Bisected, complex N-glycans and galectins in mouse mammary tumor progression and human breast cancer

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Introduction

The functions of complex N-glycans attached to cell surface proteins range from modulating protein stability and turnover to mediating cell–cell or cell–matrix interactions via glycan-binding proteins, including galectins (Varki et al. 2009). Galectins bind to glycans that contain β-galactosides through carbohydrate recognition domains (CRDs) (Leffler et al. 2004; Di Lella et al. 2011). Galectins are synthesized in the cytoplasm, secreted via an unconventional pathway and then bound to the glycans of cell surface glycoproteins (Seelenmeyer et al. 2005; Seelenmeyer et al. 2008). Complex N-glycans offer multiple galectin-binding sites on (poly)N-acetyllactosamine (LacNAc) units ([Galβ1,4GlcNAc]n) of their branches (Figure 1). Multivalency is a key feature of galectin binding, responsible for crosslinking multiple targets and forming a galectin–glycoprotein lattice on the cell surface (Brewer et al. 2002; Dennis and Brewer 2013). Prototypical galectins contain one CRD and typically dimerize to establish bivalency. In contrast, the tandem repeat subtype contains two distinctive CRDs per molecule and thus achieves bivalency without multimerization. Finally, the chimera type (galectin-3) forms a pentamer in the presence of ligands (Ahmad et al. 2004).

A galectin lattice has been implicated in tumor progression through modulation of growth factor signaling (Lau and Dennis 2008; Boscher et al. 2011). For example, galectin-3 is proposed to cluster glycosylated growth factor receptors on the cell surface and inhibit constitutive endocytosis, prolonging downstream signaling and thereby promoting cell proliferation and survival through enhanced extracellular signal-regulated kinase (ERK) and protein kinase B (AKT) pathways (Partridge et al. 2004; Lau et al. 2007). Galectin-1, on the other hand, contributes to immunosuppression and enhancement of angiogenesis, two key factors that promote tumor growth and metastasis (Thijssen et al. 2006; Thijssen et al. 2010; Banh et al. 2011; Dalotto-Moreno et al. 2013). In contrast, galectin-9 is believed to be antimetastatic by inhibiting tumor cell adhesion to vascular endothelium and extracellular matrices (Nobumoto et al. 2008). Therefore, it is important to determine the number of different galectins expressed in tumors in order to ascertain roles for galectins in tumor progression.
metastasis, as originally observed in the mixed genetic back- 
tumor burden, increased glucose uptake and promoted lung females promoted mammary tumor appearance, enhanced Here, we show that loss of MGA T3 in C57BL/6 MMTV-PyMT 
and N-glycan branching GlcNAc-transferase gene (MGAT3) to 
the core mannose on a complex (or hybrid) N-glycan to generate a bisected, complex N-glycan. MGAT4A, MGAT4B, MGAT5 and MGAT5B add GlcNAc to generate branched, complex N-glycans. In Chinese hamster ovary (CHO) cells a Gal residue is added to each GlcNAc residue, except the bisecting GlcNAc (North et al. 2010). Each Gal may be extended by the addition of many (poly)LacNAc units ([Galβ1,4GlcNAc]n). CHO mutant cells that express N-glycans with a bisecting GlcNAc (LEC10) exhibit reduced binding of ricin and galectin-1, -3 and -8, but increased binding of the lectin E-PHA compared with CHO cells that do not express MGAT3.

The bisecting GlcNAc is a unique N-glycan modification that occurs on complex or hybrid N-glycans following the action of β1,4-N-acetylgalcosaminyltransferase III (GlcNAcT-III or MGAT3), encoded by the MgaT3 gene (Figure 1). We have shown previously that the bisecting GlcNAc modulates plant lectin binding (Stanley et al. 1975; Campbell and Stanley 1984) and reduces the binding of galectin-1, -3 and -8 (Patnaik et al. 2006; Miwa et al. 2012). This is thought to be due to an effect of the bisecting GlcNAc on glycan conformation that reduces the accessibility of β-galactosides (Brisson and Carver 1983; Andre et al. 2004; André et al. 2007) and potentially due to a reduction in the numbers of Galβ1,4GlcNAc (LacNAc) units that are synthesized on N-glycans in the presence of the bisecting GlcNAc (North et al. 2010).

Previously, we reported the tumor suppressive roles of the bisecting GlcNAc and MGAT3 in the mouse mammary tumor virus Polyoma middle T antigen (MMTV-PyMT) model of mammary tumor formation (Song et al. 2010). Loss of MGAT3 from MMTV-PyMT tumor cells enhanced tumor progression and lung metastasis and increased growth factor signaling via ERK1/2. It remains to be determined whether the bisecting GlcNAc affects tumor growth via interactions with galectins, and if so, which galectins are most important. In this paper, we identify the galectins that are expressed in MMTV-PyMT mammary tumors and tumor cell lines and also in human breast cancers. During this work, the cohort of MMTV-PyMT females in the FVB/N and C57BL/6 (FVB/C57BL/6) mixed genetic background began to exhibit variable tumor latency in females in the FVB/N and C57BL/6 (FVB/C57BL/6) mixed background (Song et al. 2010). In addition, analysis of galectin and N-glycan branching GlcNAc-transferase gene (MGAT) expression in data reported for different breast cancer subtypes by The Cancer Genome Atlas (TCGA) (Network CGA 2012) revealed complex expression patterns, some of which are prognostic for enhanced relapse-free survival (RFS).

Results

Genetic background affects mammary tumor formation in the absence of MGAT3

We previously reported that loss of MGAT3 and the bisecting GlcNAc on complex N-glycans enhances tumor progression in the mammary glands (MG) of MgaT3−/−/MMTV-PyMT females (Song et al. 2010). In continuing studies however, we noticed that the time of tumor appearance in MG became highly variable in the FVB/C57BL/6 genetic background (Figure 2). This variability occurred in all MgaT3 genotypes, resulting in no overall difference in the timing of palpable tumor appearance in MgaT3 wild-type, heterozygotes and null females (Figure 2A–D). To reduce genetic heterogeneity, we investigated the model in a C57BL/6 background generated from MMTV-PyMT transgenic and MgaT3−/− mice that were both backcrossed to C57BL/6 >10 times. Mouse-to-mouse variability was less in the C57BL/6 background (Figure 2E–H). As observed previously (Song et al. 2010), the absence of MGAT3 accelerated tumor development and increased both tumor numbers and tumor mass at ~20 weeks (Figure 3A–C).

To determine if enhanced mammary tumor formation correlated with increased lung metastasis, lungs and lung sections from ~20-week C57BL/6 MMTV-PyMT females were examined for metastatic foci. Visible foci were rare at either the macro- or microscopic level and thus PyMT transcript levels were determined. Since MMTV-PyMT is expressed in mammary gland but not lung, the presence of PyMT transcripts in lung should reflect lung metastases. PyMT transcript levels were significantly higher in lungs from females lacking MGAT3 (Figure 3D).

Glucose uptake is increased in MgaT3−/− mammary tumors

Tumor cells increase glucose uptake and glycolysis to accommodate their rapid growth (Warburg et al. 1927; Gatenby and Gillies 2004). To determine whether MgaT3−/− mammary tumors took up more glucose than those expressing MgaT3, we used micro Positron Emission Tomography (microPET) to investigate regional glucose uptake in MMTV-PyMT-induced mammary tumors as described (Landskroner-Eiger et al. 2009). Mice of 16–17 and 19–20 weeks were analyzed after overnight fasting. Six mammary gland regions were selected as described in the Materials and methods section. Flattened three-dimensional (3D) views of representative images are shown with the six regions marked (Figure 4A). Consistent with a larger tumor burden, the maximum standardized-uptake value (SUVmax) of selected mammary gland regions of MgaT3−/− mice was significantly higher than control mice (Figure 4B). We have also analyzed the number of regions that had a SUVmax over the defined threshold value of 1 (above the whole body average) in each animal. These results showed that MgaT3−/− mice also had a larger number of mammary gland regions with high metabolic activity compared with wild-type (Figure 4C).

ERK and AKT activation were reduced in ~20-week mammary tumors lacking MGAT3

ERK and AKT activation have been implicated in cell proliferation and survival of MMTV-PyMT mammary tumor cells

Fig. 1. Relationships between N-glycans, GlcNAc-transferases and galectins. MGAT1 and MGAT2 GlcNAc-transferases generate a biantennary, complex N-glycan. MGAT3 transfers GlcNAc from uridine diphosphate (UDP)-GlcNAc to the core mannose on a complex (or hybrid) N-glycan to generate a bisected, complex N-glycan. MGAT4A, MGAT4B, MGAT5 and MGAT5B add GlcNAc to generate branched, complex N-glycans. In Chinese hamster ovary (CHO) cells a Gal residue is added to each GlcNAc residue, except the bisecting GlcNAc (North et al. 2010). Each Gal may be extended by the addition of many (poly)LacNAc units ([Galβ1,4GlcNAc]n). CHO mutant cells that express N-glycans with a bisecting GlcNAc (LEC10) exhibit reduced binding of ricin and galectin-1, -3 and -8, but increased binding of the lectin E-PHA compared with CHO cells that do not express MGAT3.
We previously showed that loss of MGA T3 in cultured cells and in MMTV-PyMT mammary tumors correlates with increased ERK1/2 phosphorylation (Song et al. 2010). Thus, we investigated ERK1/2 and AKT phosphorylation in tumor lysates from $\approx 20$-week C57BL/6/MMTV-PyMT females. The average sizes of selected tumors ($\text{Mgat}^3^{+/+}$ 0.08 ± 0.03 g; $\text{Mgat}^3^{-/-}$ 0.16 ± 0.04 g, mean ± standard deviation) were close to the median value of the four largest tumors from each genotype ($\text{Mgat}^3^{+/+}$ 0.06 g, $n = 11$; $\text{Mgat}^3^{-/-}$ 0.16 g, $n = 13$; $P < 0.0001$, Mann–Whitney test). The presence of the bisecting GlcNAc on $\text{Mgat}^3^{+/+}$ tumor glycoproteins was demonstrated by enhanced binding of Phaseolus vulgaris erythroagglutinin (E-PHA) lectin compared with $\text{Mgat}^3^{-/-}$ tumor glycoproteins (Figure 5A). In contrast, binding of Phaseolus vulgaris leukoagglutinin (L-PHA) or Datura stramonium agglutinin (DSA) lectins, which preferentially bind to $\beta_1,6$-branched and (poly)LacNAc-containing N-glycans, respectively, was indistinguishable between the

Fig. 2. Genetic background effects on MMTV-PyMT mammary tumor growth in the absence of MGAT3. KM plots of tumor-free MG vs. time for individual mice. (A–C) FVB/C57BL/6 mixed background $\text{Mgat}^3^{+/+}$ ($n = 5$), $\text{Mgat}^3^{-/-}$ ($n = 22$), $\text{Mgat}^3^{-/-}$ ($n = 12$). (D) Combined MG free of tumors with time in the FVB/C57BL/6 background for all mice shown in (A–C). Not significant, log-rank (Mantel–Cox) test. (E–G) C57BL/6 congenic background $\text{Mgat}^3^{+/+}$ ($n = 12$), $\text{Mgat}^3^{-/-}$ ($n = 21$), $\text{Mgat}^3^{-/-}$ ($n = 15$). (H) Combined MG free of tumors with time in the C57BL/6 background for mice shown in E and G. $\text{Mgat}^3^{-/-}$ MG differ significantly from $\text{Mgat}^3^{+/+}$ and $\text{Mgat}^3^{-/-}$, $P < 0.0001$, log-rank (Mantel–Cox) test.
Fig. 3. Loss of MGAT3 accelerates tumor progression in C57BL/6 MMTV-PyMT females. (A) Mice free of tumors over time in the C57BL/6 background (Mgat3+/+ (n = 12), Mgat3+/− (n = 21), Mgat3−/− (n = 15)). Mgat3−/− females were significantly different from Mgat3+/+ and Mgat3+/−, P < 0.05, log-rank (Mantel–Cox) test. (B) Combined mass of three largest tumors from ~20-week C57BL/6/PyMT mice (Mgat3+/+ (n = 11), Mgat3+/− (n = 21), Mgat3−/− (n = 13)). Bar indicates the median, *P < 0.05, nonparametric Mann–Whitney test. (C) 100% stacked bar graph comparing the relative population of ~20-week C57BL/6/MMTV-PyMT mice with respective number of MG with solid tumors typically ≥10 mm3 (Mgat3+/+ (n = 11), Mgat3+/− (n = 21), Mgat3−/− (n = 13)). (D) Quantitative real-time PCR was used to determine the relative PyMT/β-actin transcript levels in total RNA from lungs of Mgat3+/+ (n = 11) and Mgat3−/− (n = 13) females. Graph represents the fold-change of each sample against the control sample (see Materials and methods section). Bar indicates the median (*P < 0.05, Mann–Whitney test).

Fig. 4. Glucose analog uptake is increased in Mgat3−/− mammary tumors. (A) SUVmax was measured in six ROI per mouse. Representative flattened projection 3D images of a dorsal view of Mgat3+/+ and Mgat3−/− littermates are shown. (B) SUVmax of six mammary gland regions per mouse (16–17 weeks: Mgat3+/+ (n = 2), Mgat3−/− (n = 4), 19–20 weeks: Mgat3+/+ (n = 6), Mgat3+/− (n = 5), Mgat3−/− (n = 3)). Bar indicates the median (*P < 0.05, **P < 0.01, Mann–Whitney test). (C) 100% stacked bar graph comparing the relative population of mice with mammary gland regions having SUVmax >1. Data from mice of 16–17 and 19–20 weeks were combined (Mgat3+/+ (n = 8), Mgat3+/− (n = 5) and Mgat3−/− (n = 7)).
two genotypes (Figure 5A). Unexpectedly, phosphorylation of both ERK1/2 and AKT were reduced in 
Mgat3−/− mammary tumors compared with wild-type tumor lysates (Figure 5B and C). In contrast, activation of p38 mitogen-activated protein kinase (p38MAPK), Src and focal adhesion kinase (FAK) in the same tumor lysates was not significantly different between 
Mgat3+/+ and Mgat3−/− tumors (data not shown). Acute ERK1 activation in response to epidermal growth factor (EGF) stimulation was enhanced in one Mgat3 null tumor epithelial cell (TEC) line compared with controls (Figure 6A and C). In addition, the deactivation of ERK1/2, assessed by the level of phosphorylated ERK1/2 at 10 min post-EGF stimulation, was delayed in both 
Mgat3−/− TECs (Figure 6A and C), indicating prolonged growth factor signaling in the absence of MGA T3, reflected by their reduced E-PHA signal (Figure 6B). Thus, Mgat3−/− cultured TECs responded somewhat better than wild-type to EGF stimulation. However, the data from tumor lysates suggest that Mgat3−/− MMTV-PyMT tumors had reached a point of reduced growth factor signaling via ERK1/2 or AKT, compared with their Mgat3+/+ counterparts by ~20 weeks. In contrast, at a similar stage of tumor progression, Mgat3−/− MMTV-PyMT FVB/C57BL/6 tumors and TECs exhibited enhanced ERK1/2 activation (Song et al. 2010).

MGAT3 and galectins in mammary tumor progression

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MMTV-PyMT mammary tumors and TECs express eight galectin genes

The bisecting GlcNAc transferred by MGAT3 is a significant modulator of galectin binding to glycoproteins (Andre et al. 2004; Patnaik et al. 2006; Andre et al. 2007; Miwa et al. 2012). Up to 15 galectins have been identified, including 11 in human and 8 in mice (Houzelstein et al. 2008). Whereas galectin-3 has been implicated as a cross-linker of complex N-glycans that prolongs growth factor signaling and enhances mammary tumor progression in the MMTV-PyMT model (Partridge et al. 2004; Lau et al. 2007), the deletion of galectin-3 has no effect on mammary tumor progression in MMTV-PyMT tumors (Eude-Le Parco et al. 2009). We, therefore, investigated the range of galectin genes (Lgals) expressed in mouse MMTV-PyMT mammary tumors. Reverse transcription–polymerase chain reaction (RT–PCR) was performed on total RNA harvested from three independent tumors per genotype using the primer sets given in Supplementary data Table SII. PCR products were initially confirmed by sequencing. Mgat3 transcripts were present in RNA from Mgat3+/+ but not Mgat3−/− tumors, while transcripts of PyMT and Mgat5, which encode a distinct branching glycosyltransferase (Figure 1), were similar in all tumors (Figure 7A). Interestingly, transcripts from all the eight mouse galectin genes were detected in MMTV-PyMT mammary tumors (Eude-Le Parco et al. 2009). We, therefore, investigated the range of galectin genes (Lgals) expressed in mouse MMTV-PyMT mammary tumors. Reverse transcription–polymerase chain reaction (RT–PCR) was performed on total RNA harvested from three independent tumors per genotype using the primer sets given in Supplementary data Table SII. PCR products were initially confirmed by sequencing. Mgat3 transcripts were present in RNA from Mgat3+/+ but not Mgat3−/− tumors, while transcripts of PyMT and Mgat5, which encode a distinct branching glycosyltransferase (Figure 1), were similar in all tumors (Figure 7A). Interestingly, transcripts from all the eight mouse galectin genes were detected in MMTV-PyMT mammary tumors (Figure 7A). While most galectins were also detected by western analysis, galectin-2 and -12 were not, although bands of the expected molecular weight were obtained in control tissues (Figure 7B). Similar results were obtained by RT–PCR of cDNA from mammary tumors of FVB/C57BL/6-MMTV-PyMT females (data not shown).

Because tumor tissue is composed of a heterogeneous mixture including tumor cells, immune cells and adipocytes, we also investigated the galectins expressed in cultured TEC
lines to determine galectins intrinsic to the tumor cells. *Mgat3* transcripts and *E-PHA* and *L-PHA*-binding glycoproteins were present in *Mgat3*+/- but not *Mgat3*-/- TECs, while transcripts of *Mgat5* were similar in *Mgat3*+/- and *Mgat3*-/- TECs (Figure 8A and B). Although, PyMT expression was higher in *Mgat3*-/- TECs (Figure 8A), such differences were not observed in tumors (Figure 7A), or in comparisons of 3 *Mgat3*+/- and 3 *Mgat3*-/- TEC lines generated from mammary tumors of FVB/C57BL/6 females (data not shown), nor in TEC lines developed previously (Song et al. 2010). Importantly, all the eight galectin genes were expressed in *Mgat3*+/- and *Mgat3*-/- TECs (Figure 8A). Western analyses showed that galectin-2 and -12 were not detected, as observed for tumor tissue, and galectin-7 was detected only in one of the four cell lines (Figure 8B). Since galectin-7 was readily detected in western blots of all tumor tissues, the fact that most TECs have no detectable galectin-7 protein suggests that it is mainly expressed in stromal cells of the tumor. Galectin-4 and -8 protein isoforms were observed in both tumors and TECs (42 vs. 45 kDa). We also noted for galectin-9 that tumor tissues contained predominantly an ~36 kDa species, but an ~45 kDa species was more prominent in TECs. Similar results were obtained following western analyses of TEC lines derived from FVB/C57BL/6 females (data not shown). In conclusion, galectins-1, -3, -4, -8 and -9 were the galectins most robustly expressed at both transcript and protein levels in tumor tissues and TECs. However, all the eight mouse galectin genes were expressed in TECs, and thus the full range of galectins may influence tumor progression in the MMTV-PyMT mammary tumor model.

**Galectins and complex N-glycans in human breast cancer**

In order to develop mouse models that may provide insights into roles for galectins and the glycosyltransferases responsible for the synthesis of complex N-glycans in human breast cancer, we analyzed published microarray data reported by TCGA for various cohorts of women with breast cancers of different subtypes (Network CGA 2012). The expression data are presented as a heat map with statistically significant differences between breast tumor subtypes shown in an adjacent table (Figure 9A). Among the galectins, basal-like breast cancer (BLBC) differed from each of the other types, having increased galectin-2 and -7 expression but decreased galectin-8 expression, compared with overall median expression levels. Compared with Luminal A, BLBC differed in the expression of two additional galectins, galectin-9 and -12. Compared with Luminal B, expression of galectin-2, -4, -7 and -8 were significantly different in BLBC. Galectin-3 expression was significantly lower in Luminal B than in human epidermal growth factor receptor 2 (HER2) and Luminal A tumors. In general, galectin-2, -7, -8 and -12 exhibited the most variation in expression. Among the glycosyltransferase genes that generate complex N-glycans, multiple...
MGAT3 and galectins in mammary tumor progression

expression differences were observed between BLBC and HER2 (MGAT3, MGAT4A and MGAT5), BLBC and Luminal A (MGAT3, MGAT4A, MGAT4B, MGAT5 and MGAT5B) and BLBC and Luminal B (MGAT2, MGAT3, MGAT4A and MGAT5). Compared with the overall average, BLBC cancers have markedly increased expression of MGAT3 and decreased expression of MGAT4A. Based on mouse studies, increased bisected N-glycans caused by MGAT3 and reduced branched N-glycans due to reduced MGAT4A would be expected to retard tumor progression (Lau and Dennis 2008; Song et al. 2010; Miwa et al. 2012; this work). HER2-amplified tumors exhibit increased expression of MGAT4A and MGAT5B, which should both increase complex N-glycan branching and enhance tumor progression (Lau and Dennis 2008). Luminal A cancers have no differences from the overall average, and Luminal B only one difference in reduced expression of MGAT3 which should enhance tumor progression.

Investigations of LGALS and MGAT genes in the breast cancer database Kaplan–Meier (KM) plotter (Gyorffy et al. 2010) showed that in data from all types of breast cancer (n = 2978), higher expression of MGAT3 correlates with better RFS (hazard ratio (HR) < 1; Table I), as expected from mouse models (Song et al. 2010; Miwa et al. 2012; this work). Expression of MGAT5 does not correlate with worse RFS, contrary to expectations from mouse models (Partridge et al. 2004; Lau et al. 2007; Guo et al. 2012). However, higher expression of MGAT2 correlates with worse RFS (Table I), and this should lead to increased substrate for MGAT5, thereby enhancing N-glycan branching by an alternative mechanism to upregulation of MGAT5. With respect to LGALS transcripts, patients with higher expression of LGALS4, LGALS8, LGALS10, LGALS13 and LGALS14 have better RFS (Table I). Other galectins that correlate positively with RFS are LGALS2 and LGALS9, whereas higher LGALS1 expression correlates with worse RFS (HR > 1; Table I). From these data, it seems that augmenting MGAT3 and galectin-4, -8, -10, -13 and -14 and reducing MGAT2 and galectin-1 would be beneficial to breast cancer patients.

Discussion

In C57BL/6 MMTV-PyMT/Mgat3+/− females, the absence of MGAT3 and the bisecting GlcNAc on N-glycans enhanced tumor progression and lung metastasis as we observed initially (Song et al. 2010), though the effect became variable in the mixed genetic background, as reported here. Others have observed similar genetic background effects. For example, tumor progression and lung metastasis of MMTV-PyMT mice deficient in inducible nitric oxide synthase is significantly reduced in the congenic C57BL/6 background, but not in the
FVB/N background (Davie et al. 2007). Similarly, Lipocalin-2 null mice show reduced tumorigenesis in the C57BL/6 background (Berger et al. 2010), but not in the FVB/N background (Cramer et al. 2012). We also uncovered differences with respect to growth factor signaling and genetic background. Thus, while EGF- or platelet-derived growth factor-AB-induced ERK activation was reduced by the presence of MGA T3 and the bisecting GlcNAc in Chinese hamster ovary cells and TECs isolated from FVB/C57BL/6 MMTV-PyMT tumors (Song et al. 2010), the opposite effect was found in C57BL/6 mammary tumors examined at an equivalent time of tumor development (Figure 5). In the absence of MGA T3, ERK1/2 and AKT activation in tumor lysates were reduced, rather than enhanced, compared with control lysates. The levels of activated p38MAPK, Src and FAK were no different between wild-type mice and \( \text{Mgat3}^{-/-} \) tumors. However, in \( \text{Mgat3}^{+/-} \) TECs, the levels of phosphorylated ERK1/2 induced by EGF were maintained slightly longer compared with controls. Therefore, it appears that the underlying mechanism by which mammary tumorigenesis is increased in C57BL/6 \( \text{Mgat3}^{-/-} \) mice is not simply due to increased signaling via a predominant growth factor signaling pathway. While the \( \text{Mgat3}^{+/-} \) TEC lines examined here expressed fewer PyMT transcripts than the \( \text{Mgat3}^{-/-} \) TEC lines (Figure 8A), such differences were not observed in other comparisons of TEC lines, nor in MMTV-PyMT tumors excised from control vs. \( \text{Mgat3}^{-/-} \) null mice.

microPET analysis revealed that \( \text{Mgat3}^{-/-} \) tumors exhibited greater glucose uptake than \( \text{Mgat3}^{+/-} \) tumors. In mouse mammary tumor cells, glucose uptake is mediated predominantly by glucose transporter 1 (GLUT1) as reduction of GLUT1 expression caused reduced glucose usage and tumor cell growth (Young et al. 2011). Increases in the number of N-acetyllactosamine (LacNAc) repeats in the N-glycan on GLUT1 have been observed in tumor cells (Kitagawa et al. 1995), which could be due to increased glucose metabolism leading to increased uridine diphosphate-GlcNAc levels (Ying et al. 2012) and thereby, increases in N-glycan branching and LacNAc repeats (Lau et al. 2007). Similarly, cell surface expression of GLUT2 and GLUT4 are regulated by N-glycans (Ohtsubo et al. 2005; Lau et al. 2007; Haga et al. 2011), and a critical role of the galectin lattice has been demonstrated for GLUT2 cell surface expression. Thus, the presence of the bisecting GlcNAc on the N-glycans of GLUT1 in mammary tumor cells may lower glucose uptake by reducing the amount of GLUT1 associated with a galectin lattice at the cell surface.

In order to investigate roles for galectins on the effects of MGAT3 and other branching GlcNAc-transferases in mammary tumor progression, we determined their expression in MMTV-PyMT mouse mammary tumors and cultured TECs.
Based on RT–PCR analysis, tumors and TECs express all the eight mouse galectin genes. According to western blot analyses, galectin-1, -3, -4, -7, -8 and -9 were detected in tumor lysates and galectin-1, -3, -4, -8 and -9 in TECs. Galectin-7 may, therefore, be a component of the stromal microenvironment, which is known to influence tumor progression (Joyce and Pollard 2009). Clearly, however, a number of galectins may play roles in mammary tumor progression and some may share overlapping roles. Thus, removal of galectin-3 does not alter mammary tumor progression in a MMTV-PyMT mouse model (Eude-Le Parco et al. 2009), despite compelling evidence suggesting a major involvement of galectin-3 in promoting tumor growth and metastasis (Partridge et al. 2004; Dennis et al. 2009). It would be interesting to know whether other galectins are upregulated in galectin-3 null mammary tumors or TECs (Eude-Le Parco et al. 2009).

Galectins may regulate tumor cell growth and metastasis not only through regulation of growth factor and cytokine signaling, but also by promoting angiogenesis and immunosuppression, as shown for galectin-1 (Thijssen et al. 2006; Thijssen et al. 2010; Banh et al. 2011; Dalotto-Moreno et al. 2013). Thus, disruption of galectin-1 and N-glycan interactions suppresses hypoxia-induced angiogenesis and tumorigenesis in Kaposi’s sarcoma (Croci et al. 2012). On the other hand, not all members of the galectin family act to promote tumorigenesis. In stark contrast to galectin-1 and -3, galectin-9 is proposed to

Fig. 9. LGALS and MGAT genes in human breast cancers. Expression of genes encoding galectins (LGALS) and N-glycan branching GlcNAc-transferases (MGAT) were analyzed in gene expression microarray data from 458 breast tumors in the TCGA study (Network CGA 2012). The heat map represents the fold-change between the median expression level of that gene in a given breast cancer subtype compared with the median expression level of that gene across all samples (log2 scale). All of the data were used to test for significant differences between the median expression levels for each gene in each subtype (Kruskal–Wallis test with Dunn’s posttest). Statistically significant P-values are indicated by asterisks in the adjacent table.

Table I. LGALS and MGAT genes and human breast cancer RFS

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<td>1.15</td>
<td>1.02–1.31</td>
<td>*</td>
</tr>
<tr>
<td>MGAT5</td>
<td>1.08</td>
<td>0.95–1.22</td>
<td></td>
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\*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 and ****P ≤ 0.0001.
inhibit metastasis in human breast cancer, through blocking adhesion to endothelium and extracellular matrices (Irie et al. 2005; Nobumoto et al. 2008). Consistent with such reports, investigation of the data from 286 samples of invasive breast tumors (Wang et al. 2005) showed that high levels of galectin-2 and -9 expression correlated with lower recurrence of distant metastasis in patients with node-negative breast cancer. The expression of other galectins, including galectin-3, or MGAT3, MGAT5 and the other branching GlcNAc-transferase genes did not indicate prognostic value from the same data (Wang et al. 2005). In contrast, investigation of the data of 295 breast cancer patients of the Netherlands Cancer Institute (NKI-295) (van de Vijver et al. 2002; van de Vijver 2005) indicated that high levels of galectin-9 correlate with poor survival. Interestingly, however, estrogen receptor (ER) negative status appears to correlate with higher levels of galectin-9 and thus, when survival was compared within a group with the same ER status (i.e. positive or negative), no correlation was detected. Clearly, the situation is complex given the changes in expression levels determined from the most recent TCGA data (Network CGA 2012) of several LGALS and MGAT genes in different breast cancer subtypes (Figure 9). In addition, based on microarray data in KM plotter, the increased expression of several galectin and MGAT genes correlates with RFS in breast cancer patients (Table I). Thus, reducing expression of galectin-1 and MGAT2 and enhancing expression of MGAT3 and galectins -9, -6, -12, -13 and -14 may potentially be beneficial to breast cancer patients. However, the molecular mechanisms underlying human breast tumor biology are complex, and with the likelihood of functional redundancy, it will be a challenge to ascertain roles for individual galectins in vivo. On the other hand, the caveat that galectin expression may differ between humans and mice, predictions could be tested by generating mouse models using the efficient CRISPR/Cas method of genomic engineering (Wang et al. 2013) to conditionally delete multiple galectin genes in mammary epithelial or stromal cells.

### Materials and methods

**Mice**

*Mgat3+/−* or *Mgat3−/−* mice (*Mgat3tm1Jxm*) (Priatel et al. 1997) backcrossed >10 generations to C57BL/6 mice were mated with MMTV-PyMT transgenic male mice in the FVB/N (FVB) background (line #634) (Guy et al. 1992) (a gift from Dr. Jeffery Pollard, Albert Einstein College of Medicine, Bronx, NY) or the C57BL/6 background (Basu et al. 2004) (a gift from Dr. Sandra Gendler, Mayo Clinic, Rochester, MN). *Mgat3+/−* or *Mgat3−/−* females in the C57BL/6 background were mated with *Mgat3+/−*/MMTV-PyMT males in either the FVB/C57BL/6 or C57BL/6 background. This mating strategy resulted in experimental female mice with an ~25% FVB and ~75% C57BL/6 genome (termed FVB/C57BL/6) or congenic C57BL/6 females, respectively. Genotypes were determined by PCR using the primers shown in Supplementary Table SI (Song et al. 2010) under the following reaction conditions: 94°C 2 min, 94°C 30 s, 58°C 45 s, 72°C 1 min, 72°C 10 min for 35 cycles. PCR products were analyzed on a 1.0% agarose gel. All experimental protocols pertaining to animal studies were approved by the Institute for Animal Studies of the Albert Einstein College of Medicine.

### Antibodies and reagents

The following antibodies were used for western analyses at the concentrations indicated. Rabbit monoclonal anti-galectin-1 (EPR3205; 1/5000) and anti-galectin-8 (EPR4857; 1/5000) antibodies were from Epitomics (Burlingame, CA), rat monoclonal anti-galectin-3 (M3/38) (1/5000) antibody was prepared in conditioned medium from the M3/38 hybridoma line kindly provided by Dr. Timothy Springer (Harvard Medical School, Boston, MA) and guinea pig polyclonal anti-galectin-2 (1/2000) and rabbit polyclonal anti-galectin-4 (1/1000) antibodies were generous gifts from Dr. Junko Nio-Kobayashi (University of Hokkaido, Sapporo, Japan) (Nio-Kobayashi et al. 2009). Rabbit polyclonal anti-galectin-7 antibody (1/10,000) was purchased from Bethyl Laboratories (Montgomery, TX). Goat polyclonal anti-galectin-9 antibody (sc-19294; 1/500), rabbit polyclonal anti-phosphorylated FAK (Tyr925) (sc-11766-R; 1/500), anti-FAK (sc-557; 1/500) antibodies and donkey anti-goat IgG-horse radish peroxidase (HRP) secondary antibody (sc-2020; 1/2500) were from Santa Cruz Biotechnology (Dallas, TX). Mouse polyclonal anti-galectin-12 antibody (1/1000) and Lgals12+/− and Lgals12−/− adipose tissue lysates were gifts from Dr. Fu-Tong Liu (UC Davis School of Medicine, Davis, CA) (Yang et al. 2011). Rabbit anti-phosphorylated p44/42 MAPK (pERK1/2) (Thr202/Tyr204) (D13.14.4E) XP® (4370; 1/2000) and mouse anti-ERK1/2 monoclonal antibody (L34F12) (4696; 1/2000), rabbit polyclonal anti-phospho-Akt (Ser473) (9271; 1/1000), anti-Akt (9272; 1/1000), anti-phosphorylated p38 MAPK (Thr180/Tyr182) (9211; 1/500) and anti-p38MAPK (9212; 1/500) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-β-actin antibody (AC-15) (ab6276; 1/5000) was purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-phosphorylated Src (Tyr418) antibody (44660G; 1/500), anti-rabbit, anti-rat and anti-guinea pig IgG-HRP secondary antibodies (1/10,000) and Alexa Fluor 680 goat anti-mouse IgG-H + L (1/10,000) secondary antibodies were purchased from Life Technologies (Grand Island, NY). IRDye 800-conjugated goat anti-rabbit IgG-H + L (1/10,000) was from Rockland Immunochemicals (Gilbertsville, PA). Anti-mouse IgG-HRP secondary antibody (1/10,000) was from Thermo Scientific (Rockford, IL). Mouse monoclonal anti-Src antibody (05-184; 1/500) was from Millipore (Billerica, MA). Biotinylated E-PHA, L-PHA, DSA and Streptavidin-HRP were from Vector Labs (Burlingame, CA). All chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Rockville, MD), unless indicated otherwise.

### Tumor analysis

All 10 MG of each mouse were palpated twice a week, beginning at 6 weeks of age, and the date of the first palpable mass in each mammary gland was recorded. Both C57BL/6 and FVB/C57BL/6 cohorts were investigated simultaneously. Mice of 15–17 weeks (FVB/C57BL/6) or 19–20 weeks (C57BL/6) were euthanized and individual solid tumor masses were excised and weighed. Some tumors were used to establish TEC lines or frozen on dry ice and stored at −80°C for protein, DNA or RNA extraction.
Lung metastasis

Preliminary gross anatomical and histological examinations of lungs from MMTV-PyMT mice at ~20 weeks were performed by visual inspection of surface metastatic nodules of intact lung or by examination of three serial sections sampled at 100 μm intervals of paraffin-embedded lung tissue stained with hematoxylin and eosin. Lung metastasis was also investigated by real-time qPCR analysis of PyMT transcripts as described previously (Song et al. 2010) with some modifications. Briefly, total RNA was extracted from a whole lung in TRIzol (1 mL per 100 mg lung), treated with DNasel (QIAGEN, Valencia, CA) and purified by RNA Protect mini kit (QIAGEN). The cDNA was synthesized as described (Song et al. 2010). Real-time PCR was performed in an iQ5 (Bio-Rad, Hercules, CA) with POWER SYBR green master mix (Life Technologies), the cDNA derived from 40 ng of original RNA material and the primers for PyMT and β-actin were as previously described (Song et al. 2010). ΔCt values were obtained by subtracting the Ct value of β-actin from that of PyMT in the same cDNA sample. The relative abundance of PyMT transcripts between two genotypes was compared by using the formula \((\Delta C_t\text{sample}) - \Delta C_t\text{(control)})^\circ\), in which ΔCt(sample) represents the ΔCt value of each cDNA sample and ΔCt (control) represents the sample with the highest ΔCt in the control group (also an overall highest). All reactions were run in duplicate in two independent experiments.

\[^{18}F\]Fluoro-2-deoxyglucose PET imaging

The mice were fasted overnight (18–20 h) and injected with 300–400 μCi (12–15 MBq) of \[^{18}F\] fluoro-2-deoxyglucose in 0.1 mL normal saline via the tail vein 1 h before imaging. The mice were secured to the imaging palette with a breathing tube affixed over their snout to supply 1.5% isoflurane–oxygen mixture anesthesia throughout the imaging portion of the procedure. Each mouse was placed on a heating pad before and during scanning to maintain normal body temperature. The mice were imaged by an Inveon Multimodality scanner (Siemens, Knoxville, TN) using its PET module. PET imaging is performed using the PET gantry, which provides a 12.7 cm axial and 10 cm transaxial active field of view. The PET scanner has no septa, and acquisitions are performed in the 3D list mode. A reconstructed full-width-half-max resolution of <1.4 mm is achievable in the center of the axial field of view. List mode acquisition of data is performed to permit dynamic re-framing for kinetic evaluation of the radiotracer uptake, where indicated. After each acquisition, data were sorted into 3D sinograms, and images were reconstructed using a two dimensional (2D)-Ordered Subset Expectation Maximization algorithm. Data were corrected for decay, dead time counting losses, random coincidences and the measured nonuniformity of detector response (i.e. normalized), but not for attenuation or scatter. Analysis was performed by using the ASIPRO software (Siemens). All image studies were inspected visually in a rotating 3D projection display to identify interpretability and image artifacts. Regions of interest (ROI) were manually defined around areas of individual MG with radiotracer uptake. Successive scrolling through 2D slices (each 1.2 mm thick in the axial images) permitted measurement of radioactivity within defined volumes. Corrected counts per cc within this volume divided by the counts per gram of total body mass of injected radioactivity determined the SUV. SUV_max, the maximum value of SUV within each tumor volume was determined in six mammary gland regions (a pair of first, second/third and fifth) per mouse, as shown in Figure 4. The second and third mammary gland regions were analyzed as one region, as it was often hard to distinguish the boundaries of tumors developed in that region. The fourth mammary gland was excluded as it was often overwrapped with hot spots resulting from radioactivity within the digestive tracts.

Cell culture

TECs were isolated from mammary tumors as described (Song et al. 2010), and cells were passaged at least eight times and no >11 times. TECs were grown in growth medium (20% heat inactivated fetal bovine serum; Gemini, West Sacramento, CA), 1x penicillin/streptomycin (Life Technologies), 1.25 µg/mL Fungizone (Life Technologies), 20 ng/mL recombinant mouse EGF (Peprotech, Rocky Hill, NJ), 10 µg/mL insulin (Sigma-Aldrich or Gemini)/DMEM (Life Technologies; #11965) at 37°C, 5% CO2.

Protein extraction, western and lectin blot analyses

About 30–100 mg solid tumors were mechanically homogenized in 300–1000 μL of tumor lysis buffer (10 mL Tris–HCl (pH 7.5), 300 mM lactose, 1 mM ethylenediaminetetraacetic acid (EDTA) and COMPLETE protease and PhosStop phosphatase inhibitors (Roche, Indianapolis, IN)). Homogenates were centrifuged at 1800 rpm for 10 min at 4°C and the supernatant was stored at −80°C. TEC lysates were prepared by scraping the cells off the culture plate in 250 μL/10 cm plate of TEC lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100) with 300 mM lactose and 1% COMPLETE protease inhibitor (Roche). Lysates were microcentrifuged at 3000 rpm for 10 min at 4°C and the supernatant was stored at −80°C. As for control lysates, mouse small intestine lysates containing galectin-2 were prepared from C57BL/6 mouse as described (Nio-Kobayashi et al. 2009). Skin lysates containing galectin-7 were prepared by homogenizing the epidermis of C57BL/6 mice in TEC lysis buffer and microcentrifuged at 14,000 rpm for 15 min at 4°C. Protein concentration was measured by a Bio-Rad DC protein assay kit (Bio-Rad). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, equal amounts of protein lysates (10–80 μg) were solubilized in SDS–PAGE loading buffer containing β-mercaptoethanol at 5% (final), heated at 95°C for 10 min and then separated on a 10, 12 or 15% SDS–PAGE gel along with molecular standards (Precision Plus Protein Dual Color Standards, Bio-Rad). Proteins were transferred to a PolyScreen polyvinylidene difluoride (PVDF) membrane (PerkinElmer, Waltham, MA) in Tris-glycine buffer containing 5% methanol, and western blot analysis was conducted as described previously (Song et al. 2010). Briefly, membranes were blocked in blocking buffer Tris-buffered saline and Tween 20 (TBS-T) (25 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) with either 3% bovine serum albumin (BSA) (for signaling molecules) or 3% nonfat dry milk (for all other antibodies) for over 1 h, and then incubated with primary antibody diluted in 3% Cold Fish Gelatin, 1% BSA, 0.001% Thimerosal/TBS-T at room
temperature for 1–3 h. After washing with TBS-T, membranes were incubated with HRP-conjugated secondary antibody diluted in the appropriate blocking buffer for 1 h. After washing with TBS-T, bands were visualized using an enhanced chemiluminescence (ECL) kit (Thermo Scientific) and exposed to film (Denville scientific, South Plainfield, NJ) and scanned or imaged with Fujifilm LAS-3000 (Valhalla, NY). When applicable, band intensity was quantified using NIH ImageJ (Bethesda, MD). For lectin blotting, membranes were blocked in 3% nonfat dry milk/TBS-T, incubated with biotinylated E-PHA, L-PHA or DSA (Vector Labs) at 10 or 15 µg/mL diluted in blocking buffer at room temperature for 1 h or overnight at 4°C. Membranes were washed with TBS-T, incubated with Streptavidin-HRP (1/5000; Vector Labs) for 1 h, washed with TBS-T and visualized using an ECL kit and exposed onto films.

RNA extraction and RT–PCR
Total RNA was extracted from ~50 mg of solid tumor by homogenization in 1 mL of TRIzol and purified according to the manufacturer’s protocol. Residual genomic DNA was removed using a TURBO DNA-free kit (Life Technologies). For TEC, cells (80–90% confluent) were lysed in 500 µl (per 10 cm plate) of TRIzol. RNA was purified according to the manufacturer’s protocol, followed by DNaseI treatment (QIAGEN) and purification using an RNA Protect mini kit (QIAGEN). The cDNA was synthesized using random hexamers and the SuperScript III first-strand synthesis system (Life Technologies). cDNA derived from 50 or 100 ng of RNA starting material was used as template with primers specific to each galectin gene or Mgat3, Mgat5 and PyMT as described (Song et al. 2010) (Supplementary Table S1). Galectin primers were designed using the online program, Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012). The following reaction conditions were used: 94°C 2 min, 94°C 30 s, 58°C 45 s, 72°C 1 min, 72°C 10 min for 30–35 cycles. PCR products were visualized on 1 or 2% agarose gels.

Signaling assays in TECs
TECs (85–90% confluent in 24-well plates) were serum-starved for 24 h and stimulated with 50 ng/mL of recombinant mouse EGF (R&D Systems, Minneapolis, MN) at 37°C for the indicated times. To terminate the reaction, plates were placed on an ice slurry, and then washed once with 1 mL ice-cold phosphate-buffered saline (PBS), pH 7.2, lysed in 2× SDS–PAGE buffer diluted with TEC lysis buffer containing COMPLETE protease and PhosStop phosphatase inhibitors (Roche), incubated at 95°C for 10 min and then electrophoresed on 12% SDS–PAGE gels and transferred to PVDF membranes. Membranes were blocked in Odyssey blocking buffer and then incubated with rabbit anti-phosphorylated p44/42 MAPK (pERK1/2) antibody (Thr202/Tyr204) and mouse anti-ERK1/2 mAb (L34F12) in blocking buffer at 4°C overnight. Following washes with PBS/0.025% Tween-20 (PBS-T), pH 7.2, IRDye800-conjugated goat anti-rabbit IgG-H + L and Alexa Fluor 680 goat anti-mouse IgG-H + L diluted in blocking buffer were added for 1 h at room temperature, the membranes were washed with PBS-T, rinsed with PBS and bands were visualized and quantified by the ODYSSEY IR Imaging System (LI-COR BioSciences, Lincoln, NE).

LGALS and MGAT gene expression data from human breast cancer databases
Gene expression profiles of breast tumors with matched exome sequencing data (n = 466) were obtained from the TCGA study (Network CGA 2012). These included 81 basal-like, 53 HER2, 210 Luminal A, 114 Luminal B and 8 Normal-like tumors. Samples assigned to the Normal-like subtype were excluded as they are believed to be substantially contaminated by normal tissue. Because the TCGA data lack extensive clinical follow-up, we examined additional datasets (van de Vijver et al. 2002; Wang et al. 2005; Gyorffy et al. 2010) to test for association between our genes of interest and patient outcomes. KM plotter was used to analyze RFS in breast cancer patients (Gyorffy et al. 2010). Where multiple Affymetrix probes were available for a given gene, the optimal probe was selected using Jetset (Li et al. 2011).

Statistical analysis
Statistical analyses of experimental data were performed using either GraphPad Prism (La Jolla, CA) or the Microsoft® Excel software (Redmond, WA).

Supplementary Data
Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Abbreviations
AKT, Protein kinase B; BLBC, Basal-like breast cancer; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CRD, Carbohydrate recognition domain; DSA, Datura stramonium agglutinin; ECL, enhanced chemiluminescence; EDTA: ethylene-diaminetetraacetic acid; EGF, epidermal growth factor; E-PHA, Phaseolus vulgaris erythroagglutinin; ER, estrogen receptor; ERK, Extracellular signal-regulated kinase; FAK, focal adhesion kinase; FVB, FVB/N; GcNAc, N-acetylgalactosamine; GLUT1, Glucose transporter 1; HER2, human epidermal growth factor...
References


