is regulated by miRNA-21/AKT and TGF- β 2 pathways** (Submitted manuscript-2013)

Campagnolo Paola, Tsung-Neng Tsai, John Paul Kirton, Andriana Margariti, **Elisabetta Di Bernardini**, MeiMei Wong, Yanhua Hu, and Qingbo Xu. **Isolation of a novel stem cell-derived vasculogenic population able to generate endothelial lineage through stimulation of Wnt/Klf4 pathway** (Submitted manuscript-2013)

In preparation

Paola Campagnolo, Xuechong Hong, Ioannis Smyrniyas, **Elisabetta Di Bernardini**, Yanhua Hu, Qingbo Xu. **Resveratrol reduces vessel-graft neointimal formation by inducing endothelial differentiation of resident progenitor cells**

MEETING ABSTRACTS

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Yanhua Hu, Qingbo Xu. **Endothelial lineage differentiation from iPS cells is regulated by miRNA-21/AKT and TGF- β 2 pathways**. Symposium on Vascular Signalling Mechanisms, Lund (Sweden) 13-15 June 2013 (Oral and Poster presentation)

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Yanhua Hu, Qingbo Xu. **Endothelial lineage differentiation from iPS cells is regulated by miRNA-21/AKT and TGF- β 2 pathways.** BAS/BSCR Annual Conference, ExCel London (UK) 3-5 June 2013 (Poster presentation)

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Qingbo Xu. **VEGF and shear stress synergistically induce stem cell differentiation into endothelial cell through microRNA dependent mechanism.** SmArt Symposium on Vascular Progenitors in biology and Medicine, Fribourg (Switzerland) 13-15 September 2012 (Oral and Poster presentation)

Campagnolo P, Kirton JP, Tsai TN, **Di Bernardini E**, Hu Y, Xu Q. **Isolation of a population of a c-Kit⁺ vascular progenitor cell population from ESC and iPS able to differentiate into endothelial and smooth muscle cells.** SmArt Symposium on Vascular Progenitors in biology and Medicine, Fribourg (Switzerland) 13-15 September 2012 (Oral and Poster presentation)

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Qingbo Xu. **VEGF and shear stress synergistically induce stem cell differentiation into endothelial cell through microRNA dependent mechanism.** King's BHF Centre of Research Excellence Postgraduate Symposium, King's College London (UK) 17 April 2012 (Oral presentation)

Elisabetta Di Bernardini, Paola Campagnolo, Andriana Margariti, Anna Zampetaki, Qingbo Xu. **VEGF and shear stress synergistically induce stem cell differentiation into endothelial cell regulated by microRNA.** SmArt meeting on Light Microscopy of small arterial vessels, Munich (Germany) 10-11 October 2011 (Poster presentation)

Campagnolo P, Kirton JP, Tsai TN, **Di Bernardini E**, Hu Y, Xu Q. **Isolation of a population of a c-Kit⁺ vascular progenitor cell population from ESC and iPS able to differentiate into endothelial and smooth muscle cells.** BAS/BSCR Meeting, Manchester (UK) 13-15 June 2011 (Poster presentation)

Elisabetta Di Bernardini, Paola Campagnolo, John Paul Kirton, Andriana Margariti, Qingbo Xu. **Shear stress-induced stem cell differentiation into endothelial cells.** SmArt meeting on Extracellular Matrix of the Vessel Wall, Muenster (Germany) 2-10 October 2010 (Oral presentation)

John Paul Kirton, Paola Campagnolo, **Elisabetta Di Bernardini**, Qingbo Xu. **Klf4 acts as a molecular switch during stem cell differentiation into endothelial and smooth muscle cells.** American Heart Association Meeting, Chicago (IL, USA) 2010 (Poster presentation)

CHAPTER 6. REFERENCES

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