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Review

The extended ppGalNAc-T family and their functional involvement in the metastatic cascade

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Summary. O-linked glycosylation of proteins begins with the attachment of a single N-acetylgalactosamine (GalNAc) residue to a serine or threonine residue of the polypeptide and glycosylation of proteins can dramatically change their properties, interactions and activities. This initial attachment is catalysed by members of a family of 20 isoenzymes, the UDP-N-α-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferases or ppGalNAc-Ts. Why such a large family of isoenzymes are required to perform, apparently, a single function has been the subject of intense interest. The ppGalNAc-Ts, in fact, have overlapping, but distinct, substrate specificities and are differentially expressed in different cells and tissues and under different conditions of differentiation and development, allowing subtle and complex control of cellular glycosylation. Intriguingly, there is a growing body of evidence showing that altered expression of members of this transferase family are a common feature of many types of cancer and, crucially, that the resulting aberrant glycosylation has functional effects. Here, we review what is known of the expression and distribution of these intriguing transferases in health and in malignancy and, for the first time, bring together what is known of the functional and molecular effects of their disregulation in each step of the complex cascade of cancer metastasis.

Key words: ppGalNAc-T, Metastasis, Cancer, O-linked glycosylation, *Helix pomatia* agglutinin (HPA)

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Introduction

UDP-N-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferases (aliases include: ppGalNAc-Ts, polypeptide GalNAc transferases, polypeptide GalNAc transferase protein-UDPs, Pp-GaNTases, GALNAC-Ts, *GALNTs*, GalNAc Ts and GalNAcTs), are a family of 20 enzymes, all of which catalyse the addition of the first N-acetylgalactosamine (GalNAc) monosaccharide to serine (Ser) and threonine (Thr) residues of polypeptides at the initiation of O-linked mucin-type glycosylation. There are many documented examples of aberrant O-linked glycosylation associated with cancer progression. Most interestingly, disregulation of ppGalNAc-Ts in a number of epithelial cancers have been shown to be important in the different stages of the metastatic cascade; exploration of their potential role in mechanisms of metastasis is the primary focus of this review.

Metastasis

Metastasis is the dissemination of cancer cells from a primary tumour forming secondary lesions at distant anatomical sites. Metastasis is a significant clinical problem as it is responsible for the majority of cancer related deaths owing to the fact that metastatic disease is poorly responsive to chemotherapeutic drugs and is often difficult to excise surgically - for review see Brooks et al. (2010).

Although metastasis is a multifaceted molecular process, it is often easier to consider the progression towards a secondary tumour as a cascade of several distinct events, which in reality are heavily

interconnected. The steps in the cascade have been defined as follows, and are illustrated in Fig. 1: (1) angiogenesis, the formation of a tumoural blood supply; (2) disaggregation of cancer cells from the primary tumour mass; (3) cancer cell invasion of the surrounding basement membrane (BM) and extracellular matrix (ECM); (4) cancer cell intravasation of local blood vessels; (5) hematogenous spread of the cancer cell and (6) their adhesion to the endothelial lining of the blood vessels; (7) their extravasation through the BM and ECM of the blood vessels and finally (8) development of a secondary tumour at a new site. In order for cancer cells to be able to metastasise the entire cascade must be completed successfully, and it is widely accepted that most cancer cells are unable to do this (Fidler, 1970; Luzzi et al., 1998; Wong et al., 2001). For example, as few as <0.01% of cancer cells which successfully enter the blood circulation go on to form secondary tumours (Liotta and Kohn, 2001).

There are several theories as to why the small numbers of cancer cells which do establish secondary tumours are able to do so successfully. One such idea arises from the inherent genetic instability of cancer cells - as the tumour progresses, single cells acquire characteristics which leads to them being more metastatically competent (Fidler, 2003). More recently the paradigm has been more focused on the idea of gene signatures associated with metastatic ability (Harrell et al., 2012; Landermaine et al., 2008) as well as the idea of cancer stem cells (reviewed in Reya et al., 2001).

O-linked glycosylation: exposure of GalNAc

It is widely accepted that cancer cells undergo many genetic changes during the process of becoming metastatic and that some of these genetic changes result in phenotypic alterations which aid in the ability of a cancer cell to metastasise. One such example of this is the exposure of N-acetylgalactomsamine (GalNAc) residues in O-linked mucin-type glycosylation. O-linked glycosylation is a post-translational event in which monosaccharides are attached to proteins. As shown in Fig. 2., O-linked glycosylation begins with the addition of the first monosaccharide, a GalNAc, to an oxygen molecule (hence the term O-linked) of a Ser or Thr amino acid. O-glycosylation is catalyzed by the enzyme family UDP-N-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts). This initial GalNAc residue is then always further extended by the action of other glycosyltransferases in healthy cells and is therefore normally cryptic. For review of O-glycosylation and ppGalNAc-Ts see Bennett et al. (2012); Brooks et al. (2008) and Raman et al. (2012).

Leathem and Brooks (1987) first reported that binding of the GalNAc-recognising lectin *Helix pomatia* agglutinin (HPA) to the cancer cells of clinical tumour samples, demonstrated using immunohistochemistry, correlated with metastatic ability and consequent poor

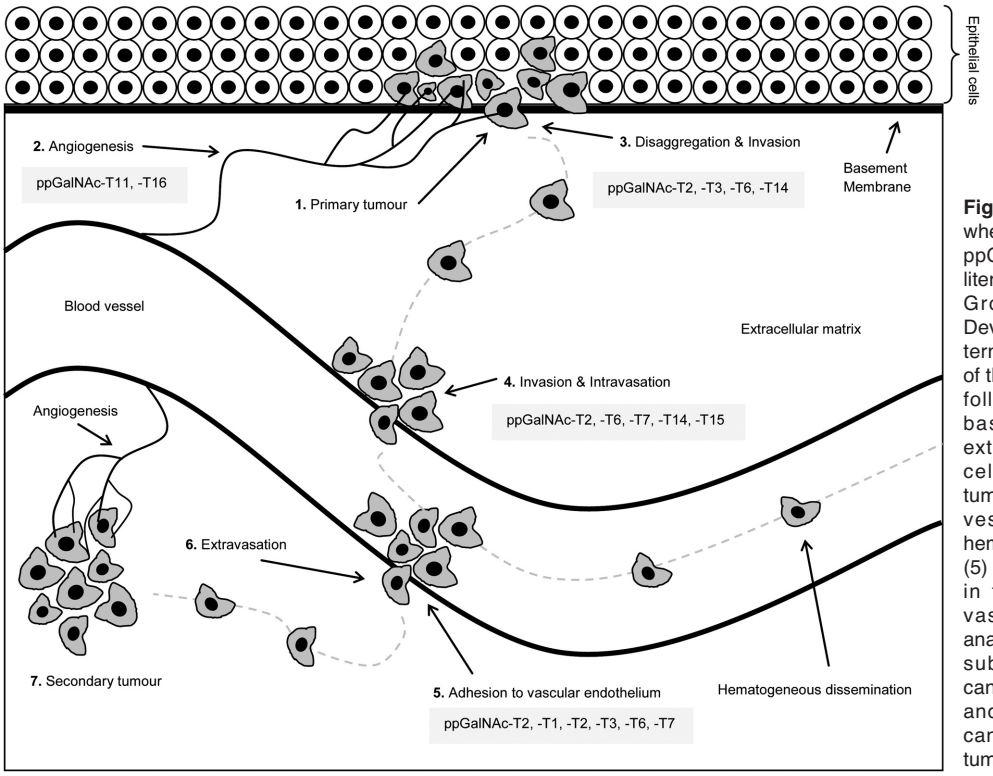

Fig. 1. The metastatic cascade, detailing where in the cascade deregulation of the ppGalNAc-Ts have been reported in the literature - indicated by the grey boxes. (1) Growth of a primary tumour. (2) Development of a tumoural blood supply, termed angiogenesis. (3) Disaggregation of the cancer cells from the primary tumour followed by invasion through the local basement membrane (BM) and extracellular matrix (ECM). (4) Cancer cells invade the ECM and BM of the tumoural blood supply and into the blood vessel lumen (intravasation) where hematogeneous dissemination can occur. (5) Cancer cells arrest at a particular point in the circulation and adhere to the vascular endothelium in a process analogous to leukocyte rolling capture and subsequent firm adhesion (6) Finally cancer cells extravasate through the BM and ECM at a secondary site. (7) The cancer cells colonise and a secondary tumour forms at a new site.

patient prognosis in breast cancer (Brooks and Leathem, 1991). Later, Brooks et al. (1993) demonstrated, again using imunohistochemistry, an association between HPA binding to exposed GalNAc-O-Ser/Thr, also termed the Tn epitope, and tumour spread to local lymph nodes, a physical indication of metastatic competence. Since then, numerous immunohistochemical studies have concurred that HPA binding, and hence exposure of GalNAc, is associated with metastatic ability and poor prognosis in breast cancer (Fenlon et al., 1987; Fukutomi et al., 1989; Alam et al., 1990; Thomas et al., 1993) and adenocarcinomas at other sites, including lung (Thöm et al., 2007), prostate (Shiraishi et al., 1992), colorectum (Schumacher et al., 1994, 1995), thyroid (Parameswaran et al., 2011) and stomach (Kakeji et al., 1991) - for a review see Brooks (2000). The unusual glycosylation recognised by HPA (the Tn epitope), has also been considered as a possible target for anti-tumour therapies (Brooks et al., 2008). However, Brooks and Leathem (1995) and Brooks et al. (2001), using both lectin/immunohistochemistry and Western blotting demonstrated that HPA recognises not only Tn but also a more heterogeneous array of glycan structures with terminal GalNAc, and that this unusual glycosylation is a feature of a heterogeneous array of different cellular glycoproteins. The functional role of the exposed GalNAc glycans in metastatic mechanisms remain uncharacterised (Brooks and Hall, 2002). Moreover, the molecular mechanisms underlying this aberrant glycosylation has not been completely defined; however it is assumed that it is a result of disruption of glycosylation enzymes, including, critically, the ppGalNAc-Ts.

ppGalNAc-Ts

It is thought that all of the ppGalNAc-T gene family, except for ppGalNAc-T4, arose from an ancestral ppGalNAc-T gene and that throughout evolution various paralogs formed. Fig. 3. depicts the proposed phylogenetic tree for the ppGalNAc-T gene family based on sub-family clusters. To date, 20 ppGalNAc-T isozymes have been identified and 17 of these characterised functionally in humans. ppGalNAc-Ts are located throughout the Golgi apparatus. All of the ppGalNAc-Ts except for -T20 share a type II membrane

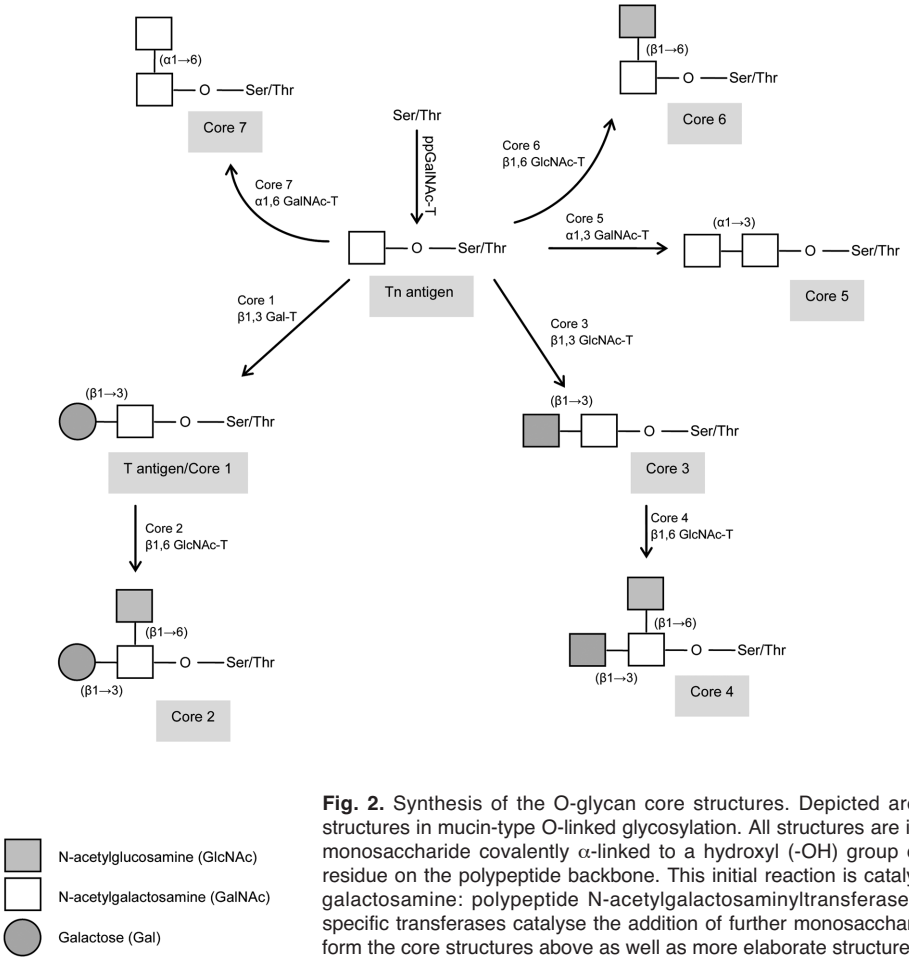

Fig. 2. Synthesis of the O-glycan core structures. Depicted are seven of the most commonly occurring core structures in mucin-type O-linked glycosylation. All structures are initiated with an N-acetylgalactosamine (GalNAc) monosaccharide covalently α-linked to a hydroxyl (-OH) group of a serine (Ser) or threonine (Thr) amino acid residue on the polypeptide backbone. This initial reaction is catalysed by a family of enzymes called UDP-N-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts). As many as up to 30 other specific transferases catalyse the addition of further monosaccharides to the structures in a sequential manner to form the core structures above as well as more elaborate structures.

structure composed of a short N-terminal cytoplasmic tail, a hydrophobic membrane spanning domain, a stem region (90-470 amino acids in length), a luminal catalytic domain (~230 amino acids long) and, unique to the ppGalNAc-Ts, a C-terminal ricin-like lectin domain (~120 amino acids in length) which has a binding affinity for α-GalNAc monosaccharides. This common structure is illustrated in Fig. 4. ppGalNAc-T20 does not have the lectin domain but does share the other structural components of this family. Several of the isoenzymes are expressed ubiquitously throughout the body: ppGalNAc-T1 (White et al., 1995), -T2 (White et al., 1995), -T4 (Bennett et al., 1998), -T5, -T7 (Bennett et al., 1999a), -T8 (White et al., 2000), -T10 (Cheng et al., 2002), -T14 (Wang et al., 2003), -T15 (Cheng et al., 2004) and -T18 (Raman et al., 2012). Whilst some are highly regulated and have only been described in specific organs: for example, -T3 in pancreas and testis (Bennett et al., 1996), -T6 in placenta and trachea (Bennett et al., 1999b), -T9 in brain and spinal cord (Toba et al., 2000), -T11 in kidneys (Schwientek et al., 2002), -T12 in digestive organs including the stomach, small intestine and in the colon (Guo et al., 2002), -T13 in neurons (Zhang et al., 2003), -T16 in heart (Peng et al., 2010), -T17 in brain and testis (Raman et al., 2012), -T19 in cerebellum and cerebral cortex (Nakamura et al., 2005) and -T20 in testis (Raman et al., 2012) - see Table 1 for an overview of the enzyme nomenclature and gene location. The ppGalNAc-Ts show different, albeit partly overlapping, substrate specificities, catalytic activities, genomic organisation and are expressed differentially in cells and tissues, displaying incomplete functional redundancy, posing the question why have so many enzymes seemingly performing the same function?

Aberrant glycosylation, ppGalNAc-Ts and cancer

It is this oddity of the existence of such a large family of related transferases that leads us to believe that disregulation of the ppGalNAc-T genes may be causing the increased exposure of GalNAc (Tn epitope and other structures with terminal GalNAc) seen in the aggressive epithelial cancers and therefore may play a role in the ability of those cancers to metastasise. Altered expression of ppGalNAc-Ts, investigated using approaches including immunohistochemistry and molecular approaches, has been reported to be useful as a prognostic marker of metastatic disease in a number of cancers including; colorectal (Kohsaki et al., 2000; Shibao et al., 2002; Guo et al., 2004; Koyanagi et al., 2008; Guda et al., 2009; Gray-McGuire et al., 2010; Abuli et al., 2011; Clarke et al., 2012), gastric (Onitsuka et al., 2003; Gao et al., 2013), breast (Cavallo et al., 2005; Berois et al., 2006; Freire et al., 2006; Patani et al., 2008; Wu et al., 2010;), neuroblastoma (Berois et al., 2006, 2013), pancreatic (Li et al., 2011), gallbladder (Miyahara et al., 2004), prostate (Landers et al., 2005), bladder (Ding et al., 2012), lung adenocarcinoma (Gu et al., 2004), oesophageal cancer (Ishikawa et al., 2005) and lymphoma (Gibson et al., 2012). This therefore reinforces the notion that ppGalNAc-Ts may be involved in mechanisms underlying the metastatic process. Furthermore, there are many documented examples of aberrant O-linked glycosylation associated with cancer progression. Most interestingly, disregulation of ppGalNAc-Ts in a number of epithelial cancers have been shown to be important at different stages of the metastatic cascade, as described in the following section, and illustrated in Fig. 1.

ppGalNAc-Ts in the metastatic cascade

Angiogenesis

For tumours to grow any larger than 2 mm in diameter a tumoural blood supply from pre-existing vasculature must be formed in a process termed angiogenesis (Duffy, 1996). In cancer, the normally fine balance of angio-inhibitory and pro-angiogenic factors are tipped in favour of uncontrolled endothelial cell

Fig. 3. Phylogenetic tree depicting the evolutionary divergence of the ppGalNAc-T family from the hypothesised ancestral ppGalNAc-T gene (adapted from Bennett et al. (2012)).

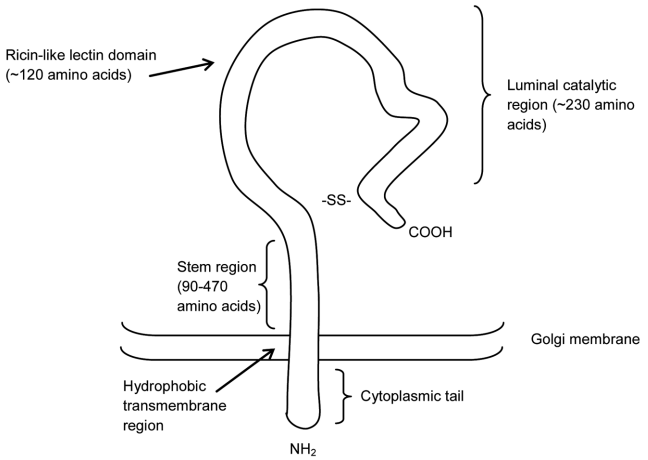

Fig. 4. Schematic representation of the shared type II membrane structure of the ppGalNAc-Ts, located in Golgi apparatus. The luminal portion consists of a catalytic domain, a ricin-like lectin domain and a variable length stem region. They are held in place by a hydrophobic transmembrane domain followed by a short N-terminal tail.

proliferation, migration and cell differentiation (Liotta et al., 1991). The degree of vascularisation of a tumour is a significant clinical prognostic indicator, associated with more advanced disease and poor prognosis. Measures of angiogenesis are therefore important. Lectin/immunohistochemistry to detect a number of markers of endothelial cells, for example *Ulex europaeus* lectin, von Willebrand factor (vWF), and the ABH blood group antigens have been widely used (Craft and Harris, 1994). Furthermore, cancer cells which have a 'plastic' phenotype have been shown to gain access to a blood supply through a process termed vasculogenic mimicry, where cancer cells have the ability to mimic endothelial cell functions allowing the tumour cells to replicate blood vessel-like structures therefore creating a tumoural blood supply (for a review see Hendrix et al., 2003). Maniotis et al. (1999) elegantly demonstrated this process using light and transmission electron microscopy (TEM) in human uveal and cutaneous melanoma cancer cells and showed that more aggressive "stem-cell-like" cells have the ability to construct vascular channels to allow blood perfusion to the primary tumour.

Herr et al. (2008) showed that ppGalNAc-T16 (also known as ppGalNAc-TL1) regulates transforming growth factor ß (TGF-ß), a pro-angiogeneic factor through interference with binding of ActR-IIB, a common TGF-ß type II receptor. It is therefore possible that differential expression of ppGalNAc-T16 could alter the balance of angio-inhibitory and pro-angiogeneic factors aiding conditions for tumoural blood supply from pre-existing vessels. Tian et al. (2007) demonstrated that mutations in ppGalNAc-T11 (*Drosophila* ortholog

Table 1. Members of the human ppGalNAc-T family, which add the first GalNAc monosaccharide in O-linked glycosylation.

ppGalNac-T	Alternative Names	Accession Number (Human)	Chromosome locus	Reference (Human)
1		X85018	18q12.1	White et al., 1995
2		X85019	1q41-q42	White et al., 1995
3		X92689	2q24-q31	Bennett et al., 1996
4	POC1B	Y08564	12q21.33	Bennett et al., 1998
5		AJ245539	2q24.1	Bennett et al., 1999b
6		Y08565	12q13	Bennett et al., 1999a
7		AJ002744	4q34.1	Bennett et al., 1999b
8		AJ271385	12p13.3	White et al., 2000
9		AB040672	12q24.33	Toba et al., 2000
10		AJ505950	5q33.2	Cheng et al., 2002
11		Y12434	7q36.1	Schwientek et al., 2002
12		AJ132365	9q22.33	Guo et al., 2002
13		AJ505991	2q24.1	Zhang et al., 2003
14	FLJ12691	Y09324	2q23.1	Wang et al., 2003
15	ppGalNAc-TL2, ppGalNAc-T13, ppGalNAc-T7, PIH5	NM_054110	3q25.1	Cheng et al., 2004
16	ppGalNAc-TL1, KIAA1130	AJ505951	14q24.1	Peng et al., 2010
17	ppGalNAc-TL6, ppGalNAc-T20	AJ626725	4q34.1	Raman et al 2012
18	ppGalNAc-TL4, ppGalNAc-T15, MGC71806	AJ626724	11p15.3	Raman et al., 2012
19	ppGalNAc-TL3, ppGalNAc-T16, ppGalNAc-T20, WBSCR17	AJ626726	7q11.23	Nakamura et al., 2005
20	ppGalNAc-TL5, ppGalNAc-T15	NM_145292	7q36.1	Raman et al., 2012

Information adapted from Bennett et al. (2012).

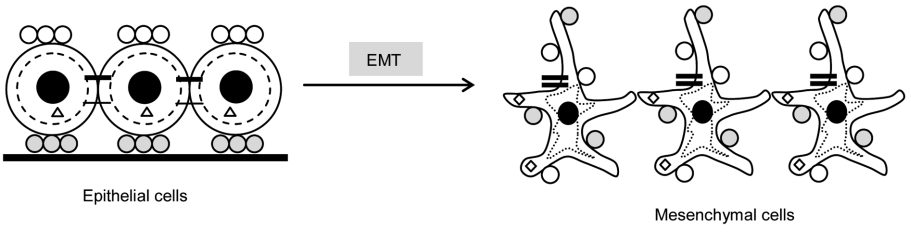

Fig. 5. Epithelial-to-mesenchymal transition (EMT) is the process by which epithelial cells transdifferentiate into mesenchymal cells. During EMT, epithelial cells down-regulate cell-cell adhesion structures (thick line) such as: gap junctions, tight junctions and adherins junctions. Epithelial-specific cell adhesion molecule E-cadherin (thin line) and integrins are replaced by extracellular specific N-cadherin (double thick lines) and integrins. Loss of cell-cell adhesion alters the cell polarity from apico-basal to end-end, changing the cell morphology from cuboidal to spindle-shaped. This change in cell polarity allows apical (clear circles) and basolateral (shaded circles) membrane components to merge. The actin cytoskeleton (triangles) is transformed into stress fibres (diamonds), which accumulate at cell protrusions. Epithelial intermediate filaments, the cytokeratins (dashed line) are replaced by vimentin (dotted line), which is specific to mesenchymal cells. The basement membrane (thick black line) is dissolved allowing the newly formed mesenchymal cells then invade the surrounding stroma. (Figure adapted from Micalizzi et al. (2010)).

pgant35a) gene results in irregular embryonic tracheal tube formation in *Drosophila*, this work poses interesting implications to disregulation or mutation of ppGalNAc-T11 in mammalian epithelial tube formation, for example aiding in tumoural vasculature mimicry.

Disaggregation

Once a blood supply has been established, the next step in the cascade is disaggregation of cancer cells from the primary tumour mass. This involves the loss of cell-cell adhesion through changes in expression of cell adhesion molecules such as cadherins and catenins. Behrens et al. (1993) used a v-SRC temperature sensitive MDCK epithelial cell line to illustrate how E-cadherin and ß-catenin cell adhesion molecules become activated through phosphorylation when cells are in a mesenchymal state compared to that of an epithelial state. Mesenchymal cells with greater phosphorylated E-cadherin and ß-catenin were also consequently more invasive.

Epithelial-mesenchymal transition (EMT) is the transdifferentiation of epithelial cells from an epithelial phenotype which are not motile and provide structural integrity, to a mesenchymal cell phenotype which appear unspecialised in form and which are highly motile and invasive, as depicted in Fig. 5. EMT and its reciprocal process mesenchymal-epithelial transition (MET) are key processes during normal foetal development and whilst the process is normally quiescent in the adult, EMT does occur during wound healing and, critically, in cancer. It is presumed that EMT in cancer is a result of switching-on of normally tightly regulated developmental signalling pathways. EMT is characterised by several phenotypic features including loss of apical-basal cell polarity, as seen in epithelial cells, to the end-end polarity of mesenchymal cells. Polarity is lost due to the dissolution of tight junctions which allows intermingling of apical and basolateral membrane components. Additional cell adhesion structures such as gap junctions and adherens junctions are removed. Cell surface proteins, including E-cadherin and cell surface specific integrins - which mediate cell-cell connections and cell-BM adhesion, respectively - are exchanged for N-cadherin and extracellular specific integrins which impart a more transient adhesion (Klymkowsky and Savagner, 2009). The actin cytoskeleton of epithelial cells is re-organised and replaced by stress fibres which collect at cell protrusions, whilst cytokeratin intermediate filaments are exchanged for vimentin. Changes to the cytoskeleton of the cell alters the cell morphology from cuboidal to spindle-shaped. Finally, the underlying basement membrane is degraded, and the cell acquires the ability to move and invade the ECM and surrounding stroma. In the process of becoming mesenchymal, the cell also gains resistance to a specific type of programmed cell death termed anoikis "the state of being without a home", where cells which lose cell-cell adhesion with one another are destroyed. Cells also begin to respond to extracellular signals leading them to migrate to specific destinations (Micalizzi et al., 2010). Damonte et al. (2007) demonstrated in a mouse model that tumours which have a spindle-cell phenotype, which most commonly mestastasise have undergone EMT or are in the process of doing so. This was shown using immunohistochemisty to label E-cadherin and vimentin (to distinguish between epithelial cells and mesenchymal cells, respectively) where in most cases both markers were present on the spindle cells. Freire-de-Lima et al. (2011) demonstrated that down regulation of ppGalNAc-T6 and -T3 in prostate cancer cells inhibited the effect of TGF-ß on oncofetal fibronectin, a well used mesenchymal marker that is known to be up-regulated in the EMT process. Maupin et al. (2010) showed that in EMT-induced pancreatic cancer cell lines ppGalNAc-T3 expression and protein levels are increased, suggesting a potential role for ppGalNAc-T3 in the EMT process.

Matrix metalloproteinases (MMPs) are a family of proteases which have the ability to degrade almost all of the BM and ECM components and their expression is generally enhanced during tumoural invasion. MMP-2 has been shown to be prolific in invading tumours. TGF-ß is a cytokine which is a key player in the ability of cancer cells to metastasise as it both promotes tumour migration and improves cell survival therefore helping in

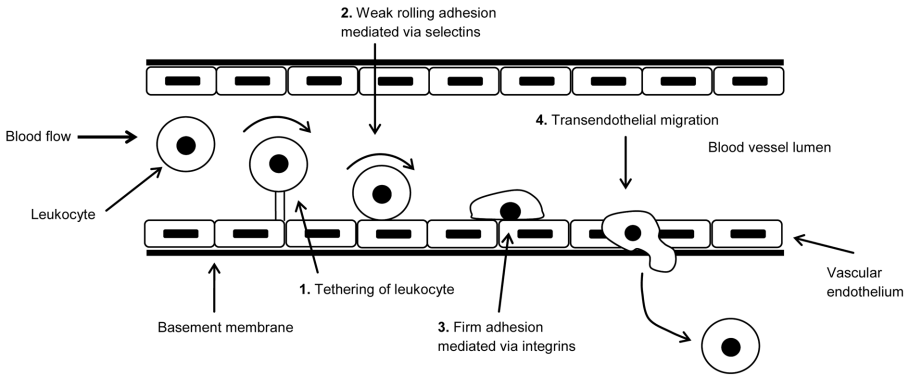

Fig. 6. The process of leukocyte recruitment. (1) Tethering and rolling of leukocytes to vascular endothelium mediated via an inflammatory stimulus. (2) Weak rolling adhesion of leukocytes along vascular endothelium mediated by selectins. (3) Firm adhesion of leukocytes to vascular endothelium mediated via integrins. (4) The leukocyte passes through the blood vessel endothelial cells in a process termed transendothelial migration which is mediated mainly by integrins. (Figure adapted from Brooks et al. (2010)).

tumour cell invasion. Hua et al. (2012) demonstrated that down-regulation of ppGalNAc-T2 in gastric carcinoma cell lines increased proliferative, adhesive and invasive characteristics by the up-regulation of both MMP-2 and TGF-ß.

Epidermal growth factor receptor (EGFR) is a member ERB family of receptor tyrosine kinases. Activation of EGFR is initiated through the binding of its cognate ligand, epidermal growth factor (EGF) and subsequent dimerization of EGFRs leads to numerous down-stream signalling cascades controlling cell migration, adhesion and proliferation. Wu et al. (2011) reported that ppGalNAc-T2 is a critical mediator of malignancy in hepatocellular carcinoma cell lines. Down-regulation of ppGalNAc-T2 alters the O-glycosylation of EGFR which changes the effect of EGF binding to EGFR giving rise to increased cell migration, invasion and growth.

Insulin-like growth factor binding protein 3 (IGFBP-3) regulates the mitogenic and anti-apoptotic effects of insulin-like growth factor-1 (IGF-1), although little is known about the IGF-1 independent function of IGFBP-3 and its binding with other proteins. Wu et al. (2009, 2012) identified ppGalNAc-T14 as a novel binding partner for IGFBP-3 suggesting that ppGalNAc-T14 may play an important role in the functional regulation of IGFBP-3.

Invasion and intravasation

Cells that have become "independent" from the primary tumour mass have the capability of disaggregation and it is the enhancement of cell motility which allows the initial spread and further movement of the metastatic cancer cells. Singular cells or groups of several cancer cells migrate through the BM and ECM surrounding the tumour epithelium and then through the BM and ECM of local tumoural vasculature. The often incompletely formed and fenestrated tumoural blood vessels facilitate the entry of cancer cells into the blood stream and then hematogenous dissemination can occur. Friedl et al. (2004) have established numerous systems in which cell migration can be observed. These elegant systems include 2D and 3D models which allow the visualisation of different modes of cell migration *in vitro*. Most recently Alexander et al. (2008) from Friedl's lab have developed an assay in which intravital imaging can take place within a living test animal to film *in vivo* cell migration.

Integrins are transmemebrane receptors which act to anchor cells to the BM and ECM mediating adhesion through a specific ligand. In cancer, loss of cell attachment to the BM and ECM allows cell invasion to occur. Lotz et al. (1990) showed that down-regulation of ppGalNAc-T15 (also known as P1H5) in colon carcinoma cell lines inhibited integrin $\alpha 2\beta 1$ and $\alpha 3\beta 1$ adhesion to laminin, a major component of basal lamina of the BM, therefore allowing cancer cell invasion of the BM.

Liu et al. (2011) showed that up-regulation of ppGalNAc-T2 inhibits the ability of glioma cells to invade and the expression of MMP-9 and TGF-ß is reduced, whilst down-regulation of ppGalNAc-T2 increased the expression of MMP-2 suggesting a potential role for ppGalNAc-T2 expression in the ability of cancer cells to invade.

Park et al. (2011) proposed that fibronectin, a major constituent of the ECM, is O-glycosylated by overexpressed ppGalNAc-T6 in breast cancer cell lines resulting in invasive characteristics. Park et al. (2011) cultured MCF10A ppGalNAc-T6 expressing stable transfectants in a 3D culture system to model the structure of the mammary gland and demonstrated that the well-organised acinar structure was disrupted as a result of over-expression of ppGalNAc-T6.

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is a cytokine which acts as a ligand to pro-apoptotic receptors DR4 and DR5 stimulating cancer cell death. Wagner et al. (2007) demonstrated that a number of cancer cell lines which had over-expression of ppGalNAc-T14 showed reduced TRAIL sensitivity and so were more likely to evade cell death through TRAIL induced apoptosis. The process of TRAIL induced apoptosis in cancer is well reviewed in Thorburn et al. (2008).

microRNAs (miRs) are small non-coding RNA molecules which function in transcriptional and post-transcriptional regulation of gene expression and are frequently disregulated in cancer (Iorio and Croce, 2012). Peng et al. (2012) demonstrated that miR-214 is commonly down-regulated and ppGalNAc-T7 is frequently up-regulated in cervical cancer cells. miR-214 binds specifically to the 3'-UTR of ppGalNAc-T7, and represses the expression of ppGalNAc-T7. Inhibiting the expression of miR-214, thereby removing its repressive effects on ppGalNAc-T7, enhances proliferation, migration and invasiveness of cervical cancer cells. Furthermore, when ppGalNAc-T7 is knocked down in these cells there is a significant inhibition of cell proliferation, migration and invasion.

Adhesion to vascular endothelium

Once cancer cells have penetrated the vasculature they are spread around the circulatory system through the sheer force of blood-flow. The majority of cancer cells may not progress any further than this stage and will remain dormant in the blood flow until they die or are recognised by the immune system and destroyed. How cancer cells arrive and arrest at a potential secondary destination can be explained in a number of ways. The most straightforward is simply that they become physically trapped in the microvasculature of the next major organ down-stream. Another solution, first proposed as the 'seed and soil' hypothesis of Paget (1889), is the idea that cancer cells (the 'seeds') require a favourable environment (the 'soil') for them to flourish, and therefore that cancer cells metastasise to

specific environments in an organ-selective process (Fidler, 2003). There is strong evidence that some cancer cells, at least, bind specifically to the endothelium of a blood vessel at a new site via a process analogous to leukocyte tethering, rolling and firm adhesion, as shown in Fig. 6, using well characterised molecular mechanisms based on selectins, integrins and members of the immunoglobulin superfamily (IgSF) (Miles et al., 2008). Giavazzi et al. (1993), for example, was able to visualise and quantify cancer cells rolling, tethering and adhering to a pseudo-vascular endothelium using a flow chamber assay system.

Meyts et al. (2007) have suggested that ppGalNAc-T3, which has been found to be abundantly present in spermatozoa, may be important in mediating the adhesion of spermatozoa to the zona pellucida in sperm-egg fertilization through the glycosylation of a testis-specific protein, zonadhesin. Zonadhesin is evolutionarily related to the blood glycoprotein vWF; whose function is to bind adhesion molecules in the blood. These findings suggest a mechanism for vascular endothelial binding of maverick cancer cells by glycosylation of vWF and subsequent binding to cancer cells in the blood.

Mucin 1 (MUC1) is a transmembrane protein commonly over-expressed in a number of epithelial cancers, most notably breast cancer. It is an oncoprotein which contributes to carcinogenesis through interaction with EGFRs, p53, ß-catenin and IL-7. Schumacher et al. (2001) used radiolabelled chelate in conjunction with PET imaging to increase contrast when visualising MUC-1, a clinical marker of breast cancer, using anti-MUC1 antibodies in *in vivo* mouse models. Park et al. (2010) have demonstrated that ppGalNAc-T6 glycosylates MUC1, thereby stabilizing MUC1 and allowing downstream interactions with above-mentioned targets contributing to cell proliferation and invasion.

Interleukin-4 (IL-4) is a cytokine which stimulates naïve helper T-cells to differentiate, playing an important role in humoral immunity. Kanoh et al. (2008) showed that during inflammation of epithelial colonic cells, more aberrant mucins are stimulated to be produced by IL-4 expression, the majority of this glycosylation being performed by ppGalNAc-T2.

Taniuchi et al. (2011) demonstrated that down-regulation of ppGalNAc-T3 expression using RNA interference (RNAi), a technique that inhibits gene expression through the targeted destruction of specific mRNA molecules, suppresses growth in pancreatic cancer cells. Furthermore, ppGalNAc-T3 is commonly over-expressed in pancreatic cancer resulting in invasive growth characteristics.

Fibroblast growth factor (FGF) is a pro-angiogenic factor and encourages cell growth. Tian et al. (2012) demonstrated that mice deficient in ppGalNAc-T1 accumulated BM proteins, affecting integrin and FGF signalling as well as cell proliferation and growth. Loss of ppGalNAc-T1 specifically controls the composition of the cellular microenvironment during early organ development leading to abnormal growth. If ppGalNAc-T1 was to be down regulated in cancer its effects on cell growth and important adhesion molecules such as integrins could help in adhesion to vasculature endothelial cells and subsequent formation of secondary tumours.

Gaziel-Sovran et al. (2011) demonstrated that in melanoma cells, where miR-30b/30d are expressed at a high level, immunosuppression through reduced recruitment and immune cell activation occurs due to increased production of the cytokine IL-10. miR30b/30d acts directly on ppGalNAc-T7, by interacting with a recognition site in the 3' end of the gene. Binding leads to the high level of IL-10 production. Immuno-suppression reduces the likelihood of a migrating cancer cell being identified and targeted for destruction, thus increasing the chances of the cell surviving and completing the metastatic cascade successfully.

Block et al. (2012) demonstrated that ppGalNAc-T1 is important in attaching O-linked glycans to selectin ligands, most notably P-selectin glycoprotein ligand-1 (PSGL-1) which functions in leukocyte recruitment. In ppGalNAc-T1-deficient mice, leukocyte rolling adhesion was significantly reduced and L-selectin-dependent leukocyte rolling was completely eliminated, whilst leukocyte rolling velocity was increased thus preventing attachment of leukocytes to vascular endothelium at sites of inflammation. It has already been mentioned previously that cancer cells attach to, and migrate through, vascular endothelium in a process analogous to leukocyte adhesion and extravasation. If ppGalNAc-T1 was to be up-regulated in metastatic cancer cells, they also would be better able to adhere to the endothelium, therefore aiding in extravasation at a secondary site.

Conclusion

It is pertinent here to return to a question we asked earlier in this review: why are so many enzymes seemingly performing the same function? ppGalNAc-Ts are the largest glycosyltransferase family and catalyse a single glycosidic linkage in humans. At first consideration, the existence of 20 isoenzymes all similarly initiating O-glycosylation seems extravagant; however, on further investigation, it becomes clear that different ppGalNAc-Ts are differentially expressed within tissues, between cells within a single tissue, and in different patterns at different stages in development and differentiation. ppGalNAc-T isoenzymes have overlapping but somewhat distinct substrate specificity, as exemplified by disorders caused by their deficiency.

One example is deficiency in ppGalNAc-T3 causing a rare condition called tumoural calcinosis, which is characterised by calcium deposition in soft tissues. Interestingly, deletion of a single ppGalNAc-T, while sometimes leading to pathologies, has never been documented to be lethal in mouse models. The overlapping yet distinct functions of the ppGalNAc-T

family provide dynamic regulation of O-glycosylation which allows for the differential functional modification of proteins.

As there are so many ppGalNAc-Ts, all performing distinct but interrelated functions, it is easy to imagine that there is considerable scope for misregulation of O-glycosylation leading to favourable characteristics for disease formation. Altered expression of several ppGalNAc-Ts has repeatedly been reported to be a feature of many different types of cancer, often correlated with more aggressive disease or poor prognosis. Critically, as described in this review, there is mounting evidence of how the resulting changes in glycosylation may be functionally contributing to metastatic mechanisms through diverse pathways. These findings parallel the historic and well documented observation that lectin/immunohistochemical labelling of cancers by the GalNAc-recognising lectin HPA is associated with aggressive biological behaviour, metastasis and poor patient prognosis, and suggest myriad mechanisms by which the altered glycosylation may be functionally implicated.

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