Universitat de Girona

ROLE OF ALPHA 2,3-SIALYLTRANSFERASES ST3Gal III AND ST3Gal IV IN PANCREATIC DUCTAL ADENOCARCINOMA

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Tesi Doctoral

Role of alpha-2,3-sialyltransferases ST3Gal III and ST3Gal IV in pancreatic ductal adenocarcinoma

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Rosa Peracaula Mirò, professora titular del Departament de Biologia de la Universitat de Girona,

CERTIFICO:

Que aquest treball, titulat "Role of alpha-2,3-sialyltransferases ST3Gal III and ST3Gal IV in pancreatic ductal adenocarcinoma", que presenta Marta Pérez Garay per a l'obtenció del títol de Doctora per la Universitat de Girona, ha estat realitzat sota la meva direcció.

Signatura

Girona, 15 de septembre de 2010
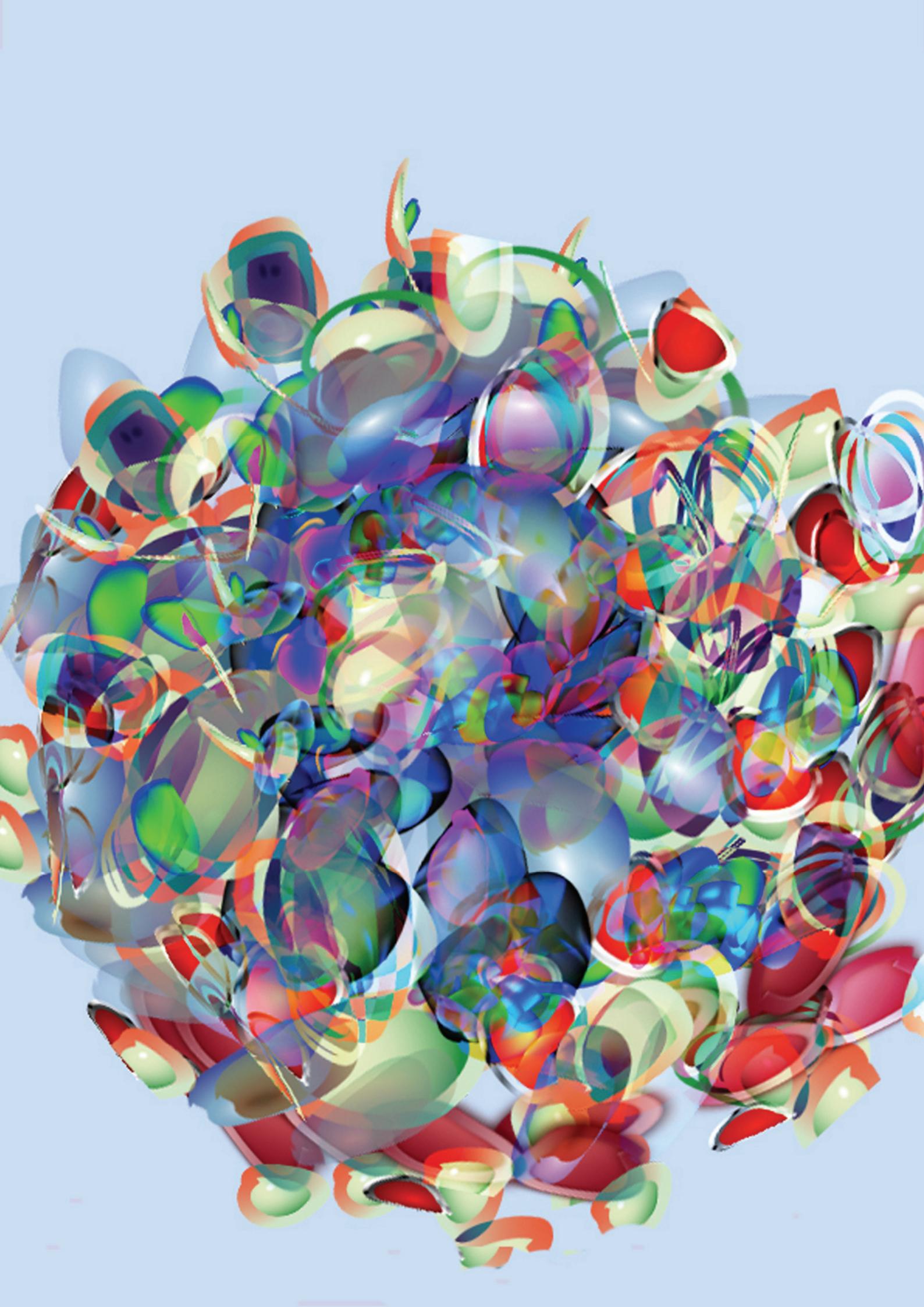
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Publications derived from the thesis

The work presented in this Doctoral Thesis is giving rise to the following research articles:

1. Pérez-Garay M, Arteta B, Pagès L, de Llorens R, de Bolòs C, Vidal-Vanaclocha F, Peracaula R (2010) α2,3-Sialyltransferase ST3Gal III Modulates Pancreatic Cancer Cell Motility and Adhesion *In Vitro* and Enhances Its Metastatic Potential *In Vivo* PloS ONE 5(9)

   PLoS ONE (ISSN: 1932-6203): With an Impact Factor of 4.351; ranks 10th in the "Biology" category (Q1).

2. Pérez-Garay M, de Llorens R, de Bolòs C, Peracaula R, Vidal-Vanaclocha F. Role of Reactive Oxygen Species as α2,3-Sialyltransferase ST3Gal III and sialyl-Lewis^x expression modulators in pancreatic adenocarcinoma cell lines

   In revision in *International journal of biochemistry & cell biology* (ISSN: 1357-2725): With an impact Factor of 4.887, ranks **56th in** "Biochemistry and Molecular Biology" category (Q1)

3. Pérez-Garay M, de Llorens R, Peracaula R, Vidal-Vanaclocha F Soluble E-Selectin depending VEGF secretion and migration in human pancreatic adenocarcinoma**.**

   In revision in *Journal of Translational Medicine* (ISSN: 1479-5876): With an impact Factor of 3.407, ranks 22nd in the "Medicine, Research & Experimental" category (Q1)

4. <u>Pérez-Garay M,</u> Arteta B, Pagès L, de Llorens R, de Bolòs C, Vidal-Vanaclocha F, Peracaula R α2,3-Sialyltransferase ST3Gal III is more metastatic than ST3Gal IV *In Vitro* and *In vivo.*

   To be sent to Glycobiolgy (ISSN: 0959-6658): Impact Factor of 3.929, ranks 77th in the "Biochemistry and Molecular Biology" category (Q2).

In addition, parts of this work have been presented in nine national and international congresses as written or as oral selected communications.

Abbreviations

Aa	amino acid
Abs	absorbance
ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
BSO	l-buthionine- (S,R)-sulfoximine
C31	ST3Gal III gene transfected Capan-1 cells
C32	ST3Gal III gene transfected Capan-1 cells
CA 19.9	carbohydrate antigen 19.9
CD62	E-Selectin
cDNA	complementary DNA
Cer	ceramide
CM-H$_2$DCFH-DA	5-(6)-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate acetyl ester
CMP	cytidine monophosphate
CP	Empty pcDNA3.1 transfected Capan-1 cells
Ct	threshold cycle
Da	dalton
DEPC	diethyl pyrocarbonate
DMEM	Dubelcco's Eagle modified medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FSC	forward scatter
Fuc	fucose
FucT	fucosyltransferase enzyme

FUT	fucosyltransferase encoding gene
Fw	forward
GAG	glycosaminoglycan
Gal	galactose
GalA	galacturonic acid
GalN	galactosamine
GalNAc	*N*-acetylgalactosamine
GAR-POD	goat anti-rabbit horseradish peroxidase conjugate
Glc	glucose
GlcA	glucuronic acid
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
GlcNAcT	*N*-acetylglucosaminyltransferase enzyme
GPI	glycosylphosphatidylinositol
GSH	reduced glutathione
GT	glycosyltransferase
H&E	haematoxylin-eosin
HBSS	Hank's buffered salt solution
HCC	hepatocarcinoma cells
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HSE	hepatic sinusoidal endothelium
HSEC	hepatic sinusoidal endothelial cells
HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon γ
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IgM	immunoglobulin M
IL1-β	interleukin 1 beta
IPMN	intraductal papillary mucinous neoplasm
kb	kilo base pairs
LacNAc	Galβ1-4GlcNAc or *N*-acetyllactosamine or Type II glycan unit
Le^a	Lewis a
Le^b	Lewis b
Le^x	Lewis x
Le^y	Lewis y
LPS	lipopolysaccharide
M33	ST3Gal III gene transfected MDAPanc-28 cells
M34	ST3Gal III gene transfected MDAPanc-28 cells
M42	ST3Gal IV gene transfected MDAPanc-28 cells

MAA	*Maackia amurensis* agglutinin
MAb	monoclonal antibody
Man	mannose
MCN	mucinous cystic neoplasm
MFI	mean fluorescence intensity
MGAT5	*N*-acetylglucosaminyltransferase V encoding gene
min	minute
MP	Empty pcDNA3.1 transfected MDAPanc-28 cells
MTT	3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide
MUC	mucin
NAc	*N*-acetylcysteine
neo-LacNAc	Galβ1-3GlcNAc or Type I glycan unit
Neu	neuraminic acid
NeuAc	*N*-acetylneuraminic acid
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
N-glycan	*N*-(Asn)-linked oligosaccharide
NK	natural killer cells
nm	nanometer
O.D.	optical density
O/N	overnight
O-GlcNAc	*O*-linked *N*-acetylglucosamine
O-glycan	*O*-linked oligosaccharide or O-GalNAc glycan
P	statistical significance level
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyviylidene fluoride
PSC	pancreatic stellate cells
qPCR	quantitative PCR
r	Pearson correlation coefficient
rh-E-Selectin	recombinant human E-Selectin
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park memorial institute medium
Rv	reverse
S1	stage 1

S2	stage 2
S3	stage 3
SD	standard desviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sE-Selectin	soluble E-Selectin
Sia	sialic acid
SLe^a	sialyl-Lewis^a
SLe^x	sialyl-Lewis^x
SNA	*Sambucus nigra* agglutinin
SPSS	statistical package for the social sciences
SSC	side scatter
ST	sialyltransferase (gene or enzyme)
ST3Gal III	beta-galactoside alpha-2,3-sialyltransferase 3 or Galβ1,3(4)GlcNAc-alpha2,3-sialyltransferase
ST3Gal IV	beta-galactoside alpha-2,3-sialyltransferase 4
TBE	tris/borate/EDTA buffer solution
TBP	TATA box binding protein
TBST	tris-buffered saline tween-20
TEMED	tetramethylethylenediamine
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
Thr	threonine
TNF-α	tumour necrosis factor α
Tris	tris-hydroxymethyl-aminomethane
Type I	Galβ1-3GlcNAc
Type II	Galβ1-4GlcNAc
Type III	Galβ1-3GalNAc
U	unit
UV-light	ultraviolet light
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
Vs.	Versus [Latin: in opposition to, against]
α1,2-FucT	alpha-1,2-fucosyltransferase enzyme
α1,3/4-FucT	alpha-1,3/4-fucosyltransferase enzyme
α1,6-FucT	alpha-1,6-fucosyltransferase enzyme
α2,3-ST	alpha-2,3-sialyltransferase enzyme
α2,6-ST	alpha-2,6-sialyltransferase enzyme
α2,8-ST	alpha-2,8-sialyltransferase enzyme
ΔRn	logarithmic increase in fluorescence signal

Symbols

SYMBOLIC REPRESENTATIONS OF COMMON MONOSACCHARIDES

⬤	Galactose (Gal)	★	Xylose (Xyl)
⬛	N-Acetylgalactosamine (GalNAc)	◆	N-Acetylneuraminic acid (Neu5Ac)
◺	Galactosamine (GalN)	◆	N-Glycolylneuraminic acid (Neu5Gc)
⬤	Glucose (Glc)	◆	2-Keto-3-deoxynononic acid (Kdn)
⬛	N-Acetylglucosamine (GlcNAc)	▲	Fucose (Fuc)
◤	Glucosamine (GlcN)	◇	Glucuronic acid (GlcA)
⬤	Mannose (Man)	◇	Iduronic acid (IdoA)
⬛	N-Acetylmannosamine (ManNAc)	◇	Galacturonic acid (GalA)
◺	Mannosamine (ManN)	◇	Mannuronic acid (ManA)

FULL REPRESENTATION

9-O-Ac-Neu5Ac$p\alpha$2-3Gal$p\beta$1-4GlcNAc$p\beta$1-2Man$p\alpha$1   Fuc$p\alpha$1

Man$p\beta$1-4GlcNAc$p\beta$1-4GlcNAc$p\beta$1-Asn

3-O-SO$_3$Gal$p\beta$1-4GlcNAc$p\beta$1-2Man$p\alpha$1

SYMBOLIC REPRESENTATIONS
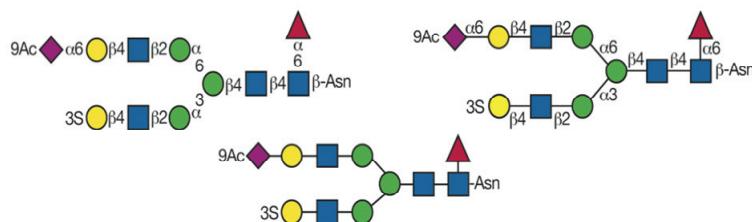
Symbols and conventions for drawing glycan structures recommended by the European Carbohydrate Databases (EUROCarbDB) and the Essentials of Glycobiology book (Varki et al. 2008).

Index

Summary

Pancreatic ductal adenocarcinoma (PDAC) has the highest mortality/incidence ratio of all major cancers and presents an extremely poor prognosis. Due to its extreme aggressiveness and rapid progression PDAC frequently results in metastasis by the time of diagnosis. The better understanding of metastatic process is a primary goal in PDAC research, since only a deeper comprehension will lead to improvements in the design of a more effective therapy.

In this regard, one of the common PDAC features is an altered glycosylation on their cell surface glycoconjugates, such as the overexpression of sialyl-Lewis^x (SLe^x) and related antigens. These sialyl-Lewis antigens have demonstrated to have an important *in vivo* role. The carbohydrate determinants SLe^x or SLe^a located on tumour cell

surface serve as ligands for the E-Selectin located on vascular endothelial cells. Thus, this interaction is thought to be crucial during the initial adhesion steps of haematogenous metastasis.

In the last steps of SLe^x and SLe^a biosynthetic pathway, sialyltransferases and fucosyltransferases compete to transfer sialic acid or fucose to terminal moieties of glycan units. Previous studies in our group had shown that sialyltransferase activity was correlated to sialyl-Lewis antigen expression on pancreatic cancer cell surface. Setting from this background, it was studied whether the overexpression of beta-galactoside alpha-2,3-sialyltransferase 3 (ST3Gal III) and beta-galactoside alpha-2,3-sialyltransferase 4 (ST3Gal IV) influenced the biosynthesis of sialylated Lewis antigens and whether they were involved in key steps of the tumourigenic process.

ST3Gal III and ST3Gal IV overexpressing Capan-1 and MDAPanc-28 pancreatic adenocarcinoma cell lines showed a subsequent increase in SLe^x expression. The transfectants binding capacity to E-Selectin was proportional to cell surface SLe^x levels. Cellular migration positively correlated with ST3Gal III/ SLe^x and ST3 Gal IV/SLe^x levels. Moreover, intrasplenic injection of the ST3Gal III and ST3Gal IV transfected cells into athymic nude mice provoked a decrease in mice survival and higher metastasis formation when compared to the intrasplenic injection of control cells.

When the ST3Gal III transfected cells were compared to the ST3Gal IV transfected cells, the former showed higher SLe^x surface expression and higher E-Selectin binding capacity. Moreover, ST3Gal III transfectants were endowed with higher migration and metastasis formation

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capabilities, leading to lower survival when intrasplenically injected into nude mice.

ST3Gal III mRNA expression levels in the pancreatic adenocarcinoma cell lines Capan-1 and MDAPanc-28 were modulated through cell proliferation and differentiation processes. As a consequence, these processes were able to regulate SLe^x determinant expression and E-Selectin adhesion. However, ST3Gal IV mRNA levels were not significantly modulated through cell proliferation and differentiation.

Cell proliferation and differentiation processes were accompanied by a decrease in intracellular glutathione anti-oxidant levels and intracellular Reactive Oxygen Species (ROS) generation. In fact, when cell cultures were treated with the oxidant H_2O_2, intracellular ROS levels increased along with ST3Gal III and SLe^x levels. Moreover, when H_2O_2 was added to *N*-Acetylcysteine pre-treated cells, ROS production was partially abrogated and a decrease in ST3Gal III and SLe^x expression was observed. These data demonstrated that ROS were involved in the control of ST3Gal III and SLe^x levels and, therefore in the acquisition of a more aggressive phenotype.

Finally, SLe^x-E-Selectin interaction in pancreatic adenocarcinoma cells was studied to determine its potential for up-regulating other metastatic capabilities such as migration and angiogenesis. The addition of recombinant soluble E-Selectin (sE-Selectin) was chemotactic for human pancreatic adenocarcinoma cells and significantly increased Vascular Endothelial Growth Factor (VEGF) secretion through a SLe^x depending mechanism.

Summary

In summary, this work shows that ST3Gal III, and in a lower extent ST3Gal IV, are directly implicated in key steps of tumour progression such as adhesion, migration and metastasis formation. ROS, generated in Capan-1 and MDAPanc-28 cell lines during cell proliferation-differentiation processes or by external oxidant stimuli such as H_2O_2, play a role in the control of ST3Gal III and SLe^x levels and in the acquisition of a more aggressive phenotype. And, together with the proadhesive role of E-Selectin for circulating cells, this work uncovers sE-Selectin dependent migration and VEGF secretion through a SLe^x depending mechanism, supporting additional prometastatic effects for sE-Selectin-SLe^x interaction.

Resumen

El adenocarcinoma ductal de páncreas (PDAC), con un ratio incidencia/mortalidad próximo a uno, es uno de los cánceres de peor pronóstico. Con frecuencia, en el momento del diagnóstico el cáncer ya se encuentra en un estadío metastático. Este hecho se explica en parte dada la extrema agresividad y rápida progresión que caracteriza el PDAC. En este sentido, uno de los objetivos fundamentales en la investigación del PDAC es llegar a conocer los mecanismos responsables del proceso metastático; conocimiento imprescindible para el diseño de terapias efectivas.

Desde este punto de vista, una de las características del PDAC es su glicosilación alterada de los glicoconjugados de superficie celular, como es la sobreexpresión de antígenos sialil-Lewis^x (SLe^x) y sus derivados.

Estos determinantes antigénicos, localizados en la superficie de células tumorales, son ligandos de adhesión de la molécula E-Selectina localizada en la superficie del endotelio vascular. Su interacción se considera clave en la extravasación que tiene lugar durante el proceso de metástasis.

Sialiltransferasas y fucosiltransferasas son enzimas clave en la biosíntesis de estos antígenos sialidados. En las últimas etapas de la ruta biosintética de SLe^x y SLe^a, ambos tipos de enzimas compiten por transferir ácido siálico o fucosa a los precursores de los glicoconjugados. Estudios previos de nuestro grupo han demostrado que la actividad sialiltransferasa correlaciona con la expresión de antígenos Lewis en la superficie de las células de cáncer de páncreas. Partiendo de esta base, se estudió el efecto de los enzimas beta-galactosido alfa-2,3-sialiltransferasa 3 (ST3Gal III) y beta-galactosido alfa-2,3-sialiltransferasa 4 (ST3Gal IV) en la biosíntesis de los antígenos Lewis y su posible implicación en etapas clave del proceso tumorigénico.

La sobreexpresión de ST3Gal III y ST3Gal IV, en las líneas de adenocarcinoma pancreático Capan-1 y MDAPanc-28, aumentó los niveles de SLe^x en la superficie de dichas células; dando lugar a un incremento proporcional en su capacidad adhesión a E-Selectina. También se observó una correlación positiva entre los niveles de ST3Gal III/SLe^x o ST3Gal IV/SLe^x y la capacidad migratoria de las células. Además, la inyección intraesplénica en ratones atímicos de las células que sobreexpresaban ST3Gal III o ST3Gal IV produjo una disminución en la supervivencia de los ratones y una mayor capacidad metastática respecto a la inyección intraesplénica de las células control. Al comparar los transfectantes de ST3Gal III con los transfectantes de

ST3Gal IV se observó que estos últimos presentaban niveles inferiores de SLe^x en superficie, menor capacidad de unión a la E-Selectina y menor migración; así como una mayor supervivencia y menor formación de metastasis tras la injección intraesplénica en ratones atímicos.

Por otra parte, se observó que los procesos de proliferación y diferenciación celular de las líneas de adenocarcinoma pancreático Capan-1 y MDAPanc-28 modulaban la expresión de ST3Gal III a nivel de mRNA, y por lo tanto la expresión de SLe^x en la superficie celular y su capacidad de adhesión a E-Selectina. Sin embargo, estos procesos no modularon de manera significativa la expresión de ST3Gal IV.

Simultáneamente, se observó que estos procesos de proliferación y diferenciación iban acompañados de una disminución del antioxidante intracelular glutatión (GSH) y de un incremento en la síntesis de Especies Reactivas del Oxígeno (ROS) intracelulares. De hecho, cuando los cultivos se trataron con el oxidante H_2O_2, los niveles intracelulares de ROS aumentaron junto con los de ST3Gal III y SLe^x; y cuando el H_2O_2 se añadió a cultivos pre-tratados con *N*-Acetilcisteina (un precursor del GSH) la producción de ROS y los niveles de ST3Gal III y SLe^x no se vieron significativamente alterados. Estos experimentos demostraron que ROS desempeña un papel significativo en la regulación de los niveles de ST3Gal III y SLe^x, y por lo tanto en la adquisición de un fenotipo más agresivo.

Por último, se estudió como las células de adenocarcinoma pancreático se sirven de la interacción SLe^x–E-Selectina para regular otras capacidades metastáticas tales como la migración y la angiogénesis. La adición de E-Selectina soluble indujo la quimiotaxis de las células de

adenocarcinoma pancreático. Además, incrementó de manera significativa los niveles de secreción del Factor de Crecimiento del Endotelio Vascular (VEGF) implicado en procesos angiogénicos a través de un mecanismo dependiente de SLe^x.

En resumen, este trabajo demuestra que los enzimas ST3Gal III, y en menor medida ST3Gal IV, están directamente implicados en etapas clave de la progresión tumoral como la adhesión, la migración y la formación de metástasis en las líneas de adenocarcinoma pancreático humano estudiadas. También, que las Especies Reactivas del Oxígeno generadas durante los procesos de proliferación y diferenciación celular o debido a estímulos oxidantes externos, desempeñan un importante papel en el control de la síntesis de ST3Gal III y SLe^x, y por lo tanto en la regulación del fenotipo metastático. Además, junto al papel pro-adhesivo de la E-Selectina, este trabajo ha descrito efectos prometastáticos adicionales para esta molécula como inductora de la migración y de la secreción de VEGF a través de un mecanismo E-Selectina-SLe^x dependiente.

Introduction

1. Cancer

Cancer is the third leading cause of death worldwide, with more than 12 million new cases and 7.6 million cancer deaths estimated to have occurred in 2007. Furthermore, by 2030 it is calculated that there will be more than double new cancer cases and 17 million cancer deaths per year (American_Cancer_Society. 2007; Boyle and Levin 2008). Those data evidence the need to develop efficient therapies for cancer, which will be achieved with a better understanding of its genesis and progression. Understanding such processes is a main objective in cancer research.

Cancer encompasses a group of diseases in which cells of an organ or tissue in the body become abnormal, failing to respond to normal growth control mechanisms. When cells grow out of control, they form a mass, called a tumour. Some tumours grow and enlarge only at the site where they begin and these are referred to as benign tumours, which are generally curable by surgical resection. Malignant tumours, not only enlarge locally but also have the potential to detach from the original (primary) tumour, to invade and destroy the normal tissue around them, to spread to distant parts of the body and to establish themselves in the new site as a metastasis (or secondary tumour) and, at the end of the process, cause death.

Although there are more than 100 distinct types of cancer, and subtypes of tumours can be found within specific organs, the vast catalogue of cancer cell genotypes is a manifestation of ten essential alterations that collectively dictate malignant growth and progression: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000), inflammation (Colotta et al. 2009), altered glycosylation (Fuster and Esko 2005), altered metabolism (Tennant et al. 2009) and interactions with tumour stroma (Pietras and Ostman 2010).

1.1. Stages of tumour progression

Tumourigenesis and tumour progression are extremely complex processes consisting of a long series of sequential interrelated steps that can be summarized as follows (Figure 1)(Fidler 1990; Langley and Fidler 2007):
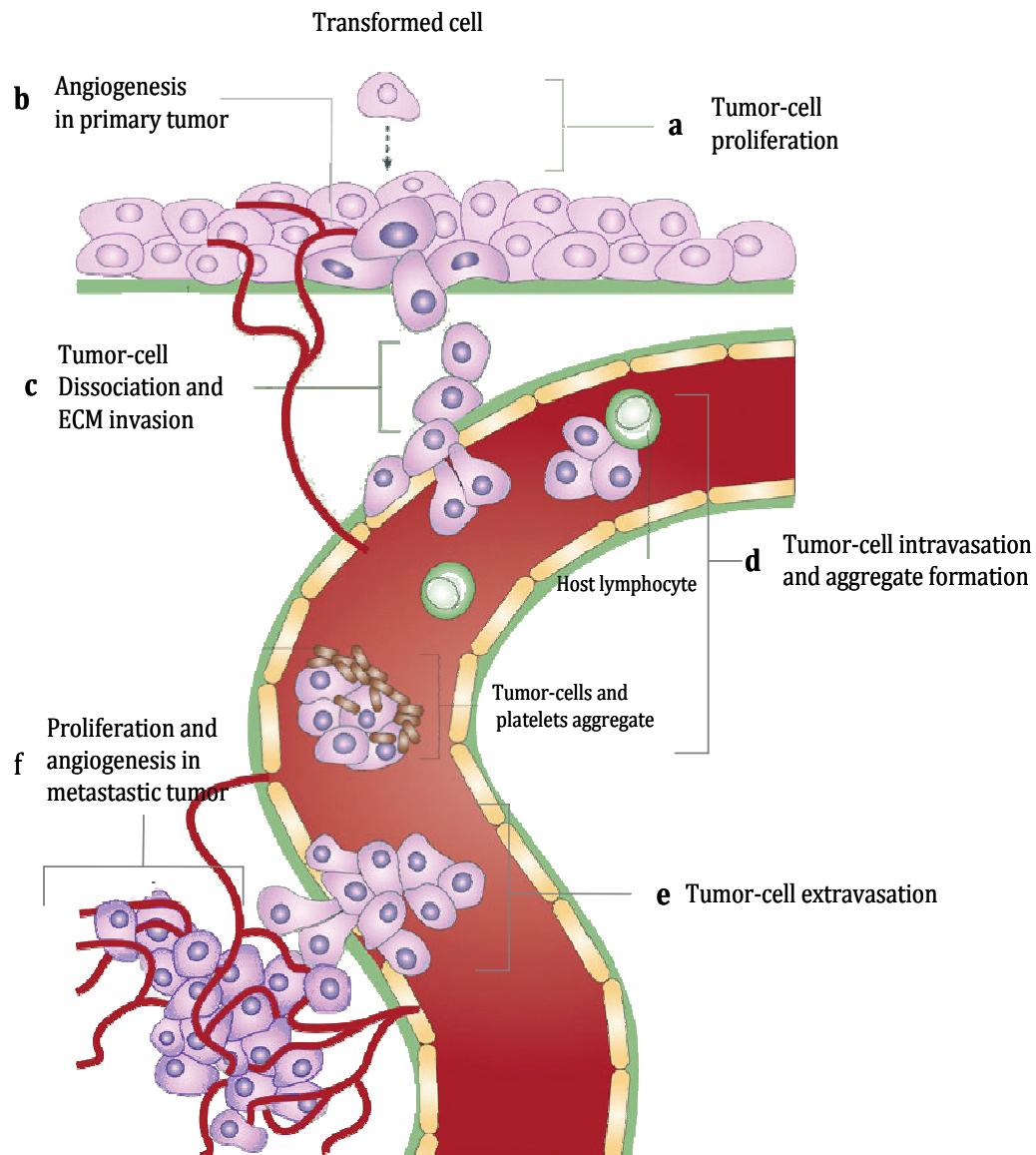
Figure 1: Stages of tumour progression: a) Uncontrolled proliferation of a transformed cell is a crucial step at early stages b) Tumour angiogenesis is required for pathological growth of the primary tumour c) During invasion, tumour cells gain the capacity to degrade and migrate through the basement membranes and extracellular matrix d) Tumour-cells extravasate and disseminate through the bloodstream and form aggregates with blood cells e) Tumour-cells mimic the homing of lymphocytes in order to extravasate f) After extravasation, tumour-cells proliferate and develop a vascular network forming secondary tumours (metastasis). See text for further details. Extracted and modified from Fuster and Esko (Fuster and Esko 2005).

a) The initial transforming event is a mutation in proto-oncogenes, tumour suppressor genes or DNA repair genes involved in cell proliferation control, which lead to a progressive neoplastic cell proliferation. It is though to be a slow process that occurs for years before becoming apparent.

b) Continuous proliferation engenders a tumour mass, which if exceeding 2 mm in diameter extensively vascularizates (Folkman 1984). The synthesis and secretion of several angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), play a key role in establishing a neocapillary network from the surrounding host tissue (Folkman and Klagsbrun 1987).

c) During local invasion of host stroma, tumour cells detach from one another and from the Extracellular Matrix (ECM) and migrate through surrounding tissue. This requires the remodelling of ECM, through the secretion of proteolytic enzymes and through changes in cell-surface adhesion receptors.

d) Tumour intravasation occurs when tumour cells invade the thin-walled capillaries (haematogenous metastasis) or lymphatic channels (lymphatic metastasis). After entry into the vasculature, small tumour cells form large aggregates with platelets and leukocytes to facilitate their survival in the circulation (Tsuruo and Fujita 2008).

e) Tumour cells that survive the circulation must arrest and extravasate in the capillary beds of organs. In order to do so, cancer cells mimic the homing of leukocytes into inflammatory sites (Carlos and Harlan 1994; Kannagi 1997).

f) Proliferation within the new organ completes the metastatic process. To produce detectable lesions, the metastases must develop a vascular network (angiogenesis), evade the host immune system, and respond to organ specific factors that influence their growth (Fidler et al. 1978; Horak et al. 1986; Nicolson 1988).

Each one of these sequential interrelated steps can be rate limiting since a failure or an insufficiency at any of the steps aborts the process (Poste et al. 1979). The resulting course of action is dependent on both, the intrinsic properties of the tumour cells and the host responses (Fidler et al. 1978). Since tumour cells do not act in isolation, but rather subsist in a rich microenvironment provided by the host, functional and structural interactions between tumour and host cells promote cancer growth, invasion and metastasis (Cheng and Weiner 2003; Pietras and Ostman 2010).

2. Glycans and glycoconjugates

Glycans are covalent assemblies of monosaccharides, oligosaccharides or polysaccharides that could be found in either free form or in covalent complexes with proteins or lipids (the aglycone) constituting the glycoconjugates.

Since many glycans are found on the outer surface of cells and in secreted macromolecules, they are in a position to modulate or mediate a wide variety of events in cell-cell, cell-matrix and cell-molecule interactions (Varki et al. 2008). Thus, they are considered to be

particularly important for mediating crucial physiopathological events during tumour progression (Fuster and Esko 2005).

2.1. Major classes of glycans and glycoconjugates

The common classes of glycans are defined according to the nature of the linkage to the aglycone (Varki et al. 2008) (Figure 2):
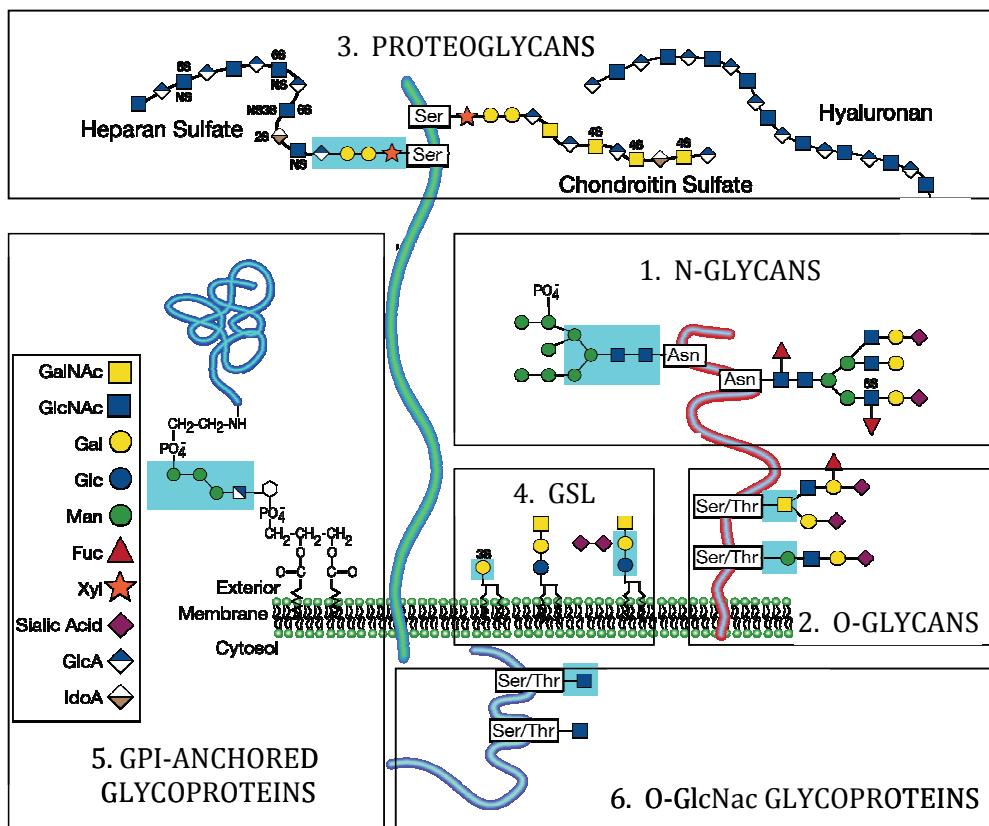
Figure 2: Major common classes of glycans and glycoconjugates: 1. *N*-Glycans 2. *O*-Glycans 3. Proteoglycans as heparin Sulphate or free glycosaminoglycans such as hyaluronan 4. GSL = glycosphingolipids 5. Glycosylphosphatidylinositol (GPI) anchored glycoproteins 6. *O*-GlcNAc glycoproteins. See text for further details. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

1. A *N*-glycan (*N*-linked oligosaccharide, *N*-(Asn)-linked oligosaccharide) is a sugar chain covalently linked to an asparagine residue of a polypeptide chain, where an *N*-Acetylglucosamine (GlcNAc) residue is attached to the amide nitrogens of asparagine side chains in the consensus peptide sequence : Asn-X-Ser/Thr. All *N*-glycans share a common pentasaccharide core region Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr, and can be divided into three main classes: *oligomannose type*, in which only mannose residues are attached to the core; *complex type* in which "antennae" initiated by *N*-Acetylglucosaminyltransferases are attached to the core and *hybrid type,* in which mannose residues are attached to the Manα1–6 arm of the core and one or two antennae are initiated by *N*-Acetylglucosaminyltransferases on the Manα1–3 arm of the core (Figure 3).
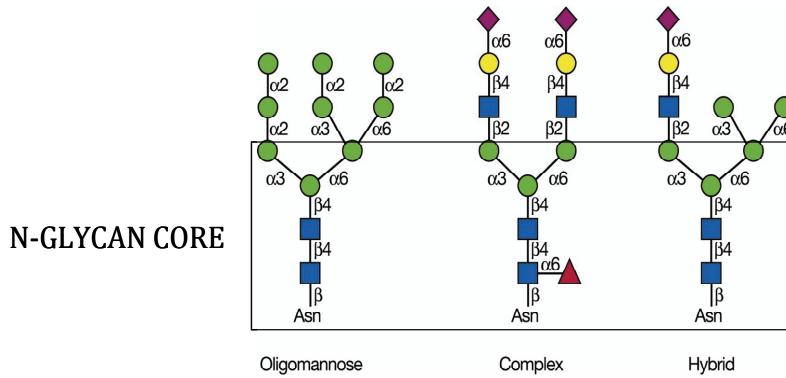
Figure 3: General types of *N*-Glycans: Oligomanose, Complex and Hybrid. All three, contain the common core $Man_3GlcNAc_2Asn$. See text for further detail. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

2. An *O*-glycan (*O*-linked oligosaccharide, *O*-GalNAc glycan) is frequently α-linked via an *N*-Acetylgalactosamine (GalNAc) moiety to the hydroxyl group of a serine or threonine residue. Nevertheless, other types of *O*-Glycans also exist including α-linked *O*-fucose, β-

linked *O*-xylose, α-linked *O*-mannose, β-linked *O*-GlcNAc, α- or β-linked O-galactose and α- or β-linked O-glucose glycans. *O*-glycans can be extended in a variety of different structural core classes (see Table 1) and are common in many glycoproteins, especially on secreted and membrane bound mucins.

O-Glycan core	Structure
Tn antigen	GalNAcαSer/Thr
Sialyl-Tn antigen	Siaα2-6GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr
Core 5	GalNAcα1-3GalNAcαSer/Thr
Core 6	GlcNAcβ1-6GalNAcαSer/Thr
Core 7	GalNAcα1-6GalNAcαSer/Thr
Core 8	Galα1-3GalNAcαSer/T

Table 1: Structures of *O*-Glycan cores. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

3. A proteoglycan (such as heparan sulphate or chondroitin sulphate) is a glycoconjugate that has one or more glycosaminoglycan (GAG) chains attached to a core protein through a core region ending in a xylose residue that is linked to the hydroxyl group of a serine residue. GAGs are linear polysaccharides, whose disaccharide building blocks consist of an amino sugar and an uronic acid or galactose. Some glycosaminoglycans could be present as free polysaccharides (such as hyaluronan). Virtually all mammalian cells produce proteoglycans and secrete them into the ECM or insert them into the plasma membrane. There is a tremendous structural variation of proteoglycans due to the large number of existing core proteins.

4. A glycosphingolipid (often called a glycolipid) consist of a glycan usually attached via glucose of galactose to the terminal primary hydroxyl group of the lipid moiety ceramide, which is composed of a long chain base (sphingosine) and a fatty acid. Further extensions of the glycan generate a series of neutral core structures that form the basis for the nomenclature of glycosphigolipids (Table 2).

Subfamily series	Glycosphingolipids core Structures
Lacto	GlcNAcβ1-3Galβ1-4GlcβCer
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcβCer
Neolacto	Galβ1-4GlcNAcβ1-3Galβ1-4GlcβCer
	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcβCer
Ganglio	GalNAcβ1-4Galβ1-4GlcβCer
	Galβ1-3GalNAcβ1-4Galβ1-4GlcβCer
Globo	Galα1-4Galβ1-4GlcβCer
	GalNAcβ1-3Galα1-4Galβ1-4GlcβCer

Table 2: Names and structures of major core vertebrate glycosphingolipids. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

5. A glycosylphosphatidylinositol (GPI)-anchor is a glycan bridge between phosphatidylinositol and ethanolamine that is in amide linkage to the carboxyl terminus of a protein. This structure typically constitutes the only anchor to the lipid bilayer membrane for such proteins. All characterized GPI anchors share a common core consisting of ethanolamine-PO_4-6Man α1–2Manα1–6Manα1–4GlcNα1–6myo-inositol-1-PO_4-lipid. Heterogeneity in GPI anchors is derived from substitutions of this core structure.

6. Nuclear and cytoplasmic proteins can bear the monosaccharide *O*-linked *N*-Acetylglucosamine (*O*-GlcNAc) linked to serine.

2.2. Type I and Type II glycan units

Although glycans have unique core regions by which they are distinguished, certain outer structural sequences are often shared. Frequently these outer terminal structures determinate the function or recognition properties of a glycoconjugate. *N*-glycans, *O*-glycans and glycosphingolipids often contain terminal *N*-Acetylglucosamine (GlcNAc) residues. The subsequent addition of galactose in β1–4 linkage generates a Type II unit, which is composed of the disaccharide Galβ1–4GlcNAc, also called *N*-Acetyllactosamine (LacNAc) (Figure 4).
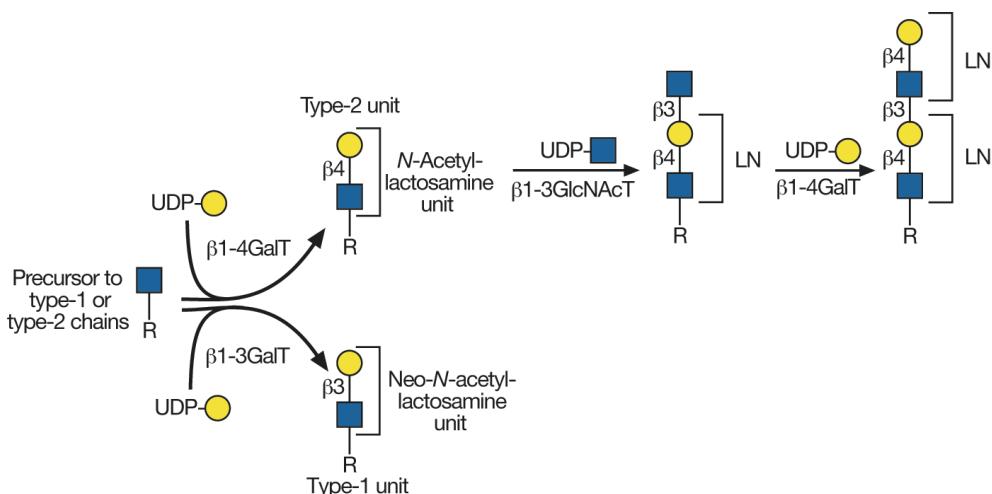
Figure 4: Type I (Type-1 unit) or Type II (Type-2 unit) glycan units. In *N*-glycans, *O*-glycans and glycosphingolipids terminal GlcNAc residues are usually galactosylated. If the reaction is catalyzed by the β1-4 galactosyltransferase (β1-4GalT) yields a unit termed Type II. If catalyzed by the β1-3 galactosyltransferase (β1-3GalT) yields a unit termed Type I. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

The terminal galactose so generated can be modified by the addition of a GlcNAc residue, which in turn can receive a galactose in β1–4 linkage, thus forming two LacNAc units. These reactions may continue to form poly-*N*-Acetyllactosamine [Galβ 1–4GlcNAc]n. Alternatively, GlcNAc

residues in *N*-glycans, *O*-glycans, and glycolipids may instead be modified by galactose in β1–3 linkage to obtain a Type I unit composed of a disaccharide termed neo-*N*-Acetyllactosamine (neo-LacNAc) (Varki et al. 2008).

2.3. Modifications of Type I and Type II glycan units by glycosyltransferases

Outer Type I and Type II in *N*-glycans, *O*-glycans and glycosphingolipids can be further modified by other glycosyltransferases. The glycosyltransferases (GT) constitute a very large family of enzymes catalyzing a group-transfer reaction in which the monosaccharide moiety of a simple nucleotide sugar donor substrate is transferred to the acceptor substrate. Traditionally those enzymes have been classified in families based on their substrate specificity (Table 3).

Glycosyltranferase families	Sugar donor substrate
Sialytransferases (ST)	CMP- sialic acid
Fucosyltransferases (FucT)	GDP-Fucose
Galactosyltransferases	UDP-Galactose
N-Acetylglucosaminyltransferases	UDP-GlcNAc
N-Acetylgalactosaminyltransferases	UDP-GalNAc
Manosyltransferases	GDP-Mannose

Table 3: Main glycosyltransferase families. Classified as function of their substrate specificity according to the International Union of Biochemistry and Molecular Biology

This way, sialyltransferases (ST) are a family of glycosyltransferases capable of transferring sialic acid to terminal moieties of glycoconjugates, and fucosyltransferases (FucT) a family of glycosyltransferases responsible for transferring fucose.

2.3.1. Fucosyltransferases

Fucosylated glycans are synthesized by fucosyltransferases which, based on the site of fucose addition, are classified in four subfamilies: *alpha-1,2-fucosyltransferases (α1,2-FucTs), alpha-1,3/4-fucosyltransferases (α1,3/4-FucTs), alpha-1,6-fucosyltransferases (α1,6-FucTs)* and *O-fucosyltransferases (O-FucTs) (Ma et al. 2006).* To date thirteen fucosyltransferase genes have been identified in the human genome. FUT1 and FUT2 encode for the *alpha-1,2-fucosyltransferases* FucT I and FucT II (Kelly et al. 1995; Larsen et al. 1990). FUT3-FUT7 and FUT9-FUT11 genes have been identified in the *alpha-1,3/4-fucosyltransferase* subfamily (Roos et al. 2002). All the enzymes they encode present *alpha-1,3-fucosyltransferase* activity. In addition, the enzymes encoded by FUT3 and FUT5 have been reported to present both *alpha-1,3-and alpha 1,4-fucosyltransferase* activity (Kaneko et al. 1999; Natsuka et al. 1994). FUT7 encodes for an *alpha-1,3-fucosyltransferase* that synthesizes only SLe^x. FUT8 encodes for an *alpha-1,6-fucosyltransferase* that directs addition of fucose to the inner core of asparagine-linked GlcNAc moieties (Miyoshi et al. 1999). Finally, FUT12 and FU13 genes encode for the human *O-fucosyltransferases* O-FUT1 and O-FUT2 (also named POFUT1 and POFUT2) that add fucose directly to the hydroxyl group of Ser and Thr residues on glycoprotein acceptors (Ma et al. 2006; Wang et al. 2001).

2.3.2. Sialyltransferases

In mammals, 20 enzymes capable of catalyzing the transfer of sialic acid residues from the donor substrate CMP-sialic acid to the oligosaccharide side chains of glycoconjugates have been identified (Harduin-Lepers et al. 2001). These various intracellular, Golgi membrane-bound sialyltransferases (STs) were grouped in three

subfamilies according to the sialyl linkages they form (Tsuji et al. 1996). *Alpha-2,6-sialyltransferases (α2,6-STs)* mediate the transfer of sialic acid with an alpha 2,6-linkage to terminal Gal (ST6Gal I, II) or GalNAc residues (ST6GalNAc I-VI). *Alpha-2,8 sialyltransferases (α2,8-STs)* mediate the transfer of sialic acid with an alpha 2,8-linkage (ST8Sia I-IV). Finally, *alpha-2,3 sialyltransferases (α2,3-STs)* mediate the transfer of sialic acid with an alpha 2,3-linkage to terminal Gal residues. Focusing in this subfamily members, ST3Gal I-II and IV catalyze the transference to the Gal residue located on terminal Galβ1-3GalNAc structures. ST3Gal IV and VI transfer sialic acid to the Gal residue located on terminal Galβ1-4GlcNAc structures. ST3Gal V acts on the Gal residue located on terminal Galβ1-4Glc-Cer structures. Finally, ST3Gal III catalyzes the transfer of sialic acid with an alpha 2,3-linkage to terminal Gal residues located on either Galβ1-3GlcNAc or Galβ1-4GlcNAc structures (Harduin-Lepers et al. 2001). Due to the importance of the ST3Gal III and ST3Gal IV enzymes in the present work, they will be next described in detail.

2.3.2.1. Alpha-2,3-sialyltransferase ST3Gal III

The enzyme beta-galactoside alpha-2,3-sialyltransferase 3 or Galβ1,3(4)GlcNAc-alpha2,3-sialyltransferase (ST3Gal III or alpha 2,3-sialyltransferase ST3Gal III) catalyzes the synthesis of NeuAc2,3Galβ1,3(4)GlcNAc structures on terminal moieties of Type I (Gal(β1-3)GlcNAc) or Type II (Gal(β1-4)GlcNAc) units of *N*-glycans, *O*-glycans and glycolipids. ST3Gal III acts preferably on Type I chains participating on SLe[a] synthesis (see 2.4), however it can catalyze the sialylation of Type II chains and therefore participate in SLe[x] synthesis (Kitagawa and Paulson 1993; Kono et al. 1997; Weinstein et al. 1982; Wen et al. 1992).

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The first ST3Gal III gene was cloned by Wen (Wen et al. 1992) from a rat liver cDNA library and a year later the same authors cloned the human gene from a placenta cDNA library and reported a 91% homology for nucleotide sequence and a 97% homology for aminoacid sequence with the previously cloned rat gene (Kitagawa and Paulson 1993). The human Galβ1,3(4)GlcNAc-alpha2,3-sialyltransferase gene (hST3Gal III) is localized to human chromosome 1(p34-q33). It spans more than 223 kilobases of human genomic DNA and is distributed over 15 exons (Kitagawa et al. 1996). Characterization of cDNAs encoding the hST3Gal III revealed that the gene produces at least three transcripts in human placenta differing at the 5' ends, but that code for identical protein sequences (Kitagawa and Paulson 1994). Recently new isotranscripts, generated via alternative splicing, have been reported for leukocytes (Grahn et al. 2002) and embryo brain (Grahn et al. 2004).

2.3.2.2. Alpha-2,3-sialyltransferase ST3Gal IV

The enzyme beta-galactoside alpha-2,3-sialyltransferase 4 (ST3Gal IV or alpha 2,3-sialyltransferase ST3Gal IV) catalyzes the transference of sialic acid with an alpha 2,3-linkage to terminal Gal residues on Type III (Gal(β1-3)GalNAc) structures. It also acts on Type II units participating on Sialyl-Lewis^x (SLe^x) synthesis (see 2.4). It has a very low or negligible preference for Type I units.

The human gene (hST3Gal IV) is localized to human chromosome 11(q23-q24). It spans more than 25 kilobases of human genomic DNA and is distributed over 14 exons (Kitagawa et al. 1996). Two independent groups reported the cloning of the human gene codifying for this enzyme obtained from different sources: a placenta cDNA library (Kitagawa and Paulson 1994) and a melanoma cell line (Sasaki

et al. 1993). Characterization of cDNAs encoding the hST3Gal IV revealed that the gene produces at least five transcripts (Kitagawa et al. 1996; Kitagawa and Paulson 1994). Recently seven new isotranscripts have been reported from leukocytes (Grahn and Larson 2001).

2.4. Lewis blood group determinants.

In contrast to core *N*-glycan synthesis, which is constitutive in most cell types; terminal sugars biosynthesis is function of the consecutive action of several GT, often regulated in a tissue or cell lineage–specific manner. This fact explains the enormous variety of *N*-glycans diferring in their terminal moieties found in nature. Many of these terminal N-glycan modifications described so far have not yet been related to defined functions. However, for some of them it has been possible to establish such relation, as, for instance ABO blood groups, the Forssman antigen or the Lewis blood groups. Members of the Lewis blood group family accomplish important functions in selectin-dependent tumour cell adhesion processes (see 3.4). Thus, they deserve a detailed review in the present work.

The term Lewis derives from a family who suffered from a red blood cell incompatibility, which lead to the discovery of this blood group. The Lewis blood group antigens are glycans presented on the Type I or Type II Acetyllactosamines on N-glycans, O-glycans or glycosphingolipids. In the Lewis antigens biosynthetic pathway (Figure 5), the alpha 1,3/4-fucosyltransferases FucT III and FucT V, encoded by FUT3 and FUT5 genes, transfer alpha 1,4-linked fucose to the outer GlcNAc of the Type I precursor to form the Lewis a (Le[a]) antigen. The alpha-1,2-fucosyltransferase FucT II, and with lower catalytic efficiency FucT I (Barreaud et al. 2000), transfer alpha 1,2-linked fucose to the terminal

galactose of the Type I precursor to form the Type-H1 antigen. Sequential action of 1,3/4-fucosyltransferases FucT III or FucT V on Type-H1 give place to Lewis b (Le[b]) antigen.
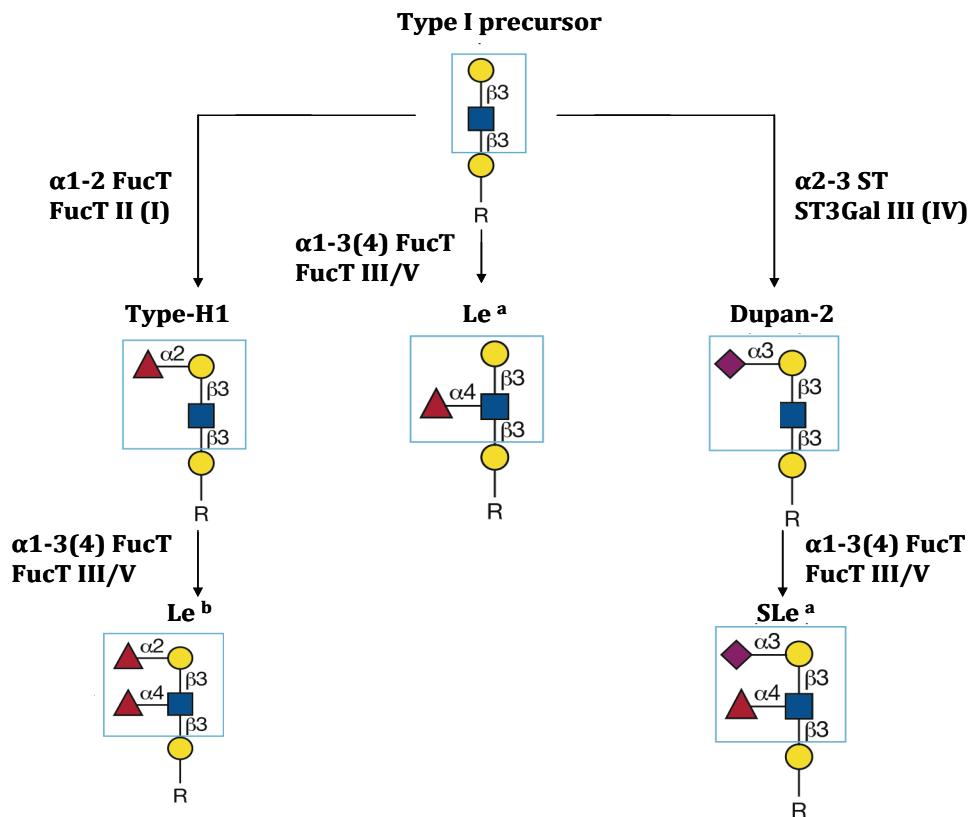
Figure 5: Enzymes involved in Lewis antigens synthesis from Type I precursor. α1-2 FucT: alpha-1,2-fucosyltransferases FucT II and FucT I. α1-3/4 FucT: alpha-1,3/4-fucosyltransferase FucT III and FucT V. α2-3 ST: alpha-2,3-sialyltransferases ST3Gal III and ST3Gal IV. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

Alternatively, the alpha 2,3 sialyltransferases ST3Gal III and, with very low catalytic efficiency ST3Gal IV, transfer alpha 2,3-linked acid sialic to the terminal galatose of the Type I precursor to form the Dupan-2 antigen. Sequential action on Dupan-2 antigen of alpha-3/4-fucosyltransferases FucT III or FucT IV form the SLe[a] antigen.

Similarly, the alpha-1,3/4 fucosyltransferases FucT III, FucT IV, FucT V, FucT VI and FucT IX transfer alpha 1,3-linked fucose to the outer GlcNAc of the Type II precursor to form the Lewis x antigen (Figure 6).
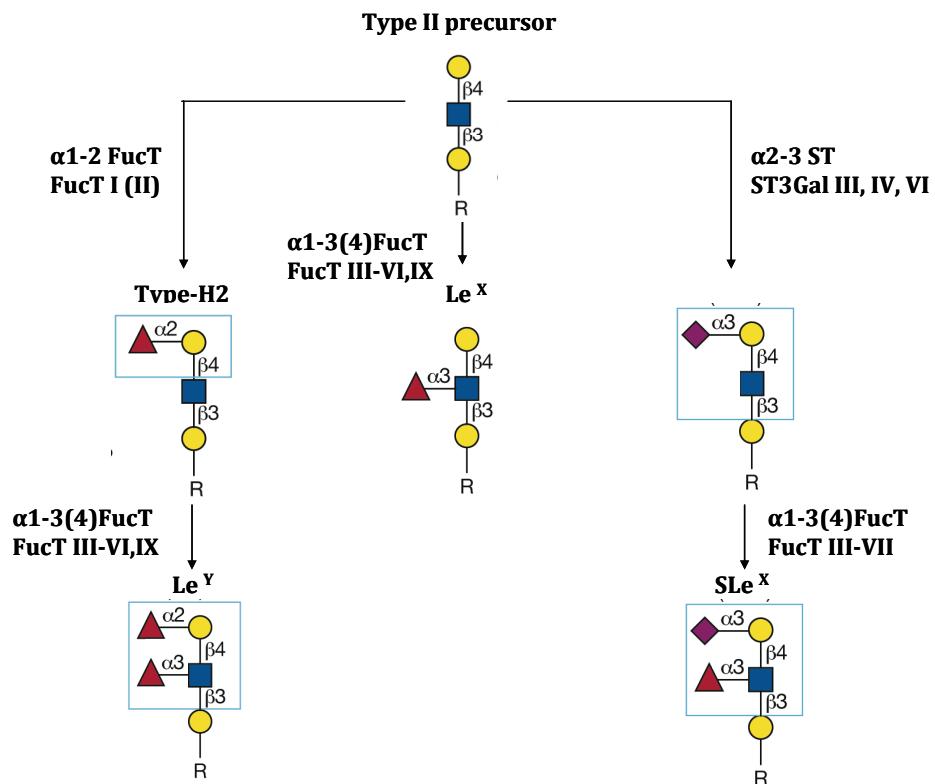
Figure 6: Enzymes involved in Lewis antigens synthesis from Type II precursor. α1-2 FucT: alpha-1,2-fucosyltransferases FucT I and FucT II. α1-3/4 FucT: alpha-1,3/4-fucosyltransferases FucT III, FucT IV, FucT V, FucT VI and FucT IX. In SLe^x formation FucT VII could also be implicated. α2-3 ST: alpha-2,3-sialyltransferases ST3Gal III and ST3Gal IV. Extracted and modified from the *Essentials of Glycobiology* book (Varki et al. 2008)

The alpha-1,2-fucosyltransferases FucT I (and FucT II, in lower degree), transfer alpha 1,2-linked fucose to the terminal galactose of the Type II precursor to form the Type-H2 antigen. Sequential action of 1,3/4-fucosyltransferases (FucT III-VI and IX) give place to Lewis y (Le^y)

antigen. Alpha-2,3 sialyltransferases ST3Gal IV, ST3Gal VI and ST3Gal III transfer alpha 2,3-linked acid sialic to the terminal galatose of the Type II precursor. Sequential action of alpha-1–3/4 fucosyltransferases FucT III-VII is able to form the SLe[x] antigen.

3. Glycans in tumour progression

Glycans are particularly important for mediating key events during tumour progression. In the tumour environment, changes in glycosylation allow tumour cells to appropriate many of the events that occur in normal cells during embryogenesis, such as adhesion to a variety of other cell types and capacity to spread throughout the organism (Varki et al. 2008). There is a profuse bibliography on how aberrant glycosylation of tumour cells affects different steps of tumour progression, such as proliferation, angiogenesis, cell detachment, invasion and extravasation, which are explained below.

3.1. Glycans in tumour proliferation

Although the initial tumour cell proliferation is attributed to mutations in cell proliferation controlling proto-oncogenes and tumour suppressor genes, glycosylation changes in key molecules involved in tumour proliferation can also regulate this process.

Aberrant glycosylation of Epidermal Growth Factor Receptor (EGFR) is able to modify tumour pathways that lead to tumour cell proliferation. This way, deglycosylated EGFR is unable to become fully phosphorylated and to trigger cell proliferation (Pratt and Pastan 1978), while overexpresion of MGAT5 in tumour cells (which transfers GlcNAc to N-glycans giving rise to tetranantennary structures)

enhances EGF recruitment to galectin lattice domains that limit its diffusion and results in a positive feed-back to growth signalling (Lau et al. 2007).

Extensive literature exists about the roles of O-linked glycans and mucins in proliferation. Mucins are glycoproteins that contain numerous O-glycans in clustered domains along the core protein. Most carcinomas express mucins, either as transmembrane proteins on the cell surface or as secreted proteins. MUC expression often gets altered in the tumour. For example, endometrial adenocarcinoma has increased levels of MUC4, MUC5AC, and MUC6 when compared to normal endometrium (Alameda et al. 2007). In addition, recent experiments have shown that the overexpression of MUC4 in melanoma cells induces rapid cellular growth and the suppression of tumour apoptosis in human melanoma-bearing nude mice (Komatsu et al. 2001).

In addition, some proteoglycans are able to function as co-receptors for soluble tumour growth factors. Negatively charged sulphate groups of glycosaminoglycans facilitate interactions with basic amino-acid residues on protein ligands and are involved in binding a wide array of bioactive factors such as fibroblast growth factors (FGFs), transforming growth factor-β (TGFβ), and numerous members of the interleukin, chemokine an growth factor families (Bernfield et al. 1999; Kleeff et al. 1998; Varki et al. 2008). This way, glycosaminoglycans are able to facilitate the formation of ligand-receptor complexes, lowering the effective concentration of ligand required for receptor activation.

Finally, is well-known that tumour matrices are especially rich in hyaluronan, a large anionic glycosaminoglycan which is polymerized at the plasma membrane and secreted into the ECM. Interactions between

hyaluronan and its main receptor CD44, wich is a cell surface protein, changes in cell motility and growth (Turley et al. 2002). Moreover, changes in CD44 expression, including isoforms and glycoforms alterations, are associated to a variety of tumours and to the metastatic spread of cancer.

3.2. Glycans in tumour angiogenesis

Angiogenesis is the developing of blood-vessel endothelial cells in response to pro-angiogenic factors such as the Vascular Endothelial Growth Factor (VEGF). Recent studies reported that the heparan sulphate proteoglycan on the endothelial-cell surface facilitates VEGF binding and VEGFR activation on the endothelial cells, which leads to their mitogenesis and sprouting (Iozzo and San Antonio 2001; Jiang and Couchman 2003). On the other hand, cell adhesion mediated by selectins and their carbohydrate ligands such as SLe^x and SLe^a was suggested to be involved in the process of angiogenesis in bovine endothelial cells (Nguyen et al. 1993). This study was confined to angiogenesis by endothelial cells *per se;* a recent study, however, uncovers the interaction of cancer cells with endothelial cells through selectins and their ligands (SLe^x and SLe^a) as a critical factor for *in vivo* tumour angiomorphogenesis (Tei et al. 2002).

3.3. Glycans in tumour cell detachment and invasion

Tumour cells frequently overexpress specific glycosyltransferases capable of synthesizing glycans that facilitate cell-cell detachment and ECM invasion. Classic reports of increased size of tumour cell-derived glycopeptides have now been convincingly explained by an increase in β1,6-branching of *N*-glycans, which results from an enhanced

expression of the GlcNAcT-V (Dennis et al. 2002; Hakomori 1996; Varki et al. 2008). Increased expression of GlcNAcT-V along with the presence of β1,6-branched *N*-glycans on tumour-cell N-cadherin (an adhesion molecule that normally mediates cell aggregation through homotypic interactions) has been associated to reduction in tumour cell–cell adhesion and to the promotion of cell detachment and invasion (Guo et al. 2003).

Another common feature of tumour cells is a tendency to produce increased levels of glycoconjugates containing sialic acid (Figure 7), an acidic sugar transferred to oligosaccharides by sialyltransferases (see 2.3.2.). Those increased levels of glycoconjugates containing sialic acid are often associated with the increased invasive potential of tumour cells, in both cultured cell lines as well as in clinical tumours, and its expression correlates with poor prognosis (Fuster and Esko 2005). Sialic acid imparts a negative charge to the glycan chain, which may promote cell detachment from the tumour mass through charge repulsion (Seidenfaden et al. 2003).
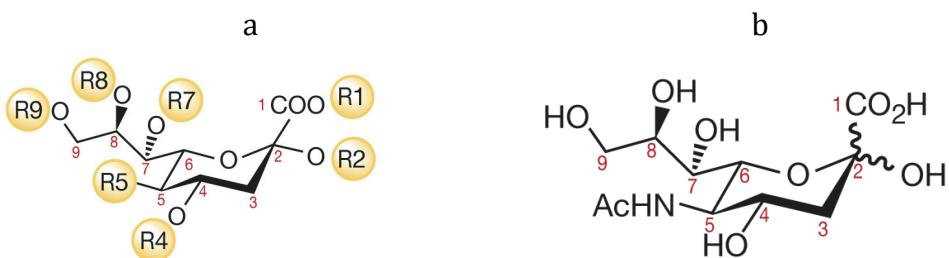
Figure 7. Sialic acids. a) The nine-carbon backbone common to all known Sias is shown in the α configuration; variations can occur at the carbon positions indicated b) The most common "primary" sialic acid is *N*-Acetylneuraminic acid, Neu5Ac. Glycosidically bound sialic acids in naturally occurring glycans are in the α form, and free sialic acids in solution are mainly in the β-form.

Moreover, sialic acid can also potentiate invasiveness by receptor-dependent processes or by facilitating interactions between tumour sialic acids and matrix proteins. For instance, the sialyl Tn antigen (a disaccharide commonly overexpressed in the mucin-rich surfaces of cancer cells) potentiates tumour invasiveness (Julien et al. 2001) and increased sialylation of CD44 in cancer cells reduces its binding to hyaluronan leading to a more migratory phenotype.

Invasion by tumour cells involves the interaction of cell-matrix interactions, which are mediated by adhesion molecules present on the tumour cells that bind to ECM components. Integrins represent a particularly important class of cell-surface adhesion receptors that mediate attachment to important ECM protein ligands, such as collagen, fibronectin and laminin. Changes in integrins glycosylation have been reported to influence their function. For instance, hipersialylation of β1 integrin adhesion molecule up-regulates the attachment to certain extracellular matrix milieus such as collagen and laminin and stimulates cell migration (Christie et al. 2008; Seales et al. 2005; Shaikh et al. 2008; Yamamoto et al. 2001).

3.4. Glycans in tumour cell extravasation

Selectins are C-type calcium-dependent lectins (glycan-binding proteins) expressed by endothelial cells, leukocytes, and platelets. The three known selectins are *L-Selectin* (which is expressed on all leukocytes), *E-Selectin* (which is expressed by cytokine-activated endothelial cells) and *P-Selectin* (which is expressed constitutively in α-granules of platelets, in Weibel–Palade bodies of endothelial cells, and on the surface of activated platelets and endothelial cells).

The most important selectin carbohydrate ligands are the Lewis Type blood antigens SLe^x and SLe^a (see 2.4)(Varki et al. 2008). Unlike their normal cell counterparts, tumour cells frequently overexpress SLe^x and SLe^a on surface glycoproteins or glycosphingolipids (Kim et al. 1988; Varki et al. 2008). After entry into the vasculature, circulating tumour cells are exposed to shear forces and to the immune system attack. The overexpression of sialylated Lewis antigens allows tumour cells to form large aggregates with circulating platelets and leukocytes by way of Selectin–SLe^x/^{a} interaction. Those aggregates increase tumour cell survival in the bloodstream by avoiding tumour cells destruction by the shear forces or the immune system.

In addition, tumour cell-induced aggregation has been reported to facilitate haematogenous metastasis by increasing tumour cells arrest and extravasation in the microcirculation (Pearlstein et al. 1980; Tsuruo and Fujita 2008). Carbohydrate determinants SLe^x and SLe^a located in the tumour cell surface serve as ligands for the E-Selectin located on inflamed vascular endothelial cells (Figure 8). These interactions promote tethering (capture) and rolling of tumour cells on vascular surface. Rolling is a form of adhesion that requires rapid formation and dissociation of bonds between selectins and their ligands. Rolling adhesion enables tumour cells to encounter endothelium-bound chemokines. Signalling through chemokine receptors cooperates with signalling through selectin ligands to activate tumour cell integrins, which bind to immunoglobulin superfamily ligands on endothelial cells to slow rolling velocities and arrest tumour cells on vascular surfaces. Arrested tumour cells then are able to transmigrate across the vascular wall into the underlying tissues (Hosono et al. 1998; Izawa et al. 2000; Kannagi 1997; Lowe et al. 1990; Takada et al. 1993; Varki 2007)
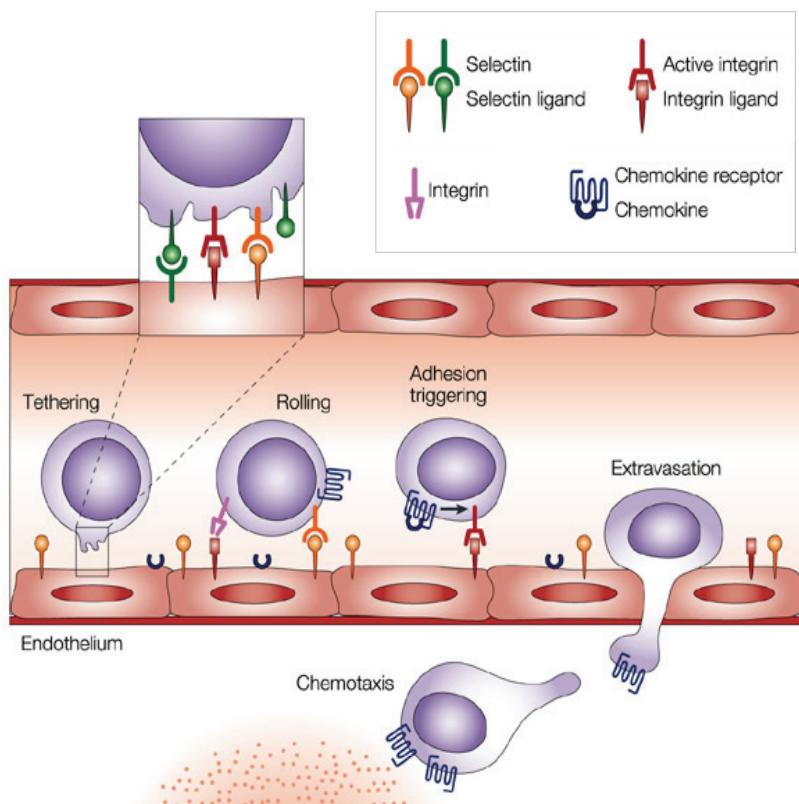
Figure 8. Tumour cell extravasation: *1) Capture or tethering*: First contact of a tumour cell with the activated endothelium. It allows cells to move close to the activated endothelium, away from the central blood stream *2) Rolling:* Once captured, tumour cells may transiently adhere to the venular endothelium and begin to roll. Interaction between S receptors on endothelial cell surfaces and their ligands (SLe^x and SLe^a) on tumour cell surfaces mediate this process *3) Adhesion:* Tumour cells firmly adhere to the endothelium *4) Transmigration or extravasation*: Tumour cells migrate across resting endothelium, generally by chemotaxis.

4. Pancreatic ductal adenocarcinoma

Cancer represents not a single disease but a group of heterogeneous diseases. Although different cancers share the common biological properties aforementioned, we must take into account that tumour progression and metastasis regulation are tumour-specific and organ-specific processes. The individual study of each tumour type and its

specific cellular and molecular surrounding (microenvironment) are required to improve the prevention and therapeutic control of cancer. Thus, we will now focus on the distinctive characteristics of the pancreatic ductal adenocarcinoma (PDAC).

PDAC is the most common neoplasia of exocrine pancreas thus most investigators use the terms pancreatic cancer and PDAC synonymously. It is one of the most aggressive human malignancies with the highest mortality/incidence ratio of all major cancers (Parkin et al. 2005). Due to the absence of specific symptoms and its exceptionally rapid progression (Bardeesy and DePinho 2002; Real 2003), it is typically detected late in the course of disease, and 93% of patients present metastasis at the time of diagnosis. As a result, PDAC patients have an extremely poor prognosis being the 5-year survival rate only 5% (Jemal et al. 2009).

4.1. Pathology

PDAC produces a firm, highly sclerotic mass with poorly defined edges, with long tongues of carcinoma extending beyond the main tumour. At light-microscopic level, it is composed of an infiltrating gland, forming a dense nonneoplastic stroma, with numerous inflammatory cells and extracellular matrix components admixed with the tumour cells. This nonneoplastic host response named desmoplasia is characteristic of PDAC and it is usually so intense that only a minority of the cells in the mass formed by pancreatic cancer are neoplastic cells. Pancreatic cancers are also extremely infiltrating neoplasms. Vascular and perineural invasion are present in the majority of surgically resected cancers, and metastases to regional lymph nodes, the liver, peritoneo and distant sites such as lungs are common (Maitra and Hruban 2008).

4.1.1. Altered glycosylation in PDAC

The majority of PDAC express a number of mucins including MUC1, MUC3, MUC4 and MUC5AC (Adsay et al. 2005) and immuno-histochemically detectable altered glycosylation such as the overexpression of SLe[x], Le[x] and related antigens (Hosono et al. 1998; Kim et al. 1988; Mas et al. 1998; Peracaula et al. 2005; Satomura et al. 1991; Sinn et al. 1992). This altered glycosylation could be used to develop new serologic markers and recently, several efforts using hightroughput techniques have addressed this issue, results are still preliminary however (An et al. 2009). Nowadays, CA19-9 (which is based on the carbohydrate structure SLe[a]) is the best available PDAC marker for following the disease progression. Unfortunately it has low predictive value for identifying patients with pancreatic cancer (Misek et al. 2007; Steinberg 1990). As it has shown before, the expression of tumour associated carbohydrate antigens has been correlated to tumour progression in several cancers, such as colorectal and gastric cancer (Amado et al. 1998; Nakamori et al. 1997). Therefore, altered glycosylation is also expected to give a selective advantage to the PDAC.

4.2. PDAC precursor lesions

PDAC is thought to arise from pancreatic ductal cells; however this still remains an area of ongoing study (Bardeesy and DePinho 2002). Traditionally, the ductal morphology of PDAC led to postulate that ductal cells were at the origin of transformation. Nevertheless, proofs were lacking because the pancreas is so infrequently biopsied that were no longitudinal studies showing disease progression. The strongest evidence of a ductal origin aroused from the characterization of the precursor lesions, so-named Pancreatic Intraepithelial Neoplasia (PanINs) (Maitra et al. 2006). PanINs are microscopic lesions in the

smaller pancreatic ducts, which can be papillary or flat, and they are composed of columnar to cubical cells with varying amounts of mucins (Maitra and Hruban 2008). PanINs are subclassified into *PanIN-1 lesions,* presenting hyperplasia without dysplasia; *PanIN-2 lesions*, which are variably dysplastic and *PanIN-3 lesions*, corresponding to carcinoma in situ (Real 2003). When the molecular genetic alterations defining PDAC were identified, almost all the genetic changes that characterised invasive pancreatic cancer were demonstrated in PanINs, concluding that PanINs gave rise to PDAC. Those data led to propose a progression model for PDAC in which low grade PanIN-1 progress to PaIN-2, which in turn progress to PaIN-3 and this to PDAC (Maitra et al. 2006). However, this linear progression model has been surprisingly difficult to prove (Hernandez-Munoz et al. 2008) and recently alternative models have been proposed (Real 2003) (Figure 9).
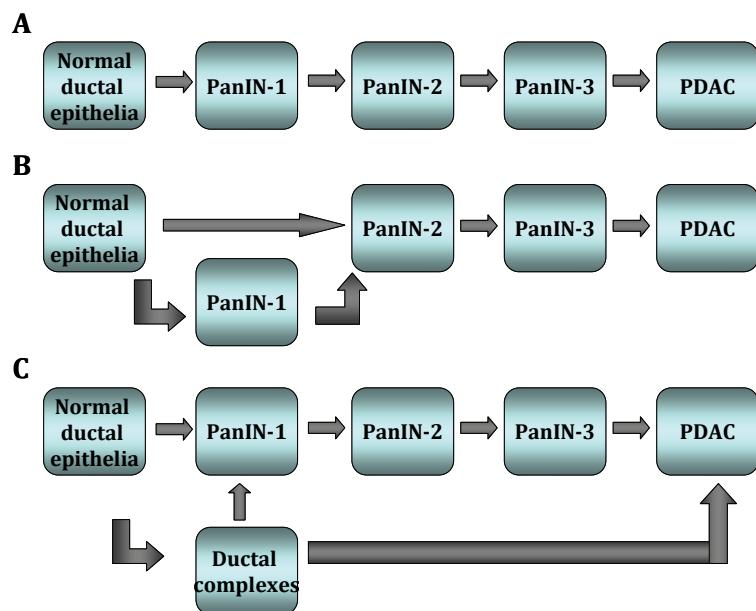
Figure 9. Models of PanIN progression to PDAC. A) The most commonly depicted linear model **B)** Alternative model proposing direct progression from normal ductal epithelium to PanIN-2 **C)** Alternative model proposing the appearance of ductal complexes as an intermediate state that may lead to PDAC. Extracted and modified from Real and Hernandez-Munoz (Hernandez-Munoz et al. 2008; Real 2003).

4.3. Molecular genetics of PDAC

A compendium of signature mutations defines PDAC and differentiates this malignancy from other neoplasms (Figure 10) (Bardeesy and DePinho 2002; Ghaneh et al. 2007; Maitra and Hruban 2008):
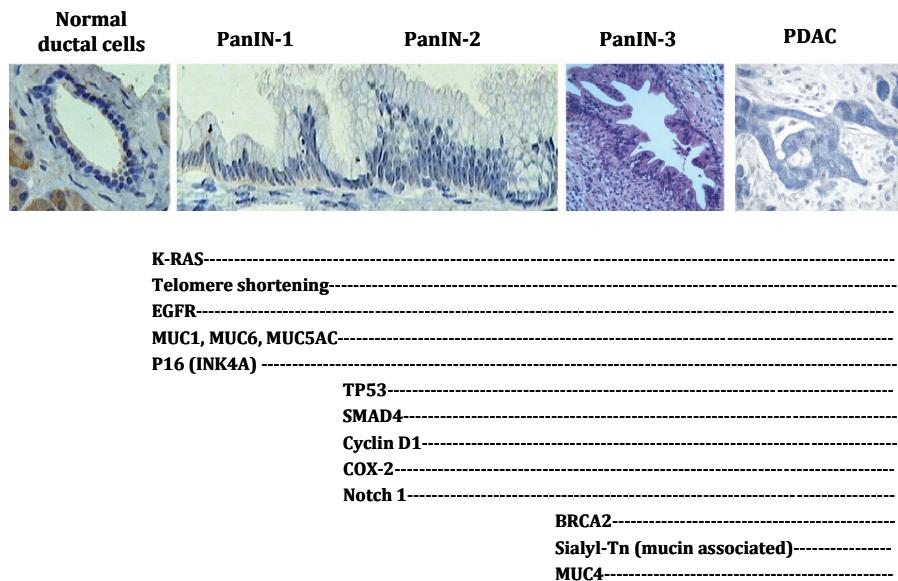
Figure 10. Genetic progression model of pancreatic adenocarcinoma. Histological images of bening pancreatic ductal epithelial cells, intraepithelial neoplasias (PanINs) and invasive carcinoma (PDAC). The genetic alterations documented in PDAC also occurred in PanIN in what seems a temporal sequence, although these alterations have not been correlated with the acquisition of specific histopathological features. Extracted and modified from (Bardeesy and DePinho 2002; Ghaneh et al. 2007).

4.3.1. Alterations in oncogenic molecular pathways

Activating mutations of the K-RAS oncogene is one of the most common genetic abnormalities observed in PDAC, being present in 95% of cases. Mutations of the K-RAS gene are one of the earliest genetic abnormalities observed in the progression model of pancreatic cancer and are demonstrable in 36% PanIN-1, 44% PanIN-2 and 87% of

PanIN-3. Another common feature in pancreatic cancer is the overexpression of the EGFR and their principal ligands EGF and TGF-α. The lipid kinase phosphoinositide 3-OH kinase (PI3K/Akt) pathway regulates cell survival, proliferation and resistance to apoptosis and it is activated in up to 60% of PDACs.

4.3.2. Alterations in tumour suppressor genes

The gene p16/CDKN2A also known as INK4A is the most commonly inactivated tumour suppressor gene in pancreatic cancer and loss of INK4A function is observed in approximately 90% of PDAC. Other common tumour suppressor gen mutations occur in TP53 gene (transcription factor p53) and Smad4 genes.

4.3.3. Reactivation of developmental signalling

The upregulation of a number of genes involved in the Notch pathway and Hedgehog signalling pathways occurs in pre-neoplastic lesions and PDAC, having important roles in PDAC initiation and invasion.

4.3.4. Altered mucin glycosilation

Overexpression of MUC1 (epithelial mucin), MUC6 (pyloric-gland mucin) and *de novo* expression of MUC5AC (gastric foveolar mucin) are observed in all stages of PanINs and invasive ductal adenocarcinoma. In contrast, the expression of mucin-associated carbohydrate antigen sialyl-Tn is markedly increased only in PanlN-3 and invasive ductal adenocarcinoma (Itzkowitz et al. 1991; Kim et al. 2002).

4.3.5. Other common genetic alterations

Telomere shortening is one of the earliest demonstrable genetic aberrations in pancreatic cancer, with >90% of even PanIN-1 lesions

showing marked shortening of telomeres. Moreover, epigenetic silencing is frequently observed in PDAC and tends to involve genes that function in tumour suppression and critical homeostatic pathways (Fukushima et al. 2002; Jansen et al. 2002; Sato et al. 2006; Sato and Goggins 2006). Finally, several reports of altered MicroRNAs expression have been reported for PDAC (Hampton 2007; Szafranska et al. 2007; Volinia et al. 2006).

In conclusion, pre-neoplastic lesions contain many of the genetic changes that characterize PDAC (Bardeesy and DePinho 2002; Maitra et al. 2006), which could partially explain why early/small tumours progress so rapidly and are associated with a disproportionate rate of metastasis. This behaviour, however, not solely corresponds to structural genetic alterations but to the accumulation of a number of genetic and epigenetic changes and to the activation of a coordinated transcriptional program (Real 2003), which may be regulated in part by the PDAC microenvironment (Farrow et al. 2008; Ghaneh et al. 2007).

4.4. Tumour microenvironment in PDAC

In considering the biology of any cancer, the interplay between cancer cells and the surrounding supporting host cells (known as tumour stroma) plays a key role in tumour initiation, blood vessel formation, invasion, metastasis and evasion of the host immune system. One of the most important characteristics of PDAC is a particularly intense desmoplastic stroma, which is not observed in other neoplasias (Ghaneh et al. 2007; Mahadevan and Von Hoff 2007). PanIN-1 and PanIN-2 lesions are associated with small amounts of normal stroma surrounding the normal pancreatic ducts from which the PanINs arise. With PanIN-3 lesions, the enhancement of stroma formation begins.

Progression to PDAC is often associated with an evident increase in stroma, that ultimately results in extensive stroma associated to an inflammatory infiltrate (Korc 2007). This data suggests an active role played by ductal adenocarcinoma cells in influencing modifications in the surrounding host tissue elements, in order to create a favourable microenvironment for their growth and spreading.

4.4.1. The stromal structure in PDAC

The stroma in PDAC is a complex structure (Figure 11). It consists of proliferating fibroblasts and pancreatic stellate cells (PSC) that produce and deposit fibronectin and collagens I and III (Bachem et al. 2005).
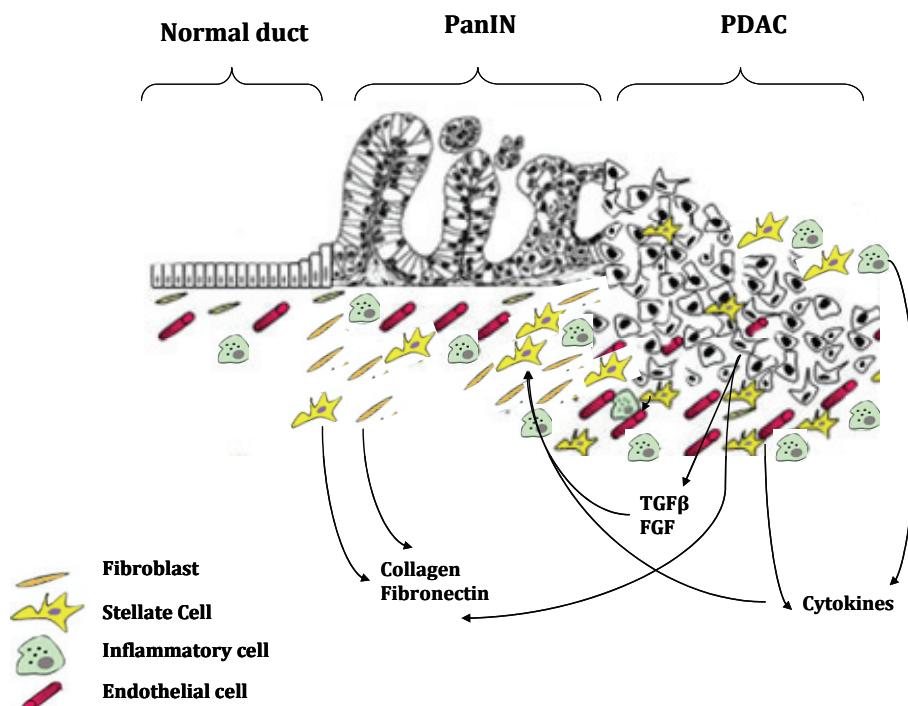
Figure 11. Progression model of pancreatic cancer that taking into account the influence of microenvironment on tumour cells. **TGF-β**: Transforming growth factor beta **FGF**: Fibroblast Growth Factor. Extracted and modified from (Kleeff et al. 2007).

Cancer cells are capable of synthesizing and releasing collagens (type I and III) and growth factors (such as FGF or TGF-β). In addition, the matrix contains aberrant endothelial cells, foci of inflammatory cells and macrophages that produce cytokines, many of which are mitogenic towards both fibroblasts and stellate cells. The stroma also contains nerve fibers that release nerve growth factors (NGFs), and bone marrow derived stem cells that may have the capacity to differentiate into PSC and fibroblasts. The result is a unique microenvironment in which the PDAC cells can prosper, and from which they can promptly metastasize (Korc 2007).

4.4.2. The role of the stroma in PDAC pathogenesis

The notion that the stroma can influence PDAC genesis is now widely accepted (Hernandez-Munoz et al. 2008). Chronic inflammatory conditions induce stromal activation and have been frequently associated with cancer risk in numerous tissues. This also holds true for PDAC, where hereditary and chronic pancreatitis produce pancreatic inflammation associated with a 53 times increased risk of developing PDAC (Whitcomb et al. 1999). Pancreatitis provides an inflammatory environment associated with ROS production, cytokine release (TNF-α, IL-6, IL-8 and interferon-α) and upregulation of pro-inflammatory transcription factors such as NF-κB. Those inflammatory response mediators can induce malignant transformation and eventually, the accumulative effect of the inflammatory response could result in PDAC (Farrow and Evers 2002; Hernandez-Munoz et al. 2008).

4.4.3. The role of the stroma in PDAC progression

Besides its role in PDAC genesis, stroma is also implicated in promoting PDAC. Similarly to pancreatic inflammation, in early PanINs fibroblasts

proliferate in the surrounding stroma. In addition, new blood vessels are generated, which allow inflammatory cells to infiltrate the tissue. Those inflammatory immune cells express high levels of profibrotic and proangiogenic factors. This way, PSC and fibroblast can proliferate and contribute to tumour desmoplasia with an extensive deposition of extracellular matrix components (Hernandez-Munoz et al. 2008).

Several types of tumour-stroma interactions have been described as having the potential to promote PDAC invasion and metastasis. Tumour cells produce growth factors (FGF, TGF-β, IGF-1, PDGF-BB) which become sequestered within the stroma. When invading cancer cells produce matrix metalloproteinases these growth factors are released and promote cancer cell proliferation. Moreover, there is an altered gene expression profile in the cancer-associated stroma, including altered integrin expression that may act to promote cancer cell motility, increased expression of cyclooxygenase-2, vascular endothelial factor (VEGF) and collagen I that enhance stromal neovascularisation and promote cancer cell growth (Korc 2007; Langley and Fidler 2007).

In summary, along with the changes observed in cancer cells during the tumorigenic process there is also an altered cancer-associated stroma. Those aberrant tumour-stroma interactions may promote cancer cell proliferation and invasiveness, enhancing tumour spread.

Hypothesis and Objectives

1. Working Hypothesis

Glycans are particularly important for mediating key events during tumour progression in many cancers, such as proliferation, angiogenesis, cell detachment, invasion and extravasation. Focusing on PDAC, one of the features that has an important *in vivo* role is the overexpression of sialyl-Lewis antigens.

Since sialyltransferase activity can be correlated to sialyl-Lewis antigen expression on pancreatic cancer cell surface, sialyltransfesases such as ST3Gal III and ST3Gal IV are expected to influence the biosynthesis of

sialylated Lewis antigens and to be involved in key steps of the tumourigenic process.

The mechanisms that may regulate pancreatic ductal adenocarcinoma (PDAC) aberrant glycosylation are still not known, but it is tempting to postulate that the specific surrounding cellular and molecular elements of PDAC, such as the high inflammatory environment, could be implicated.

2. Objectives

The general objective of this work is to study how altered glycosylation is involved in pancreatic adenocarcinoma tumour progression. In order to do so, this general objective has been concretized in three specific objectives that are as follows:

1. To investigate the specific role of beta-galactoside alpha-2,3-sialyltransferase 3 (ST3Gal III) and beta-galactoside alpha-2,3-sialyltransferase 4 (ST3Gal IV) in the adhesive, migratory and metastatic capabilities of pancreatic adenocarcinoma cells.

2. To study whether cell proliferation and differentiation and some specific molecules produced along these processes, such as ROS, could act as possible regulatory mechanisms for the alpha-2,3-sialyltransferases ST3Gal III and ST3Gal IV.

3. To investigate additional prometastatic effects of E-Selectin such as VEGF secretion and migration in pancreatic adenocarcinoma cells.

Materials and Methods

Ethics statement

All procedures working with animals were performed according to protocols approved by the Basque Country University Ethics Commission on Research and Education in compliance with the national and international laws and policies.

1. Cell culture

A detailed description of the cell culture techniques is provided in order to stably transfect, routinely culture or culture for further use in cell based experiments the human pancreatic adenocarcinoma cell lines used in this study. The techniques used to isolate and primary culture

the murine hepatic sinusoidal endothelial cells used in this work are also described.

1.1. Human pancreatic adenocarcinoma cell lines

The human pancreatic adenocarcinoma cell lines used in this study were Capan-1 and MDAPanc-28. Capan-1 (ATCC nºHTB-79, Rockville, MD) is a well-differentiated cell line from a pancreatic adenocarcinoma liver metastasis. This cell line has medium levels of endogenous ST3Gal III and SLe^x and spontaneously differentiates on becoming confluent (Fanjul et al. 1991; Hollande et al. 1990; Levrat et al. 1988). MDAPanc-28, a generous gift from Dr. Frazier from M.D. Anderson Cancer Center (Houston), is a poor-differentiated cell line from a pancreatic adenocarcinoma of the body of the pancreas presenting local invasion. This cell line has very low levels of endogenous ST3Gal III and SLe^x and also spontaneously differentiates on becoming confluent (Frazier et al. 1996; Peracaula et al. 2005).

1.2. Stable transfection

Capan-1 and MDAPanc-28 cells stably transfected with the pcDNA 3.1 expression vector encoding the ratST3Gal III gene, the pcDNA 3.1 expression vector encoding the human ST3Gal IV gene and the empty pcDNA™ 3.1. expression vector were used (Pagès-Pons 2006).

1.3. Routine culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM)_GlutaMAX-I containing 10% Fetal Bovine Serum, 100 U/mL Penicillin G, 100 μg/mL Streptomycin and 0.25 μg/mL Amphotericin B

(all of them from Gibco, Paisley, UK) and kept at 37ºC in humidified atmosphere containing 5% CO_2. Stable transfectants were supplemented with 400 µg/mL (Capan-1) or 800 µg/mL (MDAPanc-28) Geneticine® G-418 (Gibco, Paisley, UK). Cell growth and morphology were daily assessed under the field microscope. For routine culture, 3.5 x 10^5 Capan-1 parental and transfectant cells and 5.5 x 10^5 MDAPanc-28 parental and transfectant cells were seeded in 75 cm² flasks (Nunc; Roskilde, Denmark) and cultured for 84h. Cells were maintained for up to 8 passages by successive trypsinization and seeding. After 8 passages new cells were expanded from the frozen stock. Cell viability was assessed at each trypsinization by trypan blue staining (only cultures displaying > 90% viability were used for further work). Possible *Mycoplasma* contamination was routinely checked using the Venor®GeM *Mycoplasma* Dection Kit (Minerva Biolab GmnH Germany).

1.4. Cell culture and cell treatments in the experiments

In the experiments with the transfectant cells, cells were seeded and cultured as previously described (see 1.3), after trypsinization viable cells were physically counted (Ng et al. 2005). Then, cells were collected for mRNA extraction and posterior gene expression analysis, flow cytometry analysis, binding assays or *in vivo* assays.

In cell confluence assays, 3.5 x 10^5 Capan-1 or 5.5 x 10^5 MDAPanc-28 parental cells were seeded in 75 cm² flasks (Nunc; Roskilde, Denmark). At 24, 48, 72, 96, 120, 144 and 168 h in culture morphology was recorded, trypsinized and viable cells were physically counted (Ng et al. 2005). Then, cells were collected for mRNA extraction and posterior gene expression analysis or flow cytometry analysis. Supernatants were collected, centrifuged at 4 ºC 30 min 13000 rpm and kept a -20 ºC.

In H_2O_2 treatment assays, 2.25 x 10^4 Capan-1 parental cells or 3.35 x 10^4 MDAPanc-28 parental cells were seeded in six-well plates (Nunc; Roskilde, Denmark) and cultured for 72h or 96 h. Cells were treated for 24 h with 50 μM H_2O_2 (Panreac, BCN, Spain) and collected for mRNA extraction and posterior gene expression analysis or flow cytometry analysis. Supernatants were collected, centrifuged at 4ºC 30 min 13000 rpm and kept a -20 ºC. In certain assays, cells were pre-treated overnight with 200 μM *N*-Acetylcysteine (NAc).

In the sE-Selectin treatment assays, 2.25 x 10^4 Capan-1 parental and transfectants cells were seeded in six-well plates (Nunc; Roskilde, Denmark) and cultured for 72h. Recombinant human soluble E-Selectin (sE-Selectin) from R&D systems (Minneapolis, MN) was diluted to a final concentration of 150 ng/mL or 300 ng/mL in basal medium and applied to the cell cultures for 16 h. Supernatants were collected, centrifuged at 4 ºC 30 min 13000 rpm and kept a -20ºC. In some experiments, after supernatant collection new basal medium was added to the cells and a new supernatant was collected 24 h later. In other experiments, cells were incubated with MAb KM93 (anti-SLe^x) (Calbiochem, EMD Chemicals, CA) 30 min prior to sE-Selectin challenge.

1.5. Isolation and primary culture of Hepatic Sinusoidal Endothelial cells

Syngenic Balb/c mice (male, 6–8 weeks old) were obtained from Harlan Iberica (Barcelona, Spain). HSE cells were separated from these mice, identified, and cultured (Vidal-Vanaclocha et al. 1993). Briefly, hepatic tissue digestion was performed by sequential perfusion of pronase, collagenase, and DNase. Sinusoidal cells were separated in a 17.5% (wt/vol) metrizamide gradient and incubated in glutaraldehyde-treated

human albumin-coated dishes for 30 min, as a selective adherence step for Kupffer cell depletion. Non-adherent sinusoidal cells were re-plated on Type I collagen-coated 24-well plates, at 1×10^6 cells/mL/well, and 2h later were washed. HSE cell purity of resulting adherent sinusoidal cells was around 95% as checked by previously used identification parameters: positive endocytosis (acetylated low density lipoprotein, ovalbumin); negative phagocytosis (1 μm latex particles) and CD45 antigen expression; positive lectin binding-site expression (wheat germ and viscum album agglutinins); and negative vitamin A storage (revealed by 328 nm of UV fluorescence). Cultures of HSE cells were established and maintained until adhesion experimets were performed (24 hours) in pyrogen-free RPMI (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO) at 37°C in a humidified atmosphere with 5% CO_2.

2. Molecular biology

The following molecular biology techniques were used for total RNA isolation, single-stranded cDNA synthesis, and cDNA amplification by semi-quantitative PCR and quantitative PCR (qPCR).

2.1. Total RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy® RNA isolation kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol, including on-column DNase digestion using the RNAse-Free DNase Set (Quiagen GmbH, Hilden, Germany). RNA yield and purity were spectrophotometrically determinate using a Nanodrop (ND-1000,

Thermo Scientific, Wilmington, DE). Single-stranded cDNA was synthesized from 2.0 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA) according to the manufacturer's instructions.

2.2. Semi-quantitative PCR

ST3Gal III and ST3Gal IV expression were examined by semi-quantitative PCR using the β-actin expression as internal reference. PCR were performed using 1.3 μL of cDNA, 2 mM MgCl$_2$, 50 μM each dNTP, 18 $p$mol each forward (Fw) and reverse (Rv) oligonucleotides, and 1 unit μL^{-1} Biotools DNA polymerase (Biotools B&M Labs, Madrid, Spain) in 20 μL reactions. ST3 Gal III primers to specifically amplify the rat ST3Gal III cloned gene were designed with Primer3 software (Rozen and Skaletsky 2000). Primer sequences were 5'-CTGCATGGCTGTGATGAAGT-3'(Fw) and 5'-CAACAGATGGCTGGCAACTA-3' (Rv). PCR product size was 272 bp and thermal cycling parameters were as follows: 1 cycle at 90°C for 2 min, 35 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and 30 s, 1 cycle at 72 ºC for 10 min; using a MyCycler™ thermocycler (Bio-Rad Labs Hercules, CA). ST3 Gal III, ST3Gal IV and β-actin primers and thermal cycling conditions were previously described by (Mas et al. 1998; Peracaula et al. 2005) and the sizes of the PCR products obtained were 485 bp and 838 bp respectively. 15μL aliquots of amplified cDNAs were run in 1.5% agarose gels, stained with 0.5 μg/mL ethidium bromide, and visualized under UV-light. The intensity of the amplified cDNA bands of ST3Gal III and ST3Gal IV were measured using the *Quantity-One* software package (Bio-Rad Labs Hercules, CA) and normalized to the housekeeping gene (β-actin) for each cell line.

2.3. Quantitative PCR

Primers and probes sequences for the TATA box binding protein (TBP) (reference Hs99999910_m1) and ST3Gal IV (reference Hs00920871_m1) were Taqman Pre-designed Gene Expression Assays™, whereas primers and probes sequences for β-actin and ST3Gal III were Custom Taqman Gene Expression Assays™, all of them from Applied Biosystems-Applera Hispania SA, Spain. ST3Gal III primers and probe were specifically designed along highly conserved regions of both rat and human ST3Gal III transcripts (positions 318 or 330). All PCRs were performed in optical 96-well plates with an ABI PRISM 7300 Sequence Detector System in a total volume of 20 μL containing 9 μL of cDNA diluted in RNAse free water, 10 μL of TaqMan® Universal Master Mix, No AmpErase® and 1 μl of the corresponding Custom Taqman Gene Expression Assay™. The following standard thermal profile was used for all PCRs: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

All data were processed and analyzed with 7300 SDS 1.3.1 software (Applied Biosystems). For each sample dilution, logarithmic increase in fluorescence signal (ΔRn) was obtained. ΔRn threshold was set at 0.02 to obtain the corresponding Ct (threshold cycle) values. The relative concentrations of the target and the reference gene were calculated by interpolation in the corresponding standard curve. To estimate the intra-assay variability, six technical replicates were performed for each sample and gene. To estimate the inter-assay variation, PCR assays were performed in triplicate. Results were expressed as mean ± SD values of the relative ST3Gal III or ST3Gal IV transcript abundance normalized with corresponding endogenous housekeeping genes β-actin or TBP.

3. Cell based assays

Flow cytometry assays, cell adhesion assays, transwell migration assays and evaluation of cells VEGF secretion to the culture medium were the cell based assays used in this work.

3.1. Flow cytometry

Flow cytometry was used to quantify glycan structures on pancreatic adenocarcinoma cell surface, to quantify cells intracellular Reactive Oxygen Species (ROS) and intracellular glutathione (GSH) content.

3.1.1. Glycan structures by flow cytometry

Monoclonal antibodies (MAb) T-218 (anti-Lewis b) and T174 (anti-Lewis a) (Sakamoto et al. 1986) were used at 1/2 dilution hybridoma supernatant; 19-OLE (anti -H type 2), anti-H type1 and DUPAN-2 (Rouger et al. 1987) were used diluted at 1/1000. MAb KM93 (anti-SLe^x) was used at 1/40 dilution and MAb P12 (anti-Le^x), MAb KM231 (anti- SLe^a), MAb F3 (anti-Le^y) (all from Calbiochem, USA), and Biotinilated *Sambucus Nigra* (Elderberry) Bark Lectin (Vector Laboratories, USA) were used diluted at 1/20. Finally, Biotinilated *Maackia amurensis* (Vector Laboratories, USA) was used diluted 1/400. Detection of oligosaccharide epitopes on the surface of exponential growing cells was perfomed by indirect fluorescence. 5 x 10^5 viable cells were incubated with the antibodies or the lectins and after a wash; cells were incubated with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG or Streptavidin Alexa Fluor 488 (Invitrogen Life Technologies, Frederick, MD). Fluorescent analyses were carried out using a FACSCalibur (BD Biosciences). For each sample three independent assays were undertaken.

3.1.2. Intracellular Rective Oxygen Species by flow cytometry

Intracellular Reactive Oxygen Species (ROS) levels were measured by flow cytometry based on a previously described method (Anasagasti et al. 1996). Cells were incubated at dark for 30 min at 37 ºC with 10 µM of the redox-sensitive dye CM-H$_2$DCFH-DA (Molecular Probes, USA). Fluorescence intensity, indicative of ROS content, was determined by excitation at 490 nm and emission at 520 nm using a FACSCalibur (BD Biosciences, USA). Corrections for autofluorescence were made by measuring fluorescence in unstained cells. For each sample three independent assays were undertaken.

3.1.3. Intracellular Glutathione levels by flow cytometry

Monochlorobimane (Sigma-Aldrich, MO, USA) was added to the cell suspension to a final concentration of 40 µM and cells were maintained in the dark at 37 ºC for 30 min. After a wash, intracellular GSH content was measured by excitation at 350 nm an emission at 460 nm using a BD™ LSR Flow Cytometer (BD Biosciences, USA). Corrections for autofluorescence were made measuring fluorescence in unstained cells. For each sample at least two independent assays were undertaken.

3.2. Cell adhesion assays

Cell adhesion assays were performed to study the capabilities of pancreatic adenocarcinoma cells to bind to rh-E-Selectin and to fresh primary cultured Hepatic Sinusoidal Endothelial (HSE) cells.

3.2.1. Tumour cell adhesion assays to rh- E-Selectin

Adhesion of pancreatic adenocarcinoma cells to rh-E-Selectin was performed based on a previously described method (Mejias-Luque et al.

2007). 96-well microplates were coated with rh-E-Selectin (R & D Systems, Minneapolis, USA) or 1% BSA. Plates were blocked with PBS-1% BSA for 1 h. At each well 5 x 10^4 viable Capan-1 parental or transfectant cells or 1 x 10^5 MDAPanc-28 parental or transfectant cells were added and the plate was incubated at room temperature for 1 h. In selected experiments, cells were previously incubated with specific antibodies for 30 min at 4 ºC. After two washes, adherent cells were estimated by adding 0.5 mg/mL Thiazolyl Blue (Sigma-Aldrich, St. Louis, MO) for 2 h. Formazan crystals were solubilized with Dimethyl Sulfoxide and optical density was measured at 570 nm. All the experiments were carried out in quintuplicate, and three independent assays were undertaken. Results were expressed as the mean ± SD values of specific binding to E-Selectin (O.D. 570 nm of cells bonded to E-Selectin – O.D. 570 nm of cells bonded to PBS-1% BSA).

3.2.2. Tumour cell adhesion assay to HSE cells

Adhesion assays were performed using a quantitative method based on a previously described fluorescence measurement system (Vidal-Vanaclocha et al. 1994). Freshly isolated HSE cells were incubated for 16 hours, with 10 ng/mL recombinant Interleukin IL1-β, 10 ng/mL recombinant TNF-α or 10 ng/mL LPS before addition of cancer cells. 2 μg/ mL anti-murine CD62 (E-Selectin) MAb (Acris Antibodies GmbH, Herford, Germany) was added to HSE cells 30 min before tumour cell addition. Anti-murine IgG antibody was added at a similar concentration and time to check the specificity of the anti-murine E-Selectin antibody. Exponentially growing Capan-1 parental or transfectant cells were trypsinized and resuspended in 20 μg Calcein, AM (Invitrogen Life Technologies, Frederick, MD) DMEM solution. After washing, cells were resuspended in HEPES-buffered DMEM without phenol red at a concentration of 2 x 10^6 cells per millilitre. HSE cells

were washed and basal autofluorescence was determined using a CytoFluor-2350 system (Millipor Co., Bedford, MA).

Labelled Capan-1 parental or transfectant cells (0.1 mL per well) were added to 24-well-plate cultured HSE cells or to collagen pre-coated control wells. To determine the fluorescence of the added number of cells in each well, a second determination was performed on the CytoFluor system. The plates were then incubated at 37ºC, and 15 min later, wells were washed three times with fresh medium and read for a third time for fluorescence. Each experiment was performed in triplicate wells and three independents assays were undertaken. The number of adhering cells was quantified in arbitrary fluorescence units based on the percentage of the initial number of cancer cells added to the endothelia and results were expressed as the mean ± SD values of % Specific adhesion to HSE cells (Vidal-Vanaclocha et al. 1994).

3.3. Transswell *in vitro* migration assays

Cell migration was determined using modified Boyden chambers (Olaso et al. 2003). Cells were detached and resuspended in serum-free medium, 1×10^4 Capan-1 parental or Capan-1 transfectant cells or 2.5×10^4 MDAPanc-28 parental or MDA-Panc-28 transfectant cells were seeded onto Type I-Collagen coated inserts with 8 μm-pores and placed on top of 2 cm^2 wells (Greiner Bio-One GmbH, Kremsmünster, Austria) containing 300 μL DMEM or DMEM plus 1% FBS or DMEM plus 0.3 μg/mL sE-Selectin.

After incubation at 37ºC, 6 h for Capan-1 cells and 18 h for MDAPanc-28, non-migrated cells on the upper surface of the filter were carefully and thoroughly wiped from the top surface of the filter and the

migrated cells were fixed, stained with H&E and counted in × 40 high-power light microscopy. Results were expressed as the average number of migrated cells per well obtained from three separate experiments done in triplicate.

3.4. Measurement of VEGF in culture medium

Vascular endothelial growth factor (VEGF) secretion levels in culture medium were measured using an ELISA kit based on specific human VEGF monoclonal antibody (R&D Systems, Minneapolis, MN) following manufacturer instructions. Individual cell counts were made using a haemocytometer, and the amount of secreted VEGF protein was calculated in units of pg per 10^6 pancreatic carcinoma cells. Values are expressed as mean values ± SD obtained from three separate experiments done in triplicate.

4. *In vivo* metastasis assays in athymic nude mice

In vivo assays with athymic mice were performed to establish an experimental metastasis model for the pancreatic adenocarcinoma cell lines.

4.1. *In vivo* metastasis assay conditions assessment

Athymic Nude-Fox n1 nu/nu mice (male, 6-8 weeks old) weighing 21.9-24.8 g were obtained from Charles River (Barcelona, Spain). *In vivo* assay optimal conditions were assessed by intrasplenic injection into anesthetized mice (50 mg/kg pentobarbital i.p) of exponentially growing (84 h in culture) $1x10^6$, $1,5x10^6$, $3x10^6$ and $5x10^6$ Capan-1 viable cells and $7x10^6$ MDAPanc-28 viable cells suspended in 0.1 mL

Hanks'Balanced solution (Gibco, UK). A small left abdominal flank incision was created, spleen was exteriorized and then 0.1 mL of tumour cell suspension was injected into the spleen with a 30-gauge needle. The spleen was returned to the abdomen, and the wound was closed. Mice were daily examined for survival and sacrificed when looked sick or when the presence/absence of internals sings of malignancy needed to be studied.

4.2. *In vivo* metastasis assay for MDAPanc-28 model

MDAPanc-28 model *in vivo* metastasis assay was performed by intrasplenic injection of exponentially growing (84 h in culture) $7x10^6$ MP, M34 and M42 viable cells into anesthetized mice (n= 8 per group) as described in section 4.3. Mice were daily examined for survival and sacrificed when looked sick or when the presence/absence of internals sings of malignancy needed to be studied. Healthy mice from three experimental groups were also sacrificed to check for internal signs of malignancy and censored in the Kaplan-Meier survival analysis. Animals were necropsized and macroscopical analysis was performed by registering the incidence of tumour lesions in spleen, liver, pancreas, lung, heart, lymph nodes, kidneys, ganglia, suprarenal gland, and other peritoneal organs. At different time points, tumour-bearing mice were sacrificed and spleen, pancreas, right kidney, left kidney, adrenal gland and lymph nodes were collected for microscopic analyses. The organs were washed with an isotonic solution and fixed by immersion in formaldehide-Zinc solution for 24 h at room temperature, paraffin-embedded and 10 μm-thick tissue sections from each organ were stained with haematoxylin-eosin (H&E) to detect the presence of metastatic foci.

5. Statistical Analysis

Data (x) were expressed as means ± standard deviation (SD). Statistical analyses were performed using SigmaStat 3.5 for Windows (Systat Sofware, Inc., San José, CA) and SPSS statistical software for Windows (version 15.0; SPSS Inc., Chicago, IL). Normality of data (x) was tested using the Kolmogorov-Smirnov test and the homogeneity of variances was checked using the Levene's test. Data with normal distribution and homogeneous variances were analyzed with Student's t test, one-way or three-way ANOVA using Tukey's test for multiple comparisons. For heterocedastic data, ANOVA on ranks (Kruskal-Wallis test) was run using Dunn's method for pairwise multiple comparisons. On the other hand, time to survival data in experimental metastasis assay were analyzed by Kaplan-Meier method and compared by the long-rank test. The criterion for significance was set at *P<0.01*.

Results

1. Role of ST3Gal III and ST3Gal IV in PDAC progression

The specific role of beta-galactoside alpha-2,3-sialyltransferase 3 (ST3Gal III) and beta-galactoside alpha-2,3-sialyltransferase 4 (ST3Gal IV) in the biosynthesis of sialylated Lewis antigens and its implications in key steps of pancreatic adenocarcinoma cells tumourigenic process were studied.

First, the role of ST3Gal III was studied. Second, the role of ST3Gal IV was studied. Third, comparisons between ST3Gal III and ST3Gal IV were established.

1.1. Role of ST3Gal III in PDAC progression

The role of ST3Gal III on cell surface Lewis antigen expression and its effects in the adhesive, migratory and metastatic capabilities for the pancreatic adenocarcinoma cell lines Capan-1 and MDAPanc-28 was investigated (Perez-Garay et al. 2010).

1.1.1. Stable overexpression of ST3Gal III in Capan-1 and MDAPanc-28 cells

To explore the mechanistic role of ST3Gal III, the rat ST3Gal III gene, exhibiting virtually identical acceptor specificity and enzymatic activity as human ST3Gal III gene (Kitagawa and Paulson 1993), was used. Capan-1 and MDAPanc-28 cells were transfected with the pcDNA 3.1 vector encoding rat ST3Gal III gene. Parental cells were concomitantly transfected with the empty pcDNA3.1 vector.

Several stable cell clones were selected in the presence of geneticine and ST3Gal III mRNA overexpression was analysed by semi-quantitative PCR. ST3Gal III mRNA overexpressing clones, C31 and C32 (for the Capan-1 model) and M33 and M34 (for the MDAPanc-28 model) were selected for further studies. As controls, parental cell lines (Capan-1 and MDAPanc-28) and mock transfected clones (CP for Capan-1 and MP for MDAPanc-28) were used. ST3Gal III mRNA expression was quantified by real-time PCR (qPCR) (Figure 12).

Capan-1 parental cells had 3-fold higher ($P<0.001$) ST3Gal III expression than MDAPanc-28 parental cells. Regarding the Capan-1 model, ST3Gal III mRNA expression was 140-fold higher ($P<0.001$) in C31 clone and 100-fold higher ($P <0.001$) in C32 clone than in Capan-1 and CP control cells. For the MDAPanc-28 model, ST3Gal III mRNA

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expression was 7-fold higher ($P < 0.001$) in M34 clone and 3.6-fold higher ($P <0.001$) in M33 clone than in both control cells, MDAPanc-28 and MP.
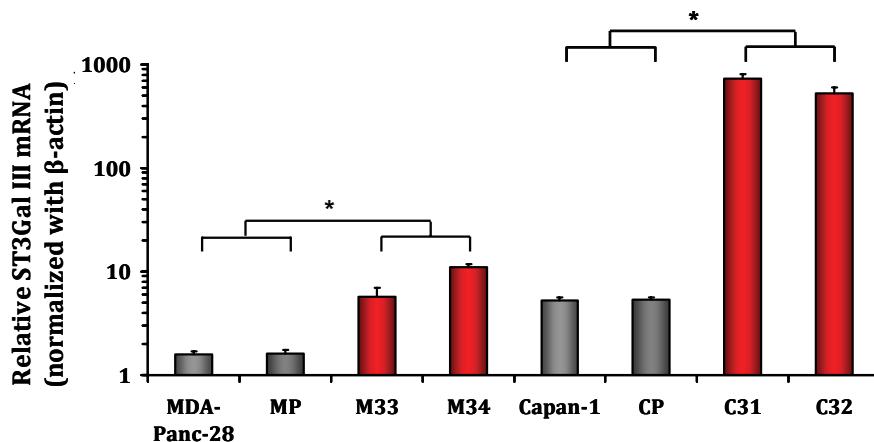
Figure 12: ST3Gal III expression normalized to β-actin for the pancreatic adenocarcinoma cells studied. MDAPanc-28 parental cells, MP: mock cells, M34 and M33: cells transfected with the ST3Gal III gene. Capan-1: parental cells, CP: mock cells, C31 and C32: cells transfected with the ST3Gal III gene. Data represents the mean ± SD of 3 separate experiments, in six replicates (n=18). * Significantly different ($P < 0.001$).

1.1.2. ST3Gal III increased SLe^x surface levels by enzymatic competition

The Lewis antigens expression pattern for both models was studied by flow cytometry (Figure 13). The analysis of Type II Lewis antigens in the Capan-1 model revealed that Capan-1 and CP cells had high-medium levels of the Le^x, SLe^x and H2 antigens and high levels of the Le^y antigen. When compared to controls, C31 and C32 clones displayed a large increase in SLe^x expression at the expense of completely losing the expression of non-sialylated antigens (Le^x, H2 and Le^y). Moreover, accompanying the increase in SLe^x, a decrease in α2-6 sialic acid was observed, detected by *Sambucus nigra* agglutinin (SNA). Maackia amurensis agglutinin (MAA) did not show differences among clones,

probably due to the fact that is unable to bind to SLe^x estructure. Type I Lewis antigens SLe^a, Le^a, Le^b and H1 were not detected in the Capan-1 model (data not shown). Thus, these results evidenced a multiple enzymatic competition for Type II chains.
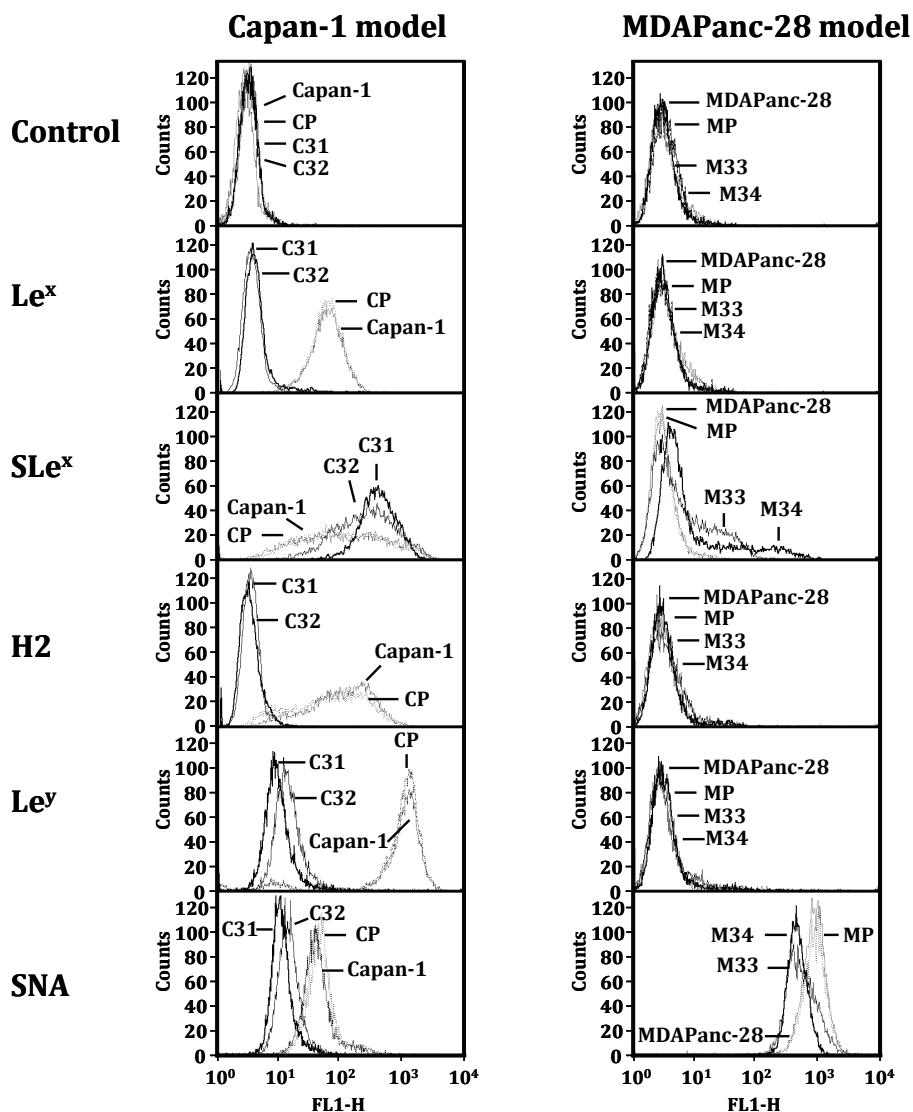
Figure 13: Flow cytometry profiles of the cell surface glycan structures for the pancreatic adenocarcinoma cells. **Capan-1 model:** Capan-1 (dot outline), CP (dot outline), C31 (bold outline), C32 (plain outline). **MDAPanc-28 model:** MDAPanc-28 (dot outline), MP (dot outline), M34 (bold outline), M33 (plain outline). Experiments were performed in triplicate. Representative flow cytometry histograms are shown.

The analysis of MDAPanc-28 model showed that MDAPanc-28 and MP control cells had very low SLe^x expression and high α2-6 sialic acid expression. They also lacked of Type I Lewis antigens. When M33 and M34 clones were compared to controls an increase in surface SLe^x levels with a simultaneous decrease in α2-6 sialic acid was detected. As for Capan-1 model, MAA did not show differences between MDAPanc-28 clones., wich is explainable since MAA does not recognize SLe^x. Since ST3Gal III transfected clones C31 and C32 behaved similarly in the Capan-1 model as well as the M33 and M34 ST3Gal III transfected clones in the MDAPanc-28 model, the following *in vitro* and *in vivo* studies were performed only with C31 for the Capan-1 model and M34 for the MDAPanc-28 model.

1.1.3. ST3Gal III enhanced pancreatic adenocarcinoma cells adhesion to rh-E-Selectin

E-Selectin is a cell adhesion molecule expressed on activated endothelial cells that recognizes and binds to specific carbohydrate determinants, such as SLe^x and SLe^a, present on surface glycoconjugates (Barthel et al. 2007). Binding assays to rh-E-Selectin were performed to analyse whether the different pattern of the Lewis antigen expression was able to induce changes in the adhesion. Both models showed different adhesion patterns to rh-E-Selectin (Figure 14). MDAPanc-28 parental cells displayed lower adhesion to rh-E-Selectin than Capan-1 parental cells, which is consistent with the lower expression level of ST3Gal III and SLe^x of MDAPanc-28 compared to Capan-1 cells. In the Capan-1 model the ST3Gal III overexpressing clone, C31, tripled the adhesion (*P <0.001*) to rh-E-Selectin when compared to corresponding controls Capan-1 and CP. To confirm that SLe^x expression induced by ST3Gal III caused the up-regulated E-Selectin binding, cells were previously incubated with the anti-SLe^x MAb and the binding to E-

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Selectin was inhibited. In the MDAPanc-28 model the ST3Gal III overexpressing clone, M34, quadrupled (*P*<0.001) the adhesion to rh-E-Selectin when compared to the corresponding controls MDAPanc-28 and MP. When cells were previously incubated with the anti-SLe^x MAb, the binding to E-Selectin was completely abrogated. These results demonstrated that, in these models, ST3Gal III levels modulated the *in vitro* binding to rh-E-Selectin via SLe^x expression.
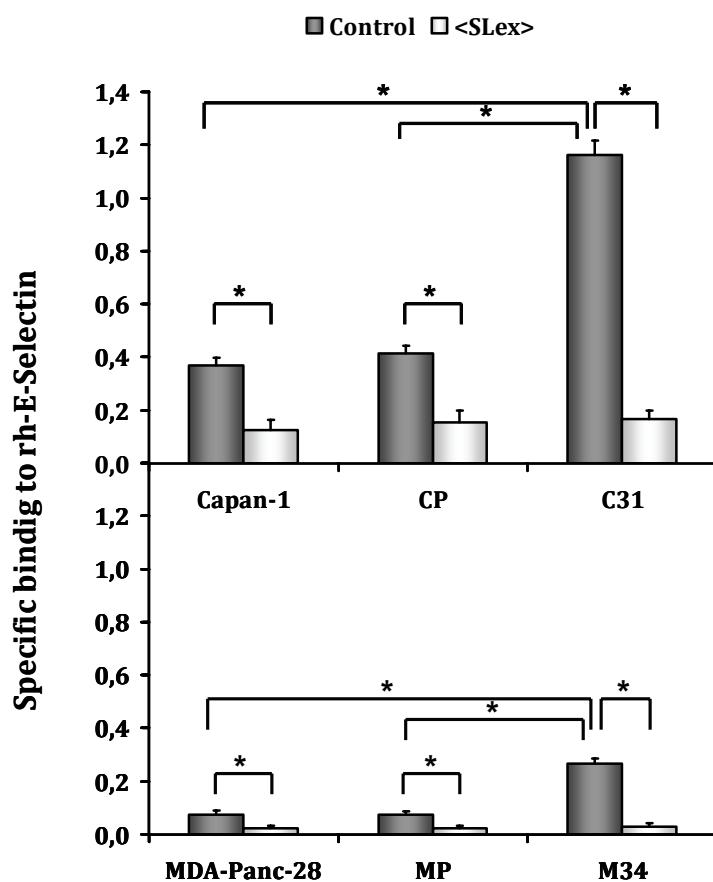
Figure 14: Binding assay to rh-E-Selectin. Capan-1 variant cells (*Upper graphic*) and MDAPanc-28 variant cells (*Lower graphic*), previously incubated with PBS-1% BSA (light bars) or anti-SLe^x MAb (dark bars), were added to microplates coated with rh-E-Selectin or PBS-1% BSA (negative control). Adherent cells were estimated with a MTT-based colorimetric assay. Data represents the mean ± SD of 3 separate experiments, each in five replicates (n=15). * Significantly different (*P < 0.001*).

1.1.4. The effect of cytokines on pancreatic cancer cell adhesion to HSE cells

Because ST3Gal III and SLe^x expression levels were directly proportional to *in vitro* rh-E-Selectin adhesion, the binding of pancreatic cancer cells to primary cultured HSE cells was evaluated. The Capan-1 model was selected for this experiment due its higher adhesion to rh-E-Selectin. First, the specific adhesion of Capan-1 parental cells to HSE cells (stimulated with TNF-α, IL1-β or LPS for 16 h before the assay) was determined (Figure 15).
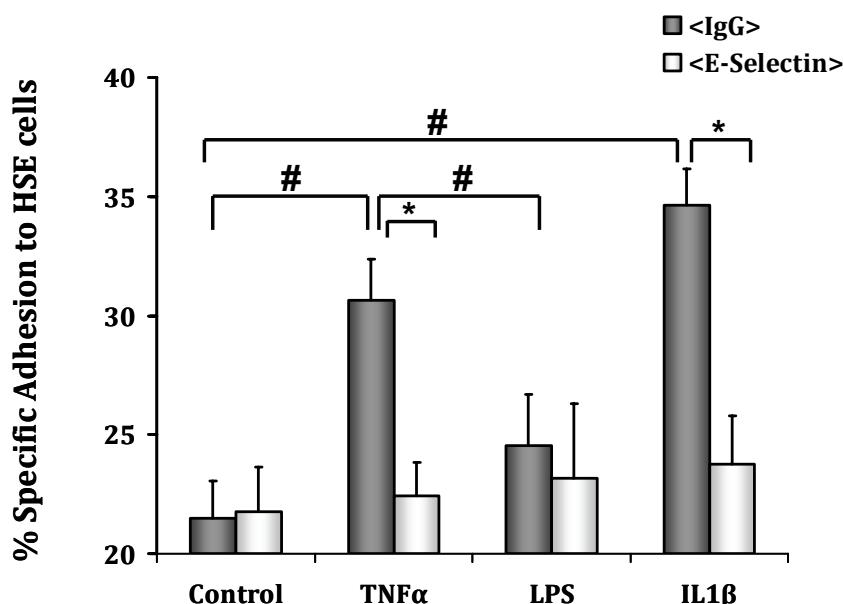
Figure 15. E-Selectin induction on Primary Cultured Hepatic Sinusoidal endothelial cells. HSE cells were incubated with <Sel> = anti-murine CD62 E (E-Selectin) MAb or <IgG>= isotype-matched control antibody. Parental Capan-1 cells were labelled with calcein and added to HSE control cells, TNF-α stimulated HSE cells, LPS stimulated HSE cells or IL-1β stimulated HSE cells. Results are expressed as the % Specific adhesion to HSE cells (Vidal-Vanaclocha et al. 1994). Data represents the mean ± SD of 3 separate experiments, each in three replicates (n=9). * Significantly different (*P < 0.001*) when comparing anti-E-Selectin incubated HSE cells to control cells. # Significantly different (*P < 0.001*) when comparing treatments.

Treatment with TNF-α and IL1-β greatly increased the number of adherent cells, being IL1-β the best stimuli for our model. When incubating with anti-E-Selectin MAb, the increase in adhesion was completely abrogated. Those data showed that HSE cell cytokine pre-treatment, especially IL-1β, promoted E-Selectin expression, which caused an increase of Capan-1 adhesion.

1.1.5. ST3Gal III increased tumour cell adhesion to HSEC

Next, the adhesion of different ST3Gal III and SLe^x levels expressing Capan-1 cells to IL-1β pre-treated or control HSE cells was determined (Figure 16).
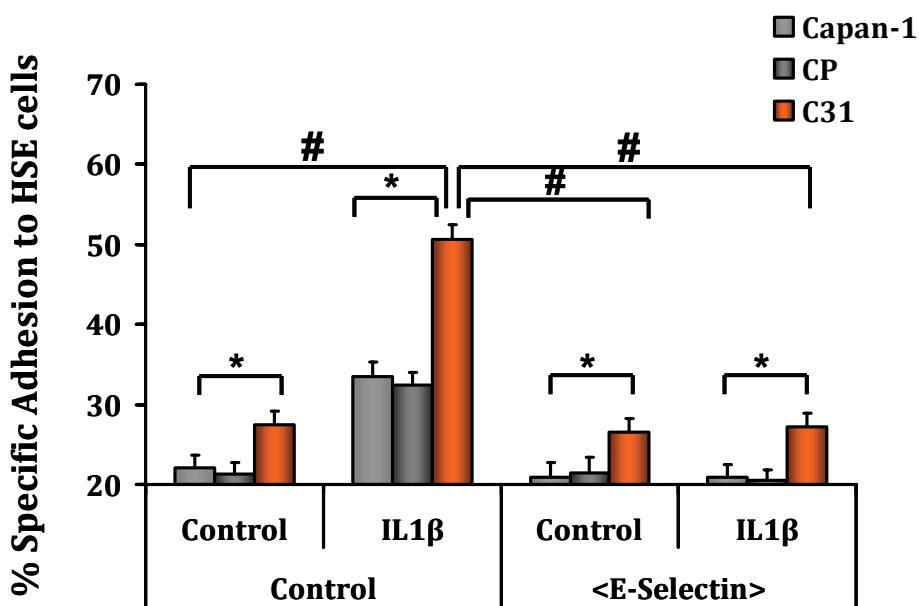
Figure 16. Tumour cell adhesion assay to HSE cells. Capan-1 variant cells were labelled with Calcein and added to IL-1β stimulated HSE cells or not stimulated HSE control cells. <Sel> = anti-murine E-Selectin MAb was added to IL-1β stimulated HSE cells or control HSE cells before tumour cell addition. * Significantly different (P < 0.001) when comparing clones. # Significantly different (P < 0.001) when comparing the adhesion of each clon to different HSE cell treatments.

Capan-1 and CP control cells showed a basal adhesion to HSE cells, which significantly increased in IL-1β pre-treated HSE cells and returned to basal levels in anti-E-Selectin MAb pre-incubated HSE cells. C31 showed a basal adhesion to HSE cells, which significantly increased in IL-1β pre-treated HSE cells and also returned to basal levels in anti-E-Selectin MAb preincubated HSE cells.

Those data showed that E-Selectin played an important role mediating adhesion to HSE cells, although a non-E-Selectin dependent basal adhesion (higher for C31 than for control cells) existed. Moreover, high ST3Gal III and SLe^x expressing C31 cells presented higher adhesion to HSE cells than medium ST3Gal III and SLe^x expressing control cells.

1.1.6. ST3Gal III overexpression increased migration

Cell migration on collagen Type-I using a transwell migration assay was evaluated and results are shown in Figure 17.
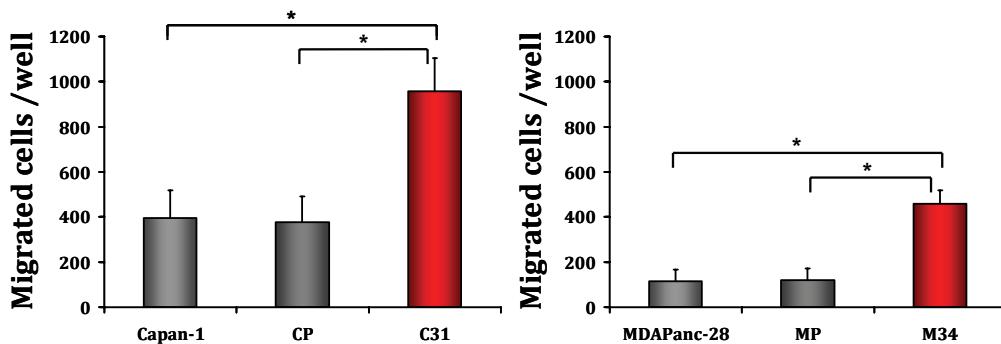
Figure 17: Cell migration assay. Capan-1 variant cells (Capan-1, CP and C31) *(left)* and MDAPanc-28 variant cells (MDAPanc-28, MP and M34) *(right)* were seeded onto 8 μm-pores inserts on top of wells containing DMEM-1% FBS and incubated at 37ºC. Results are expressed as migrated cells per well. Data represents the mean ± SD of the values obtained in 3 separate experiments, (n=9). * Significantly different (*P < 0.001*).

Both cell models showed different migratory capabilities, with the Capan-1 model being nine times more migratory than the MDAPanc-28 model. Regarding the Capan-1 model, the ST3Gal III overexpressing clone C31 was twice (*P<0.01*) more migratory than control cells CP and Capan-1. For the MDAPanc-28 model, the ST3Gal III and SLe^x overexpressing clone M34 increased migration by four with respect to the control cells MDAPanc-28 and MP. These results demonstrate a positive correlation between ST3Gal III and SLe^x levels and migratory capabilities.

1.1.7. ST3Gal III overexpression increased metastasis formation and decreased survival in athymic nude mice

Since the *in vitro* results described above suggested an important role for the ST3Gal III gene in tumour progression, *in vivo* assays were performed to study whether ST3Gal III could be important in tumour metastasis.

First, the metastasis development and survival of athymic nude mice after intrasplenic injection with different amounts of the Capan-1 and MDAPanc-28 parental cells were assayed. In our hands, when $1.5x10^6$, $3x10^6$ or $5x10^6$ Capan-1 cells were injected, the nude mice died owing to emboli formation, probably caused by the Capan-1 size and aggregation capacity. Injection of $1x10^6$ Capan-1 cells avoid fatal emboli while spleen tumours were generated. However, under these conditions, no metastatic spread to other organs was observed. Since MDAPanc-28 cells are 4-5 times smaller than Capan-1 cells, $7x10^6$ MDAPanc-28 cells were intrasplenically injected into nude mice which led to metastatic spread. Thus, MDAPanc-28 model was chosen to study the influence of ST3Gal III overexpression in metastasis formation and nude mice survival.

Exponentially growing 7×10^6 viable MP and M34 cells, which share the same morphological characteristics, were injected in the spleen of athymic nude mice (n = 8 /group) and survival analysis was performed using the Kaplan-Meier method (Figure 18).
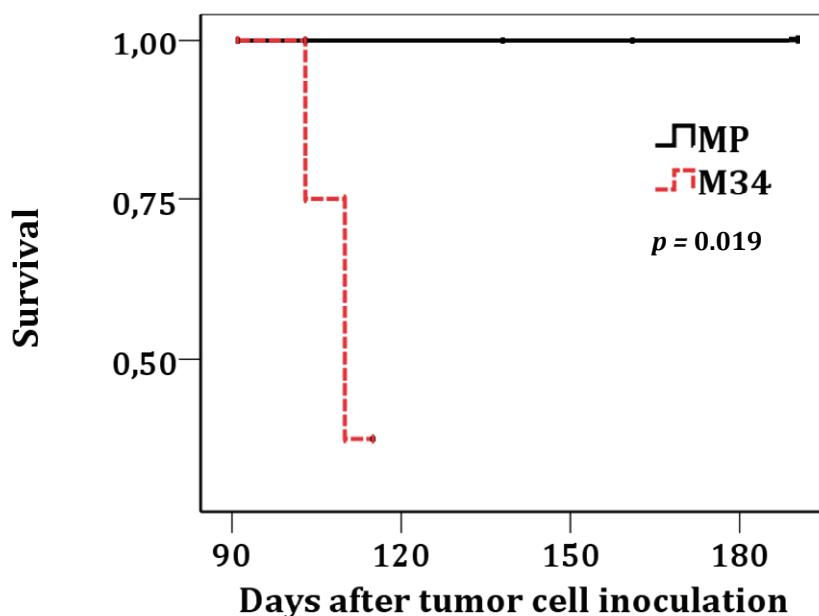
Figure 18: Kaplan-Meier plot of estimated survival after injection of MP (MDAPanc-28 mock cells) and M34 (MDAPanc-28 ST3Gal III transfected cells). Cells (7×10^6) were intrasplenically injected in nude mice on day 1 of the experiment. Mice were daily examined and sacrificed when they looked sick. The differences between groups were assessed by the long-rank test (P = 0.019; n = 7-8/group).

Mice injected with ST3Gal III overexpressing cells (M34) showed a large decrease in survival (*P=0.019*) when compared with mice injected with control MP cells. Most of M34 injected mice (5/8) died after 103-110 days post-injection, while MP injected mice survived until the end of the study (190 days).

All M34 injected mice were necropsied and the macroscopic analysis showed spleen tumours in 62.5% of the mice (5/8) and metastatic spread to some other organs in 75 % of the mice (6/8). On the contrary, none of MP injected mice (8/8) showed macroscopic tumour masses neither in spleen nor other organs (examined on days 103, 138, 161 and 190 post-injection).

Thus, the overexpression of ST3Gal III in MDAPanc-28 cells, which leads to a higher expression of SLe^x and a higher adhesion and migration, could be directly correlated with a decrease in survival when injected in nude mice. Furthermore, it endowed cells with a greater tumour formation capability and metastatic potential when intrasplenically injected.

1.2. Role of ST3Gal IV in PDAC progression

The role of ST3Gal IV on cell surface Lewis antigen expression and its effects in the adhesive, migratory and metastatic capabilities for the pancreatic adenocarcinoma cell line MDAPanc-28 was investigated.

1.2.1. Stable overexpression of ST3Gal IV

To investigate the function of ST3Gal IV in pancreatic adenocarcinoma progression, MDAPanc-28 cells were transfected with the pcDNA 3.1 vector encoding the ST3Gal IV gene or the empty vector pcDNA3.1. Several stable cell clones were selected in the presence of geneticine and ST3Gal IV mRNA overexpression was first analysed by semiquantitative PCR and then by qPCR. M42 clone, with 415-fold higher ST3Gal IV mRNA expression (*P< 0.001*) than control MP and

parental MDAPanc-28 cells showed the highest overexpression and was selected for further studies (Figure 19).
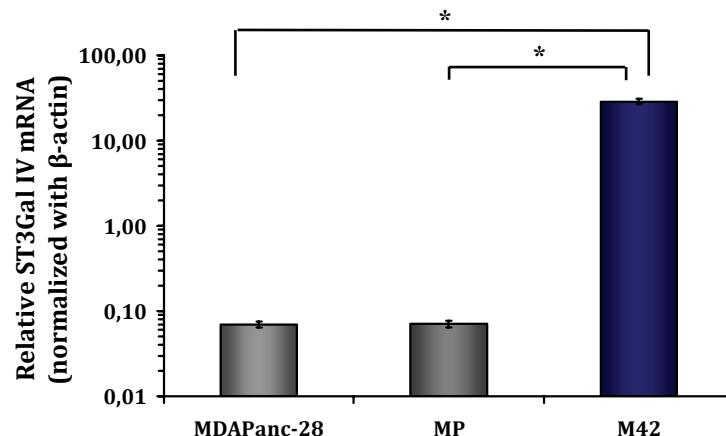
Figure 19: qPCR for relative ST3Gal IV mRNA expression normalized to β-actin.
MDAPanc-28**:** parental cells, MP: MDAPanc-28 mock cells, M42: MDAPanc-28 cells transfected with the ST3Gal IV gene. Mean ± SD of (n=18). * Significantly different (*P < 0.001*).

1.2.2. ST3Gal IV overexpression led to *de novo* synthesis of SLe^x and decreased α2-6 sialic acid

The surface glycan expression pattern was studied by flow cytometry (Figure 20). MDAPanc-28 and MP exponentially growing control cells presented high α2-6 sialic acid expression, very low levels of sialyl-Lewis ˣ (SLeˣ) and a negligible expression of other Type II or Type I Lewis antigens.

ST3Gal IV mRNA overexpressing clone M42 displayed medium SLeˣ levels and a decrease in α2-6 sialic acid when compared to controls.

These results pointed to an enzymatic competition between alpha-2,3- and alpha-2,6-sialyltransferases for Type II precursor.
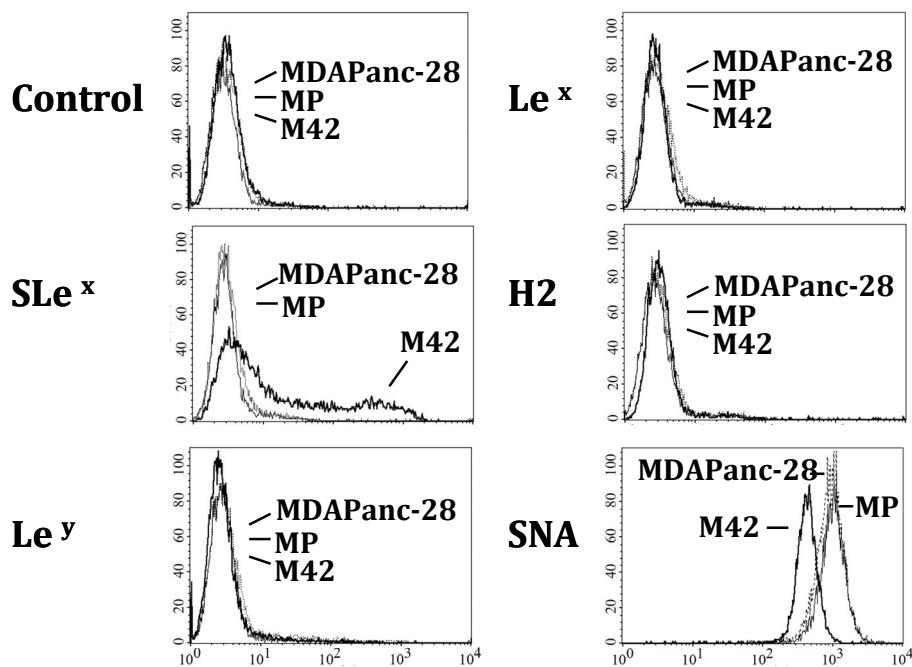
Figure 20. Flow cytometry analysis for glycan structures on the surface of MDAPanc-28 cells. 5 x 10^5 cells were incubated with antibodies or lectins. Experiments were performed for triplicate; here representative cytometry histograms are shown. MDAPanc-28: parental cells (plain outline), MP cells (dot outline), M42 cells (bold outline) Anti-Le^x MAb binds to Galβ1,4[Fucα1,3]GlcNAc-; anti-SLe^x MAb binds to NeuAc2,3Galβ1,4 [Fucα1,3]GlcNAc-; anti-H2 MAb binds to [Fucα1,2]Galβ1,4GlcNAc-; anti-Le^y MAb binds to [Fucα1,2]Galβ1,4[Fucα1,3]GlcNAc-; SNA lectin (*Sambucus nigra agglutinin*) binds to NeuAc α 2-6Gal β- structures.

1.2.3. ST3Gal IV overexpression increased pancreatic cancer cell adhesion to E-Selectin via SLe^x

Binding assays were performed to analyze whether *de novo* synthesis of SLe^x was able to induce functional functional changes in the adhesion to recombinant human E-Selectin and results are shown in Figure 21. The

ST3Gal IV overexpressing clone M42 showed 1.7-fold times increase (*P < 0.001*) in the adhesion to rh-E-Selectin when compared to the corresponding controls. To confirm that SLe^x expression caused the up-regulated E-Selectin binding, cells were previously incubated with the anti-SLe^x MAb and the binding to rh-E-Selectin was completely abrogated. These results demonstrate that ST3Gal IV levels modulate cells *in vitro* binding ability to rh-E-Selectin via SLe^x expression.
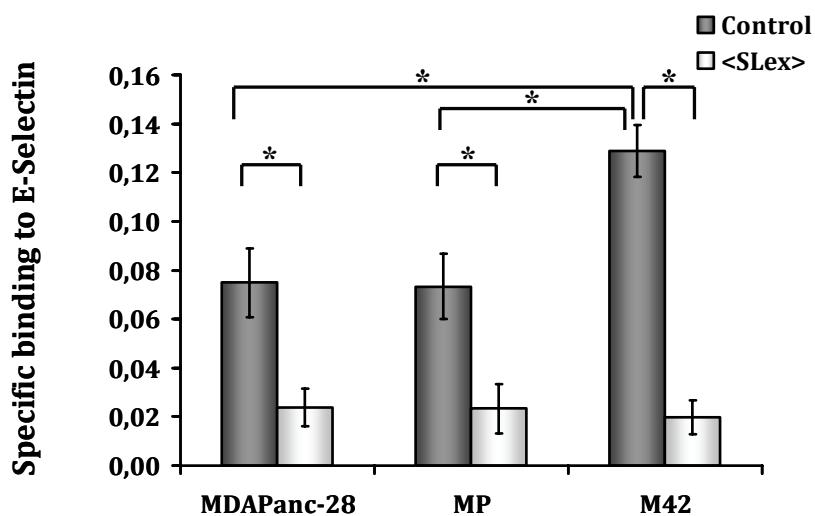
Figure 21. Binding assay to rh-E-Selectin. MDAPanc-28 variant cells, previously incubated with anti-SLex MAb (light bars)or PBS-1% BSA as a control (dark bars), were added to rh E-Selectin or PBS-1% BSA coated microplates. Adherent cells were estimated with a MTT-based colorimetric assay. Results are expressed as the Specific binding to E-Selectin. Data represents the mean ± SD of three separate experiments, each in five replicates (n=15). * Significantly different (*P* < 0.001).

1.2.4. ST3Gal IV overexpression increased migration

To determine whether ST3Gal IV induced changes in the glycan expression pattern might conduct to the achievement of a more migratory cell phenotype, cell migration on collagen Type-I assays were performed and results are shown in Figure 22. The ST3Gal IV

overexpressing clone M42 multiplied by 2.7 (P < 0.001) migration capabilities of control cells MDAPanc-28 and MP. The results demonstrate a positive correlation between ST3Gal IV and SLe^x levels and migratory capabilities.
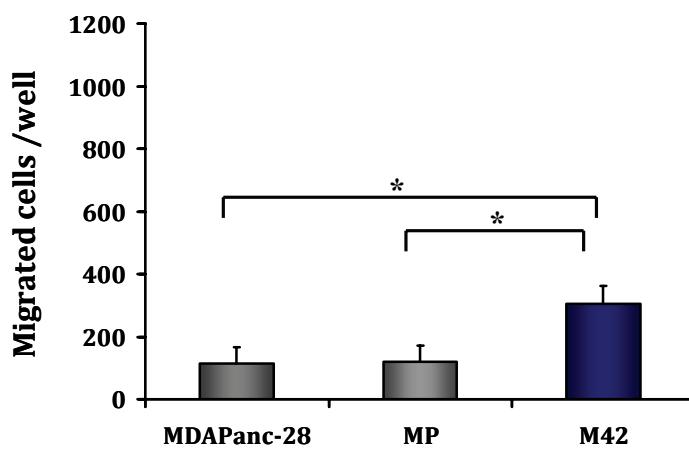
Figure 22. Cell migration assay. MDAPanc-28 variant cells (MDAPanc-28, MP and M42) were seeded onto 8 μm-pores- coated inserts, placed on top of wells containing DMEM-1% FBS and incubated at 37ºC. Results are expressed as migrated cells per well. Data represents the mean ± SD of the values obtained in 3 separate experiments, (n=9). * Significantly different (*P* < 0.001).

1.2.5. ST3Gal IV overexpression increased metastasis formation and decreased survival in athymic nude mice

Since the *in vitro* results described above suggested an important role for the ST3Gal IV gene in tumour progression, *in vivo* assays were performed to study whether it be important in tumour metastasis. Exponentially growing 7x10^6 viable MP and M42 cells, which share the same morphological characteristics, were injected in the spleen of athymic nude mice (n = 8/group) and survival analysis was performed using the Kaplan-Meier method (Figure 23). Mice injected with ST3Gal IV overexpressing cells (M42) showed a decrease in survival

when compared with mice injected with control MP cells (*P=0.018*). Half of M42 injected mice died between 125-138 days post-injection, while all MP injected mice survived until the end of the study (190 days).

Figure 23: Kaplan-Meier plots of estimated survival after injection of MP (MDAPanc-28 mock cells) and M42 (MDAPanc-28 ST3Gal IV transfected cells). Cells (7 x 10^6) were intrasplenically injected in nude mice on day 1 of the experiment. Mice were daily examined and sacrificed when they looked sick. The differences between groups were assessed by the long-rank test (*P* = 0.012; n = 7-8/group).

All M42 injected mice were necropsied and the macroscopic analysis showed metastatic spread in 38 % of the mice (3/8). However, none of the MP injected mice (8/8) showed macroscopic tumour masses neither in spleen nor other organs. Thus, the overexpression of ST3Gal IV in MDAPanc-28 cells could be correlated with a decrease in survival when injected in nude mice. Moreover, it endowed cells with a greater metastatic potential when intrasplenically injected.

1.3. Specific role of ST3Gal III and ST3Gal IV in PDAC

The results obtained so far suggested that both ST3Gal III and ST3Gal IV could be considered metastasis related genes. In order to analyze the specific role of each gene in the biosynthesis of sialylated Lewis antigens and its implications in the adhesive, migratory and metastatic capabilities, comparisons were established between ST3Gal III and ST3Gal IV MDAPanc-28 transfected cells.

1.3.1. ST3Gal III and ST3Gal IV overexpression differently increased SLe^{x}synthesis, E-selectin adhesion and migration

MDAPanc-28 parental and MP cells had low levels of endogenous ST3Gal III mRNA and negligible levels of endogenous ST3Gal IV mRNA (Figure 24). The ST3Gal III mRNA expression was 7-fold higher ($P<$ 0.001) in M34 clone than in control cells; while the ST3Gal IV mRNA expression was 415-fold higher ($P < 0.001$) in M42 clone than in control cells.
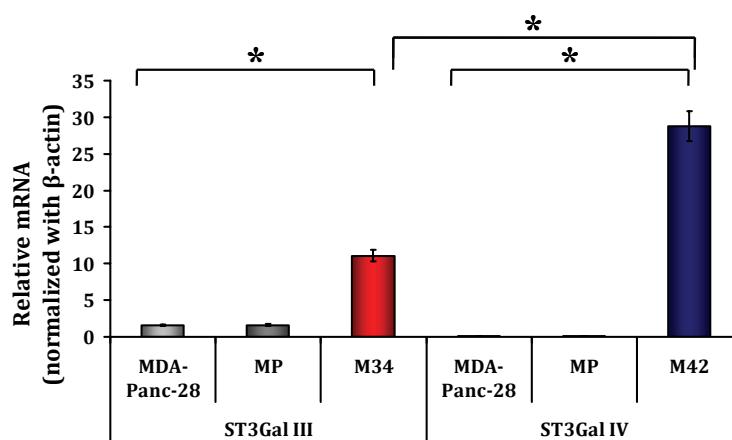
Figure 24: Relative ST3Gal III and ST3Gal IV mRNA expression normalized to β-actin. MDAPanc-28**:** parental cells, MP: MDAPanc-28 mock cells, M34: MDAPanc-28 cells transfected with the ST3Gal III gene, M42: MDAPanc-28 cells transfected with the ST3Gal IV gene. Mean ± SD of (n=18). * Significantly different (*P < 0.001*).

Regarding SLe[x] expression, M34 clone displays a significant 1.7-fold increase in SLe[x] levels when compared to clone M42 (Figure 25A). In contrast, the decrease in α2-6 sialic acid (detected by SNA lectin) was not significantly different between M34 and M42 clones (Figure 25B).
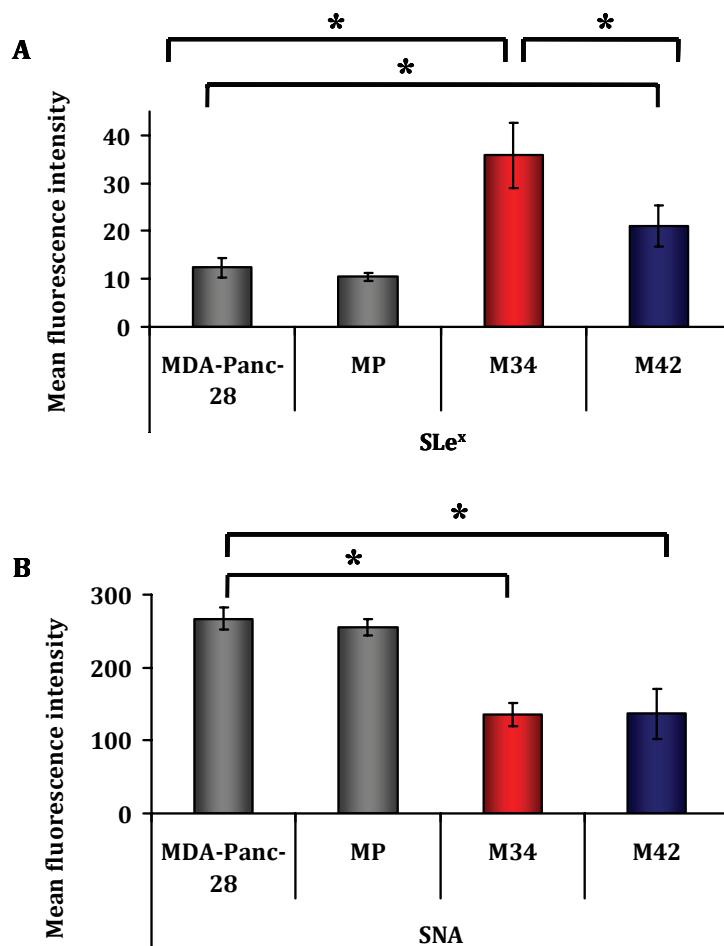
Figure 25: Flow cytometry analysis for the surface SLe[x] (Figure 25A) and surface SNA (Figure 25B) in MDAPanc-28 cells. MDAPanc-28**: parental cells, MP: mock cells, M34: ST3Gal III transfected cells M42: ST3Gal IIV transfected cells. Data represents Mean Fluorescence Intensity (MFI) ± SD of at least three independent experiments. * Significantly different (*P < 0.01*)

With respect to E-Selectin adhesion and migration (Figure 26), ST3Gal III overexpressing clone M34 doubled (*P<0.001)* the adhesion to rh-E-

Selectin when compared to the ST3Gal IV overexpressing clone M42. When the binding to rh-E-Selectin was performed with cells previously incubated with the anti-SLe^x MAb, the binding to rh-E-Selectin was abrogated in both cases. In addition, ST3Gal III overexpressing M34 cells were 1.5 fold (*P<0.001)* more migratory than ST3Gal IV overexpressing M42 cells.
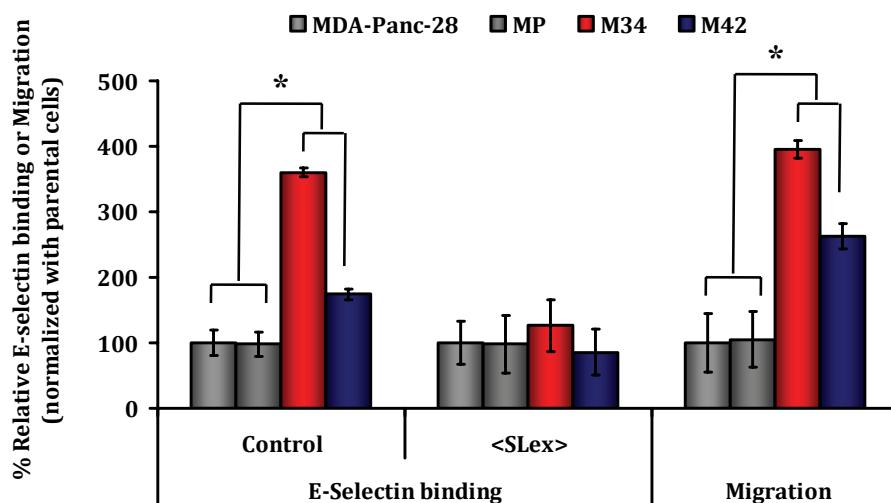
Figure 26. % of Relative E-Selectin binding and % of Relative migration, normalized with parental MDAPanc-28 cells. MDAPanc-28 parental cells, MP mock transfected cells, M34: ST3Gal III overexpressing cells and M42: ST3Gal IV overexpressing cells. Control: cells incubated with PBS-1% BSA. < SLe^x >: cell incubated with anti-SLe^x MAb. Results are expressed as % of parental MDAPanc-28 cells. Data represents the mean ± SD of the values * statistical significance of *P < 0.001*.

1.3.2. ST3Gal III and ST3Gal IV overexpression differently influenced survival and metastasis formation in nude mice

The Kaplan-Meier method was used to compare the survival of athymic nude mice (Figure 27). Mice injected with M42 showed higher survival (*P=0.012*) than M34 cells injected ones.

Most of the M34 injected mice died between 103-110 days post-injection, while all M42 injected mice survived at least fifteen days more (day 125 post-injection). In addition, three of M42 injected mice survived at least 160 days post-injection and one of them survived until the end of the study (day 190 post-injection).
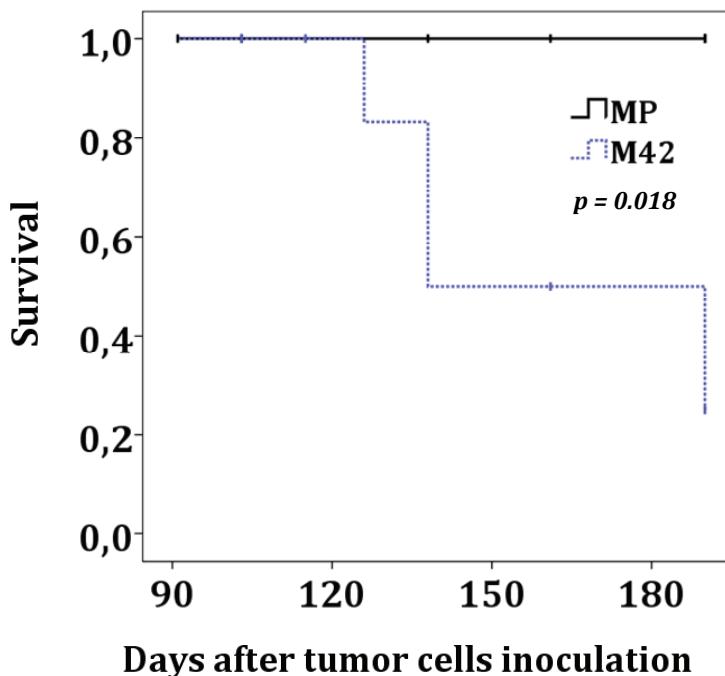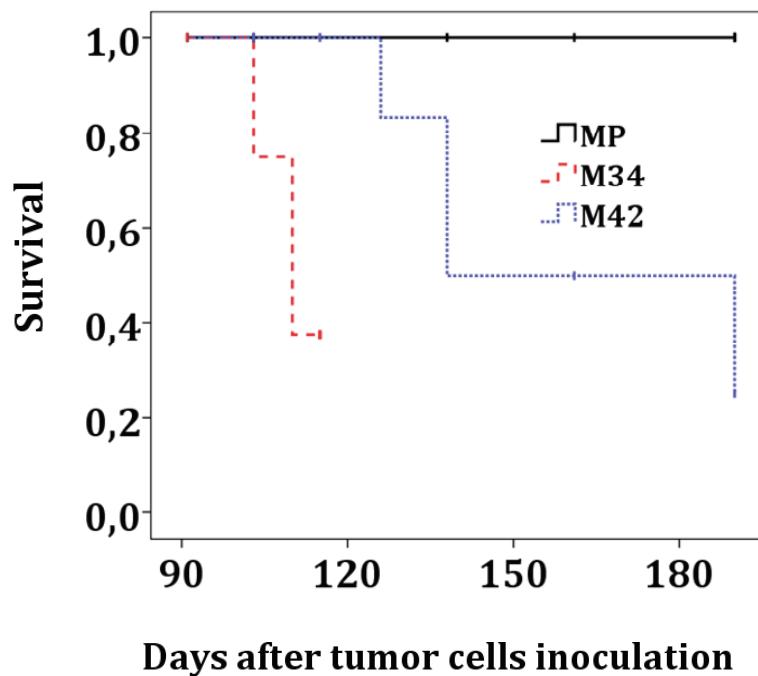

Figure 27: Kaplan-Meier plots of estimated survival after injection. MP (MDAPanc-28 mock cells), M34 (MDAPanc-28 ST3Gal III transfected cells) and M42 (MDAPanc-28 ST3Gal IV transfected cells

All mice were necropsied and the macroscopical analysis showed that 62.5% of M34 injected mice presented spleen tumours while none of the M42 injected mice did (Table 4). With regard to the metastatic spread to other organs, lymph node mestastasis was observed in 50% of M34 injected mice while it was observed in just 25% of M42 injected mice. Moreover, some of M34 bearing mice presented metastatic spread to kidneys, liver and lungs while none of M42 injected mice did. Both

M34 and M42 injected mice presented similar amounts of macroscopic metastasis foci to suprarenal glands.

	MP	M34	M42
Spleen	n.d	62.5 %	n.d
Pancreas	n.d.	37.5 %.	12.5%
Lymph nodes	n.d	50 %	25 %
Kidneys	n.d	37.5%	n.d
Suprarenal glands	n.d	25 %	25 %
Liver	n.d	12.5%	n.d
Lungs	n.d	12.5%	n.d

Table 4: Percentage of mice presenting macroscopic metastastatic focus. MP: mice injected with MDAPanc-28 mock transfected control cells; M34: mice injected with ST3Gal III overexpressing MDAPanc-28 cells; M42: mice injected with ST3Gal IV overexpressing MDAPanc-28 cells. n.d : not detected at any mice.

In order to perform the microscopical analyses, spleen, pancreas, lymph nodes, kidneys and suprarenal glands from all mice were collected and stained with Haematoxylin-Eosin (H&E) (Table 5).

	MP	M34	M42
Spleen	12.5 %	75 %	25 %
Pancreas	12.5 %	75 %	50 %
Lymph nodes	25 %	87.5%	50 %
Kidneys	12.5 %	50 %	37.5 %
Suprarenal glands	25 %	37.5 %	37.5 %

Table 5: Percentage of mice presenting microscopical metastatic focus. MP: mice injected with MDAPanc-28 mock transfected control cells; M34: mice injected with ST3Gal III overexpressing MDAPanc-28 cells; M42: mice injected with ST3Gal IV overexpressing MDAPanc-28 cells. n.d : not detected at any mice.

In MDAPanc-28 control cells (MP) bearing mice, one out of eight animals (12.5%) showed microscopical metastatic foci in spleen, pancreas and kidneys and two out of eight (25%) presented metastatic foci in lymph nodes and suprarenal glands.

In M34 bearing mice the incidence of metastatic development was as follows: 87.5% of mice presented metastatic foci in lymph nodes, 75% of mice presented metastatic foci in pancreas and spleen, 50% in both kidneys and 37.5 % in suprarenal glands. In those mice bearing M42, 50% of the mice showed the pancreas and lymph nodes affected by the tumour, 37.5 % showed kidney and suprarenal glands affectation and 25% of the mice presented spleen affectation

With regard to pancreas tumour histology (Figure 28), M34 and M42 bearing mice showed lesions which occupied variable areas of the pancreas. Such lesions were irregularly shaped within an amount of normal pancreas that contained well-organized acinar, ductal, and islet tissue. In M42 injected mice most pancreas infiltrating ductal adenocarcinoma formed poorly to moderately defined glands, where the gland formation was less well-defined and included some necrotic areas. In M34 injected mice, pancreatic carcinomatous tissue showed moderate to well differentiated glands with some invasive areas, desmoplastic reaction and polar development. Both M34 and M42 showed the typical morphology of pancreatic ductal adenocarcinoma and were also richly distributed with a stromal component.

With regard to kidneys, M34 tumours also presented gross tumour growth. The kidney involvement seen in M42 bearing mice was similar in extension; however, it was clearly less differentiated.

With regard to spleens, the tumour development seen in mice bearing M34 cells was clearly higher than in those bearing M42 cells, not only in degree of differentiation but also in the extent of invasion and aggressiveness. However, both tumours demonstrated aggressive metastasis and/or direct invasion in which the spindle-shaped cells were spreading along with the ductal tumour cells.
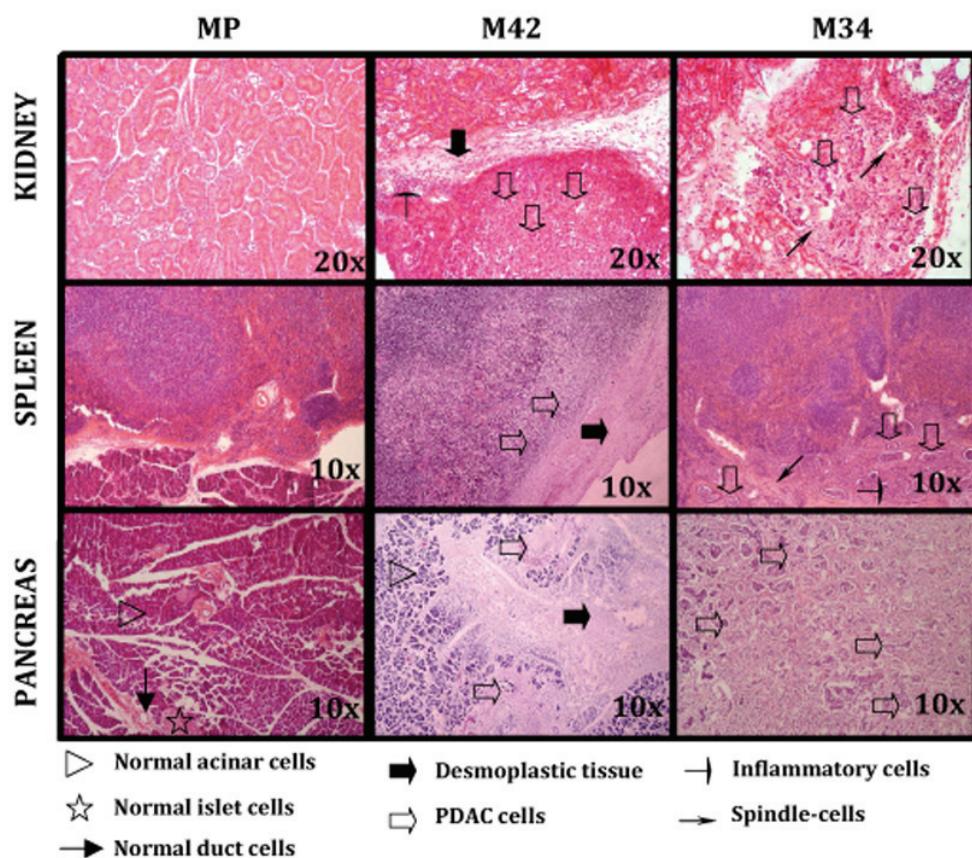
Figure 28: Microscopic analysis of spleen, pancreas and kidney. Mice injected with MDAPanc-28 mock transfected cells (MP); mice injected with ST3Gal III overexpressing MDAPanc-28 cells (M34); mice injected with ST3Gal IV overexpressing MDAPanc-28 cells (M42).

2. ROS as ST3Gal III and SLe^x modulators in pancreatic adenocarcinoma cell lines

Next, Capan-1 and MDAPanc-28 proliferation and differentiation processes, associated to ST3Gal III mRNA and surface SLe^x expression were investigated.

2.1. Cell growth associated changes in ST3Gal III levels and SLe^x cell surface expression

Pancreatic adenocarcinoma cells lines Capan-1 and MDAPanc-28 were cultured and cell number, culture morphology, ST3Gal III mRNA levels and SLe^x surface expression levels were studied. Visual inspection of Capan-1 cells in culture (Figure 29) showed that cells become confluent after 96 hours (indicated by a vertical line marked "C" in Figure 30). Between 96 and 144 hours some cells spontaneously differentiated and dome formation occurred. After 144 hours, cells appeared non-proliferating (indicated by a vertical line marked "NP" in Figure 30).

Cell number (Figure 30A) grew logarithmically until 144 h, and then it slightly decreased. From these data, three distinct stages of cell growth were defined: Stage 1 (S1) from seeding until visual confluence (96 hours), with an 18.3 hours doubling time; Stage 2 (S2) from 96 to 144 h, where differentiation and dome formation occurred with a 33.3 h doubling time; and non-proliferative Stage 3 (S3) after 144 h. Results for MDAPanc-28 were similar to Capan-1 and the same stages of cell growth were defined: S1 with a 17.9 h doubling time, S2 with a 56.5 h doubling time and dome formation, and non-proliferative stage S3. In both cell lines, there was a positive correlation between ST3Gal III mRNA levels and cell proliferation at S1 (Figure 30B). At the beginning
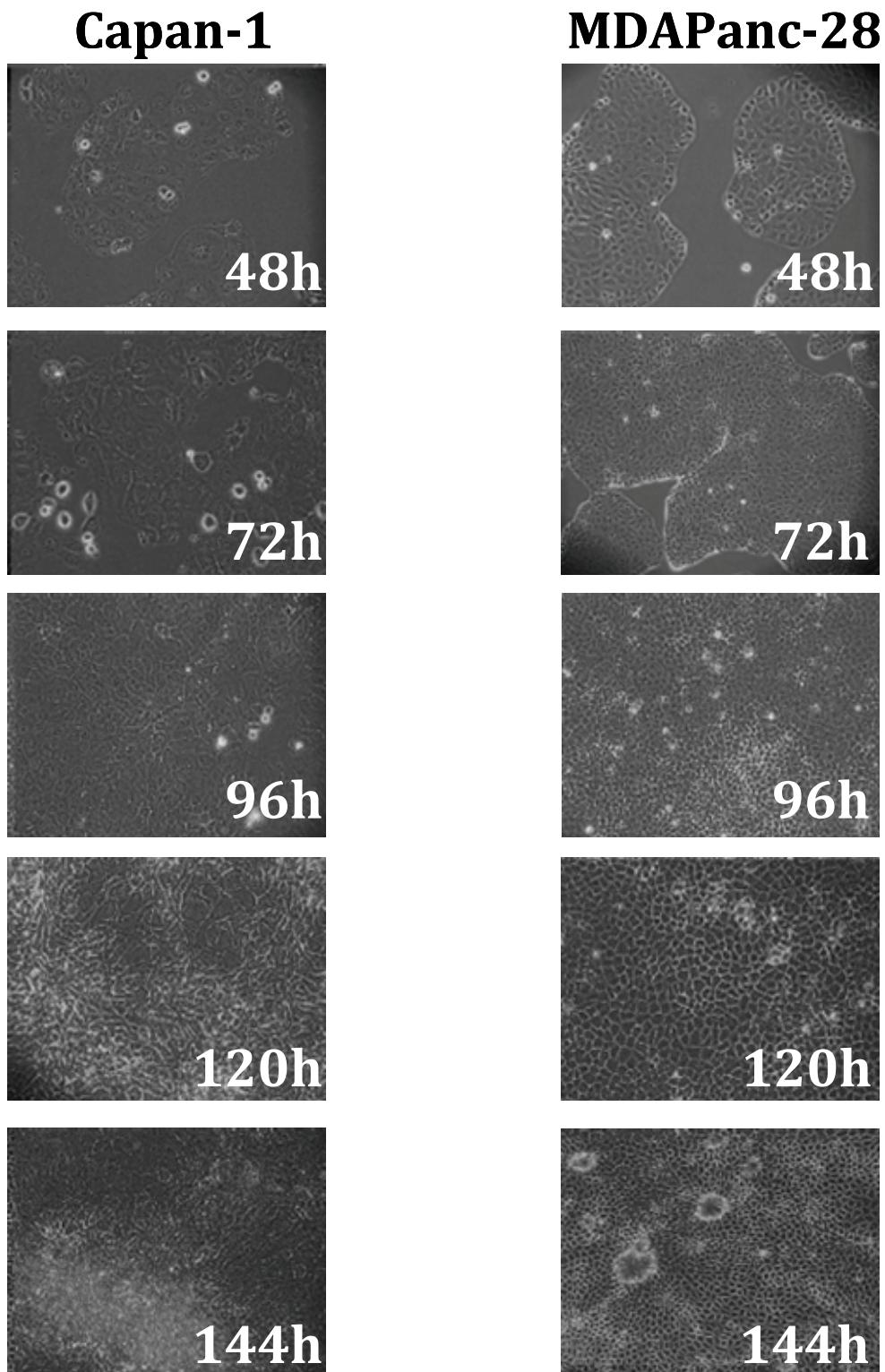
Capan-1   MDAPanc-28

Figure 29: Visual inspection of Capan-1 and MDAPanc-28 cells from 48 until 144 h in culture. See the text for further detail.

of S2, cell proliferation continued and ST3Gal III expression reached its maximum levels. Then, as cell differentiation and dome formation occurred, ST3Gal III levels decreased. In all three stages ST3Gal III mRNA levels correlated with SLe^x cell surface expression for both cell lines (Figure 30C).
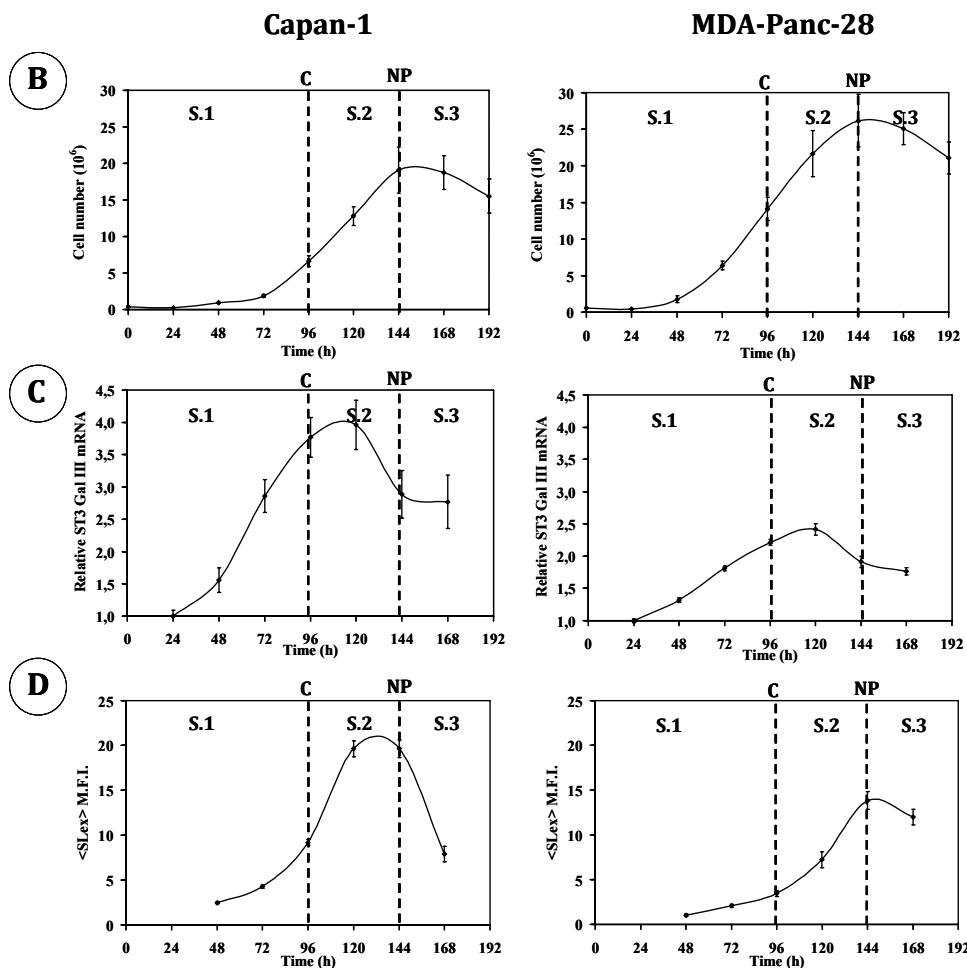
Figure 30A: Viable cell number versus hours in culture for Capan-1 and MDAPanc-28 cell lines. C: Visual confluence, NP: visual non-proliferating state, S1: Stage, S2: Stage 2, S3: Stage 3 *Figure 30B: Relative expression of ST3Gal III* by q-PCR, normalized with TBP for the Capan-1 cells and with β-actin for the MDAPanc-28 cells. *Figure 30C: Flow cytometry results for the SLe^x* antibody expressed as Mean fluorescence intensity (MFI) for the Capan-1 and MDAPanc-28 cell lines. Data represents the mean ± SD of three independent experiments.

2.2. Cell growth-associated changes in E-Selectin binding

Binding assays were performed to analyze whether growth-associated changes in SLe^x surface expression were able to induce changes in the adhesion to rh-E-Selectin. S1 growing period, with the highest correlations amongst these parameters was selected for this experiment (Figure 31).
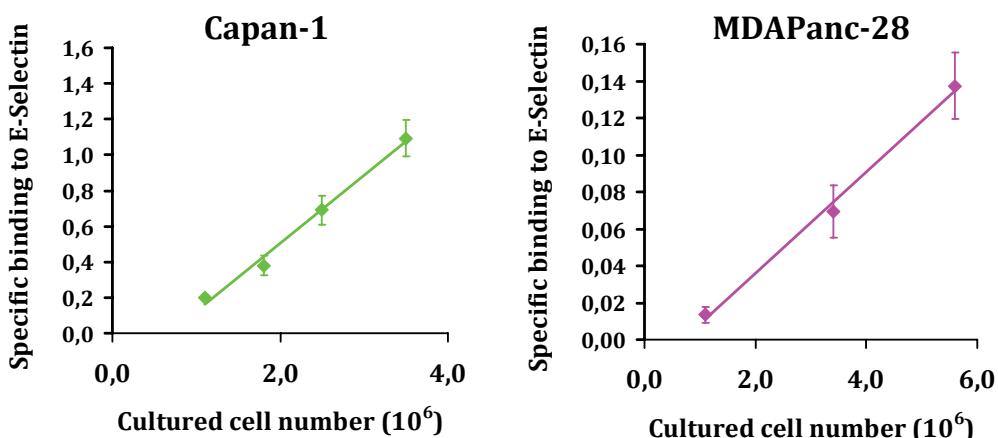
Figure 31. Time course binding assay to rh-E-Selectin for the Capan-1 and MDAPanc-28. Results are expressed as the Specific binding to E-Selectin (O.D. 570 nm of cells bounded to E-Selectin – O.D. 570 nm of cells bonded to PBS-1% BSA) vs. cell number. Data represents the mean ± SD of two separate experiments, each in five replicates.

As cell number in culture increased an enhanced adhesion to rh-E-Selectin was observed for both cell lines. When compared to Capan-1 sparse cultures (1.1 x 10^6 cells), denser cultures containing 1.8 x 10^6 cells, 2.5 x 10^6 cells and 3.5 x 10^6 cells, doubled, tripled or quintupled respectively (*P <0.001*) the adhesion to rh-E-Selectin. Similar results were obtained for MDAPanc-28, where cultures with 3.4 x 10^6 cells and 5.6 x 10^6 cells quintupled and multiplied per ten (*P <0.001*) the

adhesion to rh-E-Selectin when compared to sparse cultures (1.1 x 10^6 cells). MDAPanc-28 cells, with lower ST3Gal III and SLe^x expression than Capan-1 cell, displayed lower adhesion to rh-E-Selectin.

2.3. Cell growth-associated changes in cells redox status

When analyzing GSH and ROS intracellular levels (Figure 32), we observed for both cell lines that GSH levels were maximum at initial stages of cell growth and then gradually decreased over the proliferation and differentiation processes. Inversely, ROS levels were minimum at initial stages and gradually increased while GSH levels decreased. This inverse correlation between GSH and ROS levels was also observed when comparing Capan-1 and MDAPanc-28 cell lines. Capan-1 cells, with low GSH levels, had significantly higher ROS than MDAPanc-28 cells, with a higher GSH content.
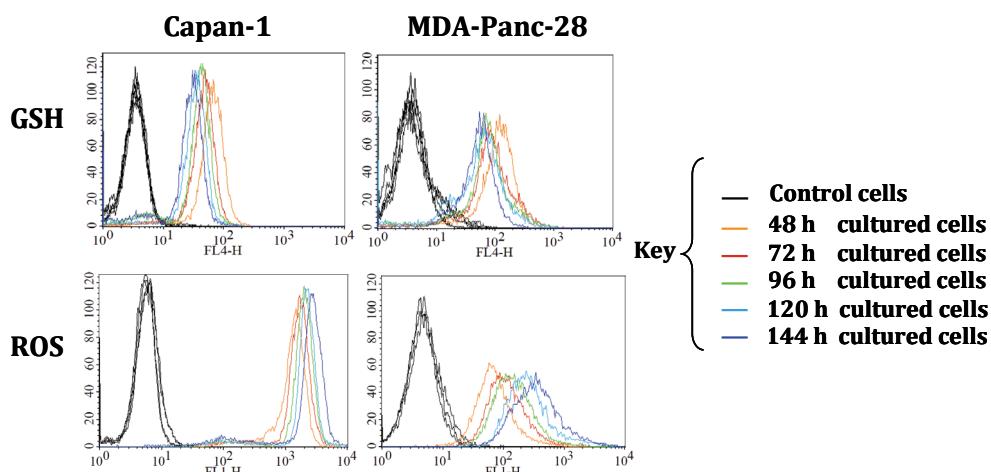
Figure 32. Representative flow cytometry profiles of the intracellular GSH (reduced glutathione) and ROS (Reactive Oxygen Species) for the Capan-1 and MDAPanc-28 cells from 48 until 144 h in culture. At least two independent experiments were taken for each cell line and time.

2.4. ROS generated after exogen H$_2$O$_2$ addition increased ST3Gal III levels and SLe^x expression

Sparse (S1, 72 hours in culture) and confluent (S1, 96 hours in culture) Capan-1 and MDAPanc-28 cells, pretreated or not with *N*-Acetylcysteine (NAc), were treated with 50 µM H$_2$O$_2$ for 24 h. Intracellular ROS content and SLe^x surface expression was evaluated by flow cytometry and results are shown in Figure 33A and Figure 33B and ST3Gal III mRNA levels were measured by qPCR and results are shown in Figure 34.
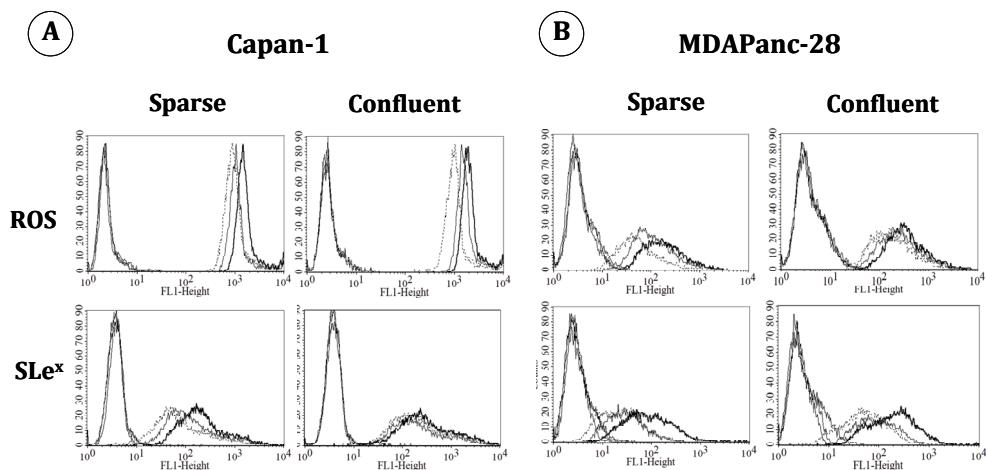
Figure 33. Representative flow cytometry profiles of intracellular ROS (Reactive Oxygen Species) content and surface SLe^x expression for sparse and confluent Capan-1 (Figure 33A) and MDAPanc-28 (Figure 33B). control cells (dot line), cells treated with 50 µM H$_2$O$_2$ (bold line) and for control cells treated with 100 µM *N*-Acetylcysteine (NAc) before H$_2$O$_2$ treatment (plain line). At least three independent experiments were taken for each cell line and treatment.

H$_2$O$_2$ treated Capan-1 cells (bold outline) showed a higher ROS content and SLe^x surface expression than non-treated control cells (dot outline). When Capan-1 cells were pre-treated with NAc and then treated with

H_2O_2 (plain outline) the increase in ROS levels and the increase in SLe^x expression was partially abrogated. Regarding to ST3Gal III mRNA levels, H_2O_2 treatment led to an increase in ST3Gal III levels while NAc pre-treatment prevented the enhancement (Figure 34 left). Both observations were more noticeable for sparse cultures, with lower ROS and SLe^x basal levels, than for confluent cultures.
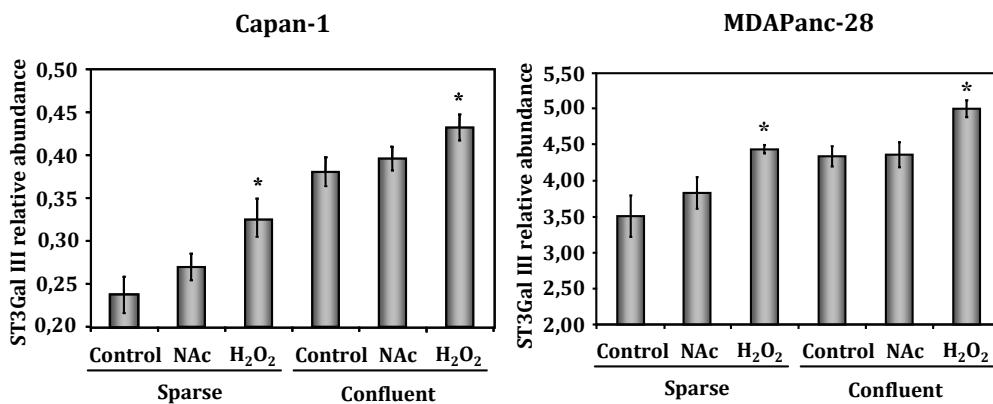
Figure 34. Relative expression of ST3Gal III by q-PCR, normalized with TBP for the Capan-1 cells (left) and with β-actin for the MDAPanc-28 cells (right) for control, H_2O_2 treated and *N*-Acetylcysteine (NAc) pre-treated sparse and confluent cells. Data represents the mean ± SD of three separate experiments, each in six replicates. * Significantly different from control and NAc pre-treated cells.

Similar behaviour was observed for MDAPanc-28 cells with overall lower ROS, ST3Gal III and SLe^x levels than Capan-1 cells. When MDAPanc-28 cells were pre-treated with NAc and then treated with H_2O_2 (plain outline) the increase in ROS levels and the increase in SLe^x expression was partially abrogated. In addition, H_2O_2 treatment led to an increase in ST3Gal III levels while NAc pre-treatment prevented the enhancement. However, for this cell line increases in ROS, ST3Gal III and SLe^x levels were equally noticeable for sparse and confluent cultures.

3. Soluble E-Selectin depending VEGF secretion and migration

Finally, additional prometastatic effects for sE-Selectin, such as VEGF secretion and migration, were investigated in pancreatic adenocarcinoma cells.

3.1. VEGF secretion after sE-Selectin treatments

To determine whether soluble E-Selectin (sE-Selectin) had a role in the *in vitro* angiogenic process, VEGF secretion after sE-Selectin treatments was determined in Capan-1 and C31 cell lines (Figure 35). C31 cells, showed a significant ($P<0.01$) lower basal VEGF secretion than Capan-1 cells. VEGF secretion was significantly ($P<0.01$) increased in both
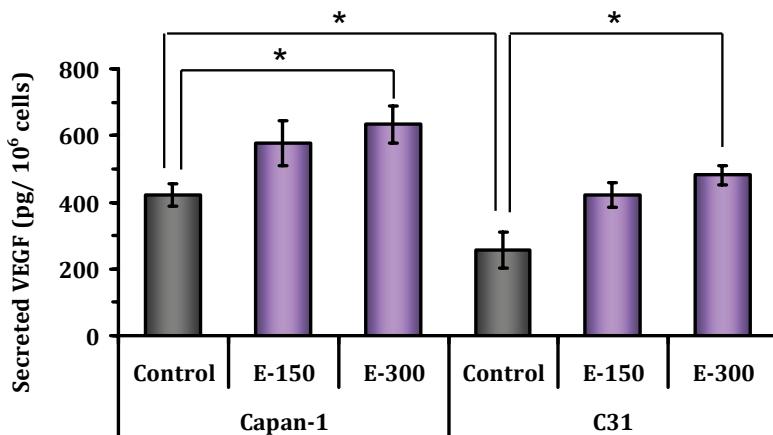
Figure 35. VEGF secretion after sE-Selectin treatment. Capan-1 and C31 cells were cultured and then treated for 16 h with basal medium (control), 150 ng/mL sE-Selectin (E-150) or 300 ng/mL sE-Selectin (E-300). Secreted VEGF was calculated in units of pg per 10^6 pancreatic carcinoma cells. Data represents the mean ± SD of three separate experiments, each in triplicate. * Significantly different (*P < 0.01*)

Capan-1 and C31 cells after 16 hours treatment with 150 ng/mL or 300 ng/mL sE-Selectin.Although no significant differences (*P*>0.01) were observed between 150 ng/mL or 300 ng/mL sE-Selectin treatment, a dose dependent trend was observed. When compared to corresponding non-treated controls, sE-Selectin induced VEGF secretion by approximately 2 folds in higher SLe^x expressing C31 cells while induced VEGF secretion by 1.3 folds in medium SLe^x expressing Capan-1 cells.

3.2. VEGF secretion after sE-Selectin treatment removal

Then, we studied VEGF secretion 24 h after treatment removal (Figure 36). We did not observe significant (*P*>0.01) differences for treated and non-treated Capan-1 and C31 cells. This result suggested that after the stimuli removal, VEGF secretion returns to basal levels. Although no significant differences (*P*>0.01) were observed between Capan-1 and C31, VEGF secretion tended to be lower for C31 cell line.
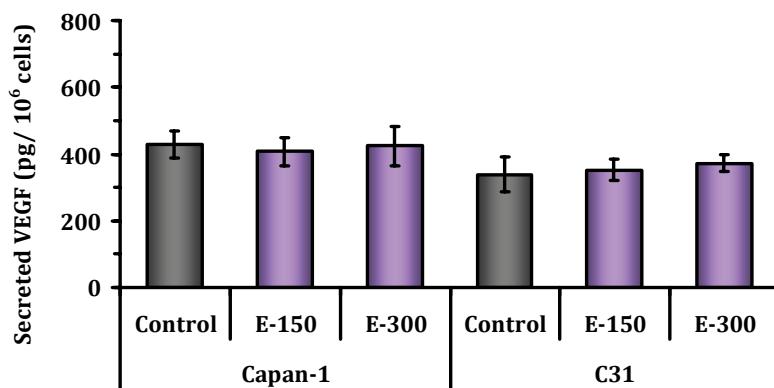
Figure 36. VEGF secretion after sE-Selectin treatment removal. Capan-1 and C31 cells were cultured and then treated with basal medium (control), 150 ng/mL sE-Selectin (E-150) or 300 ng/mL sE-Selectin (E-300). Treatment was replaced for basal medium and new supernatant collected 24 hours later. Secreted VEGF was calculated in units of pg per 10^6 pancreatic carcinoma cells. Data represents the mean ± SD of three separate experiments, each in triplicate. * Significantly different (*P < 0.01*)

3.3. VEGF secretion after sE-Selectin treatment in anti-SLe^x antibody pre-incubated cells

To investigate whether VEGF secretion on pancreatic adenocarcinoma cancer cells after sE-Selectin treatment was mediated through a SLe^x depending mechanism, Capan-1 and C31 cells were incubated with an anti-SLe^x antibody previous to sE-Selectin treatment (Figure 37). In anti-SLe^x antibody pre-treated Capan-1 cells, sE-Selectin addition did not increase VEGF secretion and was not significantly different from sE-Selectin non-treated control Capan-1 cells or anti-SLe^x antibody pre-incubated cells. Similar results were observed for C31 cells, where the 2 folds increase in VEGF secretion after sE-Selectin treatment was also completely abrogated by pre-incubating cells with anti-SLe^x antibody. These results demonstrated that sE-Selectin induced VEGF secretion in Capan-1 and C31 cells through a SLe^x depending mechanism.
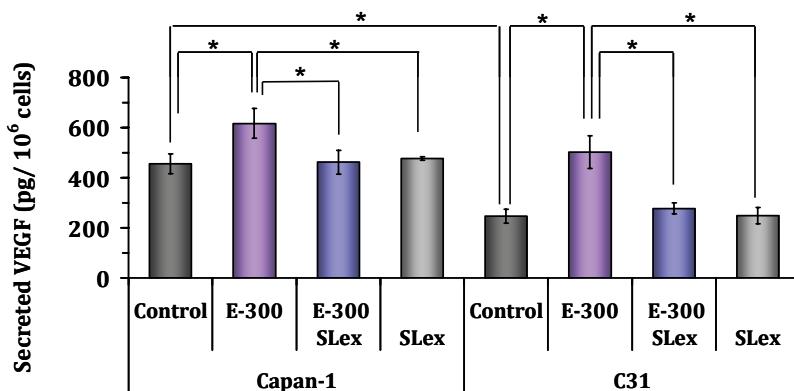
Figure 37. VEGF secretion after sE-Selectin treatment in anti-SLe^x antibody pre-incubated cells. <SLe^x> and E-300-<SLe^x> Capan-1 and C31 cells were incubated with an anti-SLe^x MAb while Control and E-300 cells were incubated with PBS-1% BSA. Cells were treated with basal medium (control) or 300 ng/mL sE-Selectin (E-300). Secreted VEGF was calculated in units of pg per 10^6 pancreatic carcinoma cells. Data represents the mean ± SD of three separate experiments, each in triplicate. * Significantly different (*P < 0.01*)

3.4. Effect of sE-Selectin on cell migration.

To study the role of sE-Selectin as a chemoattractant, 300 ng/mL of sE-Selectin were added to the lower compartment of a modified Boyden chamber (Figure 38). sE-Selectin significantly (*P*<0.01) increased parental Capan-1 cells migration by 1.3 fold. Furthermore, sE-Selectin was more chemotactic for higher SLe^x expressing C31 cells by significantly (*P*<0.01) increasing migration by 1.8 fold.
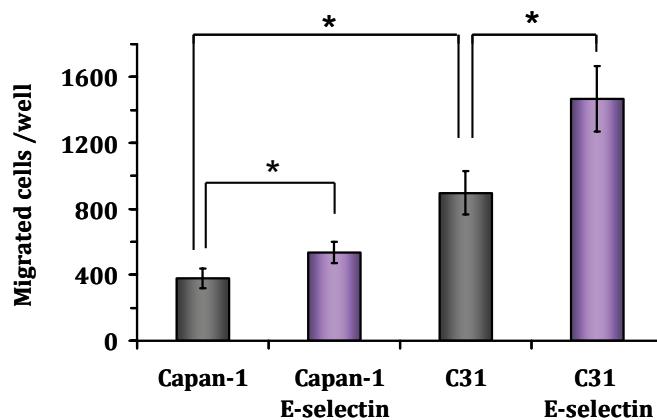
Figure 38. Effect of sE-Selectin on cell migration. cells were seeded onto 8 μm pores inserts and placed on top wells containing 300 μL of basal medium or 300ng/mL sE-Selectin in basal medium. After 6 h incubation non-migrated cells were wiped and migrated cells were fixed, stained with H&E and. Data represents the mean ± SD of three separate experiments, each in triplicate. * Significantly different (*P < 0.01*).

Discussion

1. Role of ST3Gal III and ST3Gal IV in PDAC progression

ST3Gal III, and in a lower extent ST3Gal IV, are implicated in key steps of tumour progression such as adhesion, migration and metastasis formation in Capan-1 and MDAPanc-28 PDAC cell lines.

1.1. Role of ST3Gal III and ST3Gal IV in cell surface glycosylation

Capan-1 and MDAPanc-28 transfection with ST3Gal III or ST3Gal IV genes led to an increased expression or *de novo* synthesis of SLe^x

antigen accompanied with a surface alpha 2,6 sialic decrease and a loss of non-sialylated Type II Lewis antigens when present. However, ST3Gal III overexpresion gave place to higher surface SLe^x than ST3Gal IV overexpression.

1.1.1. Increased expression or *de novo* synthesis of SLe^x

Capan-1 and MDAPanc-28 cell lines, were defective in Type I antigens which is attributed to the absence of Type I precursor structures (Galβ1-3GlcNAc) in these cells. The increased expression or *de novo* synthesis of SLe^x, could be easily explainable since ST3Gal III or ST3Gal IV acting on Type II structures (Galβ1-4GlcNAc) form the terminal NeuAcα2-3Galβ1-4GlcNAc structure, which is the SLe^x precursor. In agreement with our results, human Burkitt lymphoma cell line Namalwa transfection with ST3Gal IV (Sasaki et al. 1993) and MKN45 gastrointestinal cancer cell line transfection with ST3Gal III or ST3Gal IV (Carvalho et al. 2010) also conferred increased expression or *de novo* synthesis of SLe^x.

With regard to the surface α2,6 sialic acid decrease, it is likely explainable by the fact that the Type II structure (Galβ1-4GlcNAc) is a shared substrate for ST3Gal III, ST3Gal IV, ST6Gal I and ST6Gal II enzymes. Thus, when those enzymes are expressed in the same cell, an enzymatic competition to form NeuAcα2,3Galβ1-4GlcNAc and NeuAcα2,6Galβ1-4GlcNAc structures is expected (Dall'Olio and Chiricolo 2001; Harduin-Lepers et al. 2001; Kitagawa and Paulson 1994). In agreement with our results, some reports in the literature have previously suggested a compensation between α2,3 and α2,6 sialylation for given cells (Ulloa and Real 2001) and proteins (Ellies et al. 2002), although it has also been reported increased α2,6 sialylation

after ST6Gal I transfection without changes in overall α2,3 sialic acid expression (Yamamoto et al. 2001).

In addition to the glycosylation changes mentioned above, transfection with ST3Gal III of Capan-1 cells lead to a loss of non-sialylated Type II Lewis antigens (Le^x, H2 and Le^y). Since alpha-2,3-sialyltransferases, alpha-1,2-fucosyltransferases and alpha-1,3/4 fucosyltransferases compete for Type II structures (Galβ1-4GlcNAc) (Aubert et al. 2000; Aubert et al. 2000; Mas et al. 1998; Mejias-Luque et al. 2007; Toivonen et al. 2002), the increased expression of sialylated Type II structures due to the ST3Gal III overexpression is probably responsible for the decrease of non-sialylated Type II structures.

1.1.2. ST3Gal III and ST3Gal IV differently increased surface SLe^x levels

ST3Gal III and ST3Gal IV transfected MDAPanc-28 cells expressed respectively 7-fold and 415-fold higher levels of mRNA than corresponding control cells. Surprisingly, this higher ST3Gal IV mRNA levels did not increase surface SLe^x expression as much as ST3Gal III mRNA overexpression did; while the decrease in α2,6 sialic acid was not significantly different between both cell lines.

Both ST3Gal III and ST3Gal IV have reported to sialylate Type I Galβ1-3GlcNac, Type II Galβ1-4GlcNac and Type III Galβ1-3-GalNAc substrates with different affinity and catalytic efficiency. With regard to ST3Gal III, Type I substrates are the preferred acceptors, Type II substrates are secondary acceptors and Type III substrates are very poor acceptors (Kitagawa and Paulson 1993; Kitagawa and Paulson 1994). For ST3Gal IV, the reported substrate specificities have shown a preferential

enzymatic activity towards Type III, followed by Type II, and finally a very minor or negligible activity towards Type I substrates (Carvalho et al. 2010; Kitagawa and Paulson 1994; Sasaki et al. 1993).

MDAPanc-28 cells do not posses Type I precursors thus, ST3Gal III and ST3Gal IV enzymes compete for Type II structures to form SLe^x. When comparing both enzymes, Kitagawa and Paulson had previously observed that ST3Gal III presented higher NeuAc incorporation rate to Type II disaccharides; while ST3Gal IV showed higher NeuAc incorporation rate to Type II oligosaccharides (Kitagawa and Paulson 1994). In addition, ST3Gal III had higher specific activity towards Type II disaccharides, while presented lower affinity for them with an V_{max} = 45 pmol/h/mL and K_m = 3 mM for ST3Gal III on natural Type II disaccharides and Vmax = 0.68 nml/mL/min and K_m = 3.9 mM on synthetic Type II disaccharides. In contrast, ST3Gal IV presented an V_{max} = 43 pmol/h/mL and K_m = 0.22 mM on natural Type II disaccharides and V_{max} = 0.0028 nml/mL/min and K_m = 0.81 mM on synthetic Type II disaccharides (Kono et al. 1997; Rohfritsch et al. 2006). At the light of these data, ST3Gal III and ST3Gal IV acting on Type II disaccharides (rather than acting on longer Type II containing oligosaccharides) as well as not limited substrate conditions, could explain the higher SLe^x levels observed in M34 cells when compared to M42 ones.

In addition, ST3Gal IV may use Type III determinants as preferred substrate to form NeuAcα2-3Galβ1-4GalNAc structures (which can not be detected with the anti-SLe^x antibody), while ST3Gal III has shown little or not activity towards this substrate. This way, while ST3Gal III may be exclusively sialylating Type II structures, ST3Gal IV may be acting on both Type II and Type III structures, which could also explain the lower SLe^x levels detected in M42 cells compared to M34 ones.

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1.2. Role of ST3Gal III and ST3Gal IV in E-Selectin adhesion

Although the molecular mechanisms regulating PDAC metastasis are still poorly understood, SLe^x-E-Selectin interaction is thought to be a key step in the extravasation process of hematogenous metastatasis. SLe^x located on pancreatic adenocarcinoma cell surface could serve as ligand for the E-Selectin located on activated endothelium. This interaction may facilitate the rolling and attachment of tumour cells to the endothelial cells, promoting extravasation (Kannagi 1997; Kannagi et al. 2004; Kobayashi et al. 2007; Magnani 2004; Varki 2007).

In agreement with this, overexpression of either ST3Gal III or ST3Gal IV in Capan-1 or MDAPanc-28 cells led to an increased adhesion to rh-E-Selectin when compared to control and parental cells. In fact, the adhesion was proportional to cell surface SLe^x levels and was completely abrogated when pre-incubated cells with anti-SLe^x antibody. Furthermore, when comparing the adhesion of ST3Gal III and SLe^x overexpressing cells to Hepatic Sinusoidal Endothelial cells (HSEC), an enhanced ability to adhere to IL-1β stimulated HSE cells was observed. In agreement with our results, a direct correlation between ST3Gal III levels and colon cancer cell adhesion to IL-1β activated human umbilical vein endothelial cells (HUVEC) has been described (Dimitroff et al. 1999). Moreover, SLe^x expressing colon cancer cells have been also reported to adhere to HSEC via E-Selectin-SLe^x interaction (Matsushita et al. 1998). Furthermore, several works reported a correlation between SLe^x, and the adhesion to cytokine stimulated HUVEC in H7721 hepatocarcinoma (Liu et al. 2001; Liu et al. 2002; Wu et al. 2003; Zhang et al. 2002) and in lung adenocarcinoma cells (Martin-Satue et al. 1999).

HSEC pre-treatment with IL1-β and TNF-α cytokines significantly increased pancreatic adenocarcinoma cell adhesion to HSE cells and returned to basal levels in anti-E-Selectin pre-incubated HSE cells. Those results are consistent with previous work reporting that E-Selectin expression is low or absent in HSEC under normal conditions, but can be up-regulated by cytokines (Lalor et al. 2002), by hepatocytes (Edwards et al. 2005) or as a response to metastatic tumour cells (Khatib et al. 1999).

Adhesion of ST3Gal III and SLe^x overexpressing C31 cells to non-stimulated HSE cells was significantly greater when compared to controls. This adhesion was not reverted when pre-incubating HSE cells with anti-E-Selectin. Therefore, there is a non-E-Selectin dependent, but SLe^x-mediated, pancreatic cancer cell adhesion to non-stimulated HSE cells. This interaction may be mediated by other SLe^x binding cell adhesion molecules such as P-Selectin (Khatib et al. 1999; Paschos et al. 2010) or I-Type lectins. In agreement with our results, a previous study reported the binding of ST3Gal III overexpressing colon cancer cells to non-activated HUVEC, and the authors also suggested that this non-E-Selectin dependent SLe^x mediated adhesion could be due to other I-Type lectins (Dimitroff et al. 1999).

1.3. Role of ST3Gal III and ST3Gal IV in cell migration

Cell migration is a multistep process that plays a pivotal role in metastasis. Tumour cells need to migrate through Extracellular Matrix (ECM) in order to get out the primary tumour and through a new organ stroma to form a metastatic lesion.

To our knowledge, this is the first work on a positive correlation between cell migration and ST3Gal III/IV via SLe^x expression in PDAC. In our work, ST3Gal III and ST3Gal IV transfected cells, with higher SLe^x levels and lower surface alpha 2,6 sialic acid than parental and mock control cells, were endowed with a higher migration capability. With regard to M34 and M42 cells, which have the same surface alpha 2,6 sialic and only differ on surface SLe^x, the 1.5-fold increased migration of M34 in relation to M42 could be attributed to a 1.7-fold higher SLe^x levels in M34.

Despite the absence of studies on pancreatic cancer cells, some investigations support our findings. ST3Gal III overexpression on the glioma cell line U-374 resulted in a more invasive phenotype, through a surface α2,3-linked sialic acid increase (Yamamoto et al. 2001). Whereas, ST3Gal IV activity depression led to a decreased cell migration on the breast cancer cell line MDA_MB-231 by lowering surface α2,3 acid sialic levels (Hsu et al. 2005). Moreover, SLe^x was crucial for H7721 migration, since migration was inhibited by anti-SLe^x Mab and after sialic acid residues elimination (Zhang et al. 2002). Moreover, downregulation of 1,3FucT and ST3Gal I, III, IV in human hepatocarcinoma through *nm*23-H1 gene transfection, inhibited surface SLe^x expression causing a proportional decrease in cell migration (Duan et al. 2005; Liu et al. 2001; Liu et al. 2001; Liu et al. 2002).

The initial response of migratory cells to a migration-promoting agent is to polarize and extend protrusions. These protrusions are stabilized by extracellular matrix adhesion molecules, such as integrins, and serve as traction sites for migration as the cell moves forward over them (Casey et al. 2003). There is a rapidly growing body of evidence that demonstrates that sialylation influences migration capabilities by

modulating the integrin function (Ridley et al. 2003). Thus, we hypothesize that ST3Gal III/ST3Gal IV could be influencing cell migration by altering integrin sialylation and modulating their extracellular matrix adhesion. Nevertheless, further investigations are required to address this issue.

1.4. Role of ST3Gal III and ST3Gal IV in PDAC cells metastastic capabilities

The *in vivo* model showed higher metastatic capabilities for either ST3Gal III or ST3Gal IV overexpressing PDAC cells when compared to control cells. However, the specific role of each gene was different since ST3Gal III overexpression, which led to higher SLe^x surface levels, accounted for a more metastatic phenotype than ST3Gal IV overexpression.

1.4.1. Role of SLe^x levels in metastasis

Several studies have previously described a correlation between SLe^x levels and metastasis or poor survival in patients for different types of cancer, such as colon cancer (Nakamori et al. 1997), gastric carcinoma (Amado et al. 1998), breast cancer (Matsuura et al. 1997) and pancreatic cancer (Kishimoto et al. 1996). Along with those clinical observations, the expression levels of SLe^x on colon cancer cell surface (Bresalier et al. 1996; Cho et al. 1997; Nakamori et al. 1997) and gastric cancer cell surface (Nakashio et al. 1997) correlated with metastatic potential in animal models.

Focusing on pancreatic cancer, the SLe^x antigen has an important *in vivo* role, since an inhibitory effect in tumour establishment and metastatic

colonies growth was observed when mice were treated with anti-SLe^x antibody (Kawarada et al. 2000). In addition, SLe^x cell surface levels have also been found to be important in determining the degree of metastasis formation. Ohyama *et al.* have described how excessive expression of SLe^x in tumour cells leads to rejection by natural killer cells rather than tumour formation in a melanoma model, while moderate amounts of SLe^x lead to tumour metastasis (Ohyama et al. 2002; Ohyama et al. 1999).

In our hands, low number of Capan-1 cells with high-medium levels of SLe^x did not generate metastasis when intrasplenically injected into athymic nude mice. In contrast, MDAPanc-28 cells with low levels of SLe^x were able to form metastases in several organs, when injected at high number. However, the different behavior of these two cell lines in tumour metastasis generation may be explained by a combination of multiple factors, including gene expression pattern. Unfortunately, there are not many works comparing Capan-1 and MDAPanc-28 gene expression pattern. So far, just a differential expression of the tumour suppressor gene *DPC4* has been described in this cell lines (Grau et al. 1997). Nevertheless, genes affecting tumour progression and metastasis (Chiang and Massague 2008) such as genes codifying for cell-cell adhesion molecules (members of the immunoglobulin and calcium-dependent cadherin families and integrins), extracellular proteases and matrix metalloproteases and genes related to angionegesis (Keleg et al. 2003) would be expected to be differentially expressed in both cell lines.

1.4.2. Role of ST3Gal III and ST3Gal IV in athymic mice metastasis

When compared the survival of athymic mice injected with either ST3Gal III or ST3Gal IV overexpressing cells a large decrease in survival

with respect to mice injected with control MP cells was observed. Animal necropsy revealed macroscopical metastasis in pancreas, lymph nodes and suprarenal glands for ST3Gal III or ST3Gal IV overexpressing cells injected mice, while none of control MP injected mice did. In agreement with these data, the microscopical analysis confirmed much higher incidence of metastasis in ST3Gal III or ST3Gal IV overexpressing cells injected mice than in the MP control cells injected ones.

When comparing the role of each sialyltransferase overexpression, mice injected with M42 cells showed higher survival than mice injected with M34 cells. In adition, animal necropsy revealed macroscopic metastasic foci in spleen, kidneys, liver or lungs of M34 injected mice, while they were not observed in M42 injected mice. Although the posterior microscopical analysis revealed metastatic foci in those organs for some of M42 injected mice; microscopic metastatic incidence was much higher in ST3Gal III overexpressing cells injected mice than in ST3Gal IV injected ones. In addition, there appears to be a clear difference among these three lines tumour histology. By the time the first invasive tumours developed in MP line, more invasive tumour developed in M34 and M42 bearing mice. When comparing M34 and M42 tumour histology, tumour development seen in those mice bearing M34 cells was clearly higher in the differentiation degree and the extent of invasion than in mice bearing M42 cells.

Taken together this data, both ST3Gal III and ST3Gal IV genes seem to increase the aggressiveness of pancreatic ductal adenocarcinoma carcinoma cells. However, the overexpression of ST3Gal III, which leads to higher SLe^x surface levels, increased endothelial E-Selectin adhesion and more migratory capabilities than ST3Gal IV overexpression, accounts for an increased ability to tumour establishment, higher

ability at the time of spreading to secondary organs and decreased survival in athymic mice. Although this is the first study that shows a direct implication of ST3Gal III and ST3Gal IV in tumour and metastasis formation, other studies had pointed in this direction. The overexpression of FUT1 which competes for the same substrate as ST3Gal III and IV, showed a decrease in the metastatic potential of pancreatic adenocarcinoma (Aubert et al. 2000) and colon cancer (Mejias-Luque et al. 2007) cells after injection into nude mice.

1.4.3. Clinical relevance of ST3Gal III and ST3Gal IV

Recently, the importance of ST3Gal III in cancer progression, metastasis and poor survival for different cancers has been highlighted in some clinical studies. In the extrahepatic bile duct carcinoma ST3Gal III levels correlated with tumour advancement, differentiation and metastasis (Jin et al. 2004) while in breast cancer high ST3Gal III /ST6Gal I ratio correlated with a shorter overall survival and bad prognosis (Hebbar et al. 2003; Recchi et al. 1998). In gastric cancer, high levels of ST3Gal III in tumour tissue correlated with secondary tumour recurrence (Gretschel et al. 2003). Finally, in cervix squamous cell carcinoma ST3Gal III mRNA expression levels were significantly increased in patients with lymph node metastasis when compared to those without metastasis (Wang et al. 2002).

There is not an agreement with regard to ST3Gal IV clinical significance. While some authors reported decreased ST3Gal IV mRNA expression in gastrointestinal, ovarian and renal carcinoma (Ito et al. 1997; Wang et al. 2005; Zhang et al. 1997) others showed significant up-regulations in poorly differentiated colorectal carcinoma and cervical intraneoplasia (Kudo et al. 1998; Lopez-Morales et al. 2009). Thus, ST3Gal IV up

regulation seems to be restricted to certain types of cancers and may be related to high ST3Gal IV expression in healthy tissues (Kemmner et al. 2003). Unfortunately specific studies of the role of ST3Gal IV in pancreatic cancer tissues have not been performed up to date, but will deserve further study in light of the present results.

2. ROS as ST3Gal III and SLe^x modulators in PDAC

ROS, generated in Capan-1 and MDAPanc-28 cell lines during cell proliferation-differentiation or by external oxidant stimuli such as H_2O_2, play a role in the control of ST3Gal III and SLe^x levels and in the acquisition of a more aggressive phenotype.

2.1. Cell growth associated changes in ST3Gal III levels

In culture, both MDAPanc-28 and Capan-1 cell lines are relatively undifferentiated during the exponential growth phase and spontaneously differentiate and form domes on becoming confluent (Frazier et al., 1996; Fanjul et al., 1991; Hollande et al., 1990; Levrat et al., 1988). In these cell lines we have observed a positive correlation between ST3Gal III mRNA levels and cell proliferation; and a negative correlation between ST3Gal III and differentiation (during dome formation). As expected from our previous results, cell proliferation related increase in ST3Gal III also correlated with higher SLe^x surface expression and E-Selectin adhesion capability. Proliferation or differentiation dependent sialyltransferase and Lewis antigens levels regulation have been shown in other tissues and cell lines in agreement with our results. With regard to proliferation related changes, gastrointestinal carcinoma MKN45 confluent cultures showed higher

ST3Gal III and SLe^x / SLe^a expression than sparse ones (Carvalho et al. 2010). With regard to differentiation related changes, when inducing differentiation in the myeloid HL-60 cell line ST3Gal IV mRNA levels dramatically decreased (Taniguchi et al. 1999) and differentiation of the PHK16 immortalized keratinocyte cell line was accompanied with a decrease in hST3O/N (ST3Gal IV) mRNA levels (Taniguchi and Matsumoto 1998). Our results, and the aforementioned, collaborate in constructing a growing awareness of growth-associated changes as critical factors that should be taken into account when investigating sialyltransferases, sialyl-Lewis epitopes and selectin related cell adhesion.

2.2. Cell growth associated changes in intracellular ROS and GSH levels

For both cell lines, GSH levels were highest at initial stages of cell culture and gradually decreased over the proliferation and differentiation process. Previous studies reported GSH intracellular levels as a dynamic event function of cell proliferation and differentiation. There is not an agreement regarding to GSH-proliferation related changes, since some authors showed that actively growing cells were associated with increases in cellular GSH (Attene-Ramos et al. 2005; Estrela et al. 1992; Terradez et al. 1993), while others showed a decrease in GSH levels during the proliferation (Carretero et al. 1999; Hutter et al. 1997; Kirlin et al. 1999; Lu and Ge 1992; Nkabyo et al. 2002; Schnelldorfer et al. 2000). Regarding to GSH-differentiation related changes, several authors have reported a decrease in GSH levels associated with differentiation, in agreement with our results (Attene-Ramos et al. 2005; Benard and

Balasubramanian 1997; Kirlin et al. 1999; Liu et al. 2000; Singh et al. 2006; Stewart et al. 1997). GSH is considered the major intracellular redox buffer and plays an essential role in the maintenance of the redox homeostasis (Meister 1994). Our results showed a gradual increase in intracellular ROS levels, as GHS levels decreased, and several authors have reported a decrease in intracellular GSH content leading to an inability of cells to deal with pro-oxidant conditions (oxidative stress), and therefore to an increase in redox potential or an increase ROS production (Attene-Ramos et al. 2005; Kirlin et al. 1999; Nkabyo et al. 2002).

The decrease in GSH levels and ROS generation in Capan-1 and MDAPanc-28 cell proliferation-differentiation processes occurred simultaneously with an increase in ST3Gal III levels and SLex expression. Interestingly, a similar phenomenon could be deduced from the studies of Nkabyo et al. (Nkabyo et al. 2002) and Dall'Olio et al. (Dall'Olio et al. 1996; Dall'Olio et al. 1992) on the colon adenocarcinoma CaCo-2 cell line. The first ones described that proliferation and spontaneous differentiation processes of CaCo-2 in culture were accompanied by a continuous decrease in GSH content related to an increase in redox potential. The second ones reported an important increase in ST6Gal I levels and an enhanced alpha 2,6-sialylation of membrane glycoconjugates related to the above processes.

2.3. ROS increases ST3Gal III levels

To establish a possible link between both phenomena, the cell oxidative state and sialyltransferase expression level, the addition of nontoxic concentration of exogenous H_2O_2, that mimics the physiopathological generation of ROS, was added to sparse and confluent Capan-1 and

MDAPanc-28 cells. In both cell lines, there was a subsequent increase in ST3Gal III and SLex levels. Moreover, NAc which is a well know precursor of GHS and has been widely reported to diminish ROS levels in pancreatic cancer cells (Shi et al. 2008; Vaquero et al. 2004; Wang et al. 2009) was able to reduce ROS levels together with a decrease in ST3Gal III and SLex levels.

To our knowledge this is the first work to report how external oxidants induce ST3Gal III expression via ROS. Although little is known about the stimuli and physiological signals capable to induce sialyltransferases expression, several studies have demonstrated transcriptional regulation in cancer cells (Taniguchi 2008; Taniguchi et al. 2003) induced by some hormones (Peyrat et al. 2000), inflammation related factors (Higai et al. 2006; Yasukawa et al. 2005) and hypoxia (Koike et al. 2004). Among others, a family of factors whose regulation and function appears to be intimately linked to ROS is the NF-κB family or transcription factors (Bubici et al. 2006). In this regard, NF-κB-p65 dependent transcriptional up-regulation of the sialyltransferase ST3Gal I in human colon adenocarcinoma cell line has been described (Higai et al. 2006) and it is also known that NF-κB activation is influenced by cell confluence (Hellweg et al. 2006). These data suggest that NF-κB could be a common mediator that may relate the aforementioned processes i.e. cells redox state (ROS), sialyltransferases expression and cell confluence.

In summary, our results demonstrated that GSH (reduced glutathione) intracellular content, which is high in sparse cultures and low in confluent cultures, decreases with cell proliferation and differentiation and increases with NAc (*N*-Acetylcysteine) addition. Moreover, ROS, either generated during tumour cell metabolism or coming from an

oxidative environment, increases sialyltransferase expression and the consequent sialylated carbohydrate epitopes, which contribute to the acquisition of a more aggressive phenotype.

3. Soluble E-Selectin depending VEGF secretion and migration

Some authors have previously shown that sE-Selectin is potently angiogenic, in part through a SLe^x dependent mechanism, of human umbilical vein endothelial cells and human dermal microvascular endothelial cells (Koch et al. 1995; Kumar et al. 2003). Others, suggested that endothelial cell adhesion mediated by selectins and their carbohydrate ligands was involved in the process of angiogenesis in bovine endothelial cells and human umbilical vein endothelial cells (Kobrin et al. 1994; Nguyen et al. 1993). These studies were focused on studying angiogenesis by endothelial cells *per se*, recently, however Tei et al (Tei et al. 2002) have reported that the interaction between E-Selectin located on endothelial cells and SLe^x/SLe^a located on cancer cells surface was involved in tumour growth and angiogenesis. In agreement with Tei results, when Mathieu et al (Mathieu et al. 2007) were studying the *in vivo* impact of inhibiting SLe^x synthesis by overexpressing FUT1 on hepatocarcinoma cells (HCC) they observed, that among all parameters studied, vasculogenesis was the most clearly inhibited, corroborating that tumour angiomorphogenesis may depend on E-Selectin-SLe^x mediated interaction. To our knowledge, our data demonstrated for the first time how the direct stimulation of pancreatic cancer cells with sE-Selectin is able to induce VEGF secretion through a SLe^x depending mechanism. Supporting our findings, several authors, have previously reported that after E-Selectin-SLe^x interaction, several

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signalling transduction pathways are initiated inside the cell; and the increase in tyrosine phosphorylation of a number of proteins related to the metastatic phenotype occurs (Flugy et al. 2002; Kumar et al. 2003; Kumar et al. 2001). With regard to the chemotactic role for E-Selectin, sE-Selectin was reported to be potently chemotactic of human umbilical vein endothelial cells (Koch et al. 1995) and monocytes (Kumar et al. 2001). In agreement with our results recently a work reported that sE-Selectin induced cell migration in colon carcinoma cells in a SLe^x depending manner (Flugy et al. 2002). Taken together, our data corroborate the sE-Selectin role in cancer cell migration and is the first to describe sE-Selectin as pancreatic cancer cells chemoattractant.

Conclusions

The analysis of the results obtained in this work, has made possible to extract the following conclusions:

1. Beta-galactoside alpha-2,3-sialyltransferase 3 (ST3Gal III) and beta-galactoside alpha-2,3-sialyltransferase 4 (ST3Gal IV) overexpression lead to increased expression or *de novo* synthesis of SLe^x in the pancreatic adenocarcinoma cell lines Capan-1 and MDAPanc-28. However, ST3Gal IV overexpression does not increase surface SLe^x levels as much as ST3Gal III overexpression does.

2. ST3Gal III or ST3Gal IV overexpression induced SLe^x enhances pancreatic cancer cell adhesion to recombinant human E-Selectin (rh-E-Selectin) and to IL1-β pre-stimulated Hepatic Sinusoidal

Endothelial (HSE) cells trough a SLe^x-E-Selectin interaction. ST3Gal III or ST3Gal IV overexpression induces pancreatic cancer cells *in vitro* migration in a surface-SLe^x-levels proportional manner.

3. The *in vivo* model shows higher metastatic capabilities for either ST3Gal III or ST3Gal IV overexpressing PDAC cells when compared to the intrasplenic injection of control cells. However, ST3Gal III overexpression, leading to higher SLe^x surface levels than ST3Gal IV overexpression, accounts for a more metastatic phenotype.

4. Capan-1 and MDAPanc-28 cell lines proliferation and differentiation processes are associated to changes in ST3Gal III mRNA levels, SLe^x surface expression and a differential adhesion to rh-E-Selectin. Therefore, growth-associated changes should be taken into account as critical factors, when investigating sialyltransferases, sialyl-Lewis epitopes and selectin related adhesion.

5. Capan-1 and MDAPanc-28 pancreatic adenocarcinoma cell lines proliferation and differentiation processes are associated to changes in intracellular glutathione and Reactive Oxygen Species levels.

6. ROS generated in Capan-1 and MDAPanc-28 cell lines during cell proliferation-differentiation or by external oxidant stimuli such as H_2O_2 increase ST3Gal III and SLe^x levels which give cells a more aggressive phenotype.

7. And, together with the proadhesive role of E-Selectin for circulating cells, this work uncovers sE-Selectin dependent migration and VEGF secretion through a SLe^x depending mechanism, supporting additional prometastatic effects for sE-Selectin- SLe^x interaction.

Proposed Model

The assembly of conclusions about the role of sialyltransferases in PDAC progression derived from this thesis could fit in the tumour progression model proposed by Fidler (Fidler et al. 1978; Langley and Fidler 2007) and Fuster *et al.* (Fuster and Esko 2005) depicted on Figure 1, as follows:

a) Role of sialyltransferases in primary tumour growth and angiogenesis.

As continuous tumour cell proliferation occurs in primary tumour, reduced glutathione (GSH) content —one of the major intracellular anti-oxidants—decreases in tumour cells (Figure 39). Thus, tumour cells are not longer able to maintain redox homeostasis and their

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intracellular Reactive Oxygen species (ROS) levels increase. Increased ROS levels (which are critical signal transduction pathways regulators) activate ST3Gal III transcriptional regulation. This might occur through the nuclear factor kB (NF-kB) signal transduction pathway.
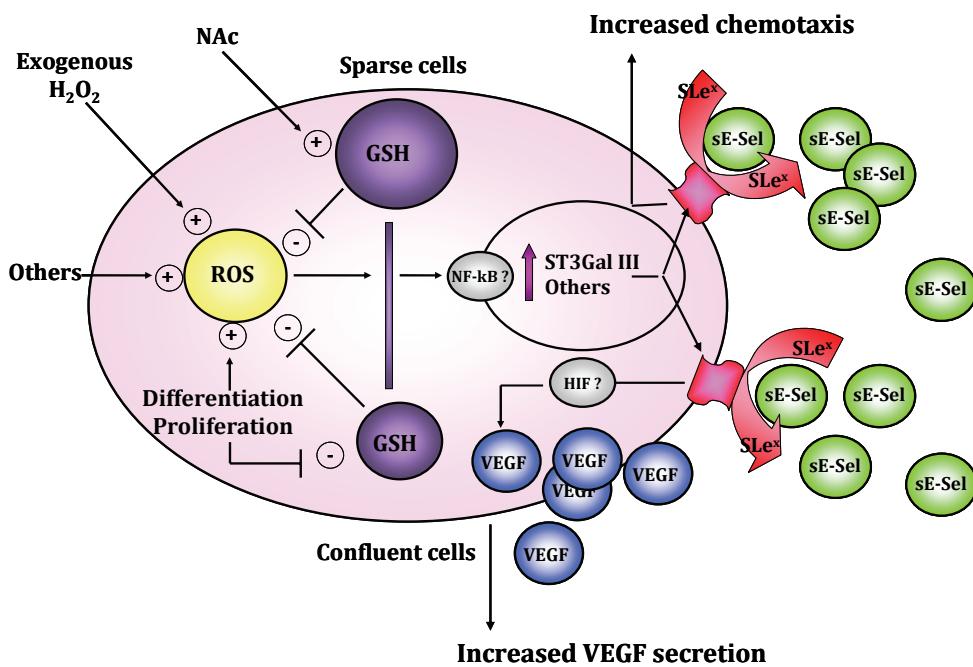
Figure 39. Proposed model to explain the role of sialyltransferases in tumour growth, angiogenesis and invasion. GSH (reduced glutathione) intracellular content, which is high in sparse cultures and low in confluent cultures, decreases with cell proliferation and differentiation and increases with NAc (*N*-Acetylcysteine) addition. In turn, intracellular ROS (Reactive Oxygen Species) levels increase leading to higher ST3Gal III mRNA levels, maybe throught NF-kB pathway activation. ST3Gal III and, in a minor extent, ST3Gal IV sialyltransferases overexpression give place to an increase in SLe^x surface levels, which correlate with the acquisition of key metastatic capabilities such as: *enhanced adhesion to soluble E-Selectin (sE-Sel)*, which in turn increases Vascular Endothelial Growth Factor (VEGF) secretion maybe throught HIF (Hypoxia inducible factor) activation and *increased cell chemotaxis* through a sE-Selectin-SLe^x depending mechanism.

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Concurrently, highly inflammatory primary tumour microenvironment is associated with ROS production (such as H_2O_2 species). These extracellular oxidants are able to diffuse into tumour cells, increasing intracellular ROS content and activating ST3Gal III transcriptional regulation. ST3Gal III and, although in a lower extent ST3Gal IV overexpression give place to an aberrant glycosylation pattern, where *N*-glycans, *O*-glycans and/or glycosphingolipids on tumour cell surface showed an increased SLe^x expression.

In addition, cytokines present in the inflamed microenvironment are able to up-regulate E-Selectin expression on vascular endothelial cell surface. Soluble isoforms of this adhesion molecule (sE-Selectin) are rapidly shed from activated endothelial surfaces and are found free on tumour microenvironment. This way, microenvironmental sE-Selectin is able to interact with its natural ligand SLe^x found in tumour cell surface.

The interaction between sE-Selectin and SLe^x is capable to activate VEGF secretion by tumour cells. This might occur through Hypoxia Inducible Factor (HIF) activation. Secreted VEGF plays a key role in establishing a neocapillary network from the surrounding endothelial capilars. Such network is crucial, since promotes primary tumour growth by supplying oxygen and nutrients and also provides thin-walled capillaries which are able to promote tumour intravasation.

b) Role of sialyltransferases in tumour cell dissociation and invasion

Cells endowed with higher levels of surface SLe^x are more migratory. Increased SLe^x on tumour cell surface imparts a negative charge which might promote cell detachment from the tumour mass through charge

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repulsion. Moreover, increased SLe^x on certain surface glycoproteins, such as integrins, could modify the interaction between tumour cells and ECM components explaining the increased migration. In addition, sE-Selectin in tumour microenvironment is chemotactic for tumour cells, inducing tumour migration towards E-Selectin expressing activated vascular endothelia.

c) Tumour cell aggregation

After entry into the vasculature, cells with higher SLe^x surface levels have an enhanced capability to form aggregates with platelets and leukocytes after Selectin-SLe^x interaction. Those aggregates increase tumour cell survival in the bloodstream by avoiding their destruction by the shear forces or the immune system. However, excessive SLe^x surface levels may conduct to emboli formation or to tumour cells rejection by Natural Killer cells.

d) Tumour cell extravasation

SLe^x-E-Selectin interaction is a key step in the extravasation process of hematogenous metastasis. SLe^x located on pancreatic adenocarcinoma cell surface promotes transient adhesion to activated venular endothelium via SLe^x-E-Selectin interaction. Tumour cell rolling velocity dramatically drops until complete arrest on vascular surfaces. The arrested tumour cells are then able to transmigrate across the vascular wall into underlying tissues.

e) Tumour cell metastasis formation

As a result of ST3Gal III and ST3Gal IV overexpression, PDAC cells present higher surface SLe^x levels and are endowed with higher growth

and angiogenic capabilites. They are also able to more easily dissociate from primary tumour and to invade the surrounding stroma. Moreover, they can aggregate with plateles and leukocytes within the circulation surviving to the immunological assault, and are capable of more easily extravasate by attaching to activated endothelia. By affecting all the tumour progression steps aforementioned, ST3Gal III and ST3Gal IV overexpression conducts ultimately to higher metastasis formation.

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