Control of expression of glycosyltransferases involved in O-linked glycosylation in breast cancer.

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AIM

The aim of this PhD project was to investigate the control of expression of ST3Gal-I (and C2GnT1) in breast cancer and to determine the involvement of COX-2 in the control of their expression.

Abstract.

Aberrant glycosylation is a common phenotypic change observed in malignancy. Changes in mucin-type O-glycosylation in breast cancer can result in the expression of truncated core 1-based sialylated glycans rather than core 2-based glycans found in the normal gland. This is partly due to changes in the expression of glycosyltransferases. C2GnT1 expression, the glycosyltransferase that initiates branching in normal mammary epithelial cells, can be decreased in tumour cells while, in contrast, the expression of the sialyltransferase ST3Gal-I, which causes chain termination, increases in breast cancer.

When dendritic cells mature, prior to migration to lymph nodes, their O-glycosylation changes by decreasing C2GnT1 expression and increasing ST3Gal-I expression. This can be controlled by PGE2, the major product of COX-2. The project described in this thesis investigated the control by PGE2/COX-2 of ST3Gal-I and C2GnT1 expression in breast carcinomas. Importantly, COX-2 is normally only expressed during inflammation but is found to be upregulated by many carcinomas including those of the breast.

In the breast cancer line T47D, mRNA expression of ST3Gal-I was induced by PGE2, resulting in increased sialyltransferase activity. Induction of COX-2 in the MDA-MB-231 breast cancer cell line also resulted in increased expression of ST3Gal-I and increased sialylation of the ST3Gal-I substrate. This effect on sialylation could be reversed by the selective COX-2 inhibitor celecoxib. The use of siRNA to knock-down COX-2 and the over-expression of COX-2 in MDA-MD-231 confirmed the involvement of COX-2 in the upregulation of ST3Gal-I. Moreover, analysis of the expression of ST3Gal-I and COX-2 in 78 primary breast cancers showed a significant correlation between the two enzymes.

COX-2 expression in breast cancer has been associated with poor prognosis. Thus these results suggest the intriguing possibility that some of the malignant characteristics associated with COX-2 may be via the influence that COX-2 exerts on the glycosylation of tumour cells.

Abbreviations

Arg: Arginin

Asn: Asparagine

ALG: asparagine-linked glycosylation

BSA: Bovine serum albumin

CFG: Consortium of Functional Glycomics

CHO: Chinese hamster ovary cell line

CHO K1: Chinese hamster ovary cell line (parental line)

CK5/6: cytokeratins 5/6.

COX: cyclooxygenase

DCs: dendritic cells

DCIS: Ductal Carcinoma In Situ

α-DG: α-dystroglycan

DP: double positive-immature T cells

EDTA: Ethylenediaminetetraacetic acid

EGF: epidermal growth factor

ER α: estrogen receptor α

ER: endoplasmatic reticulum

Fuc: Fucose

GAGs: glycosaminoglycans

Gal: galactose

GalCer: Galactosylceramide

GalNac: N-acetylgalactosamine

GI:gastrointestinal tract

Glc:glucose

GlcCer: Glucosylceramide

GlcNAc: N-acetylglucosamine

GPI: glycophosphatidylinositol

HER-2: human epidermal growth factor receptor 2

HIV-1: human-immunodeficiency-virus-I

HMFG1: anti-MUC1 antibody

HMFG2: anti-MUC1 antibody

ICAM-1: intercellular adhesion molecule-1

IgG:immunoglobulins G

IL: interleukine

LacCer: lactosylceramide

LPS: Lipopolysaccharide

MAA: Maackia amurensis lectin

mAb:monoclonal antibody

Man: mannose

MCF7: Breast cancer cell line

MHC: Major histocompatability complex

MUC1: Mucin 1

Neu5Ac: Sialic acid

PF: paraformaldehyde

PGE2:Prostaglandin E2

Phe: Phenylalanine

ppGalNAcT-peptidylGalNActransferase

PNA: Peanut agglutinin lectin

PMTs-O-mannosyltransferases

PTM: posttranslational modifications

OGT: O-GlcNAc transferase

O-Ser/Thr: Serine/Threonine

OST: oligosaccharyltransferase

NSAIDs: non-steroidal anti-inflammatory drugs

Ser/Thr: Serine/Threonine

SLex :SialylLewis x

SM3: anti-MUC1 antibody

SP: single positive-mature T cells

T47D: Breast cancer cell line

TAA: Tumour associated antigen

TCR: T cell receptor

Th1: Differentiated CD4 cell associated with acute inflammation and a CTL response

Th17: Differentiated CD4 cell associated with chronic inflammation

Th2: Differentiated CD4 cell associated with a humoral response.

TNF-α: tumour necrosis factor-α,

TPA:12-O-tetradecanoylphorbol-13-acetate

TSP-1: Thrombospondin-1

TSS:transcription start site

Xyl: xylose

Val:Valine

VEGF-C: vascular endothelial growth factor

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Chapter 1. Thesis introduction

1.1 Introduction.

ST3Gal-I is a glycosyltransferase overexpressed in breast cancer that is involved in tumorigenesis (Burchell et al.1999, Picco et al. 2010). This chapter will firstly describe breast cancer and the role of glycosylation in this disease, in which the different classes of glycans will be discussed with a particular focus on O-glycans and ST3Gal-I structure and function.
COX-2 is the target of NSAIDs (anti-inflammatory non steroidal drugs) (Minghetti, 2004) and its mechanism and regulation in inflammation and cancer will be discussed. The final section will show glycosylation involvement in inflammation and possible interactions between ST3Gal-I and COX-2.

1.2 Breast Cancer.

1.2.1 The epidemiology of breast cancer.

Breast cancer is the most common form of malignant disease among women in Western countries, and it is second to lung cancer as the cause of cancer death. The frequency of this disease varies widely between countries and is about five times higher in North America than in Japan. Wide risk variations occur also within some countries. For example, within Italy the risk in the North is twice than the South and in Israel, Jews have a risk of breast cancer about four times higher than non-Jews. The increasing rates of disease in “low risk countries” (especially in Asia and in Africa) and the changing rates of disease for migrants indicate that environmental factors, such as diets with a high intake of meat, cheese and milk, lack of physical activity, exposure to mutagens (radiations) and obesity, have a strong influence on the aetiology of breast cancer. Other factors that influence the risk of breast cancer include age at menarche and menopause, age at first live birth, and other variables related to pregnancy and lactation. These risk factors suggest that hormones play an important role in the aetiology of breast cancer. This hypothesis can be demonstrated by studies on hormone levels and on the modest increase of breast cancer risk associated with long-term use of hormone replacement therapy (Beral, 2003).

According to a report published in the BMJ, breast cancer mortality has dropped over the past two decades in many European countries. This study was carried out on breast cancer mortality for all women and by age group (<50, 50-69, and ≥70 years) and it was found that England and Wales had the second greatest reduction in mortality. However in central European countries mortality has not declined but showed an increase during the same period (Autier et al. 2010).

1.2.2 Histopathology of breast cancer and classification.

The two major classifications of breast cancer are ductal and lobular carcinoma. Traditionally it was thought that ductal carcinoma starts in the ducts, that move milk from the lobules to the nipple, and lobular carcinoma starts in lobules, that produce milk (Pubmed Health- http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001911/).

Breast cancer can also be classified as carcinoma in situ and invasive cancer. Carcinoma in situ is defined as being confined within an intact basement membrane in which the lumen can become filled with cancer cells. Carcinoma in situ may become invasive, but the transition between these two stages is not well understood (Cichon et al. 2010). An extensive effort is being placed on defining markers that could identify carcinoma in situ that develops into invasive carcinoma.

In invasive ductal carcinoma the basement membrane is ruptured and the tumour cells invade the stroma. In advanced breast carcinoma, cancer cells invade the dense and fibrous stroma and the sorrounding area (see figure 1.1) (Kalluri et al. 2006). Malignant cells can metastasize initially through the lymph nodes or directly via the bloodstream. Metastatic breast cancer may affect almost any organ in the body, especially lungs, liver, bone, brain, and skin. There are about 20 histological examples of invasive breast carcinoma (including medullary type, mucinous carcinoma, papillary carcinoma, tubular carcinoma, adenoid cystic carcinoma, secretory carcinoma, inflammatory carcinoma), but invasive ductal (IDCs) and invasive lobular carcinomas (ILCs) account for 90% of all breast tumours (Bertucci et al. 2009).

Breast cancer is also classified by stage and grade. Staging refers to the severity of the cancer, basing this evaluation on three parameters: the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of distant metastasis (M). This system is called TNM, acronym for tumour, nodes and metastasis. A number is added to each letter to indicate the size or extent of the primary tumor and the extent of

cancer spread. Most tumors can be described as stage 0, stage I, stage II, stage III, or stage IV. This system has been accepted by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). For example, breast cancer classified as T3 N2 M0 refers to a large tumor that has spread outside the breast to nearby lymph nodes but not to other parts of the body.

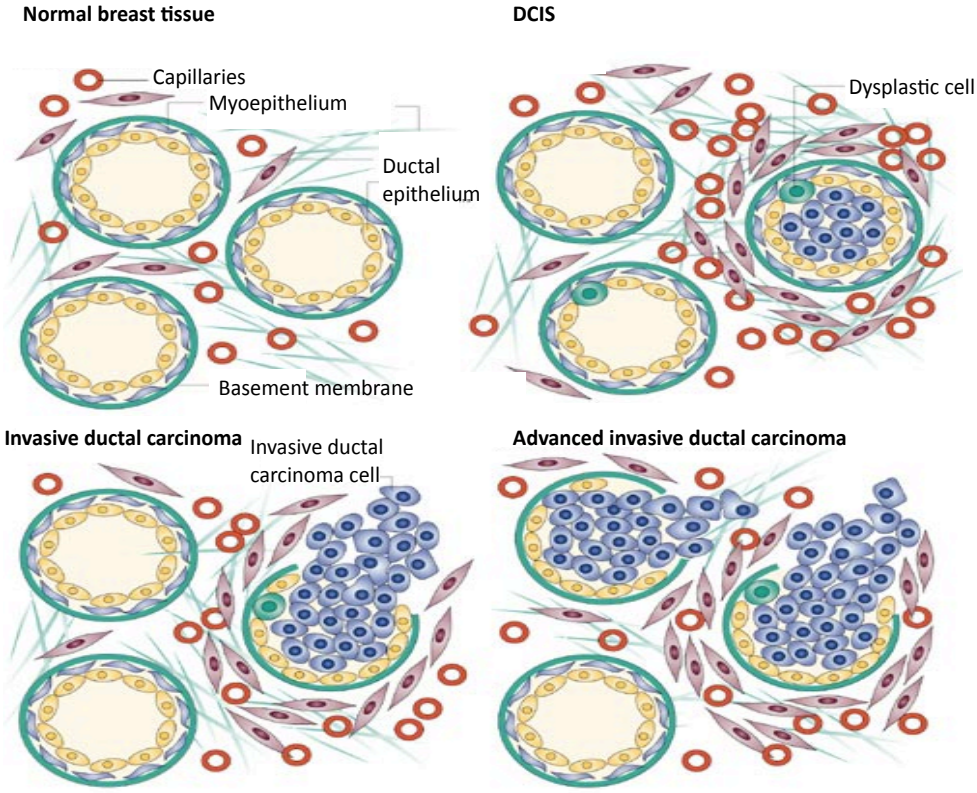


Figure 1.1 Different stages of mammary ductal carcinoma progression (Kalluri et al. 2006 Nature Reviews Cancer 6, 392-401).

Tumor grade is a system to classify cancer cells in terms of differentiation and how abnormal appearance of the cells. This evaluation can be done at the microscope by a pathologist. There are four degrees of severity: Grades 1 (well differentiated), 2 (moderately differentiated), 3, and 4 (undifferentiated tend to grow rapidly and spread faster). The Grading system is different for each type of cancer (American Joint Committee on Cancer, 2002).

Breast cancers can also be classified by their estrogen receptor status (ERα) as being ER negative or ER positive.

1.2.3 Molecular classification of breast cancer.

In 2001 Sorlie & Perou reported a study of gene expression on dissected human breast cancers. This study provided a new classification of tumours based on their gene expression which was also associated with prognosis. A total of 85 cDNA microarray experiments, representing 78 cancers, 3 fibroadenomas, and 4 normal breast tissues, were analyzed by hierarchical clustering using a 476 cDNA intrinsic clone set. From this paper and a subsequent paper from the same group (Sorlie et al. 2003), five main subgroups of breast cancer were identified (see figure 1.2).
These consist of:

1. Luminal A (ER+ve and PR+ve, HER-2-ve, low Ki67)
2. Lumimal B (ER+ve, some can be HER-2 positive, high Ki67)
3. HER-2 positive (ER-ve, PR-ve HER-2+ve)
4. Basal
5. Normal like

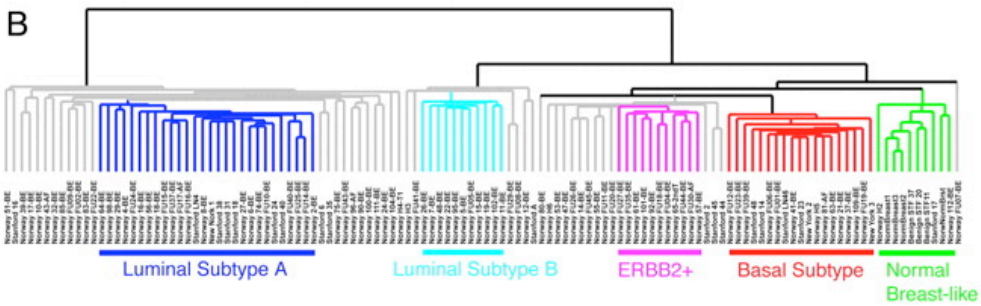


Figure 1.2 Gene expression patterns of 85 experimental samples analyzed by hierarchical clustering. The tumour specimens were divided into five subtypes based on differences in gene expression (dark blue: luminal subtype A, light blue: luminal subtype B, purple: ERBB2, red: basal-like, green: normal breast like) (Sorlie et al. 2003, Proc Natl Acad Sci USA;100(14):8418-23.)

The five molecular subsets of breast cancer defined by gene expression profiling are shown in figure 1.2 (Sorlie et al 2003).
Surrogate immunohistochemical markers such as the expression of estrogen receptor α (ER α), progesterone receptor (PR), HER2, EGFR and cytokeratins 5/6 (CK5/6), can be used to classify tumours (Blows et al. 2010, Ali et al. 2011). Using these markers, breast cancers have been classified into molecular subtypes and defined as luminal A (ER or PgR positive, HER2 negative, EGFR negative, cytokeratins 5/6 negative); luminal B (ER positive or PR positive, HER2 negative or positive, CK5/6 positive or

EGFR positive), basal phenotype (ER negative and PR negative, HER2 negative, CK5/6 positive or EGFR positive) or triple negative (ER negative, PgR negative, HER2 negative).

These subtypes have distinctive behavioural characteristics and responses to therapy. Thus, the molecular classifications of breast cancer can be used for prognostic and predictive evaluation. Statistically, patients with the ER positive phenotype receiving endocrine therapy have the best survival, although resistance to therapy develops in many patients. Triple negative breast cancer patients (ER negative, PR negative, HER-2 negative) have a poor prognosis and specific targeted therapy is not available. Triple negative tumours may or may not also be basal.

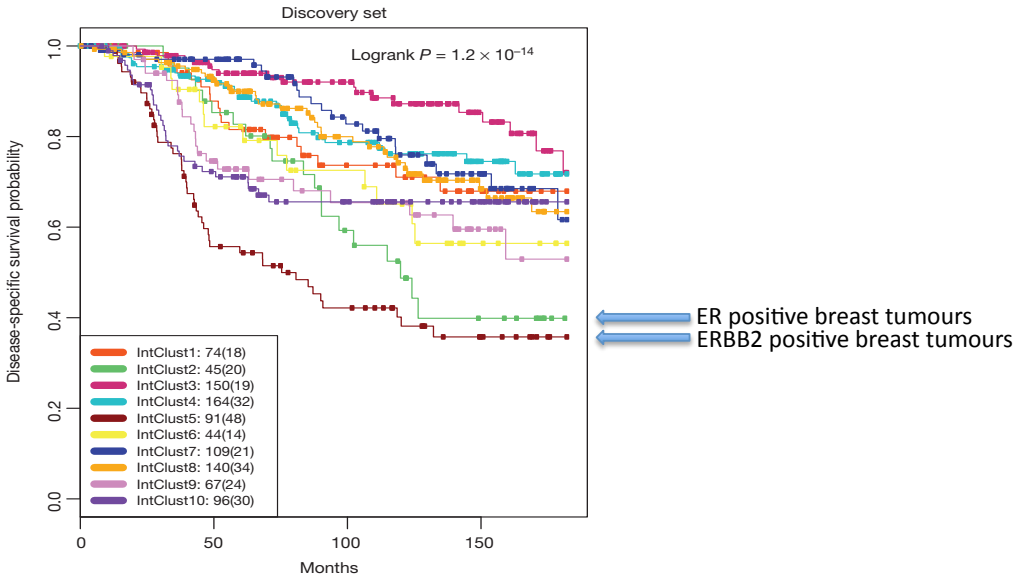


Figure 1.3 Kaplan–Meier plot of disease-specific survival (truncated at 15 years) for ten subgroups in the discovery cohort. Each cluster is indicated with a different colour. The number of samples at risk is indicated as well as the total number of deaths (in brackets). The two groups with worst disease specific survival probability are marked with an arrow (Curtis et al. 2012, *Nature*; **486**, 346–352).

Recently, within this broader classification of subtypes, other molecular features, which relate to prognosis, have been identified (Curtis et al. 2012). Using a large cohort of 2,000 breast tumours, ten subgroups were identified with distinct clinical outcomes (see figure 1.3). Importantly within this study a cohort of ER positive luminal epithelial cancers have been identified (on the basis of acquired somatic copy number aberrations and gene expression profiling) as having a very bad outcome (Curtis et al. 2012) unexpected for ER positive breast cancer patients. This group is represented by the green line (Intclust 2) in figure 1.3. From figure 1.3 Intclust 5 (brown line) seems to have a worse disease specific survival probability compared to intclust2 (green line). Intclust 5 is composed of ERBB2 enriched and luminal cases. These patients were

enrolled before the general availability of Trastuzumab and when treated with this drug the disease survival increased.

1.3 Glycobiology in breast cancer.

Breast cancer can be affected by changes in posttranslational modifications (PTM) of proteins that occur during neoplastic transformation. As well as being important for biomarker detection, understanding PTM changes that drive or contribute to oncogenic progression will allow development of agents that inhibit these specific PTMs (Krueger et al. 2006).

Glycosylation is one of the most common and important modification of proteins and lipids. It is defined as the reaction during which saccharide units are covalently attached to the target structures (proteins or lipids) and then, sequentially elongated and branched. Changes in the composition of glycans, added to glycoproteins and glycolipids, are common events in malignancy (Hakomori et al. 2002) and these changes can affect the course of the disease (see figure 1.4). The change in glycosylation can affect cell-cell interaction and signaling, adhesion properties (important for invasion of the tumour and metastasis) and, very importantly, tumour interaction with the immune system (Ohtsubo et al. 2006). These changes have been reported in numerous studies (Burchell et al. 2001; Hakomori et al. 2002; Ohtsubo et al. 2006;). The changes of glycosylation in malignancy can result in a) the appearance of novel structures, b) the persistence of incomplete or truncated structures, c) the accumulation of precursors, and d) loss of expression or excessive expression of certain structures. Changes in the first few branches in the normal biosynthesis can affect the relative amount of one class of structures, causing the dominance of another.

According to Potapenko et al., glycans are classified in four main groups that differ in their target attachment and the type of saccharides comprising the mature structure: N-linked glycans, O-linked glycans, glycosaminoglycans (GAGs) (including glycosphingolipids (GSLs) and glycophosphatidylinositol (GPI) anchors) and polysaccharides (glycans without lipid or protein backbone) (Potapenko et al. 2010). The N-linked, O-linked glycans and GAGs, are attached to polypeptide chains (but also to lipids). The N-linked glycans are considered to be a co-translational modification. Glycosphingolipids are considered the majority of glycolipids. Glycosylation pathways have been better defined in the last decades, partly due to the cloning of the enzymes involved (glycosyltransferases) (Furukawa et al. 2001; Marth, 1996). These enzymes

are type II transmembrane proteins with a short cytoplasmic tail, a transmembrane domain and a catalytic domain facing into the Golgi lumen. There are more than 100 glycosyltransferases involved in all processes and they are grouped into families, based on the type of sugar they transfer (galactosyltransferases, sialyltransferases, fucosyltransferases, N-acetylgalactosaminyltransferase) (Paulson et al. 1989). The number in the nomenclature of the glycosyltransferases represents the linkage, the named sugar represents the acceptor substrate sugar.

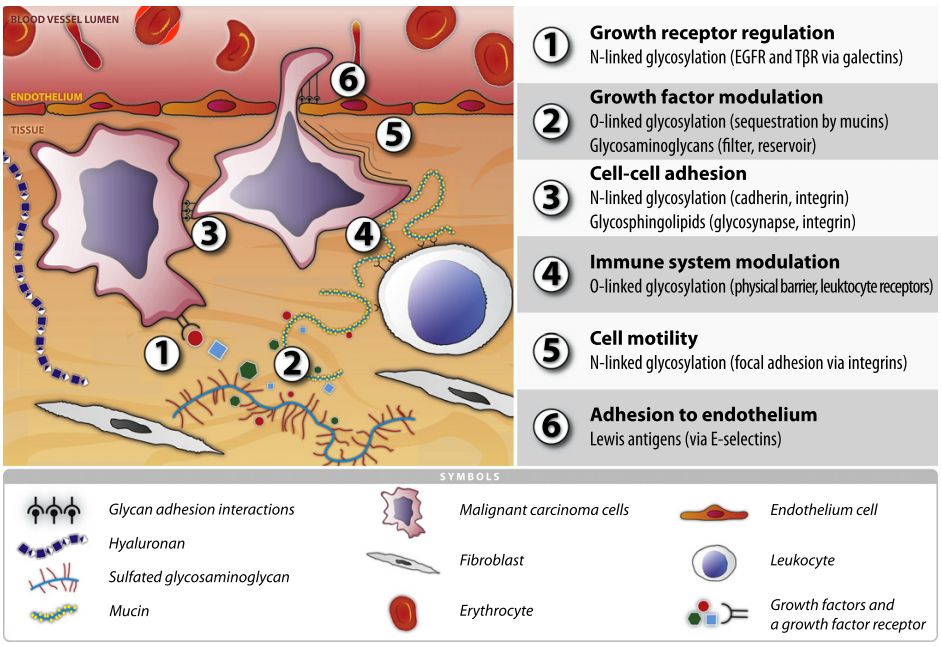


Figure 1.4 Glycosylation mechanism in carcinogenesis. This figure illustrates six important processes of glycosylation that influence the metastasis of carcinoma cells (Potapenko et al. 2010)

This thesis focuses on the change of expression of glycosyltransferases in O-linked glycosylation seen in breast cancer. The following sections will explain briefly glycosphingolipids, N-glycans and O-glycans in physiological conditions. Then mucin type O-linked glycosylation and its changes in cancer will be described in more detail. The Consortium for Functional Glycomics (CFG) has established a symbol system with coloured geometric shape, for each sugar, illustrated in figure 1.5. In the next figures these symbols will be used.

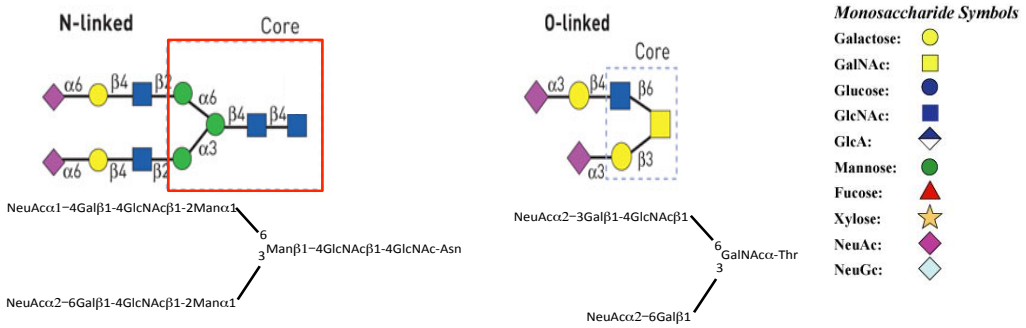


Figure 1.5 Monosaccharide symbols (Varki et al. 2009, Proteomics 2009, 9, 5398–5399) and examples of how the monosaccharides are assembled together in N- and O-linked glycosylation.

1.3.1 Glycolipids.

A glycolipid consists of one or more monosaccharide residues covalently linked to a hydrophobic moiety, such as an acylglycerol, a sphingoid or a prenyl phosphate. In mammals the majority of glycolipids are glycosphingolipids (GSLs), a large and widely varying family of amphipathic lipids, based on the ceramide N-acylsphingoid lipid moiety (Kolter et al. 2002). Glycosphingolipids are present in cellular membranes (typically in the plasma membrane) and they play a role in organizing role in the cell membrane, in cell–cell recognition, cell–matrix interactions, and cell surface receptor and messaging, in the association with certain cell membrane receptors (eg. the tyrosine phosphorylation of the EGF receptor is specifically down-regulated by adding the ganglioside GM3) (Varki et al. 2009).

Glycosphingolipid synthesis starts on the cytosolic face of the ER with the formation of ceramide (that consists of sphingosine-2-amino-4-octadecene-1,3-diol- and a fatty acid). Ceramide then crosses the endoplasmatic reticulum (ER) membrane and causes two possible reactions that lead to the formation of the core of all mammalian GSLs: Galactosylceramide (GalCer) and Glucosylceramide (GlcCer). The enzymes involved in these reactions are ceramide glucosyltransferases (Merrill et al. 2002). The GalCer undergoes few reactions (like sulphation that generates sulphatides) and its extention is limited. Since GalCer is expressed in specific cell types, like oligodendrocytes, Schwann cells, epithelial cells of renal tubules and the gastrointestinal tract, it has been used as a marker for oligodendrocyte differentiation (Satoh et al. 1996).

Table 1.1 Nomenclature for classification of glycosphingolipids based on Glc-Cer. The abbreviations and the partial structure with the sugar position is illustrated in the table (Merrill and Sandhoff, 2002).

Table 1
Nomenclature for classification of glycosphingolipids

Root name	Abbreviation	Structure
Ganglio	Gg	Galβ1–3GalNacβ1–4Galβ1–4Glcβ1–1′Cer
Lacto	Lc	Galβ1–3GlcNacβ1–3Galβ1–4Glcβ1–1′Cer
Neolacto	nLc	Galβ1–4GlcNacβ1–3Galβ1–4Glcβ1–1′Cer
Globo	Gb	GalNacβ1–3Galα1–4Galβ1–4Glcβ1–1′Cer
Isoglobo	iGb	GalNacβ1–3Galα1–3Galβ1–4Glcβ1–1′Cer

On the other hand, the next step for GlcCer is the formation of lactosylceramide (LacCer) by adding a β4-linked galactose residue. Unlike GalCer-derived GSLs, hundreds of structures can be generated from the GlcCer core including: 1) neolactoseries and lactoseries, that begin with the addition of a β1,3-GlcNAc or β1,4-GlcNAc respectively 2) globoseries that are distinguished by the addition of an α1,4-Gal; 3) gangliosides, glycolipids that are characterized by one (GM1, GM2, GM3), two (GD1a, GD1b, GD2, GD3), three (GT1b) or four (GQ1) sialic acid residues (Yu et al. 2011) (figure 1.6).

The class of gangliosides is the most common among glycosphingolipids. The name ganglioside was first applied by Ernst Klenk in 1942 to lipids isolated from ganglion cells of brain (Yamakawa et al.1996) and gangliosides are indeed mainly present in the central nervous system. All gangliosides (with an exception for GM4) are synthesized from LacCer and continue down through two pathways: the asialo pathway (also called the o-pathway) through the addition of a GalNAc residue, or the “ganglioside proper” pathway (a-, b-, and c- pathways as shown in figure 1.6) through the addition of one or more sialic acid residues (Maccioni et al. 1999). The addition of the sialic acids is performed by sialyltransferases. The first sialyltransferase that synthesizes the GM3 structure is the ST3GAL5, by addition of a α3-sialic acid to LacCer. The sialyltransferase ST8SIA1 can add one or two sialic acid to form GD3 and GT3. Further, an addition of a GalNAc to LacCer, GM3, GD3 and GT3 results in the formation of a GA2, GM2, GD2 or GT2 structures. The transferase catalyzing this step is coded by B4GALNT1 ((1) in figure 1.6). Then a Galactose is added by B3GALT4 creating GA1, GM1, GD1b and GT1c. The terminal galactose residues of GA1, GM1, GD1b and GT1c can be sialylated by transferases ST3GAL1/-2 (number 3 in figure 1.6) (resulting in

cisGM1, GD1a and GT1b structures) and ST6GALNAC6 (creating GD1a, GT1a and GQ1ba) in all but the c-series.

Other sialyltransferases are involved in the formation of cis-GD1 like ST6GALNAC3 and ST6GALNAC5, but they are less specific (Varki et al. 2009). ST6GALNAC5 has been associated with brain metastasis in breast cancer patients as the product of this sialyltransferase enhances the adhesion of cancer cells to brain endothelial cells and their passage through the blood–brain barrier (Bos et al. 2009).

Alterations of gangliosides structures have been found in different diseases like Alzheimer's disease, Guillain-Barre syndrome but also in aggressive angiogenesis to support cancer growth. This suggests that gangliosides are important targets for prevention and cure of some diseases (Yu et al. 2011).

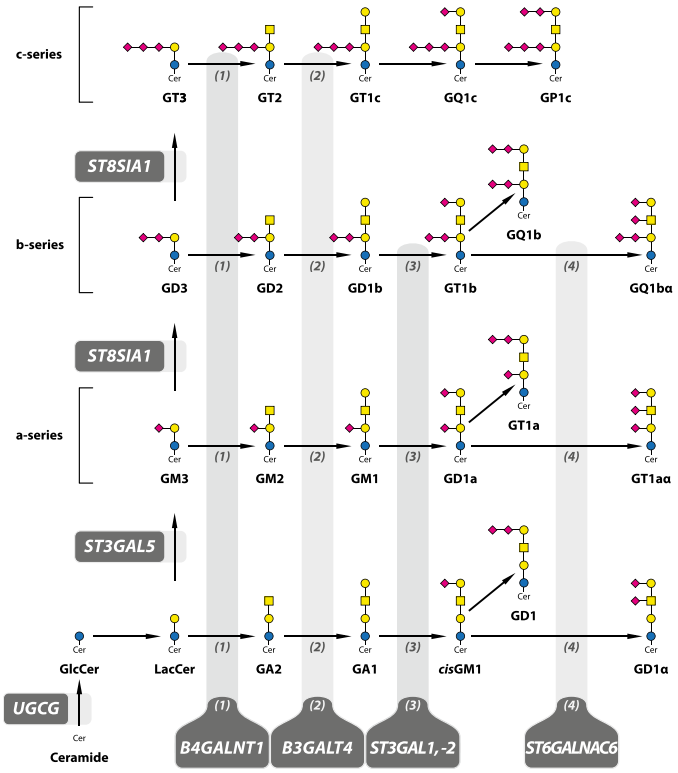


Figure 1.6 Ganglio-series glycosphingolipid synthesis pathway. The sialylltranferases involved in the pathway are indicated with numbers, because the same enzymes catalyze several steps of the pathway (modified from Varki et al. 2009, Essentials of Glycobiology-Potapenko et al. 2010).

1.3.2 N-Glycosylation.

N-glycans are a class of glycans covalently attached to asparagine with the consensus sequence of Asn-X-Ser/Thr by the N-glycosidic bond, GlcNAc β1-Asn. The N-glycans

are classified in three subfamilies that share the main core sugar sequence, as shown in figure 1.5, but differ in the chain elongation: (1) oligomannose type, in which only mannose residues are attached to the core; (2) complex type, in which “antennae”, initiated by N-acetylglucosaminyltransferases (GlcNAcTs), are attached to the core; and (3) hybrid type, in which, on one side, mannose residues are attached to the Manα1–6 arm of the core and, on the other side, one or two antennae are on the Manα1–3 arm (Varki et al. 2009).

N-Glycans are involved in the proper folding of newly synthesized polypeptides in the ER and in the maintenance of protein solubility and conformation. If some proteins are incorrectly glycosylated, they fail to fold properly and they are consigned to degradation in proteasomes (Varki et al. 2009).

The biosynthesis of N-glycans precursor is initiated by a family of transferases, encoded by the ALG (asparagine-linked glycosylation) gene family. The biosynthesis of N-glycans begins in the ER with the transfer of GlcNAc-P from UDP-GlcNAc by the enzyme GlcNAc-1-phosphotransferase to the lipid-like precursor dolichol phosphate (Dol-P). This reaction generates dolichol pyrophosphate N-acetylglucosamine (Dol-P-P-GlcNAc). A second N-acetylglucosamine is added and the enzyme responsible for transferring the second GlcNAc saccharide to the GlcNAc-P-P-Dol is ALG14. Consequentially, five mannose saccharides, four mannoses and three glucoses are added to create the mature N-glycan precursor in the cytosol. Each step is catalyzed by a different ALG (asparagine-linked glycosylation): for instance ALG3 is a transferase that attaches the second α1,3 mannose to the glycan, and ALG8 and ALG10 add two terminal glucose residues to the precursor, prior to its attachment to a polypeptide. The precursor translocates across the ER membrane bilayer, so that the glycan becomes exposed to the lumen of the ER. At this stage, the precursor is transferred to a protein. The complex that transfers the 14-sugar glycan is called oligosaccharyltransferase (OST). At this point a sequential removal of glucose and mannose by ER glucosidase and mannosidase trims the glycan to Man5GlcNAc2 that can be branched by the Mgat family of GlcNAc-transferases. Up to four branches can be added to this glycan by Mgat1, -2, -4 and 5. Cores of N-glycans may be modified with other sugar moieties along their passage through the Golgi. The main core modification of the N-glycan in mammals is the α1,6 fucosylation of the GlcNAc residue bound to asparagine (Varki et al. 2009).

N-glycosylation changes occur in cancer. One of the most common interesting changes in malignancy affects Mgat5, mannosyl (α-1,6-glycoprotein-β-1,6-N-acetyl-glucosaminyl-transferase). The growth of mammary tumours and metastases in mice,

deficient in Mgat5, crossed with polyomavirus middle T oncogene, were considerably less in the *Mgat5*$^{-/-}$ mice than in the controls expressing Mgat5 (Granovsky et al. 2000). The β1,6 branch, initiated by the Mgat5 transferase, may be elongated by a polylactosamine chain. Increase of expression of Mgat5 causes accumulation of polylactosamine chains (potentially recognized by galectins-see section 1.8) with consequential polyfucosylation and sialyl Lewis^{x} production (potentially recognized by the selectins-see section 1.8.2 selectins). Galectins are a family of lectins that bind β-galactosides and affect a variety of physiological and pathological processes, including cancer (Yang et al. 2008). The β1,6 branching by Mgat5 creates a different conformation from the normal outer antennae of N-glycans and can cause changes in the adhesive property and in the motility of a cancer cell (Saito et al. 1994). For instance, it was shown that exogenous galectin-3, added to Mgat5$^{+/+}$ epithelial mammary tumor cells, isolated from Mgat5-deficient mice crossed with mice expressing the PyMT oncogene cells, recruits conformationally active α5β1-integrin to fibrillar adhesions, increases F-actin turnover and activates FAK and PI3K (Lagana et al. 2006).

Epidermal growth factor (EGFR) and transforming growth factor-b (TbR) receptors present β1,6 branching by Mgat5, which, in concert with the galectin family does not allow the endocytosis of these receptors and thus facilitate their retention at the cell surface (Partridge et al., 2004). So, Mgat5 can control cytokine signaling involving these receptors, which are key factors in epithelial–mesenchymal transition (Partridge et al., 2004).

The change in expression of the glycosyltransferase GlcNAcT-III has been reported in certain tumors. GlcNAcT-III (coded by MGAT3 gene) is responsible for addition of a bisecting branch that competitively inhibits Mgat5 (Gu et al. 2004). Homotypic cadherin adhesion is influenced by Mgat3 glycosylation (Iijima et al. 2006). The glycosylated E-cadherin enhanced cell-cell aggregation and, in this way, it suppresses metastasis into melanoma cells (Yoshimura et al. 1996). Mgat3 transferase, in most cases, is correlated to inhibition of motility and reduction in malignancy (Bhaumik et al., 1998). The bisecting branch, created by Mgat5 and Mgat3, is involved in a more elaborate network of interactions that is still not fully understood.Potapenko et al. showed through expression data that the expression of other enzymes, like MGAT2, MGAT4A, B4GALT3 involved in the anabolic steps and MAN2A1 (involved in the catabolic steps), is changed in breast cancer.

1.3.3 O-glycosylation.

In contrast to the N-glycans, which are synthesized by en-bloc transfer of a high-mannose-type oligosaccharide to the asparagines residues within the polypeptide, O-glycans are linked to the hydroxyl groups of serines and threonines of a protein and the sugars are added individually and sequentially. Other than in O-GlcNAc and O-Man type O-linked glycosylation, the addition of each sugar is catalyzed by the action of specific resident Golgi glycosyltransferases (Burchell et al. 2001). The various type of O-glycosylation are listed in table 1.2.

Table 1.2 List of O-glycans. Sugar composition and examples of O-glycosylated proteins (http://themedicalbiochemistrypage.org/glycoproteins.php#oglycans).

O-Glycan Type	Structure of Linkage	Glycoprotein Type
O-linked GlcNAc	GlcNAc-β1–Ser/Thr	nuclear and cytoplasmic
O-linked mannose	Neu5Ac-α2–3Gal-β1–4GlcNAc-β1–2Man-α1–Ser/Thr	α-dystroglycan
O-linked fucose	Neu5Ac-α2–6Gal-β1–4GlcNAc-β1–3±Fuc-α1-Ser/Thr	EGF domains; this particular *O*-fucosylation is critical in the function of the receptor protein Notch
O-linked glucose	Xyl-α1–3Xyl-α1–3±Glc-β1–Ser	EGF domains
O-linked galactose	Glc-α1–2±Gal-β1-*O*–Lys	collagens
mucin-type	(R)-GalNAc-α1–Ser/Thr	plasma membrane and secreted

a) O-linked GlcNAc.

O-GlcNAcylation is an abundant posttranslational modification and consists of the incorporation of an O-linked β-N-acetylglucosamine (O-GlcNAc) linked to the hydroxyl group of a serine or threonine residue. O-GlcNAcylation is found on numerous cytoplasmic and nuclear proteins and in contrast to other O-glycosylation, the modification consists of a single O-linked-N-acetylglucosamine (Holt et al. 1986). The enzyme involved in the addition of O-GlcNAc is an O-GlcNAc transferase (OGT), while the enzyme that catalizes its removal is called D-N-acetylglucosaminidase (O-GlcNAcase). O-GlcNAc is dynamic and its half life is very short. It has been observed that O-GlcNAc–containing proteins are phosphoproteins (Wells et al. 2001) and mass spectrometry experiments by Hu et al. have revealed that O-GlcNAc sites can interact

with phosphorylation-mediated signalling (Hu et al. 2010). The difference is that phosphorylation is catalyzed by hundreds of distinct kinases (Venter et al. 2001), while there is only one transferase (OGT) and the beta-D-N-acetylglucosaminidase (OGA) encoded by single highly conserved genes (Shafi et al. 2000). Wells et al. supported the idea that O-GlcNAc transferase and the serine/threonine phosphatases PP1β and PP1γ, enzymes that remove phosphate from proteins, form stable and active complexes (Wells et al. 2001).

GlcNAcylation is not only found in normal biological function, but also in some diseases like neurologic disorders and diabetes. In diabetes many proteins involved in the phosphoinositide-3-kinase/AKT signaling cascade, are GlcNAcylated and the modification induces insulin resistance (Issad et al. 2010). A decrease in O-GlcNAcylation and consequent hyperphosphorylation of tau at the majority of the phosphorylation sites has been observed In patients with Alzheimer's disease. This causes tau to aggregate into the paired helical filaments that constitute the visible neurofibrillary tangles, characteristic of the disease. In an animal model of starved mice with Alzheimer’s disease, it was seen that the decrease of tau O-GlcNAcylation was probably induced by deficient brain glucose uptake metabolism (Liu et al. 2004).

GlcNAcylated proteins are also involved in cancer. Tumor-associated proteins, like c-Myc, are GlcNAcylated on Thr58, which competitively inhibits phosphorylation. This event suppresses the proteasome-mediated degradation of c-Myc (Chou et al. 1995). The p53 protein (tumor suppressor protein important for the prevention of cancer formation) is GlcNAcylated at Ser149 and this blocks ubiquitin-dependent proteolysis and stabilizes p53 (Yang et al. 2006). A report from Gu et al. showed that GlcNAcylation was significantly enhanced in metastatic lymph nodes of breast cancer compared to their corresponding primary tumor tissues. These results also suggest that GlcNAcylation might be a potential target for the diagnosis and therapy of breast cancer (Gu et al. 2010).

b) O-linked mannose.

The incorporation of mannose to Ser and/or Thr of a protein is known as O-mannosylat ion. O-α-Man was identified in yeast in the 1960s. Today it is known that most of the proteins in yeasts and fungi are substantially O- mannosylated (de Groot et al. 2005; Strahl-Bolsinger et al. 1999). Furthermore in mammals, O-mannosylation is found on proteins like α-dystroglycan (α-DG) from nerves and muscles (see figure 1.7) (Chiba et al. 1997; Sasaki et al. 1998), chondroitin sulfate proteoglycans (Finne et al.1979), and,

most recently, on neuron-specific protein tyrosine phosphatase, receptor type, zeta 1 (PTPRZ1, also known as RPTPβ) (Abbott et al. 2008). A GDP-Mannose is transferred to dolichol-phosphate forming Dol-P-Man (Sharma et al. 1974) by a family of O-mannosyltransferases (PMTs). An α-D-mannosidic linkage is formed through invertion of the anomeric configuration of the mannose through a putative flippase machinery. The PMT family is formed by three subfamilies PMT1, PMT2, and PMT4 (Girrbach et al. 2000). Coexpression of the POMT1 and POMT2 proteins and the formation of their complex are crucial for mannosyltransferase activity (Manya et al. 2004). Differently from most other O-glycosylation reactions (that take place exclusively in the Golgi apparatus), O-mannosylation occurs in the ER (Haselbeck et al. 1983). After mannosylation of the target protein, further extension of the O-linked mannose residue takes place in the Golgi apparatus. The vast majority of mammalian *O*-mannosyl glycans represent variations of the tetrasaccharide Neu5Acα2–3Galβ1–4GlcNAcβ1–2Manα1–Ser/Thr with different lengths (e.g., asialo) and variable fucose (α1,3-linked to GlcNAc) contents. The presence of mannose in the muscles and nerves and its role in α-dystroglycanopathies are not known. Tran et al. published a few months ago that the presence and specific location of O-Man can determine the site of O-GalNAc addition on α-DG, so the absence of proper O-mannosylation can alter the O-GalNAc addition. This plays a central role in the α-dystroglycanopathies (Tran et al. 2012).

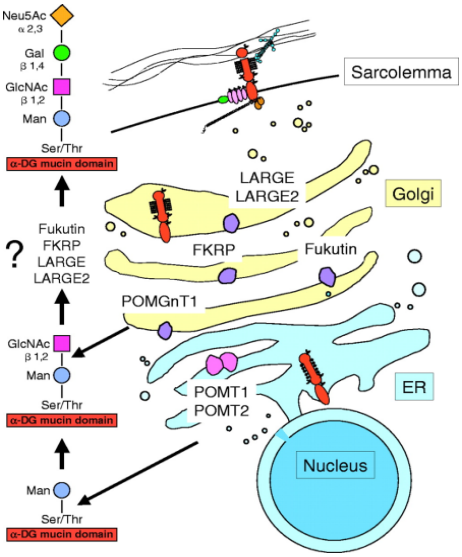


Figure 1.7 Glycosylation of α-dystroglycan. Sequential O-glycosylation pathway of α-dystroglycan within the cellular organelles.

The most-studied *O*-mannosylated protein in humans is α-DG, that is an essential component of the dystrophin-glycoprotein complex (DGC) in skeletal muscles. Most

defects in α-DG are associated with impaired *O*-mannosylation. Mutations in six glycosyltransferase genes, including the human POMT1, POMT2, and POMGnT1, have been identified to cause various α-dystroglycanopathies. Mutations in either POMT1 or POMT2 occur in about 20% of all cases of α-dystroglycanopathy patients. These diseases are called autosomal recessive muscular dystrophies or congenital muscular dystrophies (CMD), and have variable brain and ocular abnormalities (Martin et al. 2007;Yoshida et al. 2001).

c) O- linked fucose.

O-fucose (O-Fuc) is found on the EGF domains of different proteins and thrombospondin type 1 repeats (TSR). O-fucose is attached to the serine or threonine of the sequence -Cys-Xaa-Xaa-Gly-Gly-Thr/ Ser-Cys- on the EGF domain of human urokinase and in other clotting proteins, such as tissue plasminogen activator (t-PA) and clotting factor like factor VII (Bjoern et al.1991), factor XII (Harris et al.1993), and factor IX (Nishimura et al. 1992). The fucosyltransferase cDNA, responsible for the initiation of this modification, has been cloned and studied by Wang (Wang et al. 1996). In factor VII and IX, the O-fucose is extended with three additional sugars to form Neu5Acα2–6Galβ1–4GlcNAcβ1–3Fucα- and this particular tetrasaccharide is also present on many serum glycoproteins of N-glycans (Nishimura et al. 1992). The addition of the GlcNAc on the Fuc in β1-3 is catalized by a specific transferase. In contrast, the addition of sialic acid and galactose can be catalyzed by sialyltransferases and galactosyltransferases respectively, employed by other pathways.

The function of these particular O-linked glycoproteins are not totally understood. In the case of t-PA, the O-linked fucose is essential for the binding and degradation of t-PA by HepG2 cells (a hepatoma cell line). The glycosylation confers a particular function to glycoproteins in hepatic clearance. However, it has been seen that, mutating the O-Fuc attachment site, had no effect on its binding to the mannose receptor. O-fucose on urinary-type plasminogen activator (uPA) is important to activate the uPA receptor (Rabbani et al. 1992). One important contribution of O-Fuc is on the protein receptor Notch, involved in cell-fate determination.

Notch-1 has a series of 36 tandem EGF-like modules, and 12 of them have the consensus sequence for O-fucosylation. Addition of fucose residues to the hydroxyl group of serine and threonine occurs in the endoplasmic reticulum (ER) and is catalyzed by “protein O-fucosyltransferase” or Pofut1 (Stanley et al. 2007). The O-linked fucose can be elongated by sequential addition of three other sugar residues: N-

acetylglucosamine (GlcNAc), galactose (Gal) and sialic acid (SA).

Fringe is a glycosyltransferase that modifies Notch (Moloney et al. 2000). This enzyme, called Fringe in Drosophila and Lunatic, Manic and Radical Fringe in mammals, transfers GlcNAc on the added O-linked fucose (see figure 1.8) (Bruckner et al. 2000). Fringe mutations only alter some aspects of Notch signalling, in contrast the addition of O-fucose by POFUT1 is absolutely necessary for notch function (Yao et al. 2011), so POFUT1 mutants can result in loss of Notch signaling (Okajima et al. 2002).

O-fucosylation occurs also on TSP-1 (Thrombospondin-1) repeats. Specifically the O-linked disaccharide Glc-Fuc-O-Ser/Thr is found in the motif CSX(S/T)CG of TSP-1 (Hofsteenge et al. 2001). Apart from this protein the vast majority of fucosylation is found on EGF domains.

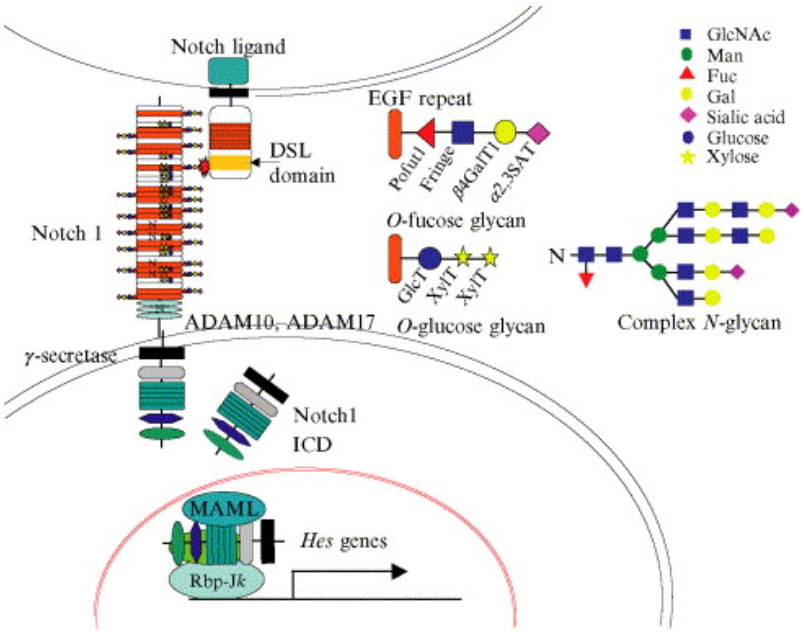


Figure 1.8 Canonical Notch signal transduction pathway and glycans of mammalian Notch1 on EGF repeats. The EGF repeats of both Notch receptors and ligands contain consensus sites for the addition of O-fucose and O-glucose glycans and AsnXSer/Thr consensus sequons for the addition of N-glycans. The glycosylation of Notch determine the interaction between receptor and ligand. In the canonical Notch signaling pathway the Notch ligands Delta-like or Jagged (in mammals) bind to the extracellular domain of Notch receptors (NECD) on apposing cells and two sequential proteolytic cleavages ensue. When ligand binds, the dimeric Notch1 receptor is cleaved by an ADAM protease. The released ECD is endocytosed by the ligand-expressing cell, and there it is cleaved by a complex with γ-secretase activity. The released NICD forms a complex with the transcriptional repressor CSL/Rbp-Jκ and activators (master-mind like; MAML) and induces the expression of target genes such as Hes genes (Lu et al. 2006).

d) O- linked glucose.

Like the O-Fuc, the O-Glc pathway is an unusual form of posttranslational modification. While O-fucosylation occurs on TSP-1 repeats and EGF-like domains, O-glucosylation and O-fucosylation together occur only on EGF domains. Several serum proteins (factor VII, factor IX, protein Z) contain O-Glc on their epidermal growth factor-like repeats. The consensus sequence on the EGF domain by O-Fuc and O-Glc is different and the presence of both glycan types on a single EGF domain has been shown only for factor VII (Bjoern et al.1991) and δ-like protein 1 (Krogh et al.1997). A trisaccharide form of O-glucose (Xyl-α1,3-Xyl-α1,3-Glc-β1-O-Ser) was found on bovine and human factor IX, human factor VII and protein Z (Nishimura et al.1989). Each of the xylose and the glucose residues is added sequentially by a separate glycosyltransferase: O-glucosyltransferase (Poglut) for transferring the glucose on proteins such as Notch, coagulation factors VII and IX, protein Z, Delta-like protein, and thrombospondin (Shao et al. 2002), β-D-glucoside α−1,3-D-xylosyltransferase is responsible for adding the α1,3-Xyl onto O-glucose (Omichi et al. 1997), the α−D-xyloside α1,3 xylosyltransferase for adding the α1,3-Xyl onto Xyl (Minamida et al.1996). A consensus sequence for the addition of glucose has been recognized on these proteins: a C^1-X-S-X-P-C^2, where C1 and C2 are the first and the second cystein of the EGF domain (Harris et al. 1993).

The Notch-1 protein also has O-glucosylation sites in human and in Drosophila. In Drosophila eighteen of the 36 EGF repeats on the Notch receptor contain the consensus O-glucosylation motif. Leonardi et al. showed that all the glycosylated sites, not just a single one, contributes to maintain robust signalling of Notch, especially at higher temperatures (Leonardi et al. 2011). The O-glucosyltransferase that regulates Notch signaling is called Rumi (Lee et al. 2010). Rumi enzymes can have also a significant protein O-xylosyltransferase activity and the donor substrate (UDP-glucose or UDP-Xyl) used depends on the amino acid sequences of the recipient EGF repeat (Leonardi et al. 2011).

In humoral defence the first step is the binding of C1q to the antibody IgG. Immunoglobulin G (IgG) antibodies are composed of two heavy chains (HC) and two light chains (LC) that form the Fab (fragment antigen binding) and Fc regions of the antibody. Within the Fc portion of the antibody, C1q binds to the CH_2 domain (Yasmeen et al. 1976). Duncan and Winter localized the binding sites of C1q to three side chains, Glu 318, Lys 320 and Lys 322 in the mouse IgG2b isotype (Duncan & Winter, 1998).

IgG is glycosylated in the Fc region at asparagine (Asn) residue 297 and the N- glycan

occuring has a biantennary complex structure with the following glycan sequence of GlcNAc2-Man3-GlcNAc2-Gal2 (Dwek et al. 1995). Significant reduction in Clq and Fc receptor binding was observed, when the quantity of galactose was decreased (Tsuchiya et al. 1989). Mimura, Church et al. reported that glycosylation in the Fc region gives CH2 domain stability, while stepwise truncation of the N-glycan sugar moieties causes decrease in thermal stability and biological function (Mimura,Church et al. 2000).

e) O-linked galactose.

O-galactosylation is a special type of O-linked glycosylation and consists of a galactose on hydroxylysines of few proteins like collagen, surfactant proteins (Larsen et al. 1999), complement factor I (Clq) and mannan-binding proteins (Iobst and Drickamer 1994) in the sequence -Gly-Xaa-Hyl-Gly- (Michaelsson et al. 1994).
Selected hydroxylysines of the collagen can be modified by adding galactose in the endoplasmic reticulum and the reaction is mediated by two β(1-O) galactosyltransferases: GLT25D1 and GLT25D2 proteins. The GLT25D1 gene is constitutively expressed in all human tissues, whereas the GLT25D2 gene is expressed only at low levels in the human nervous system. Aberrant modification of O-linked galactose on collagen can be involved in the etiology of connective tissue disorders (Schegg et al. 2009)
Complement Factor I (fI) is a protein of the complement system, part of the immune system. Galactose depleted IgG reduced Clq binding and Fc receptor binding, which imply an important biological function to the glyconutrient moiety of IgG (Tsuchiya et al. 1989).

1.4 Mucin type O-glycosylation.

Mucin type O-glycosylation is the main subject of this study and it will be described more extensively in the next paragraphs."Mucin-type" O-glycosylation is characterized by a GalNAc attached to the hydroxyl group of serine/threonine(Ser/Thr) (Hang et al. 2005). Mucin type O-linked glycosylation is the most common O-linked glycosylation and its name derives from the fact that this glycosylation is commonly found in many

secreted and membrane-bound mucins (Julenius et al. 2005). Mucin *O*-glycosylation involves the transfer of single sugars or monosaccarides from their nucleotide donors in an individual and sequential manner and can be characterized by the following steps:

a. Initiation by peptidylGalNActransferase (ppGalNAcT).
b. Elongation reactions to form core structures.
c. Termination reactions by the addition of sialic acid (SA), fucose (Fuc), galactose (Gal) or sulphate groups (Hanisch, 2001).

The cellular repertoir of glycosyltransferases with their distinct donor and acceptor sugar specificities, their sequential action and their localisation in subcompartments of the Golgi, all determine the cell specific O-glycosylation profile.

1.4.1 Initiation of mucin type O-glycosylation by peptidyl-GalNAc transferases.

Mucin-type O-glycosylation is initiated by a family of enzymes called peptidyl N-acetylgalactosaminyltransferases (ppGalNAcTs), that transfer GalNAc (derived from the donor substrate UDP-GalNAc) to serine and threonine residues on polypeptides (Clausen et al. 1996).

The first ppGalNAcT gene, to be cloned and characterised, was from bovine tissue (Hagen et al. 1993). This enzyme was found to have a human equivalent with a highly homologous nucleotide sequence that had 79 nucleotide substitutions, encoding only 6 amino acid differences (Clausen et al. 1996), and is a type II transmembrane protein with a small cytoplasmatic-oriented segment (Hennet et al. 1995).

Subsequent studies have revealed that 24 unique homologous polypeptide GalNAcT genes are present in humans (Hogenesch et al. 2001). In isozyme comparisons among human sequences, the catalytic domain of ppGalNacT2 and ppGalNacT3 exhibits 60% and 67% amino acid identity to the catalytic domain of ppGalNAcT1 (Paulson et al.1989).

Physicochemical parameters like volume, charge, hydrophobicity of the protein backbone around the glycosylation site can be crucial for the protein conformation and for the recognition by ppGalNAcTs (Kinarsky et al. 2003). This suggests that there are unique active site conformations for different ppGalNAcTs. ppGalNAcT1 and -T3 utilize only UDP-GalNAc, while GalNAc-T2 also utilizes UDP-Gal with one peptide acceptor substrate (Wandall et al. 1997). ppGalNAcT4, ppGalNAcT7 and ppGalNAcT10 seem to

require the prior addition of GalNAc to a synthetic peptide before they can catalyze sugar transfer to their substrate (Ten Hagen et al. 2003). Expression of ppGalNAcT1 RNA is ubiquitous in mammalian cells, whereas others are expressed in a cell and tissue specific manner (see table 1.3). This may reflect individual roles in cell type specific glycosylation of one or more glycoproteins (Ten Hagen et al. 2003).

Table 1.3 Some ppGaNTases and transcript expression in adult tissues (Ten Hagen et al. 2003).

ppGaNTase isoform[a]	**Strongest signal for transcript expression in adult tissues**[b]
ppGaNTase-T1	ubiquitous
ppGaNTase-T2	Liver; skeletal muscle
ppGaNTase-T3	Pancreas, testis
ppGaNTase-T4	sm. intestine; stomach
ppGaNTase-T6	Placenta; trachea
ppGaNTase-T7	Spinal cord; trachea
ppGaNTase-T9	Brain
ppGaNTase-T11	Kidney
ppGaNTase-T12	Stomach; sm. intestine[f]
ppGaNTase-T13	Brain

1.4.2 Core structures.

Following the addition of GalNAc to Ser or Thr, eight possible core structures can be formed by adding Gal, GlcNAc or GalNAc (Figure 1.9). In humans, core 1 - 4 are the most common structures produced in vivo. The core 1 subtype structure is formed by the addition of Gal in a β1-3 linkage to the GalNAc. Core 1 is also called T antigen (from Thomsen-Friedenreich antigen). The glycosyltransferase responsible for the formation of Core 1 (T) is known as the Core 1 β1-3 galactosyltransferase (Core 1 GalT).

C2GnT can generate core2 from core1 by the addition of GlcNAc to the GalNAc in a β1-6 linkage. Core 2 O-glycan may become elongated into a mono- or biantennary form with the presence of multiple lactosamine structures (Galβ1-4GlcNAc) that can be terminated with fucose and sialic acid. There are three functional forms of C2GnT that have been characterized (Stone et al. 2009).

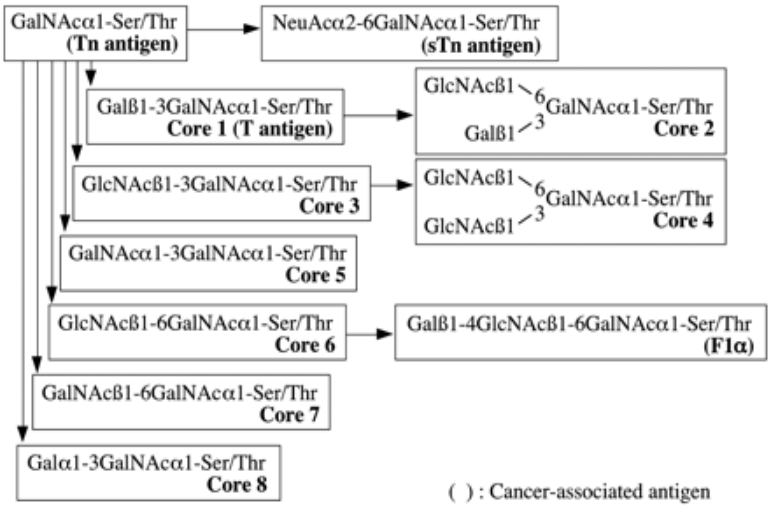


Figure 1.9: Core O-glycan structures and biosynthetic pathways for the core structures. Cancer-associated antigens are shown in parentheses (Ten Hagen et al. 2003).

C2GnT1 is the main functional form found in breast epithelial cells. Mucin O-linked glycosylation in the epithelial cells of resting, pregnant and lactating breast is Core 2 based (Dalziel et al. 2001). The C2GnT-3 enzyme is expressed in the thymus and is found in the breast but at very low levels (Schwientek et al. 2000). The C2GnT-2 enzyme can also synthesize the Core 4 structure from core 3 (see figure 1.10) but the presence of core 3 is restricted to GI tract tissues. The core 3 (GlcNAcβ1-3GalNAcα1-Ser/Thr) structure of the O-glycan, is synthesized by core 3 β3-N-acetylglucosamine (GlcNAc)-transferase (core 3 GlcNAcT) and coded for the β3GNT6 gene. B3GNT6 gene expression is restricted to mucus-secretory tissues. The core 3 structure is expressed on mucins by the stomach, small intestine, and colon, but downregulated in colon cancer. Core 3GlcNAcT is not expressed in the breast where only core 1 and 2 structures have been observed (Brockhausen et al. 2006, Iwai et al. 2002). However, Blixt et al. found a high level of autoantibodies reactive with core 3 MUC1 (MUC1 is a glycoprotein overexpressed in 90% of breast and other type of cancers- see section 1.7) in some sera from a cohort of 395 breast cancer patients compared to the healthy controls. In the same patients, it was also found a significant correlation with reduced incidence and increased time to metastasis. However, as the RT-qPCR performed in this study confirmed the lack of expression of B3GnT6 gene, the epitope recognized by these autoantibodies needs to be elucidated (Blixt et al. 2011). The above mentioned reactions and enzymes are summarized in the figure 1.10.

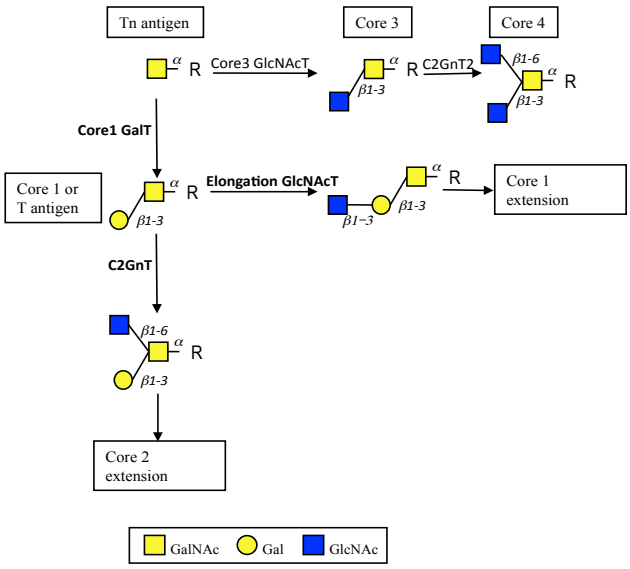


Figure 1.10 Biosynthesis of the Core structure. GalNAc (Tn antigen) can be converted to core 1 (T antigen) by core 1 GalT or to core 3 structures by core 3 GlcNAcT, which can be branched by C2GnT2 to form core 4. Core 1 can also be elongated by a β3-GlcNAcT or can be branched by C2GnT to form core 2. All core structures can be further extended to form complex *O*-glycans (Brockhausen 2006).

1.4.3 Elongation of the glycan chain.

Addition of Gal in a β1,4 or β1,3 linkage to the GlcNAc of core 2 results in the formation of a type 2 chain. Addition of Gal in a β1,4 linkage determine the formation of a precursor of Lewis^x and SialylLewis^x (Le^x and SLe^x) and addition of Gal in a β1,3 linkage determine Lewis^a and SialylLewis^a (Le^a and SLe^a) formation.

The Galβ1,4 GlcNAc disaccharide is known as lactosamine and is catalysed by β4-galactosyltransferase (β4GalT), as seen in figure 1.11. Six β4-galactosyltransferase genes (B4GALT is the gene name) have been cloned from mammalian sources. Each encodes a galactosyltransferase that utilizes the donor substrate UDP-Gal and transfers Gal in α 1,4-linkage to GlcNAc or Glc (Lo et al. 1998). It was reported that β4GalT-4 is more efficient in galactosylating mucin-type, core 2 branch oligosaccharides. It was also suggested that β4GalT-5 may function best in transferring Gal to O-glycans (Ujita et al.1998, Ujita et al.1999). Glycoproteins often bear glycans that include linear polymers of type 2 lactosamine units (polylactosamine).

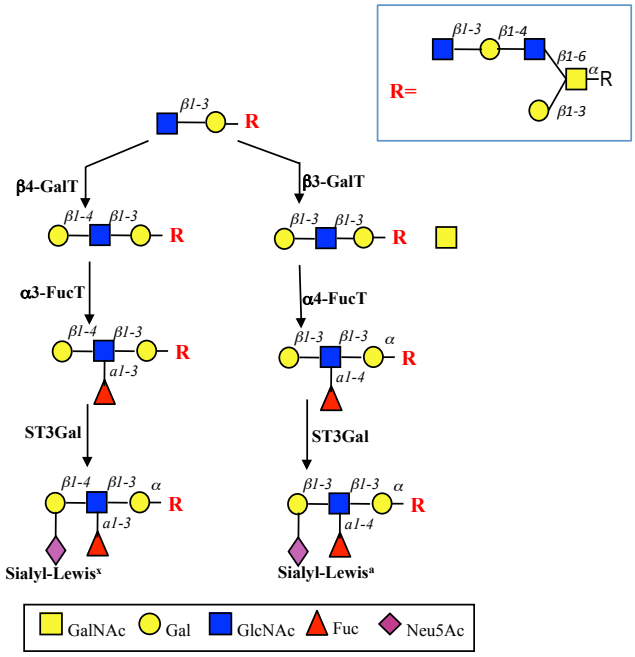


Figure 1.11 Elongation and assembly of Lewis antigens. In the first case, SialylLewis^x structure is synthesized by the addition of a β4Gal to GlcNAc (type 2 chain) by a β4-Gal-transferase (β4-GalT), an α3-Fuc to GlcNAc by an α3-Fuc-transferase (α3-FucT), followed by the addition of an α3-sialic acid to Gal by an α3-sialyltransferase (ST3Gal) to form sLe^x. SialylLewis^a antigen chains are synthesized by β3-Gal-transferase (β3-GalT), α4-Fuc-transferase (α4-FucT) and ST3Gal.

Addition of GlcNAc to the inner galactose of the backbone is mediated by several transferases, for example those encoded by the B3GNT1, -2, -3 and -5 genes. GlcNAc residues constitute the basis for the attachment of Lewis antigens as seen in paragraph 1.4.4 (see figure 1.13) (Potapenko et al. 2010).

In the case of Le^a and SLe^a, the GlcNAc sugar is modified by Gal in a β1,3 linkage by the action of β3GalT resulting in a type 1 chain. Furthermore, branching of the polylactosamine backbone can occur.

1.4.4 Termination.

The addition of sialic acid, Gal, sulfate and GalNAc in an α linkage can all result in the termination of chain extension. One of the most common termination reactions is the addition of the sialic acid N-acetyl-Neuraminic acid (Neu5Ac) to Gal by the action of sialyltransferases ST3Gal-I (figure 1.14). ST3Gal-I is a transferase that catalyzes the addition of sialic acid in an α2,3 linkage to Gal β1–3 GalNAc, and thus terminates chain extension by inhibiting core 2 formation. The only additional sugar that can be added is

sialic acid in the α2,6 position to GalNAc catalyzed by ST6GalNAcII, generating disialylT (Burchell et al. 1999). As ST3Gal-I uses the same substrate (core 1) as C2GnT1 there is competition between the two enzymes for their common substrate (Gal β1-3 GalNAc) (Dalziel et al. 2001). ST3Gal-I and its product SialylT (ST) are very important in the immune system. T cell maturation is accompanied by regulation of the sialyltransferase ST3Gal-I (Priatel et al. 2000). This subject will be discussed more in details in section 1.6.5.

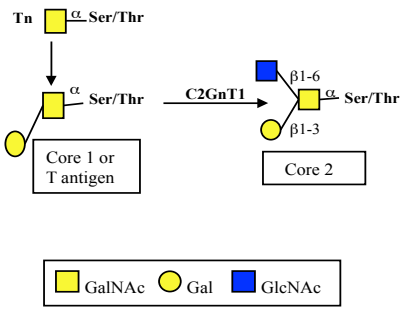


Figure 1.12 General pathways of O-linked glycosylation in normal mammary gland. In the normal mammary gland Core1 is converted to Core2.

Terminal GlcNAc residues, and especially those of core 2 structures, can be used as the basis for the attachment of Lewis antigens. These are antigens expressed on the surface of erythrocytes and a variety of cell types, including some epithelia (Narita et al. 1993). These antigens are common to many types of glycans, including N- and O-linked glycans as well as glycosphingolipids. Structurally these epitopes are composed of fucosylated three lactosamine units (Gal-GlcNAc) that can be either linked in a β1-3 or β1-4 (Figure 1.11).

β-1,3-Galactosyltransferases (B3GALT1, -2 and -5 genes) catalize the formation of the structure to be a type 1 Lewis epitope (Le^a, SLe^a and Le^b structures) as shown in figure 1.11. On the other hand, β-1,4-galactosyltransferases 1, 2, 3 and 4 (B4GALT1, -2, -3, -4) will synthesize type 2 Lewis antigens (for example Le^x and Le^y) by transferring a galactose to the GlcNAc saccharide in a β-1,4-linkage. This galactose may be fucosylated or sialylated. Fucosyltransferase 3 (FUT3) transfers a fucose in an α1,4 residue on the GlcNAc to give Le^a or Le^b structures. Fucosyltransferase 3, 7 and 9 (FUT3, FUT-7 and FUT9) transfer a fucose in an α1,3 linkage on type 2 antigens to give Le^x and Le^y. FUT3, 5 and 6 can also add a fucose on the Gal of short glycolipids. ST3GAL3 and -4 yield SLe^a by a transfer of a sialic acid in α1,3, `and ST3GAL6 activity results in sLe^x. The addition of fucose forms Le^b and Le^y epitopes, which are both

synthesized by fucosyltransferases 1 and 2 (FUT1 and FUT2) (Julien, Ivetic , Grigoriadis , QiZe , Burford , Sproviero et al. 2011;Potapenko et al. 2010). The enzymes involved in the synthesis of sLe^x on glycoproteins are in figure 1.13.

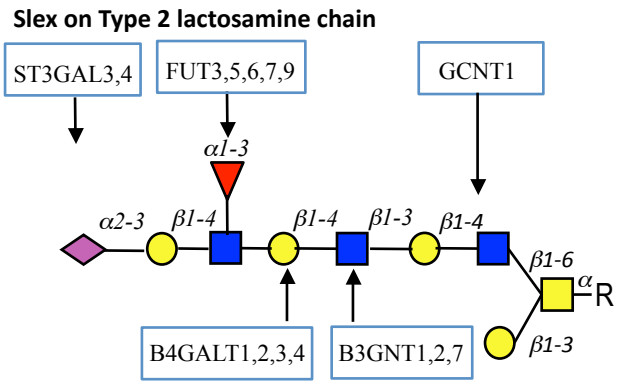


Figure 1.13 Synthesis of sLex on lactosamine chain backbone (Julien, et al. 2011).

1.5 Change of mucin type O-glycosylation in breast cancer.

The epitopes found in cancer includes 1) β6GlcNAc branching in *N-linked* structure; 2) GM2, GD3 in lipid-linked structure, 3) sialyl-Tn, Tn in *O-linked* structure; 4) sialyl-Lex, sialyl-Lea, and Ley in either *N-linked, O-linked,* or lipid-linked structure and 5) T and ST in *O-linked* structure that are tumour associated structures.

As described in section 1.3.2, β1,6 GlcNAc branching in N-linked structure added by MGAT5 are abundant in breast cancer tissues and other types of cancer (colon, leukemia etc.) with high metastatic potential (Yoshimura et al. 1995, Fernandes et al. 1991).

Gangliosides have a role in cancer progression since 1) they act as immunosuppressors, 2) GM3 and GD2 are important in angiogenesis, 3) they regulate adhesion/motility fundamental in metastasis 4) they are modulators of signal transduction (Birklé et al. 2003). Gangliosides are expressed in breast, prostate, brain, lung and other type of cancer (Steenackers et al.2012, Hatano et al. 2011, Basu et al. 2012, Segatori et al. 2012).

STn is over-expressed in many epithelial cancers with the highest frequencies in pancreas, colorectal and ovarian cancers (Julien et al. 2012). Hence STn over-expression occurs in carcinogenesis in tissues that normally don't express the antigen (Ogata et al. 1998). It can be considered a good tumor marker of carcinogenesis and potentially useful for diagnosis (Julien et al. 2012).

Sialyl Lewis antigens, sialyl Lewis a and Sialyl Lewis x, are used as tumor markers, since they are overexpressed in the majority of cancers (colon, prostate, breast, melanoma, lung, liver etc.) and they are associated with tumor progression and metastasis (by enhancement of cancer cell adhesion to endothelial E-selectin).

In a screening of 28 mucinous adenocarcinomas from different locations (stomach, ampulla of Vater, colon, lung, breast and ovary), the O-linked glycoforms in 50% of the cases detected were Tn/STn/SLea/SLex-MUC1 and STn/SLea/SLex-MUC2 glycoforms and T/SLea-MUC2, STn/T/SLea/SLex-MUC5AC and STn/T/SLea/SLex-MUC6-were identified in a variable percentage of cases from different organs (Pinto et al. 2012).

In the following section we will concentrate on the last three classes of glycans with a particular focus on the change of glycosylation in breast cancer.

1.5.1 Premature O-linked glycan chains observed in breast cancer.

Differences of cellular glycosylation are observed between normal mammary epithelial cells and breast cancers. As discussed above, in normal resting, pregnant and lactating breast, mucin O-glycans are largely extended (core 2 type) structures. The addition of GalNAc is followed by the addition of Gal to form core 1 (T epitope) and core1 (T) acts as a substrate for the C2GnT1 enzyme, leading to the formation of branched, complex, core 2 glycans.

In contrast, mucin O-glycans found in breast carcinomas is often truncated core 1 based structures. Tn and T are expressed on breast cancer cells and are associated with a poor prognosis (Imai et al. 2001, Varki et al. 2009).

The activity of Core1 GalT, the enzyme responsible for the formation of core1 (T), requires the expression of Cosmc (core 1 β1,3Gal-T-specific molecular chaperone). It was demonstrated that mutation of this chaperone results in a loss of core 1 structures (Ju et al. 2002, Wang et al. 2010) and can explain the expression of the tumour-associated structure Tn and sialylated Tn (Neu5Acα2,6GalNAc) (see figure 1.14). However, in our lab it was found that Cosmic mutations are not the mechanism responsible for the expression of Tn and STn in breast cancer (paper in preparation).

In contrast, one mechanism that is responsible for the increase in core 1 structures is a change in the expression of glycosyltransferases, particularly an increase in the expression of the sialyltransferase, ST3Gal-I, and a decrease or a loss of C2GnT1 (Burchell et al. 2001). ST3Gal-I, (Burchell et al. 1999) is not found in normal breast (see figure 1.12 and 1.14) and a high expression of this enzyme is associated with aggressive disease and may be immunosuppressive (Mungul et al. 2004). Furthermore, in around 25-30% of breast cancer the transcription of the sialyltransferase ST6GalNAc-I is turned on. This enzyme adds sialic acid, Neu5Ac, in α2,6 linkage to GalNAc linked to Ser or Thr, thus creating the STn epitope (Sewell et al. 2006). ST6GalNAc-I competes with Core1 glycosyltransferase and it has been shown to be located throughout the Golgi (Sewell et al. 2006). Of the short O-glycan expressed in carcinoma, the disaccharide, STn, is the most tumour specific (Julien et al. 2003).

In summary the glycans on breast cancer cells can consist of Tn, T, STn, ST. However carbohydrate antigens, such as sLe^x and sLe^y, that are normally found on the periphery of long side chains, have also been demonstrated to be present in breast carcinomas (Nakagoe et al. 2000; Julien, Ivetic , Grigoriadis , QiZe , Burford , Sproviero et al. 2011). These epitopes will be dealt with in detail in the next sections.

1.5.2 Abnormal glycosylation on peripheral antigens.

Although the Lewis and SialylLewis (Le and sLe) antigens can normally be found on lymphocytes that migrate from the blood stream to the tissues, they are not extensively found on mucin-like proteins expressed by epithelial cells. Sialyl Lewis^{a} (SLe^{a}) and sialyl Lewis^{x} (SLe^{x}) are abnormally found on glycoproteins in lung, colon, stomach and breast cancers and they are considered to be tumor-associated markers (Soejima & Koda, 2005). Fukushi et al. and Fukushima et al. reported decades ago that Lewis antigens were functionally important terminal glycan epitopes implicated in breast cancer development (Fukushi et al.1984; Fukushima et al.1984).

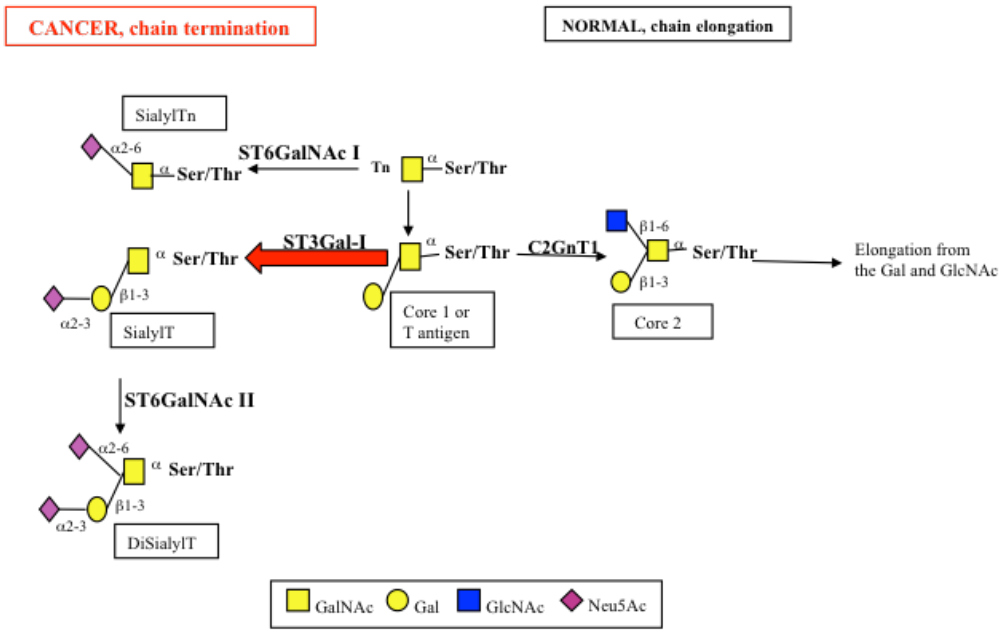


Figure 1.14 Pathways of O-glycosylation in normal breast tissue and carcinoma. In breast carcinoma shorter O-glycans are formed by the increased expression of ST6GalNAc-I or ST3Gal-I. On the other hand, in normal epithelial breast O-Glycan structures are mainly core 2-based resulting from the action of C2GnT-I on core 1 O-glycans (Sewell et al. 2006).

Among the group of several type 2 antigens, SLe^{x} has been reported to have the prevalence in breast cancer cells (Matsuura et al.1998). Potapenko et al. reported an upregulation of enzymes like B4GALT1, -2, -3, FUT5 and ST3GAL4 in breast cancer. However down-regulation of FUT4, FUT9, FUT10, ST3GAL3 and ST3GAL6 genes was also observed (Potapenko et al. 2010). According to Recchi et al. ST3Gal III is the most expressed sialyltransferase involved in sialylation of Lewis antigen (Recchi et al. 1998)

(see section 1.6.2). In this lab it was demonstrated that sLex expression is associated with ER-negative status, lymph node involvement, and high grade of breast tumors, but not with the survival of patients. Moreover, in ER-negative breast tumors there was a higher expression of glycosyltransferases FUT3, FUT4, and ST3GAL6 (Julien, Ivetic , Grigoriadis , QiZe , Burford ,Sproviero et al. 2011). Interestengly, FUT3 was one of the 16 genes signature predicting distant metastasis in lymph node negative, ER-negative breast cancer (Wang et al. 2005). However high levels of sLe^x in ER-positive cancers is correlated with bone metastasis, a metastatic site prevalent with ER positive tumours (Julien, Sproviero et al. 2011). Adhesion of cells to the endothelium can be mediated by E-selectin via sLe^x. E-selectin (also known as CD62A, ELAM-1) is a sialic acid-binding lectin (sugar binding protein-see section 1.8.2), expressed by endothelial cells, when they are activated by cytokines (Lowe et al. 1990). Importantly, E-selectin is constitutively expressed on the endothelial cells in bone marrow and in small vessels adjacent to tumor nests in primary breast cancer (Kobayashi et al. 1998).

There are therapies that inhibit sLe^x binding to selectins, like glycosyltransferase inhibitors and receptor inhibitors for the treatment of cancer.

A disaccharide precursor of sLex, GlcNAcβ1,3Galβ-O-napthalenemethanol, reduced levels of sLex on the surface of colon adenocarcinoma cells and decreased the interactions with selectins sufficiently to reduce lung metastasis in a murine tumour model (Foster et al. 2003). Shirota et al. showed that co-administration of GSC-150, an analogue of sLex, with the colon carcinoma cell line KM12-HX to mice reduced metastasis to the liver, compared with mice treated with the cells alone (Shirota et al. 2001). Recently, Rillahan et al. showed the development of inhibitors of some fucosyltransferases and sialyltransferases that reduced the expression of sialyl Lewis x on myeloid cells, resulting in loss of selectin binding and impaired leukocyte rolling. Other inhibitors of SLex, like GlycoDesign (GD39), Oxford Glycoscience (OGT719), GlycoGenesys (GCS-100), are currently under development in pharmaceutical companies. These data suggest that the sLex–selectin interactions contribute to metastasis and that selectin inhibitors might find use in cancer treatment (Dube et al. 2005).

1.6 ST3Gal-I.

ST3Gal-I is a sialyltransferase that catalyzes the addition of sialic acid in an α2,3 linkage to Galβ1–3GalNAc, and, thus, terminates chain extension by inhibiting core 2 formation (Burchell et al. 1999).. ST3Gal-I is the main subject of this thesis, so this

section will explain the class of sialyltransferases in general with an emphasis on the ST3Gal family. Specifically, ST3Gal-I (gene and protein) structure will be described and its role in the immune system, in diseases and in breast cancer will be dealt.

1.6.1 Sialic acids and sialyltransferases.

Sialic Acids (SA) form a large family of 9 carbon sugars. The most common sialic acid added to O-glycans is N-acetyl-Neuraminic acid (Neu5Ac or Neu-5-amido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid) (Varki et al.1997). Sialic acids derivatives are at the terminal positions of many oligosaccharides of glycoproteins, which determine the half-lives of many circulating glycoproteins and affect cell behavior (Zeng & Gabius,1992). In fact, these molecules are involved in cell-cell and cell-matrix interactions and, thus, in molecular recognition during tumor development, differentiation and progression (Wang et al. 2005). Sialyl residues are linked to galactose (Gal) residue via α2,6 or α2,3-linkage or linked to N-acetylgalactosamine(GalNAc) via α2,6-linkage. Sialic acid can also be linked to another sialic acid on the C8.

The transfer of Neu5Ac from CMP donors to glycoconjugates in the Golgi is catalyzed by a large family of sialyltransferases (Harduin-Lepers et al. 2001), which differ in their substrate specificity, tissue distribution and various biochemical parameters. Each sialyltransferase is linkage specific and has its own substrate specificities. There are four subgroups of these enzymes: the ST3Gal (α2,3-ST), ST6Gal (α2,6-ST), ST6GalNAc, and ST8Sia (α2,8-ST) families (Takashima et al.1999) (shown in table 1.4). Every family is further classified into other subfamilies. All ST3Gal enzymes transfer Neu5Ac residues in 〈2,3-linkage to terminal Gal in glycoproteins or glycolipids. ST3Gal-I and -II transfer Neu5Ac on the structure Galβ1,3GalNAc-R, whereas the ST3Gal-III, -IV, -V, and -VI use the oligosaccharide isomers Galβ1,3/4GlcNAc-R and the ST3Gal-V subfamily transfers the sialic acid only on the lactosylceramide (i.e. Galβ1,4GlcNAc-Cer), giving rise to the synthesis of the ganglioside GM3 (Harduin et al. 2005). There are only two sialyltransferases in ST6Gal family, ST6Gal-I and –II. They both have Galβ1,4GlcNAc-R as the acceptor substrate. The enzymes of the ST6GalNAc family transfer Neu5Ac residues in α2,6 linkage to the GalNAc residues, found in O- glycosylproteins (ST6GalNAc-I, -II and -IV) or found in glycolipids (ST6GalNAc-III, -V and -VI). Enzymes of the ST8Sia family catalyze the transfer of

Neu5Ac residues in α2,8-linkage to other Neu5Ac residues found in glycoproteins and glycolipids (Wang et al. 2005).

Table 1.4 A list of the sialyltransferase subgroups, and their linkage and acceptor substrate sugar.

Sialyltransferase subgroups	Linkage
ST3Gal	Neu5Acα2,3 to Gal
ST6Gal	Neu5Acα2,6 to Gal
ST6GalNAc	Neu5Acα2,6 to GalNAc
ST8sia	Neu5Acα2,8 to Neu5Ac

Like all glycosyltransferases, sialyltransferases are type II transmembrane glycoproteins with a short amino (NH_2) cytoplasmic tail, a 16-20 amino acid transmembrane domain, a highly variable stem region, and a large carboxyl (COOH)-catalytic domain, which resides in the lumen of the Golgi (Datta et al. 1995). One conserved Cys residue is found in each of the L- and S-sialylmotifs, which are involved in disulphide bond formation and are essential for correct conformation and activity (Geremia et al.1997).

1.6.2 ST3Gal enzymes.

There are six ST3Gal gene sub-families that have been identified in various vertebrate genomes. Recombinant enzymes of the ST3Gal family catalyze the transfer of sialic acid in ⟨2,3-linkage to the terminal Gal residues found on glycoproteins or glycolipids. There are two main subfamilies: 1) ST3Gal-I and ST3Gal-II subfamilies 2) ST3Gal III, ST3Gal IV, ST3Gal V and ST3Gal VI sub-families (Hashimoto et al. 2009). ST3Gal III, ST3Gal IV and ST3Gal VI catalyze the transfer of sialic acid on the Gal residue of the disaccharide Galβ1,3(4)GlcNAc of glycoprotein or glycolipids.

In breast cancer, ST3Gal III, involved in sialylation of Lewis antigen formation, is the most expressed sialyltransferase and correlates to tumour size and the number of axilary nodes. ST3Gal III expression was positively correlated to the expression of ST6Gal I and ST3Gal IV, to tumor size, and to the number of involved axillary nodes. Patients with high ST3Gal III expression had a shorter overall survival, although the

cohort size (n=49) was relatively small (Recchi et al.1998). ST3Gal IV and VI catalyze the transfer of sialic acid on Galβ1,3GlcNAc and preferentially on Galβ1,4GlcNAc sequence, found in the carbohydrate moieties of glycoproteins and glycolipids. ST3Gal VI is more specific in the synthesis of sLe^x on ceramide (see figure 1.13) (Okajima et al.1999). Human and mouse ST3Gal V leads to the biosynthesis of GM3 because it adds a sialic acid on lactosylceramide (LacCer, Galβ1,4GlcCer). This enzyme is also known as the GM3-synthase and it can be released in serum in a soluble form after cleavage of the N-terminus (Richardson et al.1995). ST3Gal V in placenta can use also GalCer, GA1 and GA2.50 as substrates.

In the second subfamily, ST3Gal-I and ST3Gal-II use as substrate only the disaccharide Galβ1-3 GalNAc found on glycoproteins (core1 of O-glycosylproteins) and glycolipids (asialo-GM1 and GM1a) (Hatano et al. 2011). The members of these two gene sub-families show the same number of exons and intron positions and the nucleotide and amino acid sequences of hST3Gal II show 56.3% similarity to hST3Gal-I (Kitagawa & Paulson,1994a, Kitagawa & Paulson,1994b). The human ST3Gal-II was cloned from a T-cell cDNA library and showed activity on both glycolipids and glycoproteins (Giordanenco et al.1997). hST3Gal II mRNA is highly expressed in heart, liver, skeletal muscle and various lymphoid tissues but not in brain and kidney (Giordanenco et al. 1997).

1.6.3 ST3Gal-I gene and peptide structure.

ST3Gal-I is a sialyltransferase that transfers Neu5Ac to the Galβ1,3GalNAc disaccharide found on O-glycosyl proteins and gangliosides (Varki et al. 2009; Potapenko et al. 2010; Hatano et al. 2011). ST3Gal-I also has limited activity towards type I disaccharide (Galβ1,3 GlcNAc). However, human ST3Gal-I exhibits high transfer efficiency and high affinity (K_m of 51 μM) towards the core 1 mucin type disaccharide Galβ1,3GalNAcα- (Kono et al.1997). In addition, the mouse ST3Gal-I has a preference for glycolipid acceptors leading to the biosynthesis of GM1b, GD1a and GT1b at least in in vitro (Lee et al.1993). However, a very recent study from Sturgill et al. showed that in the mouse, St3gal2 and St3gal3 are responsible for nearly all the terminal sialylation of brain gangliosides (Sturgill et al.2012).

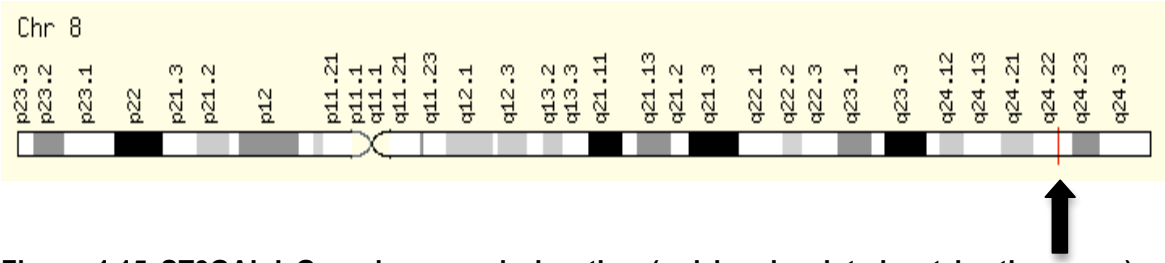


Figure 1.15 ST3GAL-I Gene in genomic location (red band pointed out by the arrow). Bands locations are according to GeneLoc, Entrez Gene, Ensembl.

The human ST3Gal-I is located on chromosomes 8 (8q24.2) (see figure 1.15) and the complete cDNA of the human ST3Gal-I gene was cloned from human placenta. Comparison with the porcine ST3Gal-I sequence showed a homology of 84% at the nucleotide level, with differences of a single amino acid deletion in the cytoplasmic tail and a two amino acid deletion in the stem region.

There are two isoforms of ST3Gal-I distinguished as 48kDa and 45kDa proteins as shown in figure 1.16. The 48 kDa isoform has an additional 16 hydrophobic amino acids near the NH_2 terminus than the 45-kDa (figure 1.16) (Reference Sequences: NM_003033.2 and NM_173344.2 for the longer and shorter form, respectively) (Gillespie et al.1992). The longer form is the active enzyme, while the shorter isoform, which is soluble, is not active (Gillespie et al.1992). Videira et al. analyzed the distribution of these two isoforms in bladder cancer cell lines and patients' specimens. Although the intensities of the PCR products differed among the analyzed cell lines and patients' samples, all samples expressed both transcripts (Videira et al. 2009).

The primary structure of this enzyme consists of a short NH_2-terminal cytoplasmic domain, a signal-membrane anchor domain, a proteolytically sensitive stem region, and a large COOH-terminal active domain (Lee et al. 1993). As described above, all eukaryotic sialyltransferases have three conserved peptide regions in the catalytic domain (sialylmotifs L, S, and VS). Sialylmotifs L and S are involved in the binding of donor and acceptor substrates, while the region between S and VS contains a stretch of four highly conserved residues. In this motif, ST3Gal-I has His299 and Tyr300, that together with His316 of the VS motif, are essential for activity of the enzyme. Also a Tyr residue (Tyr300) plays an important conformational role of ST3Gal-I. The human ST3Gal-I contains four potential N-glycan attachment sites in its catalytic domain, however, the glycosylation of these sites are not necessary for enzyme activity, but are important for the proper folding and trafficking of the enzyme (Jeanneau et al. 2004).

48 kDa peptide sequence

NH_2-Ser-Thr-Leu-Lys-Leu-His-Thr-Leu-Leu-Val- 10

11 Leu-Phe-Ile-Phe-Leu-Thr-Ser-Phe-Phe-Leu- 20

21 Asn-Tyr-COOH

45 kDa peptide sequence

NH_2-Lys-Tyr-Pro-Tyr-Arg-Pro-Thr-Thr-Thr-Thr- 10

11 Arg-Ile-Ile-Glu-Glu-Gln-Lys-Val-Ser-Ala- 20

21 Phe-Phe-COOH

Figure 1.16 NH_2-terminal amino acid sequences of the 48- and 45-kDa α2,3 sialyltransferase peptides (Gillespie et al.1992).

1.6.4 Promoter studies of ST3Gal-I.

The promoter of ST3Gal-I has not been widely studied. Taniguchi et al. cloned and sequenced the promoter of ST3Gal-I. The luciferase assay on leukaemia and colon adenocarcinoma cells showed that only the region between -304 and -145 is important for the transcriptional activity of ST3Gal-I. Transcription factor binding sites for Sp1 and USF1 are present in this region and are involved in the transcription of ST3Gal-I. Inserting base substitutions in the binding sites of both transcription factors resulted in a reduction of activity by 40-50% of ST3Gal-I (Taniguchi et al. 2001). In porcine kidney cells, transcriptional activation of this sialyltransferase can be induced by TGF-β1 (Son et al. 2011). Moreover Son et al. and Higai et al. found that NF-κb is involved in the transcription of ST3Gal-I (Son et al. 2011, Higai et al.2006). The expression of ST3Gal-I was investigated in two human colon adenocarcinoma cell lines HT-29, Colo205 and in one leukaemia cell line HL-60. NF-κb knockdown could inhibit the increase of expression of ST3Gal-I after TNF-α treatment (Higai et al. 2006).

1.6.5 ST3Gal-I and SialylT antigen in the immune system.

ST3Gal-I and its product SialylT (ST) are very important for the immune system. T cell maturation from the DP (double positive-immature T cells) to SP (single positive-mature T cells) stages is accompanied by regulation of the sialyltransferase ST3Gal-I. Analysis of ST3Gal-I knockout mice revealed that loss of this enzyme leads to disregulation of mature CD8 T cell homeostasis and to the extended survival of CD8 T (Priatel et al. 2000). A consequence of this difference in glycosylation is that immature CD4/CD8 DP

thymocytes bind MHC class I tetramers more avidly than mature CD8 SP thymocytes. Moody et al. showed that sialic acid addition to core1 of mature CD8 single-positive thymocytes decreases CD8 binding to MHCI (Moody et al. 2001).
Neuraminidase treatment of mature CD8 SPs enhanced CD8 noncognate binding (Starr et al. 2003) and ST3Gal-I up-regulation has shown to be responsible for the loss in noncognate CD8-class I binding that accompanies thymocyte maturation (Kao et al. 2006).
MUC1 carrying the sialylT (ST-MUC1) glycoform was shown to inhibit the differentiation and function of monocyte derived DCs. Human monocyte-derived DCs cultured in the presence of ST-MUC1, showed a modified phenotype with decreased expression of costimulatory molecules (CD86, CD40), antigen-presenting molecules (DR and CD1d), and differentiation markers. In this way DCs were defective in their ability to induce immune responses and may represent a mechanism by which epithelial tumors can escape immunosurveillance (Rughetti et al. 2005). However, these results should be interpreted with caution as subsequently we showed that ST-MUC1 purified from CHO cells contain small quantity of TGFβ (Beatson, Sproviero et al. 2011).

1.6.6 ST3Gal-I in different diseases.

Lebreve et al. showed a hyposialylation of O-glycans on CD43 and CD45 lymphocytes surface in HIV-1 (human-immunodeficiency-virus-I) infected T-cell lines. It was thought that a possible variation in mRNA transcription of hST3Gal-I could occur in this case (Lefebvre et al.1994). However, comparable levels of hST3Gal-I and hST3Gal II mRNA and enzymatic activity were observed in parental and HIV-1-infected T-cell lysates, so the variation of sialylation in this case is probably due to a different mechanism from an alteration of transcription and catalytic activity of ST3Gal-I (Giordanengo et al.1997).
Influenza infection starts with the recognition of the sialic acids on the host cell surface by the hemagglutinin (HA) of the virus. All avian influenza viruses recognize α2,3 sialic acid linkage on the host cell (Gambaryan et al. 2004).
Alterations of ST3Gal-I level of expression were found also in Grave's disease (Kiljański et al. 2005) and SNPs (single-nucleotide polymorphism) in ST3GAL-I gene were discovered in bipolar disorders (Zandi et al. 2008).

1.6.7 ST3Gal-I in breast cancer.

Increased expression, relative to normal tissue, of ST3Gal-I is observed in many carcinomas, especially colon (Kudo et al. 1998), bladder (Videira et al. 2009), ovarian (Wang et al. 2005) and breast cancer (Burchell et al. 1999). In primary breast cancers, ST3Gal-I is overexpressed and its level is correlated with tumour grade (Burchell et al. 1999). In contrast C2GnT1 is found to be low or absent in around 50% of carcinomas and breast cancer cell lines. ST3Gal-I activity was also found to be increased 8–10 fold in three breast cancer cells lines relative to the normal cell line (MTSV1–7), while the C2GnT enzyme activity was absent in two of the breast cancer cell lines (T47D & BT20) and reduced by 50% in MCF-7 (Brockhausen et al.1995). ST3Gal-I competes with C2GnT1 for the core 1 substrates and there is an overlap of the localization of two enzymes in the Golgi (Whitehouse et al. 1997). Although ST3Gal-I is localised mainly in the medial/trans Golgi, there are detectable levels in the cis Golgi. C2GnT1 has been located mainly in the cis Golgi, but with some expression in the medial cisternae and low levels in the trans Golgi. However, this overlap appears to be sufficient in allowing competition between the two enzymes (Dalziel et al. 2001). Since ST3Gal-I competes with C2GnT1, when ST3Gal-I is over-expressed, core1 will become sialylated and termination of the chain will occur (Dalziel et al. 2001). Importantly, over-expressing ST3Gal-I in the mammary gland can promote the early development of spontaneous mammary tumours in a murine model. Transgenic mice, expressing human ST3Gal-I from the MUC1 promoter (used to allow upregulation in the mammary gland and in mammary tumours), were crossed with Polyoma middle T mice (a model of spontaneous breast cancer). These mice showed a significant increase in the speed of mammary tumour development compared to the controls (Picco et al. 2010) (see figure 1.17).

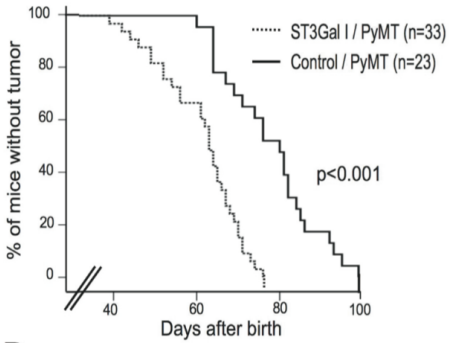


Figure 1.17: Mammary tumours develop faster in the ST3Gal-I/PyMT mice compared to the controls. Kinetics of tumour formation in ST3Gal-I/PyMT and Control/PyMT mice. The difference between ST3Gal-I/PyMT and control mice was highly significant ($P < 0.001$), evaluated by the Breslow test (Picco et al. 2010).

1.7 One O-linked glycosylation mucin, MUC1, and antibodies to its glycosylated form.

Mucin type O-glycosylation is dominant on mucin-type molecules like MUC1. MUC1 is a transmembrane glycoprotein overexpressed in many types of cancer, with a differential expression pattern of *O*-glycans in malignant compared to normal epithelial cells. O-glycosylation occurs on five potential sites (Ser or Thr), found within each tandem repeat (TR) of 20 amino acids (Tarp et al. 2008). The extracellular domain of MUC1 can contain 20 to 150 of these tandem repeats. Many monoclonal antibodies, that target MUC1, recognize epitopes within the tandem repeat domain and show differential reactivity with this mucin, expressed by normal mammary epithelium compared to that on malignant breast epithelium (Taylor-Papadimitriou et al. 1999). SM3 is a monoclonal antibody that recognizes MUC1 produced by cancer cells but not by normal cells. SM3 does not react with normal breast epithelium because its epitope is masked by the core 2 glycans (Burchell et al. 1989, Dalziel et al. 2001, Mungul et al. 2004) (see figure 1.18A). However, overexpressing ST3Gal-I in a cell line expressing C2GnT1, results in SM3 binding (Dalziel et al. 2001), demonstrating the competition that occurs between these two enzymes. Another two antibodies HMFG1 and HMFG2, also recognize a peptide epitope in MUC1 but their binding is influenced by its glycosylation. HMFG1 reacts well with the mucin expressed by normal cells but the presence of sialic acid can decrease its binding to its epitope. In contrast, HMFG2 can react with shorter glycosylated chain and can be masked by longer carbohydrate side chain on normal mucins as shown in figure 1.18 (Burchell & Taylor-Papadimitriou, 1993).

1.8 Lectins.

Plant lectins are sugar-binding receptors, that bind glycans in an oligosaccharide and linkage specific manner. Lectins were first described in 1888 by Stillmark working with castor bean extracts. Some lectins were discovered by their ability to agglutinate red blood cells in vitro and other lectins by investigating various cell-cell interaction phenomena (Sharon et al. 2004). Sugar binding proteins are present in animals and in humans and to date 79 lectin-like binding receptors have been described and they are divided in three subfamilies: C-type lectins, I-type lectin and galectins.

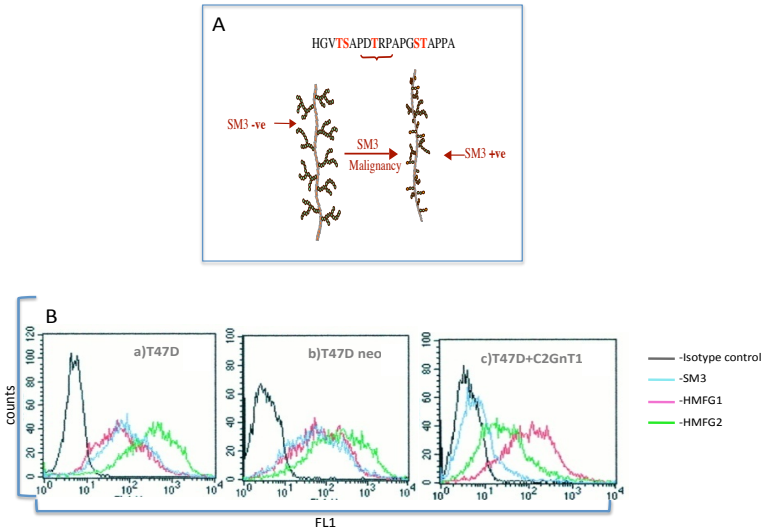


Figure 1.18: A) Reactivity of SM3 with O-glycoforms in malignancy. SM3 does not react with normal breast epithelium because the epitope is masked by the Core 2 glycans. **B) T47D transfected with C2GnT1 loses SM3 reactivity on MUC1.** a) the breast cancer cell line T47D does not express C2GnT1 and therefore the MUC1 expressed by these cells bind SM3; b) T47D transfection control; c) T47D transfected with C2GnT1 loses expression of SM3 (Dalziel et al. 2001).

C-type lectin receptors (CLRs) are calcium-dependent carbohydrate-binding proteins and consist of a large family of receptors (60–80). C-type lectins can be divided into two categories (which are further divided into other subfamilies): 1) the mannose-specific C-type lectins (which contain an EPN (Glu-Pro-Asn) amino acid motif) have a specificity for mannose- and/or fucose-terminated glycans; 2) the galactose-specific C-type lectins (which contain the QPD (Gln-Pro-Asp) sequence in the CRD- carbohydrate-recognition domains) and recognize galactose-terminated or GalNAc terminated glycan structures (Zelensky & Gready, 2005). The C-type lectins have mainly been identifed on antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs). For instance dendritic cells express DC-SIGN (dendritic cell-specific ICAM grabbing non-integrin), MGL (macrophage galactose-type C-type lectin) and dectin-1, which are involved in glycan-mediated pathogen recognition and internalization of antigen for loading on major histocompatibility complex (MHC) class I and II molecules (Figdor et al. 2002). In general, C-type lectins can be expressed by mammalian cells or by pathogens, so they have dual function in host-pathogen recognition and immune cell responses (Weis et al. 1998).

I-type lectins are glycan-binding proteins with variable numbers of immunoglobulin domains. The Siglec family is the only well-characterized group of I-type lectins. Siglecs

are sialic acid-binding immunoglobulin-like lectins and can selectively recognize sialylated structures of specific linkages (α(2-3), α(2-6) or α(2-8)) (Crocker et al. 2007). There are 13 members in the siglec family and it can be divided into two types: CD33-related siglecs (Siglecs 3, 5, 6, 7, 8, 9, 10, 11 and 14) and a group consisting of sialoadhesin (Siglec-1 or CD169), MAG (Siglec-4) and Siglec-2 (CD22). Siglecs are widely expressed on immune cells like neutrophils, monocytes, B cells, DCs, NK cells, eosinophils and basophils. Siglecs have a role in recognizing sialic acids on pathogens and, on the other hand, have a role in intercellular communication of immune cells (Crocker et al. 2007).

Galectins are a family of soluble lectins characterized by a common structural fold and a conserved CRD of about 130 AA, that recognize glycans containing the disaccharide *N*-acetyllactosamine (Gal-β(1-4)-GlcNAc) (Leffler et al. 2004). They (about 15 galectins have been identified in mammals) are classified in two main classes: 1) 'prototype' galectins' (galectins 1, 2, 5, 7, 10, 11, 13, 14 and 15) which have one CRD that can dimerize, 2) 'tandem-repeat galectins' (galectins 4, 6, 8, 9 and 12) which contain two homologous CRDs in a single polypeptide chain (Liu & Rabinovich, 2005). Galectin-3 is an exception to these two classes, since it contains a CRD connected to a non-lectin N-terminal region (about 120 amino acids) that is responsible for oligomerization of the lectin and ligand cross-linking (Nieminen et al. 2007). In the immune system galectins are expressed by activated macrophages, regulatory T cells and activated T and B cells (Liu & Rabinovich, 2005).

In this thesis lectins have been used to study the glycans found on the surface of breast cancer cell lines. The following two paragraphs will be dealing with some sialic-acid-binding lectins, plant lectins specific for Core1 and sialylated-Core1 and selectins, a c-type lectin subclass.

1.8.1 Lectins binding Core1/sialylated-Core1 and Neuraminidase.

There are two different lectins that bind with different affinities to α2,3 sialylated glycans including Core1:

1) Maackia Amurensis agglutinin (MAA) derives from a leguminous seed and binds to α2,3 linked sialic acid (Loris et al. 2002). There are two types of MAA: a) Maakia amurensis leukoagglutinin (MAL), which binds Neu5Acα2,3Galβ1,4GlcNAc with the best affinity and b) Maakia amuresis haemagglutinating (MAH), which is more specific for Neu5Ac2,3Galβ1,3(Neu5Acα2,6) GlcNAc (Boyd et al. 1961);

2) Peanut agglutinin (PNA) from Arachis hypogaea, which binds specifically the Gal β-1-3-N-acetylgalactosamine (T antigen) structures and this binding is inhibited by the presence of sialic acid (Novogrodsky et al. 1975).

These lectins are used in combination with neuraminidase, glycoside hydrolase enzymes, responsible for the cleavage of sialic acid (Klenk & Uhlenbruck, 1957). The crystal structure for influenza neuraminidase was obtained in 1983 (von Itzstein et al. 1993), and since then, many other sialidases have been characterized from other sources like Arthrobacter ureafaciens (which hydrolizes NeuAc bound in α2→3,6,8,9), Streptococcus pneumoniae (which hydrolize NeuAc bound in α 2→3), Vibrio cholerae (which hydrolize NeuAc in α2→3,6,8), Clostridium perfringens (which hydrolize NeuAc in α2→3,6) (Taylor et al. 1996).

1.8.2 Selectins

This work focused on the control of glycosyltransferase by COX-2, an enzyme mainly involved in inflammation, on O-linked glycosylation. The selectins are sugar binding proteins involved in inflammation. The C-type lectins (Ca^{2+}dependant lectins) involved in inflammation comprise of three members, E(ndothelial), L(eukocytes), and P(latelet)-selectin (Lowe et al. 2002). E-selectin is expressed on endothelial cells during inflammation but it is constitutively expressed on the endothelial cells of the microvasculature of bone marrow and skin (Sackstein, 2004). P-selectin is displayed on activated endothelial surfaces and on platelets. L-selectin is expressed constitutively by lymphocytes and neutrophils, with the highest expression being by active cells, that facilitate trafficking into tissues (Ley et al. 2004). Sialyl-Lewis^{x} (sLe^{x}) and Sialyl-Lewis^{a} (sLe^{a}) on scaffold proteins such as PSGL-1, MadCAM and CD34 are recognized by these selectins. Binding to selectins is used by malignant cells, especially colonic, during the metastatic process (Ono & Hakomori, 2004), and sialyl-Lewis^{x} was shown to be expressed by breast cancers, where in the ER positive tumours high expression is associated with bone metastasis (Julien, Ivetic, Grigoriadis, QiZe, Burford, Sproviero et al. 2011).

1.9 Principles of inflammation and role in cancer.

Inflammation is the body's immediate response to infection or injury and its main function is to eliminate pathogens and toxins and to repair damaged tissue. These

responses must be ordered and controlled. Inflammation is characterized by redness, swelling, heat and pain. These characteristics are due to increased blood flow and permeability across blood capillaries. The increased permeability of the blood capillaries allows leukocytes, antibodies and cytokines to migrate from the bloodstream into the surrounding tissue. The movement of leukocytes depends on the upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E, (P and L)-selectin (Kansas,1996) on the surface of endothelial cells, allowing leukocyte binding and subsequent diapedesis. After the leukocytes reach the tissue interstitium, they bind to extracellular matrix proteins via expressed integrins and CD44 (Hidalgo et al. 2007). The first cells that appear at the inflammation site are granulocytes, monocytes/macrophages, all involved in pathogen killing and in tissue repair. Inflammation is initiated by several factors like bacteria, chemical injury etc. that induce the release of inflammatory mediators from leukocytes, monocytes and macrophages including cytokines such as TNF-α and IL-1, eicosanoids, such as PGE2; nitric oxide; matrix metalloproteinases; and other mediators (Calder et al. 2006; Paterson et al. 2003).

Chronic irritation and inflammation can give rise to tumorigenesis and induce apoptosis, migration of tumour cells and other neoplastic processes (Coussens et al. 2002). In the IX century Virchow hypothesized inflammation could enhance cell proliferation and that chronic inflammation could trigger cancer (Balkwill et al. 2001). In 1941 Rous recognized that cancer could initiate from viral and chemical carcinogens (Rous et al. 1941). This initiation is irreversible (since it causes irreversible DNA alterations) or can last in normal tissue until a second exposure to a carcinogen, that can be also a chemical irritant (eg.phorbol esters), hormones, chronic irritation and inflammation, occurs (Coussens et al. 2002).

It is estimated that more than 15% of malignancies is caused by infections (Kuper et al. 2000). In chronic inflammation leukocytes and other immune cells induce release of free radicals of oxygen and nitrogen species, which can induce DNA damage in proliferating cells. When this process of inflammation repeats there is a risk of permanent genomic alterations such as point mutations, deletions, or rearrangements (Maeda et al. 1998).

There are various examples of chronic inflammation that cause cancer. There is a strong association of inflammatory bowel diseases like chronic ulcerative colitis and Crohn's disease with the development of colon cancer (Coussens et al. 2002). Patients with hepatitis C have higher risk of liver carcinoma; schistosomiasis is associated with bladder and colon carcinoma (Coussens et al. 2002) and Helicobacter pylori infection is the principle cause of stomach cancer (Ernst et al. 2000).

Additional evidence that inflammation is important during tumour development and progression is given by the study that aspirin and nonsteroidal anti- inflammatory drugs (NSAID) are associated with reduced risk of cancer (Garcia-Rodríguez et al. 2001; Agrawal et al. 2008;Takkouche et al. 2008), and are prophylactic for lung, oesophagus and stomach cancer (Baron et al. 2000;Garcia-Rodriguez et al. 2001). NSAIDs target and inhibit cyclooxygenases (COX-1 and -2). COX-2, in particular, is involved in inflammatory reactions and is overexpressed in cancer.

1.9.1 Cyclooxygenase and inflammation.

Cyclooxygenase (COX) or prostaglandin endoperoxidase synthase converts arachidonic acid into the unstable intermediate prostaglandin G2. Cyclooxygenase has two actions: 1) an endoperoxide synthase action, which converts the arachidonic acid (released from membrane phospholipids by phospholipase A2) into prostaglandin G2; 2) a peroxidase action that converts PGG_2 to PGH_2 (Wang & Dubois, 2006). Prostaglandin H2 is the precursor for several structurally related prostaglandins, like prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), prostaglandin F2a (PGF2a), prostaglandin I2 (prostacyclin) and thromboxane-A2, which are formed by the action of specialized prostaglandin synthases (as shown in figure 1.19).
COX enzymes are the targets of non-steroidal anti-inflammatory drugs (NSAIDs) and in 1971 Vane and collaborators demonstrated that NSAIDs could inhibit prostaglandin formation and has in vivo anti-inflammatory activity (Vane, 1971). At present, three COX isoenzymes are known: COX-1, COX-2 and COX-3. COX-1 protein was discovered over 30 years ago and cloned in 1988. Daniel Simmons and collaborators discovered COX-2 in 1991 at Brigham Young University (Simmons et al. 1989, Xie et al. 1991).

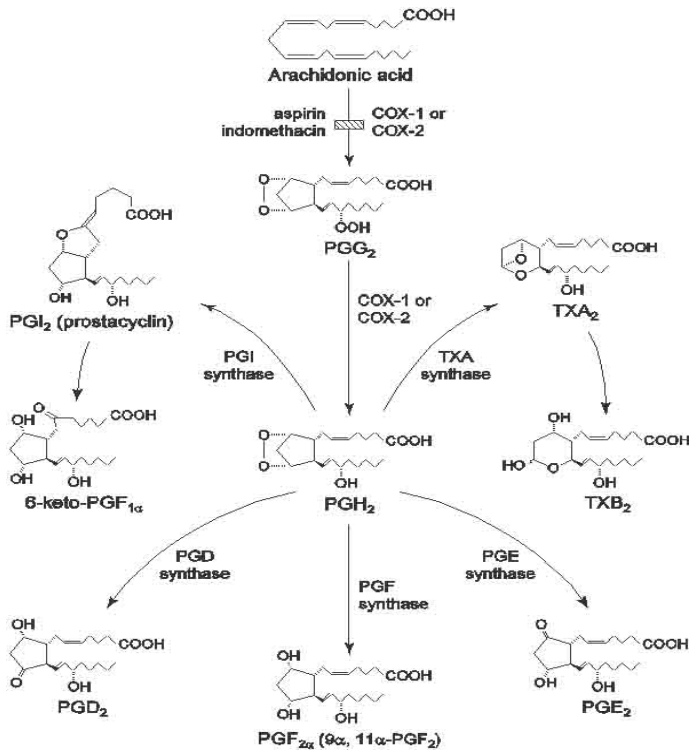


Figure 1.19: Arachidonic acid cascade. (Botting 2004, Frontiers in Bioscience 9, 956-966)

Simmons's group discovered COX-3 in 2002. COX-3 is a splice variant of COX-1, which, in humans and rodents, has a frameshift mutation. For this reason, COX-3 encodes a protein with a completely different amino acid structure from COX-1 or COX-2 and does not have COX activity (Kis et al. 2005).

1.9.2 COX-1 and COX-2: similarities and differences.

COX-1 and COX-2 are encoded by two different genes (Reference Sequence: NM_080591.1 and NM_000963.2) on different chromosomes 9q32-q33.3 and 1q25.2-q25.3. The sequence and the protein tertiary structure of the two isoforms are very similar. Numerous studies have shown that both COX-1 and COX-2 are located inside the lumen of the endoplasmic reticulum and the nuclear envelope (Morita et al.1995). The proteins are of similar molecular weight (approximately 70 and 72 kDa respectively), highly homologous in sequence (60% identity at the amino acids level) and have nearly identical three-dimensional structure.

COX-1 and COX-2 are homodimers (Kurumbail et al. 2001) and have four common domains (identified by X-ray crystallography): amino-terminal signal, an epidermal

growth factor domain or dimerization domain, a membrane-binding motif, a catalytic domain.

1. **N-Amino Terminal Signal Peptide**. COX-2 amino-terminal signal peptide region is 17 amino acids long in all species and is less hydrophobic than the COX-1 peptide, which contains 4 or more leucines and isoleucines. The amino-terminal signal peptides are important in directioning COX-1 and COX-2 into the lumen of the endoplasmic reticulum (HLa et al. 2002).

2. **Dimerization Domain**. COX-1 and COX-2 are dimers and each monomer has a dimerization domain, held together by hydrogen bonds and salt bridges. The dimerization domain consists of about 50 amino acids near the amino terminus. This domain has 3 disulphide bonds, resulting in a similar structure to epidermal growth factor structure.

3. **Membrane Binding Domain.** This region consists of 50 amino acids found just after the dimerization domain. It is composed of a tandem series of four amphipathic helices, which form a hydrophobic channel.

4. **C-terminal Catalytic Domain**. The carboxy-terminal is formed by 480 amino acid and it represents 80% of the protein (Simmons et al. 2004). The catalytic domain has two distinct lobes that are linked together: Cyclooxygenase Active Site and Peroxidase Active Site. The **Cyclooxygenase Active Site** catalizes the conversion of arachidonic acid to prostaglandin G2. This region is a hydrophobic dead-end channel and the entrance is surrounded by the four amphipathic helices of the membrane binding domain (Picot et al. 1994). Tyrosine 385 of the catalytic pocket activates arachidonyl radical that undergoes the cyclization/oxygenation reaction (Schneider et al. 2002). The **Peroxidase Active Site** reduces prostaglandin G2 to prostaglandin H2. This site contains a heme cofactor, which is linked via an iron-histidine bond (Malkowski et al. 2000). Arg-120, Glu-524 and Tyr-355 are part of the hydrogen-bonding network, which stabilizes substrate/inhibitor interactions. NSAIDs generally bind at the mouth of the COX channel between Tyr385 and Arg120 (Picot et al. 1994). The synthesized PGH2 is converted to prostaglandins (PGD2, PGE2, $PGF_{2\alpha}$), prostacyclin (PGI2), or thromboxane A2 by different synthases (Hamberg et al. 1974). Although, COX-1 and COX-2 have a very similar structure, there are some important differences between those two isoforms.

Table 1.6 Action of COX-1 and COX-2 in the stomach. (Wallace JL. 2008)

Effect (Species)	COX Isoform
Inhibition of gastric acid secretion (rat)	COX-1
Inhibition of gastric acid secretion (mouse)	COX-1
Bicarbonate secretion/juxtamucosal pH gradient in stomach (mouse)	COX-1
Maintenance of mucosal surface hydrophobicity (mouse)	COX-1
Decreased epithelial permeability to acid (rat)	COX-1
Mucosal blood flow (rat)	COX-1
Ulcer healing (rat)	COX-2
Ulcer healing (mouse)	COX-2
Resistance to ischemia/reperfusion-induced gastric damage (mouse)	COX-2

COX-1 and COX-2 are N-linked glycosylated. COX-1 is glycosylated at three asparagines, whereas COX-2 may be glycosylated at up to four asparagines. (Nemeth et al., 2001). Glycosyl moieties are of the high mannose type. The glycosylation on COX-1 promotes the right protein folding and it is essential for COX-1 catalitic activity (Otto et al., 1993).

COX-2 is frequently observed in two or three glycosylated states according to Western Blot and mass spectrometry results analysis (Nemeth et al., 2001). The most common form of COX-2 is the glycosylated form at Asn^{580} (74 kDa) and the unglycosylated (72 kDa). Sevigny et al. demonstrated that COS cells expressing COX-2 glycosylated form at Asn^{580} have five times less COX-2 activity than cells expressing the not glycosylated COX-2. Thus, COX-2 turnover appears to depend upon glycosylation of the 72kDa glycoform (Sevigny et al. 2006).

COX-1 is the constitutive isoform and its expression does not change under physiological and pathological conditions. The prostaglandins, produced by COX-1, play a central role in many physiological processes, like cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane, by the platelets (Fitzpatrick et al. 2001). It was shown that COX-1 is the dominant isoform, regulating the relatively alkaline pH at the gastric luminal surface in mouse stomach (Baumgartner et al. 2004). Table 1.6 shows how COX-1 is involved in stomach cytoprotection and how COX-2 is involved in ulcer healing and resistance in ischemia induced gastric damage.

COX-2 is normally not expressed in most cells and tissues (apart from some parts of the brain, kidneys, ovaries) and its expression, induced by cytokines and growth factors, is responsible for the high production of prostaglandins during inflammation (Wang, 2006). In fact, according to the 'COX-2 hypothesis'—the "inflammatory prostaglandins" derive principally from COX-2, while prostaglandins important in homeostatic roles, like the protection of the gastrointestinal mucosa, are derived from COX-1 (Fries et al. 2005). So, the inhibition of all prostaglandins and of thromboxanes by COX-1 and COX-2 inhibitors, (like aspirin, ibuprofen, diclofenac etc) explains the typical side effects of NSAIDs like, gastrointestinal toxicity, ulcer perforation etc.

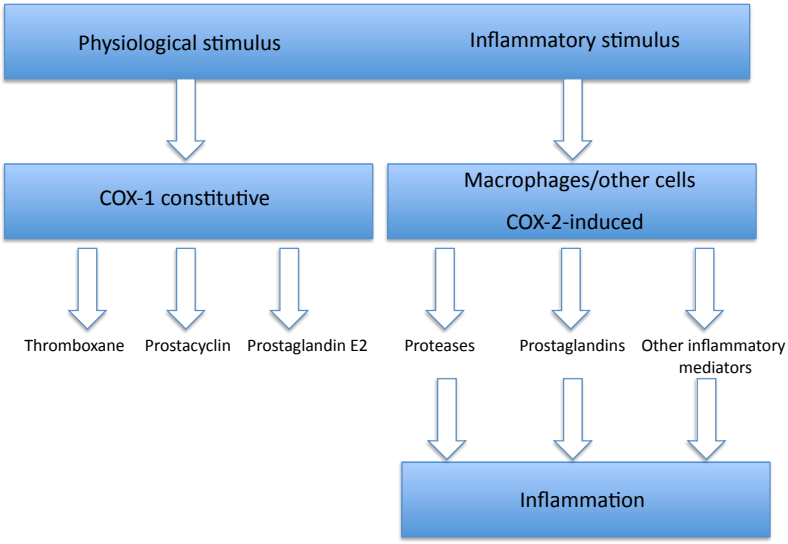


Figure 1.20: Actions of the two known isoforms of COX (PGHS). The hypothesis stemming from this scheme is that the therapeutic effects of drugs, such as aspirin, are due to inhibition of COX-2 whereas the unwanted side effects (and the action on platelets) stem from inhibition of COX-1. (Vane 1994)

Secondly, COX-2 has a hydrophobic "side pocket", created by Valine -523, hydrogen bonds to Arginin-513 and the peptide bond of Phenylalanine-518. This pocket is not present in COX-1, since the Val-523 is substituted by an isoleucine at position 523, that creates steric hindrance (Kurumbail et al.1996). The presence of the "side pocket", created by Val-523, allows the design of inhibitors selective for COX-2. These selective inhibitors block COX-2 with high affinity, but are too big to fit into the COX-1 catalytic domain (see figure 1.21). This overcomes the major side effects of the non-selective inhibitors of COX-2, which block the production of the good prostanoids from COX-1. This class of NSAIDs is called "second generation COXIBs" and comprises etoricoxib, lumiracoxib, rofecoxib, valdecoxib, celecoxib.

1.9.3 Prostaglandins and Cyclooxygenase

A prostaglandin is a lipid compound containing 20 carbon atoms including a 5 carbon ring and are derived enzymatically from fatty acids and, in particular, from arachidonic acid. Goldblatt and von Euler were the first to extract prostaglandins from semen, prostate, and seminal vesicles in the 1930s. Between 1950s and 1960s Bergström and collaborators purified the first prostaglandin isomers and identified that arachidonic acid is the precursor of prostaglandins. Samuelsson and colleagues identified the cyclooxygenase reaction that catalyzes the production of PGG_2 from arachidonic acid (Hamberg & Samuelsson, 1973).

Prostaglandins are classified into types A to I, however type A, B, C are not found in nature and are only produced artificially. PGH2 and PGG2 have the same structure but PGG2 has a hydroperoxy instead of a hydroxy group on the C15. As said above, the most important products of cyclooxygenase pathway are PGI2, PGD2, TXA2, PGE2 and PGF2α (Narumiya et al. 1999).

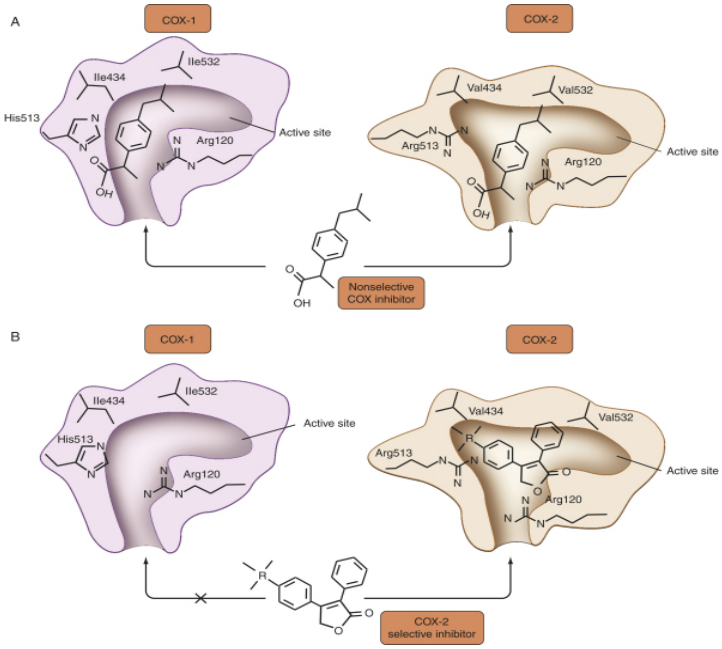


Figure 1.21: COX-1 and COX-2 substrate-binding channels. A, Nonselective inhibitors have access to the binding channels of both isoforms. B, The more voluminous residues in COX-1, Ile434, His513, and Ile532, obstruct access of the bulky side chains of the coxibs (Grosser et al. 2006).

PGI_2 is a mediator of oedema and pain and is found after tissue injury or inflammation. It causes vasodilatation, inhibition of platelets aggregation, rennin natriuresis.

PGD_2 causes vasodilatation, inhibition of platelet aggregation, uterine and gastrointestinal relaxation. $PGF_2\alpha$ is a mediator of myometral contraction in humans.

TXA_2 has vasoconstriction, bronchoconstriction, platelets aggregation effect and it is mainly produced by COX-1 (Mukherjee et al. 2001). PGE_2 can have different effects depending on the receptor it binds. There are four PGE_2 receptors (EP receptors) that can be expressed in different tissues. If PGE_2 binds to EP_1 there is contraction of bronchial and gastrointestinal smooth muscle; if it binds to EP_2 or EP_4 it causes bronchodilatation, vasodilatation, relaxation of GI smooth muscle; binding EP_3 causes inhibition of gastric acid secretion, contraction of intestinal muscle and stimulation of contraction of the uterus in pregnant women (Rang & Dale 2003).

In summary, prostaglandins are important pathological mediators, since they are implicated in inflammation, pain, pyrexia, cancer, glaucoma, male sexual dysfunction, osteoporosis, cardiovascular disease and asthma (Abramovitz et al.1998). They are also important in physiological functions such as homeostatis of the gastrointestinal tract, renal function, blood clotting, embryonic implantation and parturition (Simmons et al. 2004).

Considering that they are lipids, prostanoids are transported through a prostaglandin transporter across biological membranes. Bao et al. only ten years ago found the first known PG transporter in rats (Bao et al. 2002). The half-life of most prostaglandin is very short (between 30 seconds and 1 minute). Once prostanoids are uptaken by the carrier, there is a rapid inactivation by several intracellular enzymes, like 15-hydroprostaglandin dehydrogenase (15-PGDH). The best substrate for 15-PGDH is PGE_2 and $PGF_2\alpha$. There are two isoforms of 15-PGDH: one is found in most organs of the body (including lung, GI tract, kidney, heart) and it needs NAD^+ as a cofactor, while the second isoform is mainly in the kidney, brain and erythrocytes and the cofactor is $NADP^+$. The metabolites are secreted in the urine (Rang & Dale 2003).

1.9.4 Prostanoid receptors

There are two DP receptors (DP_1 and DP_2) for PGD2, two IP receptors (IP_1 and IP_2) for PGI2, one TP for TXA2, one FP for PGF2α and four EP receptors for PGE2. The prostanoid receptors are all G-protein-coupled receptors (GPCRs) and have seven-transmembrane regions, an extracellular N terminus, an intracellular C terminus, and three interhelical loops on each side of the membrane.

G proteins are composed by three subunits: α, β, γ. The α subunit, with its enzymatic activity, converts GTP (Guanine nucleotide) to GDP. The β and γ subunits are very hydrophobic and so they remain associated with each other within the cytoplamic mem-

brane. When an agonist binds a G coupled receptor protein, the cytoplasmic domain of the receptor acquires high affinity with the α, β, γ subunits, which, at this stage, bind a molecule of GDP. When the receptor is activated, GTP replaces GDP and the α-GTP complex dissociates from the $\beta\gamma$ subunits. α-GTP complex is the active G protein, which in turn, activates or inactivates other ion channels or enzymes (eg.like adenylate cyclase). The complex gets inactivated, when GTP is hydrolysed to GDP by the GTPase activity of the α unit, and this allows the α subunit to reassociate with $\beta\gamma$ and start a new cycle (Lodish et al. 2000, Rang & Dale 2003).

There are different G-proteins that interact with specific receptors and effectors: adenylate cyclase, phospholipase C, Rho family GTPase. There are four types of G-proteins: G_s, $G_{i/0}$, $G_{q/11}$, $G_{12/13}$. The G_s activates plasma membrane adenylyl cyclases, increasing cellular cyclic AMP (cAMP). $G_{i/0}$ inhibits adenylyl cyclases, decreasing cellular cAMP. The level of cytosolic cAMP may then determine the activity of various ion channels as well as members of the Ser/Thr specific Protein Kinase A (PKA) family. $G_{q/11}$ activates phospholipase Cβ (PLCβ), which cleaves phosphatidylinositol 4,5-biphosphate (PIP_2) at the plasma membrane, and generates inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG) that releases Ca^{2+} from intracellular stores and activates phosphorylation by protein kinase C. IP_3 acts on IP_3 receptors on the membrane of the endoplasmic reticulum (ER) to elicit Ca^{2+} release from the ER, while DAG diffuses along the plasma membrane where it may activate Protein Kinase C (PKC). Since many isoforms of PKC are also activated by increases in intracellular Ca^{2+}, these pathways can converge on each other to signal through the same secondary effector. The effectors of the $G_{12/13}$ pathway are three RhoGEFs (p115-RhoGEF, PDZ-RhoGEF, and LARG), which, when bound to $G_{12/13}$ allosterically, activate the cytosolic small GTPase, Rho.

Prostaglandin E2 (PGE2) is the most abundant prostanoid in the human body and it has various functions in reproduction, neuronal, metabolic, and immune systems. PGE2 action is mediated via four receptors (EP_1, EP_2, EP_3, EP_4) with different signalling pathways. EP_1 is mainly a G_q coupled receptor with phospholipase C signalling through Ca^{2+}; EP_2 and EP_4 are G_s coupled receptors signalling through adenylyl cyclases; EP_3 instead is mainly a G_i coupled protein and inhibits adenylyl cyclases, decreasing cellular cAMP. The EP_2 and EP_4 receptors stimulates of cyclic AMP (cAMP) and protein kinase A (PKA) (Ichikawa et al. 2010). However EP_2 is more efficient than EP_4 in inducing cAMP probably because EP_4, unlike EP_2, can also activate Phosphoinositide-3-kinase (PI3K) through G_i (Fujino & Regan. 2006). EP_2 and EP_4 differ also in their sensitivity to PGE2 and EP_4 is desensitized after short-term exposure (Nishigaki et al. 1996).

The EP_3 and EP_4 receptors are found throughout the body, (Honda et al.1993), while EP1 is expressed only in the kidney, lung, and stomach(Rang & Dale 2003). EP2 is the least expressed of the EP receptors, but it is induced by different stimuli, like LPS in a macrophage cell line and gonadotropins in the uterus (Katsuyama et al. 1997, Katsuyama et al. 1998). EP_1 and EP_3 have alternative mRNA splice variants while EP_3 has eight different isoforms in humans and it can associate with G_i, G_s, G_q, and $G_{12/13}$ (Israel & Regan et al. 2009).

This is summarized in table 1.7 (Narumiya et al. 1999). The EPs are G-protein-coupled receptors (GPCRs), so they have seven-transmembrane α helices, an extracellular N terminus and intracellular C terminus.

Table 1.7: Classification of the prostanoid EP receptors for PGE2 and signalling pathway.

Receptor	Coupled G protein	Signal
EP1	$G_{q/11}$, (G_i)	↑ $IP_3/DAG/Ca^{2+}$
EP2	G_s	↑ cAMP
EP3	G_i, (G_s, $G_{q/11}$, $G_{12/13}$)	↑ cAMP ↓ $IP_3/DAG/Ca^{2+}$
EP4	G_s	↑ cAMP

1.9.5 Synthetic Cyclooxygenase Inhibitors-Nonsteroidal antiinflammatory

The principal mechanism of action of NSAIDs (Nonsteroidal anti-inflammatory drugs) is to target cyclooxygenase. This class includes nonsteroidal anti-inflammatory, nonsteroidal analgesics and antipyretic (fever-reducing) drugs. The first NSAID discovered was aspirin. The active ingredient is a compound called salicilin, which is present in the willow tree. Although the salicilin was discovered in the XIX century by Leroux and it was introduced on the market by Bayer with the name Aspirin, bark and leaves of the willow tree were used by Hippocrates (460-377 B.C.) to heal pain and fevers. At the end of the 19th century other drugs with the same anti-inflammatory properties of the aspirin were discovered. In 1963 indomethacin was introduced as a cure for the rheumatoid arthritis and many other compounds with similar actions have

been introduced in the last few years, including the latest COX-2 selective inhibitors (COXIBs) (Rao & Knaus, 2008).

NSAIDs differ in how quickly they bind the COX active site and how they are released from the COX channel. Traditional NSAIDs (tNSAIDs), ie. nonselective inhibitors for COX-1 and COX-2, have three different modes of binding: 1) reversible binding (eg: ibuprofen), 2) slowly reversible binding-time-dependent, (eg: fluorbiprofen, indomethacin), 3) irreversible binding caused by a covalent modification of the enzyme (eg: aspirin) (Marnett et al. 1998). Furthermore, NSAIDs can be classified based on their chemical structure: a) Salicylates (aspirin, diflunisal), b) Propionic acid derivatives (Naproxen, Ibuprofen, Ketoprofen etc.), c) Acetic acid derivatives (indomethacin, diclofenac etc), d) Enolic acid (Oxicam) derivatives (Piroxicam, Meloxicam), e) Fenamic acid derivatives (Mefenamic acid, Meclofenamic acid etc.), f) Sulphonanilides (Nimesulide), g) selective COX-2 inhibitors (COXIBs) (Celecoxib, Parecoxib, Rofecoxib, Valdecoxib etc.).

As reported in the previous section, selective inhibitors of COX-2, the COXIBs, were developed to avoid or at least decrease the risk of gastrointestinal complications. Selective COX-2 inhibitors bind tightly to the COX-2 active site (Rao et al. 2008) and valdecoxib and rofecoxib are about 300 times more potent at inhibiting COX-2 than COX-1, while, Celecoxib is approximately 30 times more potent at inhibiting COX-2 than COX-1. Table 1.8 shows IC50 for COX-1 and COX-2 for selective and non-selctive COX-2 inhibitors.

Some patients using COXIBs reported superior pain relief compared to that experienced on traditional nonsteroidal anti-inflammatory drugs (tNSAIDs). This was not true for all patients and the clinical response to a particular NSAID vary from patient to patient (Fries et al. 2005). Even if gastrointestinal side effects decreased, COXIBs (rofecoxib, valdecoxib, and celecoxib) are associated with increased incidence of cardiovascular complications like stroke, myocardial infarction (Cannon et al. 2006; FitzGerald et al. 2004). Since COX-2 is the predominant source of vascular prostacyclin (PGI2) (important for vasodilatation and inhibition of platelets) and TXA2 (cause of vasoconstriction, bronchoconstriction, platelets aggregation) is mainly produced by COX-1, selective inhibition of COX-2 alters the balance of PGI2 and TXA2 synthesis more than tNSAIDs. This is the most frequently proposed mechanism to explain the adverse prothrombotic effects of COXIBs (Rabausch et al. 2005, Cheng et al. 2002).

Table 1.8: Comparison of nonsteroid anti-inflammatory drugs for their selectivity towards COX-1 and COX-2 (Vane et al. 1998)

Drug	IC_{50} COX-1 (μM)	IC_{50} COX-2 (μM)	Ratio IC_{50} COX-2/COX-1
Nonselective for COX-2			
Piroxicam	0.0005	0.3	600
Aspirin	1.67	278	166
Indomethacin	0.028	1.68	60
Diclofenac	1.57	1.1	0.7
6-MNA[a]	278	187	0.67
Selective for COX-2			
Etodolac	34	3.4	0.1
Meloxicam	4.8	0.43	0.09
Nimesulide	9.2	0.52	0.06
SC58125	38.7	0.27	0.007
NS398	16.8	0.10	0.006
L-745, 337	369	1.5	0.004
Celecoxib	15	0.04	0.003
DFU	>50	0.04	<0.001

Celecoxib Long-term Arthritis Safety Study (CLASS; 8059 patients) confirmed that COXIB drugs are associated with an increased risk of thrombotic cardiovascular events (myocardial infarction, unstable angina, cardiac thrombus, resuscitated cardiac arrest, sudden or unexplained death, ischemic stroke, and transient ischemic attacks) (Mukherjee et al. 2001).

COXIBs represent a cardiovascular hazard, so, on FDA recommendation, Rofecoxib and Valdecoxib were withdrawn from the market by Merck and Pfizer (the pharmaceutical companies that first marketed these drugs) (FDA, Center for Drug Evaluation and Research, 2008). Celecoxib is the only and the best selling COXIB on the market (Fries et al. 2005). Epidemiological and experimental studies have reported that COXIBs can reduce the risk of colonic tumors (Steinbach et al. 2000).

The FDA (Food and Drug Administration) approved the use of Celebrex (Celecoxib) in patients with familial adenomatous polyposis (FAP). Because of the increased risk of cardiovascular (CV) diseases, COXIBs may be best suited for use by young patients (with a reduced risk of CV diseases) with familial adenomatous polyposis (FAP) (Garcia Rodriguez et al. 2012).

However in a meta-analysis of randomised trials in patients receiving celecoxib, there was no increased cardiovascular risk with celecoxib relative to placebo. Furthermore, it was demonstrated that there was a comparable rate of cardiovascular risk both with the use of celecoxib and nonselective NSAIDS (White, 2007). So the association of

increased cardiovascular risk with COXIBs is still controversial and there are various clinical trials using Celecoxib for different diseases (see section 1.10.1).

1.9.6 Inducers of COX-2 expression.

The human COX-2 gene is on chromosome 1 q25.2–q25.38 (Khuri et al 2001). It is approximately 8.3 kb long and comprises 10 exons (Appleby et al. 1994). The promoter region of the COX-2 gene was well characterized. Xie and Herschman (1995) showed that the cAMP response element (CRE) on the promoter was critical for transcription (Xie & Herschman, 1995). Sequence analysis of the 5′-flanking region revealed several potential transcription regulator elements like the cAMP response element (CRE), the C/EBP-NF-IL6 ("CAAT/enhancer binding protein"), NFAT ("nuclear factor of activated T cells") and NFκB ("nuclear factor kappa B cells") sites (see figure 1.22), the E-box, SP1 (Crofford et al.1997; Herschman et al.1997; Sirois & Richards, 1993; Diaz-Munoz et al. 2012). CRE and AP-1 binding regions are located at –60 to –40 from the transcription start site and overlap with CRE binding protein (CREB), ATF, C/EBP, C-Jun, C-Fos, and USF binding sites (Schroer et al. 2002) (shown in figure 1.22).

There are several inducers of COX-2 expression. Bacterial lipopolysaccharide (LPS) was the first to be identified (Lee et al. 1992). This factor can activate COX-2 through several pathways like MEK/ERK and the TRAF6/NIK/Tpl2/IKK/NFkB pathway (Chen et al. 2005). Several cytokines such as IL-1 or IFN-γ (Caivano et al. 2000) and several growth factors including IGF (by PI3Kinase and Src/ERK), TGFα and EGF (through p38MAPK, ERK1/2 and PI3K pathways) can induce COX-2 expression (Chun et al. 2004). Moreover, UVB was shown to induce COX-2 mRNA using cancer cells signalling through CREB, ATF1, p38MAPK and PI3K (Bachelor et al. 2005). Glycogen synthase kinase 3 (GSK3) phosphorylates/inactivates β-catenin, leading to inhibition of the transcription factor TCF4, which regulates COX-2 transcription (Dihlmann et al. 2003). Cyclooxygenase 2 (COX-2) is also induced by p53 and DNA damage through Ras/Raf/MAPK cascade (Han et al. 2002).

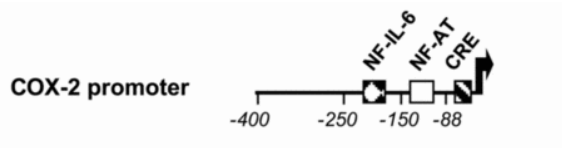


Figure 1.22: Structure of the human COX-2 promoter region. Transcription factor binding sites in the 5′-flanking sequences (promoter) of the human Cox-2 gene (Diaz-Munoz et al. 2012).

1.9.7 TPA induction of COX-2.

The phorbol ester TPA is another COX-2 inducer. TPA (12-O-Tetradecanoylphorbol-13-acetate) a tetracyclic diterpenoid, is a tumor promoter. TPA mimics the action of diacyl glycerol (DAG), an activator of protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities. TPA can influence the cell cycle. In a study on HeLa cell lines, it was seen that TPA blocked G2 phase of cell cycle (Kinzel et al. 1984). Using guinea pigs, TPA was shown to cause inflammation and epidermal hyperproliferation, by inducing DNA synthesis through prostaglandin activation, especially prostaglandin E (Bourin et al. 1982). However, TPA in high concentrations can be toxic and results in death of animals, reduced milk yield and reproduction (Forsyth et al.1968). TPA increases binding of CREB-2, C-Jun, and C-Fos to the CRE/AP-1 region and C/EBPβ to the C/EBP element at –124 to –132 of human COX-2 (Schroer et al. 2002; Saunders et al. 2001). In breast cancer cell lines, turmerone, a natural remedy with anti-inflammatory property, suppressed the TPA-induced up-regulation of COX-2 expression, by blocking NF-κB, PI3K/Akt and ERK1/2 signalling (Park et al. 2012).

Another mechanism of TPA induction of COX-2 involves PKC pathway. In glioblastoma cells, activation of protein kinase C (PKC) and extracellular signal-regulated kinases (ERKs) are involved in TPA-induced migration/invasion, COX-2 protein expression, and MMP-9 activation (see figure 1.23) (Chiu et al. 2010). The epidermis of TPA-treated mice, overexpressing PKCα, displayed increased expression of cyclooxygenase-2 (COX-2) compared to TPA treated wild type mice (Wang & Smart 1999). TNF-α induces COX-2 expression via activation of ERK1/2 and NFκB (Nakao et al. 2002), but can also induce its expression through the PKC pathway. In pulmonary carcinoma cells, a protein kinase C (PKC) inhibitor, staurosporine, attenuated TNF-α or TPA induced COX-2 promoter activity, whereas overexpression of PKCα induced COX-2 promoter activity (Huang et al. 2003).

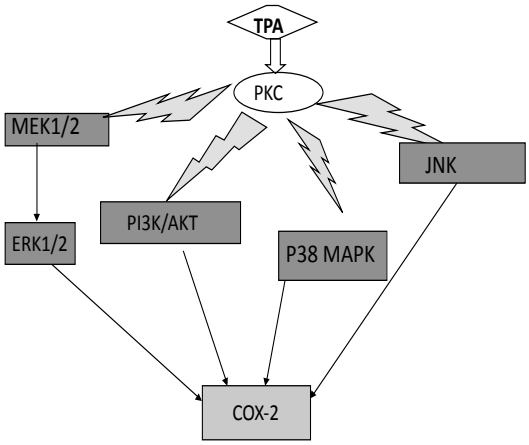


Figure 1.23: TPA induction of COX-2 expression.TPA binds to PKC and activates MEK1/2, PI3K/AKT, p38 MAPK, JNK, all of which can activate COX-2.

1.10 COX-2 in breast cancer.

Increased levels of COX-2 were reported in carcinomas of the colon (Eberhart et al. 1994), stomach (Ristimaki et al. 1997), esophagus (Zimmermann et al. 1999), lung (Hida et al. 1999), liver (Koga et al. 1999) and pancreas (Tucker et al. 1999). Moreover, COX-2 was found to be overexpressed in about 40% of cases of invasive breast carcinoma (associated with unfavorable distant disease-free survival) (Ristimaki et al. 2002) and at a higher frequency in preinvasive ductal carcinoma in situ (Boland et al. 2004). COX-1 is ubiquitously expressed in mammary tissues, while COX-2 expression was observed confined to the tumor epithelium (Simmons et al. 2004). COX-2 mediates its deleterious effects via prostaglandin synthesis. In particular, PGE2 was observed in high levels in breast tumor cells and it is synthesized by several human breast cancer cell lines (Schrey et al. 1995). PGE2 is an immunosuppressive agent and can, therefore, contribute to the evasion of tumour-specific immune responses. It was demonstrated that PGE2 shifts the tumour immune response from Th1 to Th2 (Luft et al. 2002), by downregulating Th1 cytokines (TNF-α, interferon-γ and IL-2) and increasing the secretion by dendritic cells of Th2 cytokines (IL-4, IL-10 and IL-6).

COX-2 overexpression in human breast cancers is associated with factors causing poor prognosis, such as overexpression of HER2 and overexpression of aromatase (Ranger et al. 2004; Brueggemeier et al. 1999). Overexpression of HER2 can induce COX-2 transcription in different breast cancer cell lines (Subbaramaiah et al. 2002; Half et al. 2002). Both HER2 and COX-2 are expressed at high frequencies in DCIS and so the combined targeting of COX-2 and HER-2 was suggested as a potential strategy to prevent progression of DCIS (Boland et al. 2004). It was also demonstrated that

prostaglandin E2 (PGE2) can stimulate aromatase activity and estrogen biosynthesis in the surrounding adipose tissue (Purohit et al. 2002 a; Purohit et al. 2002 b) and EP_1 and EP_2 selective antagonists decreased aromatase activity (Richards et al. 2003). Local estrogen production by breast tumors is considered to be an important mechanism by which tumors develop, evolve, and possibly metastasize (Purohit et al. 2002a).

Ristimaki et al. observed that elevated expression of COX-2 in ER-positive tumors is also associated with poor survival (Ristimaki et al. 2002), and Díaz-Cruz that COX-2 inhibitors decreased aromatase mRNA and activity in breast cancer cells (Díaz-Cruz et al. 2005). The use of combined COX-2 and aromatase inhibitor was more effective than the single agent used alone in decreasing aromatase activity and, consequently, estradiol production (Prosperi and Robertson, 2006). So, it is possible that elevated COX-2 expression in ER-positive cancers could enhance estrogen production via the aromatase pathway in the stromal cells and cause tumorigenesis and poor survival. However, there are conflicting reports as Ristimaki et al. found that elevated levels of COX-2 are associated with negative and positive hormone receptor status (Ristimaki et al. 2002), Zerkowski et al. found a correlation with negative ER status (Zerkowski et al. 2007), while Ranger did not find any correlation (Ranger et al. 2004).

In breast cancer cells, COX-2 may contribute to the enhancement of lymph node metastasis and distant metastasis. Lymph node metastasis is associated with high expression of VEGF-C (vascular endothelial growth factor) and the prostaglandin EP_1 and EP_4 receptors are involved in COX-2 mediated VEGF-C up-regulation (Su et al. 2004). COX-2 up-regulates, via EP_2 and EP_4 receptors, CCR7 (a chemokine receptor associated with breast cancer metastasis) expression in breast cancer cells. It regulates matrix metalloproteinase expression, which can digest the extracellular matrix and the basement membrane (Pan et al. 2008). Gene expression analyses and mouse model systems revealed correlations between COX-2 overexpression and breast cancer metastasis to the lung, bone, and brain (Singh et al. 2007; Minn et al. 2005; Bos et al. 2009). Moreover, COX-2 overexpression, in MDA-MB-231 breast cancer cell line, increases their motility and invasion (Singh et al. 2005), while silencing of COX-2 inhibits migration of this line in vitro and metastasis in vivo (Stasinopoulos et al. 2007).

COX-2 is also involved in angiogenesis and in migration of endothelial cells. Angiogenesis is the formation of new blood vessels from preexisting ones and it is important in tumorigenesis for cell growth and metastasis (Costa et al. 2002). In COX-2 knockout mice, the expression level of angiogenesis-related genes (*VEGF*, *Ang1*, and *Flt1)* in the mammary glands was found significantly reduced compared to that found in COX-2 wild-type (Howe et al. 2005). PGE2 stimulates VEGF secretion through the

stimulation of ERK2/JNK1 signalling pathways in endothelial cells (Pai et al. 2001) and in ovarian cancer cells the EP_2/EP_4 receptors have been shown to be involved (Spinella et al. 2004). Moreover, Timoshenko et al. demonstrated that VEGF production could be blocked by EP_1 and EP_4 inhibition in several human breast cancer cell lines (Timoshenko et al. 2003). However, other studies suggested that EP_3 receptor could play a role in angiogenesis. In a model that can mimic tumor-stromal angiogenesis, an EP_3 agonist, ONO-AEI-248, increased angiogenesis in a dose dependent manner and, in EP_3 knockout mice, angiogenesis was significantly reduced compared to wild-type mice (Amano et al. 2003). Celecoxib was shown to decrease tumor angiogenesis (reducing functional vessel density) and to reduce the growth of a variety of experimental primary tumors, including colorectal, prostate, and breast tumors (Klenke et al. 2006).

COX-2 can suppress apoptosis and inhibit the apoptosis mechanism, caused by some anti cancer drugs (Chang et al. 2001). Apoptosis (programmed cell death) is essential to mantain tissue homeostasis and any failure in this mechanism can cause excessive cell survival, essential for tumorigenesis (Hanahan and Weinberg, 2011). There are many mechanisms by which cancer cells can evade apoptosis and one of them is the overexpression of Bcl-2 protein, that can inactivate the apoptotic caspase cascade upstream of the mitochondria (Danial & Korsmeyer, 2004). It was shown that in transgenic mice overexpressing the human COX-2 gene in the mammary gland, there were reduced levels of the proapoptotic proteins Bax and Bcl-x_L and an increase of Bcl-2, suggesting that enhanced COX-2 expression is sufficient to inhibit apoptosis and induce mammary gland tumorigenesis. Therefore, inhibition of COX-2 may represent a mechanism-based chemopreventive approach for carcinogenesis (Liu et al. 2001). Indeed, the cell apoptosis induced by celecoxib in a mouse mammary tumour was associated with an activation of antiapoptotic/prosurvival kinase (Akt) and increased expression of proapoptotic protein Bax (Basu et al. 2004).

1.10.1 NSAIDs and breast cancer.

COX-2 expression associated with cancer is considered a possible target for breast cancer chemoprevention and therapy. Studies that examined the correlation between risk of breast cancer and use of NSAIDs, like aspirin and ibuprofen, published between 1966 and 2008, were analyzed by meta-analysis. Among 38 identified studies (including about 3 million people), it was found that NSAIDs are associated with

reduced risk of breast cancer (Takkouche et al. 2008; Agrawal et al. 2008). These meta-analyses included the study of Harris et al. that showed a reduced risk of breast cancer in 66% of women using ibuprofen and aspirin for more than one year (Harris et al. 1996). A reduction in incidence of breast cancer was shown in patients taking aspirin and other NSAIDs in two studies from Schreinemachers and Everson and Sharpe et al. (Schreinemachers & Everson, 1994; Sharpe et al. 2000). For its cardiovascular benefit and efficacy data, aspirin is the most likely NSAID for chemoprevention in cancer (Cuzick et al. 2009).

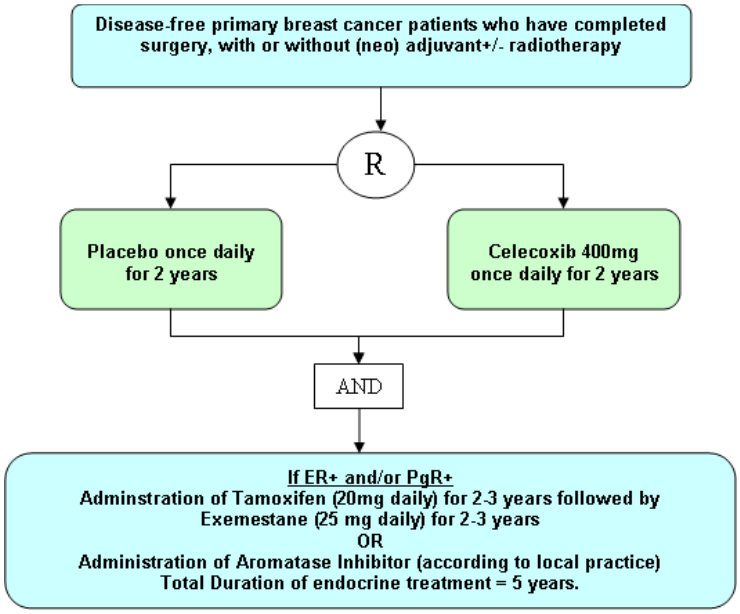


Figure 1.24 Design of phase III clinical trial REACT (Randomised European Celecoxib Trial). This diagram was taken from the website http://www1.imperial.ac.uk/surgeryandcancer/divisionofcancer/cancer/ccb/iccg/react/

A multicentre phase III clinical trial, the Randomised European Celecoxib Trial (REACT), is now underway for women with breast cancers that are either node positive or high-risk node negative. Patients are randomized and either receive two years of Celecoxib or placebo. Women with tumours that are estrogen or progesterone receptor positive receive tamoxifen, followed by exemestane for a total of 5 years. Coombes et al. showed that there is a reduction in the death rate in women ER positive, who received sequential exemestane (p=0.05) (Coombes et al. 2007).

1.11 Glycosylation and inflammation.

Functions of glycans in inflammation are well defined. As said above, L-selectin is expressed by most leukocytes, whereas E-selectin and P-selectin are expressed by inflamed endothelial cells and they are adhesion molecules for granulocytes, monocytes and T lymphocytes (Carlos et al. 1991). L-selectin, P-selectin and E-selectin recruit lymphocytes to sites of inflammation, and mediate the initial interaction of the leukocytes with the endothelial surface. The selectins bind to SLe^x, carried on specific protein or lipid backbones, and binding slows down the flow of lymphocytes allowing them to roll along the endothelial surface and bind to adhesion molecules, such as integrins. This allows their movement into the tissues, which is induced by chemokines (Ley et al. 1995). L-selectin, P-selectin and E-selectin interact with P-selectin glycoprotein ligand 1 (PSGL1) (McEver et al. 1997) and other glycosylated ligands (CD44 and E-selectin ligand 1 (ESL1) (Hidalgo et al. 2007).

The Sialyl Lewis x (sLex)-like glycans are expressed in relatively high numbers by circulating leukocytes and endothelial cells in the lymph nodes and in the endothelium of inflamed tissues (Varki et al. 1997). Munro et al. performed an analysis of sLex expression on leukocytes, monocytes and other cells in the context of inflammatory and immune processes. They analysed tissues from inflammatory disease (like appendicitis, sinovium rheumatoid, chronic dermatitis, psoriasis) and from secondary lymphoid tissues. A substantial number of monocytes/macrophages in inflamed appendiceal synovial and dermal tissues reacted with a SLex antibody (CSLEXI) compared to the uninflamed sites (Munro et al. 1992). Furthermore, other monoclonal antibodies to SLex, such as MECA-79 (which share similar specificity to L-selectin) or PNAd, which reacted with the endothelium of chronically inflamed tissues, were used (Kannagi et al. 2002; Rosen et al. 1999). Inhibitors for leukocyte migration into inflamed tissues (inhibitors for selectins and for the biosynthesis of their ligands) constitute an attractive target for anti-inflammatory therapy. Therapeutics that block selectins like selectin inhibitor PSGL-1 (Khor et al. 2000) or inhibitors of the glycosyltransferases (Chen et al. 2011; Lee et al. 2003) are presently under development. However, selectins and some glycosyltransferase are present in various part of the body, so an inhibitor would face multivalent and low-affinity interactions in vivo.

Very importantly, Kaneko et al demonstrated that IgG (immunoglobulins G) acquires anti-inflammatory properties with the sialylation of the constant domain (Fc) of immunoglobulins. Sialylation is reduced upon the induction of an antigen-specific immune response (Kaneko et al. 2006).

Some non-invasive diagnostics for sites of chronic inflammation (like inflammatory diabetes), prior to the presentation of clinical symptoms, are in the process of development. Denis et al. have reported a diagnostic for inflammatory diabetes that targets leaky vasculature, an early event in this kind of disease (Denis et al. 2004). This method consists of imaging the expression of L-selectin ligands, which probably precedes inflammatory tissue damage. Sibson et al. reported a strategy for the early detection of inflammation in the brain: an MRI-contrast reagent coupled to an sLex mimetic that binds E-selectin (Sibson et al. 2004). Understanding the early changes in glycosylation can actually improve strategies for early detection and cure of inflammatory diseases.

1.12 PGE2 changes the expression of C2GnT1 and ST3Gal-I.

The idea for this study arose from the findings of Julien et al., which reported that PGE2 changes the expression of glycosyltransferases expressed by dendritic cells: C2GnT1 was rapidly down regulated and the ST3Gal-I was up-regulated (Julien et al. 2007). Prostaglandin E2 is fundamental during monocytes derived dendritic cells (MoDC) maturation and triggers the migration of these cells from the tissue to the lymph node-derived induced by chemokines (Kalinski et al. 1998). PGE2 signals in human MoDCs through EP_2 or EP_4 either alone or in combination (Legler et al. 2006). It is reported that dendritic cells express all EP receptors (Harizi et al. 2003).

There are a few studies on the interaction between glycosyltransferases and COX-2/PGE2 pathway in colorectal and prostate cancer that associate PGE2/COX-2 pathway with ST3Gal-I expression (Hatano et al. 2011; Kakiuchi et al. 2002). Kakiuchi et al. showed that, in epithelial colorectal adenocarcinoma cells, COX-2 overexpression or PGE2 treatment reduced ST3Gal-I expression, while Celecoxib (COX-2 selective inhibitor) increased its expression (Kakiuchi et al. 2002). In contrast, Hatano et al. showed that in castration-resistant prostate cancer cell lines, PC3 and DU145 cells, the expression of ST3Gal-I could be mildly induced by phorbol-12-myristate-13-acetate (TPA), and TPA-induced expression of ST3Gal-I could be inhibited by NF-κB decoy oligodeoxynucleotides (ODN) (Hatano et al. 2011).

As PGE2 is the main metabolite of COX-2, an enzyme overexpressed in invasive breast cancer, and PGE2 can influence the expression of the glycosyltransferases C2GnT1, and ST3Gal-I in DCs, this study aims to investigate the role of PGE2/COX-2 pathway in the regulation of glycosyltransferases (GT) in breast cancer. To our

knowledge this is the first study investigating the interaction between COX-2 and the two glycosyltransferases ST3Gal-I and C2GnT1 in breast cancer.

Chapter 2 Materials and Method.

2.1 Material.

a) Cell culture.

T47D, MDA-MB-231, SKBr3 and ZR75-1 (human breast cancer cell lines) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and P/S (pen/strep) and glutamine. MCF7(human) was cultured in the same medium with the addition of 10% insulin. Cell cultures were grown at 37° C in a humidified atmosphere of 5% CO2. Cells were mainly plated in either T-80 flasks or 6 well plates and grown to subconfluency. Before treatment, the media was changed every two days.

b) Tissue samples.

All tissue samples used were from the Guy's and St. Thomas' Breast Tissue & Data Bank and had been approved for use in this study in accordance with their NHS REC approval (REC No: 07/H0874/131). These tumours are available from the Breast Tissue Bank (thanks to the Tissue Bank Manager, Dr Cheryl Gillett).

c) Antibodies

-HMFG1, HMFG2, SM-3 were produced in this lab;
-Polyclonal rabbit anti-human prostaglandin receptor 1,2,3,4 antibodies (Cayman Chemicals, Ann Arbor, MI, USA);
-Rabbit monoclonal antibody to EpCam (Abcam) ;
-Rabbit COX-2 polyclonal antibody (Oxford Biomedical Research) and Mouse COX-2 monoclonal antibody (Cayman);
-Mouse Anti-beta-Actin Monoclonal Antibody (Sigma Alrich);
-Rabbit p44/42 MAPK (Erk1/2) Monoclonal Antibody (mAb), Rabbit Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb, Rabbit FAK mAb, Rabbit Phospho-FAK mAb, Rabbit CREB, Phospho-CREB (Ser133) mAb (Cell Signalling Technology). All the antibodies are HRP Conjugate.
- PNA (Vector laboratories);
- HRP-conjugated goat anti-mouse IgG (Dako).

d) Reagents.

Prostaglandin E2 (Sigma),
TPA (12-O-tetradecanoylphorbol-13-acetate) (Sigma).

e) Inhibitors

Celecoxib (Sigma Aldrich).
NS398 (Sigma Aldrich).
Gö 6976 and Gö 6983 (kind gift from Prof. Peter Parker's lab)

2.2 Incubation of chemicals on cell lines culture.

Previous to incubation with either PGE2, TPA, COX-2 inhibitors, PKC inhibitors, cells were starved in serum free medium from 2 to 6 hours. All compounds were incubated in serum free medium.

2.3 Molecular Biology techniques: RNA

2.3.1 Extraction of total RNA from cell lines.

Cells were counted using the CASY counter. 25 µL of "RNA Later" was used for each million cell. Cells were either snapped frozen or directly used for extraction. RNA was isolated using the Nucleospin RNAII kit (Macherey-Nagel). Unfortunately, the company does not release the composition of any of the buffers contained within the kit. It is known, however, that both buffer RA1 and RA2 contain the guanidine isothiocyanate that removes the hydration shell surrounding RNA molecules, allowing them to bind the silica membranes within the columns. The presence of the guanidine isothiocyanate and the addition of β-mercaptoethanol to the lysis buffer ensures complete denaturation of RNases, preventing RNA degradation. Contaminated DNA, bound to the silica membrane, is removed by a rDNase solution, which is directly applied onto the silica membrane during the preparation. Optimal conditions for the rDNase are achieved by washing the silica membrane with a specific desalting buffer before treatment. Salts, metabolites and macromolecular cellular components are removed by simple washing

steps with two different buffers. Total RNA is finally eluted with RNase-free water supplied with the kit.

Protocol.

The pellet of cells was diluted in 350 μL Buffer RA1 containing 3.5 μL β-mercaptoethanol. Insoluble materials from the lysate were removed by filtration through a Nucleospin filter by centrifugation at 11,000g for 1 minute. The eluate was mixed with 350 μL 70% ethanol and loaded onto a Nucleo-spin RNA II column before being centrifuged at 11,000 g for 30 seconds. The eluate was discarded and the column desalted by addition of 350 μL membrane desalting buffer. The column was centrifuged at 11,000 g for 1 minute. The column was treated with 95 μL reconstituted DNase and incubated at room temperature for 15 minutes. The column was washed by the addition of 200 μL buffer RA2 prior to centrifugation at 11,000 g for 30 seconds. Following an additional wash using 600μL buffer RA3 and centrifugation at 11,000 g for 30 seconds, 250 μL buffer RA3 was added and the column subsequently centrifuged at 11,000 g for two minutes. The RNA was eluted from the column into an RNase-free 1.5mL eppendorf by the addition of 60μL RNase-free water and centrifugation at 11,000 g for one minute. The RNA was stored at -20 ^{O}C until required.

2.3.2 Extraction of total RNA from human tissue samples.

Samples were crushed using mikrodismembrator II and then homogenised in RNA1 lysis buffer or qiazol. RNA extraction was carried out using Macherey-Nagel RNA Spin L (1-10) or Qiagen Rneasy lipid tissue mini kit (Macherey-Nagel up to 200mg of tissue, Qiagen up to 100mg of tissue). The extraction of RNA was carried out as described in paragraph 2.3.1.

2.3.3 Quality of RNA and measurement of nucleic acid concentration.

The quality of the RNA extracted was checked by running an aliquot on an agarose gel stained with ethidium bromide (described in paragraph 2.5.3), and by measuring the optical density 260/280 ratio of each sample. Intact total RNA shows two sharp bands on agarose gel; these two bands represent 28S and 18S ribosomal RNA. For

quantification of DNA or RNA concentration, OD readings were taken at 260nm and 280nm of a diluted sample. Estimates of purity were obtained from the ratio OD_{260}/OD_{280}. This is because nucleic acid is detected at 260 nm, whereas protein, salt and solvents are detected at 280 nm. The ratio is typically 1.8 for DNA and 2.1 for RNA.

2.3.4 Reverse Transcription of RNA in cDNA.

The production of cDNA was performed by reverse transcriptase. This enzyme is capable of using its polymerase activity to synthesize a complementary DNA strand from RNA.

Reaction mixture A and B were produced.

1) Reaction Mixture A:

1μL Random Primers, 1μL dNTPs;

2) Reaction Mixture B

1μL Superscript II reverse transcriptase, 4 μL 5x FS Buffer, 1μL DTT, 1μL RNase Out.

All the reaction reagents were purchased from Invitrogen. RNA concentration was measured by Nanodrop and the same amount of RNA (1-2μg) was used and diluted in dH2O. RNA was mixed with 2 μL of Reaction Mixture A and incubated at 65°C for 5 minutes. This was mixed with reaction mixture B and incubated at 50°C for 1 hour. The reaction was stopped at 94°C for 5 minutes. The samples were incubated on ice for at least 1 minute. Each sample was treated with 1μL RNase H and incubated at 37^0C for 20 minutes. The samples were diluted with 80μL of nuclease free distilled water or less water, in order to give a concentration of 10-100ng/μL.

2.3.5 Amplification of ST3Gal-I, C2GnT1 and COX-2 sequence by Real Time quantitative Polymerase Chain Reaction.

Once the cDNA had been obtained, it was probed with primers specific for the sequence of interest. The RT-qPCR was performed with the primers described in table 2.2, using the Opticon RT-qPCR Analysis System (MJ Research). 10-20 ng of cDNA was used for each reaction. For independent biological experiments the same amount of cDNA for reaction was used. In quantitative RT-qPCR a measurable fluorescent signal is associated to the amount of amplification product present. The dye used in this work is SYBR® Green, a fluorescent dye that binds to the minor groove of the DNA

double helix. As the copies of the DNA template are exponentially produced with increased cycles, the amount of fluorescence detected increases exponentially. Melting curve analysis is a simple way to check qPCR reactions for contamination and to ensure reaction specificity and accurate quantification. As the temperature increases through the dissociation temperature, the double stranded DNA product separates into complementary strands and SYBR® Green molecules are released and they decrease in fluorescence. The Tm is calculated to be the point at which maximum fluorescence is lost. The amount of target is normalized to an endogenous reference gene.

The relative amount of expression is performed by the "comparative CT Method," given by the mathematical expression $2^{-\Delta CT}$ (see appendix of materials and meethods). The CT is the threshold cycle number and it indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The exponent ΔCT is given by the difference between the CT of the target gene and CT of the reference gene (Applied Biosystems User Bulletin).

The protocol used was according to Julien et al. 2007:

Denature	5 minutes at 95°C	
Denature	15 sec at 94°C	35-45 cycles
Anneal	30 sec at 60°CT_m	35-45 cycles
Extend	30 sec at 72°C	35-45 cycles
Fluorescence detection	30 sec at 78°C	35-45 cycles
Final extension	10 minutes at 72°C	
Hold	4°C	

A melting curve fluorescence analysis was performed on each sample, once the amplification cycles were completed, to verify that a single product had been amplified. Each sample was normalized to the housekeeping gene β-actin or PUM1 (Pumilio homolog 1 of Drosophila).

Protein	Forward Primer (5'–3')	Reverse Primer (5'–3')
β-actin	CCAAGTCCACACAGGGGAGGTGATAGC	CCAGGGAGACCAAAAGCCTTCATACATC
PUM1	GATTATTCAGGCACGCAGGT	AGCAGCGCTGATGATGTATG
COX-2	GTGCCTGGTCTGATGATGTAT	GTCTGGAACAACTGCTCATCA
CCR7	CAACACTGGGAAACAGAGGCTATTGTC	AAAACGATGGAGGGAGGGGTTCAGAGAG
ST3Gal I	ATGAGAGGTTCAACCAGACC	ATGGTGTCATTCAAGTTATTGG
C2GnT1	AGAAGGATACACAAAACGTACC	ACCTTTCTAGCTAACTGTGCTC

Table 2.2. Pairs of primers used in qPCR.2.4 Protein techniques.

2.4.1 Western blotting

a) Preparation of lysate from cell culture

Cells were washed with ice-cold PBS. PBS was drained, and then lysis buffer (RIPA or Laemmli SDS PAGE buffer 1x) was added. The two protocols are described below:

1.Lysis in RIPA buffer.

The RIPA buffer (Radio Immuno Precipitation Assay buffer) is composed of 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate) 50 mM Tris, pH 8.0.

The lysate was incubated for 30 minutes at 4°C and then centrifuged in a microcentrifuge at 4°C. A BCA assay was performed (ThermoFisher). Bovine serum albumin (BSA) was used for the protein standard. The same amount of protein and volume was used for all the samples. They were diluted with 6x Laemmli buffer, boiled at 95°C, and loaded on the gel with 5 μL of Prosieve protein Ladder (Amersham).

2. Lysis in Laemmli 2X SDS PAGE sample buffer

The Laemmli 2X buffer is composed by 4% SDS, 10% 2-β-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl pH 6.8.

In this case Laemmli buffer 1x (1 μL/10000 cells determined by counting a parallel well) was used directly on the cell layer. The lysate was passed through a needle, boiled at

94°C and loaded on a gel with 5 μL of Prosieve protein Ladder (Amersham) in a parallel lane.

b) Casting the gel.

The gel is formed by a resolving and stacking gel. The resolving gel contained 7.5% acrylamide, 1M Tris-HCl pH 8.8 and 0.1% SDS and was polymerised by the addition of 0.6% TEMED and 0.03% ammonium persulphate. A stacking gel was prepared and once the resolving gel was set, it was poured on top and left to polymerize around a comb. The stacking gel was composed of 5% acrylamide, 1M Tris pH 6.8, 0.1% SDS and polymerised as mentioned above.

c) SDS-electrophoresis.

The protein samples were loaded in an equal volume of sample buffer and loaded onto the gel. Rainbow molecular weight markers (Amersham International) were used for size comparison. Electrophoresis was performed in 1x SDS-PAGE running buffer at 150V. The proteins were run until fully separated and then Western blotted.

d) Western blotting

Following electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Hybond-C nitrocellulose membrane and 4 sheets of Whatmann 3mm paper were cut to the size of the gel and soaked in 1x transfer buffer.

The transfer sandwich was built in this order on the anode side of the transfer tank:

- Sponge
- Whatmann 3M paper sheet
- Nitrocellulose sheet (Pall Corporation)
- Gel (gently moved taking it by the more concentrated side = bottom)
- Whatmann 3M paper Sheet
- Sponge

The air bubbles were rolled out between the membrane and the gel. The gels were transferred at 70V for 2 hours.

e) Probing of Western blot membranes.

The membrane was washed briefly with deionised water and stained with a Ponceau solution for not more than 5 minutes to confirm efficient transfer. The Ponceau solution was removed and the membrane washed with clear water until a satisfying staining contrast was obtained. Non-specific binding of nitrocellulose membranes were blocked in wash buffer (PBSA, 0.1% Tween-20, 5% milk or 5% BSA.) for 1 hour at room temperature or overnight at 4°C. All antibodies were diluted in blocking buffer. The membrane was incubated in primary antibody at room temperature with gentle agitation at the appropriate concentration for 1 hour. The membrane was then washed 3 times for 10 minutes in wash buffer, before the HRP conjugated secondary antibody was added at a determined dilution. The membrane was incubated for 1 hour at room temperature with gentle agitation and then was washed as describe previously. The protein bands were visualised by the ECL Plus system (Amersham) as per manufacturer's instructions.

2.4.2 Stripping for reprobing protocol.

The buffer used was made of 20 ml SDS 10%,12.5 mL Tris HCl pH 6.8 0.5 M, 67.5 mL ultra pure water and 0.8 mL β-mercaptoethanol. The buffer was warmed to 50°C and it was then added to the membrane. The membrane was incubated at 50°C for up to 20 minutes with some agitation, it was rinsed for 1 hour in PBS/BSA, was again blocked and probed as described above.

2.4.3 Mouse Kidney Sample Preparation (as Positive Control for EP Receptor Antibodies).

Two mouse kidneys were weighed and 1 mL buffer (20 mM Tris ph 7.5, 5mM EDTA, with protease inhibitor-Roche) per 0.3 g tissue was added. The kidneys were ground with dry ice in a mortar and pestle and homogenized in Teflon/glass tube homogenizer. The product derived from homogenization was centrifuged at 10,000g for 15 minutes and the pellet was solubilized with ~2 mls of the buffer used to grind the tissues containing 1% TritonX-100. The derived solution was centrifuged for 10 minutes and the pellet discarded. The protein concentration was measured on supernatant by BCA-

protein assay (Thermo Scientific). Equal volume of 2x Laemmli sample buffer was added to the protein sample and set in boiling water bath for 5 minutes. 10 µg was loaded per lane of a small gel. The protocol was provided by Cayman Chemicals.

2.4.4 Human Phospho-Kinase Array Protocol.

The kit used is called Human Phospho-Kinase Array Kit from R&D systems. It consists of 46 capture antibodies of different kinases and proteins and control antibodies that have been spotted in duplicate on nitrocellulose membranes. The Human Phospho-Kinase Array is divided into two parts (A and B) to maximize sensitivity and minimize cross-reactivity. Membrane A has 28 and B 18 capture antibodies. One million cells were seeded in a six well plate and three wells were used for each condition. Cells were treated with TPA 10nM for 5 and then rinsed with cold PBS and lysis buffer (1 x 10^7cells/mL), provided by the kit, was added to solubilize the cells. The extracts were resuspended and rocked gently in 1.5 ml eppendorfs at 2-8° C for 30 minutes and then microcentrifuged at 14,000 x g for 5 minutes. The supernatant was transferred into a clean tube and a BCA assay was performed to determine the protein concentration of the lysates. 100µg of protein in a volume up to 334µL was used and this volume was adjusted to a final of 2.0mL with Lysis Buffer 6. Membrane A and B were placed in the 8-well multi dish provided and 1.0mL of Array Buffer 1, used as a block buffer, was pipetted into each well. After 1 hour of incubation, the Array Buffer 1 was removed and 1.0 mL of lysate (with 50 µg of protein) was added to both the Part A and Part B membrane and incubated overnight at 2-8°C on a rocking platform. The membranes were washed three times with 1X Wash Buffer, by soaking for 10 minutes on a rocking platform. 1 mL of Antibody Cocktail A and mL Antibody Cocktail B were added respectively to membrane A and B into the 8-Well Multi-dish and incubated for 2 hours at room temperature on a rocking platform. Each membrane was washed three times with 1X Wash Buffer by soaking for 10 minutes with rocking. 1 mL of the Streptavidin-HRP labelled second antibody was pipetted into each well of the 8-Well Multi-dish and incubated for 30 minutes at room temperature with rocking. The membranes were washed as described previously and the protein bands were visualized by the ECL Plus system (Amersham) as per manufacturer's instructions.

2.4.5 Immunocytochemistry protocol.

Cells ($1x10^5$) were plated the day prior to staining. Cells were fixed in 4% paraformaldehyde at 4°C for 10 min. Non-specific binding was blocked using 100% FCS serum. Slides were incubated with 1:100 polyclonal rabbit anti-COX-2 antibody (Oxford Biomedical) overnight at 4°C. Cells were then washed and incubated with Alexa Fluor® 546 Goat Anti-Rabbit IgG (H+L) *Highly Cross-Adsorbed* *2 mg⁄mL* secondary antibodies. The slides were then washed twice with PBSA and mounted in aqueous mounting medium. Controls included slides in which the primary antibody was omitted.

2.4.6 Flow Cytometric Analysis (FACs).

Cells were gently removed from the culture with 5 mmol/L EDTA in PBS, counted, using a CASY, and the volume adjusted to have 0.5×10^6 cells/mL for each reaction. Cells were incubated for 1 hour on ice in PBS containing 0.05% BSA (human bovine serum albumin) with corresponding antibodies. Flow cytometry and analysis were performed, using a Coulter EPICS XL cytometer with Expo32 ADC software or a FACScalibur cytometer with Cellquest Pro software. Cell monolayers were detached, using 5 mmol/L EDTA in PBS. When necessary, cells were treated with 500mU of type V neuraminidase from Clostridium perfringens (Sigma-Aldrich) in serum-free medium for 1 hour at 37° C, prior to first antibody staining. After 3 washes, samples were incubated with FITC streptavadin Ab (BD Pharmingen) (in the case of PNA staining), or FITC conjugated polyclonal rabbit anti-mouse IgG (Dako) (in the case of HMFG1, 2 and SM3) or with FITC-conjugated goat anti-rabbit IgG (Dako) (in the case of the EP receptors) for 1 hour on ice in PBS containing 0.5% BSA. Cells were then fixed in 1% formaldehyde. After three washes with PBS/0.05% BSA, cells were incubated with the secondary antibody for another hour and then fixed in 0.5% PF (paraformaldehyde) (Sigma, Deisenhofen, Germany). Appropriate isotype controls were used. Cell lines were analyzed for MUC1 expression with HMFG1, HMFG2, SM3 or for Core1 with PNA. Samples were analyzed using an EPICS XL Flow Cytometer (Beckman-Coulter) and FlowJo Software.

2.4.7 Propidium iodide treatment in FACs analysis.

Propidium iodide is a fluorescent vital dye that stains DNA and crosses the cell membrane of dead cells or cells in the late stages of apoptosis. PI is detected in the orange range between 562-588 nm band pass filter. Propidium iodide binds to DNA by intercalating between the bases with little or no sequence preference and it is used in flow cytometry to evaluate cell viability. Flow cytometry assay was run only on cells that could not be stained by PI. When necessary, 20 μL of PI were added to the solution and incubated for 15 min at RT (25°C) in the dark. Cells were analyzed by flow cytometry within 1 hr without using any paraformaldehyde.

2.4.8 Intracellular FACS staining.

Cells were fixed in 200 mL 4% paraformaldehyde, incubated for 3 minutes and spun. After 2 washes in PBSA and 1 wash in PBS/BSA/saponin/NaN_3, cells were incubated for 15 minutes with PBS/BSA/sapanonin/FCS/NaN_3. Cells were stained with EP Receptor Polyclonal Antibody diluted in PBS/BSA/sapanonin/FCS/NaN_3 (1 mg/mL) for 30 minutes. After two washes with PBS/BSA/saponin/NaN_3, cells were incubated for 30 minutes with FITC-conjugated goat anti-rabbit IgG. Pellets were then fixed with 1% formaldehyde in PBSA and PBS/BSA/sapanonin/FCS/NaN_3.

2.4.9 Enzyme-linked immunosorbent assay (ELISA) for PGE2 measurement.

The PGE2 Immunoassay is a competitive enzyme immunoassay designed to measure PGE2 in cell culture supernates, serum, plasma, and urine. It was provided by R&D. This assay is based on the competitive binding technique, in which PGE2 present in the samples competes with horseradish peroxidase (HRP)-labeled PGE2 for a limited number of binding sites on a mouse monoclonal antibody. Therefore there is an inverse relationship between the signal obtained and the concentration of the analyte in the sample, i.e. the more analyte the lower the signal. The standard curve was determined using PGE2 (provided by the R&D kit) in culture medium (DMEM). The range of concentration of the standard curve spans between 2,500 pg/ml and 39 pg/ml. To test the efficiency of the kit, an aliquot of PGE2 (Sigma-Aldrich) was diluted in the calibrant medium for a final concentration of 25 ng/ml and tested.

All the materials are furnished by the kit PGE2 Assay Catalog Number -R&D:

-**Goat Anti-mouse Microplate** - 96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.

-PGE2 Conjugate - 6mL/vial of PGE2 conjugated to horseradish peroxidase with red dye and preservatives.

-**PGE2 Standard** that is reconstituted in 1.0 mL of deionized or distilled water. The concentration is then 25,000pg/mL.

-Primary Antibody Solution - 6mL/vial of a mouse monoclonal antibody to PGE2 in buffer with blue dye and preservatives.

-Wash Buffer made of buffered protein base with preservatives.

-Color Reagent A - 12.5mL/vial of stabilized hydrogen peroxide.

-Color Reagent B - 12.5mL/vial of stabilized chromogen (tetramethylbenzidine).

Color Reagent A should be mixed to Color reagent B in equal volumes within 15 minutes of use and protected from light.

-Stop Solution - 11 mL/vial of 2N sulfuric acid.

-Calibrator Diluent RD5-56 - 21mL/vial of a buffered protein base with preservatives. Even if the kit provides this diluent, the standard was diluted in serum free medium like the samples analyzed. The instruction adviced to remove the serum in a serum separator tube. This is the reason why cells were treated in absence of serum.

After the standard curve was set up, the samples were added in duplicates. First the primary antibody was added to each well and, after an incubation of 1 hour, HRP-labeled PGE2 was allowed to bind to the antibody. Following a wash to remove unbound materials, a substrate solution was added to the wells to determine the bound enzyme activity. The color development was stopped, and the absorbance was read at 450 nm. The intensity of the color was inversely proportional to the concentration of PGE2 in the sample.

2.5 Molecular Biology techniques: DNA

2.5.1 Transformation of competent bacteria.

Competent cells were thawed on ice for 10 minutes. 50ng of plasmid, dissolved in water, were added to the cells, gently mixed and left on ice for 30 minutes with regular mixing to resuspend the cells. The cell and DNA mix was then heat-shocked at 42°C for 90 seconds and then placed on ice for at least 2 minutes. 160ml of L-Broth was added and incubated with shaking at 37°C for 30 minutes. Using aseptic techniques, the

transformed cells were plated onto L-Agar plates containing the appropriate antibiotics, inverted and incubated overnight at 37°C. Ampicillin was used at a concentration of 50μg/ml; and kanamycin was used at 25μg/ml.

2.5.2 Large scale preparation of DNA-Maxiprep.

Constructs, used for transfection, were produced using a large scale extraction of DNA. 800 ⌠L of an overnight culture of transformed bacteria was grown in 200ml of L-Broth containing the appropriate antibiotic (ampicillin 50 µg/ml) at 37°C with shaking.
The culture of transformed bacteria was centrifuged at 6000 rpm for 15 minutes at 4 °C and the pellet was resuspended in 10mL of Buffer P1. 10 mL of Buffer P2 was added and it was inverted 4-5 times. 10mL of Buffer P3 was added and it was inverted 4-5 times. The sample was centrifuged at 12000 rpm in a Beckam jA rotor for 30 minutes at 4 °C. QIAGEN-tip 500 was washed with 10ml of buffer QBT and the supernatant was allowed to enter the resin of the column by gravity flow. The column was washed twice with 30 ml of Buffer QC. The column was placed in new cylinders and 15 mL of buffer QF was applied to the resin. The DNA was precipitated by adding 10.5 mL room temperature isopropanol to the eluted DNA. It was centrifuged at 7000 rpm for an hour at 4° C. The pellet was washed with 70% ethanol and centrifuged for 20 minutes at 7000 rpm. The pellet was left for 5-10 minutes to dry and then the DNA was redissolved in 200 μL of sterile water.

2.5.3 Agarose gel electrophoresis.

A 1% (w/v) agarose gel was prepared in TBE (Tris/Borate/EDTA) by boiling the solution in a microwave oven. The solution was cooled to 50°C and then ethidium bromide was added to a final concentration of 1 mg/mL. The agarose was poured into a gel tray with a gel comb and set at room temperature. One third of DNA loading buffer was added to each DNA sample and then 20 μL was loaded into the wells of the gel (submerged in TBE running buffer).
A volume of 5 μL 1 kb ladder (Invitrogen) was used. Gels were run at 90-120 V in a horizontal gel tank at room temperature and then viewed under a UV transilluminator (Biorad) and photographed using a digital video.

2.5.4 Restriction digest of DNA.

For screening of minipreps, 5 μL of eluted DNA was used. The COX-2 plasmid was cleaved with two endonucleases (EcoRI and NotI). EcoRI and NotI are active in EcoRI NEB buffer at the same temperature (37°C) and both are inactivated at 65°C. The concentration of the two enzymes was 20 units/mL each for EcoRI and NotI. One unit is defined as the amount of enzyme required to digest 1μg of λ DNA (HindIII digest) in 1 hour at 37°C in a total reaction volume of 50 μL. For a total volume of 30 μL a volume of 1 μL of EcoRI and 1μL NotI were used. A color-coded 10X NEBuffer (New England Biolabs) was used to ensure optimal (100%) activity. Both of these restriction endonucleases require BSA at a final concentration of 100 μg/mL for optimal activity from a 10mg/mL (100X) stock. EcoRI NEB buffer (3 μL) and BSA 10x (3μL) were added to the samples. Digestions were performed for 1 hour at 37°C. Analysis of fragments, generated by the digestion, was performed by agarose gel electrophoresis.

2.5.5 Transfection of COX-2 in MDA-MB-231.

MDA-MB-231 and COS cells were transfected with the COX-2 vector using Lipofectamine LTX (Invitrogen) and the protocol was provided by Invitrogen.
Cells were plated the day prior to transfection in DMEM supplemented with 10% fetal bovine serum in either 6 well plates or T80s flasks. The day of transfection the medium was changed and the confluency was around 60-70%. For each well of cells to be transfected, 2.5 μg (18 μg for the T80) of DNA was diluted in 500 μL (3.750 mL for the T80) of Opti-MEM® I Reduced Serum Media without serum. PLUS Reagent was added to the same tube and it was incubated for 5 minutes at room temperature. The Plus reagent, according to the manufacturer's instructions, improves the plasmid delivery and gives superior performance. Invitrogen does not explain the composition of the reagent. For each well of cells, 5 μL (37.5 μL for the T80 flask) of Lipofectamine LTX-TM Reagent was added into the diluted OptiMEM-DNA-Plus Reagent solution. It was mixed gently and incubated for 30 minutes at room temperature to form DNA-Lipofectamine LTX-TM Reagent complexes. After 30 minutes incubation, the DNA-Lipofectamine LTX-TM Reagent was added to the wells or flasks and mixed gently by rocking the plate back and forth. Complexes do not have to be removed following transfection, however, the medium was changed after 6 hours. The cells were

incubated at 37°C in a CO2 incubator for 48-72 hours post-transfection, before assaying for transgene expression.

2.5.6 siRNA transfection protocol.

MDA-MB-231 (1x 10^6) were seeded in six well plates 18 hours before treatment in 2 mL DMEM supplemented with FBS but without P/S (Pen/Strep). Cells at 60-80% confluency were incubated at 37°C in a CO_2 incubator for 18-24 hours. For each transfection 6 μL of siRNA duplex (60 pmols siRNA) were diluted into 100 μL siRNA Transfection Medium Solution (Santa Cruz Biotechnology). siRNA Transfection reagent (Santa Cruz Biotechnology) (6 μL) were also diluted in 100 μL siRNA Transfection Medium Solution. No serum and antibiotics were added to the siRNA Transfection Medium. The siRNA duplex solution (Solution A) was directly added to the dilute Transfection Reagent (Solution B) using a pipette and the mixture was incubated for 30 minutes at room temperature. The Control siRNAs (Santa Cruz Biotechnology) and other 2 controls siRNA from Dharmacon were used. After 30 minutes 800 μL of siRNA transfection medium was added to the mixture and 1mL of this reaction was added to each well of cells for 5 to 8 hours.

The sequence of the three siRNA used are:

1) siRNA COX-2 SC (provided by Santa Cruz biotechnology)

Sense: CUGCUCAACACCGGAAUUUTT;

Antisense: AAAUUCCGGUGUUGAGCAGTT.

2) 1siRNA COX-2 (purchased from Dharmacon and designed by Stasinopoulos et al. (2007):

Sense: AACAUUCCCUUCCUUCGAAAU

Antisense: UUUUGUAAGGGAAGGAAGCUUUA

3) 2siRNA COX-2 (purchased from Dharmacon and designed by Stasinopoulos et al. (2007)

Sense:AACUGCUCAACACCGGAAUUUUU

Antisense:UUUUGACGAGUUGUGGCCUUAAAAA.

In 2' COX-2 siRNA mentioned in chapter 5, 1siRNA and 2siRNA were pooled.

2.6 ChIP Chromatin Immunoprecipitation.

The EpiTect ChIP OneDay kit was used. Ten million cells were plated in T75 flasks and were left to grow overnight. One flask was treated with TPA and the other with the vehicle for 5 hours and then cells were treated with 10 mL of 1% Formaldehyde and incubated at 37° C for 10 minutes. Formaldehyde is used to cross-link the proteins to the DNA as illustrated in the figure below. Excessive cross-linking reduces antigen accessibility and sonication efficiency. The reaction of formaldehyde with proteins starts with the formation of methylol adducts on amino groups. The methylol adducts of primary amino groups are partially dehydrated, yielding labile Schiff-bases, which can form cross-links with several amino acid residues, e.g. with tyrosine (Metz et al. 2004). 1.1 ml of Stop Buffer was added per plate for 5 minutes at room temperature. The stop buffer contains glycine that quenches the formaldehyde and terminates the cross-linking reaction.

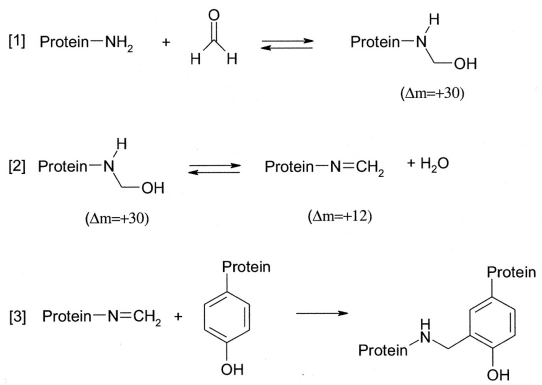


Figure 2.1 Cross linking between protein and formaldehyde

Cells were washed twice with PBS and 1.5 mL ice-cold cell harvesting buffer and collected to be sonicated. Cells were centrifuged at 800g for 10 minutes at 4° C, the supernatant was removed and the pellet was dissolved in IP Lysis Buffer and Protease Inhibitor Cocktail. The rest of the lysate was sonicated, centrifuged for 10 minutes at 4° C and the supernatant was stored at -80° C. Some of this supernatant was tested on a 1.5% agarose gel to check the correct fragment size (between 100 and 600 bp). For each IP Fraction, 100 μL of this sheared ready Chromatin was dissolved in 900 μL IP Buffer A, containing protease inhibitor. Protein A beads (50μL) for each IP fraction were added and incubate for 50 minutes at 4°C. The beads removed by centrifugation at 4000g for 1 minute at 4° C and the pre-Cleared Chromatin was transferred to a new tube. 10μL of the supernatant was stored in another eppendorf at 4° C (and used as

Input Fraction later when the elution buffer and proteinase K was added). The pre-Cleared Chromatin was incubated with the appropriate antibody on rotator at 4°C overnight. Then, 60μl Protein A Beads were added to each IP and incubated at 4°C for 1 hour. The solution was centrifuged at 4000xg for 1 min at 4°C and the supernatant discarded. The beads were washed in cold wash Buffers for three times for 4 minutes and each time the supernatant discarded after each centrifugation. Elution Buffer (30μL) and Proteinase K was added to each IP Fraction (including the Input Fraction) and they were incubated in a thermomixer at 500 rpm at 45°C for 30 minutes. DNA Extraction Beads (to remove the Proteinase K) were added to each IP Fraction incubated on thermomixer at 500rpm at 95° C for 10 minutes. Samples were then centrifuged at 14,000 g for 1 minute at room temperature. 200 μL of each supernatant was transferred to a new tube and 100 μL of elution Buffer was added to each sample. Column Binding Buffer was added to each 200 μL IP fraction sample, mixed and allowed to go through a DNA Spin Column. The column was washed, centrifuged and 100 μL of elution Buffer was added to the center of the column in a new tube. The column was centrifuged at 11,000g for 1 minute at room temperature and the same step was repeated. So at the end a final volume of 200 μL of the purified ChIP DNA samples was stored at – 20° C.

2.6.1 ChIP qPCR

For each 25 μL PCR, 12.5 μL RT² qPCR Master Mix, 6.5 μL of ddH2O, 5 μL of either undiluted or diluted ChIP DNA template, 1 μL ChIP-qPCR Assay Primers (10 μM each). The protocol used was according to Qiagen EpiTect ChIP OneDay Handbook:

- Denature 10 minutes at 95°C
- Denature 15 sec at 95°C } 40 cycle
- Anneal and extend 1 min at 60°C } 40 cycle
- Final extension 1 minutes at 95°C
  30 seconds at 55°C
  30 seconds at 95°C

The primers used were designed very close to the transcription start site and the sequence for ST3Gal-I was forward GCCCACTATGCCAGACAGTT and reverse

AAGGGGTTCGGA GGAGAATA. The GAPDH primers were designed by Qiagen Primer kit.

2.7 Statistical analysis.

Statistical analysis was carried out using the t test calculator of Graphpad software. A value of $p < 0.05$ was considered significant. For the correlation of ST3Gal-I and COX-2 expression in primary breast cancers, Spearson and Person's T test was applied. The statistics were calculated by Dr. Brian Burford.

Appendix

The following appendix explains the equation used for the calculation of the amount of target in RT-qPCR. It was taken entirely from User Bulletin #2 ABI PRISM 7700 Sequence Detection

System.

The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

Derivation of the Formula

The equation that describes the exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n$$

where:

X_n	=	number of target molecules at cycle n
X_o	=	initial number of target molecules
E_X	=	efficiency of target amplification
n	=	number of cycles

The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_o \times (1 + E_X)^{C_{T,X}} = K_X$$

where:

X_T	=	threshold number of target molecules
$C_{T,X}$	=	threshold cycle for target amplification
K_X	=	constant

A similar equation for the endogenous reference reaction is:

$$R_T = R_o \times (1 + E_R)^{C_{T,R}} = K_R$$

where:

R_T	=	threshold number of reference molecules
R_o	=	initial number of reference molecules
E_R	=	efficiency of reference amplification
$C_{T,R}$	=	threshold cycle for reference amplification
K_R	=	constant

Dividing X_T by R_T gives the following expression:

$$\frac{X_T}{R_T} = \frac{X_o \times (1 + E_X)^{C_{T,X}}}{R_o \times (1 + E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K$$

The exact values of X_T and R_T depend on a number of factors, including:

- Reporter dye used in the probe
- Sequence context effects on the fluorescence properties of the probe
- Efficiency of probe cleavage
- Purity of the probe
- Setting of the fluorescence threshold.

Therefore, the constant K does not have to be equal to one.

Assuming efficiencies of the target and the reference are the same:

$E_X = E_R = E$,

$$\frac{X_o}{R_o} \times (1 + E)^{C_{T,X} - C_{T,R}} = K$$

or

$$X_N \times (1 + E)^{\Delta C_T} = K$$

where:

X_N	=	X_o/R_o, the normalized amount of target
ΔC_T	=	$C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference

Rearranging gives the following expression:

$$X_N = K \times (1 + E)^{-\Delta C_T}$$

The final step is to divide the X_N for any sample q by the X_N for the calibrator (cb):

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1 + E)^{-\Delta C_{T,q}}}{K \times (1 + E)^{-\Delta C_{T,cb}}} = (1 + E)^{-\Delta\Delta C_T}$$

where:

$\Delta\Delta C_T$	=	$\Delta C_{T,q} - \Delta C_{T,cb}$

For amplicons designed and optimized according to Applied Biosystems guidelines (amplicon size < 150 bp), the efficiency is close to one. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

Chapter 3. Breast cancer cell lines: Characterization for the expression of COX-2 and PGE2 receptors, and PGE2 secretion

3.1 Introduction.

Julien et al. (2007) showed that prostaglandin E2 (PGE2) influences the expression of the glycosyltransferases C2GnT1 and ST3Gal-I in dendritic cells (DCs). In order to investigate if PGE2 has any effect on glycosyltransferases in breast cancer, a panel of breast cancer cell lines available in the laboratory (MCF-7, T47D, SK-Br-3, BT-20, MDA-MB-231, ZR-75-1) were first characterized for COX-2 expression, prostaglandin receptors (EP) expression and PGE2 secretion. These cell lines show a range of expression of C2GnT1 and ST3Gal-I (Dalziel et al. 2001).

3.2 Objectives

1. Define the mucin O-glycosylation core types found in six breast cancer cell lines.
2. Determine COX-2 expression by the six breast cancer cell lines by RT-qPCR.
3. Measure the PGE2 concentration in the media of the six breast cancer cell lines by ELISA.
4. Determine the EP1-4 expression at the mRNA and protein level by the six breast cancer cell lines.

3.3 Results

3.3.1 Profile of mucin O-glycosylation core types on the six breast cancer cell lines.

Initially the cell lines MCF7, T47D, MDA-MB-231, SK-BR-3, BT-20, ZR-75-1 were analyzed for their binding to the MUC1 specific monoclonal antibodies HMFG1, HMFG2 and SM3 to obtain an indication of their Core 1 and Core 2 expression. As discussed in section 1.7, SM3 reacts with an epitope on MUC1, which is masked by the Core 2 glycans on normal breast epithelium (Burchell et al. 1989; Girling et al 1989). HMFG1 reacts well with MUC1 on normal cells but the presence of sialic acid can decrease its binding to its epitope, whereas the binding of HMFG2 is inhibited by longer carbohydrate side chain on normal mucins (Burchell et al.1993). Table 3.1 shows the presence of core1 and core2 structures as determined by mass spectrometry of the six breast cancer cell lines according to Müller et al. (2002) and the reactivity of the three monoclonal antibodies.

	core1	core2	SM3	HMFG1	HMFG2
MCF7	**++**	**++**	+	++	++
T47D	**+++**	**-**	++	+	+++
MDA-MB-231 (very low MUC1 expressed)	**+**	**+**	+/-	+/-	+/-
SK-BR-3	**++**	**-**	++	+	+++
BT-20	**++**	**-**	++	+	+++
ZR-75-1	**+**	**+**	+	+++	++

Table 3.1: Expression of core 1 and core 2 in the six breast cancer cell lines analyzed. Cells ($3x10^5$) were stained with SM3, HMFG1 and HMFG2, followed by FITC-conjugated polyclonal rabbit anti-mouse IgG. As can be seen from figure 3.1, breast cancer cell lines react differently with the three antibodies in line with their core 1/core 2 expression.

All the lines reacted with SM3, but, as expected, T47D and BT-20 had the highest level of binding and MDA-MB-231 the lowest (Müller et al. 2002). MDA-MB-231 has in fact the lowest level of MUC1 among the cell lines analyzed, and this was confirmed by the low reactivity with HMFG1 and HMFG2. HMFG1 binding is greater on MCF7 and ZR-75-1 cell lines and this reflects the fact that they express a higher level of core 2 than the others. In contrast, BT-20, SK-BR-3 and T47D react more strongly with HMFG2.

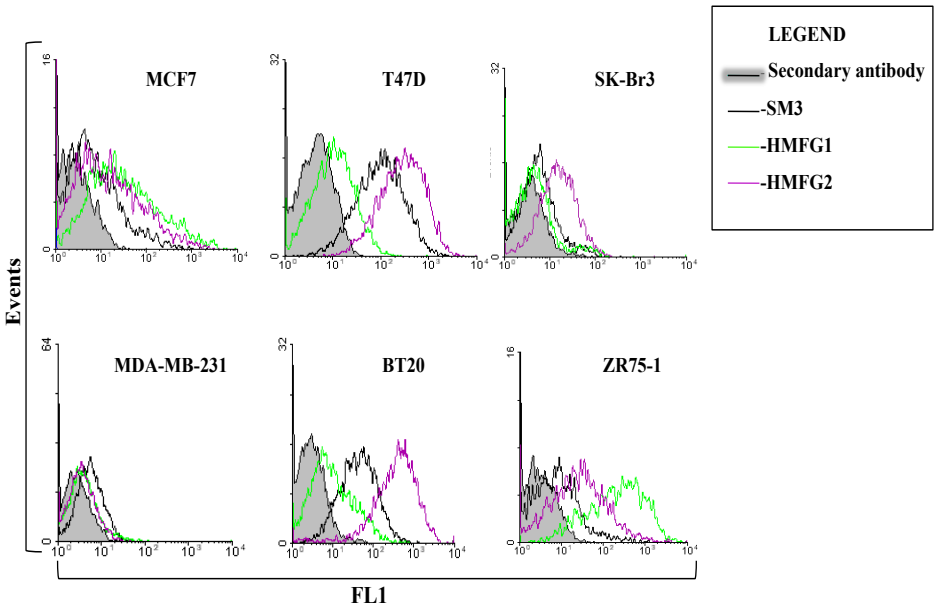
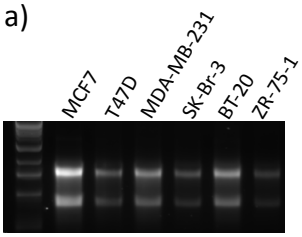


Figure 3.1: Flow cytometric analysis of the core 1 and core 2 MUC1 glycoforms on the six breast cancer cell lines. Cells were stained using the SM3, HMFG1 and HMFG2 monoclonal antibodies, followed by FITC-conjugated polyclonal rabbit anti-mouse IgG. The shaded peak is the isotope control, the black, green, purple open peaks are, respectively, the SM3, HMFG1, and HMFG2 antibody staining.

3.3.2 COX-2 expression by the six breast cancer cell lines.

The level of expression of COX-2 in MCF7, T47D, SK-BR-3, MDA-MB-231, BT-20, ZR-75-1 cell lines was analyzed by RT-qPCR. Two biological experiments were performed using the same conditions. Initially $4x10^5$ cells of each cell line were cultured and the pellets were collected after five days and stored in 50 μL of RNA Later Reagent at -20°C.

a)



b)

Cells lines	$OD_{260/280}$	
	I experiment	II experiment
MCF7	2.16	2.12
T47D	2.12	2.19
MDA-MB-231	2.15	2.18
SK-Br3	2.15	2.2
BT-20	2.16	2.15
ZR-75-1	2.16	2.18

Figure 3.2: Quality control of RNA. a) An aliquot of 5 μL of RNA was run on 1% agarose gel. The ladder used is a 1 kb ladder (New England Biolabs). b) The Nanodrop measurements of the optical density ratio for two biological experiments are shown in the table. The values for each cell line are about two.

The quality of the RNA extracted was checked by running an aliquot on an agarose gel, stained with ethidium bromide, and by measuring the optical density to obtain the 260/280 ratio of each sample, as described in material and methods (section 2.3.3).

The agarose gel showed two bands for each cell line (figure 3.2 a), confirming that in all the samples the RNA was of good quality. This was confirmed by the ratio OD_{260}/OD_{280} of the RNA from the two biological experiments used in this experiment (table b figure 3.2). (In the results of the other chapters the quality of RNA will not be shown, even though it was always analyzed). 1 μg per reaction of mRNA was used for the reverse transcription and the cDNA was then used for the qPCR.

SYBR® Green, a fluorescent dye, that binds to the minor groove of the DNA double helix (as described in appendix of materials and methods), was used in quantitative RT-qPCR and in these experiments the results were normalized to β-actin, a reference gene that does not vary in different cell lines. Figures 3.3 and 3.4 show the melting curves of the β-actin and COX-2 primers from one biological experiment for each cell line.

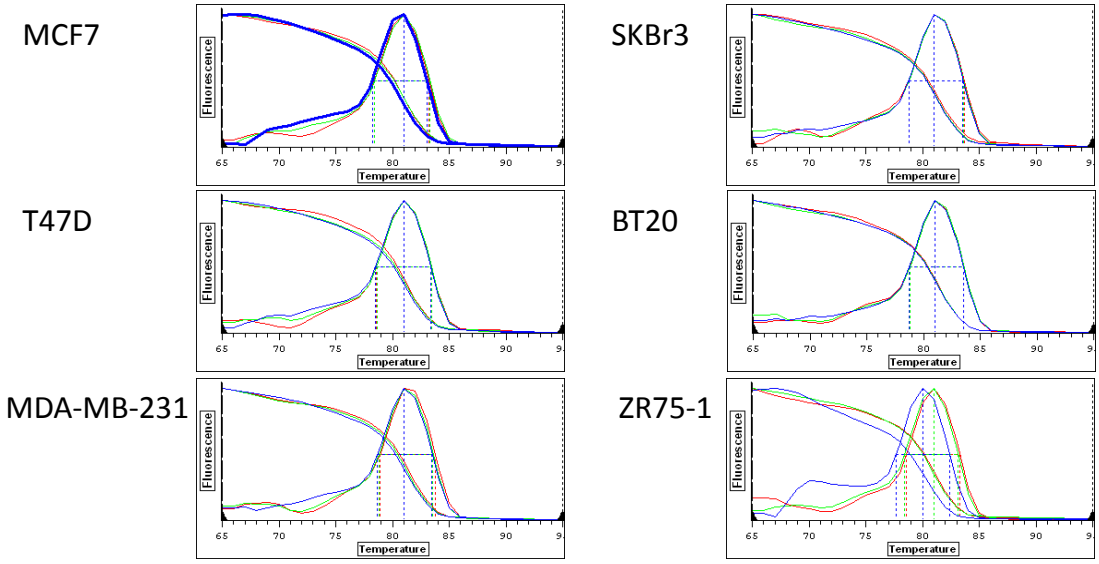


Figure 3.3: Melting curves analysis for all the cell lines cDNA with β-actin primers. The qRT-PCR reaction was performed with SYBR® Green, as described in materials and methods(section 2.3.5). Melting curve analysis was performed, after amplification, using a temperature ramp of 1°C/5 seconds between 65°C and 95°C.

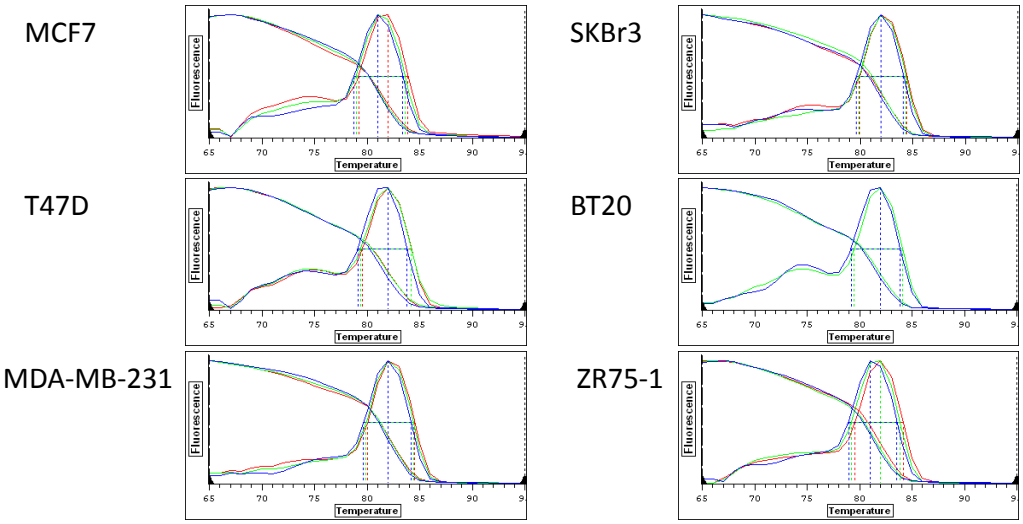


Figure 3.4: Melting curves analysis for all the cell lines cDNA with COX-2 primers. The RT-qPCR reaction as described in figure 3.3.

The relative amount of expression was performed by the "comparative CT method", given by the mathematical expression $2^{-\Delta CT}$ (see appendix of materials and methods). The values shown in figure 3.5 are the mean of 2-ΔCt of two biological experiments. MDA-MB-231 showed the highest level of COX-2 followed by SK-BR-3, BT-20 and ZR-75-1; MCF7 and T47D the lowest level. This result agrees with published data showing that MDA-MB-231 expresses a higher level of COX-2, while MCF7 a very low level.

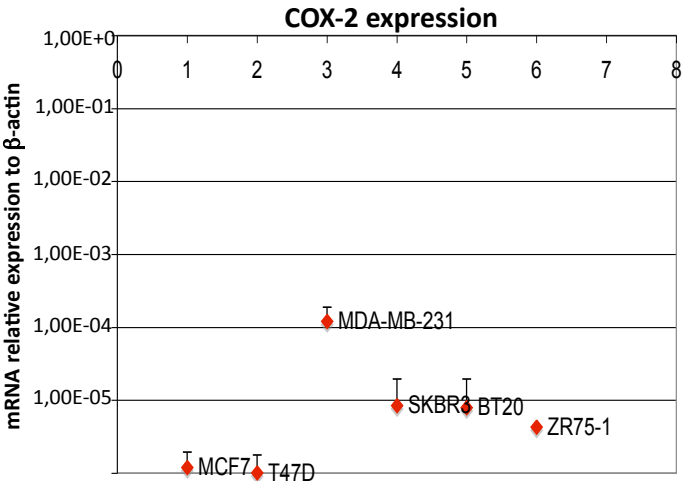


Figure 3.5: MDA-MB-231 cell line expresses the highest level of COX-2 mRNA. The dots represent the mean±SD of the 2-ΔCt of two biological experiments comparing COX-2 (the target gene) to β−actin (the reference gene) for six breast cancer cell lines.

3.3.3 Secretion of PGE2 by the breast cancer cell lines.

The level of PGE2 was determined by ELISA (see materials and methods, section 2.4.9). Two million cells, for each cell line, were cultured for 24 hours and the supernatant tested. The concentration of PGE2 in the medium, shown in table 3.6 below, was calculated from the equation of the standard curve. Since it is a competitive ELISA, there is an inverse relationship between the signal obtained and the concentration of the analyte, so, as seen in the table, the more analyte the lower the signal.

Table 3.6: Table of the concentrations of PGE2 found in the supernatant of the six breast cancer cell lines after 24 h of culture.
PGE2 concentration was calculated from the equation of the ELISA assay. MDA-MB-231 and BT-20 are the only cell lines that have a greater PGE2 concentration than the negative control (DMEM). The concentration of PGE2 was calculated from two biological experiments.

	Mean OD	**Concentration (pg/ml)**
Negative control	1.123	29.09±4.66
MDA-MB-231	1.055	41.68±5.25
BT20	1.044	44.26±4.63
ZR75-1	1.169	22.65±4.25
MCF7	1.179	21.54±3.92
SK-Br-3	1.158	24.05±3.87
T47D	1.25	14.88±4.41

This assay demonstrated that only MDA-MB-231 and BT-20 supernatant showed a concentration of PGE2 higher than the negative control used, while the PGE2 concentration of most of the cell lines was below the negative control value and therefore considered as zero.

3.3.4 PGE2 receptor expression in breast cancer cells.

PGE2 action is mediated via four receptors (EP_1, EP_2, EP_3, EP_4) with different signalling pathways. EP_1 is mainly a G_q coupled receptor with phospholipase C signalling through

Ca^{2+} and EP_2 and EP_4 are G_s coupled receptors to protein kinase A (PKA). On the other hand EP_3 is mainly a coupled G_i protein and is linked to a decrease of cAMP (discussed in section 1.9.4) (Narumiya et al.1999). The expression of the EP receptors was determined at the protein level by FACS and Western Blotting.

Rabbit polyclonal antibodies raised against the EP receptors antibodies, followed by FITC-conjugated goat anti-rabbit IgG were used for the flow cytometric analysis. The antibodies were against a synthetic peptide from the C-terminal intramembrane domain of the EP receptors, so the cells had to be permeabilized before staining. Results are shown in figure 3.7. To confirm the success of the intracellular staining, a positive control (a Rabbit monoclonal antibody to the cytoplasmic domain of EpCam-C-term) was used (data not shown).

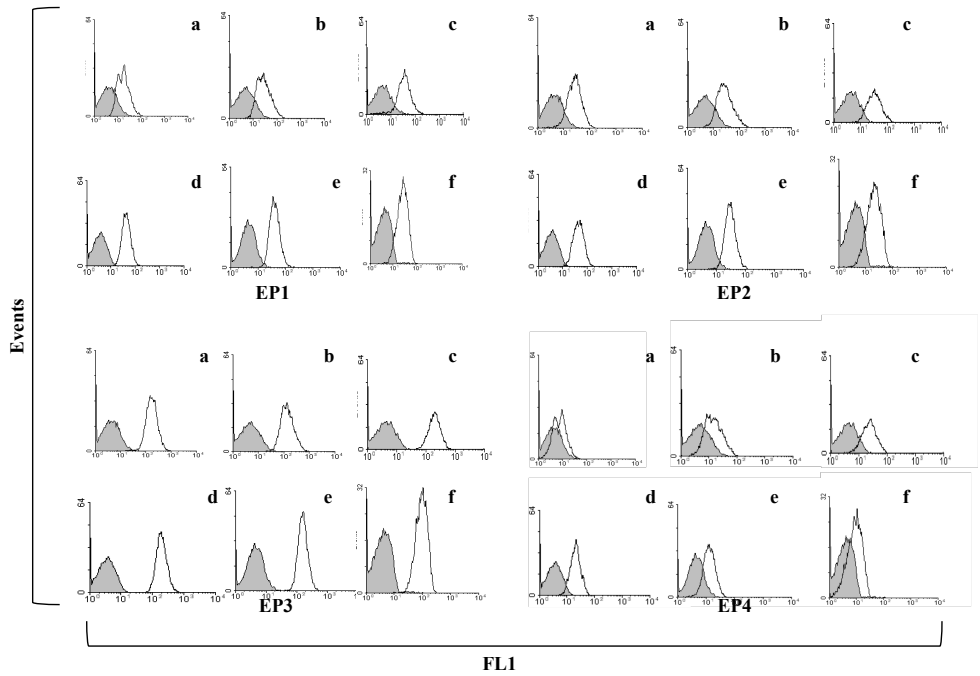


Figure 3.7: Flow cytometric analysis of EP receptors in the six breast cancer cell line. Cells ($4*10^5$) were fixed, permeabilized by PBS/BSA/saponin/NaN_3 and stained with EP Receptor Polyclonal Antibody (Cayman Chemicals) followed by FITC-conjugated goat anti-rabbit IgG (Dako) as described in Materials and Methods. Shaded peak, staining with control antibody, open clear peak, EP staining. a, b, c, d, e, f are MCF7, T47D, MDA-MB-231, SK-Br-3, BT-20, ZR-75-1 respectively.

From figure 3.7 it is evident that the positive mean fluorescence to each EP receptor was more or less the same in each cell line, which raised some concerns. Therefore, in order to check the specificity of the antibodies to each receptor, Western Blot analysis was performed with the same antibodies (according to the manufacturer's instruction).

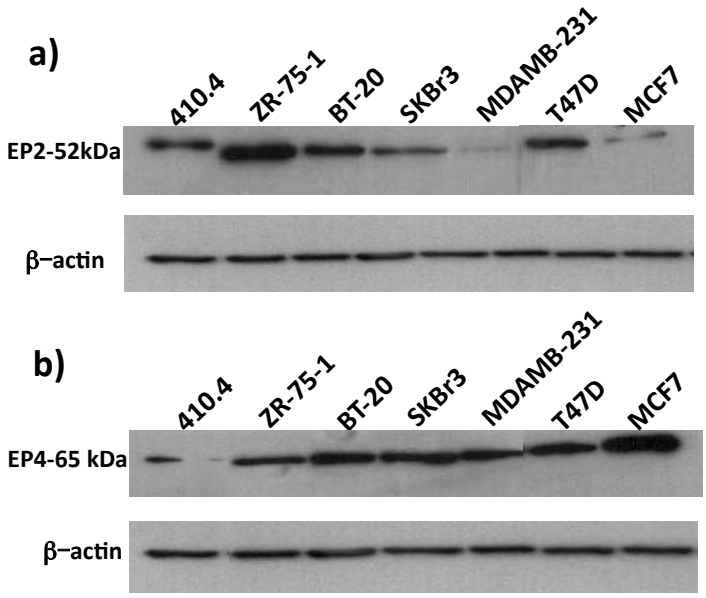


Figure 3.8: Westen Blots to determine EP_2 and EP_4 expression. 410.4, ZR-75-1, BT-20, SK-Br-3, MDA-MB-231, T47D, MCF7 protein lysate (10 μg) were immunoblotted with Rabbit polyclonal antibodies (Cayman Chemicals) to EP_2 (a) and EP_4 (b) receptors followed by HRP-conjugated swine anti-rabbit IgG (Dako). Both blots were stripped and reprobed with Mouse Anti- β−actin, followed by HRP-goat antimouse IgG (Dako), as loading control. Membranes were developed with the ECL system (Amersham), according to the manufacturer's instructions.

Cells were cultured in T80 flasks and after each cell line reached 80% of confluency, pellets were collected. Cell disruption was conducted as described in the materials and methods. Protein concentration was measured by BCA-protein assay and 10 ⌠g of protein of each sample was loaded onto a 7.5% SDS-PAGE gel. The murine mammary cell line, 410.4 was used as positive control for the EP receptors (Ma et al. 2006). In figure 3.8, it is evident that very little expression of EP_2 is seen in MCF7 and MDA-MB-231. In contrast the expression of EP_4 is expressed by all the cell lines with an increase of expression in MDA-MB-231 and MCF7. Western Blots with EP receptor antibodies showed optimum and clear results for EP_2 and EP_4, which did not agree with the flow cytometric analysis, confirming the concerns about this assay. A repeat of a Western blot with different protein lysate gave identical results.

For EP_1 and EP_3, an additional control of mouse kidney lysate was used together with 410.4. The EP_1 and EP_3 antibodies did not appear to be specific as a number of bands of varying migration were observed. As shown in figure 3.9, there was no band of the expected size of EP_1 receptor (42 kDa) in the cell lines. BT-20 and MCF7 lysates showed a band of about 30 kDa that came up in three different experiments. This band could be a breakdown product or a cross-reacting band.

EP_3 has a predicted molecular weight of 53 kDa. Figure 3.9b shows a common band of 53 kDa in all the cell lines (apart from the mouse kidney control), but together with this

band there are many other unspecific bands. This lack of specificity of the antibodies may reflect the massive shift seen in the FACS plot.

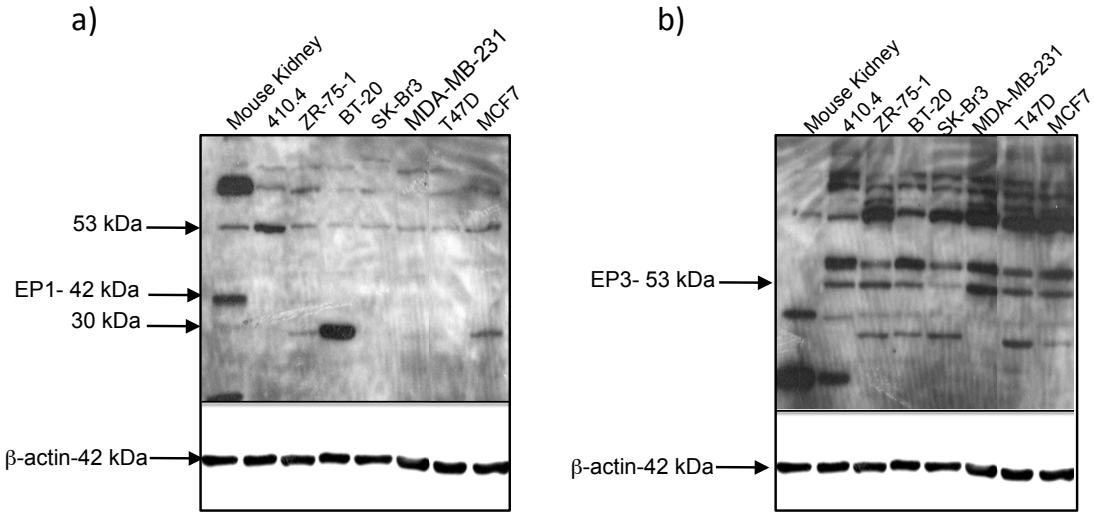


Figure 3.9: Westen Blots to determine EP1 and EP3 expression.
Mouse kidney, 410.4, ZR-75-1, BT-20, SK-Br-3, MDA-MB-231, T47D, MCF7 protein lysate (10 µg) were immunoblotted with Rabbit polyclonal antibodies (Cayman Chemicals) to EP1(a) and EP3(b) receptors followed by HRP-conjugated swine anti-rabbit IgG (Dako). Both blots were stripped and reprobed with Mouse Anti-β−actin followed by HRP-goat antimouse IgG (Dako) and were used as loading control. Membrane were developed with the ECL system (Amersham) according to the manufacturer's instructions.

In conclusion all the breast cancer cell lines examined (MCF-7, BT20, MDA-MB-231, ZR-75-1, SK-Br-3, T47D) express the EP_2 and EP_4 receptors, but at different levels. However, no clear conclusion can be drawn on the expression of the EP_1 and EP_3 receptor as non-specific binding of the antibodies were observed on the Western blots. siRNA for EP1 and EP2 can be used to validate EP antibodies. Moreover, the presence of EP1 and EP3 proteins can be confirmed by mass spectrometry of some lysate from the different cell lines.

3.4 Discussion.

The glycosylation changes that occur during maturation of dendritic cells are similar to the changes in glycosylation observed in malignant breast epithelium. In dendritic cells at maturation, C2GnT1 is rapidly down regulated and the sialyltransferases ST3Gal-I is upregulated, resulting in the change from core 2 based glycans to sialylated core 1 glycans. In our lab it has been demonstrated that prostaglandin E2 alone can induce the upregulation of ST3Gal-I and the downregulation of C2GnT1 in dendritic cells (Julien et al. 2007). Preliminary data showed the possible implication of the prostanoid receptors EP_2 and EP_4 in this process.

To study how PGE2 regulates the expression of the glycosyltransferases (GT), C2GnT1 and ST3Gal-I, in breast cancer, it was important to characterize the breast cancer cell lines available in the lab and investigate the expression of COX-2, PGE2 receptors and production of endogenous PGE2 by these lines. The six breast cancer cell lines have a different O-glycosylation profile: MCF7, ZR-75-1 have core 2 and core 1 based glycans, while T47D, BT-20 and SK-Br-3 only express core 1 (Müller et al. 2002). The O-glycosylation pattern was confirmed by RT-q-PCR which showed a very low or lack of expression of C2GnT1 in BT-20, SK-Br-3 and T47D (data not shown).

The expression of COX-2 detected by qPCR, is relatively low in all the cell lines with the highest expression being by the ER negative breast cancer cell line, MDA-MB-231. This is in agreement with Half et al., who showed that the COX-2 levels in SK-BR-3, MCF7, MDA-MB-231 is very low in comparison to a colon-cancer cell line like HCF-7; however they also showed that MDA-MB-231 expresses the highest amount of COX-2 compared to the other two breast cancer cell lines (Half et al. 2002). Moreover, Liu et al. published data showing that MDA-MB-231 expressed high levels of COX-2 compared to MCF7 (Liu et al. 1996). Although MDA-MB-231 has the highest expression of COX-2 among the breast cancer cell lines studied, the level of the metabolite PGE2 secreted is low, as detected by the ELISA assay.

Previous studies described dramatic differences in PGE2 production between cell lines and primary tumours from specific tissues, including those derived from human lung and colon tumours (Hubbard et al. 1988). Schrey and Patel showed how MCF7, T47D, BT-20, ZR-75-1 do not secrete PGE2 in the media, even when stimulated with the phorbol ester TPA or IL-1β, while MDA-MB-231 and other invasive breast cancer cell lines demonstrated a large increase of the metabolite in response to the two stimulating agents (Schrey & Patel, 1995). In the present study MDA-MB-231 and BT-20 are the only cell lines, among the panel of breast cancer cell lines considered, that showed any

secretion of PGE2. However, a concentration of about 40 pg/ml is very low, as the background detected in the medium was 29.09 pg/ml.

There are various parameters to be considered for the PGE2 production. First of all, the half-life of PGE2 in the circulatory system is approximately 30 seconds and rapid metabolic inactivation of any PGE2, formed by the cancer cells, may also limit its detection (Fitzpatrick et al. 1980). The enzyme responsible for the metabolic inactivation of prostaglandins is 15-hydroxyprostaglandin dehydrogenase. According to Wolf et al. low levels of this enzyme were detected in MDA-MB-231 cells but high levels in MCF7 (Wolf et al. 2006).

The absence of detectable accumulation of PGE2 in the cell culture medium does not necessarily mean that there is no PGE2 produced by the COX-2 enzyme. PGE2 could be bound to the four prostanoid receptors and trigger a downstream response. For this reason, the expression of all four EP isoforms was characterized in the six breast cancer cell lines. However, the western blots for EP_1 and EP_3 were unclear, as many non-specific bands were present. Different lysates were used, but they did not improve the blots. Therefore, no conclusion can be made on these two prostanoid receptors. In contrast, EP_2 and EP_4 receptors blots are very clear (Figure 3.8). The EP2 receptor is weakly expressed by MCF7 and MDA-MB-231, while it is expressed in the other cell lines and prevalently in ZR-75-1. The EP_4 receptor is present in all the cell lines and has the highest intensity in MCF7 cell line.

In the literature, it is evident that EP receptors play different roles during breast cancer progression and that EP_2 and EP_4 are the receptors likely to be involved in the regulation of mammary tumor progression and angiogenesis (Subbaramaiah et al. 2008; Chang et al. 2004). Importantly, these are the receptors that we have established to be expressed by the breast cancer cell lines studied.

Chapter 4. PGE2 and TPA can trigger ST3Gal-I expression.

4.1 Introduction.

The previous chapter described the characterization of six breast cancer cell lines (MCF-7, T47D, SK-Br-3, BT-20, MDA-MB-231, ZR-75-1) for their COX-2 expression, PGE2 secretion and EP receptors expression. The MDA-MB-231 cell line expressed the highest level of COX-2. Moreover, it was found that only media from the BT-20 and MDA-MB-231 cell lines contained PGE2 and this was at a very low concentration. Protein expression of the receptors was examined by Western blot analysis and all the breast cancer cell lines expressed EP_2, EP_4 receptors but by different degrees. No conclusion could be drawn for EP_1 and EP_3.

Julien et al. had previously shown that stimulation of dendritic cells with PGE2 could change the expression of glycosyltransferases expressed by monocyte derived dendritic cells: C2GnT1 was rapidly down regulated and the ST3Gal-I upregulated (Julien et al. 2007). Although the breast cancer cell lines expressed EP receptors, the expression of COX-2 and PGE2 was low or below the level of detection. Therefore, to be able to investigate the influence of the COX-2 pathway on the expression of glycosyltransferases ST3Gal-I and C2GnT1 in breast cancer, exogenous PGE2 and TPA (inducer of COX-2 expression) were added to the breast cancer cell lines. The expression of the two enzymes and their product were determined by RT-qPCR and FACs analysis.

4.2 Objectives.

1. Treat six breast cancer cell lines with PGE2 and determine the change in glycosyltransferase expression.
2. Determine eventual modification of PNA staining in cell lines that showed a change in expression of ST3Gal-I and/or C2GnT1 after PGE2 treatment.
3. Treat six breast cancer cell line with TPA and determine change in glycosyltransferase expression.
4. In the cell line that showed change of expression of a glycosyltransferase, measure by FACS the modification of PNA staining after TPA treatment.

4.3 Results.

4.3.1 Choice of a gene for normalization of real-time RT-PCR data for the breast cancer cell lines.

The fluorescent quantitative Real Time PCR (RT-qPCR) has become a very popular technique to quantify gene expression in basic and translational biomedical research (Bustin et al. 2009; Bustin et al. 2000).

One of the disadvantages of this technique is the possible variation in the amount of starting material among samples, so it is necessary to normalize the gene expression to an internal reference (Wong & Medrano, 2005). It is important to choose reference genes that do not vary with the possible treatment. The most common housekeeping genes used are GAPDH or β-actin. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is an enzyme that catalyzes the sixth step of glycolysis, while β-actin is a cytoskeleton protein involved in the motility and integrity of the cell and it is ubiquitously expressed in most cell types (Bustin et al. 2000). It was observed that GAPDH levels of expression vary in different developmental stages (Puissant et al. 1994), during the cell cycle and with different treatments (TPA, dexamethasone, carbon tetrachloride etc.) (Bustin et al. 2000). On the other hand, β-actin levels of transcription can vary widely in human breast epithelial cells (Spanakis et al. 1993).

Lyng et al. in their study established which reference gene could be more reliable for qPCR of breast cancer (ER positive and negative), normal breast and breast cancer cell lines data. The reference genes investigated were RPLP0, TBP, PUM1, ACTB, GUS-B, ABL1, GAPDH and B2M. The authors idendified PUM1 (homolog of Pumilio, Drosophila) as the best reference gene for all sample combinations (Lyng et al. 2008). Therefore, in this work, PUM1 was used as a reference for all experiments. For each experiment, it was confirmed that the Cts of the reference gene was the same before and after treatment.

4.3.2 Prostaglandin E2 (PGE2) enhances ST3Gal-I expression and its product α2,3 sialyl-Core1 in T47D cell line.

Most of the RT-qPCR results reported in this thesis will be plotted as fold-change. The fold change in mRNA expression is defined as the difference of the mean log treatment data and the mean log control and both values are normalized against the log value of

the house keeping gene for each sample (ΔΔCts). The values are then represented as $2^{-\Delta\Delta Cts}$ (see appendix of materials and methods).

The breast cancer cell lines, MCF-7, T47D, MDA-MB-231, SK-BR-3, BT-20 and ZR-75-1, were treated with PGE2 (0.1 μM) for 4 hours. Figure 4.1 shows the fold change of ST3Gal-I and C2GnT1 to PUM1 in two ER positive, MCF-7 and ZR75-1 and in one ER negative breast cancer cell line, MDA-MB-231. After treatment with PGE2, there was no significant change in the expression of ST3Gal-I and C2GnT1 in any of these cell lines (figure 4.1) and SK-Br-3 and BT-20 also showed no change in expression of the two glycosyltransferases . These data are not shown.

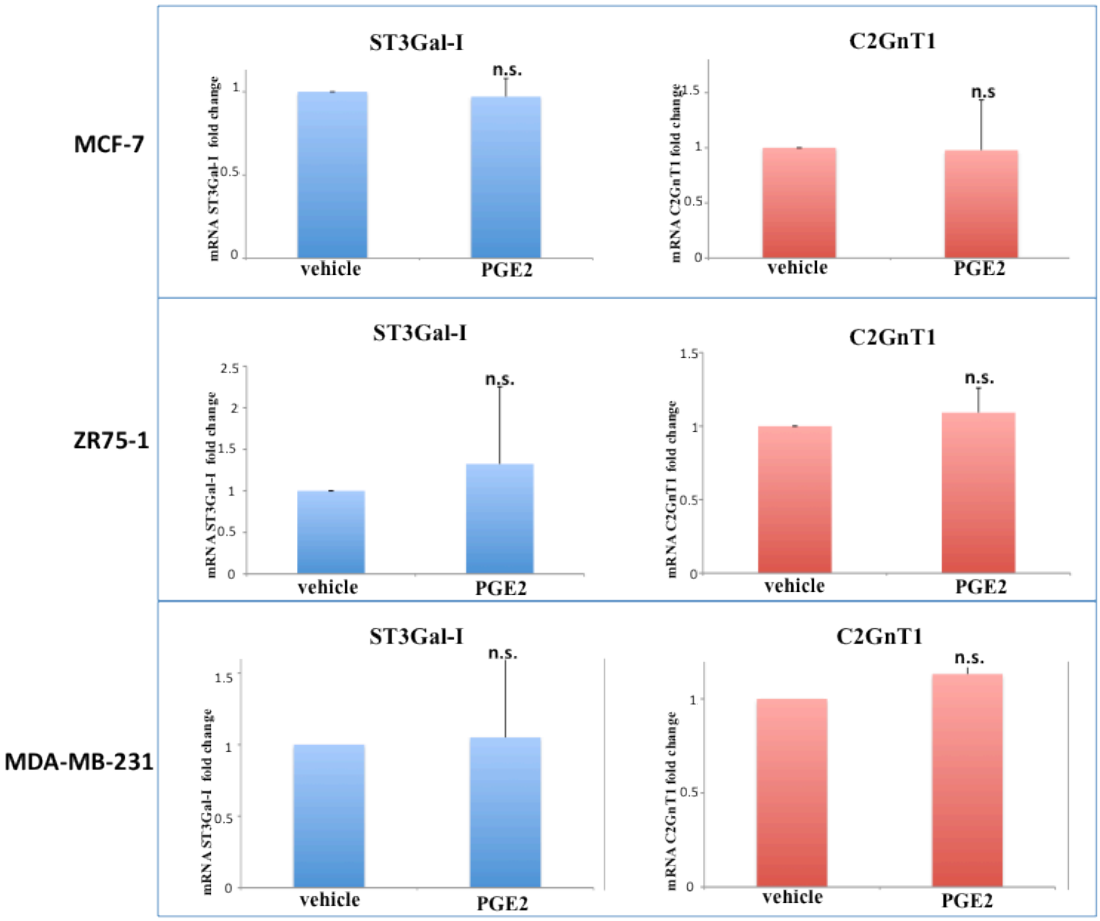


Figure 4.1: Exogenous PGE2 does not affect ST3Gal-I and C2GnT1 in MCF-7, ZR-75-1 and MDA-MB-231.Cells were treated for 4 hours in presence of 0.1 μM PGE2 or the vehicle (ethanol). RNA was extracted and the expression of ST3Gal-I and C2GnT1 determined by RT-qPCR. The histograms show the average±SD of two independent experiments. n.s. =not statistically significant.

Pan et al. showed that CCR7 has high RNA and protein expression in MDA-MB-231 cells (COX-2 expressing breast cancer cell line), while it was low in MCF-7 cells that

express low levels of COX-2. When MCF-7 were treated with PGE2 (0.1 μM for 24 hours), it was found that PGE2 increased CCR7 mRNA and protein expression (Pan et al. 2008). This positive control was used in the cell lines investigated, but no change in the expression of CCR7 was seen (data not shown).

However, when the ER positive cell line T47D was treated with PGE2, a consistent and statistically significant increase in ST3Gal-I expression was observed as shown in figure 4.2, which shows the fold change in expression of ST3Gal-I from three independent experiments.

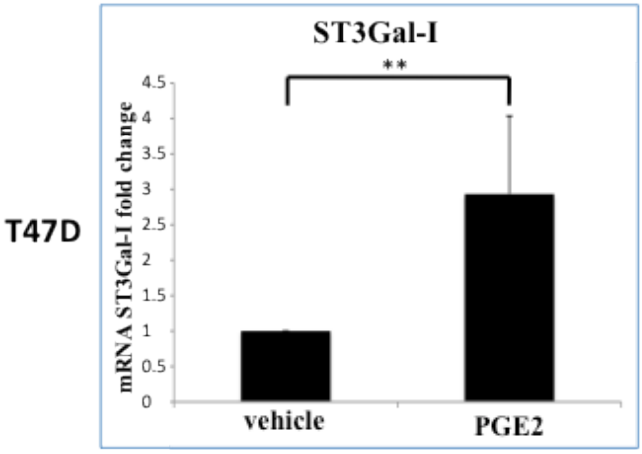


Figure 4.2: PGE2 increases ST3Gal-I expression in T47D. Cells were starved for 2 hours in serum free medium and treated for 4 hours in presence of 0.1 uM PGE2 or the vehicle (ethanol). RNA was extracted and the expression of ST3Gal-I determined by RT-qPCR. The histogram shows the average ± SD of three independent experiments. **$p<0.01$.

The time course of the induction of ST3Gal-I by PGE2 was investigated over 24 hours, by RT-qPCR. Figure 4.3 shows that the level of expression of ST3Gal-I increases by nearly 4 fold compared to the control between 2 and 4 hours (120 and 240 minutes) of PGE2 treatment. After 8 hours the level started to drop until it returned to the baseline level at 24 hours.

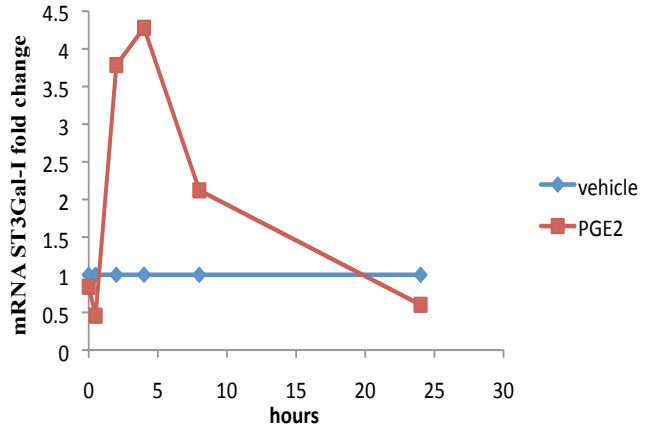


Figure 4.3: Time course of the induction of ST3Gal-I mRNA in T47D treated with PGE2. The cell line T47D was starved for 2 hours in serum free medium and treated for ½, 2, 4, 8, 24 hours with 0.1 mM PGE2 or vehicle (ethanol). RNA was extracted and the expression of ST3Gal-I determined by RT-qPCR. Red line: T47D cells treated with PGE2; Blue line: T47D treated with the vehicle.

It is not possible to quantify the change in protein expression of ST3Gal-I since there are no antibodies available to ST3Gal-I that work on Western blots. However, it is possible to quantify the change of the product of ST3Gal-I, sialylated Core1. Peanut agglutinin (PNA) is a lectin that binds to core 1 and it is inhibited by sialic acids linked in the α2,3 position to galactose. Because of the specificity of PNA, this lectin was chosen to determine ST3Gal-I activity. If the level of expression of ST3Gal-I increases, the sialyl-core 1 will increase and the PNA staining, therefore, will decrease (PNA is inhibited by sialyl-core 1). T47D cells were treated with PGE2 for 92 hours to allow turn-over of surface proteins, and cells were analysed for PNA staining by flow cytometry (FACS).

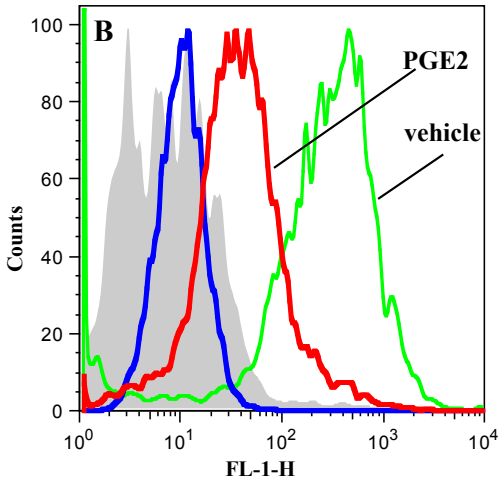


Figure 4.4: Treatment with exogenous PGE2 of T47D increases sialylated core1, demonstrated by a decrease in PNA staining.
T47D cells were treated for 92 hours with or without PGE2. The filled grey peak and blue line are the control for respectively PGE2 treated and untreated T47D (FITC streptavadin (BD Pharmingen). Green and and red lines are PNA stained T47D treated without (green) and with PGE2 (red).

The red line (PGE2 treated cells) of figure 4.4 is shifted to the left compared to the green line (untreated cells), so when the T47D cell line is treated with PGE2, PNA binding decreases, suggesting the presence of increased sialylation of core1, the product of ST3Gal-I. Taken together, these results show that in the breast cancer cell line T47D, PGE2 stimulates the expression of ST3Gal-I that results in the increase of its product sialyl-core1.

4.3.3 TPA enhances ST3Gal-I expression and its product α2,3 sialyl-Core1 in MDA-MB-231 cell line.

COX-2 is reported to be one of the early-response genes induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). The aim of this experiment was to see if inducing COX-2 over-expression could change the expression of ST3Gal-I and C2GnT1. The six breast cancer cell lines were treated with TPA (10 nM) for 18 hours following 4 hours of starvation according to Schrey et al. (1995).

The expression of mRNA for COX-2 was measured in MCF-7 and T47D. Figure 4.5 illustrates the average of three biological experiments. The expression of COX-2 after TPA treatment in MCF-7 and T47D did not change significantly, compared to the control (cells treated with the vehicle). COX-2 mRNA expression did not change in ZR-75-1, BT-20 and SK-Br-3 (data not shown).

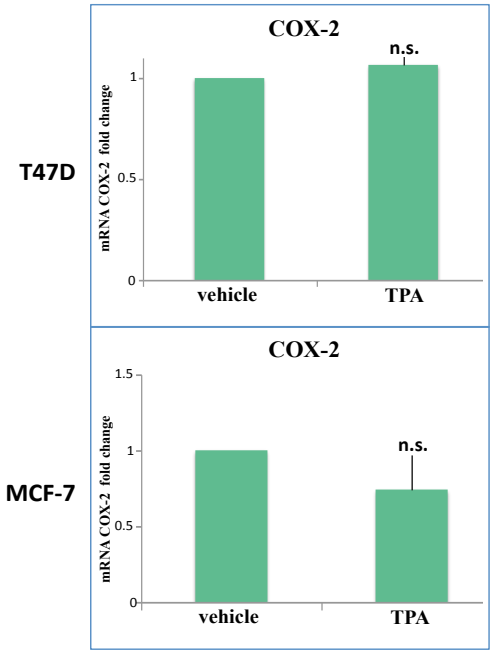


Figure 4.5: TPA treatment does not affect COX-2 levels in T47D and MCF-7. Cells were starved for 4 hours in serum free medium and treated for 18 hours with 10 nM TPA or the vehicle (ethanol). RNA was extracted and the expression of COX-2, determined by RT-qPCR. Each histogram shows the average±SD of three independent experiments for each cell line. n.s.=not statistically significant.

However, treatment of MDA-MB-231 with TPA gave a consistent and proportional increase (depending on the concentration) in the expression of COX-2 (Figure 4.6). Moreover ST3Gal-I mRNA increased and C2GnT1 decreased after TPA treatment (Figure 4.7).

MDA-MB-231

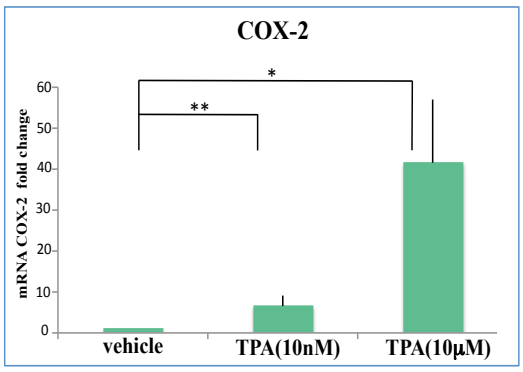


Figure 4.6: TPA induces COX-2 mRNA expression in MDA-MB-231. MDA-MB-231 cells were treated as described in figure 4.5 with 2 concentrations of TPA. RNA was extracted and the expression of COX-2 was determined by RT-qPCR. Data represent the average of three independent experiments. * *p<0.05, **p<0.01.

MDA-MB-231

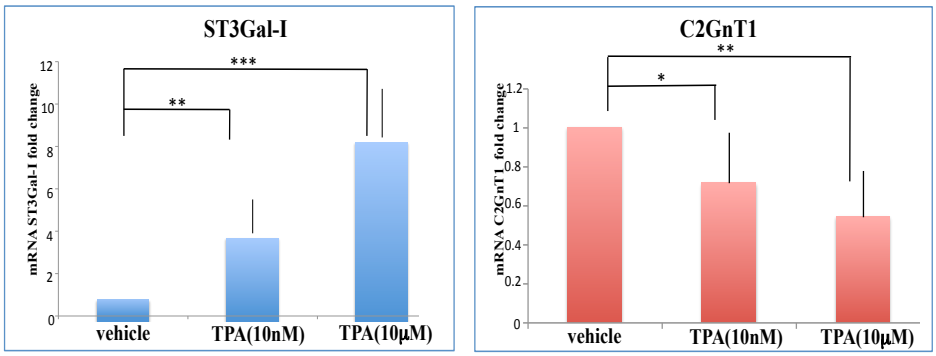


Figure 4.7: TPA induces ST3Gal-I and decreases C2GnT1 mRNA expression in MDA-MB-231. MDA-MB-231 cells were treated as described in figure 4.5 with 2 concentrations of TPA. RNA was extracted and the expression of ST3Gal-I and C2GnT1 was determined by RT-qPCR. Data represent the average of three independent experiments. * p<0.05, **p<0.01, *** p< 0.001.

TPA 10nM and 10μM could increase the basal level of COX-2 in MDA-MB-231 by up to 7 fold and 42 fold respectively. At the same time 10nM and 10μM could increase ST3Gal-I by 4 and 8 fold and decrease C2GnT1 by 1.5 and 2 fold.

A time course experiment was then carried out. 10 μM TPA was initially used and the expression of ST3Gal-I and COX-2 mRNA was determined at the times indicated in figure 4.8.

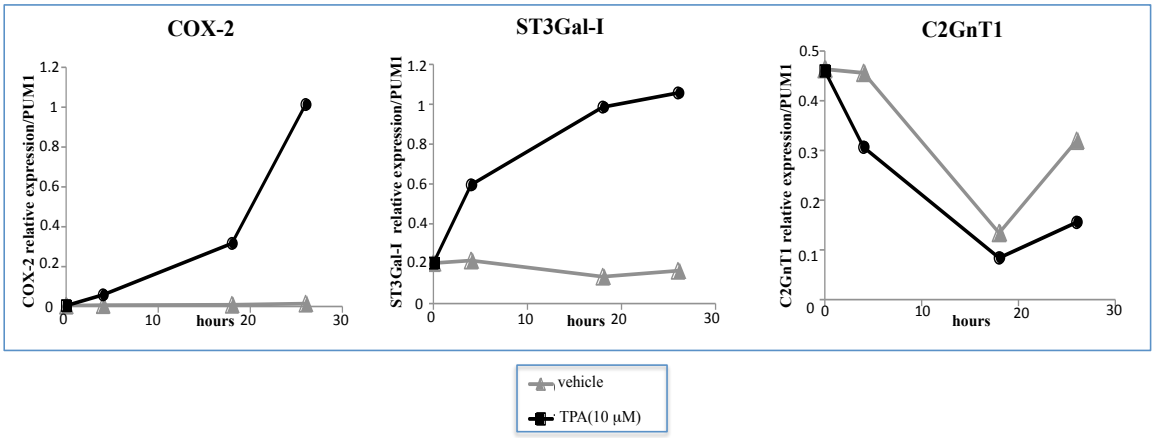


Figure 4.8: TPA (10 μM) induces the expression of COX-2 and ST3Gal-I in MDA-MB-231 cells in a range of 24 hours. MDA-MB-231 cells were starved for 4 hours and incubated with vehicle or 10 nM of TPA for the times indicated. RNA was extracted and the expression of COX-2, ST3Gal-I and C2GnT1 was determined by RT-qPCR. The black and the grey lines represent TPA and vehicle treated cells respectively.

However, incubating the cells with 10 μM TPA for 24 hours did induce some toxicity, as observed by Forsyth et al. (1968). Cells were detached from the flask and started to die already after 18 hours. In figure 4.8, it seems that the amount of vehicle affects the cells so much as to vary the control level of C2GnT1 after 18 hours. This effect can be attributed either to TPA or to the amount of vehicle used (60 μl of ethanol in 10 ml of medium).

Therefore, the time course was repeated using 10 nM TPA. As can be seen from figure 4.9, COX-2 mRNA expression started to rise between 50-100 minutes and kept on increasing till 5 hours, when the experiment was stopped. In contrast, ST3Gal-I mRNA did not start increasing until after 2 hours. This delay in ST3Gal-I expression relative to COX-2 is in agreement with the induction of ST3Gal-I being indirect through COX-2.

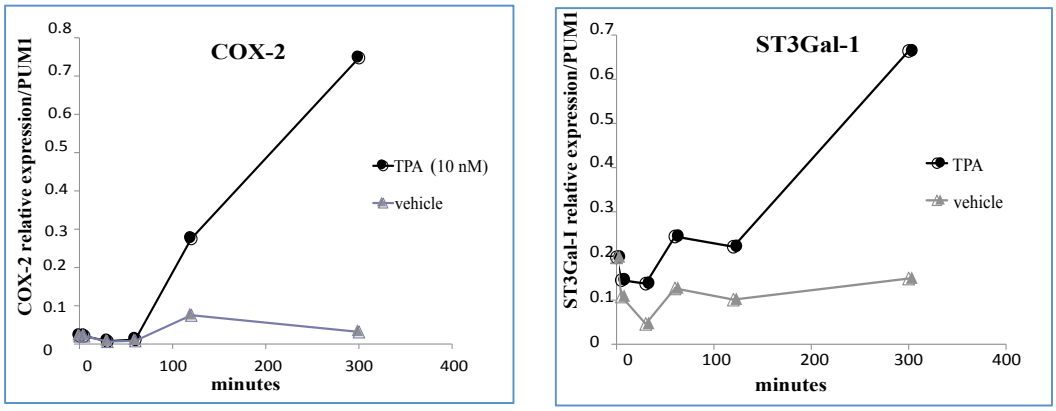


Figure 4.9: Time course of TPA (10 nM) induction of COX-2 and ST3Gal-I expression in MDA-MB-231 treated cells. MDA-MB-231 cells were starved for 4 hours and incubated with vehicle or 10 nM of TPA for the times indicated. RNA was extracted and the expression of COX-2, ST3Gal-I determined by RT-qPCR. The black and the grey lines represent TPA and vehicle treated cells respectively.

Looking at the time course in figure 4.9, MDA-MB-231, treated with 10 nM of TPA, expresses high levels of ST3Gal-I and COX-2 after 5 hours of treatment. Figure 4.10 represents the mean of four independent experiments of MDA-MB-231, treated for 5 hours with 10 nM TPA. The results show that, after 5 hours of treatment, ST3Gal-I and COX-2 expression increases nearly of 4 and 85 fold respectively.

The protein level of expression after TPA treatment was evaluated by western blot. Unfortunately we couldn't see any bands on the western blots using different antibodies (see section 5.3.3 A for western blot results).

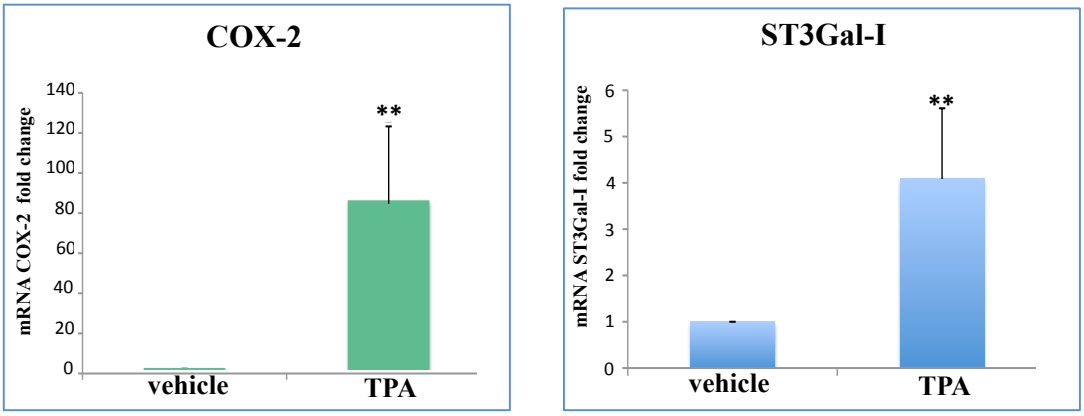


Figure 4.10: ST3Gal-I and COX-2 expression increase by 4 and 85 fold after 10 nM TPA treatement for 5 hours. MDA-MB-231 cells were starved for 4 hours and then treated for 5 hours with 10 nM TPA. RNA was extracted and the expression of COX-2 and ST3Gal-I determined by RT-qPCR. **p<0.01.

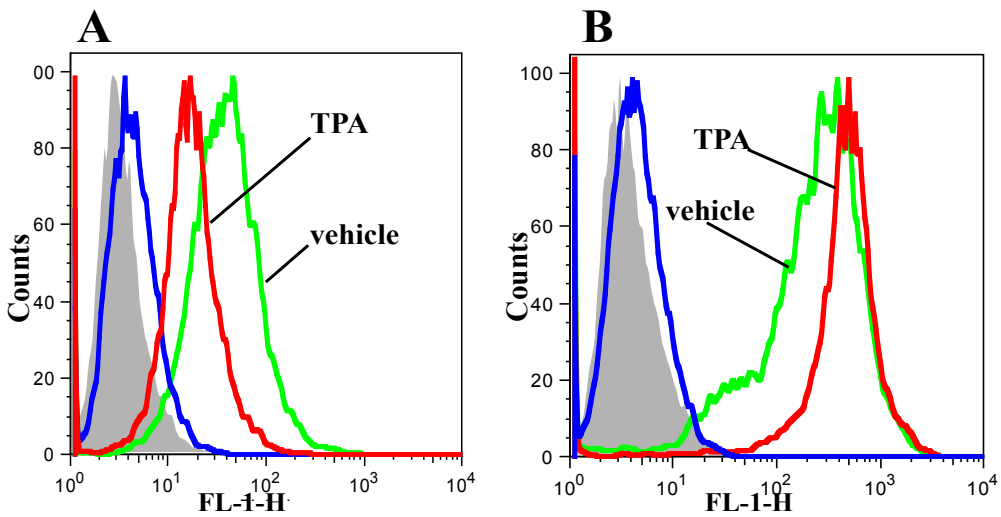


Figure 4.11: A) TPA increases the ST3Gal-I product, sialyl-core1. MDA-MB-231 cells were treated for 72 hours with and without TPA. The solid grey peak and blue line are the control for respectively TPA treated and untreated MDA-MB-231 (FITC streptavadin Ab). Light green and red lines are PNA stained MDA-MB-231 treated without (green) and with TPA (red). **B)Neuraminidase treatment restores PNA staining of TPA treated MDA-MB-231.** TPA (red line) and vehicle (green line) incubated cells were treated for 30 minutes with neuraminidase before staining with PNA and analyzing by flow cytometry. A and B are examples of two independent experiments.

To determine if TPA had increased ST3Gal-I activity, MDA-MB-231 cells were incubated with TPA for 72 hours and then stained with PNA. Cells treated with TPA (red line in figure 4.11A) showed a shift to the left compared to the control, demonstrating that there is less unsubstituted core1 (T antigen) in the TPA treated cells compared to the control.

To confirm that the decrease in PNA staining, after TPA treatment of MDA-MB-231, was dependent on the increase of sialylation of core1, the cells were treated with neuraminidase. The neuraminidase used was derived from Clostridium perfringens and it hydrolyzes α(2→3), α(2→6), and α(2→8)-glycosidic linkages of terminal sialic residues of various glycomolecules. Figure 4.11B shows PNA staining of TPA and vehicle treated MDA-MB-231 cells after treatment with Neuraminidase. The red line (TPA treated cells) overlaps with the green line (vehicle treated cells), indicating that the pool of core1 based structure was unchanged in the TPA treated cells and confirming that the decrease in PNA staining observed in figure 4.11A is due to sialic acid masking core1.

4.4 Discussion.

This chapter describes the initial data that suggest that ST3Gal-I can be induced by COX-2. We showed that in T47D cells PGE2, a product of COX-2, induced expression of ST3Gal-I mRNA. We also showed that TPA, which among its many effects has been known to induce COX-2, can also induce the mRNA expression of ST3Gal-I. In both cell lines, induction of ST3Gal-I mRNA led to increased activity of this sialyltransferase.

Different responses in two breast cancer cell lines, T47D and MDA-MB-231, to PGE2 and TPA, were observed. In T47D, PGE2 induced the expression of ST3Gal-I, resulting in increased expression of Sialyl-core1. However T47D did not respond to TPA. In MDA-MB-231 TPA induced COX-2 and ST3Gal-I expression, but these enzymes were not induced by exogenous PGE2 in this cell line.

MDA-MB-231 and T47D represent two different subtypes of breast cancer. MDA-MB-231 is an adenocarcinoma breast cancer cell line, representative of triple negative breast cancer (ER, PR and HER-2 negative-see introduction). T47D is a ductal adenocarcinoma breast epithelial tumor cell line, which is estrogen receptor positive. Both were derived from metastatic pleural effusions. The glycosylation pattern is also different. In T47D breast cancer cell lines there is no core 2 formation and this is due to the lack of expression of C2GnT1. Dalziel et al. showed, by Northern analysis, that C2GnT1 was not expressed in T47D cells, while it was expressed in a non-malignant breast cell line, MTSV1-7 (Dalziel et al. 2000). On the other hand, ST3Gal-I is very active (Lloyd et al. 1996, Whitehouse et al. 1997). MDA-MB-231 has core 1 and core 2 based structures. The core 1 structures are mostly sialylated, suggesting competition between C2GnT1 and ST3Gal-I (Muller et al. 2002).

However, the different responses to PGE2 and TPA may be due mainly to the different expression of EP receptors (PGE2 receptors) and expression of COX-2. As discussed in Chapter 3, MDA-MB-231 cells express low levels of the EP_2 PGE2 receptor, but expresses EP_4. EP_2 and EP_4 are receptors coupled to PKA/adenyl cyclase and mediate elevation in intracellular cAMP (Narumiya et al. 1999). However, in contrast to EP_2, the signal, produced by the EP_4 receptor, is lost within a few minutes because of agonist induced desensitization (Nishigaki et al. 1996). Thus, the lack of response of MDA-MB-231 to PGE2 may be explained by this line requiring a continuous PGE2 stimulus that can be provided by TPA, but not by the exogenous PGE2 which has a very short half-life (Fitzpatrick et al.1980).

TPA only induced COX-2 overexpression in MDA-MB-231, which has the highest amount of COX-2 compared to the other breast cancer cell lines (as demonstrated in chapter 3). This agrees with Liu et al. who demonstrated that MDA-MB-231 cells

expressed constitutive levels of COX-2 that increased further in the presence of TPA (Liu et al.1996).

Kakiuchi et al. (2002) found that COX-2 overexpression or PGE2 treatment reduced ST3Gal-I expression, while Celecoxib (COX-2 selective inhibitor) increased its expression in colonrectal cancer cell lines. This result is in opposition to what was found here. However, Hatano et al. (2011) showed that in castration-resistant prostate cancer cell lines, PC3 and DU145 cells, the expression of ST3Gal-I could be mildly induced by TPA, and TPA-induced expression of ST3Gal I could be inhibited by NF-κB decoy oligodeoxynucleotides (ODN).

The results presented in this chapter suggest that ST3Gal-I can be induced by COX-2 action, but more convincing data is required. Chapter 5 describes the series of experiments undertaken to confirm that ST3Gal-I is upregulated by COX-2.

Chapter 5. Induction of ST3Gal-I in MDA-MB-231 depends on COX-2.

5.1 Introduction

In the previous chapter it was demonstrated that TPA could induce the overexpression of COX-2 and of ST3Gal-I in MDA-MB-231. In the breast cancer cell line T47D, PGE2 could increase the expression of ST3Gal-I at the mRNA level and at the protein level. This chapter will focus on the MDA-MB-231 model and the involvement of COX-2 in the induction of ST3Gal-I after TPA treatment.

To confirm the involvement of COX-2, inhibitors of COX-2 were used together with TPA to see if the effect of the phorbol ester could be reversed. The enzyme was silenced or overexpressed in MDA-MB-231 and ST3Gal-I mRNA expression was determined by RT-qPCR. The expression of ST3Gal-I and COX-2 by 78 primary breast cancers was analyzed and the correlation between the two enzymes was determined.

5.2 Objectives.

1. Treat cells with TPA and the COX-2 selective inhibitors NS398 or celecoxib and determine if there is any effect on the glycosyltransferases by qPCR.
2. Determine the change of PNA staining of ST3Gal-I product after TPA and Celecoxib treatment by FACS analysis.
3. Silence COX-2 with RNAi and check for COX-2 expression and ST3Gal-I RNA expression.
4. Transfect COX-2 into MDA-MB-231 and determine the expression of ST3Gal-I.
5. Determine the correlation between the expression of ST3Gal-I and COX-2 by 78 primary breast cancers.

5.3 Results.

5.3.1 Role of COX-2 inhibitors after TPA induction of ST3Gal-I in MDA-MB-231.

The six epithelial breast cancer cell lines (MCF-7, T47D, MDA-MB-231, SKBr-3, BT20 and ZR75-1) were treated with a selective COX-2 inhibitor, NS398. NS398 can block any possible release of PGE2 in the medium. No difference was seen in ST3Gal-I and C2GnT1 expression of two biological experiments after 24 hours of NS398 treatment (figure 5.1).

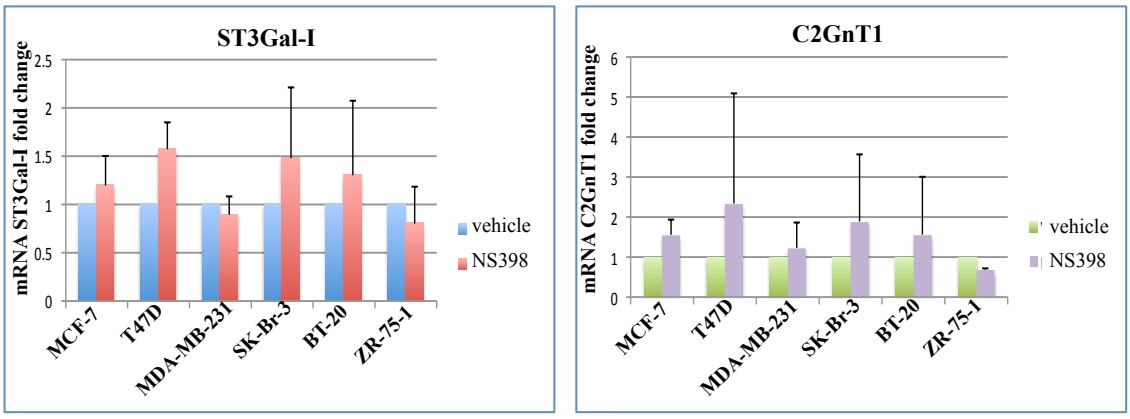


Figure 5.1: NS398 treatment in six breast cancer cell lines cannot change ST3Gal-I and C2GnT1 expression. All the breast cancer cell lines were treated for 24 hours with NS398 or the vehicle in serum free condition and the mRNA of ST3Gal-I and C2GnT1 determined by RT-q-PCR. The histograms show the mean of two biological experiments.

The expression of COX-2 and of ST3Gal-I was therefore induced with TPA in MDA-MB-231 and two selective inhibitors, NS398 and Celecoxib, were used to determine if the TPA effect could be reverted by blocking COX-2. Cells were starved for 4 hours, then treated with 10 nM TPA in the presence or absence of NS398 (20 μM) or Celecoxib (20 μM) for 18 hours.

Figure 5.2 shows that COX-2 expression increased after TPA treatment and could be decreased by NS398. Induction of ST3Gal-I expression could also be slightly decreased by NS398, but neither the reduction of COX-2 or ST3Gal-I was statistically significant. No effect of NS398 was seen on C2GnT1 mRNA. In a third biological experiment, COX-2 induction was very high (over 45 fold) but NS398 could still partially inhibit COX-2 and ST3Gal-I expression (data not shown).

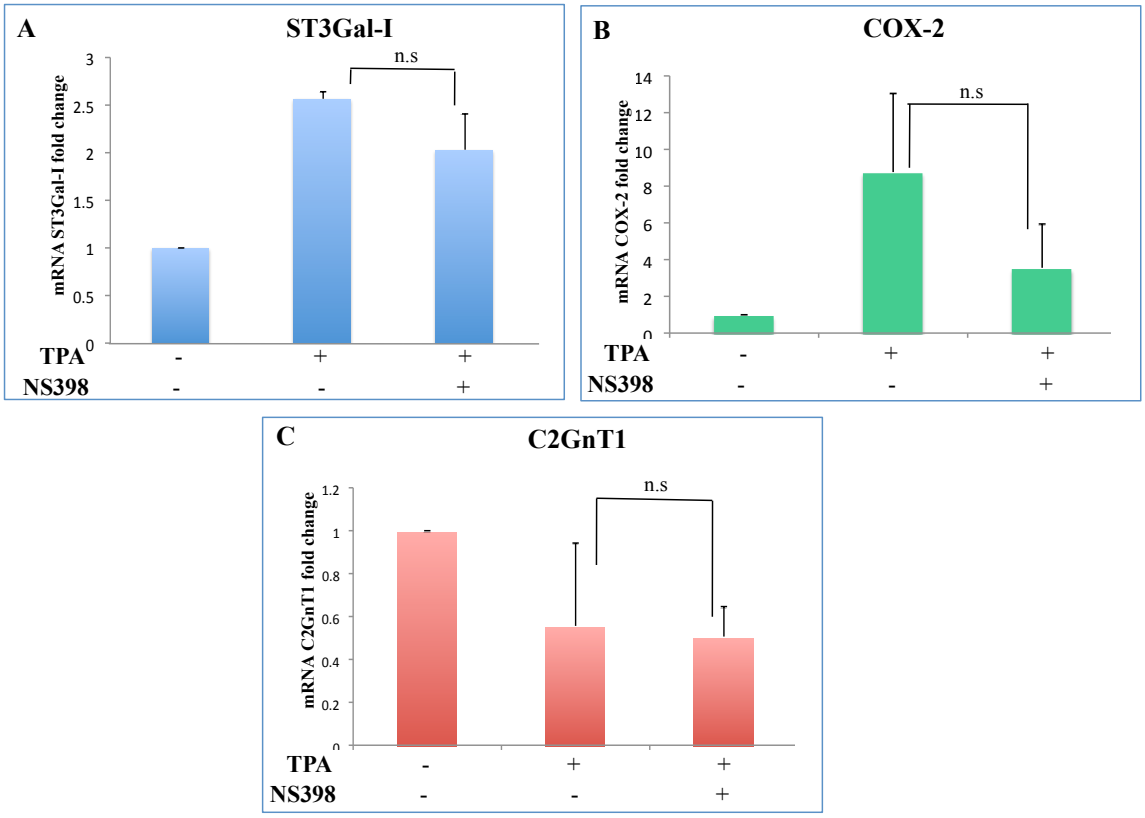


Figure 5.2: NS398 can partially reverse TPA induction of COX-2.
MDA-MB-231 were treated with TPA (10 nM) or TPA (10nM) with NS398 (20 μM) for 18 hours and the mRNA of A.ST3Gal-I; B.COX-2; C.C2GnT1 determined by RT-q-PCR. The histograms represent the mean of two biological experiments. n.s. p>0.05.

MDA-MB-231 cells were also treated with TPA in presence or absence of Celecoxib. Celecoxib is another selective COX-2 inhibitor used in chronic inflammation and management of acute pain in adults. It is also used to reduce the number of polyps in patients with familial adenomatous polyposis (FAP) (European Medicine Agency website) and in some clinical trials for breast cancer (see section 1.9.5).

Celecoxib did not inhibit the TPA effect on COX-2 and ST3Gal-I, rather unexpectedly it enhanced their mRNA expression (see figure 5.3).

MDA-MB-231 were incubated with TPA and different concentrations of Celecoxib (1, 5, 10, 20 μM). COX-2 mRNA showed a small progressive increase in expression of 27, 36, 41 fold with 1, 5, 10 μM of Celecoxib. However at 20 μM of Celecoxib, COX-2 mRNA increased by a massive 403 fold (see figure 5.4). The induction of ST3Gal-I mRNA increased by 3 fold with 1, 5, 10 μM of Celecoxib, but increased to 9 fold with 20 μM. In order to check if the production of PGE2 was also enhanced by Celecoxib, the concentration of PGE2 was measured in the media by ELISA.

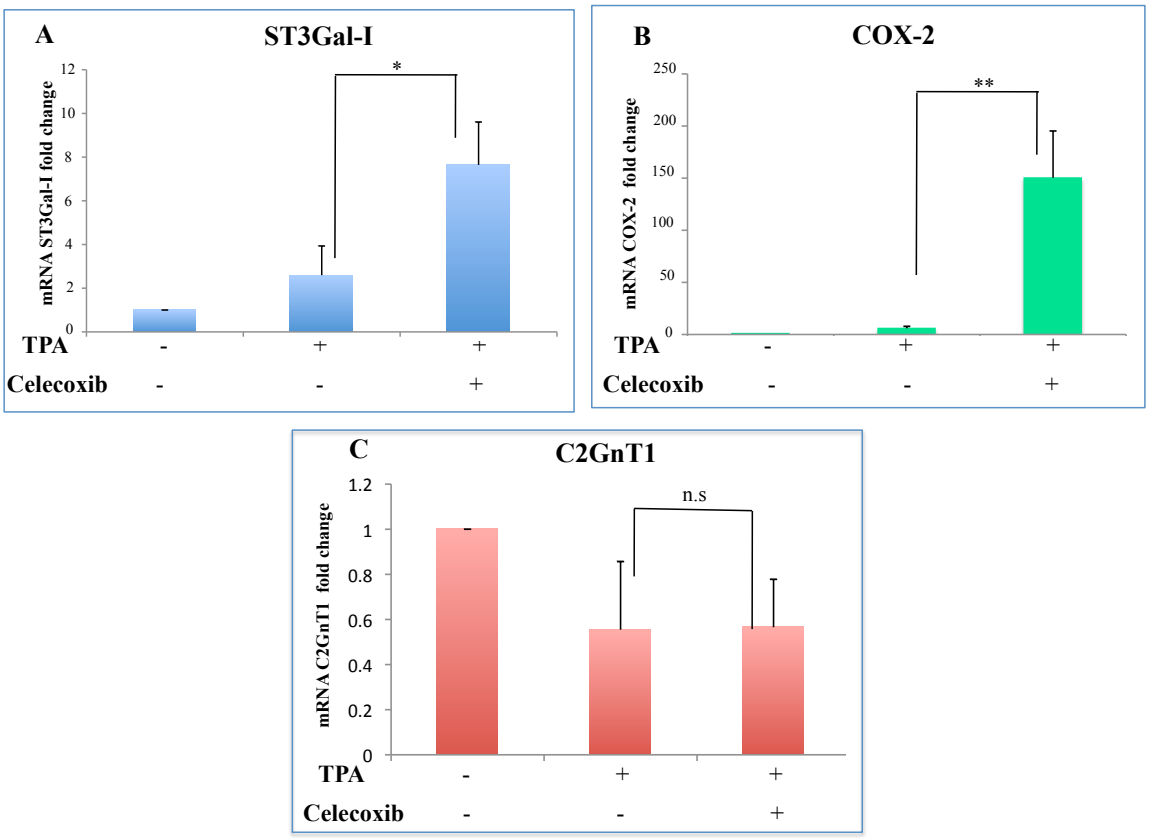


Figure 5.3: Celecoxib can enhance TPA induced expression of COX-2 and ST3Gal-I. MDA-MB-231 were treated with TPA (10 nM) on its own and TPA (10nM) and Celecoxib (20 μM) for 18 hours and the mRNA of A.ST3Gal-I; B.COX-2; C. C2GnT1 determined by RT-q-PCR. The histograms represent the mean of three biological experiments. *p<0.05, **p<0.01, n.s.p>0.05.

The concentration of PGE2 in the supernatant of MDA-MB-231 treated with TPA or different concentration of Celecoxib in the presence of TPA was higher compared to the control, ie. medium (DMEM without FCS and glutamine) from cells after 18 hours incubation (sample number 1). As can be seen in figure 5.5, Celecoxib can inhibit the TPA induced production of PGE2, with maximum inhibition being seen at 5 μM. Paradoxically, 20 μM Celecoxib (the concentration that induced a massive increase in COX-2 mRNA, see figure 5.4) still reduced PGE2 secretion by about one third, but still less than 5 μM. The increase of COX-2 and ST3Gal-I expression and the decrease in inhibition of PGE2 production after treatment with Celecoxib 20 μM can be proof of toxicity of a high dose drug on the cells.

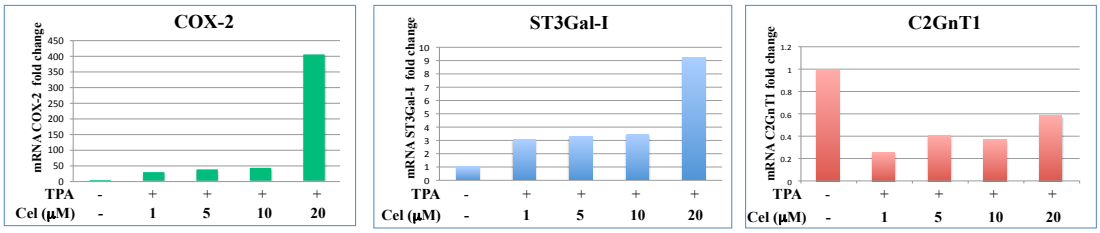


Figure 5.4: Celecoxib increases TPA induced expression of COX-2 and ST3Gal-I. MDA-MB-231 cells were treated with TPA (10nM) and Celecoxib (1, 5, 10, 20 µM) for 18 hours and the mRNA of COX-2; ST3Gal-I and C2GnT1 measured by RT-q-PCR. The histograms represent only one biological experiment. Cel stands for celecoxib.

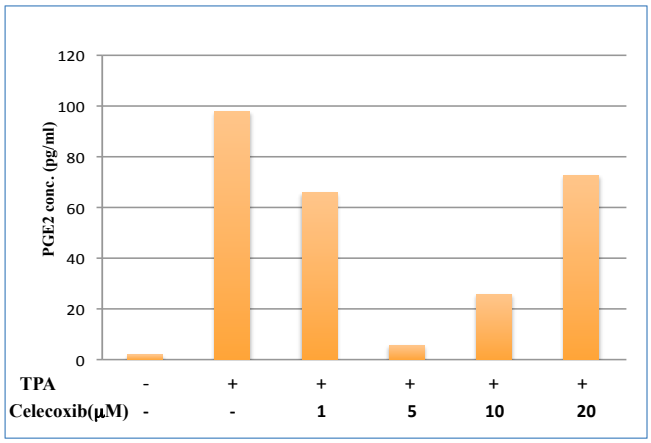


Figure 5.5: PGE2 concentration in the medium of MDA-MB-231 treated with TPA and Celecoxib. MDA-MB-231 cells were incubated with TPA in the presence of increased concentration of Celecoxib (1, 5, 10, 20 µM) and the media was collected after 18 hours of treatment. The PGE2 concentration was determined by ELISA, according to the manufacturer's instruction.

5.3.2 The effect of Celecoxib on the product of ST3Gal-I.

The results with COX-2 in the presence of TPA and Celecoxib showed that determination of mRNA expression cannot always predict enzyme activity. Therefore the formation of ST3Gal-I product was measured to determine if Celecoxib could inhibit the glycosyltransferase activity. MDA-MB-231 were treated with TPA in the presence or absence of Celecoxib (20 µM) for 92 hours to allow turn-over of surface proteins, and cells were analysed for PNA staining by flow cytometry. As shown in figure 5.6, TPA decreases PNA staining (red) compared to the control (light green), but this effect could be reverted by Celecoxib (dark green). Thus when cells were treated with both TPA and Celecoxib the shift went back to the same level of the binding observed in the absence of TPA. This means that even if the mRNA of COX-2 and ST3Gal-I increases after TPA and Celecoxib treatment, Celecoxib is able to restore the TPA effect on ST3Gal-I

product. Figure 5.6 represents two independent biological experiments. However the experiment should be repeated using other concentrations of Celecoxib (like 5 μM, concentration) that was more effective at inhibiting PGE2 production.

The use of inhibitors is always open to criticism due to their potential non-specificity. Moreover, the results obtained here with Celecoxib on COX-2 and ST3Gal-I expression were totally unexpected. Therefore to confirm that the increase of ST3Gal-I expression after TPA treatment was going via COX-2, COX-2 was knocked down after TPA treatment and ST3Gal-I expression was determined.

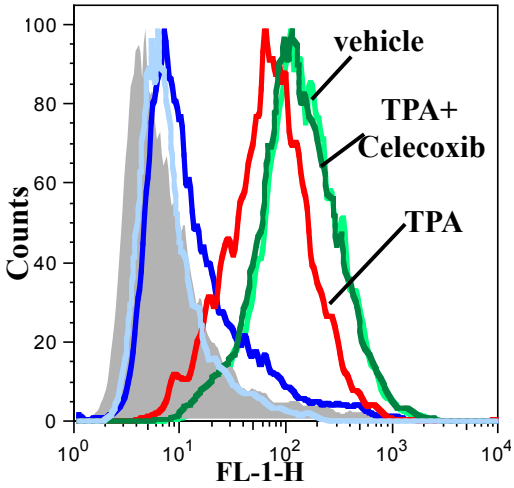


Figure 5.6: Celecoxib reversed the TPA effect on ST3Gal-I activity. MDA-MB-231 cells were treated with TPA in the presence (dark green) or absence(red) of the COX-2 inhibitor celecoxib and then analysed for PNA binding. Grey, dark blue, light blue are control cells incubated without PNA.

5.3.3 Knock down of COX-2 in MDA-MB-231 and determination of ST3Gal-I mRNA expression.

A) Characterization of the COX-2 antibody.

To determine the efficacy of siRNA the best way is to look at the protein by Western blot. The antibody to COX-2 was therefore tested on Western Blots. The expected molecular weight of COX-2 is 72 kDa. MDA-MB-231 incubated for 5 hours with TPA, were lysed in RIPA buffer according to materials and methods (see chapter 2.4.1) and run on 7.5% SDS PAGE gels.

The positive control used was MDA-MB-231 transfected for 24 hours with COX-2, lane number 3 of figure 5.7 A and B (see section 5.4). MDA-MB-231, treated with TPA for 5 hours showed a band at the same molecular weight as the positive control, that was not present in MDA-MB-231 treated with the vehicle (lane 1 of figure 5.7 A). This experiment was repeated using the same conditions and in parallel lysates for mRNA

were collected. Unfortunately a further series of western blots did not show any band after blotting with the COX-2 antibody, apart from the transfected COX-2 MDA-MB-231 (Figure 5.7 B). The loading control β-actin showed the band at the appropiate molecular weight (42 kb) indicating that the same amount of protein was used and that the problem was concerning the primary antibody against COX-2.

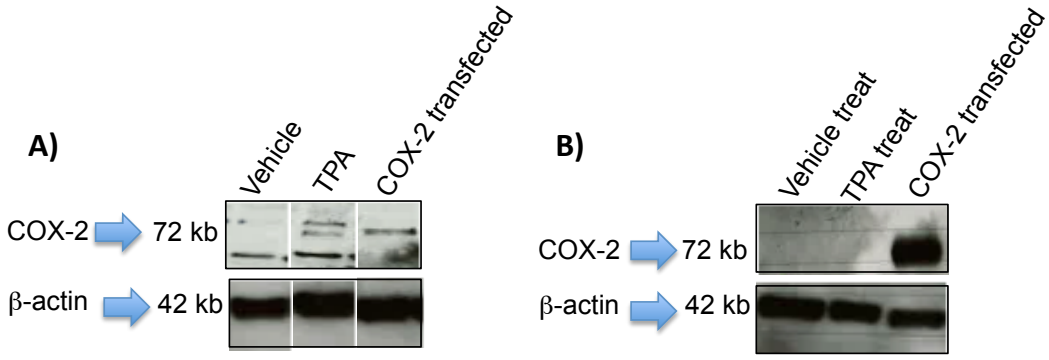


Figure 5.7: Induction of COX-2 expression in response to 5 hours of TPA treatment of MDA-MB-231. Lysates were immunoblotted with COX-2 antibody followed by HRP-conjugated goat anti-mouse IgG (Dako). Both blots were stripped and reprobed with Mouse Anti- β−actin followed by HRP-goat antimouse IgG (Dako). Membranes were developed with the ECL system (Amersham) according to the manufacturer's instructions. Lane 1, MDA-MB-231 treated with vehicle, lane 2, MDA-MB-231 treated with TPA and lane 3, MDA-MB-231 transfected with COX-2 for 24 hours (positive control). A and B are two biological replicates.

Another antibody against human COX-2 was used (Oxford Biomedical) and cells were lysed differently. In this case Laemli SDS PAGE sample buffer 1x (1 ul/10000 cells determined by counting a parallel well) was used directly on the cell layer. The concentration of antibody used was raised to 10 ug/ml. The western blot showed non-specific bands, and no difference between the different conditions.

B) Assessment of COX-2 mRNA knock down in MDA-MB-231.

Since it was not possible to consistently see endogenous expression of COX-2 in MDA-MB-231 by Western blot, the level of knock down for COX-2 was measured at the mRNA by RT-qPCR. MDA-MB-231 only express low levels of COX-2 at the mRNA level, hence the knock down was evaluated after TPA treatment (see figure 5.8, 5.9, 5.10).

Three different pairs of siRNA oligos were used to knock down COX-2 together with siRNA control. The first pair of primers from Santa Cruz Biotecnology (called siRNA COX-2 SC in figure 5.8) knocked down COX-2 only by 50% compared to the control. 24

hours after transfection, the cells were treated for 5 hours with TPA treatment (figure 5.8).

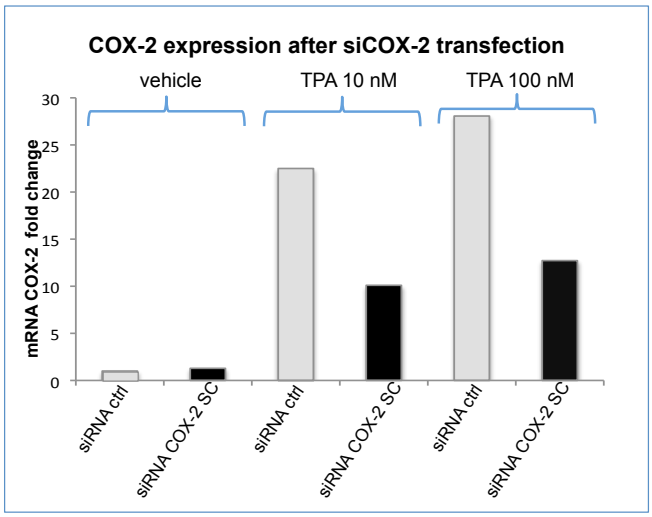


Figure 5.8: siRNA knock-down of COX-2 with Santa Cruz oligos (siRNA COX-2 SC) reduced COX-2 mRNA by 50% in MDA-MB-231 compared to si-RNA control. Cells were transfected with siRNA and 24 hours after transfection, treated for 5 hours with TPA (10 and 100 nM). COX-2 mRNA expression was then determined by demonstrated by RT-q-PCR

Another two oligos for siRNA silencing of COX-2 were used (see figure 5.9). They are called 1siRNA COX-2 and 2' siRNA COX-2. They were designed by Stasinopoulos et al. (2007) and produced by Dharmacon. In this case both siRNA oligos could knock down COX-2 in three biological experiments (Figure 5.9). Having an efficient knock down of COX-2, the expression of ST3Gal-I was subsequently measured.

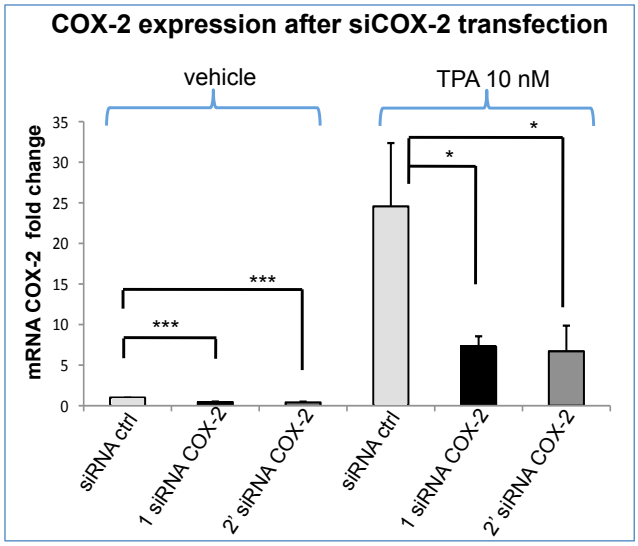


Figure 5.9: Two pairs of siRNA targeting COX-2 knocked down COX-2 in MDA-MB-231 by 70%. Cells were transfected with siRNA and 24 hours after transfection, treated for 5 hours with TPA 10 nM. COX-2 mRNA expression was then determined by RT-q-PCR. In 2' COX-2 siRNA, 1siRNA and 2siRNA described in materials and methods were pooled. Data represent three independent experiments. *p<0.05, *** p<0.001.

C) Investigation of ST3Gal-I mRNA after COX-2 knock down in MDA-MB-231.

The expression of ST3Gal-I was determined after COX-2 had been knocked-down using the siRNA shown in figure 5.9. Figure 5.10 shows that when COX-2 was knocked-down by 70%, ST3Gal-I mRNA decreased by 50 % for both siRNA oligos in three biological experiments.

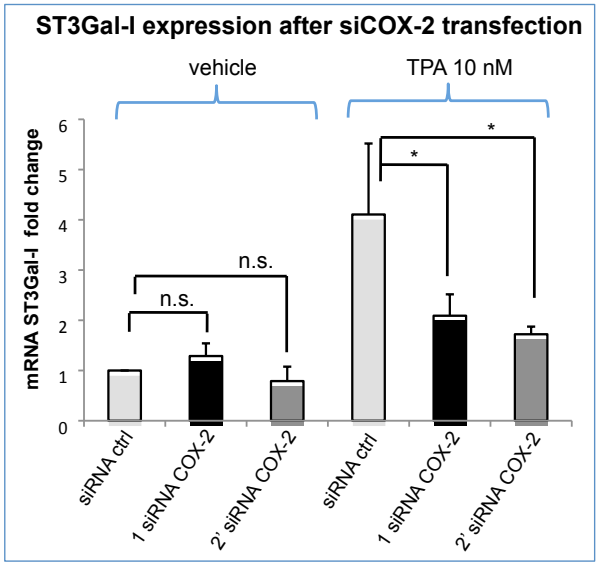


Figure 5.10: The two siRNA that knocked down COX-2 by 70% in MDA-MB-231 (figure 5.9) reverted TPA effect on ST3Gal-I by 50%. MDA-MB-231 were transfected with 1 and 2' siRNA and 24 hours after transfection, treated for 5 hours with TPA 10 nM. ST3Gal-I mRNA expression was then determined by RT-qPCR. In 2' COX-2 siRNA, 1siRNA and 2siRNA described in materials and methods were pooled. Data represent three independent experiments. *p<0.05, n.s.=not statistical significant

Moreover two pooled siRNA to COX-2, that gave a knock down of 53% in two other biological experiments (figure 5.11), were also able to decrease ST3Gal-I mRNA expression by 50% (figure 5.11).

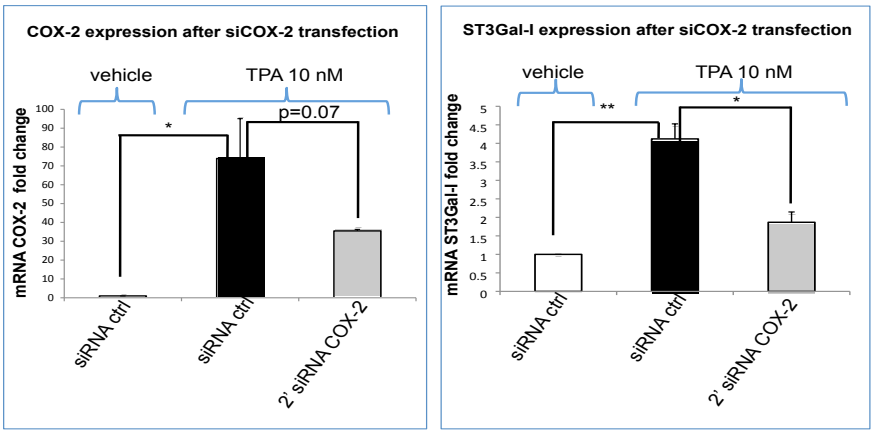


Figure 5.11: 2' siRNA, targeting COX-2, knocked down COX-2 in MDA-MB-231 by 53% in another two biological experiments and reverted TPA effect on ST3Gal-I. Cells were transfected and treated as descrive in figure 5.9 and 5.10. COX-2 mRNA expression was then determined by RT-q-PCR. Data represent two independent experiments. Asterisks denote a statistically significant difference from control: *p<0.05, ** p<0.01. In the specific case of p=0.07 the value is reported since it is very close to be significant.

In summary, two pairs of siRNA that could knock down COX-2 by 70%, could revert the TPA effect on ST3Gal-I. Furthermore, in subsequent experiment when the pooled

siRNA were used and COX-2 expression was only decreased by 50%, ST3Gal-I expression was still decreased (see figure 5.11). These data provide evidence that the increase of ST3Gal-I mRNA induced by TPA is working through COX-2.

5.3.4 Transfection of COX-2 in MDA-MB-231 and determination of ST3Gal-I mRNA expression.

A)Determination of COX-2 plasmid authenticity.

To confirm the involvement of COX-2 in regulating ST3Gal-I expression, COX-2 was overexpressed by transient transfection in MDA-MB-231.

The vector containing the cDNA for COX-2 (called pCMV6-AC) was purchased from Origene. The vector includes a strong CMV promoter, a neomycin selectable marker and the COX-2 cDNA (also called with the gene name: *PTGS*2) cloned into the vector at EcoRI and NotI sites. To check the authenticity of the construct, the plasmid was cut with restriction enzymes and an antibody against human COX-2 was used for staining transiently transfected cells.

Two plasmid maxipreps were prepared and the purified plasmid cut with EcoRI and NotI and run on a 1% agarose gel. Figure 5.12 shows that digesting the samples with EcoRI and NotI resulted in two bands of around 6 and 3.5 kb agreeing with the manufacturer's information that the size of the vector is 5.8 and the size of the insert is 3.5 kb.

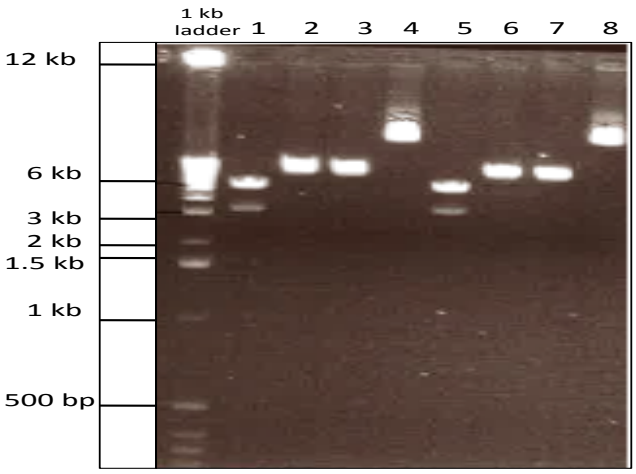


Figure 5.12: Restriction endonuclease digest of hPTGS2 vector (pCMV6-AC). Lanes 1 to 4 and 5 to 8 are two different batches derived by Maxiprep of the same vector. Lanes 1 and 5 are Not I and EcoRI digested, lane 2 and 6 Not I digested, lane 3 and 7 EcoR I digested and lane 4 and 8 are uncut. The ladder used was 1 kb ladder from (Invitrogen).

COS cells were transiently transfected with the COX-2 plasmid and after 24 hours stained with COX-2 antibody (Oxford Biomedical). Figure 5.13 shows the difference

between wild type COS cells (figure 5.13 a), COX-2 transfected COS cells stained only with secondary Alexa Fluor® 546 Goat Anti-Rabbit IgG (H+L) (figure 5.13b), and with COX-2 antibody (figure 5.13 c). The staining of COX-2 appeared to be located in the endoplasmic reticulum and around the nuclear envelope as it is expected for this enzyme (Morita et al.1995). In conclusion the plasmid pCMV6-AC was verified to express COX-2 and could be used to express COX-2 in the cell line MDA-MB-231.

Figure 5.13 COX-2 staining in COX-2 transfected COS cells. Cells ($1x10^5$) were plated the day prior to staining. Cells were fixed in 4% paraformaldehyde at 4°C for 10 min and blocked with 100% FCS. After three washes wild type COS cells were stained with anti-COX-2 antibody (Oxford Biomedical) followed by Alexa Fluor® 546 Goat Anti-Rabbit IgG (a); COX-2 transfected COS cells stained with the second antibody Alexa Fluor® 546 Goat Anti-Rabbit IgG (b); COX-2 transfected COS cells stained with antiCOX-2 antibody (Oxford Biomedical) followed by second antibody (c).

B) Transfection of COX-2 for 72 hours induced ST3Gal-I expression in MDA-MB-231

MDA-MB-231 cells were transfected with pCMV6-AC and tested after 72 hours for COX-2 and ST3Gal-I mRNA expression. COX-2 transfected MDA-MB-231 showed an increase in COX-2 and ST3Gal-I mRNA expression by RT-qPCR. Figure 5.14 shows histograms of the mean of mRNA fold change in expression of COX-2 and ST3Gal-I compared to the mock transfected cells.

5.3.5 Correlation of COX-2 and ST3Gal-I in vivo in breast cancers.

To investigate the clinical significance of our findings we determined the expression of COX-2 and ST3Gal-I mRNA by RT-PCR on 78 primary breast cancers (52 ER positive, 14 ER negative, rest unknown classification) obtained from the Guy's and St Thomas' Research Breast Tissue Bank. Before RNA extraction, sections were taken from the tissue to confirm that each sample contained at least 70% carcinoma cells (performed

by the Tissue Bank Lab). PUM1 was used as the reference gene and the level of ST3Gal-I normalized to PUM1 (see table 5.1 for the results). Figure 5.15 shows the plot of –log of the expression of COX-2 against –log of expression of ST3Gal-I.

We applied two statistical tests to the data. Firstly, Spearman's rank correlation which gave a correlation of 0.309 with a p value of 0.00593, and secondly Pearson's correlation which gave a correlation coefficient of 0.274 with a p value of 0.015. Thus both tests confirmed the presence of moderate but statistically significant correlation in the expression of the two enzymes. The statistics were calculated by Dr. Brian Burford (Breakthrough Breast Cancer, Research Oncology, King's College London, Guy's Hospital, London, UK).

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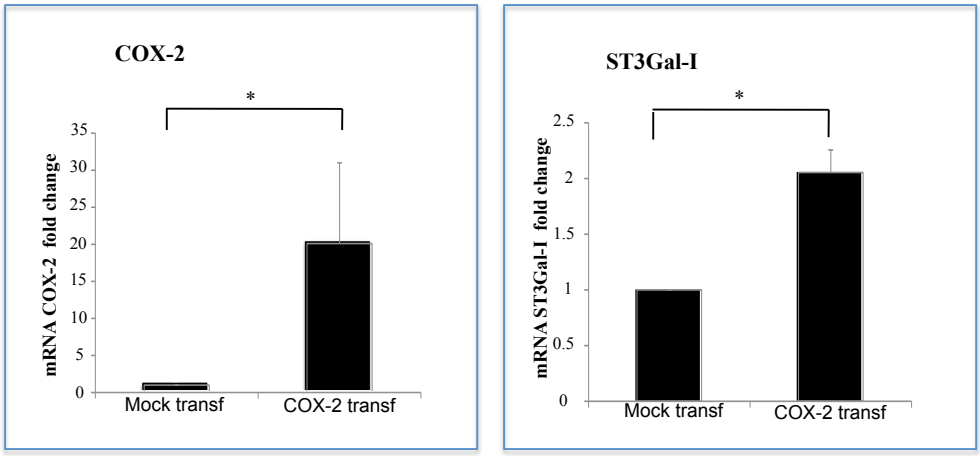


Figure 5.14: COX-2 transfection of MDA-MB-231 enhances ST3Gal-I mRNA expression by two fold. MDA-MB-231 were transfected with COX-2 cDNA and RNA extracted 72 hours past transfection. The expression of COX-2 and ST3Gal-I were determined by RT-q-PCR. The histograms represent mean of three independent experiments. Asterisks denote a statistically significant difference from control: *p<0.05.

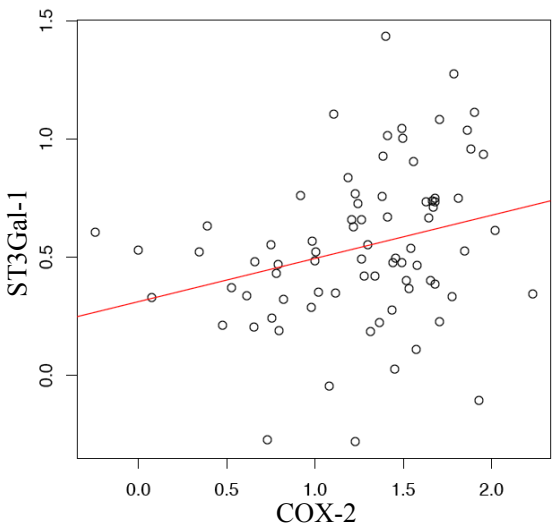


Figure 5.15: The expression of COX-2 and ST3Gal-I are correlated in primary breast cancers. RNA was extracted from 78 primary breast cancers and the expression of COX-2 and ST3Gal-I was determined by RT-PCR. The -log of the DCT was plotted and the correlation determined by the Spearman's test, correlation coefficient was 0.309, p=0.00593 and the Pearson test, correlation coefficient 0.274 p=0.0150. Table 5.1 (below) lists the data for all the 78 primary tumours.

Table 5.1: Relative expression to PUM1 of COX-2 and ST3Gal-I mRNAs in 78 specimens of primary breast cancer

	$2^{-\Delta Cts}$ relative to PUM1	
n of sample	COX-2	ST3Gal-1
1	0.02176268	0.18317993
2	0.05940247	1.91189064
3	0.0320248	0.33394135
4	0.12075599	0.17273911
5	0.18547972	1.879914
6	0.08258365	1.10867256
7	0.40650171	0.23234385
8	0.17608371	0.57130511
9	0.02221483	0.39864229
10	0.05269586	0.38165297
11	0.0227814	0.21533678
12	0.02863652	0.28964011
13	0.02665716	0.77682776
14	0.02920448	0.43077308
15	0.03521092	0.9373545
16	0.10506349	0.51536507
17	0.01426543	0.29699283
18	0.03166062	0.09961453
19	0.05419612	0.21955688
20	0.1619552	0.33836838
21	0.04136822	0.11765762
22	0.03602977	0.33463649
23	0.01971813	0.08271732
24	0.04155982	0.17559618
25	1.00138726	0.29449873
26	0.06468911	0.14549082
27	0.02090995	0.17792404
28	0.01539209	0.17722655
29	0.01964083	0.59104232
30	0.02780523	0.12453875
31	0.03874982	0.21394818
32	1.76601794	0.24810109
33	0.0210068	0.41217626
34	0.10359311	0.27093149
35	0.03962807	0.03654961
36	0.10002968	0.32657811
37	0.05037436	0.28093988
38	0.03677834	0.53120231
39	0.03052214	0.39859624

	$2^{-\Delta Cts}$ relative to PUM1	
40	0.06058061	0.2361322
41	0.24485507	0.46222483
42	0.04301549	0.59680575
43	0.02130496	0.1942967
44	0.15037813	0.47587899
45	0.2220054	0.62517558
46	0.06175358	0.21950616
47	0.09557873	0.445295
48	0.01105365	0.11614508
49	0.16072505	0.64849469
50	0.00954085	0.24400794
51	0.05480052	0.32241706
52	0.01672713	0.46377598
53	0.01309052	0.11042714
54	0.07789458	0.07839108
55	0.17825321	0.28093988
56	0.03216941	0.09037077
57	0.21915143	0.33029644
58	0.02336242	0.18349763
59	0.03903287	0.09687937
60	0.0163791	0.05294604
61	0.45360234	0.29943885
62	0.29778301	0.42479342
63	0.1658748	0.36993234
64	0.84206295	0.46943587
65	0.33564313	0.6125219
66	0.02639363	0.3428541
67	0.01251383	0.07673348
68	0.05689057	0.18685616
69	0.07668031	0.44720245
70	0.09928136	0.29992354
71	0.03507694	0.31864016
72	0.02080393	0.18445405
73	0.00583925	0.45114611
74	0.04549967	0.38068422
75	0.01364327	0.09186548
76	0.01175159	1.27869011
77	0.05904669	0.17055788
78	0.04872029	0.65474271

5.4 Discussion

In chapter 4, it was demonstrated that in the breast cancer cell line MDA-MB-231, TPA could increase ST3Gal-I and COX-2 mRNA expression and the product of both enzymes (see PNA staining by FACS and PGE2 production by ELISA). Therefore to investigate if ST3Gal-I induction was via COX-2, NS398 and Celecoxib, selective inhibitors of COX-2 were used. NS398 could revert the TPA effect on COX-2 and slightly decrease ST3Gal-I expression. However, Celecoxib unexpectedly increased ST3Gal-I and COX-2 mRNA expression. Upon searching the literature it was found that induction of COX-2 mRNA by Celecoxib has been reported by others. Indeed Niederberger et al. (2001) showed that Celecoxib increases COX-2 mRNA and protein expression in a concentration between 10 and 50 μM. We also found that Celecoxib can increase COX-2 and ST3Gal-I mRNA and slightly decrease C2GnT1 mRNA expression (see figure 5.4). Thus ST3Gal-I and COX-2 mRNA expression move in parallel, even with Celecoxib treatment. It was totally unexpected that NSAIDs extensively used in clinic and in clinical trials for cancer, could increase COX-2 mRNA. However, the results from the ELISA quantifying PGE2 secretion in the samples treated for 18 hours with TPA and Celecoxib showed there was less PGE2 secretion than when the cells were treated with TPA alone. This result means that even if Celecoxib increases COX-2 mRNA, it was inhibiting its activity. Furthermore, Celecoxib was inhibiting ST3Gal-I activity. As said in the introduction, PNA (peanut agglutinin) binds specifically the T structure (Core1) and this binding is inhibited by the presence of sialic acid (Novogrodsky et al. 1975). Celecoxib could inhibit ST3Gal-I activity since it was able to revert the PNA binding seen after TPA treatment back to the TPA untreated cells.

To confirm the ST3Gal-I mRNA was being induced by COX-2, COX-2 was silenced or over-expressed in MDA-MB-231 and ST3Gal-I expression measured by RT-qPCR. Stasinopoulos et al. (2007) showed that COX-2 expression could be knocked down in MDA-MB-231 using COX-2 targeting small interfering RNA (siRNA). The same oligos were used and they also showed a knock down of COX-2 by 70% after TPA treatment. This resulted in ST3Gal-I expression, after TPA induction, being decreased by 50% in three independent experiments. Moreover, when COX-2 was transfected into MDA-MB-231, ST3Gal-I mRNA expression increased by 2 fold. Thus, ST3Gal-I expression is affected after silencing and transfection of COX-2.

To relate these findings in breast cancer cell lines to clinical samples, the expression of COX-2 and ST3Gal-I were analyzed in 78 breast cancer samples. The statistical correlation between COX-2 and ST3Gal-I was moderate but statistically significant. However, the level of mRNA expression of an enzyme does not give us information on its activity or the possible influence of the cell environment and of competing

glycosyltransferases. This means that a moderate correlation may allow COX-2 to have even more influence on ST3Gal-I. Given all the data presented in this thesis we can conclude that ST3Gal-I expression is controlled by COX-2 in breast cancer cell lines and possibly in primary breast cancers.

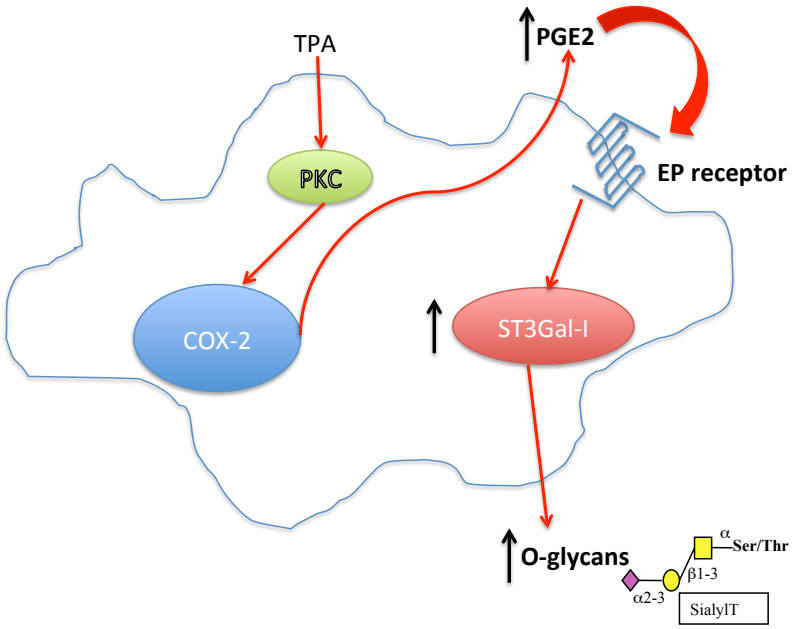


Figure 5.16 Effect of TPA and PGE2 on ST3Gal-I in breast cancer cells. TPA binds directly PKC and upregulates COX-2 expression and consequently increases PGE2 secretion. PGE2 binds EP receptors and upregulates ST3Gal-I expression and SialylT expression.

From the literature it is evident that COX-2 expression is turned on in many cancers. Increased expression has been reported in colon (Eberhart et al. 1994), bladder (Eltze et al. 2005) and breast cancers (Ristimaki et al. 2002), the same cancers that show increased expression of ST3Gal-I (Burchell et al. 1999, Potapenko et al.2010, Higai et al. 2006, Videira et al. 2009). In particular COX-2 was found overexpressed in about 40% of cases of invasive breast carcinoma (associated with unfavorable distant disease-free survival) (Ristimaki et al. 2002) and ST3Gal-I has been found over-expressed in breast carcinomas, and in primary breast cancers its increase is correlated with tumour grade (Burchell et al. 1999).

COX-2 expression has been associated with lymph node metastasis (Costa et al. 2002), distant metastasis (Larkins et al. 2006), angiogenesis (Davies et al. 2003), processes that all involve glycan interactions (Ono et al. 2004).

COX-2 is important in lymph node metastasis and distant metastasis. Lymph node metastasis is associated with high expression of VEGF-C and the prostaglandin EP_1

and EP_4 receptors are involved in COX-2 mediated VEGF-C up-regulation (Su et al. 2004, Xin et al. 2012). Moreover COX-2 up-regulates, via EP2 and EP4 receptors, CCR7 expression in breast cancer cells, a chemokine receptor associated with breast cancer metastasis. COX-2 also regulates matrix metalloproteinase expression that can digest the extracellular matrix and the basement membrane (Pan et al. 2008). Gene expression analyses and mouse model systems have also revealed correlations between COX-2 overexpression and breast cancer metastasis to the lung, bone, and brain (Bos et al. 2009; Singh et al. 2007; Minn et al. 2005). ST3Gal-I expression is associated with grade and therefore an association with lymph node expression is inferred.Julien et al. (2007) showed that when monocyte derived dendritic cells are induced to mature and migrate to the lymph nodes, the composition of their O-linked glycans changes in a way that mirrors the change in breast cancer. Upon maturation of DCs, C2GnT1 is down regulated and the sialyltransferase ST3Gal-I is up-regulated resulting in the change from core 2 based glycans to sialylated core 1 glycans. When breast cancer cells metastasize to the lymph nodes, like dendritic cells, they migrate from the tissue to the lymph nodes and it is possible that the O-linked glycans changes could be involved. Interestingly, PGE2 changed the expression of ST3Gal-I and C2GnT1 on dendritic cells (Julien et al. 2007).

It was shown that in transgenic mice overexpressing the human COX-2 gene in the mammary glands resulted in reduced levels of the proapoptotic proteins Bax and Bcl-x_L and an increase in Bcl-2, that can inactivate the apoptotic caspase cascade upstream of the mitochondria, suggesting that enhanced COX-2 expression is sufficient to inhibit apoptosis and induce mammary gland tumorigenesis. Therefore, inhibition of Cox-2 may represent a mechanism-based chemopreventive approach for carcinogenesis (Liu et al. 2001).

ST3Gal-I is important in tumorigenesis. In this lab, Picco et al. showed that ST3Gal-I promotes mammary gland carcinogenesis. Transgenic mice were developed that overexpressed ST3Gal-I from the MUC1 promoter to allow upregulation in mammary gland. ST3Gal-I transgenic mice did not show obvious change in the development of the mammary gland, but they showed increased activity and expression of the enzyme in the pregnant and lactating mammary glands, the stomach, lungs and intestine. These mice were then crossed with Polyoma middle T mice (a model of spontaneous breast cancer) running from the MMTV promoter and showed a significant increase in the speed of mammary tumour development compared to the controls (Picco et al. 2010).

The findings reported here maybe useful for the development of specific therapeutics targeting COX-2 and changes in glycosylation in breast cancer. NSAIDs (non-steroidal anti-inflammatory drug) are important in chemoprevention and are used as adjuvant for

patients under chemotherapy/radiation regimens with COX-2 expressing breast cancers. The Randomised European Celecoxib Trial (REACT) is a phase III clinical trial (now underway) based on the use of Celecoxib (and Adjuvant endocrine therapy on hormone receptor (ER and PgR) positive patients) for women with breast cancers. Moreover there are other clinical trials underway using Celecoxib for breast cancer and other types of cancer: Neoexel study, (phase III Neoadjuvant trial of pre-operative exemestane or letrozole +/- celecoxib in the treatment of ER positive postmenopausal early breast cancer);2) NCI/MSKCC (phase II study of celecoxib and herceptin in women with HER2+ metastatic breast cancer refractory to herceptin); 3) NCI phase I randomised study of 6 weeks neoadjuvant celecoxib for localised prostate cancer; 4) NCI/UCSF randomised phase II study of two years celecoxib for prevention of basal cell carcinoma in patients with basal cell naevus syndrome. However, as described in section 1.9.5, COXIBs (as well as NSAIDs) have several side effects like cardiovascular complications, which is why all patients under these clinical trials receive a cadiovascular assessment throughout the treatment (REACT PROTOCOL– version 34, 28.09.2009).

Further studies are needed on the influence of COX-2 activity on glycosylation. Eventual discoveries could overcome side effects of NSAIDs as glycosylation enzymes may be suitable therapeutic targets for cases of breast cancer that have become resistant to other therapies.

As said in section 1.2.2 Curtis et al. described a novel molecular classification of breast cancer based on the acquired somatic copy number aberrations and gene expression profiling. On a cohort of 2000 breast tumours ten groups were identified and a cohort of ER positive luminal epithelial cancers have been identified with a very bad outcome (Curtis et al. 2012). According to Gluck, Chin and other datasets ST3Gal-I is found more in invasive breast cancer (Chin et al. 2006, Gluck et al. 2012). It would be interesting to determine COX-2 and ST3Gal-I expression in the ER+ve cluster identified by Curtis et al (Curtis et al. 2012) as having a poor prognosis with the aim of developing new targeted therapies.

Chen et al. developed an inhibitor, lithocholic acid, that could block the action of different sialyltransferases, including ST3Gal-I, by lysosomal degradation. One limitation to this kind of approach is that ST3Gal-I is expressed by mature T cells and so a selective inhibitor would face multivalent and low-affinity interactions in vivo (Chen et al. 2011).

In this chapter we saw how two selective COX-2 inhibitors could affect ST3Gal-I mRNA expression and activity. It would be interesting to measure the expression of ST3Gal-I in tumours from the patients of the Clinical trials investigating the efficacy of the

selective COX-2 inhibitor, celecoxib, and see if the outcome of the patients that do express high levels of ST3Gal-I, differs from patients expressing moderate or lower levels.

The results described here suggest the intriguing possibility that some of the malignant characteristics associated with COX-2 expression may by via the influence that COX-2 exerts on the glycosylation of tumour cells.

Chapter 6. PKC can control ST3Gal-I expression.

6.1 Introduction

As discussed in the previous chapter, ST3Gal-I mRNA expression is induced by COX-2, which results in increased ST3Gal-I activity, as demonstrated by the increased

formation of sialylated core 1, the ST3Gal-I product. The aim of this chapter is to identify possible pathways that can be involved in COX-2 regulation of ST3Gal-I.

6.2 Objectives.

1. Identify the phosphoprotein activated by TPA, using a Human Phospho-Kinase Array kit.
2. Treat cells with EGF and investigate possible role in ST3Gal-I induction.
3. Use of statistic website for investigating CREB binding sites on ST3Gal-I promoter and usage of forskolin to determine any possible influence on ST3Gal-I expression.
4. Determine the effect of PKC inhibitors on ST3Gal-I and COX-2 expression after TPA and PGE2 treatment, in MDA-MB-231 and T47D, respectively.
5. Chromatin Immunoprecipitation qPCR for histone marks (H3K4Me2 and H3K4Me3) on ST3Gal-I promoter after stimulation with TPA.

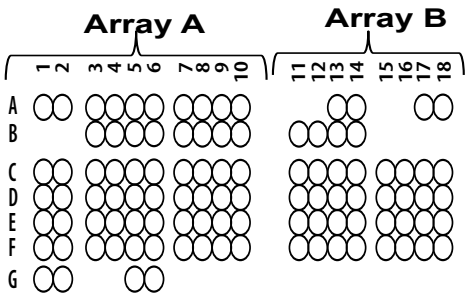
6.3 Results.

6.3.1 Phosphorylation assay after TPA treatment in MDA-MB-231.

The aim of the experiment was to analyze the phosphorylation profiles of some kinases and protein substrates in MDA-MB-231 after TPA treatment, to identify and see if any of them are involved in the upregulation of ST3Gal-I. To do so, a phosphoarray kit was

used. As explained in the materials and methods (section 2.4.4), the kit consists of 46 capture antibodies of different kinases and proteins and control antibodies that have been spotted in duplicate on nitrocellulose membranes. Figure 6.1 shows a table with all the kinases and their disposition on the membranes. The Human Phospho-Kinase Array is divided in A and B as shown in figure 6.1. The membranes are incubated with cell lysates, probed with a cocktail of antibodies and developed as western blots.

a)



b)

Membrane/Coordinate	Target/Control
A-A1, A2	Reference Spot
A-A3, A4	p38α
A-A5, A6	ERK1/2
A-A7, A8	JNK pan
A-A9, A10	GSK-3α/β
B-A13, A14	p53
B-A17, A18	Reference Spot
A-B3, B4	MEK1/2
A-B5, B6	MSK1/2
A-B7, B8	AMPKα1
A-B9, B10	Akt
B-B11, B12	Akt
B-B13, B14	p53
A-C1, C2	TOR
A-C3, C4	CREB
A-C5, C6	HSP27
A-C7, C8	AMPKα2
A-C9, C10	β-Catenin
B-C11, C12	p70 S6 Kinase
B-C13, C14	p53
B-C15, C16	p27
B-C17, C18	Paxillin
A-D1, D2	Src
A-D3, D4	Lyn
A-D5, D6	Lck
A-D7, D8	STAT2
A-D9, D10	STAT5a
B-D11, D12	p70 S6 Kinase
B-D13, D14	RSK1/2/3
B-D15, D16	p27
B-D17, D18	PLCγ-1
A-E1, E2	Fyn
A-E3, E4	Yes
A-E5, E6	Fgr
A-E7, E8	STAT3
A-E9, E10	STAT5b
B-E11, E12	p70 S6 Kinase
B-E13, E14	RSK1/2
B-E15, E16	c-Jun
B-E17, E18	Pyk2
A-F1, F2	Hck
A-F3, F4	Chk-2
A-F5, F6	FAK
A-F7, F8	STAT6
A-F9, F10	STAT5a/b
B-F11, F12	STAT1
B-F13, F14	STAT4
B-F15, F16	eNOS
B-F17, F18	PBS (Negative Control)
A-G1, G2	Reference Spot
A-G5, G6	PBS (Negative Control)

Figure 6.1: a) Human Phospho-Kinase Array Coordinates on the membranes. b) Table documenting the 46 different kinase and proteins and their coordinates on the arrays.

MDA-MB-231 was treated for 5 minutes with 10 nM TPA or vehicle. Protein lysates were collected and parallel cultures were incubated for 5 hours with TPA for RNA extraction, in order to confirm overexpression of ST3Gal-I and COX-2. RT-qPCR confirmed the upregulation of ST3Gal-I and COX-2 mRNA after 5 hours of TPA treatment (data not shown). The membranes of the array were incubated overnight with the protein lysate and the assay was run according to materials and methods. ERK 1/2, JNK, CREB, Chk-2, FAK (Spots A 5-6, A 7-8, C 3-4, F 3-4, F 5-6 squared in red in figure 6.2), showed increased phosphorylation after

treatment with TPA.

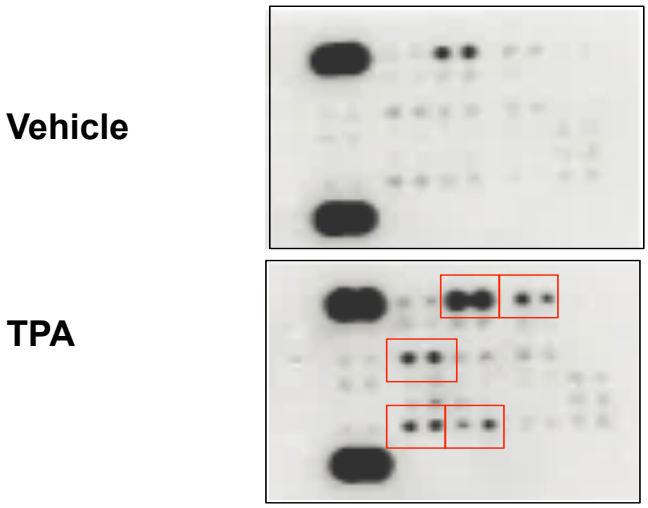


Figure 6.2: Treatment of MDA-MB-231 with TPA results in increased phosphorylation of ERK 1/2, JNK, CREB, Chk-2, FAK. MDA-MB-231 were treated with 10 nM TPA or vehicle for 5 minutes. Membranes were incubated with the lysates as described in materials and methods (section 2.4.4) and developed with the ECL system (Amersham) according to the manufacturer's instructions.

To confirm the results of the arrays, cells were treated again with 10 nM TPA for 5 minutes and lysates run on SDS-PAGE and the Western blots probed with antibodies to FAK, CREB and ERK. Figure 6.3 shows the results and confirms that TPA induces phosphorylation of FAK, CREB and ERK in MDA-MB-231 (see figure 6.3).

According to the literature (Wen-Sheng and Jun-Ming, 2005, Li et al. 2006), we found that TPA enhanced ERK and CREB phosphorylation. The involvement of these two transcription factors in the expression of ST3Gal-I was investigated in MDA-MB-231 cell line.

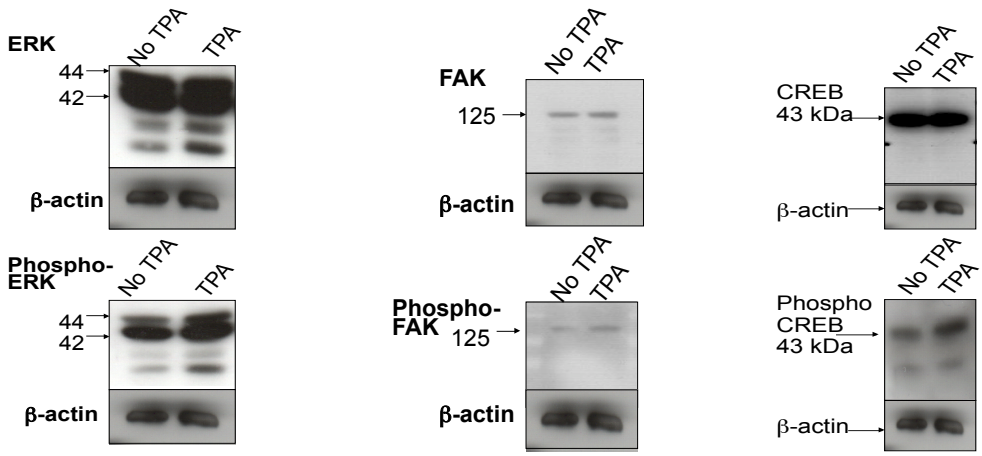


Figure 6.3: TPA increases phospho-FAK, phospho-CREB and phospho-ERK in MDA-MB-231 after 5 minutes of treament. Lysates were run on a 7.5% SDS-PAGE and the Western blots probed with antibodies to the protein or with phosphospecific antibodies. The loading control antibody used for each blot was β-actin. Membranes were developed with the ECL system according to the manufacturer's instructions.

6.3.2 ERK is not involved in ST3Gal-I and C2GnT1 activation of transcription.

The activation of EGFR (epidermal growth factor receptor) is involved in breast cancer, in particular in the process of metastasis and invasion (Nickerson et al. 2012). ERK is a downstream molecule activated by EGF–EGFR signalling. EGF binding results in the phosphorylation of EGFR and consequent activation of ERK1/2. So, exogenous EGF was used as an alternative way to TPA, to activate ERK and see if it could also induce ST3Gal-I mRNA expression. MDA-MB-231 cell line was treated with EGF (50 ng/ml and 100 ng/ml) for 2, 5 and 8 hours and ST3Gal-I and C2GnT1 mRNA expression was determined. In parallel, to confirm that ERK was phosphorylated, MDA-MB-231 were treated for 5 minutes with EGF and the phosphorylation of ERK determined by western blot (see figure 6.4).

The expression of ST3Gal-I and C2GnT1 mRNA in EGF treated cells was determined by RT-qPCR. ST3Gal-I and C2GnT1 mRNA expression did not change more than 2 fold in expression over the time course (shown in figure 6.5). However, as after 2 hours there was a two fold increase in ST3Gal-I expression, this time point was repeated a further three times.

The results showed that the induction of ST3Gal-I by EGF after 2 hours of incubation was inconsistent (figure 6.5 B) and therefore, it was concluded that EGF could not induce ST3Gal-I expression and that ERK is not involved in TPA induction of expression of ST3Gal-I.

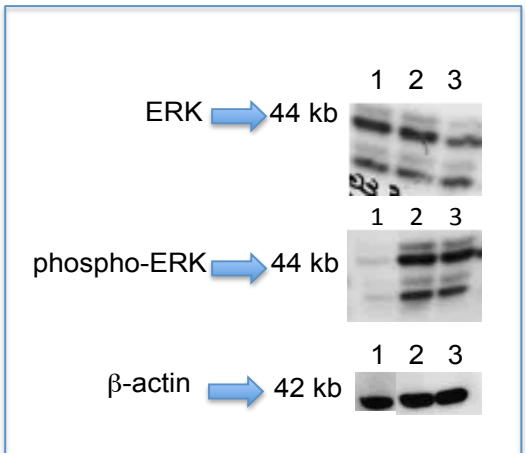
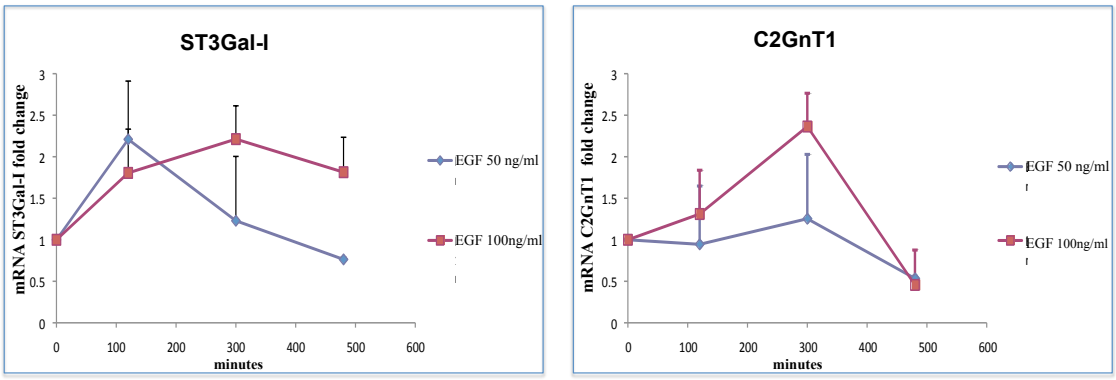


Figure 6.4: EGF treatment of MDA-MB-231 for 5 minutes can increase the phosphorylation of ERK.
MDA-MB-231 were treated with PBS (vehicle) (Lane 1) or with 50 ng/ml or 100 ng/ml EGF(Lanes 2 and 3). Lysates were run on 7.5% SDS-PAGE and the Western blots

probed with ERK and phosphoERK antibodies. The loading control antibody used was β–actin. Membranes were developed with the ECL system (Amersham) according to the manufacturer's instructions

A



B

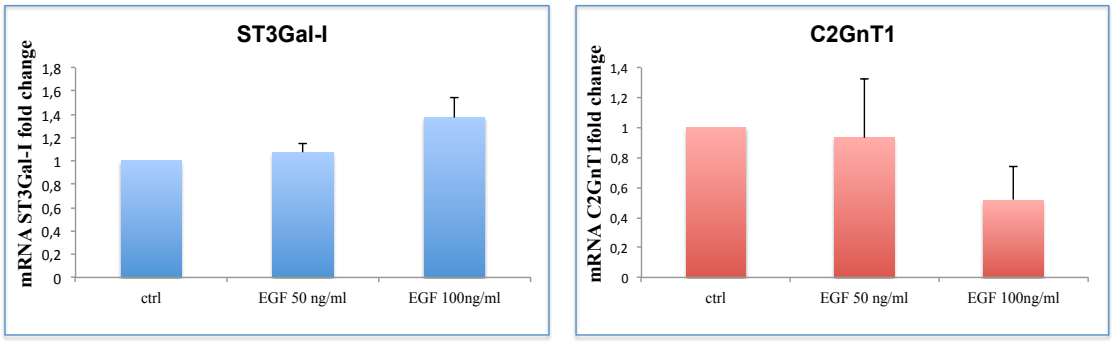


Figure 6.5: EGF does not change the expression of ST3Gal-I or C2GnT1 consistently. A. MDA-MB-231 cells were treated with EGF for the times indicated and the expression of ST3Gal-I and C2GnT1 was determined by RT-qPCR. The graphs show the average for two biological experiments. B. MDA-MB-231 cells were treated with EGF for 2 hours and the expression of ST3Gal-I and C2GnT1 was determined by RT-qPCR. The graphs show the average for three biological experiments.

6.3.3 CREB involvement on ST3Gal-I and C2GnT1 activation of transcription.

The cAMP responsive element binding protein is a phosphorylation activated trascription factor (Mayr et al. 2001). CREB can be activated by different kinases, like Protein Kinase C, Protein Kinase A, calmodulin kinases (CaMKs), Akt and pp90 ribosomal S6 kinase (pp90 RSK; also known as RSK2) (Sakamoto et al. 2009). When one of these protein kinases is activated, it translocates to the nucleus and phosphorylates the CREB protein. The activated CREB binds to a CRE region, and it is

bound by a CBP (CREB-binding protein) that works as a cofactor for the transcription of other genes (Carlezon et al. 2005).

Bioinformatic tools like Gene2promoter, MatInspector, ElDorado and FrameWorker were used to study the ST3Gal-I and C2GnT1 promoter to analyze the presence of CREB binding sites. The promoter region C2GnT1 is complex and in humans four different 5' untranslated regions have been identified, consistent with the presence of multiple tissue specific promoters for the C2GnT1 gene (Falkenberg et al. 2003). As can be seen from figure 6.6, all four promoter regions of C2GnT1 contain CRE consensus sites. The promoter region of the ST3Gal-I is less complex and although two possible promoter regions have been identified, only one appears to be in the region important for the transcriptional activity of ST3Gal-I (Taniguchi et al. 2001). However, according to Softberry (predictive transcription binding site program), the only CREB binding site on ST3Gal-I promoter has a very low affinity to CREB.

As forskolin is the most potent activator of CREB (Seternes et al.1999), it was used to activate CREB, and ST3Gal-I, C2GnT1 and COX-2 mRNA expression were determined.

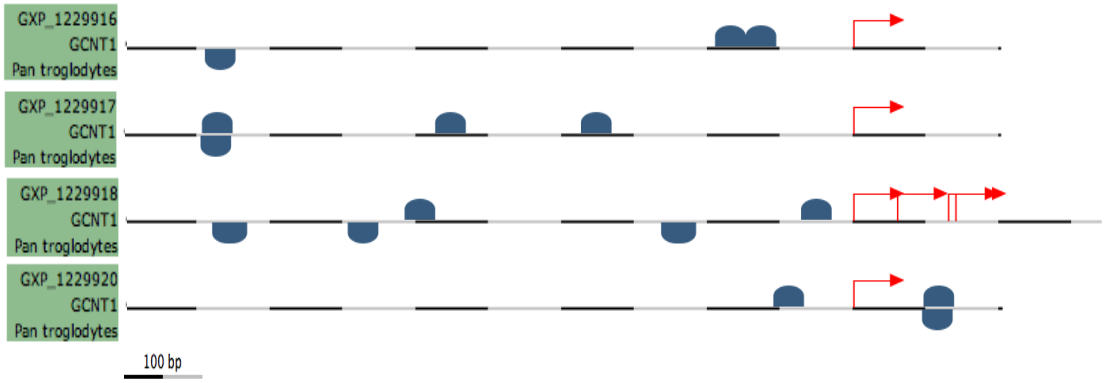


Figure 6.6: Identification of CRE sequence (blue) in the alternative promoters of human C2GnT1. Some are conserved among the species suggesting a functional relevance. (http://www.genomatix.de/online_help/help_eldorado/Gene2Promoter_Intro.html).

MDA-MB-231 were treated with forskolin for 1, 3, 5 and 20 hours. The time point at 3, 5 and 20 hours in figure 6.7 represent the mean±SD of two biological experiments. ST3Gal-I mRNA did not show any significant change in expression. On the other hand, C2GnT1 seemed to increase by about two fold. Around a three fold increase in COX-2 mRNA was observed and this increase was much lower than that seen with TPA. Moreover, the major increase in expression was seen after 20 hours, suggesting that forskolin indirectly induces COX-2 mRNA expression. The data were not suggestive of

any positive response of ST3Gal-I to forskolin. Given that the CREB binding site on the ST3Gal-I promoter is of low affinity (Softberry data base), the possible correlation with this transcription factor was not further investigated.

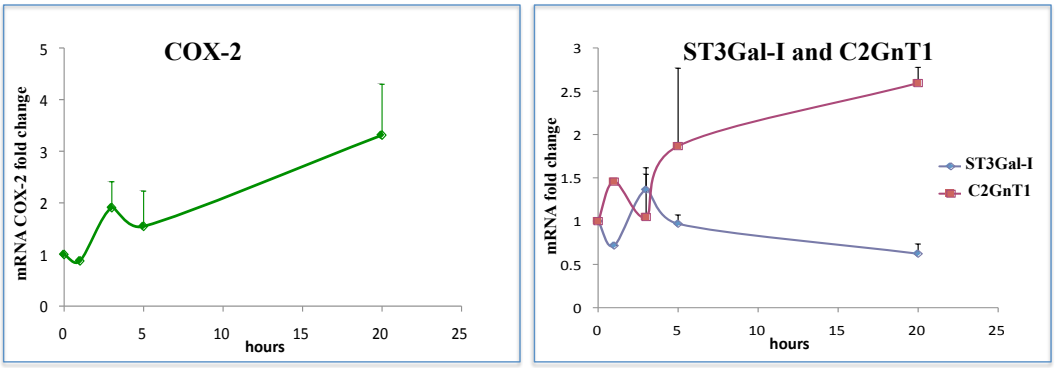


Figure 6.7: Forskolin does significantly not change ST3Gal-I mRNA expression. MDA-MB-231 were treated with forskolin (20 μM) for 1, 3, 5 and 20 hours and ST3Gal-I, C2GnT1 and COX-2 mRNA were determined by RT-qPCR. The data represent two biological experiments for the time point of 90 minutes, 3 and 8 hours.

6.3.4 Involvement of PKC in ST3Gal-I expression.

TPA is a phorbol diester that resembles the structure and the activity of diacylglycerol and so binds to Protein Kinase C (Castagna et al. 1982). It was demonstrated that TPA increased the PKC activity more than the unsaturated diacylglycerol, and decreased in the Ca2+ concentration that was necessary for enzyme activation (Castagna et al. 1982).

The PKC family consists of 12 isoenzymes classified in 3 subfamilies depending on their mechanism of activation and on structural characteristics: 1) conventional or classic PKCs (PKC α, β, γ) that require DAG and Ca2+ for activation; 2) non-classic or novel PKCs (PKC δ, ε, η, θ) that require DAG but not Ca2+ for activation and 3) atypical PKCs that do not require either DAG or Ca2+(PKC ι, ζ, μ, λ) (Newton et al. 2001).

TPA can only bind classic and novel PKC. Gö6976 inhibits PKCα, β (conventional PKC), and PKCμ (atypical PKCs). Gö6983 inhibits PKCα, PKCβ, PKCγ, (conventional PKCs), PKCδ (non-classic PKC), PKCζ, and PKCμ (atypical PKC).

ST3Gal-I in T47D responded to PGE2 but not to TPA and ST3Gal-I in MDA-MB-231 responded to TPA but not to PGE2. Thus we wanted to determine if this could be explained by differential expression of PKC isoenzymes.

MDA-MB-231 was incubated with TPA in the presence or absence of the two inhibitors Gö 6983 (6 μM) and Gö6976 (2 μM) for 5 hours, while T47D was treated for 4 hours with PGE2 (100 nM) in the presence or absence of the same PKC inhibitors. The time point used for the two cell lines was determined by the results reported in chapter 3 and 4. Figure 6.8 shows that the two PKC

inhibitors blocked the TPA effect on ST3Gal-I and COX-2. This result was expected, as TPA exerts its action through the isoforms of PKC.

Unexpected was the effect that the PKC inhibitors had on the PGE2 upregulation of ST3Gal-I in T47D. Both inhibitors (Gö6983 and Gö6976) reversed the PGE2 effect on ST3Gal-I in two biological experiments (figure 6.9 A and B). COX-2 level of expression was very low and, as observed in chapter 4, PGE2 did not increase its expression (data not shown). Thus PGE2 appears to be inducing the expression of ST3Gal-I via PKC in T47D.

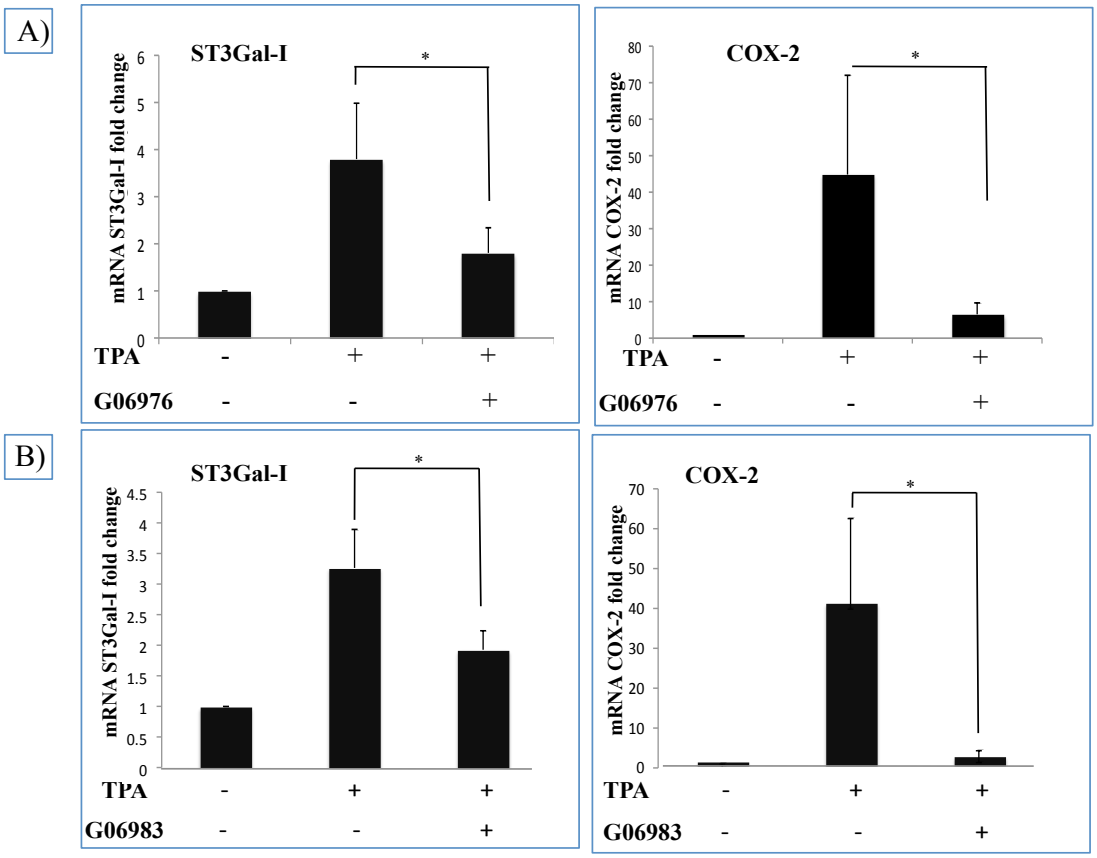


Figure 6.8 TPA effect on ST3Gal-I and COX-2 in MDA-MB-231 can be reverted by the PKC inhibitors A. Gö 6976, B. Gö 6983. MDA-MB-231 were treated with TPA or TPA and A)Gö 6976 B) Gö 6983 for 5 hours. ST3Gal-I and COX-2 mRNA expression was then determined by RT-qPCR. Data represent the mean of three independent experiments. *p<0.05.

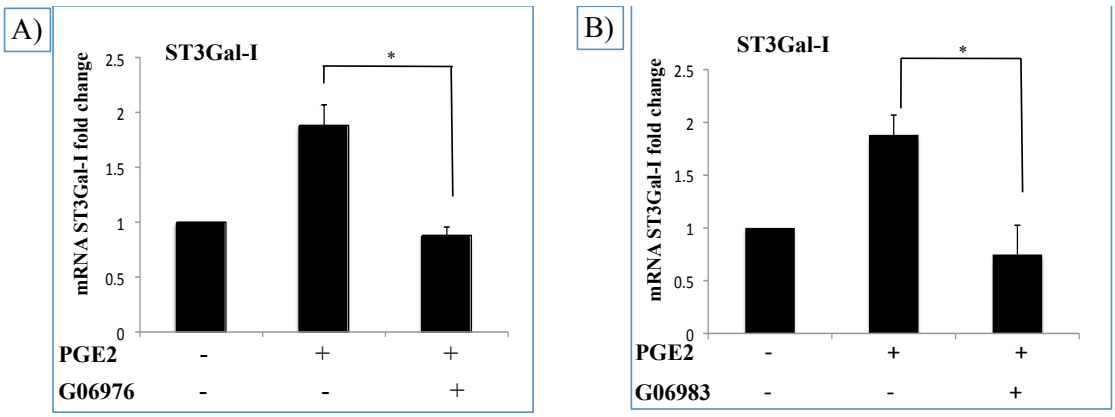


Figure 6.9 PGE2 effect on ST3Gal-I in T47D can be reverted by the PKC inhibitors Gö 6976 and Gö 6983. T47D cells were treated with PGE2 or PGE2 and A)Gö 6983 B) Gö 6976. ST3Gal-I RNA expression was then determined by RT-qPCR. Data represent the mean of three independent experiments. *p<0.05.

6.3.5 Histone marks H3K4Me3 and H3K4Me2 change on ST3Gal-I promoter region after TPA treatment.

Histones are responsible for the structure of chromatin, since the DNA is wound around them, forming nucleosomes. There are five classes of histones: H1/H5, H2A, H2B, H3, and H4. H1 and H5 are the linker histones while H2A, H2B, H3 and H4 are known as the core histones since they form the nucleosome core (two H2A-H2B dimers and a H3-H4 tetramer). Dependent upon the post-translation modifications of histones, chromatin can be condensed or more open and this will influence the expression of genes.

Histones undergo a number of posttranslational modifications and this includes lysine methylation, arginine methylation, lysine acetylation, serine/threonine/tyrosine phosphorylation and even glycosylation (O-GlcNAc). Among the lysine methylation, trimethylation and dimethylation of H3 on lysine 4 (H3K4Me3), within promoter regions, are associated with active transcription. The H3K4Me3 mark sits near the transcriptional start site of many promoters, very close to polymerase II and TFIID (transcription factor II D that binds the TATA box in the promoter gene) sites (Heintzman et al. 2007).

The methyl marks on H3K4 on the promoter of the ST3Gal-I gene was, therefore, investigated after transcription was induced by TPA, using ChIP Real Time PCR (RT-qPCR). ChIP is defined as Chromatin Immuno-Precipitation and it is a method to examine in vivo protein-DNA interactions with transcription factors, modified histones and other nuclear factors. ChIP-qPCR is Chromatin Immunoprecipitation with qPCR detection and quantification. Using the dataset http://genome.ucsc.edu/ENCODE/, we were able to confirm the presence of H3K4Me3 sites around the transcriptional start site of ST3Gal-I and primers were designed. GAPDH was used as a positive control and the primers were provided by Qiagen in their Epitect ChIP assay kit. Ten million MDA-MB-231 cells were treated for 5 hours with 10 nM TPA. The chromatin was extracted and immunoprecipitated with antibodies to H3K4Me2 and H3K4Me3, as described in material and methods. To determine any change in the H3K4Me3 and Me2 marks, the results, obtained from qPCR of the chromatin immunoprecipitate with antibodies to H3K4Me3 or Me2, were normalized to the input results and the result with the IgG

control antibody deducted. In table 6.1, the Cts of the input, treated and not treated with TPA, was subtracted from the Cts of the immunoprecipitated chromatin treated and untreated with TPA respectively. These values were then normalized against the IgG ΔCts and these values were then converted to $2^{-\Delta\Delta Cts}$.

The results obtained were totally unexpected (see figure 6.11). The H3K4Me3 and Me2 marks on ST3Gal-I promoter both decreased after TPA treatment. However, the H3K4Me3 and Me2 marks on GAPDH, used as a positive control, decreased as well. Unexpectively, the H3K4Me2 and H3K4Me3 marks of active transcription decreased by 5 fold on the ST3Gal-I promoter after TPA treatment, when ST3Gal-I transcription increased. The experiment was repeated, but the same result was obtained. Additional positive controls should be used to confirm that the decrease of the histone marks expression is not happening to all the control genes within the cell due to the action of TPA.

Table 6.1: H3K4Me3 and Me2 decreased after TPA treatment. The sixth row is the fold enrichment that normalize the Cts against the input and against the IgG**.**

	Samples	C_t	ΔCt (C_t[IP]-C_t[InputxDF])	ΔΔC_t (ΔC_t[IP]-ΔC_t[NIS])	Fold Enrichment ($2^{-\Delta\Delta Ct}$)
ST3Gal-I	Input TPA(-)	30.22			
	Input TPA(+)	31.16			
	IgG TPA(-)	36.85	13.27		
	IgG TPA(+)	35.58	11.07		
	H3K4Me2 TPA(-)	26.70	3.12	-10.2	1141.5
	H3K4Me2 TPA(+)	27.69	3.18	-7.9	237.3
	H3K4Me3 TPA(-)	28.65	5.07	-8.2	295.3
	H3K4Me3 TPA(+)	29.66	5.14	-5.9	60.6
GAPDH	Input TPA(-)	29.37			
	Input TPA(+)	30.35			
	IgG TPA(-)	36.06	13.33		
	IgG TPA(+)	33.88	10.17		
	H3K4Me2 TPA(-)	26.22	3.49	-9.8	916.5
	H3K4Me2 TPA(+)	27.13	3.42	-6.8	107.6
	H3K4Me3 TPA(-)	27.59	4.86	-8.5	354.6
	H3K4Me3 TPA(+)	28.52	4.81	-5.4	41.1

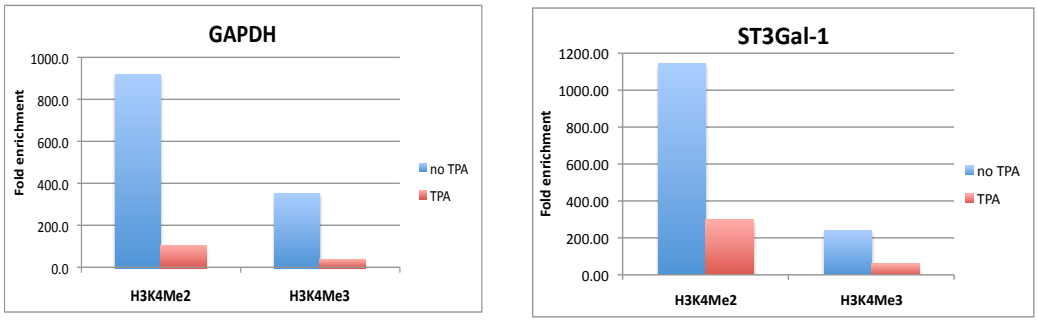


Figure 6.11: Histograms of H3K4Me2 and Me3 for GAPDH and ST3Gal-I with and without

TPA. Ten million MDA-MB-231 cells were treated for 5 hours with 10 nM TPA and the chromatin extracted and immunoprecipitated according to materials and methods. H3K4Me3 and Me3 are plotted as fold enrichment and the data are shown in table 6.1.

6.4 Discussion.

The aim of this chapter was to investigate the downstream pathways involved in ST3Gal-I induction after TPA or PGE2 treatment. The results showed that inhibition of different isoforms of PKCs leads to inhibition of the TPA and PGE2 effect on ST3Gal-I. On the other hand, ERK signalling and the transcription factor CREB do not seem to be involved in ST3Gal-I induction.

TPA is a well known phorbol esther that binds specifically PKC and therefore triggers numerous pathway. Activated PKC isoforms generally transduce through either protein kinases (MAPK) pathways, usually p44/42 MAPK and c-Jun NH2-terminal kinase (JNK) and p38, or via MAPK-independent pathways, for example the phosphatidylinositol 3-kinase (PI3-kinase). Downstream targets of phorbol esters can be transcription factors, such as AP-1, CREB, nuclear factor (NF)κ-B, or Sp1 (Chio et al. 2004). As expected, TPA treatment of MDA-MB-231 for 5 minutes triggered the phosphorylation of different transcription factors like ERK and CREB, but also FAK. The possible influence of ERK and CREB was further studied. However, treatment of MDA-MB-231 with EGF, which induces signaling of EGFR and activation of ERK, did not increase ST3Gal-I mRNA, suggesting that ERK is not involved in stimulating ST3Gal-I expression.

PGE_2 (the production of which can be increased by TPA) can bind to four receptors (EP): EP_1 is a G_q coupled receptor with phospholipase C signalling through Ca^{2+}; EP_2 and EP_4 are G_s coupled receptors to protein kinase A (PKA) signaling through elevation of cAMP level; EP_3 is mainly a coupled G_i protein and is linked to a decrease of cAMP. Thus, the two possible pathways, that can be trigerred, are PKA or PKC. Forskolin was used to activate the PKA pathway and consequently activate CREB, but no effect was obtained on ST3Gal-I expression.

Chen et al. showed that Lith-O-Asp, an inhibitor of different sialyltransferases, including ST3Gal-I, suppressed cancer cell metastasis and one of the mechanism involved was by inhibiting FAK/paxillin signalling. Lith-O-Asp decreased the ST3Gal-I protein level by 20% to 25%, inhibited migration and invasion abilities in the lung cancer cells H1299, CL1-5, and A549 cells and it delayed cancer cell metastasis in experimental metastasis assays in animal models. Importantly, Lith-O-Asp decreased the expression of phospho-FAK and phospho-paxillin in the lung cancer cell lines, H1299, CL1-5, and

A549. Inhibition of FAK activity and transwell invasion ability were similar in ST3Gal-I knocked down and Lith-O-Asp–treated cells (Chen et al. 2011). However, our study did not focus on the interaction of ST3Gal-I and FAK.

The possible role of histone marks on ST3Gal-I expression was also investigated. H3K4Me3 and H3K4Me2 are associated with active transcription, so, since TPA induced ST3Gal-I over expression, we expected an enrichment of histone marks on the TSS of ST3Gal-I gene. Unfortunately, the result obtained was totally the opposite: H3K4Me3 and Me2, marks associated with active transcription, decreased on the ST3Gal-I transcription start site after TPA treatment. These histone marks also decreased on GAPDH. Freyschuss et al. reported that TPA can vary GAPDH level of expression (Freyschuss et al. 1994), so the choice of this gene was not advantageous. It would be interesting to look at other genes and to see if they change after TPA treatment. Moreover, cells were treated only for 5 hours, so, additional time points are needed to see any possible variation in histone marks. Until these further investigations will been done, we cannot draw any conclusion.

As discussed in section 1.6.4, the promoter of ST3Gal-I has not been widely studied. Taniguchi et al. found the transcription factor binding sites for Sp1 and USF1 on the promoter of ST3Gal-I (Taniguchi et al. 2001). Higai et al. suggested the involvement of NF-κb in ST3Gal-I expression, since NF-κb knockdown, in two human colon adenocarcinoma cell line and one leukaemia cell line, inhibited the increase of ST3Gal-I expression after TNF-α treatment (Higai et al. 2006).

PKC inhibition reverted the TPA or PGE2 induction of ST3Gal-I in MDA-MB-231 and T47D. TPA binds directly PKC, so, the effect of PKC inhibitors on ST3Gal-I was expected after TPA treatment in MDA-MB-231, but not after PGE2 treatment in T47D. PGE2 can trigger PKC via EP_1 and EP_3. According to our results of chapter 3, EP_1 and EP_3 are the receptors not certainly expressed by T47D. However, Barclay et al. demonstrated that T47D cell line expresses the EP_3 receptor, but not the EP_1 (Barclay et al. 2007). Further investigation on the role of the EP (PGE2) receptors on ST3Gal-I will be needed to define PGE2 role in glycosylation. Moreover, it would be interesting to find out the specific PKC isoforms involved in ST3Gal-I expression.

To our knowledge this is the first study investigating the interaction between PKC and ST3Gal-I in breast cancer. In the last years it has been established that PKC isoenzymes are important in cell proliferation, migration, adhesion, and cancer cell metastasis and different isoforms expression are altered in various types of cancers (Schmitz et al. 2000). Also, some PKC inhibitors have been developed with significant anti-cancer activity, so, correlation of ST3Gal-I with PKC can be considered as a possible coadjuvant to PKC inhibitors in the cure and prevention of breast cancer.

Chapter 7. Summary of results and conclusion.

To our knowledge, this is the first study investigating the interaction between COX-2, ST3Gal-I and C2GnT1 in breast cancer.

We have shown that in two breast cancer cell lines, T47D and MDA-MB-231, transcription of ST3Gal-I is increased by COX-2 via PGE2. In T47D cells, PGE2 could induce expression of ST3Gal-I mRNA, but not in MDA-MB-231 cells. TPA, which induces COX-2 expression among its many effects, could also induce mRNA expression of ST3Gal-I in MDA-MB-231, but not in T47D cells. Investigation of the PGE2 receptors in MDA-MB-231 and T47D has shown that T47D expressed EP_2 and

EP_4 and MDA-MB-231 expressed the EP_4 receptor, but extremely low levels of EP_2. EP_2 and EP_4 differ in their sensitivity to PGE2. EP_4 is desensitized after short-term exposure to PGE2 and this, coupled with the rapid metabolism of PGE2 (Nishigaki et al. 1996), may explain the lack of response to exogenous PGE2 of MDA-MB-231 cells. On the other hand, TPA only induced COX-2 overexpression in MDA-MB-231, the only cell line with the highest amount of COX-2 compared to the other breast cancer cell lines.

In both cell lines, induction of ST3Gal-I mRNA has led to increased activity of this sialyltransferase, as demonstrated by decreased binding of the lectin PNA (which binds to the substrate of ST3Gal-I -T antigen). Celecoxib inhibited ST3Gal-I activity, since it was able to revert the PNA binding. A knock down of COX-2 by 70%, after TPA treatment, resulted in a 50% decrease of ST3Gal-I expression. When COX-2 was transfected into MDA-MB-231, ST3Gal-I mRNA expression increased by 2 fold. Thus, ST3Gal-I expression is affected after silencing and transfection of COX-2. Moreover, it was found that there is a significant correlation between COX-2 and ST3Gal-I expression in primary breast cancers, indicating that our findings have clinical significance (Sproviero et al. 2012).

Dendritic cells (DCs) are the most potent APC (antigen presenting cells) in the organism. The model that dendritic cell (DC) “maturation” describes the change from an immature, antigen-capturing cell to a mature, antigen-presenting cell is well-established. Dendritic cells (DC) migrate from the tissue to the lymph nodes during “maturation” and this process describes the change from an immature, antigen-capturing cell, to a mature, antigen presenting cell (Tan & O’Neill, 2005). Mature DCs are responsible for antigen presentation and stimulation of T cells. Bax et al. and Julien et al. showed that, when monocyte derived dendritic cells are induced to mature, the composition of their O-linked glycans changes in a way that mirrors the change in breast cancer: mature dendritic cells showed an up-regulation of the glycosyltransferases involved in the expression of core 1 and sialylated structures (upregulation of ST3Gal-I) and a down-regulation of genes involved in the synthesis of core 2 *O*-glycans (down-regulation of C2GnT1) (Julien et al. 2007, Bax et al. 2003). This is similar to the change seen in breast cancer cells.

As said in the introduction, DCs express C-type lectins (CLR), like DC-SIGN, MGL and dectin-1. Several carbohydrate structures (such as Tn, sialyl Tn, T (Thomsen-Friedenreich disaccharide), (sialylated) Lewis antigens) are highly upregulated in tumour cells and have been widely used as diagnostic and prognostic markers. Tn epitopes on MUC-1 is associated with poor prognosis in several types of cancer (Hakomori, 2002). Tn glycans on MUC-1 bind MGL on iDC (immature dendritic cells) and induce a Th2-mediated responses, which, unlike Th1, do not contribute to tumor

eradication (Aarnoudse et al. 2006, Saeland et al. 2007). MGL expressed on iDCs is an optimal receptor for the internalization of short GalNAcs, carrying immunogens to be delivered into HLA class I and II compartments. For this reason MGL-Tn-MUC1 has possible implications in designing cancer vaccines (Napoletano et al. 2007).

During maturation of DCs, often loss of CLR expression occurs (Figdor et al. 2002). Interestingly, it was noticed that during dendritic cells maturation there was up-regulation of sialic acid expression by mDC, correlated with an increased binding of siglec-1, -2, and -7 (Bax et al. 2007). The metastatic potential of some tumor cells has been correlated with increased sialylation of cell surface glycoproteins and consequently with an increase of sialic acid-binding lectins, such as selectins that mediate cell adhesion and extravasation during the metastatic process (Passaniti & Hart, 1988, Varki NM & Varki A., 2007, Babál et al. 2006).

The change of glycosylation that occurs during the maturation of DCs may increase the efficacy of DCs migration and possibly of breast cancer cells.

Julien et al. showed that in dendritic cells prostaglandin E2 alone was sufficient to induce the upregulation of ST3Gal-I and the down regulation of C2GnT1 (Julien et al. 2009). Moreover, it was shown that PGE2 can induce the expression of CCR7 in breast cancer cell lines and that expression of this cytokine receptor is associated with lymph node metastasis (Pan et al. 2008). We demonstrated that PGE2 and TPA can change the glycosylation of the breast cancer cell lines T47D and MDA-MB-231, so there is a real possibility that the change of glycosylation that occurs in dendritic cells and in breast cancer cells is responsible for the migration from the tissue to the lymph nodes and is central in metastasis.

Glycosylation is one of the most frequent form of posttranslation modifications and is essential for many protein and cellular functions. Changes in glycosylation are common events in malignancy and these changes can affect the course of the disease, since they play a key role in the induction of invasion, metastasis and tumorigenesis (Hakomori et al. 2002, Mungul et al. 2004, Julien, Ivetic ,Grigoriadis, QiZe, Burford, Sproviero et al. 2011).

In breast cancer overexpression of ST3Gal-I leads to the overexpression of truncated sialylated glycans, which could change the immune response and allow the progression of cancer. Picco et al. showed that over-expressing ST3Gal-I in the mammary gland promotes the early development of spontaneous mammary tumours in a murine model (Picco et al. 2010).

ST3Gal-I revealed essential roles in immune system homeostasis. Van Dyken demonstrated that deletion of the ST3Gal-I locus yields unsialylated core 1 O-glycans,

which were found to be associated with increased CD8+ T cell apoptosis (Van Dyken et al. 2007). Moreover Moody et al. showed that sialic acid addition to core1 of mature CD8 single-positive thymocytes decreases CD8 binding to MHCI (Moody et al. 2001). Hence, sialylation of cell surface glycoproteins can control the immune cell responsiveness or tolerance (Daniels et al. 2002).

Until now, very little has been known about the control of expression of ST3Gal-I in breast cancer cells. Analysis of the promoter region of ST3Gal-I identified binding sites for the transcription factors SP1, USF, NF-kB (Taniguchi et al. 2001, Higai et al. 2006). We have shown that increased expression of ST3Gal-I in breast cancer cells can be induced by COX-2 through PGE2. Further studies will be required to demonstrate the transcription factors involved.

COX-2 is induced by inflammation and is turned on in many cancers. Increased expression of COX-2 has been reported in colon, bladder and breast cancers, (Legler et al. 2006, Eltze et al. 2005, Ristimaki et al. 2002), the same cancers that overexpress ST3Gal-I. It would be interesting to measure the expression of ST3Gal-I in tumours from the patients on the Clinical trials investigating the efficacy of the selective COX-2 inhibitor, celecoxib, and seeing if the outcome of the patients that express high levels of ST3Gal-I, differs from the patients expressing moderate or lower levels.

The results described in this thesis suggest that COX-2 could affect the glycosylation of tumour cells, increasing the tumorigenesis and metastasis. Recent efforts have highlighted essential roles of glycan-binding proteins or lectins (galectins, C-type lectins and siglecs) in cancer, metastasis, host-pathogen interactions, initiation of innate immunity. This has created interest in its potential biotechnological and pharmaceutical applications. Knowing that COX-2, target of well established NSAIDs, can control the expression of ST3Gal-I, it opens up the possibility of designing new drugs.

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