Universitat de Girona

REGULATION AND FUNCTION OF SILAYLTRANSFERASES IN PANCREATIC CANCER

Sònia Bassagañas i Puigdemont

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Sònia Bassagañas i Puigdemont

2014

Programa de Doctorat
en Ciències Experimentals i Sostenibilitat

Dirigida per la Dra. Rosa Peracaula i Miró

Memòria presentada per optar al títol de
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Universitat de Girona

Rosa Peracaula i Miró, professora titular de l'Àrea de Bioquímica i Biologia Molecular del Departament de Biologia de la Universitat de Girona,

Certifica que,

Aquest treball, titulat **Regulation and function of sialyltransferases in pancreatic cancer**, que presenta **Sònia Bassagañas i Puigdemont** per a l'obtenció del Títol de Doctora per la Universitat de Girona (UdG), ha estat realitzat sota la meva direcció i que compleix els requeriments per poder optar a **Menció Internacional**.

Perquè així consti i tingui els efectes oportuns, signo el present certificat.

Vist-i-plau

La directora de Tesi

Dra. Rosa Peracaula i Miró

Als meus pares i a la meva germana
Josep, Pilar i Íngrid

I així pren, i així pren tot el fruit que et pugui donar
el camí que, poc a poc, escrius per a demà.
Que demà, que demà mancarà el fruit de cada pas;
per això, malgrat la boira, cal caminar.

Que tinguem sort (1974)
Lluís Llach

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Publications derived from the Thesis

The results presented in this Doctoral Thesis are included in the following scientific articles, whose impact factor and position whithin category are detailed according to the last update (2012) of the Journal Citation Report:

» Bassagañas, S., Pérez-Garay, M., Peracaula, R. **Cell surface sialic acid modulates extracellular matrix adhesion and migration in pancreatic adenocarcinoma cells.**
Pancreas. 2014; 43:109-117. doi: 10.1097/MPA.0b013e31829d9090.

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» Bassagañas, S., Carvalho, S., Dias, A., Pérez-Garay, M., Ortiz, R.M., Figueras, J., Reis, C.A., Pinho, S.S., Peracaula, R. **Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of α2β1 integrin and E-cadherin function.**
PLoS One. 2014; 9:e98595. doi: 10.1371/journal.pone.0098595.

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» Bassagañas, S., Allende, H., Cobler, L., Ortiz, R.M., Llop, E., de Bolós, C., Peracaula, R. **Inflammatory cytokines regulate the expression of glycosyltransferases involved in the biosynthesis of tumor-associated sialylated determinants in pancreatic cancer.**
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Abbreviations

ANOVA	analysis of variance
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albumin
C31	ST3Gal III gene transfected Capan-1 cell line
CA 19-9	carbohydrate antigen 19-9
CAM	cell adhesion molecule
CMP	cytidine monophosphate
CN	parental Capan-1 cell line
COL	collagen
CP	empty pcDNA3.1 transfected Capan-1 cell line (mock line)
Ct	threshold cycle
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
EC	Enzyme Commission number
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epithermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial to mesenchimal transition
ER	endoplasmatic reticulum
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FN	fibronectin
Fuc	fucose
FucT	fucosyltransferase enzyme
FUT	fucotyltransferase encoding gene
Fw	forward
GA	Golgi apparatus
GAG	glycosaminoglycan
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GeoMean	geometric mean
GlcNAc	*N*-acetylglucosamine
GlcNAcT	*N*-acetylglucosamine transferase
GPI	glycosylphosphatidylinositol
GT	glycosyltransferase
h	hour
HPRT	hypoxanthineguanine phospho-ribosyltransferase
HRP	horseradish peroxidase
IF	immunofluorescence
IGF1	insulin-like growth factor-1
IHC	immunohistochemistry
IL1β	interleukin 1 beta
IL6	interleukin 6
IL8	interleukin 8
kDa	kilo Dalton
Le^a	Lewis a epitope
Le^b	Lewis b epitope
Le^x	Lewis x epitope
Le^y	Lewis y epitope
LN	laminin
M34	ST3Gal III gene transfected MDAPanc-28 cell line
MAA / MAL-II	*Maackia amurensis* agglutinin II
mAb	monoclonal antibody
Man	mannose
MEC	matriu extracel·lular (Catalan) matriz extracelular (Spanish)

MFI	mean fluorescence intensity
min	minute
MN	parental MDAPanc-28 cell line
MP	empty pcDNA3.1 transfected MDAPanc-28 cell line (mock line)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mU	milliunit
MUC	mucine
NAN 1	*Streptococcus pneumoniae* sialidase
Neu	neuraminic acid
NF-kB	nuclear factor kappa β
***N*-glycan**	*N*-(Asn)-linked oligosaccharide
o/n	overnight
OD	optical density
***O*-glycan**	*O*-linked oligosaccharide or *O*-GalNAc glycan
p	statistical significance
P	Pearson's coefficient
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate buffered saline
PBST	PBS plusTween-20 buffer
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidone
pY397 FAK	FAK phosphotyrosine 397
RT-qPCR	real time quantitative PCR
Rv	Reverse
s	second
SD	standard deviation
SDS-PAGE	dodecyl sulfate polyacrylamide gel electrophoresis
Sia	sialic acid
SLe^a	sialyl Lewis a epitope
SLe^x	sialyl Lewis x epitope
SNA	*Sambucus nigra* agglutinin
SPSS	statistical package for the social sciences
ST	sialyltransferase enzyme
ST3Gal III	α2,3-sialiltransferase ST3Gal III
TBE	tris/borate/EDTA buffer
TBP	TATA box binding protein
TBS	tris-buffered saline
TBST	tris-buffered saline 0.1% Tween-20
TCL	total cell lysate
TNFα	tumour necrosis factor α
U	unit
UV-light	ultraviolet light
v/v	volume to volume
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Resum

El càncer de pàncrees presenta un pronòstic desolador degut a la seva agressivitat i tardança en el diagnòstic. S'ha descrit que canvis en l'expressió d'antígens glucídics tipus Lewis, especialment un predomini de l'estructura sialil-Lewis x (SLe^x), així com de les glicosiltransferases implicades en la seva síntesi, correlacionen amb la seva capacitat invasiva i metastàtica. Estudis previs del nostre grup demostren que l'activitat sialiltransferasa en cèl·lules d'adenocarcinoma ductal de pàncrees (PDAC) correlaciona amb l'expressió en membrana d'antígens tipus Lewis; alhora les cèl·lules transfectades amb els gens de les α2,3-sialiltransferases ST3Gal III o ST3Gal IV, que sobreexpressen l'epítop SLe^x, presenten una major capacitat migratòria i metastàtica respecte les cèl·lules control.

Aquest treball s'ha basat, per una banda, en aprofundir en la implicació dels determinants sialilats en etapes clau del procés tumorogènic del PDAC, com són els processos d'adhesió cèl·lula-matriu extracel·lular (MEC) i cèl·lula-cèl·lula, i la invasió, estudiant també la influència de la glicosilació en la funció de glicoproteïnes de membrana involucrades en aquests processos, com les integrines i la E-cadherina. D'altra banda, s'ha abordat l'estudi de la regulació de l'expressió dels gens de sialiltransferases i fucosiltransferases involucrades en la biosíntesi dels antígens Lewis i sialilats a través de citoquines presents en l'ambient tumoral.

Amb la premissa que en les línies cel·lulars de PDAC Capan-1 i MDAPanc-28 l'expressió d'ARNm de ST3Gal III es modula al llarg de la proliferació i la densitat cel·lular, s'ha avaluat per citometria de flux com varia el contingut en membrana d'epítops glucídics sialilats. Mentre que l'expressió de SLe^x augmenta en els dos models al llarg de la proliferació cel·lular, en MDAPanc-28 els nivells d'àcid α2,6-siàlic disminueixen significativament i el contingut en àcid α2,3-siàlic es manté constant. En les cèl·lules Capan-1, en canvi, augmenten els nivells d'α2,3-siàlic i d'α2,6-siàlic. Alhora amb assaigs d'adhesió es mostra que una major proporció a la superfície cel·lular d'α2,6-siàlic afavoreix l'adhesió a proteïnes de la MEC, com col·lagen tipus 1, fibronetina o laminina. A més s'observa que els nivells d'α2,3-siàlic i SLe^x correlacionen positivament amb la migració cel·lular a través de col·lagen tipus 1, mentre que els nivells d'α2,6-siàlic hi correlacionen negativament.

Les línies de Capan-1 i MDAPanc-28 transfectades amb el gen ST3Gal III, C31 i M34 respectivament, augmenten l'expressió de SLe^x i disminueixen els nivells d'α2,6-siàlic comparat amb les línies control. Aquestes línies transfectades s'han utilitzat com a model per investigar la influència dels determinants sialilats en els procesos d'adhesió i d'invasió. Els canvis de sialilació provoquen una menor capacitat adhesiva de C31 als components de la MEC; alhora que augmenten el potencial invasiu i disminueixen la capacitat d'agregació cèl·lula-cèl·lula tant de C31 com de M34 respecte les cèl·lules control. Per entendre millor l'efecte dels canvis de sialilació sobre la capacitat adhesiva i

migratòria de les cèl·lules de PDAC, la investigació s'ha centrat en dues glicoproteïnes de membrana, la integrina α2β1, involucrada en el reconeixement de col·lagen, i E-cadherina, responsable de les unions cèl·lula-cèl·lula. A nivell estructural es demostra que la sialilació d'aquestes dues proteïnes resulta modificada en les línies transfectades amb el gen ST3Gal III, i es confirmen les diferències en el patró global de sialilació. A nivell funcional, per una banda l'epítop SLe^x modula la funció biològica de la integrina α2β1, ja que en les cèl·lules C31 promou una major activació de la via de senyalització iniciada amb la fosforilació de la tirosina 397 de la proteïna FAK després del reconeixement integrina-col·lagen, que contribueix a explicar la major capacitat migratòria d'aquestes cèl·lules. D'altra banda, amb assaigs d'immunofluorescència en monocapes cel·lulars es determina que la sobreexpressió de SLe^x té un clar efecte en el fenotip de les línies transfectades, les quals presenten unions cèl·lula-cèl·lula més febles i una certa deslocalització d'E-cadherina cap al citoplasma. Mitjançant immunofluorescència en teixits humans de diferents estadis de PDAC s'observa una evolució molt clara de la desmoplàsia i alhora la desestructuració del teixit normal, a més d'un augment de l'expressió de SLe^x, el qual colocalitza en zones específiques amb E-cadherina i, focalment, amb la subunitat α2 d'integrina.

Per determinar el possible efecte regulador de citoquines proinflamatòries sobre l'expressió de gens involucrats en la biosíntesi d'antígens de membrana tipus Lewis i sialilats, s'han tractat dues línies cel·lulars de PDAC, MDAPanc-28 i MDAPanc-3, amb concentracions conegudes d' IL-1β, IL-6, IL-8 o TNFα. Els tractaments de MDAPanc-28 amb IL-1β provoquen un increment en l'expressió d'ARNm dels gens ST3Gal III, ST3Gal IV, ST6Gal I, FUT5, FUT6 i FUT7, fet que condueix a explicar l'increment dels nivells de SLe^x i α2,6-siàlic en membrana. De manera similar, en MDAPanc-3 el tractament amb IL-6 o TNFα incrementa l'expressió d'ARNm dels gens ST3Gal III, ST3Gal IV i FUT6, el que també comporta un augment en la biosíntesi de SLe^x i Le^y en aquestes cèl·lules. En teixits humans de PDAC s'observa que l'expressió dels epítops SLe^x o SLe^a sol ésser més elevada en teixits amb major presència de cèl·lules inflamatòries.

Els resultats obtinguts en aquesta Tesi Doctoral demostren que els determinants sialiats com el SLe^x modulen l'adhesió de la cèl·lula tumoral a altres cèl·lules i a components de la MEC, així com la seva capacitat migratòria i invasiva, i regulen la funció biològica d'importants receptors de membrana com E-cadherina i α2β1 integrina, involucrats en aquests procesos. Alhora, en aquest estudi es posa de manifest que les cèl·lules tumorals de càncer de pàncrees treuen profit de les característiques de l'ambient inflamatori que acompanya el tumor, ja que les citoquines acceleren la progressió tumoral regulant l'expressió de gens de glicosiltransferases específiques que contribueixen a la biosíntesi de determinats glucídics com SLe^x. És important entendre que l'estat inflamatori associat al càncer és un aspecte important a tenir en compte a l'hora de dissenyar estratègies innovadores per millorar el mal pronòstic dels pacients de càncer de pàncrees.

Resumen

El cáncer de páncreas presenta un pronóstico desolador debido a su agresividad y tardanza en el diagnóstico. Se ha descrito que cambios en la expresión de antígenos glucídicos tipo Lewis, especialmente un predominio de la estructura sialil-Lewis x (SLe^x), así como de las glicosiltransferasas implicadas en su síntesis, correlacionan con su capacidad invasiva y metastásica. Estudios previos de nuestro grupo demuestran que la actividad sialiltransferasa en células de adenocarcinoma ductal de páncreas (PDAC) correlaciona con la expresión en membrana de antígenos tipo Lewis; además, las células transfectadas con los genes de las α2,3-sialiltransferasas ST3Gal III o ST3Gal IV, que sobreexpresan el epítopo SLe^x, presentan una mayor capacidad migratoria y metastásica con respecto a las células control. Este trabajo se ha basado, por un lado, en profundizar en la implicación de los determinantes sialilados en etapas clave del proceso tumorogénico del PDAC, como los procesos de adhesión célula-matriz extracelular (MEC) y célula-célula, y la invasión, estudiando también la influencia de la glicosilación en la función de glicoproteínas de membrana involucradas en estos procesos, como las integrinas y la E-cadherina. Por otro lado, se ha abordado el estudio de la regulación de la expresión de los genes de sialil- y fucosiltransferasas responsables de la biosíntesis de antígenos Lewis y sialilados a través de citoquinas presentes en el ambiente tumoral. Con la premisa de que en las líneas celulares de PDAC Capan-1 y MDAPanc-28 la expresión de ARNm de ST3Gal III se modula a lo largo de la proliferación y la densidad celular, se ha evaluado por citometría de flujo como varía el contenido en membrana de epítopos sialilados. Mientras que la expresión de SLe^x aumenta en los dos modelos a lo largo de la proliferación celular, en MDAPanc-28 los niveles de ácido α2,6-siálico disminuyen significativamente y el contenido en ácido α2,3-siálico se mantiene constante. En las células Capan-1, en cambio, aumentan los niveles de α2,3- y α2,6-siálico. Asimismo, con ensayos de adhesión se muestra que una mayor proporción en superficie celular de α2,6-siálico favorece la adhesión a proteínas de la MEC, como colágeno tipo 1, fibronectina o laminina. Además se observa que los niveles de α2,3-siálico y SLe^x correlacionan positivamente con la migración celular a través de colágeno, mientras que los niveles de α2,6-siálico correlacionan negativamente. Las líneas de Capan-1 y MDAPanc-28 transfectadas con el gen ST3Gal III, C31 y M34 respectivamente, aumentan la expresión de SLe^x y disminuyen los niveles de α2,6-siálico comparado con las líneas control. Estas líneas transfectadas se han utilizado como modelo para investigar la influencia de los determinantes sialilados en los procesos de adhesión y de invasión. Los cambios de sialilación provocan una menor capacidad adhesiva de C31 a los componentes de la MEC, y además aumentan el potencial invasivo y disminuyen la capacidad de agregación célula-célula tanto de C31 como de M34 con respecto a las células control. Para entender mejor el efecto de los cambios de sialilación

sobre la capacidad adhesiva y migratoria de las células de PDAC, la investigación se ha centrado en dos glicoproteínas de membrana, la integrina α2β1, involucrada en el reconocimiento de colágeno, y E-cadherina, responsable de las uniones célula-célula. A nivel estructural, se demuestra que la sialilación de estas dos proteínas resulta modificada en las líneas transfectadas con el gen ST3Gal III, y se confirman las diferencias en el patrón global de sialilación. A nivel funcional, por un lado el epítopo SLe^x modula la función biológica de la integrina α2β1, ya que en las células C31 promueve una mayor activación de la vía de señalización iniciada con la fosforilación de la tirosina 397 de la proteína FAK después del reconocimiento integrina-colágeno, que contribuye a explicar la mayor capacidad migratoria de estas células. Por otro lado, con ensayos de inmunofluorescencia en monocapas celulares se determina que la sobreexpresión de SLe^x tiene un claro efecto en el fenotipo de las líneas transfectadas, las cuales presentan uniones célula-célula más débiles y una cierta deslocalización de E-cadherina hacia el citoplasma. Mediante inmunofluorescencia en tejidos humanos de diferentes estadios de PDAC se observa una evolución muy clara de la desmoplasia, a la vez que la desestructuración del tejido normal y un aumento de la expresión de SLe^x, que colocaliza en zonas específicas con E-cadherina y, focalmente, con la subunidad α2 de integrina. Para determinar el posible efecto regulador de citoquinas proinflamatorias sobre la expresión de genes involucrados en la biosíntesis de antígenos de membrana tipo Lewis y sialilados, se han tratado dos líneas celulares de PDAC, MDAPanc-28 y MDAPanc-3, con concentraciones conocidas de IL-1β, IL-6, IL-8 o TNFα. Los tratamientos de MDAPanc-28 con IL-1β provocan un incremento en la expresión de ARNm de los genes ST3Gal III, ST3Gal IV, ST6Gal I, FUT5, FUT6 y FUT7, que explicaría el aumento de los niveles de SLe^x y α2,6-siálico en membrana. De modo similar, en MDAPanc-3 el tratamiento con IL-6 o TNFα incrementa la expresión de ARNm de los genes ST3Gal III, ST3Gal IV y FUT6, lo que también comporta una mayor biosíntesis de SLe^x y Le^y en estas células. En tejidos humanos de PDAC se observa que la expresión de los epítopos SLe^x o SLe^a suele ser más elevada en tejidos con mayor presencia de células inflamatorias. Los resultados obtenidos en esta Tesis Doctoral demuestran que los determinantes sialilados como el SLe^x modulan la adhesión de la célula tumoral a otras células y a componentes de la MEC, así como su capacidad migratoria y metastásica, y regulan la función biológica de importantes receptores de membrana como E-cadherina y α2β1 integrina, involucrados en estos procesos. En este estudio también se pone de manifiesto que las células tumorales de cáncer de páncreas aprovechan las características del ambiente inflamatorio que acompaña el tumor, ya que las citoquinas aceleran la progresión tumoral regulando la expresión de genes de glicosiltransferasas específicas que contribuyen a la biosíntesis de determinantes glucídicos como SLe^x. Es importante entender que el estado inflamatorio asociado al cáncer es un aspecto importante a tener en cuenta a la hora de diseñar estrategias innovadoras para mejorar el mal pronóstico de los pacientes de cáncer de páncreas.

Summary

Pancreatic cancer presents a poor prognosis due to its aggressiveness and delay in diagnosis. Changes in the expression of Lewis-type glycan antigens, especially a predominance of sialyl-Lewis x (SLe^x) structure, and the glycosyltransferases involved in its synthesis, correlate with the invasive and metastatic capacity. Previous studies from our group have shown that the sialyltransferase activity in pancreatic ductal adenocarcinoma (PDAC) cells correlates with the expression of Lewis-type membrane antigens; in addition, the cells transfected with the genes of the α2,3-sialyltransferases ST3Gal III or ST3Gal IV, which overexpress SLe^x epitope, show higher migratory and metastatic capacity with respect to control cells.

This study focuses on the involvement of sialylated determinants on key stages of PDAC tumourigenesis, such as cell-extracellular matrix (ECM) and cell-cell adhesion, and invasion; and also on the influence of glycosylation in the function of membrane glycoproteins involved in these processes, such as integrins and E-cadherin. This Thesis also deals with the influence of the cytokines present in the tumour environment in the regulation of the expression of sialyltransferase and fucosyltransferase genes involved in the biosynthesis of Lewis-type and sialylated antigens.

Taking into account that in the PDAC cell lines Capan-1 and MDAPanc-28 the ST3Gal III mRNA expression is modulated along cell proliferation and cell density, changes in the membrane content on sialylated glycan epitopes have been evaluated by flow cytometry. While the expression of SLe^x increases in the two models along cell proliferation, in MDAPanc-28 the levels of α2,6-sialic acid significantly decrease and the α2,3-sialic acid content remains constant. In Capan-1 cells, however, there is an increase in the levels of both α2,3-sialic and α2,6-sialic acid. Moreover, the results of binding assays show that a higher content on cell surface α2,6-sialic promotes adhesion to ECM proteins, such as type 1 collagen, fibronectin or laminin. In addition, in our cell models the α2,3-sialic and SLe^x levels positively correlate with cell migration through type 1 collagen, whereas α2,6-sialic acid levels negatively correlate with this process.

Capan-1 and MDAPanc-28 cell lines transfected with the ST3Gal III gene, C31 and M34 respectively, increase the expression of SLe^x levels and decrease the levels of α2,6-sialic compared to the respective control lines. These transfectants have been used as models to investigate the influence of the sialylated determinants in adhesion and invasion processes. The changes in sialylation result in lower adhesive capacity of C31 cells to ECM components; and they increase the invasive potential and diminish the cell-cell aggregation capacity in both C31 and M34 cells, with respect to the control cells. To better understand the effect of changes in sialylation on the adhesiveness and migratory behaviour of PDAC cells, the research focuses on two membrane glycoproteins, α2β1 integrin,

involved in the recognition of collagen, and E-cadherin, responsible for cell-cell junctions. At the structural level, the sialylation status of these two proteins is shown to be modified in the ST3Gal III transfected cells, and confirmed the differences in the overall sialylation pattern. At the functional level, SLe^x epitope modulates the biological function of α2β1 integrin, since in C31 cells it promotes higher activation of the signalling pathway initiated by the phosphorylation of tyrosine 397 of FAK protein after integrin-collagen recognition, what contributes to explain the greater migratory capacity of these cells. Moreover, immunofluorescence assays with cell monolayers show that SLe^x overexpression clearly affects the phenotype of the transfected lines, which show weaker cell-cell junctions, along with E-cadherin delocalization to the cytoplasm. Immunofluorescence microscopy from human tissues at different stages of PDAC show the evolution of desmoplasia as well as a disruption of the healthy tissue organization, in addition to increased SLe^x expression, which colocalizes with E-cadherin in specific areas and, focally, with integrin α2 subunit.

To determine a possible regulatory effect of pro-inflammatory cytokines on the expression of certain genes involved in the biosynthesis of Lewis-type and sialylated membrane antigens, MDAPanc-28 and MDAPanc-3 PDAC cell lines have been treated with known concentrations of IL-1β, IL-6, IL-8 or TNFα. MDAPanc-28 treatments with IL-1β cause an increase in the mRNA expression of ST3Gal III, ST3Gal IV, ST6Gal I, FUT5, FUT6 and FUT7 genes, what explains the increased levels of SLe^x and α2,6-sialic at the cell membrane. Similarly, IL-6 and TNFα treatments in MDAPanc-3 increases mRNA expression of ST3Gal III, ST3Gal IV and FUT6 genes, what leads to an increase in the biosynthesis of SLe^x and Le^y in these cells. In human PDAC tissues, the expression of SLe^x and SLe^a epitopes is higher in the tissues with a superior degree of inflammation.

The results obtained in this Doctoral Thesis demonstrate that the sialylated determinants such as SLe^x modulate the adhesion of tumour cells to other cells and to ECM components, as well as cell migratory and invasive capacities, and thus regulate the biological function of important membrane receptors such as E-cadherin and α2β1 integrin, involved in these processes. This study also describes that pancreatic tumour cells take advantage of the inflammation environment that accompanies the tumour, given that cytokines can accelerate the tumour progression regulating the expression of specific glycosyltransferase genes that contribute to the biosynthesis of glycan determinants such as SLe^x. It is important to highlight that the inflammatory status associated with cancer is an important feature to take into consideration when designing innovative strategies to improve the poor prognosis of pancreatic cancer patients.

Introduction

1 Cancer

Cancer is a leading cause of death worldwide due to its incidence, prevalence and survival. This illness accounted for 8.2 million deaths in 2012, and is projected to continue rising to over 22 million within the next two decades (Ferlay *et al.*, 2013), influenced in part by the increase and aging of the global population. In most developed countries, cancer is the second largest cause of death after cardiovascular diseases. Currently, cancer causes 20% of deaths in the European Region and one in four deaths in the United States (Siegel *et al.*, 2014). Since at least one-third of all cancer cases are preventable, prevention and early diagnosis are the most cost-effective long-term strategies for the control of this illness (Greenberg *et al.*, 2010). There is, therefore, an urgent need for an improved understanding of the mechanisms that contribute to tumour growth and metastasis, and for the design of more effective therapies for this disorder than current ones.

Cancer is the generic term used to define a large group of diseases characterized by uncontrolled and accelerated proliferation of the cells due to a deregulation in its growth control mechanisms, contributing to a loss in tissue homeostasis. When cells grow out of control, they form a mass called **tumour**. Approximately 90% of human tumours are originated from epithelial cells, perhaps because most of the cell proliferation in the body occurs in epithelia or because epithelial tissues are most frequently exposed to carcinogens; and are known as **carcinomas**. Adenocarcinomas are carcinomas which originate in a glandular tissue, and are especially found in colon, lung, ovarian, breast, prostate and pancreatic tissues, among others.

Although there are more than 100 different types of cancer, and subtypes of tumours can be found within specific organs, ten essential alterations or skills have been suggested for cells to become malignant: 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, 2011); altered glycosylation (Dube and Bertozzi, 2005; Fuster and Esko, 2005); sustained inflammation (Colotta *et al.*, 2009); altered metabolism (Tennant *et al.*, 2009) and interactions with the tumour stroma (Pietras and Ostman, 2010).

1.1 Stages of tumourigenesis and metastasis

Tumourigenesis and tumour progression are sequential and selective processes, and also contain stochastic elements, since the outcome of the process is dependent on both the intrinsic properties of the tumour cells and the response of the organ environment. The major steps in the pathogenesis of metastasis are often described as the *metastatic cascade* and summarized as follows (Brooks *et al.*, 2010; Fuster and Esko, 2005; Gupta and Massague, 2006; Langley and Fidler, 2007) (**Figure 1**):

(a) An initial transforming event, mostly a **mutation** in protooncogenes, tumour suppressor genes and/or DNA repair genes, induces a progressive growth of the neoplastic cells, with nutrients for the expanding tumour mass initially supplied by simple diffusion. A tumour mass which enlarges only at the site where it began is known as benign tumour.

(b) The primary compensatory mechanism employed by tumour cells to offset increasing metabolic pressure in a tumour mass exceeding 2 mm in diameter involves **angiogenesis**, i.e. the recruitment of resident microvascular endothelial cells to form new vascular networks. Hypoxia is a strong selective pressure which activates genes that promote angiogenesis, such as vascular endothelial growth factor (VEGF), anaerobic metabolism, cell survival and invasion (Harris, 2002; McMahon, 2000; Plate *et al.*, 1994).

(c) Since cell division is not accompanied by basement membrane extension, piling up of epithelial cells and disorganization of normal tissue architecture leads to the selection of anchorage-independent cells and development of a malignant tumour (Ingber, 2008). Specific cells may gain the ability to detach from the primary tumour and start local invasion, by remodelling their surface adhesion receptors and the extracellular matrix (ECM) through the secretion of proteolytic enzymes such as metalloproteases.

(d) Thin-walled capillaries or lymphatic channels offer low resistance to penetration by tumour cells, and can therefore provide a common pathway for **intravasation**. The vast majority of circulating tumour cells is rapidly destroyed, but the interaction with host lymphocytes or with platelets may prolong their survival by protecting them from hemodynamic shear forces and immune-mediated killing (Biggerstaff *et al.*, 1999).

(e) The survival cells must mimic the homing of leukocytes in order to get arrested in the capillary beds of organs (Gout *et al.*, 2008; Kannagi, 1997). Afterwards they can proliferate within the vessel or extravasate.

(f) The settlement and proliferation within a new organ generates a **secondary tumour** and completes the metastatic process. Micrometastases must develop a vascular network (angiogenesis) and continue to evade the host immune system. Metastatic cells can invade, penetrate blood vessels and enter into the circulation to produce additional metastases.

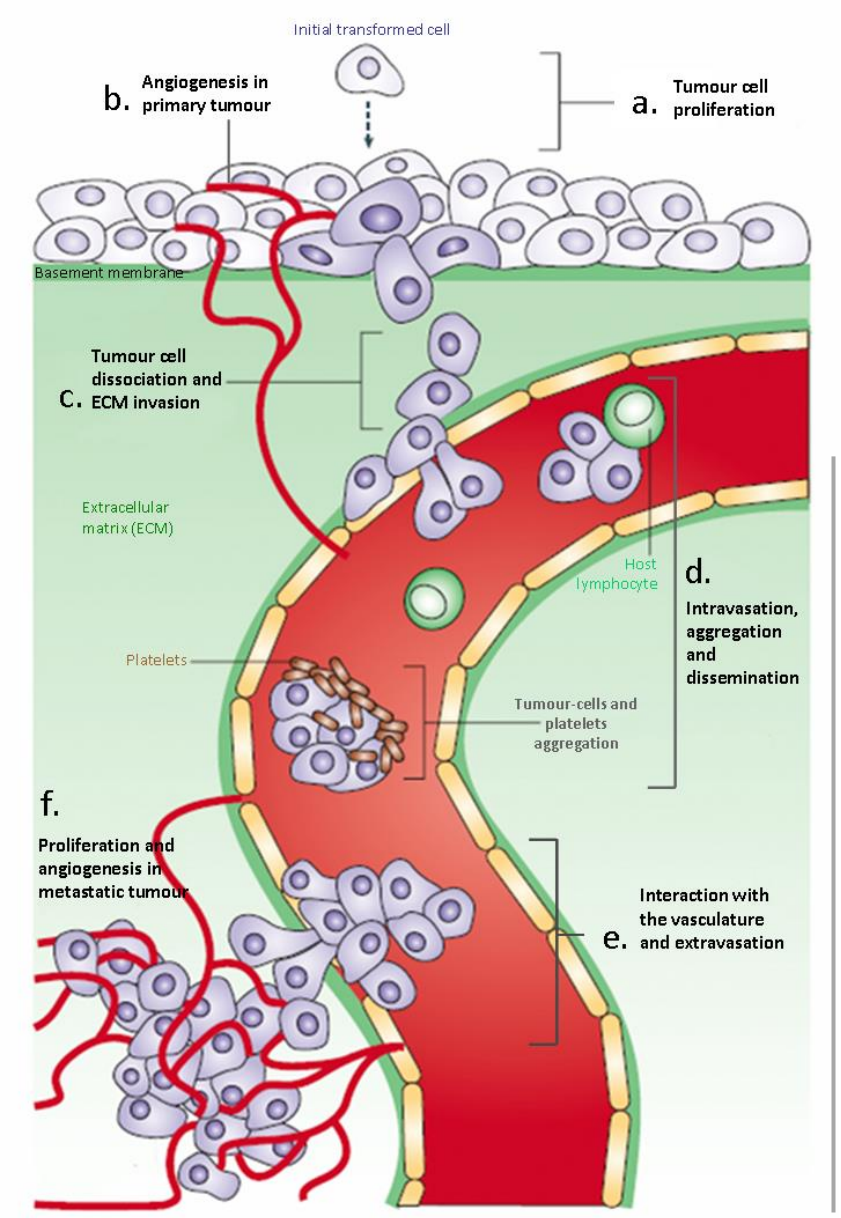


Figure 1. Stages of tumour progression.
a| Uncontrolled proliferation of a transformed cell is crucial at early stages. **b|** Tumour angiogenesis is required for pathological growth of the primary tumour. **c|** Tumour cells gain the capacity to degrade and migrate through the basement membranes and extracellular matrix. **d|** Tumour cells intravasate and disseminate through the bloodstream and form aggregates with blood cells. **e|** Tumour cells mimic the homing of lymphocytes in order to extravasate. **f|** Tumour cells proliferate and develop a vascular network forming secondary tumours (metastasis). See text for further details.
Extracted and modified from Fuster and Esko, 2005.

Metastatic cells need to successfully complete the entire *cascade*, and the vast majority is unable to do so (Wong *et al.*, 2001). Latest research has evidenced that the *metastatic competence* of tumour cells may be hardwired from an early stage, supporting the theory of **cancer stem cells** (Wicha *et al.*, 2006).

Not surprisingly, the molecular mechanisms that propel invasive growth and metastasis are also found in embryonic development and, however to a different extent, in adult tissue maintenance and repair processes (Bogenrieder and Herlyn, 2003).

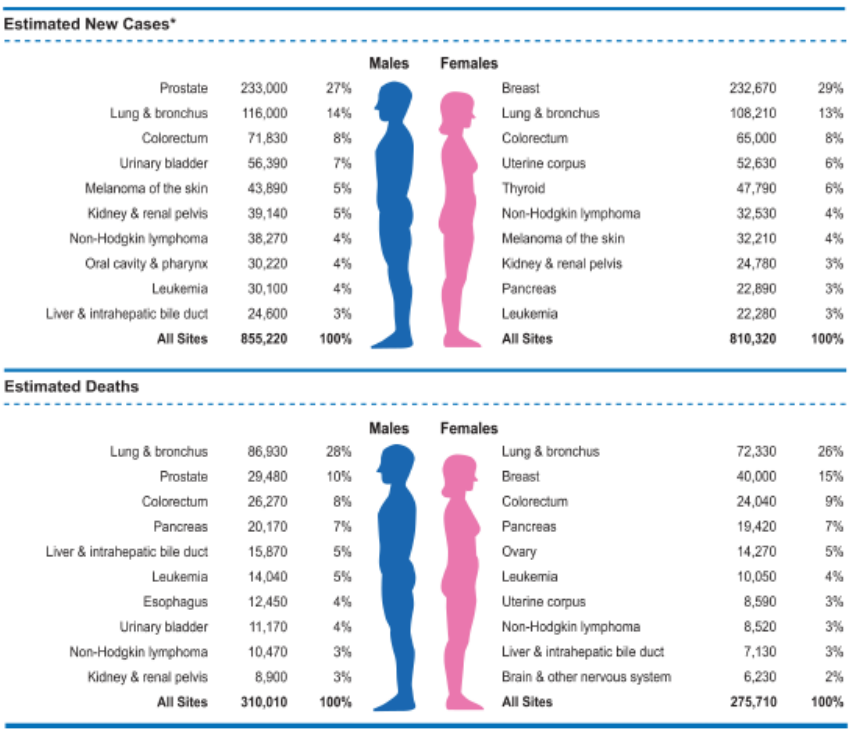
Although there are a few common features for all cancers, the outcome of metastasis is dependent on the cross-talk between tumour cells and their receptive tissues. The *seed and soil hypothesis* proposed by the English surgeon Stephen Paget in 1889 is now widely accepted, explaining that metastases result only when certain tumour cells (*the seed*) and the milieu of certain organs (*the soil*) are compatible (Fidler *et al.*, 2007; Langley and Fidler, 2011; Paget, 1889).

In general, localized tumours can be removed effectively by surgery or radiotherapy, while once tumour cells have metastized finding a cure is much more difficult because the malignity becomes systemic. In fact, metastases are the cause of 90% of solid tumour cancer deaths (Hanahan and Weinberg, 2011); but its stochastic nature does not exclude the possibility of predicting the likelihood of a successful completion.

1.2 Pancreatic cancer

The pancreas (derived from the Greek words *pan* meaning *all* and *creas* meaning *flesh*) is a glandular organ in the digestive and endocrine systems of vertebrates, extending from the cavity of the duodenum to the spleen, behind the stomach. It is a dual-function gland: on one hand, the endocrine component, which represents 1% of the total mass, is made up of different cell populations grouped into the islets of Langerhans and produces several important hormones, including insulin, glucagon and somatostatin; on the other hand, it is an exocrine gland because acinar cells secrete pancreatic juice containing digestive enzymes through the ductal system, constituted by ductal cells, into the duodenum, where assists the absorption of nutrients and the digestion. The pancreas is divided into four anatomically distinct regions: head, neck, body and tail.

Pancreatic ductal adenocarcinoma (PDAC) is the commonest cancer affecting the exocrine pancreas. While pancreatic cancer represented only around 3% of all new cancer cases in 2011, it is the fourth leading cause of cancer death in Europe and the United States of America, both in men and women. Contrarily to most types of cancer, which have improved notably their survival over the past 30 years, it is the only examined neoplasm with a negative outlook in both sexes, with the lowest 5-year survival rate (about 6%) and with a median survival of three to six months after diagnosis. This makes pancreatic cancer a priority in research and control (Malvezzi *et al.,* 2014; Schneider *et al.*, 2005; Siegel *et al.*, 2012; Siegel *et al.*, 2013; Siegel *et al.*, 2014; Vincent *et al.*, 2011) (**Figure 2**).



Estimated New Cases*

	Males			Females	
Prostate	233,000	27%		Breast	232,670	29%
Lung & bronchus	116,000	14%		Lung & bronchus	108,210	13%
Colorectum	71,830	8%		Colorectum	65,000	8%
Urinary bladder	56,390	7%		Uterine corpus	52,630	6%
Melanoma of the skin	43,890	5%		Thyroid	47,790	6%
Kidney & renal pelvis	39,140	5%		Non-Hodgkin lymphoma	32,530	4%
Non-Hodgkin lymphoma	38,270	4%		Melanoma of the skin	32,210	4%
Oral cavity & pharynx	30,220	4%		Kidney & renal pelvis	24,780	3%
Leukemia	30,100	4%		Pancreas	22,890	3%
Liver & intrahepatic bile duct	24,600	3%		Leukemia	22,280	3%
All Sites	**855,220**	**100%**		**All Sites**	**810,320**	**100%**

Estimated Deaths

	Males			Females	
Lung & bronchus	86,930	28%		Lung & bronchus	72,330	26%
Prostate	29,480	10%		Breast	40,000	15%
Colorectum	26,270	8%		Colorectum	24,040	9%
Pancreas	20,170	7%		Pancreas	19,420	7%
Liver & intrahepatic bile duct	15,870	5%		Ovary	14,270	5%
Leukemia	14,040	5%		Leukemia	10,050	4%
Esophagus	12,450	4%		Uterine corpus	8,590	3%
Urinary bladder	11,170	4%		Non-Hodgkin lymphoma	8,520	3%
Non-Hodgkin lymphoma	10,470	3%		Liver & intrahepatic bile duct	7,130	3%
Kidney & renal pelvis	8,900	3%		Brain & other nervous system	6,230	2%
All Sites	**310,010**	**100%**		**All Sites**	**275,710**	**100%**

Figure 2. Ten leading cancer types for the estimated new cancer cases and deaths by sex, USA, 2014.
* Estimates are rounded to the nearest 10 and exclude basal cell and squamous cell skin cancers and *in situ* carcinoma except urinary bladder.
Extracted from Siegel *et al.,* 2014.

The location among vital structures makes medical palpation and biopsies difficult, with the aggravating circumstance that there is no diagnostic method sensitive enough to allow detection at early stages of the disease. In addition, the high metastatic potential of tumour cells, in conjunction with their resistance to radiotherapy or chemotherapy, explain the poor outcome of pancreatic cancer (DiMagno, 1999). Except where the primary tumour is located in the head of the pancreas -the patient may present early with signs of biliary obstruction- most patients (approximately 85%) are diagnosed at advanced stages of disease, usually when cancer is locally advanced or distant metastasis is present. Therefore, only a minority of patients (10-15%) can undergo potentially curative surgery (**Figure 3**).

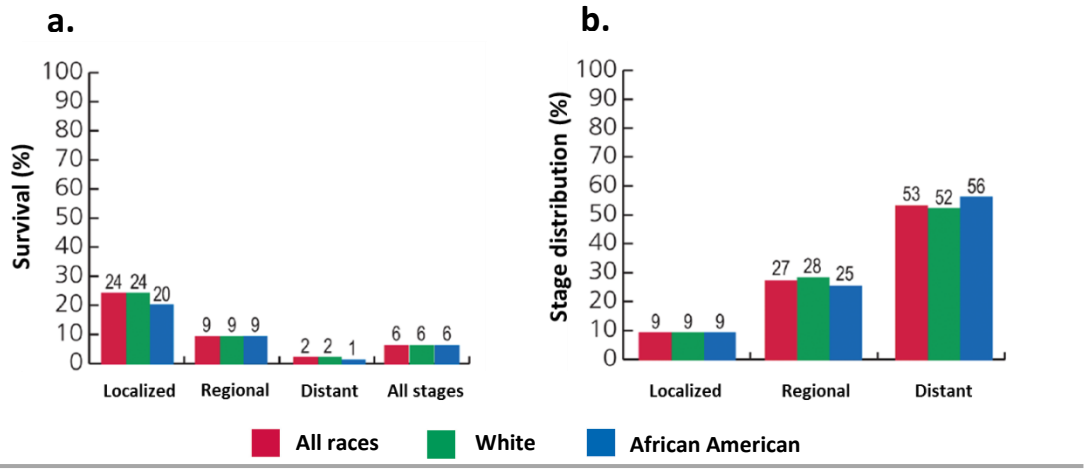


Figure 3. Pancreatic cancer survival and stage distribution statistics, United States.
a|Five-year relative survival rates for pancreatic cancer by race and stage at diagnosis, from 2003 to 2009. **b|**Stage distribution of pancreatic cancer by race, from 2003 to 2009. Extracted and modified from Siegel *et al.,* 2014.

The classical tumour markers CA 19-9 antigen -named after the monoclonal antibody used to detect the carbohidrate epitope SLe^a, mainly found on large mucine-like molecules- (Magnani *et al.*, 1983) and CEA -carcinoembryonic antigen- (Ona *et al.*, 1973) have largely contributed to the management of pancreatic cancer treatment as clinical indicators in monitoring responses to therapy, but not as diagnostic markers, due to their false positive results. Several other serologic markers have been studied, including CEACAM1 (Simeone *et al.*, 2007), macrophage inhibitory cytokine 1 (MIC1) (Koopmann *et al.*, 2006) and MUC1 (Gold *et al.*, 2006), among others. Unfortunately, none of these markers have achieved the levels of sensitivity and specificity necessary to be recommended as a screening tool for asymptomatic patients among the general population.

Current imaging studies such as abdominal Computed Tomography (CT), abdominal Magnetic Resonance Imaging (MRI) or transabdominal ultrasound are inadequate for the detection of pancreatic cancer at an early stage, because these imaging techniques do not reliably detect tumours <1-2 cm in size. To date, Endoscopic Ultrasound (EUS) and helical Computed Tomography (CT) scans are the most sensitive imaging modalities for detecting pancreatic tumours, and should be considered first line when there is a clinical suspicion of pancreatic cancer (Brand *et al.*, 2007; Chakraborty *et al.*, 2011; Goggins, 2007; Hamada and Shimosegawa, 2011; Klapman and Malafa, 2008).

PDAC produces a highly sclerotic mass, with poorly defined edges and long tongues of carcinoma extending well beyond the main tumour, and it may spread to the duodenum, bile duct or peripancreatic tissues and further into the stomach, spleen, colon or adjacent large blood vessels. Lymphatic invasion can be a feature in some cases. Distant metastases may arise mainly in liver, peritoneum and lungs (Maitra and Hruban, 2008).

Analogous to other epithelial cancers, PDAC does not arise *de novo* but rather undergoes a stepwise progression through histologically well-defined non-invasive precursor lesions, culminating in invasive neoplasia. It is also characterized by an intense fibrotic reaction, known as tumour **desmoplasia**. Therefore, an inflammatory environment may provide a landscape that promotes the transformation of epithelial pancreatic cells towards a neoplastic phenotype, which will eventually result in PDAC. In 1999, the National Cancer Institute accepted by consensus a nomenclature scheme for precursor lesions of PDAC: the **Pancreatic Intraepithelial Neoplasia** (PanIN). It was first proposed by Klimstra and Longnecker and has become a gold standard at academic centers worldwide. PanINs are microscopic lesions (<5 mm) in the smaller pancreatic ducts, which can be papillary or flat, and they are composed of columnar to cuboidal cells with varying amounts of mucins. PanINs are classified into PanIN-1 lesions, presenting hyperplasia but not dysplasia, and with the columnar cells becoming flat (1A) or with many papillary infoldings (1B); PanIN-2 lesions, with a slight loss of nuclear polarity; and finally PanIN-3 lesions,

typically papillary or micropapillary, also referred as severe dysplasia, *in situ* carcinoma or intraductal carcinoma (Hruban *et al.*, 2001; Hruban *et al.*, 2004; Koorstra *et al.*, 2008a) (**Figure 4**).

But this linear and progressive model has not been conclusively demonstrated, and alternative models propose a direct progression from normal ductal epithelium to PanIN-2 state (Real, 2003), or the appearance of ductal complexes as intermediate states that may lead to PDAC (Hernandez-Munoz *et al.*, 2008). These hypothetical models require further investigation. In addition to microscopic PanIN lesions, nowadays two recognized macroscopic/cystic precursor lesions to PDAC exist, which include intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCN) (Delpu *et al.*, 2011; Koorstra *et al.*, 2008b).

A genetic progression model for PDAC has also been developed, which incorporates genomic, transcriptomic and proteomic abnormalities implicated in the development of this malignancy. A compendium of alterations in oncogenes, tumour suppressor genes and genome-maintenance genes defines PDAC and differentiates this malignancy from other neoplasms (Koorstra *et al.*, 2008b; Maitra and Hruban, 2008) (**Figure 4**):

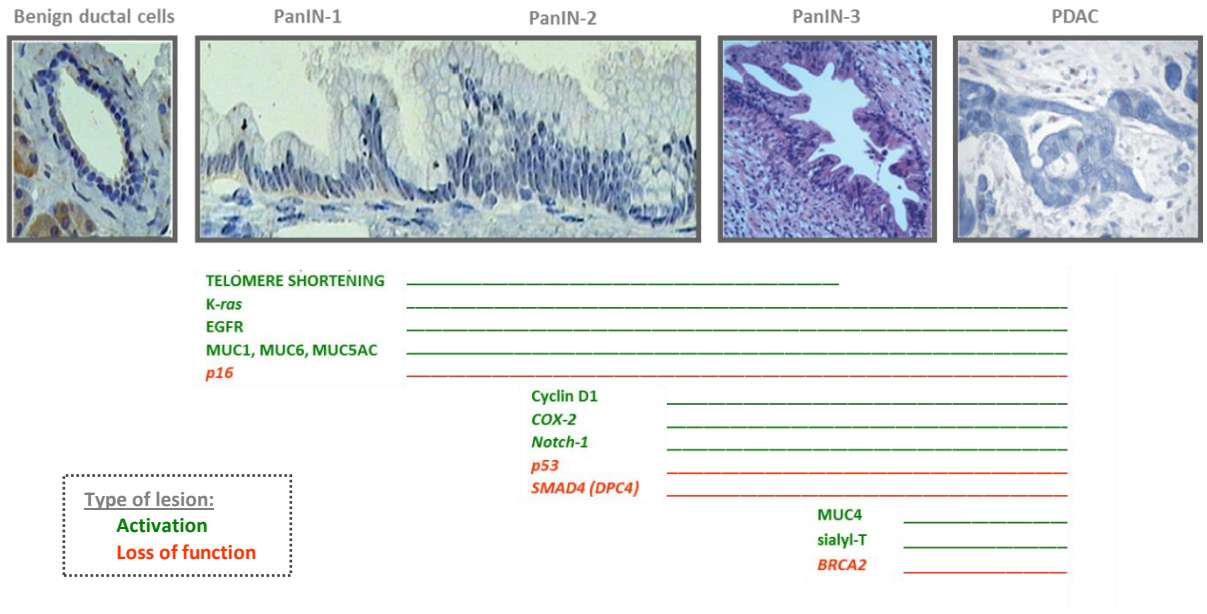


Figure 4. Histological and genetic progression models for PDAC.
The progression from histologically normal epithelium to invasive carcinoma is associated with the accumulation of specific genetic alterations. However, this progression model is specific for PanINs, and other recognized precursor lesions likely harbor a distinct compendium of genetic alterations in their path to invasive cancer. Extracted and modified from Bardeesy and DePinho, 2002; Ghaneh *et al.*, 2007; Maitra and Hruban, 2008 .

» **Oncogenes** (genes contributing to oncogenesis when mutationally activated). Activating point mutations of K-*ras* oncogene are possibly the most common and earliest genetic abnormality in pancreatic cancer, and are present in 90-95% of cases. Other oncogenes that are amplified in pancreatic cancer include the oncogenic transcription factor C-Myc, MYB, AIB1/NCOA3, Cyclin D1 and cyclo-oxygenase-2 (COX-2). Overexpression of EGFR and its main ligands (EGF and TGFα) is another common feature of PDAC.

» **Tumour suppressor genes** (genes promoting tumour growth when inactivated). p16/CDKN2A gene is the most commonly inactivated tumour suppressor gene in PDAC, and loss of INK4A protein function is described in 90% of pancreatic cancers. Other common tumour suppressor gene mutations occur in TP53 gene (transcription factor p53) and in deleted in pancreatic carcinoma 4 gene, or DPC4 (also known as SMAD4), which are present in 50-75% and 55% of PDAC cases, respectively.

» **Caretaker or genome-manteinance genes** (genes that function to identify and repair damage to DNA). The DNA mismatch repair genes hMLH1, hMSH2 and BRCA2 are examples of genome maintenance genes inactivated in pancreatic cancer.

Telomere shortening is also an early event in PDAC, occurring in >90% of PanIN-1 lesions and it may be the major cause for the chromosomal instability (van Heek *et al.*, 2002). Epigenetic abnormalities predominantly encompass methylation of CG dinucleotides in the 5' regulatory region of tumour suppression and/or critical homeostatic pathways, like p16/CDKN2A, E-cadherin, retinoic acid beta, SOCS-1, TSLC1 and *reprimo*, among others (Sato and Goggins, 2006). Moreover, upregulation of several genes related to the developmental signalling pathways Hedgehog and Notch has been further reported (Nickoloff *et al.*, 2003; Thayer *et al.*, 2003). In additon, the expression of some microRNAs is deregulated from early stages in PDAC progression, as for instance an upregulation of miR-21, miR-210, miR-155, miR-196a, miR-200b, miR-221 and miR-222, among others, has been found in an early event during the multi-stage progression of the disease (Szafranska *et al.*, 2007; Wang and Sen, 2011). Finally, changes in glycosylation are widely reported along PDAC development, and are further described in **Introduction 2.5.2**.

Altogether, pre-neoplastic lesions already contain many of the genetic changes that characterize PDAC and 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 PDAC microenvironment (Farrow *et al.*, 2004; Ghaneh *et al.*, 2007), which is described in **Introduction 3.2.1**.

2 Glycobiology

Glycobiology is the science which studies the structure, chemistry, biosynthesis and biological functions of glycans (monosaccharides and sugar chains) and its derivatives. It is a relatively new discipline since carbohydrates were primarily considered as only a source of energy or as structural materials, but the development of many new technologies for exploring their structures and functions opened a new frontier in molecular biology. The specific term **Glycobiology** was first coined in 1988 in the Oxford English Dictionary by Professor Raymond Dwek to recognize the coming together of the traditional disciplines of carbohydrate chemistry and biochemistry (Rademacher *et al.*, 1988). Nowadays it is one of the more rapidly growing fields in the natural sciences due to the importance of glycoconjugates in many biological processes (Varki and Sharon, 2009).

Glycosylation is essential for life, and it is present in secreted and membrane proteins of almost all organisms (bacteria, fungi, yeast, plants and animals), as well as in the glycolipids constituting the glycocalyx of the cell. In addition, viruses, which have no glycosylation machinery of their own, attach sugars to their envelope proteins by exploiting the biosynthetic pathways of their hosts (Rudd *et al.*, 2004).

Between 50 and 80% of all known proteins have potential glycosylation sites, and it is plausible that evolution has selected glycan molecules repeatedly because they are diverse and flexible. Owing to their mass, shape, charge and ubiquitous presence, they are considered to be involved in numerous biological roles such as cell growth and development, tumour growth and metastasis, anticoagulation, immune recognition/response, cell-cell communication and microbial pathogenesis. In general, the biological roles of glycans can be related to their structural and modulatory properties or to the specific recognition by other molecules, most commonly glycan-binding proteins. The term **glycome** describes the complete repertoire of glycans and glycoconjugates that cells produce under specific conditions of time, space and environment, and it is determined by the expression levels of specific enzymes and the availability of sugar donors and transporters required for their synthesis (Raman *et al.*, 2005; Varki and Lowe, 2009).

2.1 Glycoconjugates

Glycoconjugates form when mono-, oligo- or polysaccharides are covalently attached to a protein or lipid macromolecule (aglycone); and are classified according to the nature of this linkage (**Figure 5**). It is common to refer to the glycosidic part of glycoconjugates as **glycans** (Varki and Sharon, 2009).

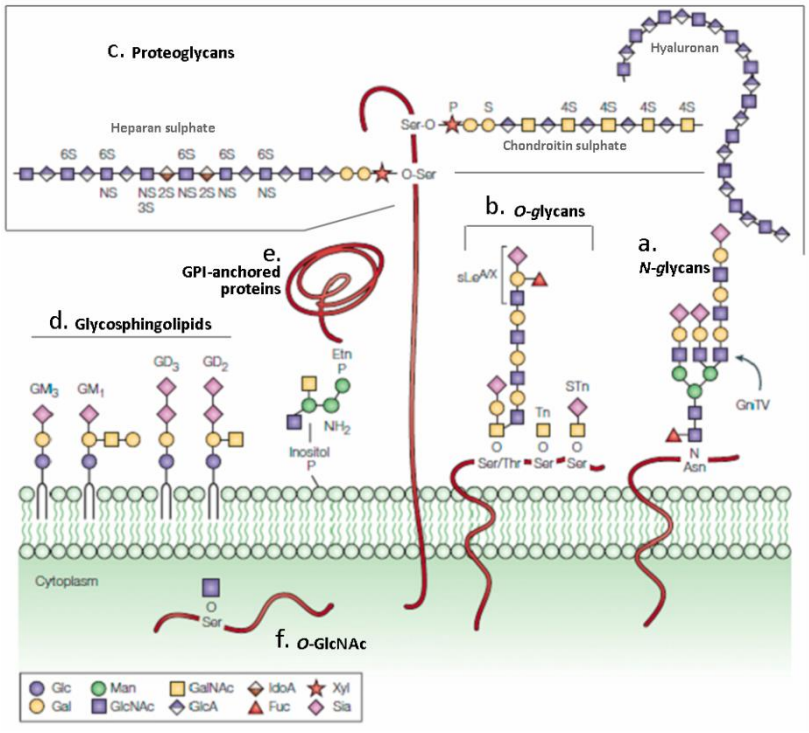


Figure 5. Major classes of glycoconjugates.
a| *N*-glycans. **b|** *O*-glycans. **c|** Proteoglycans, e.g. heparan sulphate, chondroitin sulphate and free glycosaminoglycans such as hyaluronan. **d|** Glycosphingolipids. **e|** Glycosylphosphatidylinositol (GPI)-anchored proteins. **f|** *O*-GlcNAc nuclear and cytoplasmic proteins.
Sugars are represented by coloured geometric symbols.
Extracted and modified from Fuster and Esko, 2005.

Glc, glucose; Gal, galactose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; IdoA, uronic acid; Fuc, fucose; Xyl, xylose; Sia, sialic acid.

(a) In glycoproteins, glycans may be attached to the polypeptide chain through an asparagine residue (***N*-glycans**; **Introduction 2.1.1**) or through a serine or threonine residue (***O*-glycans**; **Introduction 2.1.2**). A mucin is a large glycoprotein that carries many clustered *O*-glycans.

(b) A **proteoglycan,** i.e. heparan sulfate or chondroitin sulphate, is a glycoconjugate with one or more glycosaminoglycan (GAG) chains attached to a core protein through a typical core region ending in a xylose (Xyl) residue linked to the hydroxyl group of a serine residue. GAGs are long unbranched polysaccharides built by a repeating disaccharide unit, which consists of a galactose (Gal) or uronic acid (IdoA) linked to an amino sugar. Some GAGs can exist as free polysaccharides, i.e. hyaluronic acid.

(c) Glycolipid is a general term including different types of glycans attached to cellular membrane lipids. **Glycosphingolipids** are the most abundant glycolipids in vertebrates, and consist of a glycan attached to a ceramide (sphingosine and a fatty acid). Sialylated glycosphingolipids are known as gangliosides. **Glycosylphosphatidylinositol (GPI anchor)** is a glycolipid composed of a phosphatidylinositol group linked, on the one side to the C-terminal amino acid of a mature protein through a carbohydrate-containing linker and an ethanolamine phosphate bridge; and on the other side to the cell membrane via two fatty acids.

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

Although the same glycosylation machinery is available to all protein and lipid macromolecules that enter the secretory pathway in a given cell, most glycoconjugates emerge with characteristic

glycosylation patterns and heterogeneous populations of glycans at each glycosylation site, known as **glycoforms** (Rudd and Dwek, 1997).

2.1.1 *N*-glycans

N-linked glycans in glycoproteins are attached to the amide nitrogen of an asparagine (Asn) side chain. In animal cells, the sugar linked to Asn is almost inevitably *N*-acetylglucosamine (GlcNAc), and the linkage is always in the β configuration (GlcNAcβ1-Asn). Glycosylated Asn residues are nearly always found in the specific peptide sequence Asn-X-Ser/Thr, where X can be any amino acid except for proline. All *N*-glycans share a common core sequence ($Glc_3Man_9GlcNAc_2$) and are classified into **high mannose type**, in which only mannose (Man) residues are attached to the core; **complex type**, in which only GlcNAc residues are linked to the core; and **hybrid type**, in which only Man residues are attached to the Manα1-6 arm of the core and one or two GlcNAc are on the Manα1-3 arm. Complex type structures are subclassified according to the number of GlcNAc attached to the core (branches or *antennae*). Biantennary glycans are the most abundant ones, and tri- and tetrantennary structures are also common. Rare glycans containing five or more branches have also been documented (Raman *et al.*, 2005; Stanley *et al.*, 2009; Taylor and Drickamer, 2003) (**Figure 6**).

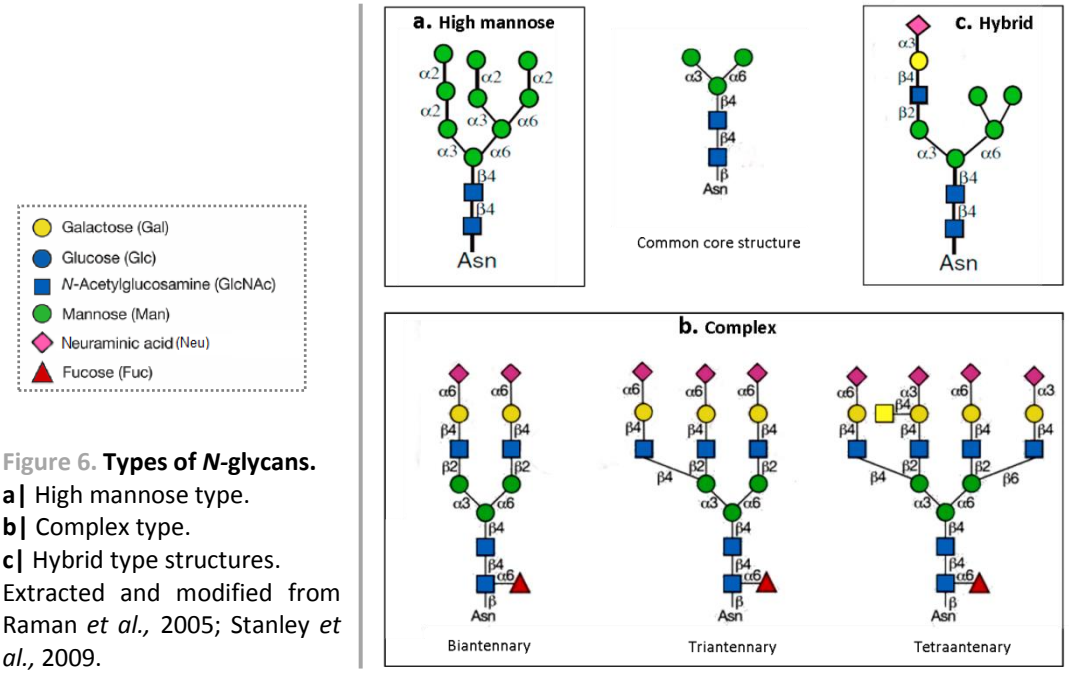


Figure 6. **Types of *N*-glycans.**
a| High mannose type.
b| Complex type.
c| Hybrid type structures.
Extracted and modified from Raman *et al.,* 2005; Stanley *et al.,* 2009.

2.1.1.1 *N*-glycans synthesis

The entire process takes place in the glycosylation pathway of the endoplasmic reticulum (ER) and Golgi apparatus (GA), where the enzymes that modify monosaccharides are specifically located. The assembly of *N*-linked glycans occurs in three major stages (Stanley *et al.*, 2009) (**Figure 7**):

» **Formation of a lipid-linked precursor oligosaccharide.** This synthesis begins in the cytoplasmic side of the ER membrane by the transfer of GlcNAc-P from UDP-GlcNAc to membrane-bound dolicol-P, forming GlcNAc-P-P-Dol. A second GlcNAc and five Man residues are subsequently transferred in a stepwise manner from UDP-GlcNAc and GDP-Man, respectively, to generate $Man_5GlcNAc_2$-P-P-Dol. It is then translocated across the ER membrane bilayer, mediated by a flippase, so that the glycan becomes exposed to the lumen of the ER. Afterwards, it is extended by the addition of four Man and three glucose (Glc) residues, generating the mature *N*-glycan precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol.

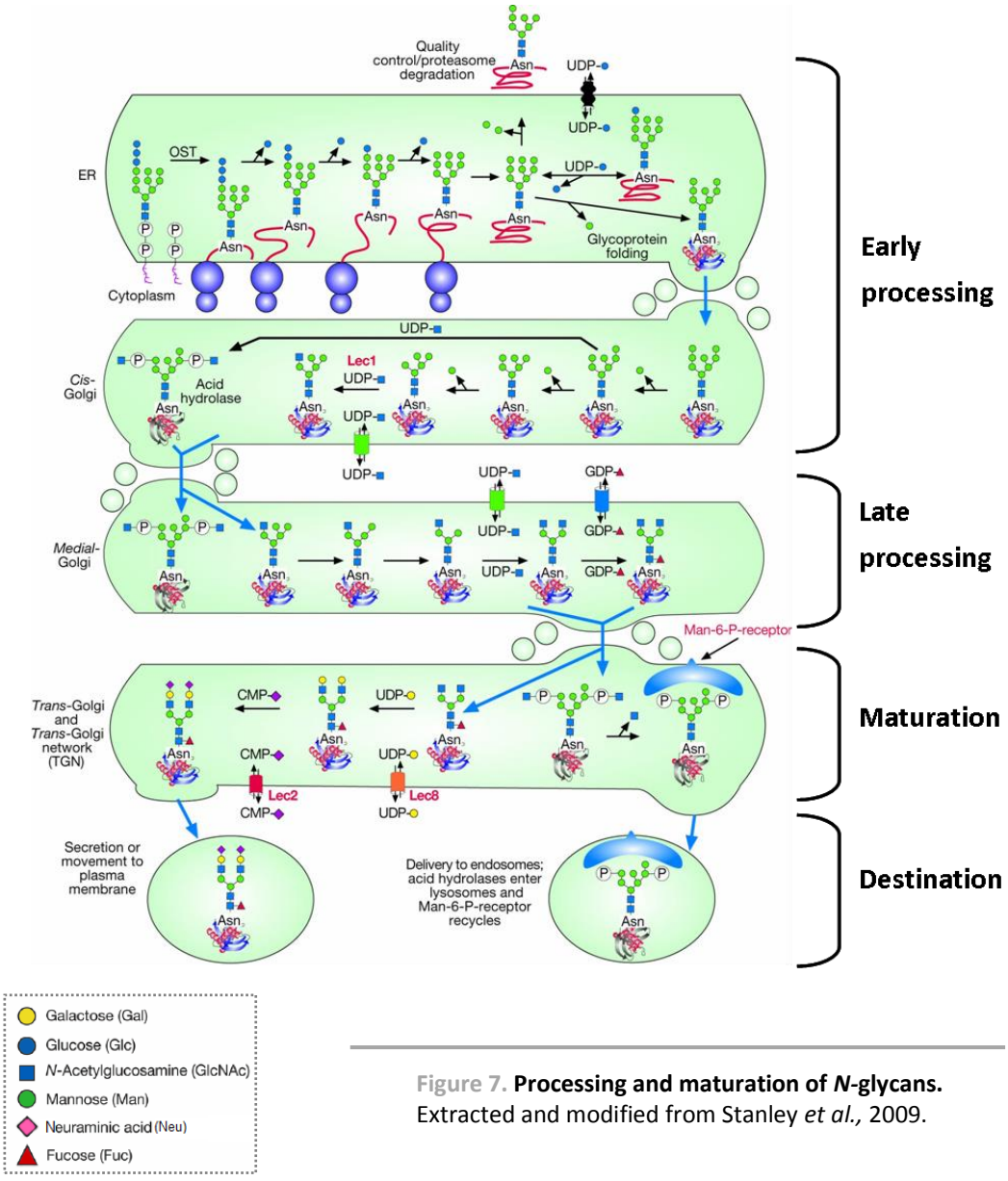


Figure 7. **Processing and maturation of *N*-glycans.** Extracted and modified from Stanley *et al.,* 2009.

» **Transfer of the dolichol-linked precursor to nascent proteins.** The multisubunit protein complex oligosaccharyltransferase (OST) in the ER membrane is responsible for catalyzing the transfer of $Glc_3Man_9GlcNAc_2$ from Dol-P-P to Asn-X-Ser/Thr (where X is not proline) in nascent proteins as they emerge from the translocon in the ER membrane.

» **Processing of the oligosaccharide.** Following the transfer to the polypeptide, the *N*-linked glycan is sequentially processed in the ER and GA to bring about the different types of *N*-glycans.

Early processing steps consist of the sequential removal of Glc residues by α-glycosidase I and II, and removal of the terminal α1,2-Man by α-mannosidase I to yield structures of nine to five Man (Man5-9GlcNAc2). The quality control system that monitors protein folding is associated with these processing reactions, and they determine whether the glycoprotein continues to the *cis*-Golgi or is degraded via interactions with ER chaperones.

Late processing steps are initiated in the *medial*-Golgi by the action of *N*-acetylglucosaminyltransferase I (GlcNAcT I), which adds a GlcNAc residue to the C-2 of the α1,3-Man in the core of Man5GlcNAc2. Afterwards, the majority of *N*-glycans are trimmed by α-mannosidase II, which removes the terminal α1,3-Man and α1,6-Man residues to form GlcNAcMan3GlcNAc2, and a second GlcNAc is added to C-2 of the α1,6-Man in the core by the action of GlcNAcT II to yield the precursor for all biantennary, complex *N*-glycans. Additional branches can be initiated at C-4 of the core α1,3-Man (by GlcNAcT IV) and C-6 of the core α1,6-Man (by GlcNAcT V) to yield tri- and tetrantennary *N*-glycans. Complex and hybrid *N*-glycans may carry a "bisecting" N-acetylglucosamine residue that is attached to the β-mannose of the core by *N*-acetylglucosaminyltransferase III (GnT-III) (**Figure 8a**).

Maturation mostly occurs in the *trans*-Golgi and converts the limited repertoire of hybrid and branched *N*-glycans into an extensive array of mature *N*-glycans. Three different processes are described:

(1) **Sugars addition to the core.** In vertebrates, the main core modification is the addition of fucose (Fuc) in α1,6 linkage to the GlcNAc adjacent to Asn in the core. The fucosyltransferase involved, FucT VIII, requires prior action of GlcNAcT I.

(2) **Elongation of branching.** The majority of complex and hybrid *N*-glycans have elongated branches made by the addition of a β-linked galactose (Gal) residue to the initiating GlcNAC in the C-4 to produce Galβ1-4GlcNAc [referred to as a type II N-acetyllactosamine (LacNAc) sequence]; or to the C-3 of the GlcNAc to yield Galβ1–3GlcNAc (referred to as a type I LacNAc sequence). Antennae can be further lengthened by the sequential addition of GlcNAc and Gal residues, resulting in tandem repeats of LacNAc(-3Galβ1–4GlcNAcβ1-)n, termed poly-N-acetyllactosamine or polyLacNAc. In some glycoproteins, β-linked GalNAc is added to GlcNAc instead of β-linked Gal, yielding antennae with a GalNAcβ1–4GlcNAc (LacdiNAc) extension (**Figure 8b**).

(3) **"Capping" or "decoration"** involves the addition of neuraminic acid (Neu), Fuc, Gal, GalNAc or sulphate to the branches. Capping sugars are most commonly α-linked.

Once *N*-glycosylated proteins have undergone the whole process successfully, and according to the signals encoded in their amino acid sequences, can be destined from the GA to endosomes, lysosomes, the plasma membrane or secretory pathways, depending on their function.

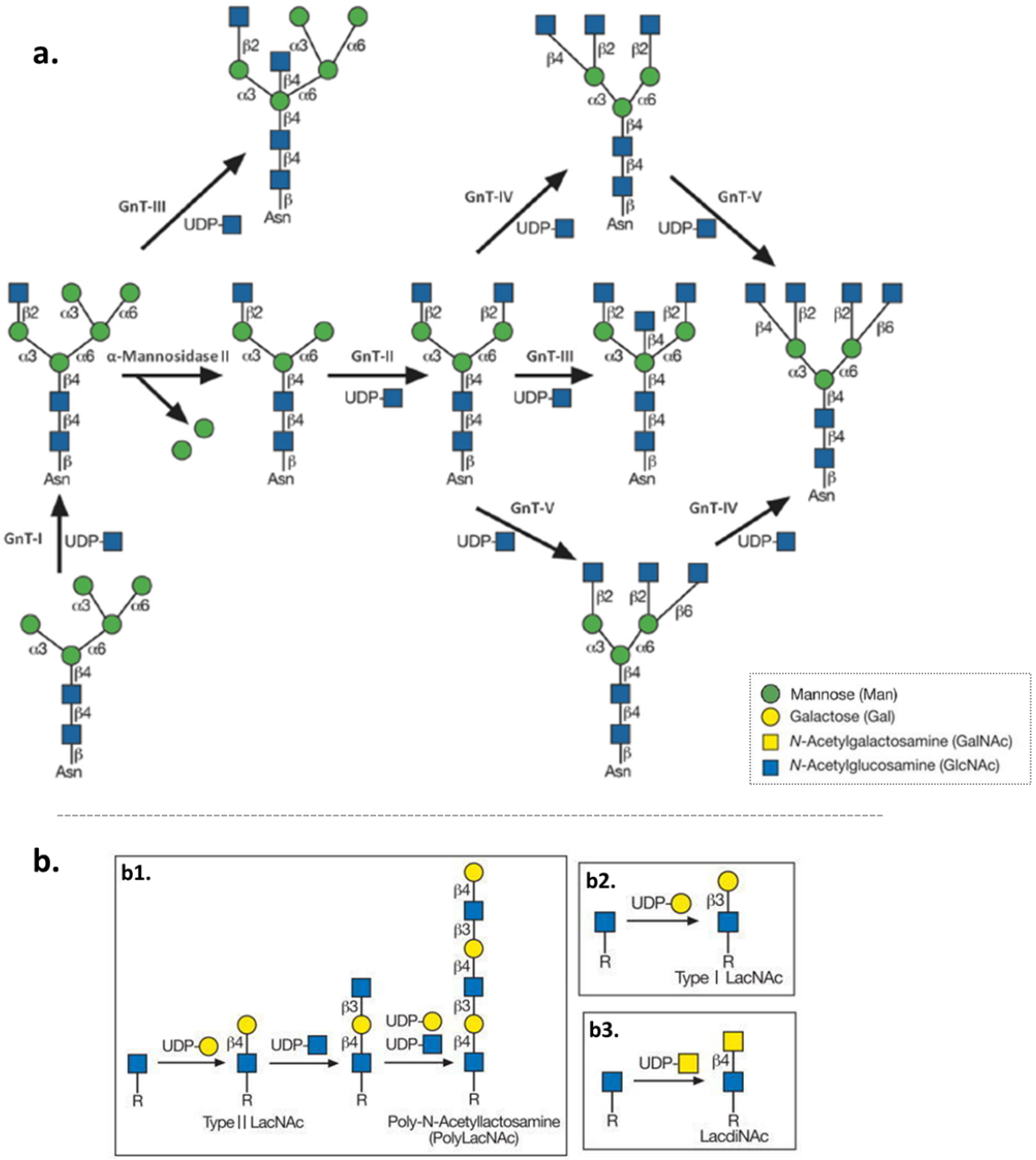


Figure 8. **Branching of complex *N*-glycans (a) and elongation of branch GlcNAc residues of *N*-glycans (b). b1|** Formation of single *N*-acetyllactosamine unit from type II LacNAc. **b2|**Type I LacNac unit. **b3|** LacdiNAc unit. Extracted and modified from Stanley *et al.,* 2009.

2.1.2 *O*-glycans

O-glycans are frequently α-linked via an *N*-acetylgalactosamine (GalNAc) moitey to the hydroxyl group of a serine (Ser) or threonine (Thr) side chain, generating the common core structure GalNAcα-Ser/Thr of mucin-type glycans, named the Tn antigen. The most common *O*-GalNAc glycan is **core 1** or T antigen, which is found in many glycoproteins and mucins. The further addition of Gal, GlcNAc or GalNAc defines **cores 2-8** (**Table 1**). Modification of *O*-GalNAc glycans include *O*-acetylation of sialic acid, and *O*-sulfation of Gal or GlcNAc, leading to structural heterogenicity. Mucins are glycoproteins that contain large

numbers of high-density clusters of *O*-linked glycans. The mucin-type glycans of these proteins frequently form cross-linked connections in aqueous solutions, resulting in a high viscosity gel (mucus). Mucins can be secreted, but may also be membrane bound and form glycan-dense areas on the cell surface.

Other existing *O*-glycans include α-linked *O*-Fuc, β-linked *O*-Xyl, α-linked *O*-Man, β-linked *O*-GlcNAc, α- or β-linked *O*-Gal, and α- or β-linked *O*-Glc glycans (Brockhausen *et al.*, 2009).

***O*-glycan core**	**Structure**
Tn antigen (Thomsen-nouvelle antigen)	GalNAcαSer/Thr
sialyl-Tn antigen	Siaα2-6GalNAcαSer/Th
Core 1 or T antigen (also TF or Thomsen-Friedenreich 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/Thr

Table 1. Structures of *O*-glycan cores found in mucins. Information extracted from Brockhausen *et al.*, 2009.

2.1.3 Outer structures common to different glycans

In contrast to core glycan synthesis, which is constitutive in most cell types, terminal sugars addition is often regulated in a tissue- or cell lineage-specific manner. Moreover, the regulation of these reactions varies in different cell conditions such as embryogenesis, differentiation, metabolic changes and malignant transformation. Frequently these outer terminal structures determine the function or recognition properties of the *N*-glycans, *O*-glycans and glycosphingolipids. Only for some of them it has been possible to establish a connection to defined functions, as for instance the ABO blood groups, the Forssman antigen or the Lewis blood groups (Stanley and Cummings, 2009), the latter further described in **Introduction 2.4**.

2.2 Sialic acids

The two major sialic acid core structures are 2-keto-3-deoxynononic acid (Kdn) and neuraminic acid (Neu), sharing nine carbons and differing at the C-5 position. Two derivatives of Neu are the most common sialic acid structures found on mammalian cells: *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, *N*-glycolylneuraminic acid (Neu5Gc) (**Figure 9**). Among mammals, humans are a known exception in their lack of Neu5Gc, due to an inactivating mutation in a hydroxylase, which

modifies CMP-Neu5Ac to CMP-Neu5Gc (Angata and Varki, 2002; Cohen and Varki, 2010; Varki and Schauer, 2009).

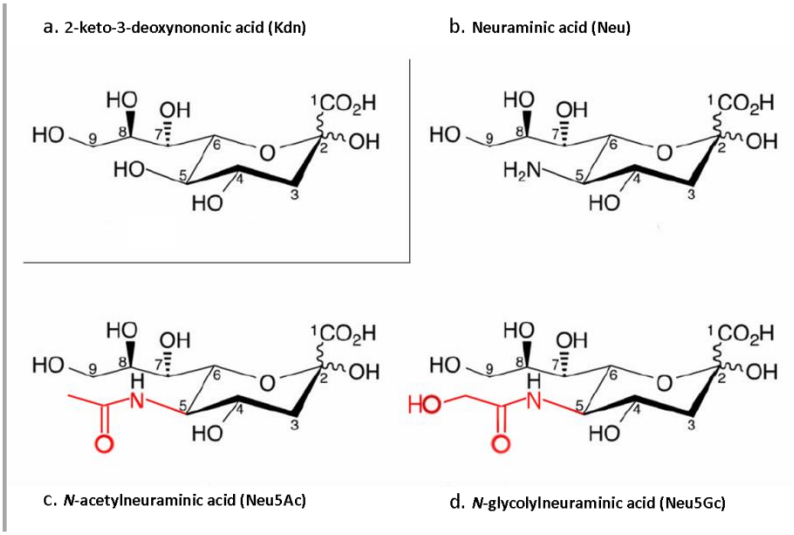


Figure 9. **Sialic acid structure diversity.** **a|** 2-keto-3-deoxynononic-acid (Kdn) and **b|** Neuraminic acid (Neu) are the most common Sia core structures. **c|** *N*-acetyl-neuraminic acid (Neu5Ac) and **d|** *N*-glycolylneuraminic acid (Neu5Gc) differ in the hydroxyl group (in red), and are the two most common Neu derivatives in mammals.
Extracted and modified from Cohen and Varki, 2010.

Natural modification of sialic acid core structures yelds over 50 variations, which include esterification (with acetyl, lactyl, sulphate or phosphate), *O*-methylation, lactonization or lactamization (Kelm and Schauer, 1997; Varki and Schauer, 2009). The carboxylate group at position 1 is normally deprotonated at physiological pH, and it confers a net negative charge that strongly influences its physicochemical properties (Buschiazzo and Alzari, 2008).

The most common linkages are α2,3 (between C-2 of Sia and C-3 of Gal), α2,6 (between C-2 of Sia and C-6 of Gal or GalNAc), and α2,8 (between C-2 of Sia and C-8 of another Sia) (**Figure 10**).

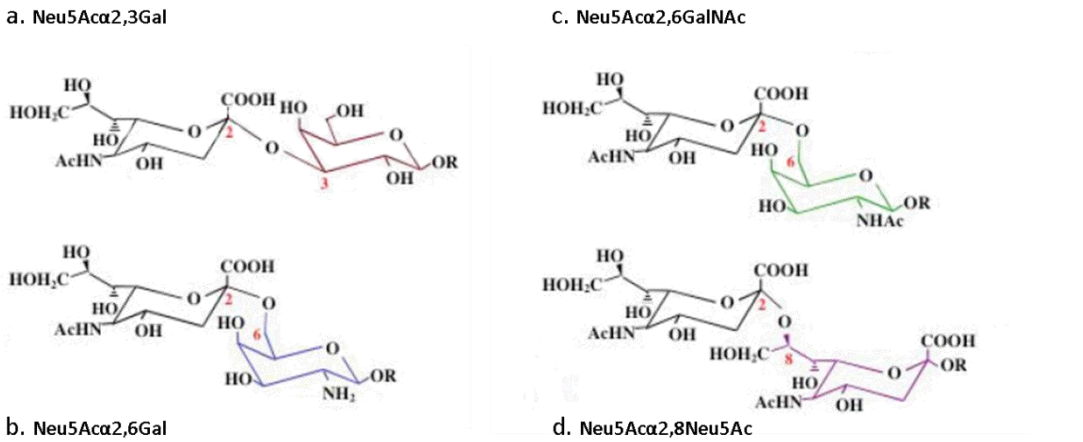


Figure 10. **Sialic acid linkage.**
The most common linkages are between C-2 of Sia and C-3 of Gal **(a),** C-6 of Gal **(b)** or GalNAc **(c)**, and C-8 of Sia **(d)**. Extracted and modified from Wang, 2005.

Sialic acid can also form α2,9-linkage to another sialic, and α2,4-linkage occur in the repeating units of some echinodermal glycans (Varki and Schauer, 2009). Each linkage results in a unique spatial

presentation of the sialic acid molecule, specifically recognized by glycan binding proteins. The underlying sugar chain can also contribute to the binding specificity of certain glycan binding proteins. Plant lectins such as *Sambucus nigra* agglutinin (SNA, Elderberry bark) (Shibuya *et al.*, 1987) and *Maackia amurensis* agglutinin (MAA or MAL II) (Wang and Cummings, 1988) are commonly used to detect sialic acid in α2,6- and α2,3-linkage, respectively, although they do not recognize all glycan structures containing these sialic acids; as for instance MAA does not recognize SLe^x epitope [Siaα2,3-Galβ1,4(Fucα1,3)GlcNAc-; see **Introduction 2.4.1**] (Varki and Schauer, 2009). Bacterial sialidase enzymes activity is highly specific and dependent on the sialic acid linkage.

Mostly found in higher eukaryotes of the Deuterostomata lineage and in particular groups of Eubacteria, sialic acids are usually terminal residues on the outermost cell surface glycoconjugates. As a result of their exposed location on molecules and cells, they play important roles in either masking recognition sites or mediating cell recognition and adhesion processes, such as in nervous system embryogenesis or in normal inflammatory and immune response pathways, thus representing ligands or counter-receptors (Kelm and Schauer, 1997).

Moreover, and due to their negative charge, sialic acids are involved in the binding and transport of positively charged molecules as well as in the attraction and repulsion of cells and molecules. This way, as components of glycoproteins, they contribute to the high viscosity of mucins protecting endothelia, and influence the organization and function of supramolecular structures in cell membranes. The negative charge of sialic acids also contributes to an anti-proteolytic effect in glycoproteins, and also prevents the action of specific endoglycosidases (Schauer, 2000). Although most bacteria are unable to synthesize sialic acids, several pathogenic species and strains do, apparently allowing those to better evade the host's immune system (Preston *et al.*, 1996).

2.3 The glycosylation machinery

Whereas in general a single gene is involved in the synthesis of a specific protein, dozens of genes take a part in the synthesis of the glycans of a glycoprotein. These are genes for glycosyltransferases, glycoside hydrolases (glycosidases), sugar nucleotide donors synthases and transporters, and, in a broad sense, molecules that recognize glycan structures such as lectins and other receptors. The specific role of several glycosyltransferases in pancreatic cancer has been studied throughout this Thesis.

Glycosyltransferases (GTs; EC 2.4.x.y) constitute a very large family of enzymes that catalyse the transfer of monosaccharide residues from a high-energy enabled donor substrate (usually a sugar-nucleotide complex) to an acceptor (lipid, protein or growing oligosaccharide), creating glycosidic bonds. These reactions require the participation of a divalent cation (Mg^{2+} or Mn^{2+}) as a cofactor, and have an optimal activity at pH between 5 and 7. The overall observed glycans in a particular cell type reflects the

set of GTs expressed, but the synthesis of oligosaccharides also depends on how these enzymes are distributed along the AG (Fukuda, 1994).

Apart from the Golgi factors, GTs expression can also be controlled at the level of RNA synthesis or turnover: several studies have indicated that although the expression pattern of some GTs' mRNAs is highly regulated in a tissue-specific and developmentally regulated manner, others have a widespread so-called *housekeeping* type of distribution. For the most, it appears that differential regulation is due to the action of specific promoter regions in the 5'-region of the corresponding genes. An additional finding of interest is that GTs' mRNAs in general have long 5'-untranslated regions with extensive secondary structure, which might result in differential message stability, especially during the cell cycle (Lowe and Varki, 1999). Epigenetic silencing and hypoxia are other mechanisms postulated to regulate the expression of some GTs during tumourigenesis (Kannagi *et al.*, 2010).

Traditionally, and according to the rules of the International Union of Biochemistry and Molecular Biology (IUBMB), GTs have been classified into families based on the type of sugar that is transferred, as shown in **Table 2**. The traditional classification system, however, has many limitations, and for this reason the Carbohidrate-Active enZymes database (CAZy; Lombard *et al.*, 2014) proposed a classification based on sequence homology. To date GTs are classified into 96 different families.

Glycosyltransferase families	Sugar donor substrate
Sialyltransferases (ST)	CMP-Sialic acid
Fucosyltransferases (FucT)	GDP-Fucose
Galactosyltransferases	UDP-Galactose
N-acetylglucosaminyltransferases	UDP-GlcNAc
N-acetylgalactosaminyltransferases	UDP-GalNAc
Mannosyltransferases	GDP-Mannose

Table 2. Main glycosyltransferase families.
Classified according to the International Union of Biochemistry and Molecular Biology (IUBMB), based on the type of sugar donor substrate.

Glycoside hydrolases or **glycosidases** (GHs; EC 3.2.1.x) are enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. In most cases, the hydrolysis of the glycosidic bond is catalyzed by two aminoacid residues of the enzyme: a general acid (proton donor) and a nucleophile/base. The IUBMB nomenclature is based on the substrate specificity of the enzymes and, occasionally, on their molecular mechanism, but this classification does not reflect their structural features. 133 families of glycosidases are presently established by CAZy database, among which neuraminidases or sialidases (Lombard *et al.*, 2014).

2.3.1 Fucosyltransferases

Fucosyltransferases (FucTs) catalyze the reaction in which a fucose (Fuc) residue is transferred from the donor GDP-Fuc to the acceptor molecules. To date, 13 FucT genes have been identified along the human genome. Based on the site of Fuc addition and the acceptor specificities, they are classified into four subfamilies, as shown in **Table 3** (Ma *et al.*, 2006).

α1,2-, α1,3/4- and α1,6-FucTs subfamilies in eukaryotic organisms are type II transmembrane Golgi-anchored proteins containing an N-terminal cytoplasmic tail, a transmembrane domain, and an extended stem region followed by a large globular C-terminal catalytic domain facing the Golgi lumen (de Vries *et al.*, 2001). *O*-FucTs, however, are ER-localized soluble proteins and catalyze *O*-fucosylation in the ER (Luo and Haltiwanger, 2005).

FUT gene expression is complex and regulated in a tissue-, cell- and stage-specific manner, and the expression pattern suggests control at both the transcriptional and post-transcriptional levels (de Vries *et al.*, 2001). As for instance, Taniguchi *et al.* (2000) found that FUT4 has two different transcription initiation sites, resulting in the production of a long and a short form of mRNA. Recently, methylation of FUT4 promoter has been pointed out to regulate its expression (Li *et al.*, 2012).

2.3.2 Sialyltransferases

Sialyltransferases (STs) catalyze the transfer of sialic acid residues from the activated donor substrate CMP-Sia to terminal nonreducing positions of oligosaccharide chains of glycoproteins and glycolipids. In mammals, 20 distinct STs have been described, and they are grouped into three subfamilies according to the sialyl linkage they form and the acceptor specificities (Harduin-Lepers *et al.*, 2001). **Table 4** includes the most relevant features of each human ST subfamily.

All vertebrate STs have a similar architecture: they are type II transmembrane glycoproteins that predominantly reside in the *trans*-Golgi compartment. They have a short N-terminal cytoplasmic tail, a unique transmembrane domain, a stem region and a large C-terminal catalytic domain. The overall homology of sequences is very limited (from 15 to 57% for human STs), but share four peptide conserved motifs in the catalytic domain called the sialylmotifs: large, small, motif III and very small motif (Geremia *et al.*, 1997; Jeanneau *et al.*, 2004).

STs spatio-temporal expression appears to be regulated mainly at the level of transcription, since multiple promoters have been found in human ST6Gal I-II and ST3Gal II, IV and VI (Kim *et al.*, 2001; Kitagawa *et al.*, 1996; Taniguchi *et al.*, 2001; Wang *et al.*, 1993). However, Taniguchi *et al.* (2003c) found that human ST3Gal III gene displays a unique promotor region, similarly to the human ST3Gal I gene, all the ST6GalNAc and all the ST8 Sia genes. More recently, epigenetic, post-translational controls and Golgi pH have also been proposed as mechanisms for the regulation of the expression, location and activity of several sialyltransferases (Harduin-Lepers *et al.*, 2012).

Table 3. Human fucosyltransferase enzymes classification based on the site and linkage of fucose addition.
Subfamilies, gene name and chromosomal location, enzyme name and activity characteristics are described. Information extracted from Ma *et al.*, 2006. α1,2-FucT: α1,2-fucosyltransferases; α1,3/4-FucT: α1,3/4-fucosyltransferases; α1,6-FucT: α1,6-fucosyltransferases; *O*-FucT: *O*-fucosyltransferases.

Subfamily	Gene name	Location	Enzyme	Characteristics
α1,2-FucTs transfer fucose with α1,2-linkage to the Gal of type I or type II structures	*FUT1*	19q13.3	α1,2-FucT I	Synthesis of H2 antigen. Mutations in this gene are a cause of the H-Bombay blood group.
	FUT2	19q13.3	α1,2-FucT II	Synthesis of H1 antigen. Expressed on epithelial cells and in body fluids (e.g. saliva).
α1,3/4-FucTs add fucose with α1,3- or α1,4-linkage to the GlcNAc moiety of type I or type II structures	*FUT3*	19p13.3	α1,3/4-FucT III	Lewis histo-blood group system.
	FUT4	11q21	α1,3-FucT IV	Synthesis of Le^x and Le^y antigens.
	FUT5	19p13.3	α1,3/4-FucT V	Lewis histo-blood group system.
	FUT6	19p13.3	α1,3-FucT VI	Synthesis of Le^x, Le^y and SLe^x antigens.
	FUT7	9q34.3	α1,3-FucT VII	Synthesis of SLe^x antigen. Expressed on leukocytes.
	FUT9	6q16	α1,3-FucT IX	Synthesis of Le^x and Le^y antigens.
	FUT10	8p12	α1,3-FucT X	Predominantly fucosylate the innermost core GlcNAc of *N*-glycans.
	FUT11	10q22.2	α1,3-FucT XI	
α1,6-FucT	*FUT8*	14q24.3	α1,6-FucT VIII	Directs addition of fucose to the inner core of Asn-linked GlcNAc moieties.
***O*-FucTs**	*FUT12*	20q11	POFUT 1	Adds *O*-fucose through an *O*-glycosidic linkage to conserved Ser or Thr residues in EGF-like repeats.
	FUT13	21q22.3	POFUT 2	Uses thrombospondin type-1 repeats as substrates.

Table 4. Human sialyltransferase enzymes classification based on the site and linkage of sialic acid addition.
Subfamilies, gene name and chromosomal location, enzyme name and activity characteristics are described. Information extracted from Harduin-Lepers *et al.*, 2001; 2012. α2,3-STs: α2,3-sialyltransferases; α2,6-STs: α2,6-sialyltransferases; α2,8-STs: α2,8-sialyltransferases. Type I chain: Galβ1,3GlcNAc; type II chain: Galβ1,4GlcNAc; type III chain: Galβ1,3GalNAc.

Subfamily		Gene name	Location	Enzyme	Characteristics
α2,3-STs (ST3Gal) transfer sialic acid with α2,3-linkage to the Gal of type I, II or III structures in glycoproteins or glycolipids		*ST3GAL1*	8q24.22	ST3O	Transfer of Sia to type III chains on glycolipids (asialo-G_{M1}, G_{M1a} and G_{D1b}) or *O*-glycoproteins [sialylated Thomsen-Friedenreich antigen (sT)].
		ST3GAL2	16q22.1	ST3Gal II	
		ST3GAL3	1p34.1	ST3Gal III	Preferentially acts on type I chains of *O*-glycoproteins or glycolipids: synthesis of SLe^a. Can also act on type II chains, but with lower catalytic efficiency.
		ST3GAL4	11q24.2	ST3Gal IV	Glycolipids or glycoproteins containing type II or type III chains: synthesis of SLe^x.
		ST3GAL5	2p11.2	ST3Gal V	Acts only on lactosyl-ceramide chains: synthesis of G_{M3} ganglioside. Mutation in this gene is associated with Amish infantile epilepsy syndrome.
		ST3GAL6	3q12.1	ST3Gal VI	Acts on type II chains found on glycolipids or glycoproteins: synthesis of SLe^x.
		LOC343705	20q11.22	*no name*	Similar to α2,3-sialyltransferase.
α2,6-STs add sialic acid with α2,6-linkage to Gal,GalNAc or GlcNAc	**ST6Gal**	*ST6GAL1*	3q27-q28	ST6Gal I	Act on type II chains found on *N*-glycoproteins and to a lesser extent, on *O*-glycoproteins, glycolipids and free oligosaccharides.
		ST6GAL2	2q11.2-q12.1	ST6Gal II	
	ST6GalNAc	*ST6GALNAC1*	17q25.1	ST6GalNAc I	Acts on *O*-glycoproteins: sialyl-Tn antigen on mucins. Wide specificity of substrate.
		ST6GALNAC2	17q25.1	ST6GalNAc II	Acts on *O*-glycoproteins. More restrictive specificity of subrate.
		ST6GALNAC3	1p31.1	ST6GalNAc III	Act on *O*-glycoproteins and glycolipids: synthesis of $G_{D1\alpha}$ ganglioside.
		ST6GALNAC4	9q34	ST6GalNAc IV	
		ST6GALNAC5	1p31.1	ST6GalNAc V	
		ST6GALNAC6	9q34.11	ST6GalNAc VI	Acts on glycolipids: synthesis gangliosides $G_{D1\alpha}$, $G_{T1a\alpha}$, $G_{Q1b\alpha}$; and di-sialyl $Lewis^a$.
		LOC390377	13q12.11	*no name*	Similar to α2,6-sialyltransferase.
α2,8-STs (ST8Sia) add sialic acid with α2,8-linkage to another sialic acid molecule		*ST8SIA1*	12p12.1-p11.2	ST8 Sia I	Synthesis of gangliosides G_{D3} and G_{T3}.
		ST8SIA2	15q26	ST8 Sia II	Mainly found attached to the neural cell adhesion molecule N-CAM.
		ST8SIA3	18q21.31	ST8 Sia III	Polisialic structures on glycoproteins or glycolypids.
		ST8SIA4	5q21	ST8 Sia IV	Polisialic structures on glycoproteins.
		ST8SIA5	18q21.1	ST8 Sia V	Synthesis of gangliosides G_{D1c}, G_{T1a}, G_{Q1b} and G_{T3}.
		ST8SIA6	10p12.33	ST8 Sia VI	Acts on *O*-glycoproteins.

2.3.2.1 β-galactoside α2,3-sialyltransferase 3 (ST3Gal III)

The enzyme β-galactoside α2,3-sialyltransferase 3 or Galβ1,3(4)GlcNAc-α2,3-sialyltransferase (ST3Gal III or α2,3-sialyltrasferase ST3Gal III) catalyzes the synthesis of NeuAcα2,3Galβ1,3(4)GlcNAc structures, present 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 in SLe^a synthesis. *In vitro* studies showed an additional catalytic activity towards type II precursors, but with lower efficiency. Therefore, this enzyme can also take a part in the synthesis of SLe^x epitope (Kitagawa and Paulson, 1993; Kono *et al.*, 1997; Perez-Garay *et al.*, 2010).

First cloned from a rat liver cDNA library by Wen *et al.* (1992a), a year later the same authors cloned the human gene from a placenta cDNA library, and reported 91% nucleotide homology and 97% aminoacid sequence similarity between the rat and human transcripts (Kitagawa and Paulson, 1993).

ST3Gal III gene resides on chromosome 1 (p34-q33), and it stretches over a gene sequence of approximately 223 kilobases of human genomic DNA. The gene is comprised of 15 exons, 12 of which contain protein encoding sequence (Hu *et al.*, 2011; Kitagawa *et al.*, 1996).

ST3Gal III mRNA expression profile in human healthy tissues from GeneNote database shows that this gene is predominantly expressed in skeletal muscle but also in several other human tissues, such as liver, pancreas, prostate, kidney and lung, heart, brain and spinal cord, thymus, bone marrow and spleen (Shmueli *et al.*, 2003). Characterization of cDNAs encoding human ST3Gal III revealed that this gene produces at least three isotranscripts in human placenta differing at the 5' ends, but that encode for identical protein sequences (Kitagawa and Paulson, 1994). Later, several new isotranscripts generated via alternative splicing were reported in peripheral blood leukocytes (Grahn *et al.*, 2002) and foetal brain (Grahn *et al.*, 2004). The whole data suggests an unknown complexity and expression profile of the ST3Gal III gene in human tissues.

2.4 Lewis blood group determinants

Type I and type II structures in *N*-glycans, *O*-glycans and glycosphingolipids can be further modified by the sequential action of various fucosyl- and sialyltransferases at different positions, giving rise to the Lewis blood group antigens. The term Lewis derives from a family who suffered from a red blood cell incompatibility that led to the discovery of this blood group (Stanley and Cummings, 2009).

» In **type I** glycan structures, α1,3/4-fucosyltransferases FucT III or FucT V can transfer α1,4-linked Fuc to the outer GlcNAc to form Lewis a antigen (Le^a). The α1,2-fucosyltransferase FucT II, or with lower catalytic efficiency FucT I (Barreaud *et al.*, 2000), can transfer α1,2-linked Fuc to the terminal Gal, giving rise to the H1 antigen, which can be further modified by the action of α1,3/4-FucT III or

FucT V, giving place to Lewis b antigen (Le^b). Alternatively, the α2,3-sialyltransferase ST3Gal III, or ST3Gal IV with very low catalytic efficiency, transfer α2,3-linked sialic acid to the terminal Gal to form the Dupan-2 antigen. Afterwards, α1,3/4-FucT III or FucT V originate sialyl-Lewis a antigen (SLe^a) (**Figure 11a**).

» In **type II** glycan structures, α1,3/4 fucosyltransferases FucT III-VI or FucT IX transfer α1,3-linked Fuc to the outer GlcNAc to form Lewis x antigen (Le^x). The α1,2-fucosyltransferase FucT I, or FucT II in lower degree, can transfer α1,2-linked Fuc to the terminal Gal, giving rise to the H2 antigen, which can be further modified by the action of α1,3/4 FucT III-VI or FucT IX, generating Lewis y antigen (Le^y). The α2,3-sialyltransferases ST3Gal III, ST3Gal IV or ST3Gal VI can transfer α2,3-linked sialic acid to the terminal Gal and, lastly, sequential action of α1,3/4 FucT III or FucT V-VII produces sialyl-Lewis x antigen (SLe^x) (**Figure 11b**).

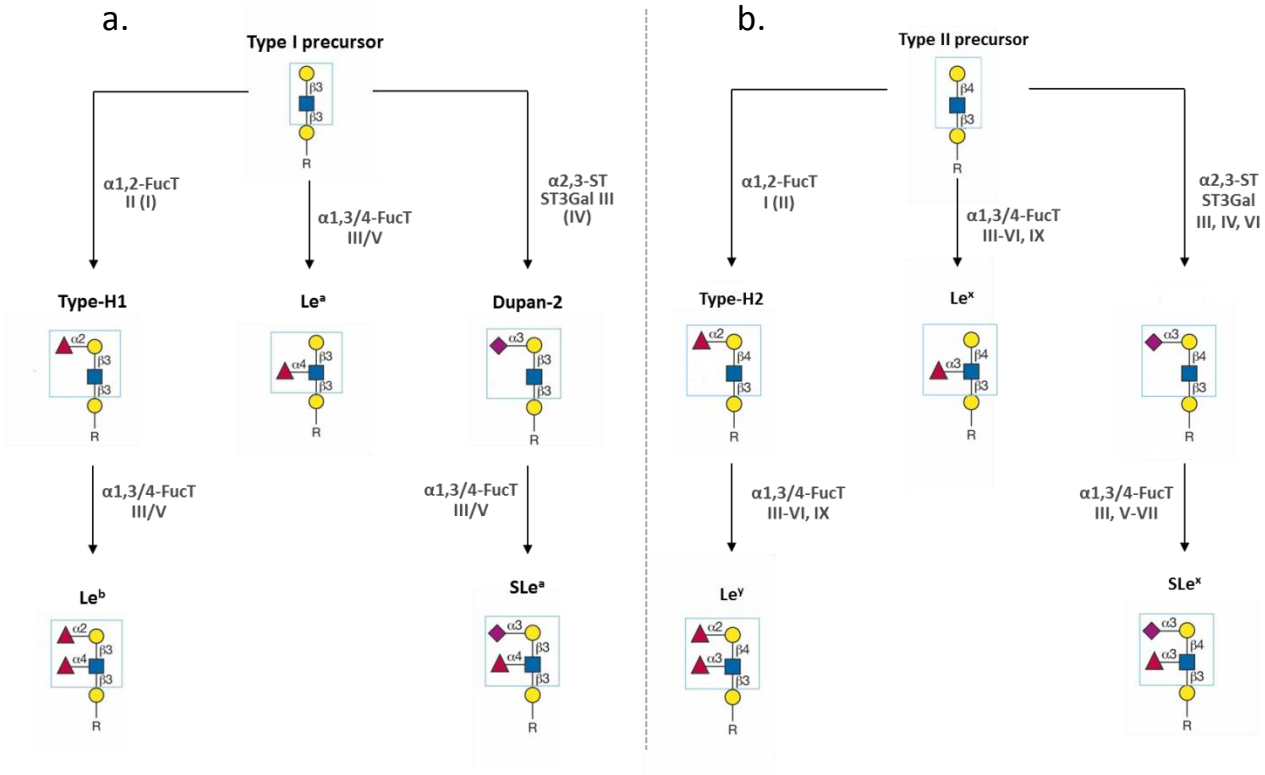


Figure 11. **Glycosyltransferases involved in Lewis antigens synthesis.**
a| From type I precursor. **b|** From type II precursor. Extracted and modified from Stanley and Cummings, 2009. α1,2-FucT: α1,2-fucosyltransferases; α1,3/4-FucT: α1,3/4-fucosyltransferases; α2,3-ST: α2,3-sialyltransferases.

2.4.1 Sialyl Lewis x (SLe^x) epitope [Siaα2,3-Galβ1,4(Fucα1,3)GlcNAc]

The sialylated lacto-*N*-fucopentose with the structure Siaα2,3-Galβ1,4(Fucα1,3)GlcNAc (**Figure 11b**) corresponds to SLe^x. SLe^x epitope was first described in the fraction of gangliosides isolated from human kidney (Rauvala, 1976), and Holmes *et al.* published its biosynthesis in 1986.

SLe^x and its counterpart SLe^a [Siaα2,3-Galβ1,3(Fucα1,4)GlcNAc] have been identified as the minimal ligands for all three types of selectins (E-, P- and L-selectin; **Introduction 2.5.1** and **Table 6**) (Varki, 1994). Hence, its best known function is in the homing and extravasation of leukocytes in the vascular endothelium during the inflammatory response. While non-malignant colonic epithelial cells preferentially express the sialyl 6-sulfo Lewis x determinant, upon malignant transformation colorectal cancer cells express the cancer associated glycan SLe^x, suggesting an impairment of sulphation at C-6 position of βGlcNAc by epigenetic silencing (Izawa *et al.*, 2000). A positive correlation between SLe^x and metastasis and poor survival has been proved in patients for several types of cancer, such as colon cancer (Nakamori *et al.*, 1997; Portela *et al.*, 2011; Vierbuchen *et al.*, 1995), gastric carcinoma (Amado *et al.*, 1998; Ura *et al.*, 1997; Wang *et al.*, 2009), breast cancer (Cui *et al.*, 2011; Jeschke *et al.*, 2005; Matsuura *et al.*, 1997) and pancreatic cancer (Kishimoto *et al.*, 1996). Tumour cells might benefit of this new SLe^x expression to mimic the mechanisms used by leukocytes to leave the blood vessels and enter the surrounding tissue (**Figure 12**).

2.5 Altered glycosylation in cancer

Like normal cells during embryogenesis, tumour cells undergo activation and rapid growth, adhere to a variety of other cell types and cell matrices, and invade tissues. In addition, embryonic development and cellular activation in vertebrates are typically accompanied by changes in cellular glycosylation profiles. Thus, it is not surprising that glycosylation changes are also a universal feature of malignant transformation and tumour progression. Glycan changes in malignant cells take a variety of forms, both in tumour cell surface, in secreted glycoconjugates and in host elements (Varki *et al.*, 2009). The most common glycan alterations are summarized in this section.

» **Increased branching of *N*-glycans**, often attributed to an increased activity of *N*-acetylglucosaminyltransferase V enzyme (GnT-V), which catalyzes the formation of β1,6-GlcNAc branching structures. The change in expression seems to result from increased transcription of its gene (also called MGAT5), but how the levels of this glycosyltransferase gene are regulated remains unclear (Taniguchi and Korekane, 2011). The precise mechanisms linking GnT-V and the metastatic phenotype may be: an increase in poly-*N*-acetyllactosamine-containing glycans (potentially recognized by galectins); alterations in the cell-surface half-life of growth factor receptors caused by changes in galectin-mediated lattice formation; increased outer-chain polyfucosylation and SLe^x production (potentially recognized by selectins), and a general biophysical effect of the branching itself on membrane protein structure.

» **Altered expression and glycosylation of mucins.** Loss of correct topology in malignant epithelial cells allows mucins to be expressed on all aspects of the cells, and soluble mucins can then enter the

extracellular space and body fluids such as the blood plasma (Bhavanandan, 1988). Apart from the specific interactions between *O*-glycans and endogenous lectins, mucins may promote displacement of the cancer cells from the primary tumour during the initiation of metastasis. Further, the aberrant overexpression and large size might provide survival advantage to cancer cells under stress or nutrient depravation by promoting autophagy (Bafna *et al.*, 2010; Baldus *et al.*, 2004). An incomplete elongation of *O*-glycan saccharide chains is typically found in carcinoma mucins, which leads to the expression of T, sialyl-Tn or Tn antigens, usually masked by additional sugar residues in normal tissues (Springer, 1995).

» **Altered expression of certain sialic acid types or their linkages** can have prognostic significance in human cancer (Dabelsteen, 1996; Hedlund *et al.*, 2008). Since sialic acid imparts a negative charge to the carrier glycan chain, this may promote cell detachment from the tumour mass through charge repulsion (Narayanan, 1994). Thus, the overall increase in cell surface sialic acid content is 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). Moreover, sialylated chains protect cells from recognition by the immune system, and also regulate the survival time of tumour cells in bloodstream (Wang, 2005). Another interesting phenomenon is the aberrant expression of *N*-glycolylneuraminic acid (Neu5Gc) in human tumour cells (Malykh *et al.*, 2001).

» **Altered expression of blood group-related structures.** Expression of A and B blood group antigens is lost in many tumours with concomitant increases in H and Le^y expression (Becker and Lowe, 2003; Kim and Varki, 1997). In addition, Le^a, SLe^a and their isomers, Le^x and SLe^x, are frequently overexpressed in carcinomas (Dabelsteen, 1996; Le Pendu *et al*., 2001). Both examples involve fucose-containing oligossacharides. Moreover, in the tumour cells the increase in β1,6-branched *N*-glycans creates new substrates that can be further sialylated and/or fucosylated by overexpressed STs and FucTs, process called *neosynthesis* (Kannagi, 2004). The expression of these antigens correlates with tumour progression, metastatic spread, poor prognosis in humans, and metastatic potential in mice (Nakamori *et al*., 1993; Saito *et al*., 2003; Sozzani *et al.,* 2008). These structures are also critical components of most natural ligands for selectins.

» **Fucosylation in cancer**. Altered levels of core-fucosylation is another of the abnormal glycosylated modifications identified in cancer. Specifically, α1, 6-fucosyltransferase (Fut8) catalyzes the transfer of fucose from guanosine diphosphate (GDP)-fucose to the innermost GlcNAc of hybrid and complex *N*-linked oligosaccharides via an α1,6-linkage, resulting in core-fucosylated glycoproteins (Noda *et al.*, 2003) and altering biological function of the resulting glycoproteins (Javaud *et al.*, 2003; Zhao *et al.*, 2008). Fut8 activity and expression is increased in several human cancers, suggesting a role for this enzyme in tumour development and progression, such as

hepatocellular carcinoma (Comunale *et al.*, 2006), colorectal cancer (Muinelo-Romay *et al.*, 2008), nonsmall cell lung cancer (Chen *et al.*, 2013), ovarian serous adenocarcinoma (Takahashi *et al.*, 2013), liver cancer (Mehta and Block, 2008) or gastric cancer (Zhao *et al.*, 2014). The levels of core-fucosylated biantennary glycans were significantly increased in prostate cancer (Saldova *et al.*, 2011), whereas the more abundant PSA subform showed a decrease in core fucosylated glycans in this type of cancer (Sarrats *et al.*, 2010a). In sera from advanced pancreatic cancer patients, a core fucosylated structure was increased on AGP (α-1-acid glycoprotein) and haptoglobin (HPT) proteins (Sarrats *et al.*, 2010b; **Introduction 2.5.2**), and an overall 40% increase in core fucosylation in the main sialylated biantennary glycans was observed in serum from patients with this cancer (Barrabés *et al.*, 2007).

Other glycan alterations commonly found in cancer consist on increased expression of gangliosides, which can be shed from the cancer cell surface and found in body fluids; loss of glycophospholipid anchor expression, involving the hematopoietic system; increased expression of galectin (especially galectin-3); increase of hyaluronan in the tumour-associated stroma; and changes in sulphated GAGs (Varki *et al.*, 2009).

2.5.1 Key steps in tumourigenesis involving glycans

Changes in glycosylation allow neoplastic cells to usurp many of the events that occur in development. A vast amount of research confirms the connection between aberrant glycosylation in tumour cells and different steps of tumour progression, including proliferation, angiogenesis, cell detachment, invasion and extravasation (Brooks *et al.*, 2010; Fuster and Esko, 2005; Rambaruth and Dwek, 2011).

(1) **Tumour proliferation** is firstly attributed to mutations in proto-oncogenes and tumour supressor genes. However, glycans have important roles in protein folding, quality control and half-life, cell-cell recognition and adhesion, and in signalling. The proliferation of tumour cells is potentiated by the ability of glycoproteins and glycosphingolipids to directly activate growth factor receptor's tyrosine kinases and by the ability of proteoglycans to function as co-receptors for soluble tumour growth factors. Some examples are as follows: de-glycosylated insulin-like growth factor 1 receptor (IGF1R) inhibits the survival of tumours that depend on IGF1R-signalling because the receptor is unable to become fully phosphorylated (Girnita *et al.*, 2000); mammary tumours overexpress MUC4 on the cell surface, which contains an epidermal growth factor (EGF)-like motif on its extracellular domain that directly interacts with ERBB2, initiating phosphorylation of the receptor's tyrosine kinases in the absence of more typical ERBB ligands, such as EGF (Hollingsworth and Swanson, 2004); some

proteoglycans are able to function as co-receptors for soluble tumour growth factors (Esko and Lindahl, 2001).

(2) A few examples exist in which glycosylation occurs in the plasma membrane, the cytoplasm or the nucleous. Tumour matrices are especially rich in hyaluronan, and the interactions between it and its main receptor, CD44, promotes cytoskeletal changes involved in cell motility and growth (Turley *et al.*, 2002). *O*-GlcNAc is an important regulatory mechanism for specific gene transcription, and *O*-GlcNAc in p53 protein appears to block its ability to bind to crucial regions of DNA, which also promotes cell growth (Chou and Hart, 2001).

(3) **Angiogenesis** is stimulated under conditions of hypoxia, when tumoural cells and the surrounding stromal cells produce soluble pro-inflammatory and pro-angiogenic cytokines, such as the vascular endothelial growth factor (VEGF), all of which bind to heparan sulphate. Specifically, it is reported that heparan sulphate-containing proteoglycans on the cell surface facilitates VEGF binding and VEGFR activation on endothelial cells, leading to their mitogenesis and sprouting (Iozzo and San Antonio, 2001).

(4) During **invasion**, cell detachment from one another and from the ECM is essential to migrate through neighbouring tissues. This requires the remodelling of cell-surface adhesion receptors and ligands, and the secretion of proteolytic enzymes and glycosidases, the activity of which gives rise to the release of sequestered growth factors. The aforementioned increased branching of *N*-glycans is commonly found on membrane glycoproteins responsible for cell-cell and cell-ECM interactions, such as E-cadherin and integrins. The increased branching disrupts the ability of these molecules to bind properly to their respective ligands, promoting cell detachment and invasion (Guo *et al.*, 2002; Guo *et al.*, 2003). Tumour sialic acids also potentiate invasiveness by promoting cell detachment from the tumour mass through charge repulsion (Narayanan, 1994) or by facilitating interactions between tumour sialic acids and matrix proteins. As for instance, the sialyl-Tn antigen potentiates tumour invasiveness (Julien *et al.*, 2001; Julien *et al.*, 2012), and an increased sialylation of CD44 in cancer cells reduces the binding to hyaluronan.

(5) After **intravasation** into a vessel, tumour cells are subjected to events that threaten their survival, including shear forces generated by the blood flow and immune response attack. Overexpression of sialylated Lewis antigens allows tumour cells to form aggregates with circulating platelets and leukocytes by the way of selectin-$SLe^{x/a}$ interaction (Nash *et al.*, 2002). These aggregates increase tumour cell survival, helping to the tumour **dissemination** through circulation.

(6) Likewise, tumour cell **extravasation** is initially mediated by the interactions of tumour cells with the activated vascular endothelia via selectin-SLe$^{x/a}$, mimicking the homing of leukocytes in immune response. Tethering and rolling of tumour cells require rapid formation-dissociation of bonds between selectins and their ligands. In addition, while rolling, tumour cells 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 upon the vascular surfaces. Arrested tumour cells can proliferate within the vessel or extravasate across the vascular wall into underlying tissues, generally by chemotaxis (Gout *et al.*, 2008; Kannagi, 1997; Varki, 2007) (**Figure 12**).

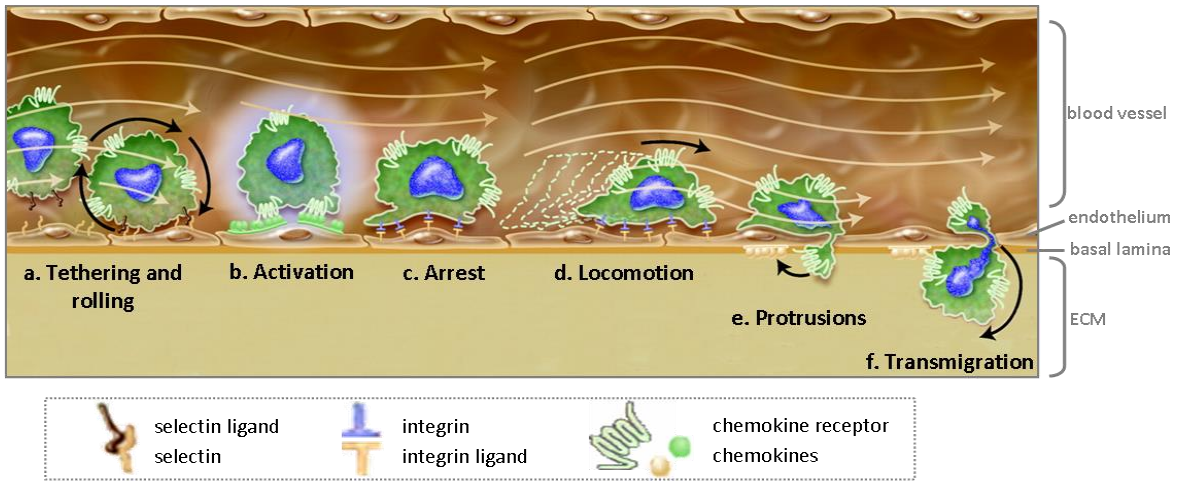


Figure 12. **Progressive steps for tumour cell extravasation.**
a| Under fluid shear forces, the cell rolls across the endothelial surface. Initial tumour cell-endothelium tethering depends mainly on selectins, expressed either by the cell or the endothelium, with carbohydrate ligands on opposing membranes. **b|** Activating signals are delivered through G-protein-coupled receptors upon encounter with ligands, e.g. chemokines. **c|** Activation induces clustering and conformational change of integrins, leading to high affinity binding to endothelial counter-receptors, with subsequent cell arrest. **d|** Apical chemokine exposure drives to locomotion along the endothelial surface in search of interendothelial junctions. **e|** Once at an interendothelial junction, the cell sends protrusions enriched with chemokine receptors to sense the basal lamina environment. **f|** Tumour cell migrates across the resting endothelium, generally by chemotaxis. Extracted and modified from Gout *et al.*, 2008; Ransohoff *et al.*, 2007.

Identifiying glycosylation changes on glycoconjugates at an early stage of cancer development may offer the potential for earlier diagnosis, for monitoring disease progression and/or as a target for biological tumour therapies (Dube and Bertozzi, 2005; Li *et al.*, 2010).

2.5.2 Altered glycosylation in pancreatic ductal adenocarcinoma (PDAC)

Important differences in the expression of glycan structures have been described in PDAC, as well as of the glycosyltransferases involved in their biosynthesis.

Immunohistochemistry studies have shown that healthy pancreatic tissues are characterized by an abundant presence of Lewis-type I antigens (Le^a, SLe^a and Le^b), and also some type II antigens like H2 and Le^y (this last one also present in tumour tissues), whereas in pancreatic tumour cells there is a predominance of Le^x and related antigens (sialylated and extended chains), SLe^a , SLe^x and sialyl-Tn antigens (Kishimoto *et al.,* 1996; Nakamori *et al*., 1999; Park *et al*., 2003; Peracaula *et al*., 2005; Satomura *et al*., 1991). As mentioned before, CA 19-9 antigen, which detects SLe^a epitope on monogangliosides, is the best marker in monitoring PDAC evolution but it lacks predictive value, since it is also detected in benign pancreatic diseases. The origin of circulating CA 19-9 antigen is still not clear and unravelling it may help its clinical applications.

The altered glycosylation pattern of PDAC is an interesting feature which can help to develop new cellular/serologic markers, and several efforts have addressed this issue, still with preliminary results (An *et al*., 2009).

Sawada *et al*. (1994) found that pancreatic cancer sera, but not sera from healthy patients, inhibit E-selectin binding of pancreatic tumour cells, suggesting that E-selectin ligands such as SLe^x could be carried by glycoproteins from the tumour that had reached the bloodstream. The identification of some serum glycoproteins with an increase in both fucosylation and sialylation in pancreatic cancer patients compared to healthy controls and pancreatitis, using glycoprotein microarray with multi-lectin detection, revealed that serum α-1-β glycoprotein and serum amyloid p-component showed an increase in SNA reactivity in PDAC patients compared to pancreatitis (Li *et al.*, 2009; Li *et al.*, 2011; Zhao *et al.*, 2007). Recently, Kontro *et al*. (2014) reported changes in the concentration of several sialylated glycopeptides derived from acute phase proteins in PDAC sera. *N*-glycan sequencing of several serum acute phase proteins showed an increase of core fucosylation in α-1-acid glycoprotein in advanced PDAC patients compared to chronic pancreatitis and healthy controls, while SLe^x was elevated in α-1-acid glycoprotein both in PDAC and chronic pancreatitis patients (Sarrats *et al*., 2010b). All of these identified proteins carrying altered glycosylation in PDAC sera are not tumour-secreted but tumour host response glycoproteins, since all of them are proteins mainly liver-derived.

Mucins are high molecular weight glycoproteins that could be carriers of altered glycosylation. Most PDACs also show changes in the expression profile of transmembrane mucines, such as an overexpression of MUC1 or *de novo* expression of MUC4; and also differences in their glycosylation, such as the presence of Tn or sialyl-Tn epitopes (Remmers *et al*., 2013). In addition, pancreatic cancers also exhibit *de novo* expression of secreted mucins, such as MUC2, MUC5AC, MUC5B, MUC7 and MUC16, which can also show an altered glycosylation pattern (Adsay *et al*., 2005; Hruban *et al*., 2006; Moniaux *et al*., 2004; Remmers *et al*., 2013; Swartz *et al*., 2002). In this regard, CA 19-9 was

detected in MUC1, MUC5AC and MUC16 from PDAC patient sera, suggesting that a biomarker pannel based on the detection of the CA 19-9 on specific proteins accurately identifies a greater percentage of cancer patients than the conventional CA 19-9 assay (Yue *et al.*, 2011).

The overexpression of some of the sialylated determinants associated to pancreatic cancer could be explained from a shift in the expression of specific glycogenes involved in their synthesis. Thus, a correlation between increased SLe^a/SLe^x antigens and diminished α1,2-fucosyltransferase activity, particularly FUT1, and with enhanced α1,3/4-fucosyltransferase FUT3 has been reported (Aubert *et al.*, 2000; Mas *et al*., 1998). In addition, PDAC tissues tend to express high levels of ST3Gal III and ST3Gal IV together with fucosyltransferase genes FUT3 and FUT6, all involved in the last steps of SLe^x biosynthesis (Perez-Garay *et al*., 2013). Furthermore, ST3Gal III has been highly correlated with pancreatic cancer bad prognosis (Amado *et al*. 1998; Kannagi *et al*., 2004; Nakamori *et al*., 1997). Apart from its strict biosynthetic role, ST3Gal III and ST3Gal IV confer pancreatic tumour cells with enhanced E-selectin adhesion and migration, and when ST3Gal III and ST3Gal IV overexpressing pancreatic cancer cells were injected into nude mice, increased metastasis and decreased survival were found (Perez-Garay *et al*., 2010; Perez-Garay *et al*., 2013). In conclusion, both α2,3-sialyltransferases are involved in key steps of pancreatic tumour progression processes and may potentiate the agressiveness of PDAC cells.

Other glycogenes involved in branching or extending glycan chains undergo changes of expression along the genesis and progression of PDAC. Maupin *et al*. (2010) demonstrated that several glycogenes were up-regulated in a panel of pancreatic cancer mesenchymal-like cells, including MGAT5B, ST3Gal II and ST6GalNAc IV. Conversely, in the study of Radhakrishnan *et al*. (2013) when PDAC cells were recovered with core 3 synthase (β3-*N*-acetylglucosaminyltransferase 6; β3Gn-T6), which is lost in pancreatic cancer, tumour growth and metastasis became suppressed. The recovery of β3Gn-T6 activity was related with the loss of the tumour-associated Tn antigen on MUC1. *N*-acetylgalactosaminyltransferases (GalNAcTs) control the initial steps of mucin *O*-glycosylation, leading to carbohydrate Tn antigen. The overexpression of the *N*-acetylgalactosaminyltransferases GalNAc-T3 and GalNAc-T6 that are involved in the first step of *O*-glycan synthesis was reported to correlate with tumour differentiation by immunohystochemistry studies in pancreatic adenocarcinoma (Li *et al*., 2011) and Taniuchi *et al*. (2011) described the participation of GalNAc-T3 in pancreatic carcinogenesis. In this regard, aberrant expression of GalNAcTs contributes to the altered *O*-glycosylation observed in cancer, usually associated to the expression of immature simple mucin-type carbohydrate antigens, and participates in the progression of cancer (Hollingsworth and Swanson, 2004).

3 Cell adhesion and the tissue stroma

Cell adhesion is the mechanism that allows the cells of multicellular organisms to be cohesive, communicate and interact among them and with the ECM. It is a dynamic system essential for normal embryonic development, morphogenesis and tissue repair, and for cell migration as well. It is also involved in pathological processes such as tumour invasion and metastasis, thrombosis and inflammation in general.

In some cases the phenomenon of adhesion is **transient** and is triggered at a particular time during the organism's life, e.g. the homing of leukocytes. Otherwise, it may be more **stable**, creating specific cell adhesion structures perfectly visible by electron microscopy. Specialized cell junctions occur at points of cell-cell and cell-matrix contact in all tissues, and are much more abundant in tissues subjected to strong traction, such as epithelia.

Although each type of junctions has its particular mechanism of formation, regulation and function, extensive communication between the different junctions influences their dynamics and signalling properties. All adhesion complexes share a similar basic architecture: a set of transmembrane components that interact on the outside with ligands (ECM components or neighbouring cells) and in the cytoplasm with generally large multimeric protein complexes of cytoskeletal linkers, regulatory components and signal-transduction proteins (Balda and Matter, 2003). The diversity of molecules involved in cell adhesion, the number of possible combinations and the complexity of the structures formed reflect the multiple functions involving cellular adhesion. A functional classification of cell junctions includes three major groups, listed in **Table 5** and illustrated in **Figure 13**.

Cell junction types and function	Classification	
OCCLUDING JUNCTIONS seal cells together in an epithelium, preventing even small molecules from leaking from one side of the sheet to the other	» Tight junctions (vertebrates only) » Septate junctions (invertebrates mainly)	
ANCHORING JUNCTIONS mechanically attach cells (and their cytoskeleton) to the neighbours or to the ECM	ACTIN FILAMENTS	CELL-CELL: adherens junctions (AJ)
		CELL-ECM: focal adhesions
	INTERMEDIATE FILAMENTS	CELL-CELL: desmosomes
		CELL-ECM: hemidesmosomes
COMMUNICATING JUNCTIONS mediate the passage of chemical or electrical signals from one interacting cell to its partner	» Gap junctions » Chemical synapses » Plasmodesmata (plants only)	

Table 5. Functional classification of cell junctions. Based on the function in which they are involved and the general components. Information extracted from Alberts *et al.,* 2002.

As early as in 1914, Theodor Boveri recognized the importance of specific changes in the adhesion of tumour cells for the development of cancer. Nowadays there is a general consensus that cell-cell and cell-MEC interactions have to be profoundly altered during tumour progression and metastasis to allow the tumoural cell to complete the whole *metastatic cascade*.

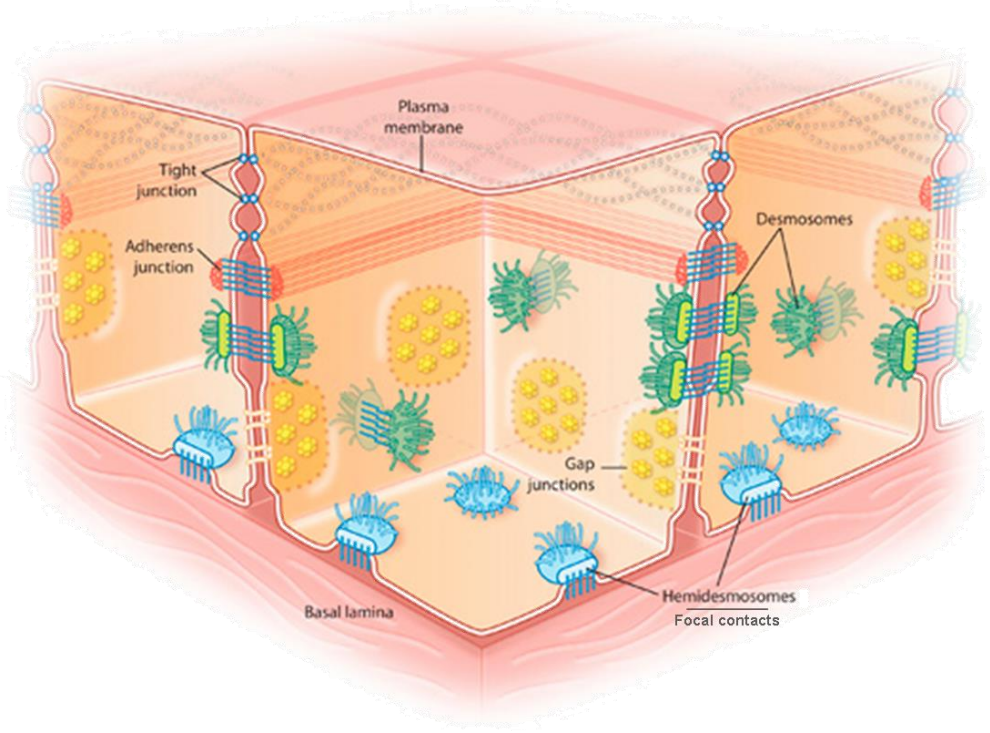


Figure 13. **Cellular location of cell-cell and cell-ECM junctions.** Extracted and modifyed from Scitable, 2012.

3.1 Cell adhesion molecules

Cell adhesion molecules (CAMs), also known as adhesion receptors, are type I transmembrane glycoproteins which mediate cell-cell or cell-ECM adhesion. Interactions can be **homophilic** (between two identical molecules in neighbouring cells), **heterophilic** (between different molecules), or mediated by a **linker molecule**. CAMs are classified into families based on their known ligands and the cell junctions in which they are involved (**Table 6**). Changes in the expression or function of CAMs can contribute to tumour progression both by altering the adhesion status of the cell and by affecting cell signalling, since CAMs may also behave as signal transducers. Therefore, adhesion receptors are a two-way link between the cell and the external environment (Cavallaro and Christofori, 2004a). Specifically, integrins and E-cadherin have been the focus of attention throughout the present Thesis.

Table 6. Structural and functional classification of cell adhesion molecules.
The type of interaction, members belonging to each family, subcellular structures formed, intracellular and extracellular ligands, and electrolytes necessary for a proper function are described. Information extracted from Alberts *et al.,* 2002; Moschos *et al.,* 2007.

Family	Type of interaction	Members	Subcellular structures	Intracellular ligands	Extracellular ligands	Electrolyte
Cadherins	Homophilic cell-cell	Classical N- (neural) P-, R-, B-, E- (epithelial) VE (vascular endothelial) Non-classical desmoglein, desmocollin, T-cadherin, protocadherin	Adherens junctions Desmosomes Hemidesmosomes	p120, plakoglobin (γ-catenin), α-catenin, β-catenin, ZO-1, vinculin, intermediate filaments	Other cadherins in *cis*- or *trans*- dimer configuration. See **Figure 17**	Ca^{2+}
Selectins	Heterophilic cell-cell	L-, E-, P-	*NP*	Anchor proteins, actin	Cell surface glycans with a specific sialyl Lewis-type structure (PSGL-1, GlyCAM-1, CD34, ESL-1)	Ca^{2+}
Integrins	Heterophilic cell-cell and cell-ECM	Heterodimers of α and β subunits	Focal contacts Hemidesmosomes	Talin, vinculin, FAK, α-actin, integrin-linked kinase, parvin, PINCH	See **Figure 15**	Ca^{2+} / Mg^{2+}
Ig-CAMs superfamily	Homophilic and heterophilic cell-cell	N-CAM/CD57, V-CAM, I-CAM, M-CAM/MUC18/CD146, MAd-CAM, PECAM, LFA-3, CD2 (...)	*NP* (Tight junctions)	SH2, ITAM, ITIM	Integrins, selectins	Ca^{2+}
Claudins	Homophilic cell-cell	1-24	Tight junctions	ZO-1, ZO-2, ZO-3,PALS1, PATJ	Claudins	-
Occludin	Homophilic cell-cell	Occludin	Tight junctions	ZO-1, ZO-2, ZO-3	Occludin	?
Syndecans	Heterophilic cell-cell and cell-ECM	1, 2, 3, 4	*NP* (Focal adhesions)	PKC, PIP2, microfilaments, tubulin	Growth factors; collagens 1,3,5; fibronectin; thrombospondin; tenascin; integrins	Ca^{2+}
ADAMs	Heterophilic cell-cell and cell-ECM	2, 7-12, 15, 17-23, 28-30, 33	*NP*	Cut off or shed extracellular portions of transmembrane proteins		

FAK: focal adhesion kinase; Ig; immunoglobulin; ITAM: immunoreceptor tyrosine-based activation motif; ITIM: immunoreceptor tyrosine-based inhibition motif; SH2: Src homology 2; ZO: proteins zonula occludens; PINCH: particularly interesting new cysteine-histidine-rich protein; PALS1: protein associated with Lin seven 1; PATJ: associated tight junction protein; ADAM: A Disintegrin And Metalloproteinase is a family of peptidase proteins; *NP*: none in particular.

3.1.1 Integrins

The name **integrin** was suggested for an integral membrane protein complex first characterized by Tamkun *et al.* in 1986. Since then, a vast amount of work has been performed to study this superfamily of CAMs.

Integrins are heterodimers formed by the noncovalent association of an α subunit of 180 kDa with a β subunit of 95 kDa, approximately, both of which are type I membrane glycoproteins (Arnaout, 2002). In vertebrates, there are 18 α and 8 β subunits that can assemble into 24 different receptors with different binding properties and different tissue distribution. The overall structure of both subunits consists on a large extracellular region which interacts with extracellular ligands (at the intersection of the β-propeller and the βA domain), and is constructed from several domains with flexible linkers between them; a transmembrane region and a short cytoplasmatic tail (except for β4 subunit) that interacts with cytosolic proteins.

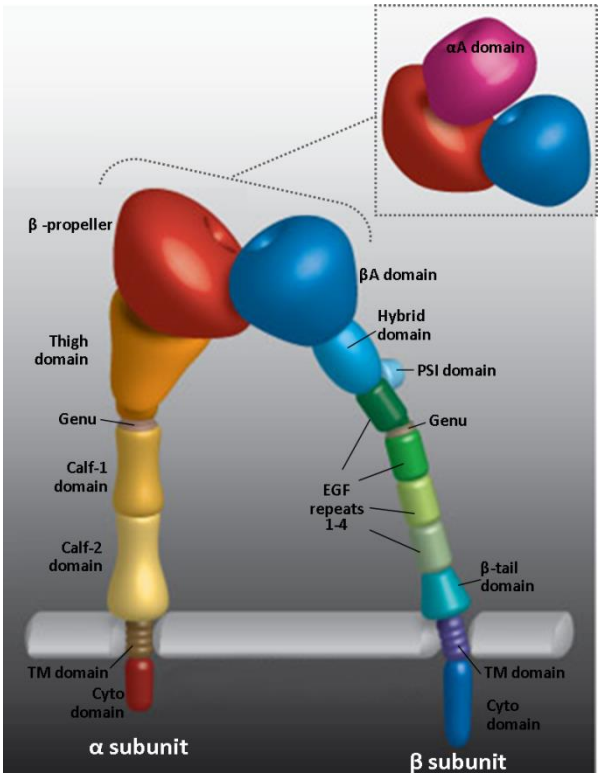


Figure 14. **Integrin architecture.**
α and β subunits specific domains in non-containing and αA-domain containing (squared) integrins. Extracted and modified from Martin Humphries Lab, 2012.

In spite of the common general structure, α and β subunits differ in their structural and functional domains (**Figure 14**). The βA domain contains an Mg^{2+} coordinating MIDAS (metal-ion-dependent adhesion site) and two adjacent Ca^{2+} binding sites (ADMIDAS) that play a crucial role in the ligand binding function (Campbell and Humphries, 2011; Luo *et al.*, 2007).

The α and β subunits show no homology to each other, but different α subunits present 30% homology among them, whereas β subunits show 45% homology. The two gene families were generated by gene duplication, and in humans the genes for both α and β subunits are located on various chromosomes (Takada *et al.*, 2007).

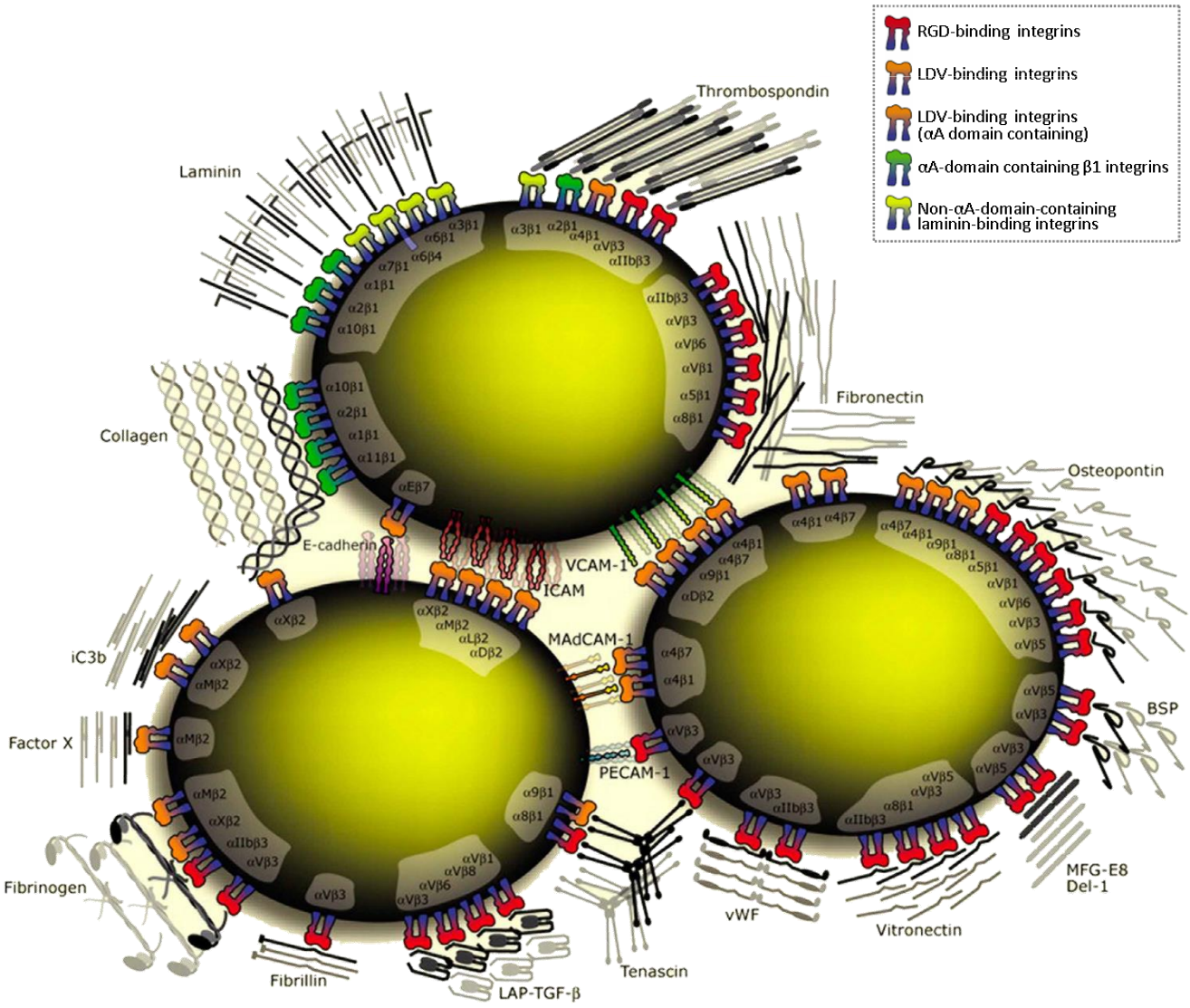


Figure 15. Major integrin-ligand combinations, using hypothetical cell surfaces.
Extracted and modified from Humphries *et al.,* 2006.

BSP: bone sialoprotein; Del-1: developmental endothelial locus-1; EGF: epidermal growth factor; ICAM: intercellular cell adhesion molecule; iC3b: inactivated complement component C3b; LAP-TGF-β: latency associated peptide transforming growth factor β; MAdCAM-1: mucosal addressin cell adhesion molecule 1; MFG-E8: milk fat globule EGF factor 8; PECAM-1: platelet endothelial cell adhesion molecule 1 (CD31); PSI: plexin/semaphorin/integrin homology; VCAM-1: vascular cell adhesion molecule 1; vWF: von Willebrand factor.

Generally, an excess of β subunit exists in the cell, and the amount of α subunit determines the amount of receptor that will go to the cell surface, for what free α and β subunits do not exist at the cell membrane (Barczyk *et al.*, 2010; Santala and Heino, 1991). While integrins are expressed in virtually all tissues, cells tend to express only those heterodimers related to the ligands present in their local microenvironment, for what no cell expresses all of them (Stupack, 2005).

Integrins are involved in a bidirectional mechanism of signalling across the plasma membrane: on one hand, ligand binding to the extracellular domain induces conformational changes and integrin clustering for next activation of signalling cascades and recruitment of multiprotein complexes to focal adhesions. Subsequently, kinase activity-lacking integrins transmit messages through a variety of intracellular protein kinases and adaptor molecules such as focal adhesion kinase (FAK), talin, paxillin, parvins, p130Cas, Src-family kinases and GTPases of the Rho family (**outside-in signalling**). The signals derived determine cellular responses such as migration, survival, differentiation and motility (Harburger and Calderwood, 2009; Hehlgans *et al.*, 2007; Travis *et al.*, 2003). Importantly, ligand binding also allows the formation of focal adhesions and hemidesmosomes by further connection to actin or intermediate filaments, respectively (Geiger *et al.*, 2009) (**Figure 16**).

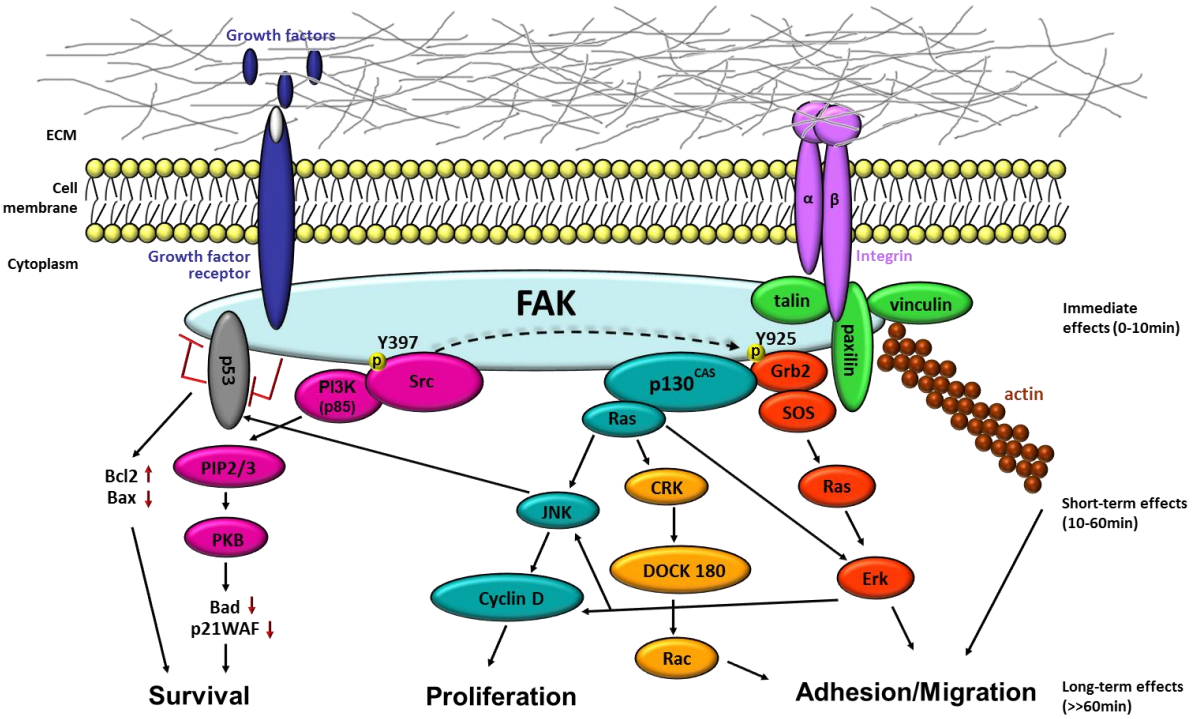


Figure 16. **Focal adhesion kinase (FAK)-mediated signalling pathways.**
Via its N-terminal domain, FAK is targeted to growth factor receptors, and via its C-terminal domain to adhesion-mediated clustered integrins. Paxillin, vinculin and talin are important for the localization of FAK to the integrins. These interactions result in the autophosphorylation of FAK tyrosine 397, which provides a binding site for Src kinase. Formation of this FAK-Src complex leads to the phosphorylation of additional FAK tyrosines. By the interaction and/or phosphoryation of a large number of targets, FAK is a mediator of survival, proliferation, adhesion and migration signalling. Extracted and modified from van Nimwegen and van de Water, 2007.

On the other hand, integrins can regulate their own affinity for extracellular ligands by signals from inside the cell (**inside-out signalling**) (Calderwood, 2004; Stupack and Cheresh, 2002). It is worth pointing here that many motogenic growth factors act in an integrin-dependent fashion; that is, EGF, VEGF and PDGF, among others, require integrin-substrate ligation to effect signalling (Stupack, 2005).

All integrins are prominent carriers of *N*-glycans, as they contain over 20 potential *N*-glycosylation sites. A great number of studies hypothesize that variant *N*-glycosylation may be a regulatory mechanism for β1 integrins (Bellis, 2004; Gu and Taniguchi, 2004; Zhao *et al.*, 2008b). But only the *N*-glycans localized in certain motifs are proposed to regulate the conformation and biological function of integrins (Isaji *et al.*, 2006; Isaji *et al.*, 2009). In particular, the modification of integrin subunits by sialyltransferases makes them to be capped with the negatively charged sugar sialic acid, and contributes to the proper function of these receptors (Litynska *et al.*, 2002; Liu *et al.*, 2008; Nadanaka *et al.*, 2001; Zhao *et al.*, 2006). In addition, alterations in the *N*-glycosylation of integrins may regulate their interactions with membrane-associated proteins such as certain growth factor receptor, e.g. EGFR; other receptors, e.g. urokinase-type plasminogen receptor activation; and the tetraspanin family.

Furthermore, some studies have related changes in the expression levels of certain integrins with several diseases, such as tumour progression (Danen, 2005; Guo and Giancotti, 2004; Varner and Cheresh, 1996). In the pancreatic cancer context, a number of integrin subunits have been shown to be both up-regulated and delocalized *in vivo,* including the α1, α2, α3 and α6 integrin subunits (Binkley *et al.*, 2004; Grzesiak *et al.*, 2007; Iacobuzio-Donahue *et al.*, 2003; Linder *et al.*, 2001; Rosendahl *et al.*, 1993; Shimoyama *et al.*, 1995; Weinel *et al.*, 1992). Furthermore, an essential role in adhesion and invasion of pancreatic carcinoma cells by β1 integrins has been reported (Arao *et al.*, 2000).

3.1.2 E-cadherin

E-cadherin, alternatively known as CAM 120/80 or uvomorulin, is a type I membrane glycoprotein, generally considered the prototype of all cadherins. Firstly described in 1977 by Takeichi, in 1984 Yoshida-Noro *et al.* introduced the name **cadherins** as a generic name for this important family of cell-cell adhesion molecules. The prefix *E*- accounts for *epithelial* to differentiate from other cadherins with distinct spatio-temporal expression patterns (van Roy and Berx, 2008).

The mature E-cadherin molecule is approximately 120 kDa and contains an ectodomain comprising five tandemly repeated domains, so-called extracellular cadherin repeats (EC1 to EC5); a single transmembrane domain and a cytoplasmic domain (**Figure 17**).

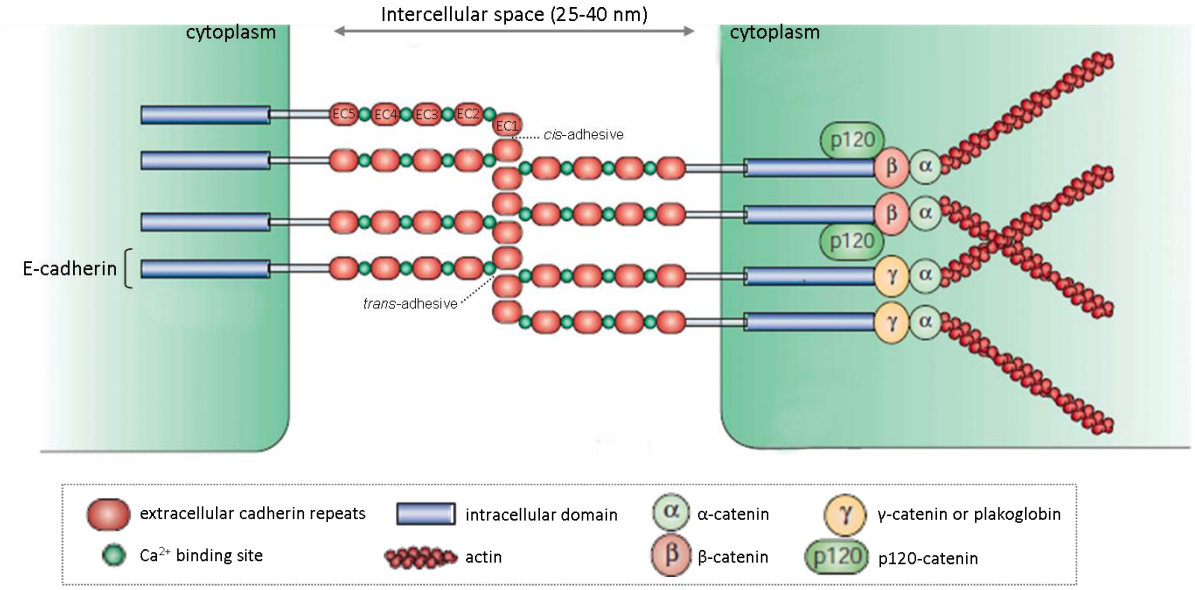


Figure 17. **Schematic overview of the E-cadherin-catenin complex (CCC) at the junction between two adjacent epithelial cells.**
β-catenin and γ-catenin (or plakoglobin) bind to the same conserved site at the carboxyl termini of E-cadherin in a mutually exclusive way, whereas p-120-catenin interacts with several sites in the cytoplasmic tail. β-catenin and γ-catenin bind directly to α-catenin, which links the CCC to the actin cytoskeleton. Extracted and modified from Cavallaro and Christofori, 2004a; Berx and van Roy, 2009.

The extracellular domain is responsible for adhesive recognition through the binding to ectodomains of others cadherins, both in *cis* (the same cell) and in *trans* (the adjacent cell), in a zipper-like fashion (Leckband and Prakasam, 2006). The binding between ectodomains of adjacent cadherins is weak, but strong cell-cell adhesion occurs during the lateral clustering and thanks to intracellular interactions. Since E-cadherin lacks intrinsic enzymatic activity, the connection with intracellular events occurs through a range of intermediary proteins. More specifically, the cytoplasmic cell-adhesion complex (CCC), consisting of α-catenin, β-catenin, γ-catenin (plakoglobin) and p120-catenin, links E-cadherin homodimers to the actin cytoskeleton. Without an intact CCC, cadherin-mediated strong cell-cell adhesion is compromised (Cavallaro and Christofori, 2004a) (**Figure 17**).

E-cadherin is the key player in inducing cell polarity and organizing an epithelium. Thus, the spatio-temporal regulation of E-cadherin expression during embryonic development allows cell migration and morphogenesis. In most, if not all, cancers of epithelial origin E-cadherin-mediated cell-cell adhesion is lost along the **Epithelial to Mesenchimal Transition** (EMT) process, leading to a fundamental change in the cellular phenotype and in the mobility behaviour. What is more, EMT is highly associated with progression from adenoma to carcinoma and the subsequent formation of tumour metastases (Kim *et al.*, 2011; Perl *et al.*, 1998; Pryczynicz *et al.*, 2010; Thiery and Sleeman, 2006). Possible inactivating mechanisms of E-cadherin include mutation and chromosomal

aberrations, epigenetic silencing, endocytosis, proteolytic degradation and increased expression of non-epithelial cadherins -the *cadherin switch* to mesenchymal cadherins, such as N-cadherin- (Cavallaro and Christofori, 2004a; van Roy and Berx, 2008). With these observations is easy to consider E-cadherin as a classical tumour supressor gene. Reciprocally, E-cadherin transcription is re-iniciated in cells undergoing **Mesenchimal to Epithelial Transition** (MET) (Halbleib and Nelson, 2006; van Roy and Berx, 2008).

The E-cadherin molecule has four potential *N*-glycosylation sites, two located in EC4 and the other two in EC5. Several studies have shown that alterations in its *N*-glycosylation can also modulate the biological functions of E-cadherin (Pinho *et al.*, 2011). In particular, *N*-glycans at Asn 633 are essential for its proper folding, trafficking and expression (Zhou *et al.*, 2008). Furthermore, *N*-glycans have been reported to influence the stability of adherens junctions (AJ) by affecting their molecular organization (Zhao *et al.*, 2008a). Liwosz *et al.* in 2006 found that the modification of E-cadherin with complex *N*-glycans was associated with the formation of dynamic but weak AJ, whereas diminished *N*-glycosylation of E-cadherin promoted the establishment of stable AJ. Consistent with this, an inverse relationship between the extent of branched *N*-glycans and the establishment of intercellular adhesion was reported by Vagin *et al.* in 2008. Moreover, Pinho *et al.* (2009) demonstrated extensive modifications of E-cadherin *N*-glycans during the acquisition of the malignant phenotype in a canine mammary tumour cell line model, consisting of an increase in β1,6-branched structures, an increase in sialylation and the presence of few high mannose structures, when compared to E-cadherin from a non malignant cell line model.

Recent data suggest that EMT may play a crucial role in the control of tumour invasion, metastasis and drug resistance in pancreatic cancer. Several factors have been proposed to trigger EMT, including citokines and specific cellular signalling pathways, as well as cell glycosylation, among others (Freire-de-Lima, 2014; Grosse-Steffen *et al.*, 2012; Maupin *et al.,* 2010; Wu *et al.*, 2012).

3.2 The tissue stroma and its involvement in tumourigenesis and cell invasion

A substantial part of tissues's volume is extracellular space, largely filled by connective tissue, namely **stroma**. It is constituted by an intricate network of macromolecules: the ECM, consisting of structural proteins (collagen and elastin), specialized proteins (fibrillin, fibronectin and elastin), and proteoglycans; cellular elements (fibroblasts, innate and adaptative immune cells); and vasculature with endothelial cells and perycites. There is a continuous and bilateral crosstalk between normal epithelial cells and cells of the stroma, mediated through direct cell-cell contacts or by secreted molecules (growth factors, cytokines, chemokines, ECM proteins, proteinases, proteinase inhibitors

and lipid products). This way, the stroma actively participates in the maintainance of the tissue integrity and in the regulation of cell functions.

The amount, exact composition and arrangement of the stroma depend and are adapted to the type of tissue and functional requirements (Bremnes *et al.*, 2011; De Wever and Mareel, 2003). The local microenvironment provides extrinsic barriers that are evolutionary conserved to preserve normal tissue structure and function. These barriers can be broadly classified as:

- **Chemical:** reactive oxygen species (ROS), hypoxia, low pH.
- **Physical:** basement membrane, interstitial pressure, tensional forces.
- **Biological:** immune surveillance, cytokines, regulatory ECM peptides.

These extrinsic barriers limit the outgrowth of the tumour at the primary site, but as tumours evolve these pressures drive the selection for traits that enable cancer cells to by-pass them (Gupta and Massague, 2006). In that sense, cancer invasion is regarded as an heterogenous and adaptative process, involving a clear plasticity in every step, since when the epithelium changes the stroma inevitably adapts and reacts to establish a permissive and supportive environment (Bishop, 1991). In addition, chronic inflammation conditions may induce stromal activation and have been frequently associated with cancer risk in numerous tissues (Coussens and Werb, 2002; Grivennikov and Karin, 2010).

Several aspects of tumour progression are ruled by a strict connection between the stroma and tumour cells, and are summarized as follows:

(1) The ECM provides a structural and molecular frame for the moving cell body and thereby impacts the mode and efficiency of cell migration -ameboid, mesenchymal, multicellular, collective or expansive growth-. The ECM dimension (2D or 3D), density and gap size, stiffness and fibers orientation are clue features that define the migratory capability of invasive cells (Friedl and Alexander, 2011; Friedl and Wolf, 2010).

(2) ECM receptors and cell-cell adhesion receptors balance the cohesion within the tumour cells and the stroma. A key stone of cell migration is the disruption of normal cell adhesions that prevent this cohesion, achieved by downregulating the expression of CAMs or by inactivating signalling pathways (Conacci-Sorrell *et al.*, 2002).

(3) In both tumour and stroma cells, multiple protease systems are upregulated with overlapping substrate specificities, which contribute to tumour invasion by proteolysis of structural ECM proteins, remodeling it and regulating the repertoire of available extracellular growth factors by enzymatic activation, inactivation or degradation.

(4) Finally, directed cell migration depends on the cells' ability to detect and move towards chemoattractant gradients of growth factors and chemokines (Ulrich and Heisenberg, 2009).

3.2.1 Tumour microenvironment in PDAC

One of the most important features of PDAC is a particularly excessive desmoplasia, which is rarely observed in other cancers of the pancreas (Ghaneh *et al.*, 2007; Kleeff *et al.*, 2007). Early PanIN lesions may be associated with small amounts of normal stroma surrounding the normal pancreatic ducts from which the PanINs arise. However, the enhancement of stroma formation begins in PanIN-3 and continues during the progression to invasive carcinoma, that ultimately results in extensive stroma with an associated inflammatory infiltrate (Korc, 2007).

Several studies have associated long-standing chronic pancreatitis and cancer in patients (Lowenfels *et al.*, 1993; Lowenfels *et al.*, 1997; Whitcomb and Pogue-Geile, 2002), likely explained by an inflammatory environment associated with ROS production, cytokine release (TNFα, IL6, IL8 and interferon-α) and upregulation of pro-inflammatory transcription factors such as NF-κβ, provided by pancreatitis. These inflammatory response mediators can induce malignant tranformation, and the accumulative effect eventually can result in PDAC (Farrow and Evers, 2002; Hernandez-Munoz *et al.*, 2008).

In their quiescent state, pancreatic stellate cells (PSC) comprise approximately 4% of the pancreatic cell population and are located in the periacinar and interlobular space. Later on, cytokines and growth factors such as PDGF, FGF, TGFβ, IGF1, released by inflammatory cells, platelets, perycites and neoplastic cells can activate PSC, which transform into activated myofibroblast-like cells. Proliferating fibroblasts and PSC, in turn, produce and deposit high amounts of ECM components (mainly type 1 and 3 collagens and fibronectin), and they can also regulate the turnover of the ECM through their ability to produce several matrix metalloproteinases (mmp) and their inhibitors (Bachem *et al.*, 2005; Duner *et al.*, 2010). Cancer cells are also capable of synthesizing and releasing type 1 and 3 collagens (**Figure 18**).

The stroma also contains nerve fibers that release nerve growth factors (NGFs), bone marrow derived stem cells that may have the capacity to differentiate into PSC and fibroblasts, and endothelial cells (Sangai *et al.*, 2005; Zhu *et al.*, 2002). Due to its location, pancreatic cancer cells are exposed to high levels of insulin deriving from the adjacent endocrine islets. In addition, an altered gene expression profile is described, including altered integrin expression that may promote tumour cell motility, increased expression of cyclooygenase-2 (COX-2), VEGF and type 1 collagen, which enhance stromal neovascularization and promote tumour cell growth (Korc, 2007; Langley and Fidler, 2007). Taken altogether, all these features create a unique microenvironment in which PDAC cells can prosper, and from which they can readily metastasize.

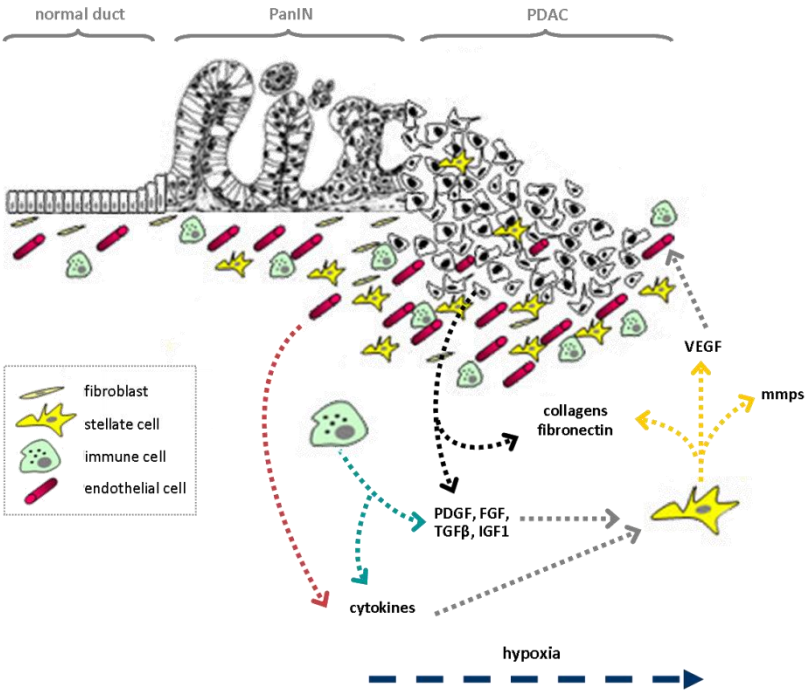


Figure 18. The stromal structure in the progression model of PDAC.
Extracted and modified from Kleeff *et al.,* 2007.

PDGF: platelet-derived growth factor; FGF: fibroblast growth factor; TGFβ: transforming growth factor beta; IGF1: insulin-like growth factor 1.

Previous research in our group showed a correlation between α2,3-sialyltransferase activity and the expression of the respective sialylated antigens on the surface of various established PDAC cell lines with different status of differentiation (Peracaula *et al.*, 2005). In addition, increased expression of ST3Gal III enzyme in stably transfected Capan-1 and MDAPanc-28 cell lines was linked to an increase in membrane SLe^x content, and resulted in greater cell adhesion to E-selectin and increased cell migration through type 1 collagen. ST3Gal III overexpression also accelerated tumourigenesis and decreased survival in athymic nude mice (Perez-Garay *et al.*, 2010). Similarly, ST3Gal IV overexpression in MDAPanc-28 cells caused an increase in SLe^x antigen, and enhanced E-selectin adhesion and migration. Furthermore, when ST3Gal III and ST3Gal IV MDAPanc-28 transfected cells were injected into nude mice, increased metastasis and decreased survival were found in both cases when compared to controls (Perez-Garay *et al.*, 2010; Perez-Garay *et al.*, 2013).

With this backgroung data, the aim of the present Doctoral Thesis was to elucidate the influence of the SLe^x antigen on membrane glycoproteins linked to key adhesion and migration steps of PDAC progression, as well as to study the potential regulation of the sialyl- and fucosyltransferase genes involved in the synthesis of this antigen and related determinants by pro-inflammatory cytokines present in the tumour microenvironment of PDAC.

Objectives

This work has been focused on the study of the altered cell membrane sialylation, in particular the overexpression of SLe^x epitope, in pancreatic adenocarcinoma to shed light on its involvement in the progression of this disease, focusing on adhesion and invasion events. In this regard, diverse methologies have been used in order to accomplish the following main objectives:

1. To determine the influence of α2,3-sialyltransferase ST3Gal III, and more specifically of SLe^x epitope, in the extracellular matrix adhesion, in migration and in the invasive behaviour of Capan-1 and MDAPanc-28 pancreatic adenocarcinoma cells.

2. To study the influence of α2,3-sialyltransferase ST3Gal III, and more specifically of SLe^x epitope, in the biological function of crucial cell membrane adhesion molecules such as integrins and E-cadherin, and their distribution in human PDAC samples.
    a. Effect on the adhesion of the cells to type 1 collagen through α2β1 integrin.
    b. Effect on the cell-cell adhesion through E-cadherin.

3. To investigate the regulation of sialyltransferase and fucosyltransferase genes involved in the synthesis of SLe^x epitope and related sialylated or Lewis-type antigens by pro-inflammatory cytokines in MDAPanc-28 and MDAPanc-3 pancreatic adenocarcinoma cells, as well as to correlate the grade of inflammation with the expression of Lewis-type antigens in human PDAC samples.

Materials and methods

1 Cell culture and cytokine treatments

Routine culture conditions for the human pancreatic adenocarcinoma cell lines used in molecular biology, cell based and biochemistry assays are described in this section. Specific culture conditions for cytokine treatments are also detailed.

1.1 Human pancreatic adenocarcinoma cell lines

The human pancreatic adenocarcinoma cell lines used throughout this work are MDAPanc-28, MDAPanc-3 and Capan-1.

» **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 ST3Gal IV enzymes, and low SLe^x expression. MDAPanc-28 cells spontaneously differentiate as they become confluent (Frazier *et al.*, 1996; Peracaula *et al.*, 2005; Perez-Garay *et al.*, 2010).

» **MDAPanc-3**, also a generous gift from Dr. Frazier, is a cell line established from a liver metastasis of a moderately differentiated adenocarcinoma of the head of the pancreas. It shows high levels of endogenous ST3Gal III and ST3Gal IV, and also high levels of SLe^x epitope (Frazier *et al.*, 1990; Peracaula *et al.*, 2005).

» **Capan-1**, obtained from the American Type Culture Collection (ATCC num. HTB-79) is a well-differentiated cell line of ductal origin established from a liver metastasis of a pancreatic adenocarcinoma. This cell line has medium levels of endogenous ST3Gal III and ST3Gal IV enzymes, and expresses high levels of SLe^x. Capan-1 cells also spontaneously differentiate on becoming confluent (Fanjul *et al.*, 1991; Fogh *et al.*, 1977; Hollande *et al.*, 1990; Kyriazis *et al.*, 1982; Levrat *et al.*, 1988; Peracaula *et al.*, 2005; Perez-Garay *et al.*, 2010).

MDAPanc-28 and Capan-1 cells stably transfected with the pcDNA^{TM} 3.1 expression vector encoding the rat ST3Gal III gene or the empty vector (Pagès-Pons, 2006) were also used. From the characterized ST3Gal III overexpressing clones (C31 and C32 for Capan-1, and M33 and M34 for MDAPanc-28) that showed similar behaviour, the highest ST3Gal III and SLe^x expressing clones for each cell line, C31 and M34, were chosen to address the assays through this work. **MN** and **CN** define the parental MDAPanc-28 and Capan-1 lines; **MP** and **CP** define the mock lines; and **M34** and **C31** correspond to the transfected lines with ST3Gal III gene, respectively.

1.2 Routine culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I containing 10% foetal bovine serum (FBS), 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 37°C in humidified atmosphere containing 5% CO_2. Stably transfected cells were supplemented with 400 µg/ml (Capan-1) or 800 µg/ml (MDAPanc-28) of Geneticine® G-418 (Gibco). Cell growth and morphology were daily assessed under the field microscope.

For routine culture, 3.5 x 10^5 Capan-1 parental and transfected cells, 5.5 x 10^5 MDAPanc-28 parental and transfected cells or 5 x 10^5 MDAPanc-3 cells were seeded in 75 cm^2 flasks (Nunc; Roskilde, Denmark) and cultured for 84 h (exponential growth). In the experiments with the transfected cells, they were also seeded and cultured for 84 h.

Cells were maintained for up to 10 passages by successive trypsinization with 0.5% trypsin-EDTA 10X (Gibco) and seeding. After 10 passages, new cells were expanded from the frozen stock. Cell viability was assessed at each trypsinization by Trypan Blue staining. Possible *Mycoplasma* contamination was routinely checked using the Venor®GeM *Mycoplasma* Detection Kit (Minerva Biolab GmnH; Germany).

1.3 Cytokine treatments

Cytokine treatment was based on a previously described method (Padro *et al.*, 2011) with minor modifications. 1.8 x 10^5 MDAPanc-3 or 1.5 x 10^5 MDAPanc-28 cells were seeded in 25 cm^2 flasks (Nunc) and cultured for 72 h in standard conditions. At subconfluence, cells were washed twice with sterile phosphate buffered saline (PBS). Next cells were serum starved for 4 h to bring them to a basal activation state. Then they were treated with or without the presence of IL-1β, IL-6, IL-8 or TNFα pro-inflammatory cytokines (all of them from R&D Systems; Minneapolis, USA) at various concentrations within the pM range, in DMEM for 4, 12 or 24 h (for glycosyltransferase mRNA evaluation) or for 40 h (for carbohydrate determinants detection by flow cytometry). Following procedures are detailed in **Materials and methods 2** and **3.1**, respectively. At least three independent experiments were performed per each treatment.

2 Molecular biology techniques

Molecular biology techniques for total RNA isolation, single-stranded cDNA synthesis and cDNA amplification by semi-quantitative PCR or quantitative Real-Time PCR (qRT-PCR) were performed in order to study the mRNA expression of specific fucosyl- and sialyltransferases in the pancreatic adenocarcinoma cell lines.

2.1 RNA isolation and cDNA synthesis

RNA was isolated from the cells using RNeasy® Mini Kit including on-column DNase digestion using the RNAse-Free DNase Set, according to the manufacturer's instructions (Qiagen GmbH; Hilden, Germany). Extraction yield and RNA purity were spectrophotometrically determined using a Nanodrop® instrument (ND-1000, Thermo Fisher Scientific; Wilmington, USA). 2000 ng of total RNA were reverse transcribed to single stranded cDNA using MultiScribe™ Reverse Transcriptase and random hexamer primers (Applied Biosystems Inc.; Foster City, USA).

2.2 Semi-quantitative PCR

ST6GAL1 and ST6GALNAC1-2 genes expression in MDAPanc-28 cells was examined by semiquantitative PCR using a MyCycler™ thermal cycler (BioRad Laboratories; Hercules, USA). The primer sequences, temperatures of annealing and products size are specified in **Table 7**.

15 µl aliquots of amplified cDNAs were run in 1.5% agarose gels with tris/borate/EDTA buffer (TBE 0.5X) stained with 0.5 µg/ml ethidium bromide and visualized under Gel Printer Plus UV-light (Scion Corporation; Maryland, USA). The intensity of the amplified cDNA bands was measured using the

Quantity-One software package (BioRad) and was normalized to β-actin. Data were expressed as the mean ± standard deviation (SD) of the intensity values of three independent PCR assays.

Gene name	Primer sequence	Annealing temperature	Product size	Reference
ST6GAL1	5'-AAAAACCTTATCCCTAGGCTGC-3' (Fw) 5'-TGGTAGTTTTTGTGCCCACA-3' (Rv)	60.8°C	379 bp	Petretti *et al.,* 2000
ST6GALNAC1	5'-GGACTATGAGTGGCTGGAAGCA-3' (Fw) 5'-CTGGTACAGCCGGATTATCCCT-3' (Rv)	65°C	421 bp	Julien *et al.,* 2001
ST6GALNAC2	5'-CTGCCAGTAAATTCAAGCTGC-3' (Fw) 3'-TTGCTTGTGATGAATCCATAGC-3' (Rv)	60.8°C	184 bp	Schneider *et al.,* 2001
ACTB	5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' (Fw) 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (Rv)	68.2°C	838 bp	Mas *et al.,* 1998

Table 7. Primer sequences, temperatures of annealing and products size in semi-quantitative PCR assays. Primer oligonucleotides were manufactured by Roche Diagnostics (Indianapolis, USA). ACTB: β-actin gene, in grey, used as internal control.

2.3 Quantitative Real-Time PCR

Quantitative Real-Time PCRs (qRT-PCR) were performed in triplicate using RNA from three biological replicas for the target genes listed in **Table 8**.

MDAPanc-28		MDAPanc-3	
Gene name	**Primers and probes**	**Gene name**	**Primers and probes**
ST3GAL3	Custom TaqMan®	ST3GAL3	Hs00544033_m1
ST3GAL4	Hs00920870_m1	ST3GAL4	Hs00920870_m1
ACTB	Custom TaqMan®	ST6GAL1	Hs00300842_m1
FUT3	5'-GCCGACCGCAAGGTGTAC-3' (Fw) 5'-TGACTTAGGGTTGGACATGATATCC-3' (Rv)	ST6GALNAC1	Hs00300842_m1
FUT4	5'-AAGCCGTTGAGGCGGTTT-3' (Fw) 5'-ACAGTTGTGTATGAGATTTGGAAGCT-3' (Rv)	FUT1	Hs00355741_m1
FUT5	5'-TGGGTGTGACCTCGGCGTGA-3' (Fw) 5'-AAACCAGCCTGCACCATCGCC-3' (Rv)	FUT2	Hs00704693_s1
FUT6	5'-CAAAGCCACATCGCATTGAA-3' (Fw) 5'-ATCCCCGTTGCAGAACCA-3' (Rv)	FUT3	Hs01868572_s1
FUT7	5'-TCCGCGTGCGACTGTTC-3' (Fw) 5'-GTGTGGGTAGCGGTCACAGA-3' (Rv)	FUT4	Hs01106466_s1
HPRT	5'-GGCCAGACTTTGTTGGATTTG-3' (Fw) 5'-TGCGCTCATCTTAGGCTTTGT-3' (Rv)	FUT5	Hs00704908_s1
		FUT6	Hs03026676_s1
		FUT7	Hs00237083_m1
		TBP	Hs99999910_m1

Table 8. Cell line, genes tested and probes used in qRT-PCR assays.
Genes used as internal control, in grey.
ACTB: β-actin gene; *HPRT*: hypoxanthineguanine phosphoribosyltransferase gene; *TBP*: TATA box binding protein gene.

Primers and probe sequences for ST3GAL3 and ACTB genes were Custom Taqman® Gene Expression Assays™ designed by Perez-Garay *et al.* (2010), and the other probes used were human pre-designed TaqMan® Gene Expression Assays, from Applied Biosystems. Data collection was performed using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems).

For MDAPanc-28 cells, α1,3/4-FucTs expression was quantified using QuantiTect™ SYBR® green qRT-PCR (Qiagen) (FUT3, FUT4, FUT6 and FUT7 probes described by Higai *et al. (*2006a); HPRT gene assay extracted from Gene-Cards database, NCBI36:X), and data collection was performed using an ABI PRISM® 7900HT system (Applied Biosystems).

The relative concentration of each gene was calculated by the comparative Ct method.

3 Cell based assays

The cell based assays of this work consist on flow cytometry analysis, cell adhesion to ECM components, type 1 collagen migration, Matrigel invasion and slow aggregation on soft agar assays. Sialidase treatments before specific cell adhesion assays are also described.

3.1 Flow cytometry analysis

Detection and quantification of carbohydrate determinants, integrin subunits and E-cadherin levels at the cell surface was performed by indirect immunofluorescence.

Cells (5 x 10^5) were incubated for 30 min at 4°C in the presence or absence of the corresponding monoclonal primary antibody (mAb) or biotinylated lectin diluted in PBS-1% bovine serum albumin (BSA) (see dilutions in **Table 9**).

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, USA) diluted 1/200 for 30 min at 4°C. Flow cytometry assays of cells treated with cytokines were performed with 2 x 10^5 cells following the same protocol. Analyses were performed using a FACSCalibur™ instrument (BD Biosciences; San Jose, USA) equipped with CELLQuest™ Pro software. Mean Fluorescence Intensity (MFI) was calculated as the quotient between the positive and negative GeoMean for each cell line. Three independent assays were undertaken for each sample. Not aggregation or loss of viability of the cells was detected after antibody or lectin incubations.

3.2 Cell adhesion assays

Cell adhesion assays were performed to study the capability of PDAC cells to bind to type 1 collagen, fibronectin or laminin, and were performed following described procedures (Grzesiak and Bouvet, 2008) with minor modifications. 96-well microplates were coated with a solution of 10 µg/ml of type

1 collagen from calf skin (COL), fibronectin from human plasma (FN) or laminin from human placenta (LN) (Sigma-Aldrich; St. Louis, USA); or PBS-1% BSA as control. Wells were blocked with PBS-1% BSA for 1 h. For each well, 2.5 x 10^4 CN, CP or C31 cells, or 4 x 10^4 MDAPanc-28 cells were seeded and incubated at 37°C for 10 min (COL and FN) or 20 min (LN) for Capan-1 model; or 40 min (COL) for MDAPanc-28. In selected experiments, cells were previously incubated with function-blocking mAb or lectin for 30 min at 4°C (dilutions in **Table 9**). Not aggregation or loss of viability of the cells was detected after antibody or lectin incubations.

	Epitope	Clone	Flow cytometry	Blocking	Collagen migration
Monoclonal antibodies (mAbs)	α2 integrin subunit	P1E6	1/200	1/50	1/50
	α5 integrin subunit	P1D6	1/200	1/50	-
	α3 integrin subunit	P1B5	1/100	1/50	-
	β1 integrin subunit	TDM29	1/10	1/5	1/5
	Le^y	F3	1/20	-	-
	Le^x	P12	1/20	-	-
	SLe^a	KM231	1/20	-	-
	SLe^x	CSLEX1	1/20	1/10	1/10
	E-cadherin	HECD-1	1/50	-	-
Lectins	α2,6-sialic acid	biotinylated *Sambucus nigra* agglutinin (SNA)	1/20 (Capan-1) 1/400 (MDAPanc-28)	1/10 (Capan-1) 1/100 (MDAPanc-28)	1/10 (Capan-1) 1/100 (MDAPanc-28)
	α2,3-sialic acid (not SLe^x)	biotinylated *Maakcia amurensis* lectin (MAA or MAL-II)	1/200	1/50	-

Table 9. Clone and dilutions of the monoclonal antibodies (mAbs) and lectins used in flow cytometry, blocking adhesion and type 1 collagen migration assays.
Clones P1E6, P1D6 and TDM29 were from Chemicon (Temecula, USA); clones P1B5, F3, P12 and KM231, from Calbiochem (EMD Chemicals Inc.; San Diego, USA); clone CSLEX1 from BD Pharmingen (Becton and Dickinson; NJ, USA); clone HECD-1 from Zymed Labs (San Francisco, USA); and lectins were from Vector Laboratories Inc. (Burlingame, CA).

After three washes, adherent cells were estimated with the MTT method (Sigma-Aldrich). Formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and optical density (OD) was measured at 570 nm using a BioTek® Synergy™ 4 microplate reader (Winooski; VT, USA). **Figure 19** outlines the process. Three independent experiments were performed in quadruplicate. Results were expressed as the mean ± SD of values of specific binding to ECM components (OD 570 nm of cells bound – OD 570 nm of cells bound to PBS-1% BSA).

3.2.1 Sialidase treatment

Enzymatic digestion of sialic acids was performed as previously described (Shaikh *et al.*, 2008) with minor modifications. Cells (2.5 x 10^4) were suspended in serum-free DMEM and were then treated with 50 mU/ml of *Vibrio cholerae* sialidase (EC 3.2.1.18, Roche Diagnostics), which digests α2,3-, α2,6- and α2,8-sialic acid linkages; or 400 mU/ml of *Streptococcus pneumoniae* sialidase (NAN 1, EC 3.2.1.18, Europa Bioproducts Ltd; Cambridgeshire, UK), which digests only α2,3-sialic acid linkages; for 1 h at 37°C. Control cells were incubated under the same conditions without sialidase. After a wash, cells were plated onto 96-well microplates previously precoated with 10 µg/ml of type 1 collagen, fibronectin or laminin. After incubating at 37°C for 10 min (COL and FN) or 20 min (LN), nonadherent cells were removed and adhesion was quantified with the MTT method, as detailed in **Materials and methods 3.2** and outlined in **Figure 19**.

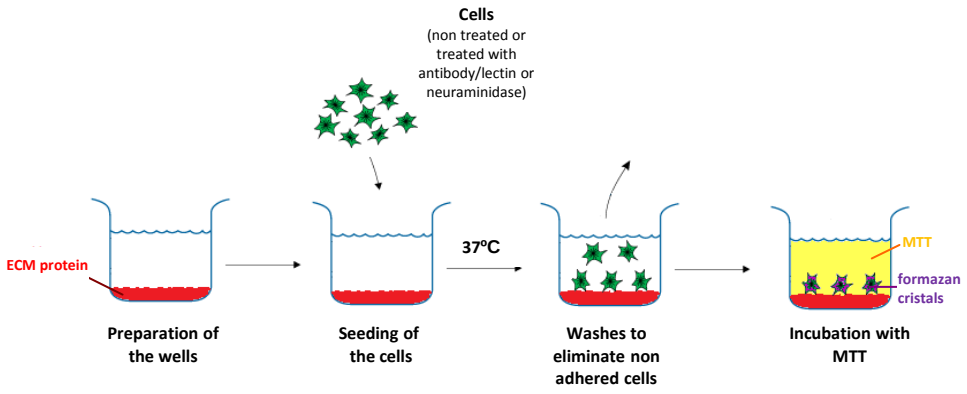


Figure 19. **Main steps in cell adhesion assays to ECM components.** Extracted and modified from Bassagañas, 2009.

3.3 Type 1 collagen migration assay

Cell migration was evaluated using modified Boyden chambers as previously described (Perez-Garay *et al.*, 2010). Serum starved cells were detached, resuspended in serum-free medium and seeded (1 x 10^4) onto type 1 collagen-coated inserts with 8 µm-pores (Greiner Bio-One GmbH; Austria) containing DMEM-1% FBS. In selected experiments, cells were previously incubated for 30 min at 4°C with function-blocking mAb or SNA lectin (see dilutions in **Table 9**). Not aggregation or loss of viability of the cells was detected after antibody or lectin incubations. After 8 h for Capan-1 model or 18 h for MDAPanc-28 model, non-migrated cells were carefully wiped from the top surface of the filter using cotton swabs, and migrated cells were fixed, stained with haematoxylin-eosin and counted under the field microscope (Telaval 31, Carl Zeiss; Jena, Germany). Results were expressed as the average number of migrated cells per well ± SD, obtained from at least two separate experiments performed in triplicate.

3.4 Matrigel invasion assay

Matrigel is a mixture generated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, constituted of several components (type IV collagen, fibronectin, laminin and proteoglycans among others), which simulate the composition of basement membrane where epithelial cells attach.

Matrigel invasion was performed following described procedures by Oliveira *et al. (*2006). 24-well Matrigel-coated invasion inserts of 8-µm pore size filters (BioCoat™ Matrigel Invasion Chambers, BD Biosciences) were rehydrated with DMEM-10% FBS for 1 h at 37°C. Cells were detached, resuspended in DMEM-10% FBS, seeded (5 x 10^4) onto the coated inserts and incubated for 24 h in standard conditions. Non-invasive cells were carefully wiped from the top surface of the filter using cotton swabs, and invasive cells were fixed with ethanol and stained with Vectashield® mounting medium with DAPI (Vector Laboratories). Finally, nuclei were counted under fluorescence microscope (Zeiss Imager Z1 AxioCam MRm, Carl Zeiss) equipped with appropriate filters and software (AxioVision 4.6). Results were expressed as the average number of invasive cells per well ± SD obtained from two separate experiments performed in duplicate.

3.5 Slow aggregation assay

Cell aggregation assays are useful to test the functionality of the cell-cell adhesion complex in epithelial tumour cells. Slow aggregation on agar assay was based on a previously described method (Boterberg *et al.*, 2004). 96-well plates were coated with 50 µl of semi-solid agar medium consisting of 100 mg agar (Bacto™ Agar, BD Biosciences) in 15 ml distilled water and sterilized through boiling three times for 10 s. After gellification, a single-cell suspension of 2 × 10^4 cells was seeded onto the agar. Cell aggregation was evaluated after 24 h with an objective of 4X on an inverted phase-contrast microscope (Olympus CKX41, Olympus; Tokyo, Japan). Particle size was measured in pixels using ImageJ 1.42q software.

4 Biochemistry techniques

The biochemistry approach has provided tools to evaluate the general content and sialylation pattern of α2β1 integrin and E-cadherin glycoproteins, as well as the phosphorylation status of focal adhesion kinase (FAK) protein after cell adhesion to type 1 collagen.

4.1 Cell lysates

Cell lysates for global sialic acid pattern and E-cadherin analysis were obtained with a general lysis protocol, whereas lysates for α2β1 integrin sialylation study were assessed with previously

biotinylated cell surfaces. N-hydroxysulfosuccinimide (NHS) esters of biotin react efficiently with primary amino groups (-NH_2) to form stable amide bonds, and proteins typically have several primary amines available for labelling, including the side chain of lysine (K) residues and the N-terminus of each polypeptide. Both streptavidin and avidin bind biotin with high affinity and specificity.

4.1.1 Total cell lysates

Cells at 70-80% of confluence were washed with cold PBS and lysed in a solution containing 1% (v/v) Triton X-100, 1% (v/v) NP-40, protease inhibitor cocktail (Roche Diagnostics), 100 mM sodium orthovanadate (Na_3VO_4) and 100 mM PMSF for 15 min on ice. After scraping the cell monolayers with a rubber policeman, the suspension was centrifuged at 14000 rpm for 10 min at 4°C. The protein content of the total cell lysates (TCL) was quantified by bicinchoninic acid (BCA) assay kit (Pierce; Rockford, USA).

4.1.2 Cell surface biotinylation and lysis

Prior to α2 integrin immunoprecipitation, cells's surface was biotinylated as described by Guo *et al.* (2002) with minor modifications. Exponential CP and C31 cells were detached, washed three times with ice-cold PBS and incubated with 1 mg/ml sulfosuccinimidyl 6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin; Pierce) in PBS for 25 min at room temperature on a rocking platform. Three washes with PBS containing 100 mM glycine were carried out to quench any unreacted biotinylation reagent, followed by a wash with PBS. Cells were lysed by incubation with lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 5 mM PMSF, 1 mM benzamidine hydrochloride hydrate, 10 mM $MgCl_2$ and 10 mM EGTA]. Lysates were cleared by centrifugation at 14000 rpm for 15 min at 4°C, supernatants were collected and protein content was determined by Bradford (BioRad).

4.2 General electrophoresis and Western/lectin blot

The overall expression of sialic acids and E-cadherin was evaluated with 50 µg or 20 µg of protein from each TCL, respectively. Samples were resuspended in Laemmli buffer and heated to 96.5°C for 5 min. Lysates were subjected to a 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking with PBS-5% BSA containing 0.05% Tween 20 (PBST) (for sialic acid blots) or 5% low-fat milk in PBS containing 0.01% Tween 20 (for E-cadherin blot) for 1 h at room temperature, membranes were incubated with mAb against human SLe^x (clone KM93; Calbiochem) or human E-cadherin (clone 36; BD Biosciences) diluted 1/100 or 1/3000 respectively, in PBST-5% low-fat milk o/n at 4°C; or with biotinylated *Sambucus nigra* agglutinin (SNA) lectin or with biotinylated *Maackia*

amurensis lectin II (MAA or MAL II) (Vector Laboratories) diluted 1/200 in PBST-1% BSA for 1 h at room temperature.

Membranes were washed and incubated for 1 h at room temperature with the secondary antibody HRP-conjugated rabbit anti-mouse IgM (Santa Cruz Biotechnology; Santa Cruz, USA) diluted 1/10000 to detect SLe^x; HPR-conjugated goat anti-mouse (Santa Cruz Biotechnology) diluted 1/2000 in PBST-1% BSA to detect E-cadherin; or Vectastain® Elite ABC kit (Vector Laboratories) to detect sialic acids. For loading control analysis, mouse antibody against human α-tubulin (Sigma-Aldrich) diluted 1/10000 in PBST for 1 h at room temperature, and HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) in the same conditions were used. Immunoreactive bands were visualized with ECL™ Reagent (GE Healthcare; Piscataway, USA). At least three independent experiments were performed.

4.3 Protein immunoprecipitation, electrophoresis and Western/lectin blot

Immunoprecipitation protocol uses protein A/G-coupled to an insoluble resin, such as agarose or Sepharose®, to capture an antibody, which recognizes the antigen (protein of interest) and physically isolates it from the rest of the lysate. Afterwards, the sample can be separated by electrophoresis and analyzed by Western/lectin blot.

4.3.1 α2β1 integrin

The protocol for α2β1 integrin immunoprecipitation was optimized according to the procedures of previous articles (Grzesiak and Bouvet, 2008; Isaji *et al.*, 2004; Isaji *et al.*, 2006; Sawhney *et al.*, 2006; Seales *et al.*, 2005; Zhao *et al.*, 2006).

50 µl of protein A Sepharose® CL-4B beads (GE Healthcare) were incubated with 0.5 µl of rabbit polyclonal antibody against α2 integrin (Chemicon) for 2 h at 4°C on a rocking platform. Then, 400 µg of biotinylated protein sample were incubated with the protein A-antibody complexes o/n at 4°C on the rocking platform. Beads were collected by rapid centrifugation and washed three times with washing buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM $MgCl_2$ and 10 mM EGTA]. Immunoprecipitates were resuspended in reducing buffer and heated to 100°C for 6 min. They were loaded and resolved on an 8% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane.

The blots were then blocked with TBST buffer (Tris-HCl 10 mM pH 7.5, NaCl 100 mM 0.1% Tween 20) with 1% BSA for SLe^x, and TBST-2% PVP for SNA. Then were probed with mAb against SLe^x (clone KM93; Calbiochem) in a dilution of 1/67 in TBST-0.5% BSA, or with fluorescein conjugated SNA lectin (Vector Laboratories) diluted 1/1000 in lectin buffer (150 mM NaCl, 0.1 M Tris-HCl pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$) for 2 h at room temperature. After three washes with TBST,

membranes were incubated with HRP-conjugated secondary antibodies goat anti-mouse (Abcam; Cambridge, UK) diluted 1/40000 in TBST-0.5% BSA; or sheep anti-fluorescein (Roche Diagnostics) diluted 1/2500 in TBST-1% BSA, respectively, for 1 h at room temperature. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate kit (EMD Millipore Corporation; MA, USA), and by the means of a FluorChem™ SP instrument (Alpha Innotech; Santa Clara, USA). Equal amounts of loaded α2 integrin were corroborated by stripping the membranes and blotting with HRP-conjugated streptavidin (GE Healthcare) diluted 1/100000 in TBST-1% BSA. Two independent experiments were performed.

4.3.2 E-cadherin

For E-cadherin immunoprecipitation, 750 µg of TCL were precleared with 25 µl of protein G-Sepharose® beads (GE Healthcare) for 1 h at 4°C, as previously described (Pinho *et al.,* 2009). After centrifugation at 13200 rpm for 5 min at 4°C, the supernatants were incubated o/n with 2.5 µg of mouse mAb against human E-cadherin (clone 36; BD Biosciences). After that, complexes were incubated with 50 µl of protein G-Sepharose® beads for 1 h at 4°C. Then the beads were washed three times with immunoprecipitation buffer [10% (v/v) lysis buffer, 5 mg/ml sodium pyrophosphate tetrabasic ($Na_4P_2O_7$), 10 mM sodium fluoride (NaF) in PBS] and immune complexes were released by boiling for 5 min at 96.5°C in Laemmli buffer, followed by centrifugation at 13200 rpm for 5 min. Western and lectin blot analyses were performed as described in **Materials and methods 4.2**. Two independent experiments were undertaken.

4.4 Tyrosine phosphorylation assay of Focal Adhesion Kinase

Tyrosine phosphorylation assays of FAK were performed as previously described (Zhao *et al.*, 2006). CP and C31 serum-starved cells at exponential growth were detached and held in suspension for 1 h to reduce the detachment-induced activation. 2 x 10^5 cells were plated onto type 1 collagen CELLCOAT® dishes (Greiner Bio-One) or kept in suspension for 20 min at 37°C. After two washes with PBS cells were lysed by incubation with RIPA B lysis buffer [20 mM phosphate buffer, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM PMSF, 1% (v/v) aprotinin, 10 µg/ml leupeptin, 250 µg/ml Na_3VO_4] for 10 min on ice. Lysates were cleared by centrifugation, supernatants were collected and protein content was determined by Bradford (BioRad).

Then, 20-25 µg of protein were resuspended in reducing buffer and heated to 70°C for 15 min. Samples were resolved on an 8% SDS-PAGE gel, electrophoretically transferred into a PVDF membrane and blotted with mAb against human FAK phosphotyrosine 397 (pY397; clone 18; BD Transduction Labs; San Jose, USA) diluted 1/1000 in TBST-1% BSA for 1 h at room temperature. Equal loading was confirmed by blotting with mAb against total human FAK (clone 77; BD Biosciences)

diluted 1/500 in TBST-5% non-fat milk. Secondary antibody was HRP-conjugated goat anti-mouse (Abcam) diluted 1/40000 in TBST-0.5% BSA. Immunoreactive bands were visualized as described in **Materials and methods 4.2**. Two independent experiments were undertaken. Bands were quantified and the relative FAK Y397 phosphorylation per cell line was calculated as the quotient between pY397 quantification and total FAK quantification.

5 Immunofluorescence and immunohistochemistry procedures

Immunofluorescence (IF) and immunohistochemistry (IHC) involve the binding of an antibody to a cellular or tissue antigen of interest and then visualization of the bound product by fluorescence or with the 3,3'-diaminobenzidine (DAB) chromogen detection system, respectively. The method relies on proper fixation of tissues or cells to retain cellular distribution of the antigen and to preserve the cellular morphology (Katikireddy and O'Sullivan, 2011). IF probes were performed both in cell monolayers and PDAC samples in order to determine the content and location of SLe^x epitope and E-cadherin molecules, whereas IHC was performed in PDAC samples to study the presence of Lewis-type antigens.

5.1 Immunofluorescence in cultured cells

Cells (4×10^4) were seeded on 24 well plates (Nunc) with coverslips on the bottom of each well and cultured for 24 h, until nearly confluent monolayers. Then cells were washed with PBS, fixed in ice-methanol for 20 min, and blocked with PBS-10% BSA for 30 min.

For E-cadherin staining, cells were incubated with mAb against E-cadherin (clone 36; BD Biosciences), and with secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen Life Technologies). For double labelling, cells were incubated with mAb against SLe^x (clone KM93; Calbiochem) and secondary antibody Texas Red-conjugated Alexa Fluor® 594 goat anti-mouse IgM diluted (Jackson Immunoresearch; PA, USA) (see dilutions in **Table 10**).

Finally, cells were washed, stained with DAPI (Sigma-Aldrich) and mounted with Vectashield® mounting medium (Vector Laboratories). Antibodies were diluted in PBS-5% BSA and incubated in a dark and humid chamber at room temperature for 1 h. Immunofluorescence images were obtained using a Zeiss Imager Z1 AxioCam MRm (Carl Zeiss) and separate images for E-cadherin, SLe^x and DAPI were captured digitally at 40X magnification. The green (for FITC), red (for Texas Red) and blue (for DAPI) components were merged and combined images were imported into Adobe® Photoshop®.

5.2 Immunofluorescence in tissue samples

The use of specimens from human subjects was approved by the Ethics Committee of *Hospital Universitari Dr. Josep Trueta* from Girona (Spain). Two control pancreatic tissue samples from healthy donors and five pancreatic adenocarcinoma tissues were obtained from patients undergoing surgical resection. The histopathologic features of the resected specimens were confirmed by the pathologists. These patients included three males and two females ranging 49-72 years with exocrine adenocarcinomas of duct cell type; four were stage IIB (two well differentiated, one moderately differentiated and one poorly differentiated), and one stage IIA (moderately differentiated) according to the Tumour Node Metastasis Classification of Malignant Tumours of the International Union Against Cancer (UICC) 7th edition (Sobin *et al.*, 2010). Tissues were fixed in 10% formalin, embedded in paraffin and cut into 5 µm serial sections.

5.2.1 E-cadherin and SLe^x

Paraffin sections were dewaxed, rehydrated and treated with Extran 0.05% (Merck; Frankfurt, Germany) in distilled water for 15 min in a microwave oven at 750 W. After cooling at room temperature, slides were rinsed twice in PBS and incubated for 20 min with rabbit non-immune serum at a dilution 1/5 in PBS-10% BSA, then incubated with mAb against E-cadherin (clone 36) o/n at 4°C and afterwards with secondary antibody FITC-conjugated rabbit anti-mouse (Dako, Denmark). Then slides were blocked with non-immune goat serum diluted 1/5 in PBS-10% BSA for 20 min, incubated with mAb against SLe^x (clone KM93) o/n at 4°C, and finally incubated for 30 minutes with Texas Red-conjugated goat anti-mouse IgM (Jackson Immunoresearch). Nuclei were stained with DAPI and slides were mounted with Vectashield® mounting medium. Antibodies were diluted in PBS-5% BSA (see dilutions in **Table 10**), and incubations were performed in a dark and humid chamber at room temperature. Microscopy images were obtained under fluorescence microscope as described in **Materials and methods 5.1**.

5.2.2 α2β1 integrin and SLe^x

Paraffin sections were dewaxed, rehydrated, treated with 10 mM sodium citrate (pH 6) and microwaved on high for 10 min. Next, they were washed three times with PBS (pH 7.4), incubated for 30 min in 0.3 M glycine in PBS for autofluorescence reduction and washed again. Next nonserum protein block (Dako) was applied for 10 min and then removed. After washing three times with PBS, 5% normal goat serum in PBS was applied for 20 min and removed by blotting. Sections were then incubated with primary antibody diluted in 5% normal goat (β1 integrin clone TDM29, or rabbit polyclonal antibody against α2 integrin AB1936; both from Chemicon) for 60 min at room

temperature and washed three times in PBS. Slides were incubated with FITC-conjugated goat anti-mouse or goat anti-rabbit (Invitrogen Life Technologies) diluted in 5% normal goat serum for 30 min, and washed with PBS. Then, sections were incubated with mAb against SLe^x (clone KM93) for 60 min, washed with PBS and finally incubated for 30 min with Texas Red-conjugated goat anti-mouse IgM (Molecular Probes) (dilutions in **Table 10**). Nuclei were stained with DAPI and slides were mounted with fluorescent mounting medium (Dako). Immunolabelled preparations were evaluated using a NIKON A1R+ confocal laser scanning microscope, following the protocol described in **Materials and methods 5.1**. For negative controls, preimmune serum instead of primary antibodies was used.

PDAC cells		**PDAC tissues**					
E-cadherin + SLe^x		**E-cadherin + SLe^x**		**α2 integrin + SLe^x**		**β1 integrin + SLe^x**	
E-cadherin (clone 36)	1/200	E-cadherin (clone 36)	1/100	α2 integrin (rabbit polyclonal)	1/1000	β1 integrin (clone TDM29)	1/20
FITC-conjugated goat anti-mouse IgG (Invitrogen)	1/500	FITC-conjugated rabbit anti-mouse IgG (Dako)	1/100	FITC-conjugated goat anti-rabbit (Invitrogen)	1/500	FITC-conjugated goat anti-mouse (Invitrogen)	1/500
SLe^x (clone KM93)	1/60	SLe^x (clone KM93)	1/60	SLe^x (clone KM93)	1/60	SLe^x (clone KM93)	1/60
Texas Red-conjugated goat anti-mouse IgM (Jackson Immuno.)	1/500	Texas Red-conjugated goat anti-mouse IgM (Jackson Immuno.)	1/50	Texas Red-conjugated goat anti-mouse IgM (Molecular Probes)	1/500	Texas Red-conjugated goat anti-mouse IgM (Molecular Probes)	1/500

Table 10. Antibodies and dilutions used for immunofluorescence staining in PDAC cells and tissues.
Primary antibodies, in grey. Clone 36 was from BD Biosciences; clone TDM29 and rabbit polyclonal against α2 integrin, from Chemicon; clone KM93, from Calbiochem; FITC-conjugated secondary antibodies were from Invitrogen or Dako; and Texas Red-conjugated goat anti-mouse IgM, from Jackson Immunoresearch or Molecular probes.

5.3 Immunohistochemistry in tissue samples

Pancreatic adenocarcinoma tissue samples (n=20) were obtained from the paraffin-embedded tissue bank Biobanc of the *Institut Hospital del Mar d'Investigacions Mèdiques* (IMIM), and the study was approved by the institution's ethical committee (CEIC). Tissue samples were processed in 4 µm sections and haematoxylin-eosin stained to be used for diagnostic purposes and characterization of the inflammatory component. For immunohistochemical analysis, the primary antibodies 57/27 against SLe^a and 77/180 against Le^y (De Bolos *et al.*, 1995) were used as supernatant at a 1/2 dilution in PBS-1% BSA, and KM93 against SLe^x (Chemicon) was used following manufacturer's instructions. The indirect immunoperoxidase technique was performed as previously described (Lopez-Ferrer *et al.*, 2000) and sections were developed using DAB (Dako).

6 Statistical analysis

Data were expressed as the mean ± SD. Statistical analyses were performed using SPSS statistical software for Windows (version 15.0, SPSS Inc.; Chicago, IL, USA). Normality of data was tested using the Kolmogorov-Smirnov test and the homogeneity of variances was checked using the Levene's test. Data with a normal distribution and homogenous variances were analyzed with Student's t test, one-way or two-way ANOVA using Tukey's test for multiple comparisons. For heterocedastic data, ANOVA on ranks (Kruskal-Wallis test) was run using Mann-Whitney's method for pairwise multiple comparisons. Pearson correlation test was used to assess the relationship between sialic acid cell surface expression and adhesion or migration capabilities. The criterion for significance was set at $p < 0.05$.

Results and discussion

The results obtained in this Doctoral Thesis are presented following a linear investigation regarding the involvement of sialyltransferases and their enzymatic products, the sialic acid determinants located on the pancreatic tumour cell surface, in key steps of PDAC. The results are divided into three chapters which stand for three scientific articles.

CHAPTER 1 corresponds to the manuscript **Cell surface sialic acid modulates extracellular matrix adhesion and migration in pancreatic adenocarcinoma cells** (Bassagañas *et al.,* 2014b) published in *Pancreas* journal in January 2014. Background data from previous studies in our group indicated a primordial role for sialic acid determinants on the migratory capabilities of pancreatic cancer cells; however, it was necessary to address the implication of each α2,3- and α2,6-sialic acid determinants on extracellular matrix (ECM) adhesion and migration. In this regard, and taking into account that tumours show an intrinsic heterogeneity, e.g. variability in tumourigenicity associated with cell-surface markers, PDAC cell models with different proportion of sialylated determinants were provided: Capan-1 cell line and its ST3Gal III transfected and SLe^x overexpressing clone (C31), and MDAPanc-28 cell line (Perez-Garay *et al*., 2010). A possible correlation between the sialic acid determinants expression along cell density and the adhesive properties of the cells on ECM components was described.

CHAPTER 2 includes the manuscript of the paper entitled **Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of α2β1 integrin and E-cadherin**

function (Bassagañas *et al.*, 2014a), published in *PLoS One* journal in May 2014. With the aim to complement the results from CHAPTER 1, next investigations elucidate the influence of sialic acid determinants, paying especial attention to SLe^x epitope, on the regulation of pancreatic cancer cell-cell adhesiveness and cell invasiveness through Matrigel. Furthermore, we also studied whether the differences in sialylation could be displayed on the α2β1 integrin (reviewed in **Introduction 3.1.1**) and E-cadherin (in **Introduction 3.1.2**) molecules, and therefore could modulate their biological function and have an effect on their intracellular pathways. Throughout this section, the ST3Gal III transfected cells of Capan-1 and MDAPanc-28, C31 and M34 respectively, were compared to their mock transfected cells, CP and MP. CHAPTER 2 also includes an histological study of the distribution of SLe^x, α2β1 integrin and E-cadherin molecules in human PDAC samples.

Finally, **CHAPTER 3** corresponds to the submitted version of the manuscript **Inflammatory cytokines regulate the expression of glycosyltransferases involved in the biosynthesis of tumor-associated sialylated determinants in pancreatic cancer**, which was submitted to *Pancreas* journal in April 2014. In this work, two PDAC cells lines, MDAPanc-28 and MDAPanc-3, were treated with pro-inflammatory cytokines to evaluate possible changes in the content of sialylated and other Lewis-type antigens at the cell membrane, as well as in the mRNA expression levels of the genes involved in their biosynthesis. A complementary study evaluates the correlation between the expression of sialylated determinants and the grade of inflammation in human PDAC samples.

Chapter 1

Cell surface sialic acid modulates extracellular matrix adhesion and migration in pancreatic adenocarcinoma cells

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Cell surface sialic acid modulates extracellular matrix adhesion and migration in pancreatic adenocarcinoma cells

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Running title: Sialic acid in pancreatic cancer cell adhesion and migration

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Abstract
Objectives: Tumor cells modulate their extracellular matrix (ECM) adhesion and migration to become more metastatic. Moreover, they show an increase in sialic acid, which could have an effect on their ECM adhesion and migration. This work describes the influence of pancreatic adenocarcinoma cell surface α2,3- and α2,6-sialic acid determinants on the aforementioned processes.
Methods: We have characterized the cell surface α2,3-, α2,6-sialic acid and sialyl-Lewis x levels and the integrin levels of two pancreatic adenocarcinoma cell lines, Capan-1 and MDAPanc-28, grown at different cell density, and also of the ST3Gal III overexpressing Capan-1 cells, C31. We have measured their adhesion to several ECM proteins and their migration through collagen, with and without blocking their sialic acid determinants.
Results: Adhesion to ECM proteins of Capan-1 and MDAPanc-28 grown at different cell density, and of C31, depended on their cell surface sialic acid determinants repertoire, correlating the higher α2,6-sialic acid levels with their increased ECM adhesion. Cell migration also depended on their sialic acid determinants expression, and in this case higher α2,3-sialic acid levels correlated with a more migratory phenotype.
Conclusion: This study shows how the intrinsic heterogeneity of cell membrane sialylation regulates the adhesive and migratory potential of pancreatic adenocarcinoma cells.

Keywords: Adhesion; Extracellular matrix; Migration; Sialic acid; Sialyltransferases; Pancreatic adenocarcinoma

Chapter 2

Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of α2β1 integrin and E-cadherin function

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Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of α2β1 integrin and E-cadherin function.

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Abstract

In our previous studies we have described that ST3Gal III transfected pancreatic adenocarcinoma Capan-1 and MDAPanc-28 cells show increased membrane expression levels of sialyl-Lewis x (SLe^x) along with a concomitant decrease in α2,6-sialic acid compared to control cells. Here we have addressed the role of this glycosylation pattern in the functional properties of two glycoproteins involved in the processes of cancer cell invasion and migration, α2β1 integrin, the main receptor for type 1 collagen, and E-cadherin, responsible for cell-cell contacts and whose deregulation determines cell invasive capabilities. Our results demonstrate that ST3Gal III transfectants showed reduced cell-cell aggregation and increased invasive capacities. ST3Gal III transfected Capan-1 cells exhibited higher SLe^x and lower α2,6-sialic acid content on the glycans of their α2β1 integrin molecules. As a consequence, higher phosphorylation of focal adhesion kinase tyrosine 397, which is recognized as one of the first steps of integrin-derived signaling pathways, was observed in these cells upon adhesion to type 1 collagen. This molecular mechanism underlies the increased migration through collagen of these cells. In addition, the pancreatic adenocarcinoma cell lines as well as human pancreatic tumor tissues showed colocalization of SLe^x and E-cadherin, which was higher in the ST3Gal III transfectants. In conclusion, changes in the sialylation pattern of α2β1 integrin and E-cadherin appear to influence the functional role of these two glycoproteins supporting the role of these glycans as an underlying mechanism regulating pancreatic cancer cell adhesion and invasion.

Introduction

Cell adhesion is a dynamic process that allows cells of multicellular organisms to be cohesive, communicate and interact among them and with the extracellular matrix (ECM), playing an essential role in many cellular functions, such as cell normal embryonic development, morphogenesis and tissue repair, as well as in many pathological processes such as tumour invasion and metastasis, thrombosis and inflammation [1]. Cancer invasion is an heterogeneous process for which the physical, cellular and molecular determinants adapt and react throughout the progression of the disease in a cell- and tissue-driven manner [2]. A key stone of cancer invasion is the disruption of the cellular junctions through the downregulation of the function and/or important signalling pathways carried out by critical cell adhesion molecules (CAMs) such as cadherins and integrins. This loss of adhesiveness allows tumour cells to disobey the social order, resulting in the alteration of the normal histological structure and dissociation from cancer nests [3].

In particular, *adherens junctions* (AJ), which are orchestrated by E-cadherin molecule, provide adhesive contacts between neighbouring epithelial cells and form intracellular interactions to the actin cytoskeleton, being involved in important signalling processes leading to the regulation of gene transcription [4,5]. It is not surprising that in most, if not all, cancers of epithelial origin E-cadherin-mediated cell-cell adhesion is downregulated or inactivated promoting cancer cell invasion and metastases. In addition, E-cadherin is one of the key molecular markers along the process of Epithelial to Mesenchymal Transition (EMT), which is a fundamental biological process associated with the progression from adenoma to carcinoma and the subsequent steps of cancer cell invasion and metastasis [6,7].

Integrins are transmembrane receptors that bind to ECM components and are involved in adhesion and migration processes. They are composed of α and β heterodimers, lack endogenous enzymatic activity and depend on signal transducers to perform their functions, such as the nonreceptor focal adhesion kinase (FAK) as well as a variety of scaffolding proteins that link integrins to the actin cytoskeleton [8]. As a result of cell adhesion to ECM components, integrins transmit information that regulates intracellular signalling. Specifically, FAK is activated via autophosphorylation at tyrosine 397 (Y397) upon integrin binding to its ligands. Phosphorylated FAK Y397 becomes a binding site for the tyrosine kinase Src, and FAK/Src complex then activates other downstream proteins, e.g. pCAS, Crk or paxillin, which in turn activate important pathways involved in cell migration progress [9].

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death, with a 5-year survival rate of less than 5%. This extremely poor outcome is mainly due to its aggressiveness and delay in diagnosis, since approximately 85% of patients are diagnosed at advanced stages of disease,

when metastasis is already present [10]. Therefore, there is an urgent need to identify the underlying molecular mechanisms of PDAC, envisioning potential clinical applications.

PDAC is characterized by an intense desmoplastic response, suggesting a role for ECM cell adhesion molecules, such as integrins, throughout the tumorigenic process [11,12]. A number of reports have described the up-regulation and delocalization of several integrin subunits, including α1, α2, α3 and α6 subunits in pancreatic cancer [13,14]. Furthermore, β1 integrins have been reported to play an essential role in promoting adhesion and invasion of pancreatic carcinoma and, in the case of α2β1 integrin, in mediating the malignant phenotype on type 1 collagen in pancreatic cancer cell lines [15,16].

On the other hand, downregulation or inactivation of E-cadherin expression has also been associated with poor survival and acquisition of invasiveness, as well as dedifferentiation of PDAC [17].

Glycosylation is one of the most important protein post-translational modification and tumour cells frequently display an altered pattern of cell surface glycosylation in relation to their normal counterparts, which directly influences several cellular processes, including cell-cell adhesion [18] and cell-ECM interaction [19,20]. In particular, several pancreatic adenocarcinoma cells have been described to exhibit an increase in the expression of the Lewis-type sialylated epitopes sialyl-Lewis a (SLe^a) and sialyl-Lewis x (SLe^x) and the correspondent glycosyltransferases involved in their biosynthesis, which have been correlated with PDAC invasiveness and metastasis [21,22,23,24].

Furthermore, several studies have reported that *N*-glycans influence the stability of AJ and E-cadherin biological functions in a variety of tumours [25,26,27]. In addition, integrins are also carriers of *N*-glycans, and changes in glycan branching and sialylation of integrins have been reported to influence integrin binding to ECM and cell migration capabilities although the mechanisms underlying these actions are still unknown [28,29].

In previous studies we have described that α2,3-sialyltransferase ST3Gal III transfection of pancreatic adenocarcinoma cell lines Capan-1 and MDAPanc-28 leads to the overexpression of SLe^x antigen and the decrease of α2,6-sialic acid in their cell surface. ST3Gal III transfectants exhibited loss of cell-ECM adhesion, increased motility rates through type 1 collagen and an enhanced metastatic phenotype *in vivo* [23,30]. To understand this enhanced metastatic phenotype *in vivo*, we here address whether cell-cell adhesiveness and cell invasion are also affected by the cell sialylation changes using the stably transfected cell line models. We have also determined whether the cell surface glycan differences between transfected and control cells could be displayed in their α2β1 integrin and E-cadherin molecules and could thus modulate their function.

Material and methods

Ethics Statement

The use of specimens from human subjects was approved by the Ethics Committee of Dr. Josep Trueta University Hospital from Girona (Spain).

Cell culture

Human pancreatic adenocarcinoma cell lines Capan-1 (ATCC nºHTB-79; MD, USA) and MDAPanc-28, and the described stable ST3Gal III transfected (C31 and M34, respectively) and mock transfected (CP and MP, respectively) [23,30] were used in this work. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAX-I containing 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin G, 100 mg/ml Streptomycin, 0.25 mg/ml Amphotericin B, and supplemented with 400 µg/ml (Capan-1 transfectants) or 800 µg/ml (MDAPanc-28 transfectants) Geneticine® G-418 (all of them from Gibco, UK), under a humidified atmosphere containing 5% CO_2. For the experiments, 3.5 x 10^5 Capan-1/CP/C31 or 5.5 x 10^5 MDAPanc-28/MP/M34 cells were seeded in 75 cm^2 flasks (Nunc, Roskilde, Denmark) and cultured for 84 h (exponential growth)

Flow cytometry analysis

Detection of integrin subunits and E-cadherin was performed by indirect immunofluorescence. Cells (5 x 10^5) were incubated at 4ºC for 30 min in the presence or absence of monoclonal antibodies (mAb) against integrin subunits β1 (clone TDM29, Chemicon, CA, USA; diluted 1/10), α2 (clone P1E6, Chemicon; 1/200) or mAb against human E-cadherin (clone HECD-1, Zymed Labs CA, USA; 1/50). After a wash, cells were incubated with the secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen Life Technologies, MD, USA). Antibodies were diluted in Phosphate Buffered Saline (PBS) containing 1% Bovine Serum Albumin (BSA). Mean Fluorescence Intensity (MFI) was calculated as the quotient between the positive and negative GeoMean for each cell line. For each sample three independent assays were performed.

Cell adhesion assay to collagen

Cell adhesion assays were performed as previously described [30]. 96-well microplates were coated with a solution of 10 µg/ml type 1 collagen from calf skin (Sigma-Aldrich; MO, USA) in PBS, or PBS-1% BSA. For each well, 2.5 x 10^4 cells were seeded and incubated at 37ºC for 20 min. In selected experiments, cells were previously incubated with function-blocking mAbs against integrin subunits α2 (clone P1E6, Chemicon; 1/50 in PBS), α3 (clone P1B5, Calbiochem, CA, USA; 1/50), α5 (clone P1D6,

Chemicon; 1/50) or β1 (clone TDM29, Chemicon; 1/5) for 30 min at 4°C. After three washes, adherent cells were estimated with the MTT method (Sigma). Three independent experiments were performed in quadruplicate. Results were expressed as the mean ± standard deviation (SD) of values of specific binding to type 1 collagen (OD 570 nm of cells bound – OD 570 nm of cells bound to PBS-1% BSA).

Type 1 collagen migration assay

Cell migration was evaluated using modified Boyden chambers as previously described [23,30]. Briefly, serum starved cells were detached, resuspended in serum-free medium and seeded onto type 1 collagen coated inserts (Greiner Bio-One GmbH; Austria) containing DMEM-1% FBS. In selected experiments, cells were previously incubated with function-blocking mAbs against α2 (clone P1E6, Chemicon; diluted 1/50 in PBS) or β1 (clone TDM29, Chemicon; 1/5) integrin subunits. After 8 h, non-migrated cells were wiped from the top surface of the filter and migrated cells were fixed, stained with hematoxylin-eosin and counted. Results were expressed as the average number of migrated cells per well ± SD, obtained from two separate experiments performed in duplicate.

Matrigel invasion assay

Matrigel invasion was performed following described procedures [31]. 24-well Matrigel-coated invasion inserts (BioCoat Matrigel Invasion Chambers; BD Biosciences, CA, USA) were rehydrated with DMEM-10% FBS for 1 h at 37°C. Cells (5×10^4) were seeded onto the coated inserts and incubated for 24 h in standard conditions. Non-invasive cells were carefully wiped from the top surface of the filter and invasive cells were fixed with ethanol and stained with Vectashield mounting medium with DAPI (Vector Laboratories, CA, USA). Results were expressed as the average number of invasive cells per well ± SD obtained from two separate experiments performed in duplicate.

Slow aggregation assay

Slow aggregation assay on agar was based on a previously described method [32]. 96-well plates were coated with 50 µl of semi-solid agar medium consisting of 100 mg agar (Bacto™ Agar, BD Biosciences) in 15 ml distilled water and sterilized through boiling three times for 10 s. After jellification, a single-cell suspension of 2×10^4 cells was seeded onto the agar. Cell aggregation was evaluated after 24 h and particle size was measured in pixels using ImageJ 1.42q software.

Western blot and lectin blot analysis of total cell lysates

Cells were washed with cold PBS and resuspended in lysis buffer containing 1% (v/v) Triton X-100, 1% (v/v) NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA), 100 mM Na_3VO_4 and 100 mM PMSF in PBS, for 15 min on ice. The protein content of the total cell lysates (TCL) was quantified by bicinchoninic acid (BCA) assay kit (Pierce, IL, USA) and afterwards the overall expression of sialic acids and E-cadherin was evaluated with 50 μg or 20 μg, respectively. Samples were resuspended in Laemmli buffer and heated to 96°C for 5 min. Lysates were subjected to a 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with PBS-5% BSA containing 0.05% Tween 20 (PBST) (for sialic acids blot) or 5% low-fat milk in PBS containing 0.01% Tween 20 (for E-cadherin blot), membranes were incubated with mAb against SLe^x (clone KM93, Calbiochem; 1/100) or E-cadherin (clone 36, BD Biosciences; 1/3000) in PBST-5% low-fat milk overnight at 4°C; or with biotinylated *Sambucus nigra* agglutinin (SNA) or biotinylated *Maackia amurensis* lectin II (MAL-II) (Vector Laboratories) diluted 1/200 in PBST-1% BSA for 1 h at room temperature. Membranes were washed and incubated with the secondary antibody HRP (horseradish peroxidase)-conjugated rabbit anti-mouse IgM (Santa Cruz Biotechnology; CA, USA) to detect SLe^x, HRP-conjugated goat anti-mouse (Santa Cruz) to detect E-cadherin or Vectastain Elite ABC kit (Vector Laboratories) to detect sialic acids. For loading control analysis, mouse antibody against human tubulin (Sigma; diluted 1/10000 in PBST), and secondary antibody HRP-conjugated goat anti-mouse (Santa Cruz) were used. Immunoreactive bands were visualized using ECL Reagent (GE Healthcare, NJ, USA). At least three independent experiments were performed.

E-cadherin immunoprecipitation

For E- cadherin immunoprecipitation, 750 μg of TCL were precleared with 25 μl of protein G-sepharose beads (GE Healthcare) for 1 h at 4°C, as previously described [33]. Briefly, after centrifugation the supernatants were incubated overnight with 2.5 μg of mAb against human E-cadherin (clone 36, BD Biosciences), and after that the immune complexes were released by boiling and subjected to 7.5% SDS-PAGE. Western blot and lectin blot analyses were performed as described above. Three independent experiments were undertaken.

Cell surface biotinylation, cell lysis and α2β1 immunoprecipitation

Cell surface biotinylation was performed following described procedures [34] with minor modifications. Exponential CP and C31 cells were detached, washed three times with ice-cold PBS and incubated with 1 mg/ml sulfo-NHS-LC-biotin (Sigma) in PBS for 25 min at room temperature on a rocking platform. After incubation, three washes with PBS-100 mM glycine were carried out to

quench any unreacted biotinylation reagent. Cells were lysed by incubation with lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 5 mM PMSF, 1 mM benzamidine hydrochloride hydrate, 10 mM $MgCl_2$ and 10 mM EGTA]. Lysates were cleared by centrifugation, supernatants were collected and protein content was determined by Bradford (Biorad). For α2 integrin immunoprecipitation, 50 μl of protein A sepharose CL-4B beads (GE Healthcare) were incubated with 0.5 μl of rabbit polyclonal antibody against α2 integrin (Chemicon) for 2 h at 4°C on a rocking platform. Afterwards, 400 μg of protein sample were incubated with the protein A-antibody complexes overnight at 4°C on the rocking platform. Beads were collected by rapid centrifugation and washed three times with washing buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM $MgCl_2$ and 10 mM EGTA].

Western blot and lectin blot analysis of the integrin immunoprecipitates

Immunoprecipitates were resuspended in reducing buffer and heated to 100°C for 6 min. Then, they were loaded and resolved on an 8% SDS-PAGE, and transferred to a PVDF membrane. The blots were probed with mAb against SLe^x [clone KM93, Calbiochem; diluted 1/67 in TBST buffer (Tris-HCl 10 mM pH 7.5, NaCl 100 mM, 0.1% Tween 20) containing 0.5% BSA)]; or with fluorescein conjugated SNA lectin [Vector Laboratories; diluted 1/1000 in lectin buffer (150 mM NaCl, 0.1 M Tris-HCl pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$)] for 2 h at room temperature. After three washes with TBST, membranes were incubated with secondary antibody HRP-conjugated goat anti-mouse (Abcam, UK; diluted 1/40000 in TBST-0.5% BSA); or sheep anti-fluorescein (Roche Diagnostics, diluted 1/2500 in TBST-1% BSA), respectively, for 1 h at room temperature. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate kit (EMD Millipore Corporation; MA, USA). Equal amounts of loaded α2 integrin were corroborated by stripping the membranes and blotting with HRP-conjugated streptavidin (GE Healthcare; diluted 1/100000 in TBST-1% BSA). Two independent experiments were performed.

Tyrosine phosphorylation assay of Focal Adhesion Kinase (FAK)

Tyrosine phosphorylation assays of FAK were performed following described procedures [35]. CP and C31 serum-starved cells at exponential growth were detached and held in suspension for 60 min to reduce the detachment-induced activation. To perform this assay, 2×10^5 cells were plated onto type 1 collagen CELLCOAT® 24-well dishes (Greiner Bio-One) or kept in suspension for 20 min at 37°C. After two washes with PBS the cells were lysed by incubation with RIPA B lysis buffer [20 mM phosphate buffer, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM PMSF, 1% (v/v) aprotinin, 10 μg/ml leupeptin, 250 μg/ml Na_3VO_4]. Lysates were cleared by centrifugation, supernatants were

collected and protein content was determined. Then 20-25 μg of protein were resuspended in reducing buffer and heated to 70°C for 15 min. Samples were resolved on an 8% SDS-PAGE, electrophoretically transferred into a PVDF membrane and blotted with mAb against human FAK phosphotyrosine 397 (clone 18, BD Biosciences; diluted 1/1000 in TBST-1% BSA) for 1 h at room temperature. Equal loading was confirmed by blotting with mAb against total human FAK (clone 77, BD Biosciences; 1/500 in TBST-5% non-fat milk). Secondary antibody was HRP-conjugated goat anti-mouse (Abcam; 1/40000 in TBST-0.5% BSA). Immunoreactive bands were visualized as described above. Two independent experiments were undertaken. Relative FAK Y397 phosphorylation per cell line was calculated as the quotient between pY397 FAK quantification and total FAK quantification.

Immunofluorescent double-labelling of cultured cells

Cells (4 x 10^4) were seeded on 24 well plates (Nunc) with coverslips on the bottom of each well and cultured for 24 h, until nearly confluent monolayers. Then cells were washed with PBS, fixed in ice-methanol for 20 min, and blocked with PBS-10% BSA for 30 min. For E-cadherin staining, cells were incubated with mAb against E-cadherin (clone 36, BD Biosciences; 1/200) and with secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen Life Technologies, 1/500). For double labelling, cells were incubated with mAb against SLe^x (clone KM93, Calbiochem; 1/60) and secondary antibody Alexa Fluor® 594 goat anti-mouse IgM (Texas Red-conjugated; Invitrogen Life Technologies; 1/500). Finally, cells were washed, stained with DAPI (Sigma) and mounted with Vectashield mounting medium (Vector Laboratories). Antibodies were diluted in PBS-5% BSA, and incubated in a dark and humid chamber at room temperature. Separate images for E-cadherin, SLe^x and DAPI were captured digitally at 40X or 63X magnification. The green (for FITC), red (for Texas Red) and blue (for DAPI) components were merged and combined images were imported into Adobe® Photoshop®.

Tissue specimens immunofluorescence

Two control pancreatic tissue samples from healthy donors and five pancreatic adenocarcinoma tissues were obtained from patients undergoing surgical resection. The histopathologic features of the resected specimens were confirmed by the pathologists. These patients included three male and two females ranging 49-72 years with exocrine adenocarcinomas of duct cell type; four were stage IIB (two well differentiated, one moderately differentiated and one poorly differentiated), and one stage IIA (moderately differentiated) according to the Tumor Node Metastasis Classification of Malignant Tumors of the International Union Against Cancer (UICC) 7th edition [36]. Tissues were fixed in 10% formalin, embedded in paraffin and cut into 5 μm serial sections.

For E-cadherin and SLe^x immunofluorescence, paraffin sections were dewaxed, rehydrated and treated with Extran 0.05% (Merck, Germany) in distilled water for 15 min in a microwave oven at 750 W. After cooling at room temperature, slides were rinsed twice in PBS and incubated for 20 min with rabbit non-immune serum at a dilution 1/5 in PBS-10% BSA, then incubated with mAb against E-cadherin (clone 36, BD Biosciences, 1/100) overnight at 4°C and afterwards with secondary antibody FITC-conjugated rabbit anti-mouse (Dako, Denmark, 1/100). Then slides were blocked with non-immune goat serum diluted 1/5 in PBS-10% BSA for 20 min, incubated with mAb against SLe^x (clone KM93, Calbiochem; 1/60) overnight at 4°C, and finally incubated for 30 minutes with Texas Red-conjugated goat anti-mouse IgM (Jackson Immunoresearch, PA, USA; 1/50). Nuclei were stained with DAPI and slides were mounted with Vectashield mounting medium. Antibodies were diluted in PBS-5% BSA, and incubations were performed in a dark and humid chamber at room temperature. Microscopy images were obtained under fluorescence microscope as described in the above protocol. For α2β1 integrin and SLe^x immunofluorescence, paraffin sections were dewaxed, rehydrated and treated with 10 mM sodium citrate (pH 6) and microwaved on high for 10 min. Next, they were washed three times with PBS (pH 7.4), incubated for 30 min in 0.3M glycine in PBS for autofluorescence reduction and washed again. Next nonserum protein block (Dako) was applied for 10 min and then removed. After washing three times with PBS, 5% normal goat serum in PBS was applied for 20 min and removed by blotting. Sections were then incubated with primary antibody diluted in 5% normal goat serum (1/20 for mAb against β1 integrin, clone TDM29, Chemicon; and 1/1000 for rabbit polyclonal antibody against α2 integrin, AB1936, Chemicon) for 60 min at room temperature and washed three times in PBS. Afterwards the slides were incubated with FITC-conjugated goat anti-mouse or goat anti-rabbit (Invitrogen Life Technologies) diluted 1/500 in 5% normal goat serum for 30 min, and washed with PBS. Then, slides were incubated with mAb against SLe^x (clone KM93, Calbiochem; 1/60) for 60 min, washed with PBS and finally incubated for 30 minutes with Texas Red-conjugated goat anti-mouse IgM (Molecular Probes), diluted 1/500 in normal goat serum . Nuclei were stained with DAPI and slides were mounted with Fluorescent mounting medium (Dako). Immunolabeled preparations were evaluated using a NIKON A1R+ confocal laser scanning microscope as described in the above protocol. For negative controls, preimmune serum instead of primary antibodies was used.

Statistical analysis

Data were expressed as mean ± SD. Data was normalized using the Kolmogorov-Smirnov test and the homogeneity of variances was checked using the Levene's test. Mean scores were compared with Student's t-test or one-way ANOVA and Tukey's test for multiple comparisons, using SPSS statistical

software for Windows (version 15.0, SPSS Inc.; Chicago, IL, USA). The criterion for significance was set at $p<0.05$.

Results

Characterization of SLe^x and α2,6-sialic acid content in total cell lysates from ST3Gal III and mock transfected Capan-1 and MDAPanc-28 clones

ST3Gal III transfectants of Capan-1 and MDAPanc-28 have been described to increase cell surface SLe^x levels concomitantly with a decrease in α2,6-sialic acid [23]. From the characterized ST3Gal III overexpressing clones (C31 and C32 for Capan-1, and M33 and M34 for MDAPanc-28) that showed similar behaviour, the highest ST3Gal III and SLe^x expressing clones from each cell line, C31 and M34, were chosen to address the influence of sialylated determinants in cell adhesion and invasion processes, as well as in E-cadherin and α2β1integrin function. They were compared to the control mock clones, CP and MP, respectively.

In order to evaluate the changes in protein glycosylation, the overall SLe^x and α2,6-sialic acid content of total cell lysates (TCL) from the four transfected cell lines was analyzed by Western and lectin blot. Higher expression of SLe^x was detected in Capan-1 cell model compared to MDAPanc-28 model; conversely, higher expression of α2,6-sialic acid structures was present in the MDAPanc-28 model (**Figure 1**). ST3Gal III transfected clones C31 and M34 showed higher SLe^x levels compared to their respective controls, with marked differences in C31 cells (**Figure 1A**). At the same time, a concomitant decrease in the expression of α2,6-sialic acid structures was observed in the ST3Gal III transfected clones compared to the controls (**Figure 1B**), which was more relevant in M34 cells. Arrows in **Figure 1** indicate the protein bands that show increases in SLe^x or α2,6-sialic acid levels between the ST3Gal III transfectants and their corresponding mock cells. These results showed a competitive expression between α2,3- and α2,6-sialic acid, which are in accordance with the competition among α2,3-sialyltransferases ST3Gal III-IV and α2,6-sialyltransferase ST6Gal I enzymes to sialylate type II glycan chains previously described [23].

It is important to notice that the differences in sialic acid content between ST3Gal III and mock transfected cells occurred mainly at high molecular weight bands, which are likely to correspond to cell membrane glycoproteins, such as integrins or E-cadherin, among others.

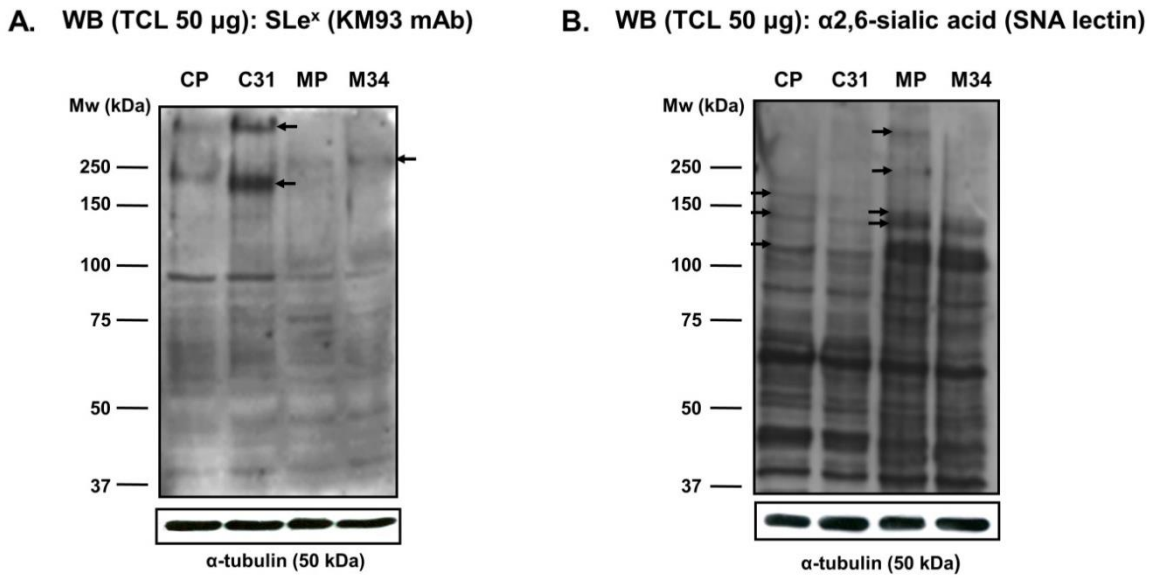

Figure 1. SLe^x and α2,6-sialic acid determinants content in total cell lysates.
SLe^x and α2,6-sialic acid content of total cell lysates (TCL) from the transfected cell lines (C31 and M34) and their respective controls (CP and MP) was analyzed by Western and lectin blot. Blots were probed with clone KM93 mAb against SLe^x epitope (**A**) or *Sambucus nigra* agglutinin (SNA), which detects α2,6-sialic acid structures (**B**). α-tubulin (50 kDa) was used as loading control in each cell line. Arrows indicate the protein bands that show increases in SLe^x or α2,6-sialic acid levels between the ST3Gal III transfectants and their corresponding mock cells.

Expression of α2β1 integrin and E-cadherin in Capan-1 and MDAPanc-28 clones upon ST3Gal III transfection

Cell surface expression levels of α2β1 integrin and E-cadherin molecules were assessed in Capan-1 and MDAPanc-28 transfected clones by flow cytometry.

CP and C31 cells showed similar levels of α2 integrin subunit, as well as of β1 subunit (**Figure 2A**). We had previously described that the levels of α2 subunit found in the MDAPanc-28 model were extremely low compared to Capan-1 model [30], what precluded further analysis of the α2β1 integrin glycosylation pattern influence on collagen type 1 adhesion using the MDAPanc-28 cell model. The corresponding study was then conducted with CP and C31 cells.

Concerning cell surface E-cadherin expression, the results showed similar levels between CP and C31, as well as between M34 and MP cells, with mean fluorescence intensity (MFI) values of 65.5 and 61.9 for CP and C31 cells and of 107.7 and 109.5 for MP and M34 cells. MDAPanc-28 model exhibited higher E-cadherin levels than Capan-1 model with an average of 1.7-fold increase in the MFI values (**Figure 2B**). A further comparison of E-cadherin protein content was performed with TCL and Western blot, and no significant differences among the four cell lines were detected (**Figure 2C**)

ST3Gal III overexpression did not modify either α2β1integrin or E-cadherin protein expression in C31 and M34 cells; therefore these clones are good models to study the potential influence of the differential glycosylation in the function of these two membrane glycoproteins.

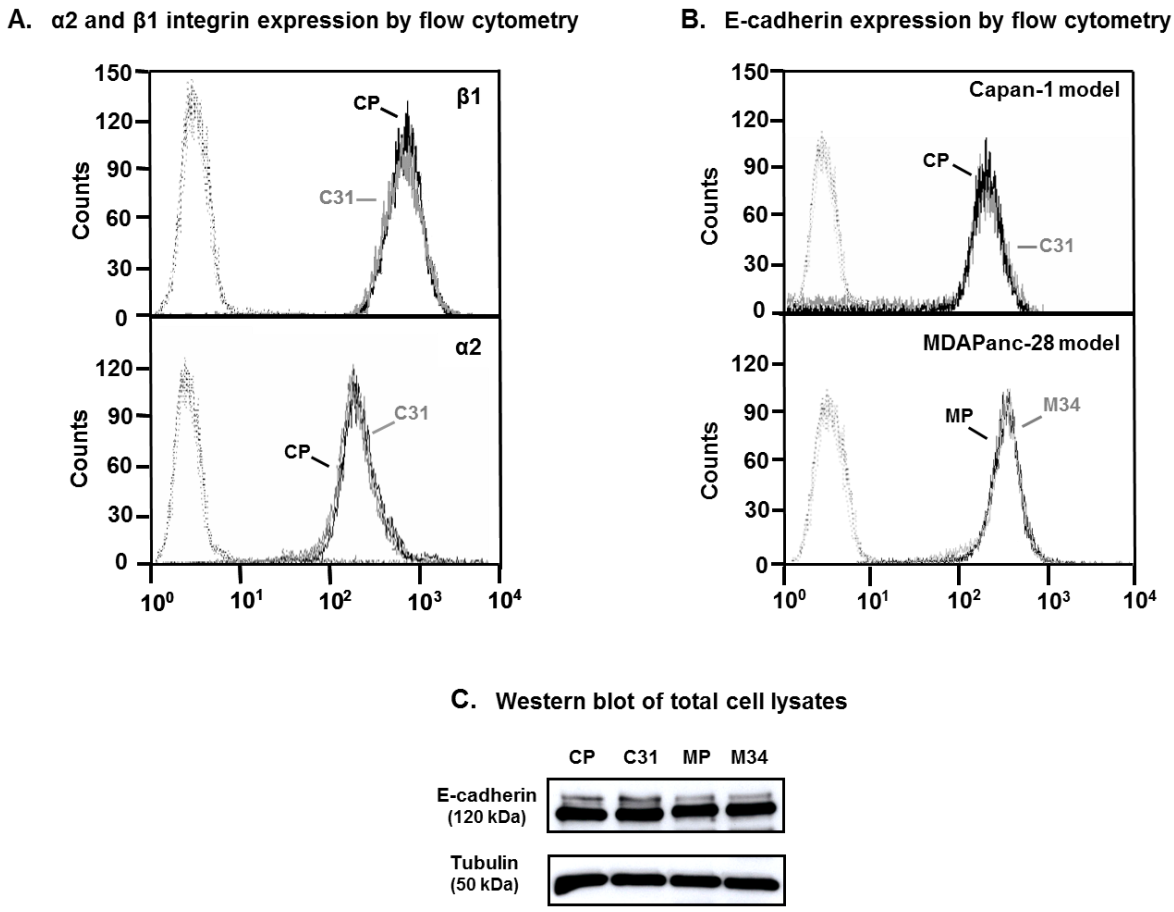

Figure 2. Expression of β1 and α2 integrin subunits, and E-cadherin in Capan-1 and MDAPanc-28 clones. **A.** Representative flow cytometry histograms of β1 and α2 integrin subunits surface expression in CP and C31 cells. Secondary antibody controls, dotted. **B.** Representative flow cytometry histograms of E-cadherin surface expression in CP, C31, M34 and MP cells. **C.** E-cadherin content of total cell lysates (TCL) from CP, C31, MP and M34 was analyzed by Western blotting. Tubulin was used as loading control in each cell line.

CP and C31 cellular adhesion to type 1 collagen and migration through collagen coated transwells is dependent on α2β1 integrin expression

To evaluate whether α2β1integrin is the main contributor to type 1 collagen adhesion and migration in Capan-1 model, function-blocking monoclonal antibodies (mAb) against specific integrin subunits were used in adhesion and migration assays.

CP and C31cells previously incubated with mAbs against α2 or β1 subunits showed extremely significant reduced rates of adhesion to type 1 collagen in a 92% and 73% for CP; and in a 89% and 69% for C31, respectively (p<0.001); whereas mAbs against other important integrin subunits (α3, α5) did not change the adhesion rates significantly (**Figure 3A**).

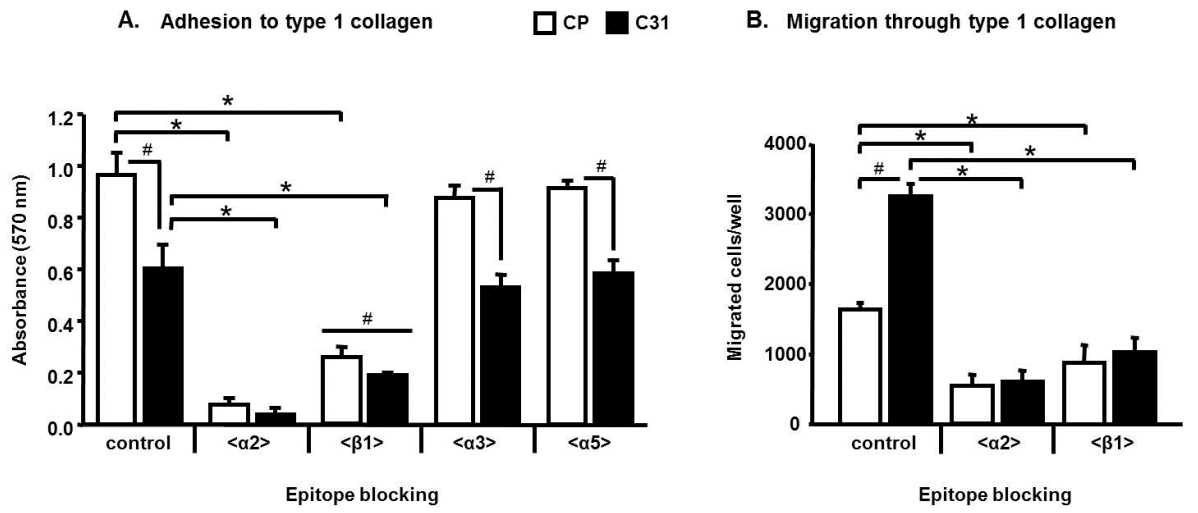

Figure 3. Influence of α2β1 surface expression in the adhesion and migration of CP and C31 cells.
Adhesion (**A**) and migration (**B**) of CP and C31 cells to type 1 collagen using mAbs against α2, α3, α5 or β1 integrin subunits or α2 or β1 integrin subunits, respectively. Bars represent mean ± SD. * Significantly different compared within the same cell line (p<0.05); # significantly different compared within CP and C31 cells (p<0.001).

Blocking of other receptors that could be involved in type 1 collagen recognition such as α1β1 integrin was not studied since Capan-1 has been reported to barely express this integrin [15,16]. Likewise, neither α10β1 nor α11β1 integrins were studied due to their restricted expression in chondrocytes [37] and mesenchymal cells [38], respectively.

Migration assays through collagen coated transwells were performed with CP and C31 cells previously incubated with mAbs against α2 or β1 integrin subunits. Migration rates were significantly diminished, in a 66% and 46% for CP, and in an 81% and 68% for C31, respectively (p<0.001) (**Figure 3B**).

These results show the involvement of α2β1 integrin in the adhesion to collagen and migration in CP and C31. Since the same expression levels of α2 integrin subunit, as well as of β1, were found in C31 and CP cells (**Figure 2A**), next experiments were addressed to determine whether α2β1 integrin glycosylation was different between these clones and could thus explain the changes in adhesion and migration previously described [23,30] and corroborated again in this study where C31 cells show lower adhesion to type 1 collagen and increased migration through collagen compared to CP cells .

Differences in α2β1 integrin sialylation pattern between CP and C31 cells

Human α2 integrin subunit has 10 potential *N*-glycosylation sites, one of them (Asn-343) identified by mass spectrometry analysis [39,40]; whereas β1integrin subunit has 14 potential sites, six of which (Asn-212, 403, 406, 411, 481 and 669) have also been identified [39,40,41]. To determine the SLe^x

and α2,6-sialic acid content on α2β1 integrin molecules, biotinylated C31 and CP α2 integrin immunoprecipitate blots were analyzed with mAb against SLe^x and with SNA lectin.

Two typical bands for the α2 subunit at 170 kDa, and a band of approximately 120 kDa, which corresponds to the β1 subunit coprecipitated with α2, were detected. A slight increase of SLe^x staining on the α2 subunit of C31 cells, together with a major decrease of α2,6-sialic acid on both α2 and β1 subunits, compared to CP was found (**Figure 4A**; *upper panels*). Equivalent amounts of loaded protein were verified by reprobing the corresponding membranes with HRP-conjugated streptavidin, which in addition provided information of the α2 and β1 subunit levels (**Figure 4A**; *lower panels*).
The blots also showed that α2,6-sialic acid is predominantly expressed on the coprecipitated β1 subunit, while SLe^x is basically found on the α2 subunit. The results lead us to conclude that the α2β1 integrin molecules of C31 cells show a much lower content of α2,6-sialic acid and slightly higher SLe^x levels than that of CP mock cells.

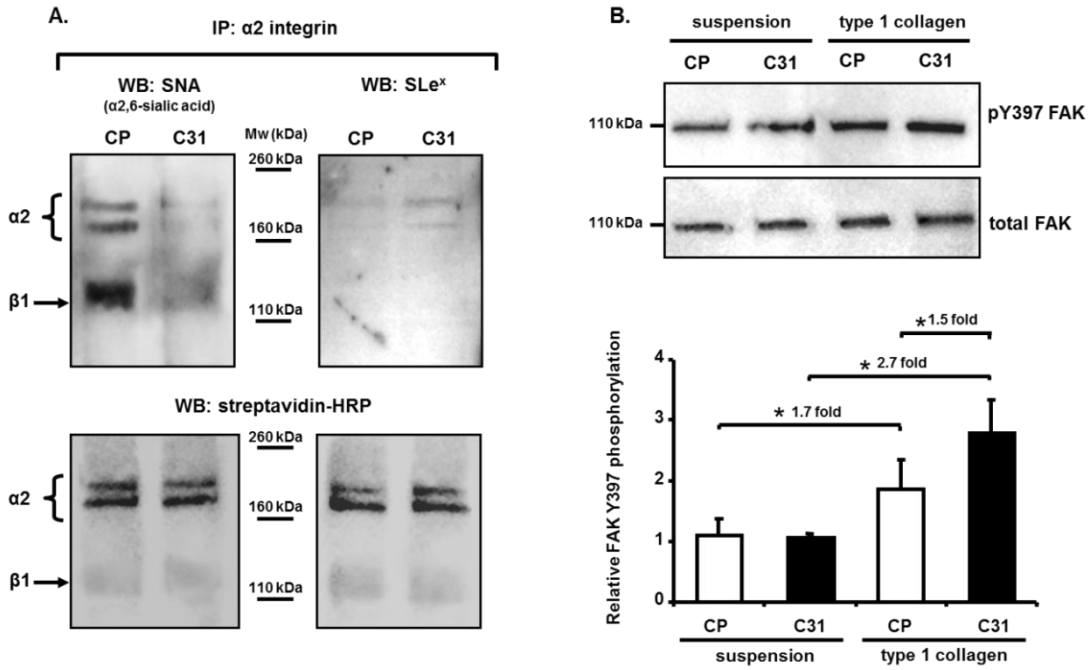

Figure 4. Sialylation of α2β1 integrin and FAK tyrosine 397 phosphorylation in CP and C31 cells.
A. α2 integrin subunit immunoprecipitates from biotinylated cells were blotted and analyzed with mAb against SLe^x (*right upper panel*) or with *Sambucus nigra* agglutinin (SNA; *left upper panel*). Equivalent amounts of loaded protein were verified by reprobing the corresponding membranes with HRP-conjugated streptavidin. **B.** Lysates from cells plated onto type 1 collagen coated dishes or kept in suspension were blotted and incubated with mAb against phosphorylated tyrosine 397 FAK (pY397 FAK) and with mAb against total human FAK. Bands were quantified and the relative FAK Y397 phosphorylation per cell line was calculated as the quotient between pY397 FAK quantification and total FAK quantification (graph). Bars represent mean ± SD. * Significantly different (p<0.05).

Overexpression of SLe^x induced alterations in intracellular signalling pathway derived from integrin-collagen binding

To address whether changes in α2β1 integrin glycosylation could affect the integrin-dependent signalling pathway, the autophosphorylation of focal adhesion kinase (FAK) at tyrosine 397 was analyzed. Both CP and C31 cells bound to collagen showed significant increased phosphorylation of FAK Y397 compared with CP and C31 kept in suspension, which showed lower levels of endogenous phosphorylation. Interestingly, relative Y397 phosphorylation levels in C31 cells bound to collagen raised 2.7-fold (p<0.001) compared with C31 cells in suspension, in a significantly higher degree (p<0.01) than the increase of Y397 phosphorylation in collagen bound CP cells compared with CP cells in suspension, which was of 1.7-fold (p<0.01) (**Figure 4B**). Since Y397 phosphorylation promotes cell motility through the formation of FAK/Src complex and the subsequent activation of different pathways related to cell migration [9,42], the higher increase of FAK Y397 phosphorylation in C31 cells could, at least in part, contribute to explain the higher migration capacity of C31 compared to CP cells.

ST3Gal III transfected cells showed decreased cell-cell aggregation and increased invasion capability

The role of the ST3Gal III sialylation on cell-cell aggregation and invasion capacity was assessed. A significant decrease in cell-cell aggregation of the ST3Gal III transfected cells compared to mock was shown after 24 h of cell seeding. MP cells formed 10-fold larger cellular aggregates than M34 (p<0.01), and CP cells formed about 3-fold larger aggregates than C31 (p<0.01). In general, Capan-1 model showed more disperse aggregates than MDAPanc-28 model (**Figure 5A**).

The invasive potential was assessed by *in vitro* invasion assay through Matrigel, which consists on a solubilized basement membrane-like preparation, mainly composed by laminin-111, collagen IV, heparan sulfate proteoglycan, various growth factors and additional components [43]. The results showed that Capan-1 model was around 6-times more invasive than MDAPanc-28 model (**Figure 5B**). Concerning ST3Gal III transfectants, there was a significant increase in the rate of cellular invasion when comparing to the corresponding controls cells. In particular, C31 cells exhibited 2.5-fold higher invasion rates than CP (p<0.001) and M34 cells were 3-fold more invasive with respect to MP (p<0.01). Interestingly, the described assay also measures the ability of the cells to attach to the ECM, invade into and through it, along with their migratory capacity toward a chemoattractant, crucial steps during the metastatic cascade [44], which were higher in the ST3Gal III transfectants.

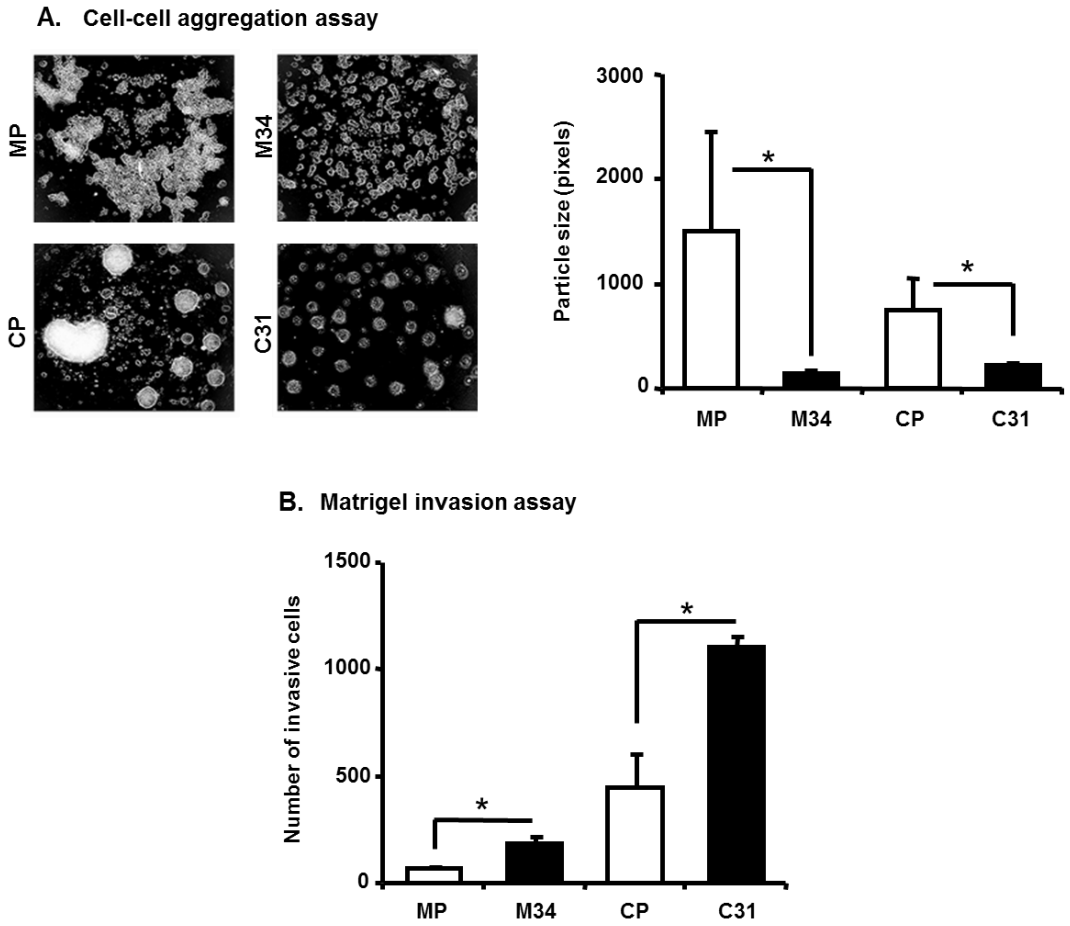

Figure 5. Aggregation on agar and Matrigel invasion assays.
A. Representative microscopy images (40X) of cellular aggregates after 24 h of seeding the cells on soft agar coated wells. Mean particle size quantification in pixels (*right graph*). **B.** Invasion of CP, C31, MP and M34 cells through Matrigel coated inserts for 24 h. Bars represent mean ± SD. * Significantly different (p<0.01).

SLe^x and E-cadherin cellular expression in the ST3Gal III transfected pancreatic cancer cells

Immunofluorescence labelling of cell monolayers with mAbs against E-cadherin and/or SLe^x showed similar E-cadherin levels among ST3Gal III and mock transfected cells, as observed by western blot and flow cytometry analysis. However, a slight delocalization of this protein to the cytoplasm was observed mainly in the ST3GalIII transfected cells M34 and, to a lesser extent, in C31 cells (white triangles in **Figure 6**; *left column*) when compared to their corresponding mock cells.

As expected, a significant increase in SLe^x staining was detected in the ST3Gal III overexpressing clones of both cell models (**Figure 6**; *mid column*). Colocalization of E-cadherin and SLe^x at the cell membrane was shown in Capan-1 model (CP and C31) and also in M34 cells (white triangles in **Figure 6**; *right column*).

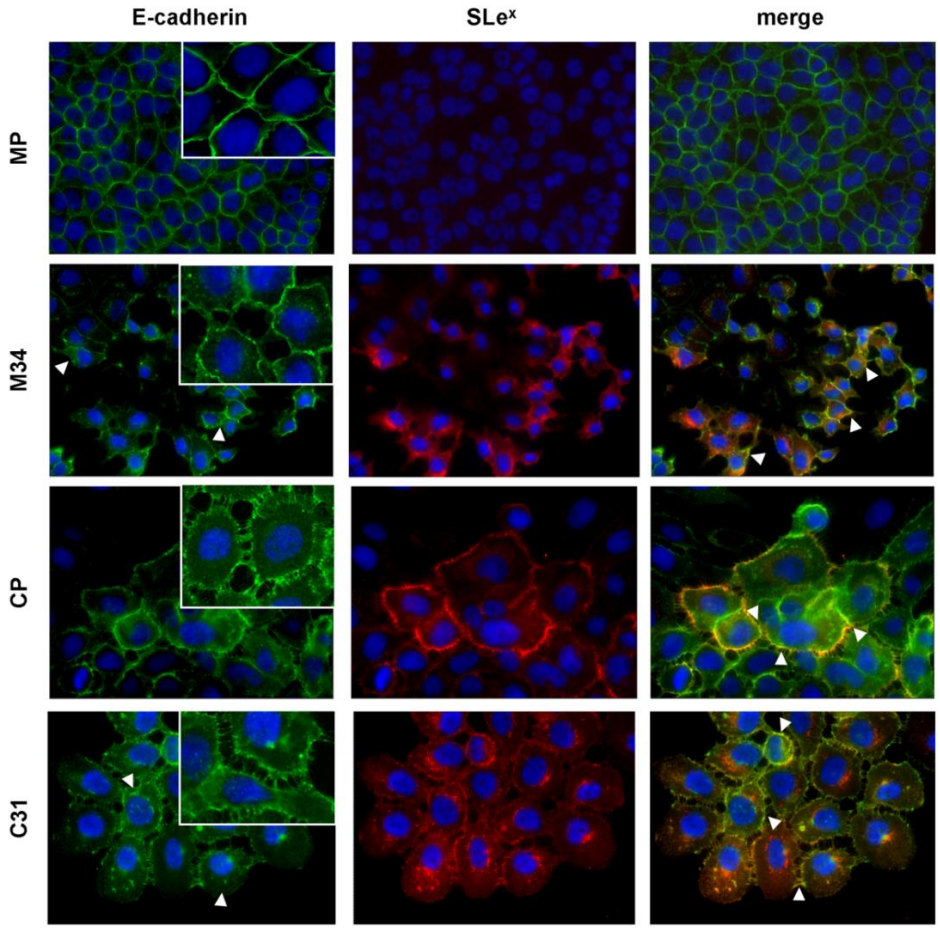

Figure 6. E-cadherin and SLe^x immunolabelling in cell monolayers.
Representative fluorescence microscopy images (400X) of E-cadherin (*left column*), SLe^x epitope (*mid column*) and merge (*right column*) in cell monolayers. Nuclei were stained with DAPI (blue). White triangles highlight areas of E-cadherin delocalization (left column) and areas of SLe^x and E-cadherin colocalization (right column). Close-up in inserts of E-cadherin localization are shown (630X).

Morphological analysis revealed important differences in the cellular phenotype and in cell-cell contacts between both models and between the ST3 Gal III transfectants and their corresponding controls. MP cells grew in compact spherical aggregates with tight cell-cell adhesion and expression of E-cadherin at the cells membrane. Upon ST3Gal III transfection (M34), alteration of the cellular morphology was observed with cells showing a more disperse phenotype with loss of intercellular contacts concomitantly with delocalization of E-cadherin into the cytoplasm. CP and C31 cells, in their turn, showed stellate morphology with faint contacts among cells, and especially in C31 notable holes were present in the midst of the monolayer (**Figure 6**).

Although the protein levels of E-cadherin did not show significant differences among the four cell lines (**Figure 2B** and **2C**), morphologic changes in cell-cell contacts with E-cadherin delocalization in the ST3Gal III transfected clones suggested a possible alteration of the adhesive function in these cells, which could contribute to explain their loss of cell-cell aggregation capacity and higher invasion.

To evaluate whether changes in SLe^x and/or α2,6-sialic acid determinants in the ST3Gal III transfected cells could occur on the E-cadherin molecule we analyzed the sialylation pattern of E-cadherin glycan chains.

E-cadherin sialylation profile in the ST3Gal III transfected pancreatic cancer cells

N-glycosylation contributes up to 20% of E-cadherin total mass, and several reports support the involvement of *N*-glycans in the modulation of E-cadherin-mediated tumour cell-cell adhesion [25,26,27]. E-cadherin sialylation pattern was evaluated by immunoprecipitation followed by lectin blot analysis. E-cadherin from the four cell lines was detected at 120 kDa range, and its sialylation profile was analyzed using mAb against SLe^x, and with SNA and MAL-II lectins, which detect α2,6-sialic acid and some α2,3- sialic acid determinants excluding SLe^x [45], respectively. SLe^x epitope could not be detected on E-cadherin (data not shown), whereas α2,3- and α2,6-sialic acid levels were faintly detected. To assess the changes in E-cadherin sialylation, the levels of sialic acid of each cell line were normalized with the corresponding E-cadherin levels. ST3Gal III transfected cells showed a slight decrease of α2,6-sialic acid levels compared to the corresponding controls, predominantly in the M34 clone (**Figure 7A**), together with an increase in α2,3-sialic acid determinants (**Figure 7B**).

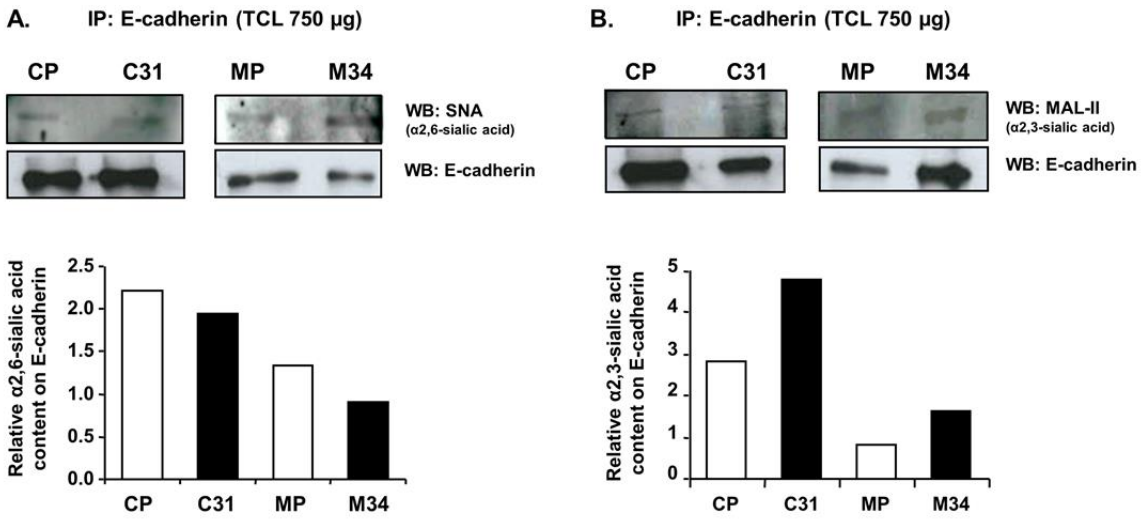

Figure 7. E-cadherin sialylation status.
Immunoprecipiation of E-cadherin followed by α2,6- or α2,3-sialylation recognition using *Sambucus nigra* agglutinin (**A**) or *Maackia amurensis* lectin II (MAL-II) (**B**) lectins, respectively.

Taken together, these differences in the overall sialylation, and in E-cadherin sialylation in particular, are in accordance with the observed changes on the cellular morphology and E-cadherin cellular

distribution and function observed in the ST3Gal III transfected cells, and could account for the differences in *in vitro* cellular aggregation and invasion assays.

E-cadherin, α2β1 integrin and SLe^x expression in human pancreatic ductal adenocarcinoma tissues

The expression of E-cadherin, α2β1 integrin and SLe^x molecules was evaluated in healthy pancreas and PDAC tissues from human patients (**Figures 8** and **9**). With regards to E-cadherin, healthy tissues displayed a strong and organized expression of E-cadherin at the cell contacts along the typical pancreatic acinus (**Figure 8**; *upper panel*), whereas PDAC samples showed clear disorganization of the tissue structure, along with the characteristic intense fibrotic response or desmoplasia. Moreover, in the tumour tissues a progressive delocalization of E-cadherin was observed, as well as loss of E-cadherin expression in a number of tumour cells in some of the samples (**Figure 8**; *lower panel*).

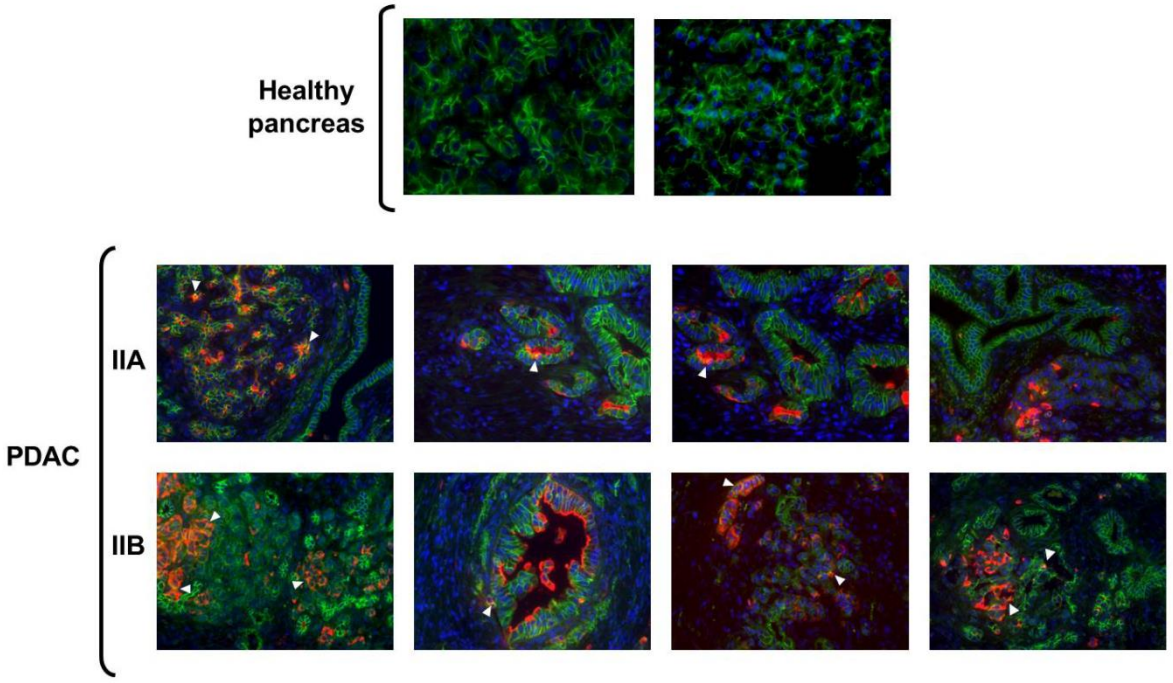

Figure 8. Immunohistological study of SLe^x and E-cadherin expression in human PDAC tissues.
Representative images (400X) of E-cadherin (green), SLe^x (red) and nucleus (blue) staining in human healthy and PDAC tissues from stages IIA and IIB. Whereas healthy tissues displayed a strong and organized expression of E-cadherin at the cell contacts, PDAC samples showed clear disorganization of the tissue structure and a progressive delocalization as well as loss of E-cadherin expression in a number of tumour cells. *De novo* expression of SLe^x is visible in every PDAC tissue. White triangles denote areas of SLe^x and E-cadherin colocalization.

Faint stain of α2 and β1 integrin subunits was found in the duct cells of normal pancreas, and β1 integrin also stained the endothelial blood vessel cells (data not shown). In PDAC samples, higher expression of α2 and β1 subunits was found in the tumour cells and in the desmoplastic stroma (**Figure 9**). β1 subunit was also expressed by the endothelial cells (**Figure 9**; *lower panel*). *De novo* expression of SLe^x determinant was found in the tumour cells of PDAC tissues throughout the dense stroma (**Figure 8**; *lower panel* and **Figure 9**). Areas of SLe^x and E-cadherin colocalization were detected in the tumour areas (**Figure 8**; white arrows), and colocalization of α2 integrin subunit and SLe^x was found in a few tumour cells (**Figure 9**, white arrows). No colocalization of β1 subunit and SLe^x could be detected.

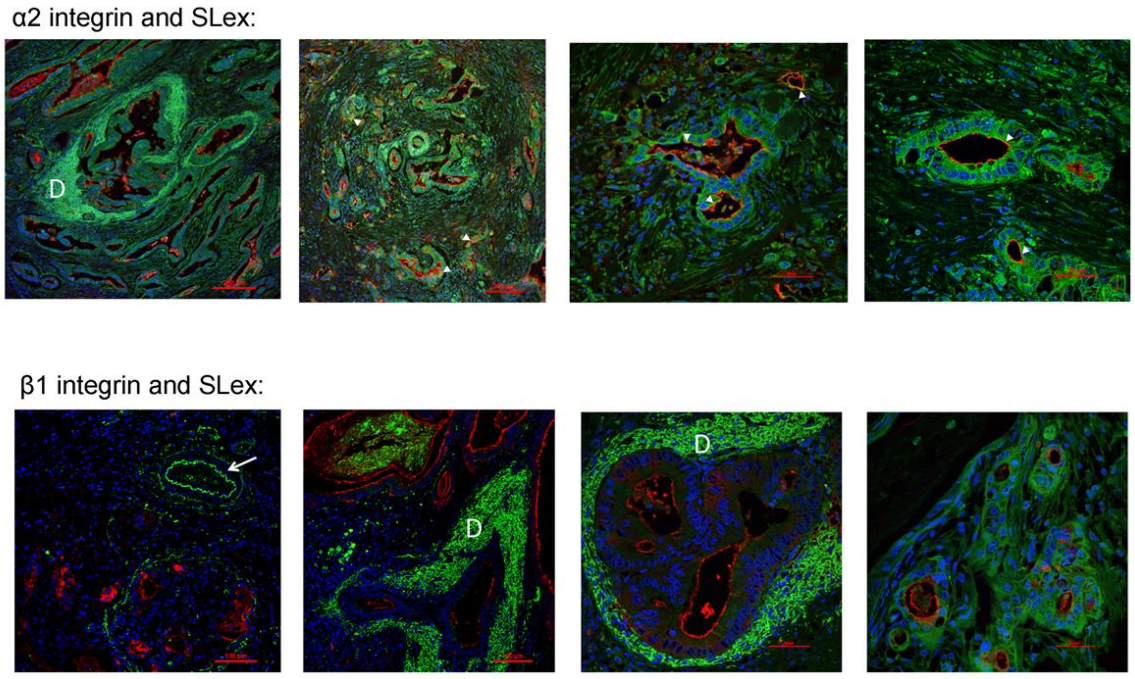

Figure 9. SLe^x and α2 and β1 integrin subunits expression in human PDAC tissues.
Representative images of merged immunofluorescence staining of α2 integrin (green, upper panel; 100X and 400X) or β1 integrin (green, lower panel; 200X and 400X) with SLe^x (red) and nucleus (blue) in pancreatic ductal adenocarcinoma (PDAC) tissues. α2 and β1 integrins are displayed in tumour cells. They also stain the stromal cells indicated as D, for desmoplasia) and β1 integrin stains the endothelial cells of blood vessels (see arrow). SLe^x is found in the lumen of tumour cells and in some secretions. No colocalization of β1 integrin and SLe^x could be detected. White triangles indicate points of α2 subunit and SLe^x colocalization.

Discussion

The local microenvironment provides tissues with extrinsic barriers to limit the outgrowth of tumours at the primary site. But as tumours evolve, these pressures drive the selection for traits that enable cancerous cells to by-pass them [46]. Dissemination of carcinomas from their original sites of

development to distant organs in the body is the cause for the major part of cancer morbidity and mortality. Although the molecular mechanisms underlying the cellular changes that take place during the invasive process are still not fully understood, there is a general consensus that cell-cell and cell-matrix interactions have to be profoundly altered [2,47]. In fact, homophilic cell adhesion and integrin signalling are among the core signalling pathways that are altered in most pancreatic cancers, including genetically altered genes such as E-cadherin and integrins [48].

In previous studies we have demonstrated the influence of sialic acid determinants in cell-ECM adhesion and in migratory processes of various human cancer models, including gastric cancer cells [49,50], and in pancreatic Capan-1 and MDAPanc-28 cell lines and their stably ST3Gal III transfected clones, C31 and M34 [25,31]. Specifically, cell surface α2,6-sialic acid levels correlated with higher cell adhesion to ECM components, such as collagen, fibronectin and laminin, which are important components of the tumour stroma, while higher α2,3-sialic acid levels favoured migration and metastasis [23,30].

In the present work we have evaluated whether ST3Gal III overexpression and the subsequent changes in the pattern of sialylation have a role in cell-cell adhesiveness and invasion in the MDAPanc-28 and Capan-1 pancreatic cancer cell lines. In addition, we have evaluated the impact of sialylation in the regulation of E-cadherin and α2β1 integrin functions.

The human pancreatic adenocarcinoma Capan-1 and MDAPanc-28 ST3Gal III transfected cells have been shown to exhibit a reduced cell-cell aggregation capacity and a high migration and invasion capability when compared with their respective mock cells, which is in agreement with our previous works reporting their increased migration through collagen and *in vivo* metastatic potential in mice [23,30]. In addition, Capan-1 cells display higher expression of SLe^x levels than MDAPanc-28 cells, which consequently show a higher invasive potential and lower aggregation rates than MDAPanc-28. These results reinforce the importance of α2,3-sialic acid in potentiating cell invasion and metastasis. In accordance, major α2,3-sialic acid residue expression was associated with higher invasive and metastatic potential of gastric and breast cancer cells [50,51,52] and, conversely, decreased α2,3-sialic acid levels of a lung cancer cell model resulted in invasion and metastasis suppression [53]. Likewise, induction of a more invasive phenotype by the terminal glycan structures containing α2,3-sialic acid through the activation of invasion-related signalling pathways has been recently reported in gastric carcinoma cells [50].

Pancreatic adenocarcinoma is characterized by a particularly high desmoplasia [12,54], and several studies have converged on the hypothesis that type 1 collagen plays an active role *in vitro* and *in vivo* in the pathophysiology of this neoplasia [15,55]. Our results showed that adhesion to type 1 collagen and migration through this ECM protein is dependent on α2β1 integrin in CP and C31 cells, which is

in agreement with other reports in pancreatic cancer cell lines [15]. Moreover, we have also shown that α2 and β1 integrins are expressed in the tumour cells and in the desmoplastic stroma of PDAC, in agreement with published studies that describe the expression of α2β1 integrin in pancreatic cancer cells and its interaction with type IV collagen in PDAC tissues [56].

Several studies have hypothesized that alteration on *N*-glycosylation may act as a regulatory mechanism for β1 integrins function [57,58,59]. The presence of *N*-glycans on the α5β1 heterodimer, which is the best-characterized integrin molecule, and on the β4 subunit has been reported to be crucial for proper integrin-ECM interactions [60,61]. However, only the *N*-glycans localized in certain motifs are proposed to regulate the conformation and biological function of these glycoproteins, either facilitating the subunit association and/or regulating the integrin activation state [62]. In addition, the modification of integrin *N*-glycans by sialyltransferases enzymatic activity results in integrin subunits being capped with the negatively charged sugar sialic acid, which can modulate integrin function [63].

In our study we have shown that α2β1 integrin glycosylation was different between pancreatic cancer Capan-1 cells overexpressing ST3Gal III and mock cells. The results showed a slight increase in SLe^x glycans expression in the α2 subunit and a significant decrease in α2,6-sialic acid content in both α2 and β1 subunits of C31 cells. Since higher α2,6-sialic acid levels in pancreatic cancer cells correlated with increased ECM adhesion [30], we here suggest that the decrease in α2,6-sialic on the α2β1 integrin molecule appears to contribute for the reduced adhesion of C31 cells to type 1 collagen. This hypothesis is consistent with several reports stating that the downregulation of α2,6-sialyltransferase ST6Gal I inhibited cell adhesion to collagen and that, conversely, the hypersialylation of the β1 integrin subunit with α2,6-sialic acid promoted adhesion to collagen of several cancer cells [64,65,66]. C31 cells also showed a more migratory phenotype. Similarly, Guo et al. [34] described that human fibrosarcoma cells MGAT5 transfected, which showed reduced attachment to fibronectin due to glycosylation changes in their α5β1integrins, increased their migration.

Recent studies have demonstrated that SLe^x can be determinant for the behaviour of cancer cells by modulating tyrosine kinase receptors [50]. In the present work we demonstrate for the first time the functional role of SLe^x in integrin mediated function. Here we show that the Capan-1 cells overexpressing ST3Gal III glycosyltransferase, C31 cells, display an enhanced SLe^x pattern of expression in general and particularly on the α2 integrin molecule compared with the CP control cells (**Figure 4A**), suggesting a regulatory effect of SLe^x on α2β1 integrin-dependent migration. In PDAC tissues, α2 and β1 integrin subunits were expressed in some of the tumour cells all over the cell surface. SLe^x antigen was also expressed in some tumour cells and was specially found at the ductal

lumen and in foci of tumour cells, as largely described by other authors [21,22,67]. Although the pattern of staining of the integrin molecules and SLe^x antigen was different, some areas of α2 integrin and SLe^x colocalization were detected in a few tumour cells.

The importance of integrin glycosylation in the activation of FAK has been described by several authors. The level of FAK tyrosine phosphorylation was shown to be reduced in α1,6-fucosyltransferase (Fut8) deficient mouse embryonic fibroblasts [35], as well as in HeLa S3 cells transfected with β1,4-*N*-acetylglucosaminyltransferase (GnT-III) [68]. Likewise, a dependence on integrin sialylation has been reported for FAK/paxillin-mediated signalling, and for cancer angiogenesis and metastasis pathways [69]. In our Capan-1 clones, FAK Y397 was more highly phosphorylated in C31 than in CP upon binding to collagen, which contributes for a higher motility of C31, that is in agreement with previous reports describing that autophosphorylation of FAK Y397 is elevated in highly motile and invasive cancer cells [70]. Since the decrease of α 2,6-sialylation β1 integrin subunit has been reported to reduce tumour migration [71], we suggest that the increase of SLe^x in α2β1 integrin may favour the higher phosphorylation of FAKY397 upon collagen adhesion (**Figure 4B**), and therefore the more migratory and invasive phenotype of the human pancreatic adenocarcinoma cells C31 versus CP cells (**Figures 3B** and **5B**) . In line with this, recent studies have described that changes in cell glycosylation may alter the cell phosphoproteome, and particularly the SLe^x overexpressing gastric cancer cells increase the phosphorylation of FAK Y397 and contributed to explain their higher invasive capacity [50].

Interestingly, we have further demonstrated that E-cadherin is a carrier of sialylation in the pancreatic cancer cell lines, being a target of modification by the ST3Gal III enzyme. In particular, an increase in α2,3-sialic acid and a decrease in α2,6-sialic acid was shown in the E-cadherin molecule of the ST3Gal III overexpressing cells. This specific modification of E-cadherin with terminal sialylated structures was concomitantly observed with alterations in cellular morphology together with alterations on E-cadherin cellular localization compared with control cells. In addition, these alterations could account for the observed decrease in cell-cell aggregation of the ST3Gal III transfectants together with their increased invasive potential. In PDAC clinical samples, E-cadherin expression was found in some tumour areas, with points of E-cadherin and SLe^x colocalization where a potential interface between both molecules could exist.

In conclusion, we have demonstrated that the alteration of the membrane sialylation pattern of PDAC cells has a modulatory effect in the proper function of important membrane adhesive molecules such as α2β1 integrin and E-cadherin, influencing cell adhesion and invasion processes. In

particular, increase in SLe^x and decrease in α2,6-sialic acid as a consequence of ST3Gal III transfection led to reduced cell-cell adhesiveness, and endowed the cells with a more invasive phenotype. Glycosylation of E-cadherin and α2β1 integrin molecules was also modified as a result of the ST3Gal III transfection, with impact in the modulation of their functions and thus underlying the observed differences in the adhesive and motile phenotype. Specifically, glycan changes in α2β1 integrin of ST3Gal III transfected cells were further shown to activate integrin-mediated signalling pathways through FAK phosphorylation and therefore contributing to increase cell migration.

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Chapter 3

Inflammatory cytokines regulate the expression of glycosyltransferases involved in the biosynthesis of tumour-associated sialylated determinants in pancreatic cancer

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Inflammatory cytokines regulate the expression of glycosyltransferases involved in the biosynthesis of tumour-associated sialylated determinants in pancreatic cancer.

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Abstract

Objectives: Pancreatic ductal adenocarcinoma (PDAC) is characterized by an abundant stroma contaning several pro-inflammatory cytokines. Here we investigate the potential role of these cytokines in the biosynthesis of the tumor-associated carbohydrate antigens such as sialyl-Lewis x (SLe^x) through the up-regulation of specific glycosyltransferase genes.

Methods: Two human PDAC cell lines, MDAPanc-3 and MDAPanc-28, were treated with IL-1β, TNFα, IL-6 or IL-8 cytokines, and the content of the aforementioned antigens was studied by flow cytometry. In addition, the mRNA expression of the sialyltransferases (STs) and fucosyltransferases (FUTs) involved in their biosynthesis was determined. The inflammatory microenvironment of PDAC tissues and the expression of sialyl-Lewis antigens were also analyzed by immunohistochemistry.

Results: IL-1β increased SLe^x and α2,6-sialic acid levels in MDAPanc-28 cells and enhanced the mRNA levels of ST3Gal III-IV and FUT5-7 related to SLe^x biosynthesis and of ST6Gal I. IL-6 and TNFα increased the levels of SLe^x and $Lewis^y$ in MDPanc-3 cells and similarly, mRNA expression of ST3Gal III-IV, FUT1-2 and FUT6 related to these Lewis antigens biosynthesis were increased. Most PDAC tissues stained for sialyl-Lewis antigens and tended to be expressed in the tissues with a higher presence of inflammatory immune cells.

Conclusions: The inflammatory microenvironment can modulate the glycosylation of PDAC cells, increasing the expression of sialylated antigens such as SLe^x, which contributes to pancreatic tumor malignancy.

Keywords: cytokines; fucosyltransferases; Lewis-type antigens; pancreatic adenocarcinoma; α2,6-sialic acid; sialyltransferases.

General discussion

1 Cell surface sialic acid modulates the adhesive and invasive capacity of pancreatic ductal adenocarcinoma cells

One of the most important features of pancreatic ductal adenocarcinoma (PDAC) is a high desmoplasia, which constitutes a microenvironment that promotes tumour growth and invasion (Ghaneh *et al.*, 2007; Kleeff *et al.*, 2007). In addition, the carbohydrate antigen sialyl-Lewis x (SLe^x) has also been shown to play an important role in several steps of PDAC progression including migration and metastatic potential (Perez-Garay *et al.*, 2010; Perez-Garay *et al.*, 2013). Although the molecular mechanisms underlying the cellular changes that take place during the invasive process are still not fully understood, there is a general consensus that cell-cell and cell-matrix interactions have to be profoundly altered (Cavallaro and Christofori, 2004b; Friedl and Alexander, 2011). In this context the aim of this study has been to deepen in the involvement of sialic acid determinants such as SLe^x in the regulation of PDAC cells' adhesion to the stroma and in adherens junctions, as well as in PDAC invasive behaviour. In order to accomplish this goal, various pancreatic cancer cell models with different pattern of cell surface sialylation have been developed and their adhesive and invasion potentials have been evaluated.

Pancreatic ductal adenocarcinoma cell models

On one hand, and since cell proliferation and differentiation are described to regulate the surface level of SLe^x antigen in cancer cells (Christie *et al.*, 2008), the membrane expression of α2,3- and

α2,6-sialic acid determinants, and of SLe^x epitope in particular, was evaluated by flow cytometry in the two PDAC cell lines Capan-1 and MDAPanc-28 at different stages of cell culture. The results showed that while SLe^x surface expression increased along with cell proliferation in both cell models, the α2,3- and α2,6-sialic acids patterns behaved differently. In particular, α2,6-sialic acid levels in MDAPanc-28 cells decreased significantly, while α2,3-sialic acids remained constant in this cell model. In contrast, Capan-1 cells at increasing cell density enhanced both α2,3- and α2,6-sialic acid levels (summarized in **Table 11**).

Several reports in the literature conclude that proliferation or differentiation-dependent regulation of α2,3- and α2,6-linked sialic acids levels on oligosaccharide chains is associated with the regulation of α2,3- and α2,6-ST genes' mRNA expression (Taniguchi *et al.*, 1998; Taniguchi and Matsumoto, 1998; Wen *et al.*, 1992b): human colon carcinoma CaCo-2 cells, which differentiate spontaneously into enterocytes when maintained confluent for several days, exhibited a dramatic elevation in their α2,6-ST activity (Dall'Olio *et al.*, 1996; Dall'Olio *et al.*, 1992); and gastrointestinal carcinoma MKN45 confluent culture showed higher ST3Gal III expression than sparse ones (Carvalho *et al.*, 2010). Oppositely, when inducing differentiation to the myeloid HL-60 cell line, their ST6Gal I mRNA levels, α2,6-ST activity and α2,6-sialic acid expression decreased extremely (Taniguchi and Matsumoto, 1998). However, the differences in the pattern of expression between our two pancreatic models Capan-1 and MDAPanc-28 has complicated interpretation due to the genomic complexity of the α2,3- and α2,6-ST genes, with multiple promoters generating tissue or cell-specific and developmentally regulated transcripts (Aasheim *et al.*, 1993; Harduin-Lepers *et al.*, 2001; Taniguchi, 2008).

On the other hand, Capan-1 and MDAPanc-28 cell lines were compared to their stably transfected ST3Gal III cell lines C31 and M34, respectively. In addition, the two mock transfected cells CP and MP were used as control lines. The increase in SLe^x expression and decrease in α2,6-sialic acid levels in C31 and M34 versus their control cells (Capan-1 and CP for the Capan-1 cell model; and MDAPanc-28 and MP, for the MDAPanc-28 cell model), was either detected by flow cytometry or by Western/lectin blot in their total cell lysates. These glycosylation changes are likely explained by enzymatic competition between ST3Gal III and α2,6-STs enzymes, such as ST6Gal I, to sialylate type II glycan chains and generate SLe^x or terminal α2,6-sialylated determinants, respectively (Dall'Olio and Chiricolo, 2001; Harduin-Lepers *et al.*, 2001; Perez-Garay *et al.*, 2010).

These characterized PDAC cell models were assayed to find a possible correlation between the pattern of sialylation and changes in their adhesion and invasion capabilities.

Adhesion to extracellular matrix (ECM) components

Results of the ECM binding assays for MDAPanc-28 cells showed a significant progressive lower adhesion to type 1 collagen along cell proliferation, in contrast to Capan-1 progressive higher adhesion. Capan-1 and the control mock transfected cells (CP) showed higher adhesion to type 1 collagen than the Capan-1 ST3Gal III transfected cell line (C31). The variation in cell surface sialylation and the changes in adhesion obtained after the incubation of the cells with SNA or MAA lectins, which block α2,6- and α2,3-sialic acids respectively, may explain the contribution of each type of sialic acid determinants to the differences in the adhesive behaviour among the cell models. A higher proportion of cell surface α2,6-sialic acid levels seems to favour adhesion to collagen, and vice versa, in all cell models (**Table 11**). The influence of SLe^x or α2,3-sialic acid on adhesion was more significant in Capan-1 model, where the α2,6-sialic acid levels are much lower than α2,3-sialic acid or SLe^x. The impact of α2,6-sialic acids on collagen adhesion has been reported in other cell types by several authors when inhibiting or up-regulating ST6Gal I gene. As for instance, colon and ovarian epithelial cells forced to α2,6-hypersialylation resulted in higher adhesion rates to this ECM protein (Christie *et al.*, 2008; Seales *et al.*, 2005). Otherwise, downregulated expression of α2,6-sialic acids in colon epithelial cells resulted in lower adhesion to type 1 collagen (Shaikh *et al.*, 2008).

The adhesion of Capan-1 and C31 cells to fibronectin or laminin showed similar results as to type 1 collagen, and data from the literature follows the same direction: an increase in β1 integrin α2,6-sialylation enhanced the adhesion of colon cancer cells to fibronectin (Lee *et al.*, 2010); and colon and ovarian cancer cells with increased α2,6-sialic acid levels resulted in higher adhesion rates to laminin (Seales *et al.*, 2005).

Some studies have shown that the enzymatic removal of sialic acids from the cell surface can modulate adhesion to ECM ligands (Pretzlaff *et al.*, 2000; Semel *et al.*, 2002; Shaikh *et al.*, 2008). In this work, sialidase treatments were performed in order to better understand the involvement of sialic acids in Capan-1 model adhesion. When treating cells with *Vibrio cholerae* sialidase, which digests both α2,6- and α2,3-sialic acids, the adhesive capability of the cells was completely reversed, demonstrating that sialic acids are required for ECM adhesion. This could also indicate that the sialylation may be in part on the integrin subunits responsible for this recognition, and that they are essential for the proper function of these glycoreceptors, as will be discussed later. However, the role played by each type of sialic acid was shown to be different. Thus, digestion of only α2,3-sialic acids with NAN 1 sialidase increased CP cells (Capan-1 mock transfected) adhesion, while C31 adhesion was not changed. These results suggest that when α2,3-sialic acids are lost, the role played by α2,6-sialic acids becomes more important. That is, C31 lower α2,6-sialic acid content compared to Capan-1, either before or after NAN 1 treatment, could probably contribute to the lower adhesion of C31 to type 1 collagen.

		MDAPanc-28 along proliferation	Capan-1 along proliferation	C31 with respect to CP	M34 with respect to MP
Cell membrane content	α2,6-sialic acid (SNA)	↓	↑	↓	↓ (*)
	α2,3-sialic acid (MAA)	=	↑	=	= (*)
	SLe^x	↑	↑	↑	↑
Rates	ECM adhesion	↓	↑	↓	n.d.
	Migration	↑	↑	↑	↑(*)
	Invasion	n.d.	n.d.	↑	↑
	Aggregation	n.d.	n.d.	↓	↓

↑ α2,6-Sia correlates positively with ECM adhesion

↓ α2,6-Sia and ↑SLe^x correlates negatively with cell aggregation

↓ α2,6-Sia and ↑ α2,3-Sia or SLe^x correlates positively with cell migration and invasion

Table 11. Cell surface sialic acid modulates the adhesive and motile behaviour of PDAC cells.
Sialic acid cell content in MDAPanc-28 and Capan-1 cell lines along cell proliferation, and in the ST3Gal III transfected cell lines (C31 and M34) compared to mock cell lines (CP and MP, respectively) are summarized, as well as the ECM adhesion, migration, invasion and aggregation rates.
Hypothetic formulas intend to summarize the main results obtained: ↑, enhancement; ↓, decrease; = (equal) no change; n.d., not determined. (*) results from Perez-Garay *et al.* (2010).

To go further, the characterized cell models and the ST3Gal III transfected cells of Capan-1 and MDAPanc-28 were used to evaluate the influence of SLe^x in other important steps of the *metastatic cascade*, such as cell-cell adhesiveness, migration and Matrigel invasion.

Cell-cell adhesion

The cell-cell adhesion behaviour is generally evaluated by the capacity of the cells to aggregate. Both Capan-1 and MDAPanc-28 ST3Gal III transfected cells (C31 and M34, respectively) exhibited a reduced cell-cell aggregation capacity compared to the respective control cells (CP and MP, respectively). In addition, parental Capan-1 cells, which display much higher SLe^x levels than parental MDAPanc-28 cells, had lower aggregation rates than the latter cell line. Our results show that the α2,3-sialic acid and SLe^x levels have a negative correlation with the aggregation capacity of the PDAC cell models, and are summarized in **Table 11**.

Cell migration and invasion

Regarding the study of cell motile behaviour, previous studies from the group described enhanced motility for the MDAPanc-28 and Capan-1 cells transfected with ST3Gal III gene, C31 and M34 cells, respectively, compared to the parental ones (Perez-Garay *et al.*, 2010). In the present work, enhanced migratory rates are also presented for both MDAPanc-28 and Capan-1 models at increasing cell confluence. In addition, Capan-1 and MDAPanc-28 ST3Gal III transfected cells (C31 and M34) exhibited a superior invasion capability through Matrigel compared to the respective control cells (CP and MP), in agreement with previous results of the group showing an enhance of the *in vivo* metastatic potential in mice (Perez-Garay *et al.*, 2010; Perez-Garay *et al.*, 2013). Capan-1 model also displayed higher invasion rates than MDAPanc-28 model.

In general, in the assayed PDAC cell models the migratory and invasive potentials have a positive correlation with the α2,3-sialic acid and SLe^x content on the cell membrane (**Table 11**). These findings reinforce the importance of α2,3-sialic acids in potentiating cell invasion and metastasis, as reported in a variety of cancer cell lines: major α2,3-sialylated residues expression was associated with higher invasive and metastatic potentials of gastric and breast cancer cells (Cui *et al.*, 2011; Gomes *et al.*, 2013; Wang *et al.*, 2009) and, conversely, the decrease in α2,3-sialic acids resulted in diminished cell migration of breast cancer cells and murine melanoma cells (Chang *et al.*, 2006; Hsu *et al.*, 2005), as well as in invasion and metastasis suppression of a lung cancer cell model (Chen *et al.*, 2011).

Despite that many other unknown aspects of the cell phenotype may contribute to explain the observed differences in adhesive and motility capabilities, the results of blocking and digestion of sialylated determinants show how the participation of every type of sialic acid determinant in ECM adhesion and migration is, first of all, cell-type specific, and depends on the cell surface global sialic acid repertoire, what is modulated along cell density. Within a cell model, higher α2,6-sialic acid levels accounted for increased ECM adhesion, while negatively correlated with cell migration. α2,3-sialic acid or SLe^x levels, in their turn, positively correlated with migration and invasion, and negatively with the aggregation capacity, whereas they did not influence significantly the cell adhesion behaviour.

Integrins and E-cadherin levels in the cell models

Since variation in cell surface expression of integrins could influence the adhesive and migratory behaviour of the cells, flow cytometry analyses were performed to evaluate the α2β1 (for type 1 collagen), α5β1 (for fibronectin) and α3β1 (for laminin) integrin surface levels, which are the main integrins described in several PDAC cell lines (Grzesiak and Bouvet, 2006; Grzesiak *et al.*, 2007). Results revealed no significant differences in the α2 integrin levels of Capan-1 cells grown at different

cell density, and the same levels of α2, α3, α5 or β1 subunits in Capan-1, CP and C31 cells. Thus, neither cell confluence in Capan-1 cells nor overexpression of ST3Gal III enzyme in C31 cells had an impact on integrin membrane expression. MDAPanc-28 cells did not express α2 integrin molecule at detectable levels by flow cytometry, so that adhesion to type 1 collagen is probably via other adhesive molecules, and it may also explain the need for a longer time in type 1 collagen adhesion and migration of this cell line compared to Capan-1 cell model.

The adhesion to type 1 collagen and migration through this ECM protein strongly depended on α2β1 integrin in CP and C31 cells, as shown by blocking these processes with monoclonal antibodies against the two integrin subunits, and in agreement with other reported pancreatic cancer cell lines (Grzesiak and Bouvet, 2006). Considering that free α and β integrin subunits do not exist at the cell surface, and that the α2 subunit only heterodimerizes with the β1 subunit (Barczyk *et al.*, 2010), it can be stated that CP and C31 cells display similar amounts of the α2β1 receptor at their cell membrane, so excluding receptor availability to explain their adhesive and migratory differences on type 1 collagen.

As well as the integrins levels for cell-ECM adhesion, the E-cadherin levels, which is the main glycoprotein involved in the cell-cell aggregation process, were determined in MDAPanc-28 and Capan-1 clones (MP and M34; and CP and C31, respectively), and no differences among them were observed, either by flow cytometry or Western blot of the total cell lysates.

α2β1 integrin and E-cadherin sialylation

The hypothesis that sialylation may modulate the function of specific membrane receptors involved in adhesive processes prompted us to study the sialylation status of α2β1 integrin, main molecule responsible for type 1 collagen adhesion in our cell models, and E-cadherin, main molecule responsible for cell adherens junctions, as previously discussed.

The α2β1 integrin glycosylation, determined by immunoprecipitation against α2 integrin subunit and subsequent Western blot with lectins or antibody against SLe^x, was different between C31 and CP cells, and reflected the overall differences in the cell surface glycosylation pattern. In particular, there was a slight increase in SLe^x glycan expression in the α2 subunit, along with a significant decrease in α2,6-sialic acids content in both α2 and β1 subunits of C31 cells. Since higher α2,6-sialic acid levels in pancreatic cancer cells correlated with increased ECM adhesion, as discussed above, the decrease in α2,6-sialic acids on the α2β1 integrin molecule appears to contribute for the reduced adhesion of C31 cells to type 1 collagen. In addition, C31 enhanced SLe^x levels on the α2 integrin molecule reveal a possible regulatory effect of SLe^x upon α2β1 integrin functions.

Several reports state that the downregulation of α2,6-ST ST6Gal I inhibits cell adhesion to collagen and that, conversely, the α2,6-hypersialylation of the β1 integrin subunit promotes adhesion to

collagen in several cancer cell types (Christie *et al.*, 2008; Hedlund *et al.*, 2008; Seales *et al.*, 2005). In this work, the ST3Gal III transfected cells also showed a more migratory phenotype through type 1 collagen (**Table 11**). Similarly, Guo *et al.* (2002) described that human fibrosarcoma cells MGAT5 transfected, which showed reduced attachment to fibronectin due to glycosylation changes in their α5β1 integrins, increased their migration.

It has been largely reported a dependence on integrin glycosylation, and more specifically on integrin sialylation, for the FAK/paxillin-mediated signalling, and for cancer angiogenesis and metastasis pathways (Isaji *et al.*, 2004; Mitra and Schlaepfer, 2006; Zhao *et al.*, 2006). In our Capan-1 clones, FAK Y397 was more highly phosphorylated in C31 cells than in the mock transfected CP cells upon binding to collagen. Since the decrease of α2,6-sialylation in the β1 integrin subunit has been reported to reduce tumour migration (Shaikh *et al.*, 2008), the increase of SLe^x in the α2β1 integrin of C31 cells may favour the higher phosphorylation of FAK Y397 upon collagen adhesion of these cells. Therefore, the more invasive phenotype of the ST3Gal III transfected cells is in agreement with previous reports describing that autophosphorylation of FAK Y397 is elevated in highly mobile and invasive cancer cells (McLean *et al.*, 2005). In line with our results, Gomes *et al.* (2013) described that changes in cell glycosylation may alter the cell phosphoproteome, and particularly the SLe^x overexpressing gastric cancer cells had increased levels of FAK Y397 phosphorylation, and contributed to explain their higher invasive capacity.

E-cadherin molecule was also shown to be a carrier of sialylation in the two PDAC cell lines, being a target of modification by the ST3Gal III enzyme. In particular, a slightly increase in α2,3-sialic acids and decrease in α2,6-sialic acids were demonstrated in the E-cadherin molecule of the ST3Gal III overexpressing cells, C31 and M34. This specific modification of E-cadherin molecules could be linked to alterations in the cellular morphology observed by immunofluorescence of cell monolayers, together with alterations on E-cadherin cellular location when comparing to control cells. Furthermore, the sialylation status of E-cadherin molecules could also account for the decrease in cell-cell aggregation of the ST3Gal III transfectants together with their increased invasive potential.

PDAC tissues

To a greater extent, the histological distribution of SLe^x, E-cadherin and α2β1 integrin molecules was evaluated in human PDAC clinical samples. *De novo* expression of SLe^x epitope was specially found at the ductal lumen and in foci of tumour cells throughout the dense stroma, as largely described in the literature (Kishimoto *et al.*, 1996; Park *et al.*, 2003; Peracaula *et al.*, 2005). E-cadherin was clearly delocalized compared to the well-organized expression in pancreatic healthy tissues, with a loss of expression in a number of tumour cells in some of the samples. Regarding α2 and β1 integrin

subunits, a high expression was found in the tumour cells and in the desmoplastic stroma. β1 subunit was also expressed by the endothelial cells. Areas of SLe^x and E-cadherin colocalization were detected in the tumour areas, and colocalization of α2 integrin subunit and SLe^x was found in a few tumour cells, whereas no colocalization of β1 subunit and SLe^x could be detected.

Over decades it has been accepted that atypical glycosylation may be detected in all types of experimental and human cancers, and it has, without doubt, a function in every step of the malignant transformation of the cells and in the overall progression of cancer (Freire-de-Lima, 2014). However, although many tumour-associated antigens have been used as tumour glycomarkers, their specific role on cancer evolution has not been widely addressed (Büll *et al.*, 2014; Freire-de-Lima, 2014). Focusing on pancreatic cancer, the metastatic dissemination requires cells to break away from the epithelial ductal structures and adquire characteristics of migratory, mesenchymal cells. This major phenotypic switch in pancreatic cancer cells may be driven by the epithelial-mesenchymal transition (EMT), which is highly characterized by changes in glycosylation (Maupin *et al.*, 2010).

The described results so far reinforce the theory that the cell-cell adhesion, cell-ECM adhesion and invasion result profoundly modified as a consequence of the ST3Gal III transfection in PDAC cells, and highlight the fact that sialylation is a very powerful tool for the cells to acquire the described invasive potential, by finely regulating the biological function of specific cell adhesion molecules such as E-cadherin and α2β1 integrin. **Figure 20** intends to summarize the proposed model for the effect of stable ST3Gal III gene transfection to PDAC cells on modulating the biological function of the mentioned glycoreceptors.

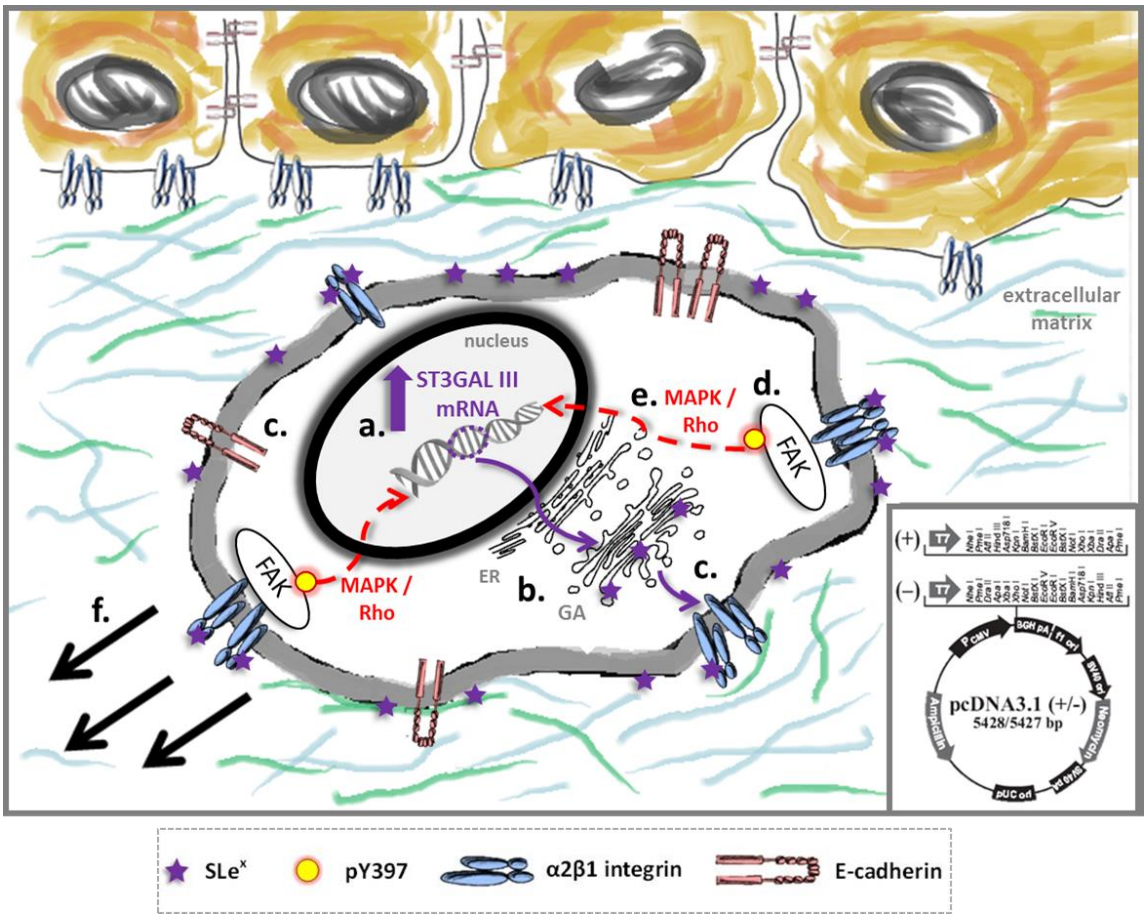

Figure 20. Proposed model for the effect of stable ST3Gal III gene transfection in PDAC cells on α2β1 integrin and E-cadherin biological functions.

a| Stable transfection of PDAC cells with pcDNA 3.1 vector encoding ST3Gal III gene. **b|** Translation of the mRNA to protein contributes to higher α2,3-ST activity and to an enhanced SLe^x and diminished α2,6-sialic acid content in the cell membrane and secreted glycoproteins. **c|** Specific sialylated glycoproteins of the cell membrane, such as α2β1 integrin and E-cadherin, alter their glycosylation pattern. **d|** α2β1 integrin with higher SLe^x and lower α2,6-sialic acid content show diminished adhesion rates to type 1 collagen (ECM component). In a similar way, SLe^x also interferes in the formation of adherens junctions between neighbouring cells through E-cadherin. **e|** After integrin-collagen recognition, FAK becomes phosphorylated at Y397, what triggers the initial steps of several signalling pathways, such as MAPK and Rho signalling pathways. **f|** Loss of cell-cell and cell-ECM adhesion, and enhanced migration rates result in a more invasive phenotype.

ECM: extracellular matrix; ER: endoplasmic reticulum; FAK: focal adhesion kinase; GA: Golgi apparatus; MAPK: mitogen-activated protein kinase; Rho: subgroup of the Ras superfamily of GTP hydrolases; ST: sialyltransferase; ST3GAL3: α2,3-sialyltransferase ST3Gal III gene.

2 Inflammatory cytokines regulate the expression of glycosyltransferases involved in the biosynthesis of tumour-associated sialylated determinants in pancreatic cancer

The intense desmoplastic reaction in PDAC leads to a stroma composed by several cell types including fibroblasts, pancreatic stellate cells, endothelial cells, immune cells and an altered ECM, constituting a microenvironment that promotes tumour growth and invasion (Hernandez-Munoz *et al.*, 2008). Inflammatory cells like macrophages and infiltrating mast cells can produce pro-inflammatory cytokines as well as pro-angiogenic molecules and basic fibroblast growth factor, which are known to activate important oncogenic signalling pathways on the tumour cells, regulating the expression of genes involved in tumour promotion and progression (Esposito *et al.*, 2004; Mahadevan and Von Hoff, 2007).

Focusing on pro-inflammatory cytokines, IL1-β and TNFα signalling are described to promote the activation of several transcription factors, including nuclear factor kappa beta (NF-kB) and activator protein-1 (AP-1), which act as transcription factors of a number of target genes (Weber *et al.*, 2010). In PDAC cells, NF-kB activation exerts anti-apoptotic effects, and it is thought to play a key role in the acquisition of chemoresistance by cancer cells (Fujioka *et al.*, 2012; Neesse *et al.*, 2012). For this reason, it has been suggested as a potential molecular target for pancreatic cancer therapy (Holcomb *et al.*, 2008). Similarly, IL-6 binding to the IL-6 receptor can activate Janus Kinases (JAKs)/signals transducers and activators of transcription (STATs, particularly STAT3), what results in the transcription of a key set of target genes (Scheller *et al.*, 2006); and particularly in PDAC, IL-6 has been reported to play a role in cell invasion, tumour progression and chemoresistance (Huang *et al.*, 2010; Toyonaga *et al.*, 2003).

Pro-inflammatory cytokine treatments in PDAC cells

In this work, two PDAC cell lines, MDAPanc-3 and MDAPanc-28, have been treated with the pro-inflammatory cytokines IL1-β, IL-6, IL-8 or TNFα to study their influence on the regulation of the expression of fucosyltransferase and sialyltransferase genes involved in the biosynthesis of Lewis-type and α2,6-sialylated antigens, which are related to PDAC tumourigenesis.

SLe^x and Le^y levels

Both MDAPanc-28 and MDAPanc-3 cells had enhanced SLe^x epitope levels after treatment with pro-inflammatory cytokines. In MDAPanc-28 cells, IL-1β treatment increased ST3Gal III, ST3Gal IV, FUT5, FUT6 and FUT7 mRNA levels at 4 and 24 h, except for ST3Gal IV whose increase was detected only at 4 h. The highest transcript increases were found for ST3Gal IV and FUT6 genes. In agreement with

our results, IL-1β stimulated hepatocellular carcinoma cells also increased their levels of SLe^x antigen, as well as the ST3Gal IV (at 4 h of treatment) and FUT6 (late in time) gene transcription (Azuma *et al.*, 2000; Higai *et al.*, 2006b).

Likewise, IL-6 and TNFα caused an increase of SLe^x in MDAPanc-3 cell membrane, likely explained by enhanced expression of ST3Gal III and ST3Gal IV genes along time course. FUT6 gene, which is constitutively expressed at much higher levels than other FucT genes (FUT3, FUT5 and FUT7) in this cell line, also increased the expression after either IL-6 or TNFα treatments. Thus, in MDAPanc-3 cells the higher expression of ST3Gal III, ST3Gal IV and FUT6 may account for the higher SLe^x levels at the cell membrane, in accordance with other authors: the treatment of lung cancer cells with TNFα enhanced SLe^x membrane levels (Kuninaka *et al.*, 2000) as well as ST3Gal IV and FUT3 genes expression (Ishibashi *et al.*, 2005). A moderate transcriptional up-regulation of ST3Gal III and FUT3 mRNA by TNFα was also demonstrated in cells derived from human tracheal glands (Delmotte *et al.*, 2001), and of ST3Gal III in human colon cells (Higai *et al.*, 2006a). An increase in SLe^x and 6-sulfo-SLe^x epitopes in human bronchial mucosa tissues, specially located in mucins, was observed after IL-6 or IL-8 treatments, what was mainly explained by the enhanced expression of ST3Gal VI, FUT3 and FUT11 genes (Groux-Degroote *et al.*, 2008).

Although little information exists regarding the regulation of sialyltransferases and fucosyltransferases expression, the analyses of the promoter regions of ST3Gal III and ST3Gal IV reveal the presence of consensus sites for NF-κB and AP-1 transcription factors (Colomb *et al.*, 2012; Taniguchi *et al.*, 2003c). Colomb *et al.* (2012) identified in the upstream regulatory region of the ST3Gal IV BX transcript expressed in lung cells several consensus sites for NF-kB and AP-1, and described that TNFα controls the activity of this putative promoter region. Regarding FUT6 promoter analysis, no consensus sequences for NF-κB or AP-1 could be detected (Higai *et al.*, 2008). Interestingly, Colomb *et al.* (2012) demonstrated that FUT6 gene expression is regulated by Hepatocyte Nuclear Factor-4α (HNF-4α) and Oct-1 transcription factors in HuH-7 hepatocarcinoma cells, and that HNF-4α promoter activity can be affected by Chicken Ovalbumin Upstream Promoter Transcription Factors (COUP-TFs), whose promoter just contains NF-kB and AP-1 binding sites.

In MDAPanc-3 cells the pro-inflammatory cytokine stimuli also resulted in enhanced levels of Le^y epitope at the cell membrane. FUT1 mRNA was up-regulated after IL-6 and TNFα treatments along time; whereas increase in FUT2 was only detected at 24 h after TNFα treatment. Similarly, a positive regulation for FUT1 and FUT2 genes was already described in MKN45 gastric cancer cells after ILβ-1 treatment, while IL-6 only activated FUT1 expression (Padro *et al.*, 2011). Interestingly, FUT1 and FUT2 genes are located at the same chromosome cluster (19q13.3) (Rouquier *et al.*, 1995), what

could explain a comparable gene regulation. Recently, Taniuchi *et al.* (2013) described that FUT1 promoter contains several putative binding sites for transcription factors such as Elk-1, c-Rel, NF-κB, AREB6 and CREB, and demonstrated that FUT1 is transcriptionally regulated by Elk-1 in colon cancer cells.

α2,6-sialic acid levels

A significant increment in α2,6-sialic acid levels was observed in MDAPanc-28 cell membrane after IL-1β treatment, what can be explained by the enhanced expression of ST6Gal I mRNA levels at 24 h stimuli. The genomic segment 5'-adjacent to the transcription initiation point of ST6Gal I is described to contain two potential NF-κB/Rel binding sites (Lo and Lau, 1996; Wang *et al.*, 1993), hence IL-1β may possibly modulate ST6Gal I gene expression in these cells. Accordingly to our results, TNFα and IL-1β treatments also increased ST6Gal I mRNA levels and sialyltransferase activity in human endothelial cells (Hanasaki *et al.*, 1994).

The expression of fucosyl- and sialyltransferase genes is tissue-, cell type- and stage-specific dependent and varies during malignant transformation (Harduin-Lepers *et al.*, 2005; Ma *et al.*, 2006), contributing to the intrinsic heterogeneity of tumours and leading to the synthesis of tumour-associated carbohydrate antigens (TACAs), such as SLe^x. Our results show that the pro-inflammatory cytokines present in the tumour microenvironment can act as a driving force in accelerating the tumour progression, by up-regulating several tumour promoting genes, which may include some fucosyl- and sialyltransferase genes, as schematized in **Figure 21**.

Not surprisingly, several authors have linked the results of cytokine treatments to enhanced motility rates of the tumour cells. For example, colon carcinoma cell lines showed higher adhesion to E-selectin via SLe^x and SLe^a epitopes after TNFα stimulation (Majuri *et al.*, 1995), as well as prostate cancer cells, which in turn had enhanced motility and invasiveness rates (Radhakrishnan *et al.*, 2011). Furthermore, cytokines have also been described to modulate the adhesion of PDAC cells to ECM proteins (Stefani *et al.*, 1999). In that sense, and since cytokines can modify the cell glycosylation, it is plausible to hypothesize that the pro-inflammatory microambient may help to modulate the adhesive and invasive phenotype of PDAC cells, through changes in the pattern of sialylation on specific glycopoteins of the cell membrane.

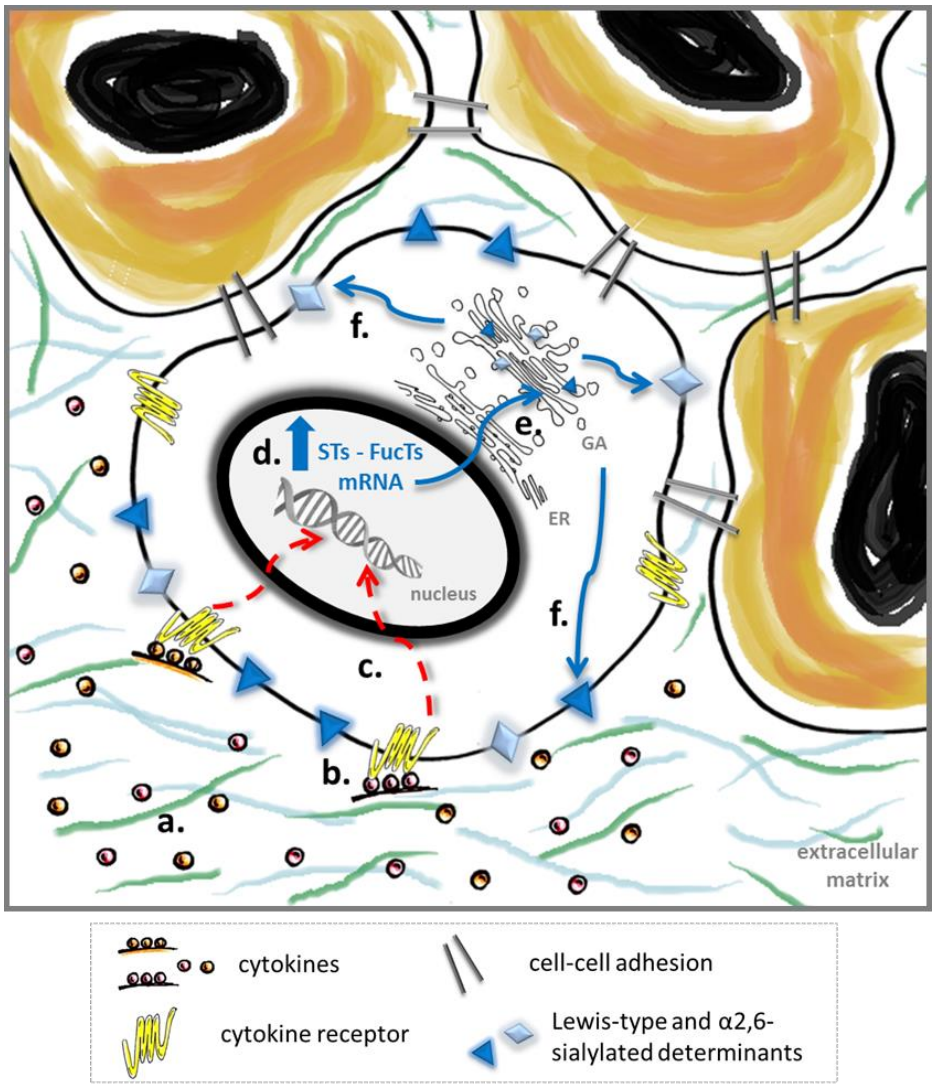

Figure 21. **Proposed model for the effect of pro-inflammatory cytokines on PDAC cell sialylation.**
a| PDAC cells with high concentrations of specific cytokines in the medium. **b|** Cytokines bind to their respective receptors at the cell membrane. **c|** Binding of cytokines to their receptors triggers intracellular pathways leading to the activation of transcription factors, such as NF-kβ. **d|** Activated transcription factors regulate the expression of several ST and FucT genes, increasing their mRNA transcription. **e|** Translation from mRNA to protein at the ER contributes to higher ST and FucT activity. **f|** Glycoconjugates show higher levels of Lewis-type and/or 2,6-sialylated determinants.

ER: endoplasmic reticulum; GA: Golgi apparatus; NF-kβ: nuclear factor kappa β; ST: sialyltransferase; FucT: fucosyltransferase.

Inflammation and Lewis-type antigens expression in PDAC tissues

The expression of sialylated epitopes was analyzed in human PDAC tissues with different grade of inflammation to find a possible correlation between the inflammed tissue grade and the level of sialylated Lewis antigens. SLe^x and SLe^a expression was in the high and moderate inflamed tissues, as well as in some poor inflamed tissues. In contrast, Le^y expression, which is not so generally expressed in PDAC, was found in both high and low inflamed tissues. The broad expression of SLe^x and SLe^a

correlates with the reported high expression tendency of α2,3-STs ST3Gal III and ST3Gal IV and of α1,3/4-FucTs FUT3 and FUT6 genes in PDAC tissues compared to control tissues (Perez-Garay *et al.*, 2013). Accordingly, the MDAPanc-3 and MDAPanc-28 PDAC cell lines treated with cytokines had also up-regulated ST3Gal III, ST3Gal IV and FUT6 mRNA expression; but FUT3 transcript levels, which is poorly expressed in these cell lines, did not show changes of expression after treatments.

The influence of inflammation in SLe^x biosynthesis has been reported in several serum acute-phase proteins. As for instance, haptoglobin, fetuin, α1-antitrypsin and transferrin showed an increase in their SLe^x content in chronic pancreatitis patients compared to control patients, while SLe^x in α1-acid-glycoprotein (AGP) was increased in chronic pancreatitis and advanced PDAC patients compared to control patients (Sarrats *et al.*, 2010b).

This Doctoral Thesis highlights the involvement of **sialylated determinants** such as SLe^x in the biology and progression of PDAC, modulating important **cellular adhesion and invasion events** via the regulation of cell adhesion molecules such as integrins and E-cadherin. This study also reinforces the theory that pancreatic tumour takes advantage of its **cancer-associated inflammation microenvironment**, which is known to play an important role in pancreatic cancer tumourigenesis, leading to the biosynthesis of the sialylated epitopes involved in PDAC progression.

Altogether, the herein proposed models open new avenues to design strategies which could target ST3Gal III enzyme and/or SLe^x epitope, or that could interrupt the inflammatory status, to improve the poor outcome of PDAC.

Conclusions

The following conclusions can be extracted from the results presented along this Doctoral Thesis:

1. Cell surface sialic acid content varies along with cell density in PDAC cell lines; however their variation is different in every cell model. SLe^x expression is enhanced in both MDAPanc-28 and Capan-1 cell lines at increasing cell density, whereas in MDAPanc-28 cells α2,6-sialic acid levels decrease significantly and α2,3-sialics remain constant. In contrast, Capan-1 cells at increasing cell density enhance both α2,3- and α2,6-sialic acid levels.

2. A higher proportion of cell surface α2,6-sialic levels in Capan-1 and MDAPanc-28 cells favours PDAC cell adhesion to type 1 collagen. In general, α2,3-sialic acid and SLe^x levels positively correlate with cell migration through collagen, whereas α2,6-sialic acid levels negatively correlate with this process. α2,3-sialic acid and SLe^x levels have also a positive relation with the invasion capacity of the cells, whereas they correlate negatively with the aggregation capacity.

3. The ST3Gal III transfected cell lines of Capan-1 and MDAPanc-28, C31 and M34 respectively, increase SLe^x expression and decrease α2,6-sialic acid levels compared to the control lines. The changes in sialylation account for the lower adhesion to type 1 collagen, fibronectin and laminin of C31, as well as for the higher invasive potential and lower cell-cell aggregation rates, and are not explained by changes in the expression levels of α2β1 integrin or E-cadherin molecules.

4. The sialylation of α2β1 integrin and E-cadherin molecules is modified as a result of the ST3Gal III transfection, and reflects the differences in cell surface and general sialylation pattern. SLe^x epitope modulates the biological function of α2β1 integrin molecule, shown by changes in the intracellular pathway derived from integrin-collagen binding, as well as the adhesive properties of E-cadherin, affecting the morphology of cell-cell contacts.

5. *De novo* expression of SLe^x is found in PDAC human tissues, especially at the ductal lumen and in foci of tumour cells. A potential interface between E-cadherin or α2 integrin subunit and SLe^x molecules is present in some tumour areas of PDAC tissues, with zones of colocalization.

6. The treatment of PDAC cells with pro-inflammatory cytokines modulates the expression of SLe^x and related epitopes at the cell membrane, through the regulation of specific glycosyltransferase genes expression. More specifically, IL-1β treatments enhance ST3Gal III, ST3Gal IV, ST6Gal I, FUT5, FUT6 and FUT7 mRNA levels along time-course, with a concomitant cell membrane SLe^x and α2,6-sialic acid overexpression in MDAPanc-28 cells. Similarly, in MDAPanc-3 cells the SLe^x and Le^y cell membrane levels are enhanced after IL-6 or TNFα treatments due to the overexpression of ST3Gal III, ST3Gal IV, FUT1, FUT2 and FUT6 genes.

7. The sialylated SLe^a and SLe^x epitopes tend to be predominantly expressed in human inflammed PDAC tissues, while the neutral Le^y epitope is not so widely expressed in PDAC tissues and tend to predominate in low inflamed tissues.

References

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