Aberrant Glycosylation Promotes Lung Cancer Metastasis through Adhesion to Galectins in the Metastatic Niche

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ABSTRACT

Metastasis is the leading cause of cancer-associated deaths. Although dissemination of tumor cells likely occurs early in tumorigenesis, the constituents of the microenvironment play essential rate-limiting roles in determining whether these cells will form clinically relevant tumors. Recent studies have uncovered many molecular factors that contribute to the establishment of a protumorigenic metastatic niche. Here, we demonstrate that galectin-3, whose expression has clinical associations with advanced malignancy and poor outcome, contributes to metastatic niche formation by binding to carbohydrates on metastatic cells. We show that galectin-3 is expressed early during tumorigenesis by both CD11b+Gr-1+ and CD11b+Ly-6C+ leukocytes. Tumors mobilize these myeloid populations through secretion of soluble factors, including IL6. We find that metastatic cancer cells exhibit elevated presentation of the oncofetal galectin-3 carbohydrate ligand, the Thomsen–Friedenreich antigen, on their surfaces as a result of altered C2GnT2 and St6GalNAc4 glycosyltransferase activity that inhibits further glycosylation of this carbohydrate motif and promotes metastasis.

SIGNIFICANCE: Although clinical observations of elevated serum galectin-3 levels and altered glycosylation have been associated with malignancy, we identify novel roles for glycosylation in promoting adhesion to galectins in the metastatic niche. This identification of a cytokine–leukocyte–glycosylation axis in metastasis provides mechanistic explanations for clinical associations between malignancy and aberrant glycosylation. Cancer Discov; 5(2); 168–81. © 2014 AACR.

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INTRODUCTION

Galectins are a family of β-galactoside–binding lectins whose pleiotropic roles in tumorigenesis have been reported to range from interactions with oncoproteins, to immunomodulation, to promotion of angiogenesis, to eliciting antiapoptotic effects through BCL2 homologies (1–3). Despite the fact that galectins lack a signal sequence or transmembrane domain, they can be incorporated into the extracellular matrix (ECM), secreted as soluble factors into circulation, presented on cell surfaces, or can act intracellularly. In particular, the presence of galectin-3 as an extracellular molecule (circulating in serum or localized on cells within tissues) has been associated with metastasis.

Elevated levels of galectin-3 have been reported in patients with advanced stages of a variety of cancers (4, 5), and treatment of cells within tissues) has been associated with metastasis. Recent studies have uncovered many molecular factors that contribute to the establishment of a protumorigenic metastatic niche. Here, we demonstrate that galectin-3, whose expression has clinical associations with advanced malignancy and poor outcome, contributes to metastatic niche formation by binding to carbohydrates on metastatic cells. We show that galectin-3 is expressed early during tumorigenesis by both CD11b+Gr-1+ and CD11b+Ly-6C+ leukocytes. Tumors mobilize these myeloid populations through secretion of soluble factors, including IL6. We find that metastatic cancer cells exhibit elevated presentation of the oncofetal galectin-3 carbohydrate ligand, the Thomsen–Friedenreich antigen, on their surfaces as a result of altered C2GnT2 and St6GalNAc4 glycosyltransferase activity that inhibits further glycosylation of this carbohydrate motif and promotes metastasis.

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Recently, the role that dynamic remodeling of the tumor microenvironment plays in the promotion of metastasis has been partially elucidated (11–13). Soluble factors released from tumors act to mobilize bone marrow–derived cells (BMDC), and in particular myeloid cells, into the peripheral blood and recruit them to the early metastatic niche, a site where metastases have yet to reach a detectable size. Frequently, these myeloid cells consist of CD11b+ cells that are Ly-6C+ or Gr-1+. The former population typically consists of inflammatory monocytes, whereas the latter may represent neutrophils or a heterogeneous poorly differentiated granulocyte population that exhibits suppressor functions, sometimes referred to as myeloid-derived suppressor cells (MDSC). Crosstalk between primary or metastatic tumors and these or other nonparenchymal populations induces the secretion of factors in autocrine, paracrine, and endocrine fashions in a manner that promotes remodeling of the primary tumor, bone marrow, and metastases to promote malignant progression. Among the molecules that are involved in metastatic niche formation, many are ECM molecules and their modifiers, including fibronectin, collagen IV, tenascin-c, osteopontin, periostin, versican, lysyl oxidase (LOX), and a variety of matrix metalloproteinases (14–21). The role of cell-surface
galectins, however, as components of the early metastatic niche, remains to be investigated.

Alterations in gene expression, cytokine secretion, and ECM and soluble factor production have all been associated with improved interactions between metastatic cells and specific microenvironments (14–16, 18, 19, 22–26). To determine the involvement of galectin-3 within the metastatic niche and of aberrant glycosylation of cancer-cell–associated surface glycoproteins, we used a mouse model of lung adenocarcinoma metastasis (27–29). Previously, we used our ECM microarray platform to identify adhesion ligands associated with metastasis and identified galectin-3 and galectin-8 as exhibiting preferential adhesion to metastatic cells (30). Here, we identify galectin-3 as a component of the metastatic niche through expression on myeloid cells. We find that changes in glycosylation by the cancer cells mediate their increased interactions with galectins, and that these alterations play essential roles in conferring metastatic potential. In particular, we find that the upregulation of the sialyltransferase St6GalNAc4 and downregulation of the core 2 N-acetylgalosaminytransferase C2GnT2 (GCNT3) are key mediators of the changes in glycosylation that potentiate metastatic proclivity by preserving presentation of the T-Antigen.

RESULTS

Tumor-Bearing Mice Exhibit Elevated Levels of Galectin-3 in the Early Metastatic Niche

To determine the role of galectin-3 in metastasis, we used a genetic mouse model of lung adenocarcinoma metastasis (Kras<sup>LSL-G12D/</sup>^+/p53<sup>fl ox/fl ox</sup>) from which cell lines representing distinct stages of metastatic progression were derived (27–29). Following Cre-mediated recombination and the development of autochthonous lung tumors and metastases, lines were generated from primary tumors that did not give rise to metastases (T<sub>nonMet</sub>), primary tumors that did give rise to metastases (T<sub>Met</sub>), lymph node metastases (N), and liver metastases (M).

Previously, we found that increasingly metastatic lines exhibited increased adhesion to galectin-3<sub>in vitro</sub> (Fig. 1A; ref. 30). As ECM molecules and their modification by BMDCs play...
important roles in the development of the metastatic niche, we asked whether galectin-3 has a role in its establishment. Livers were harvested from tumor-bearing mice before the detection of overt metastases, and examined for accumulation of galectin-3. Western blot analysis of liver protein lysates revealed an elevation in galectin-3 in the livers of tumor-bearing mice compared with naïve mice (Fig. 1B). To identify the source of this galectin, we examined both the tumor cells and recruited leukocytes. Gene expression microarray and Western blot analyses of galectin-3 in the tumor cell lines revealed equivalent expression of the molecules across all four classes of lines, indicating that galectin-3 expression by tumors is not differentially regulated during tumor progression (Supplementary Fig. S1A and S1B).

In light of recent reports of myeloid-derived populations supporting outgrowth of metastatic tumors, however, we asked whether such populations might also be presenting galectin-3 within the early metastatic niche. Staining for macrophages and galectin-3 in liver sections of mice bearing either Tumor-Bearing or M tumors but no detectable overt metastases revealed colocalization of galectin-3 with F4/80+ macrophages (Fig. 1C). Although galectin-3+ macrophages are present in the livers of tumor-free mice as well, their numbers are two to four times lower than those observed in tumor-bearing mice (Fig. 1C). Furthermore, immunostaining of the engrafted tumors also revealed a dense macrophage infiltration that colocalized with galectin-3 staining (Supplementary Fig. S1C). Although other sources of galectin-3 could include secretion directly by the tumors, these findings suggested the possibility that leukocytes might be expressing galectin-3 themselves.

**Tumor-Bearing Mice Exhibit Circulating CD11b+galectin-3+ Myeloid Cells Early in Tumorigenesis**

Given our observation of galectin-3–expressing macrophages in the metastatic niche, we asked whether tumors induce the mobilization of leukocytes expressing galectin-3. We harvested peripheral blood from naïve mice or mice bearing tumors generated by either Tumor-Bearing or M lines (Supplementary Fig. S2A–S2C) and analyzed it for the presence of galectin-3+ and CD11b+ myeloid cells. Analysis of peripheral blood cells by flow cytometry revealed a marked increase in galectin-3+ and CD11b+ leukocytes in the circulation of mice bearing both the M and Tumor-Bearing tumors (Fig. 2A–C and Supplementary Fig. S2D–S2F). Consistent with the findings of galectin-3+ macrophages in the liver, there was an enrichment in galectin-3+CD11b+ double-positive populations in the blood of tumor-bearing mice (Fig. 2A and D). In agreement with previous reports of increases in inflammatory myeloid cells in tumor-bearing mice, there was a significant increase in the frequency of CD11b+Gr-1+ and CD11b+Ly-6C+ populations (Fig. 2E and F and Supplementary Fig. S2G and S2H), which may represent neutrophils or MDSCs and inflammatory monocytes (IM), respectively, with the latter population being CD11b+ (Supplementary Fig. S2I). Comparisons of the galectin-3 levels on the CD11b+Ly-6C+ and CD11b+Ly-6C+ populations reveal 3- to 4-fold higher expression on the enriched population (Fig. 2G). In contrast, galectin-3 levels on the various subpopulations of CD11b+ leukocytes were not affected by the presence of tumors (Supplementary Fig. S3A–S3C), suggesting that there is increased mobilization of multiple galectin-3+ populations into the circulation rather than increased expression on individual leukocytes.

Galectin-3 has been reported to bind a variety of macrophage cell-surface glycoproteins (31). To determine whether the galectin-3 is bound to leukocytes through carbohydrate interactions or is presented in a manner that still permits interactions with the CRD, we isolated CD11b+ cells from the blood of tumor-bearing mice and incubated them in the presence of a competitive galectin-3 ligand, lactose, or a control, sucrose. Subsequent analysis revealed no significant difference in galectin-3 binding, suggesting that galectin-3 is presented on the surfaces of these populations in a carbohydrate-independent manner, thus preserving the availability of the CRD to interact with other glycan ligands (Supplementary Fig. S4). Taken together, these data support the hypothesis that the presence of tumors induces the mobilization of CD11b+galectin-3+ myeloid-derived cells to the blood and that these leukocytes present galectin-3 in a manner that permits additional carbohydrate interactions with the lectin.

**Tumor-Derived IL6 Mobilizes CD11b+–galectin-3+ Leukocytes into the Peripheral Blood**

Tumor-derived soluble factors frequently induce the mobilization and recruitment of prometastatic CD11b+ populations (17, 19, 20, 25, 26, 32). To determine whether tumor-secreted factors were responsible for the observed mobilization of CD11b+galectin-3+ populations, we collected conditioned media (CM) from the cell lines and injected it into the circulation of wild-type mice. Two hours following injection of the CM, we harvested peripheral blood and analyzed the populations by flow cytometry. This analysis revealed pronounced increases in circulating CD11b+galectin-3+ leukocytes and relevant subpopulations (Fig. 3A and Supplementary Fig. S5A–S5D). Again, differences in the level of galectin-3 expression on individual cells were not detectable in the peripheral blood between the two conditions (Supplementary Fig. S5E), suggesting that the elevated galectin-3 levels were due to increased mobilization of galectin-3+ populations rather than increased expression of galectin-3 by the mobilized leukocytes.

Soluble galectin-3 has been reported to act as a chemoattractant for macrophages, monocytes, and neutrophils (1, 33, 34). The galectin-3 that we have observed has primarily been presented on the surfaces of leukocytes (Figs. 1C and 2). As the tumors also secrete galectin-3, however, we asked whether galectin-3 itself is capable of inducing the rapid mobilization of CD11b+galectin-3+ leukocytes. Injections of recombinant murine galectin-3 in control medium, however, failed to elicit any increased mobilization of the CD11b+galectin-3+ populations (Fig. 3A and Supplementary Fig. S5). Nonetheless, it is possible that galectin-3 might act in combination with other secreted factors to mobilize these populations. To determine whether galectin-3 is necessary to induce mobilization, we knocked down galectin-3 in the M line using short hairpins (Supplementary Fig. S6A). Although this knockdown reduced the CM concentrations of galectin-3 by approximately 80%, there was no observable difference in the mobilization of any of the galectin-3+ leukocytes or their surface levels of galectin-3 (Fig. 3B and Supplementary Fig. S6B–S6F). Furthermore, mice bearing tumors formed by the knockdown lines exhibited no
Figure 2. Tumor-derived soluble factors induce the mobilization of galectin-3+ myeloid cells into peripheral circulation early in tumorigenesis. Wild-type mice or mice bearing T nonMet (802T4) or M (393M1) tumors were analyzed for the presence of galectin-3+ leukocytes in their peripheral blood by flow cytometry. A, analysis of staining for galectin-3 and CD11b on all leukocytes from mice with no tumors (NT), T nonMet tumors, or M tumors. Numbers represent percentages of all leukocytes that were double positive. B, percentage of all leukocytes that were galectin-3+ (Gal-3+). C, percentage of all cells that were CD11b+. D, percentage of all cells that were CD11b+ galectin-3+. E, percentage of all leukocytes that were Gr-1+. F, ratio of CD11b+Ly-6C hi cells to CD11b+Ly-6C lo cells. G, galectin-3 expression on Ly-6C lo and Ly-6C hi cells. P values in B to F were determined by one-way ANOVA with the Tukey multiple comparison test. P values in G were determined by two-way ANOVA with the Bonferroni post-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Differences in circulating galectin-3+ leukocytes in the peripheral blood or the number of galectin-3+ F4/80+ macrophages in their livers (data not shown and Supplementary Fig. S6G). Taken together, these data suggest that tumor-derived galectin-3 has no effect on the mobilization of galectin-3+ leukocytes or their presentation of galectin-3, nor does it act in an autocrine fashion on tumor cells to induce secretion of other inflammatory cytokines.

Although galectin-3 does not affect mobilization of leukocytes, we asked whether its expression by metastatic cells is necessary for seeding or colonization. To determine whether tumor-derived galectin-3 acts in a cell-autonomous manner to support growth of disseminated tumor cells, we performed experimental metastasis assays with the galectin-3 knockdown lines. Two weeks following intrasplenic inoculation of tumor cells, we queried mice for hematogenous dissemination to the liver and subsequent colonization. Analysis of GFP+ tumor nodules on livers of mice injected with the M cell line containing the shRNAs revealed no differences in metastasis formation between the control hairpin (shLuc) and the galectin-3 hairpins (Supplementary Fig. S6H), suggesting that galectin-3 production by the metastases does not affect colonization. Thus, any interactions with galectin-3 that promote metastasis likely result from its expression by stromal populations.
St6galnac4, Gcnt3, and Galectins as Drivers of Metastasis

Significantly elevated copy numbers of the IL6 gene (Fig. 3E, \( P = 5.04 \times 10^{-27} \)). Thus, we asked whether IL6 induces the mobilization of the galectin-3 leukocytes in our system. In contrast with galectin-3, injections of recombinant IL6 induced the rapid mobilization of CD11b\(^+\)galectin-3\(^+\) leukocytes and the relevant subpopulations into the circulation in a manner that mirrored CM injections (Fig. 3F and G and Supplementary Fig. S7A–S7D). To identify the set of leukocytes responsible for galectin-3 presentation, we analyzed the granulocytes, as they may represent a large portion of the mobilized CD11b\(^+\)Gr-1\(^+\) cells. Thus, we repeated the IL6 experiments in Csf3r\(^{−/−}\) (G-CSF-R) knockout mice, which exhibit impaired granulocyte production and maturation. Although IL6 was still capable of inducing CD11b\(^+\)Ly-6C\(^{hi}\) cell mobilization, Csf3r\(^{−/−}\) mice exhibited a profound reduction in CD11b\(^+\)galectin-3\(^+\) and CD11b\(^+\)Gr-1\(^+\) leukocytes compared with wild-type mice (Supplementary Fig. S8A–S8D). These findings suggest that tumor-derived IL6 is sufficient to induce mobilization of galectin-3\(^+\) leukocytes.

To determine whether galectin-3 expression by the myeloid cells or another nonparenchymal population is a prerequisite to their mobilization into circulation, we repeated the IL6 injections in Lgals3\(^{−/−}\) (galectin-3) knockout mice and observed no differences in CD11b\(^+\) myeloid cell mobilization with wild-type mice (Fig. 3H and Supplementary Fig. S9A–S9E). The relevant subpopulations also demonstrated similar mobilization (Supplementary Fig. S9A–S9D). Taken together, these data suggest that IL6, and not galectin-3, acts systemically as a factor to induce the mobilization of CD11b\(^+\)galectin-3\(^+\) leukocytes from the bone marrow and that display of galectin-3 by the BMDCs is not a prerequisite to their mobilization.
Increasingly Metastatic Cells Exhibit Elevated T-Antigen Surface Presentation That Mediates Galectin-3 Binding

Our previous studies demonstrated that cells increase their adhesion to galectin-3 as they gain metastatic potential (30). In light of the enrichment of galectin-3⁺ leukocytes in the peripheral blood (Fig. 2) and metastatic niche (Fig. 1D), we asked how the metastatic populations achieve enhanced adhesion to galectin-3. As galectins bind β-galactoside glycans through their CRD, we asked whether changes in glycan presentation by tumor cells confer their adhesion to galectin-3 on recruited leukocytes during metastasis. Analysis of glycan microarray datasets (35) and existing literature reveal a variety of carbohydrate ligands for these galectins (Fig. 4A). Although all galectins bind lactosamines, galectin-3 exhibits specific affinities for the T-Antigen (Galβ1-3GalNAcα1-O-S/T), a pan-carcinoma marker expressed on many glycoproteins (Fig. 4B; ref. 36). Galectin-3 is unable to interact with the T-Antigen if the disaccharide is further glycosylated. Thus, we asked whether metastatic cells exhibit elevated T-Antigen presentation by tumor cells confer their adhesion to galectin-3 on recruited leukocytes during metastasis. Analysis of glycan microarray datasets (35) and existing literature reveal a variety of carbohydrate ligands for these galectins (Fig. 4A).

Figure 4. Elevated T-Antigen presentation promotes increased galectin-3 adhesion in metastatic populations. A, potential carbohydrate ligands for the galectin-3 CRD. The T-Antigen (Galβ1-3GalNAcα1-0-S/T) is specific for the CRD of galectin-3 and galectin-8. N-Acetyllactosamine (LacNAc) binds the CRD of all galectins and exhibits increased affinities when in a polymeric form. The A- and B-type 2 blood group antigens have reported affinities for a variety of galectins, including galectin-3 by glycan array analysis (35). B, on normal cells, binding of galectin-3 to the T-Antigen (T-Ag) on glycoproteins is occluded as the epitope is masked by further glycan extension. On malignant cells, aberrant expression of the disaccharide without further glycosylation permits galectin-3 binding. C, peanut agglutinin (PNA) labeling of the T-Antigen on representative cell lines from the T nonMet (blue), T Met (green), and M (red) classes as determined by flow cytometry. D, binding of galectin-3 fluorophore conjugates in the presence of a glycan competitor for galectin-3, LacNAc, or control disaccharide (sucrose). E and F, human non–small cell lung cancer (NSCLC) tissue microarrays were analyzed for surface T-Antigen presentation by PNA staining. G, quantification of tissue microarray spots for PNA staining: N, noncancerous tissue; C, cancer tissue. P values in F determined by the Fisher exact test. Scale bars in C are 400 μm (100 μm for insets).
levels on their surfaces that might mediate interactions with galectin-3. Staining of T_nonMet, T_Met, and M lines with a T-Antigen–specific lectin, peanut agglutinin (PNA), revealed that the levels of T-Antigen surface presentation (20-fold increase) correlate with metastatic potential (Fig. 4C and Supplementary Fig. S10A). To determine whether metastatic cell adhesion to galectin-3 is indeed carbohydrate mediated, we incubated the cells with fluorescent galectin-3 in the presence of a competitive binder, N-Acetyllactosamine (LacNAc). Flow cytometry revealed a reduction in binding in the presence of LacNAc compared with control (sucrose), suggesting that galectin-3 adhesion to the metastatic cells is carbohydrate mediated (Fig. 4D).

To investigate whether these alterations are observed in the human disease, we stained human non–small cell lung cancer (NSCLC) tissue microarrays of 100 tissue samples with PNA to assess the presence of the T-Antigen. This staining revealed elevated levels of cell-surface T-Antigen presentation in cancerous tissue compared with noncancerous tissue, with a notable enrichment in the lymph node metastases (Fig. 4E and F and Supplementary Fig. S10B). In addition, we examined four common NSCLC cell lines for their relative expression of the T-Antigen. Flow cytometry revealed that, indeed, three of the four lines bound PNA at comparable levels to the M line, with the most invasive of the lines, A549 (37), exhibiting the highest degree of staining (Fig. 4G). Taken together, these data suggest that lung tumors bind galectin-3 through carbohydrate epitopes, such as the T-Antigen, and that this binding is mediated through a reduction in carbohydrate chain elongation.

**Differential Glycosyltransferase Expression Promotes Increased T-Antigen Presentation through Reduction of Glycan Chain Elongation and Increased Capping**

A variety of O-linked glycoproteins, such as MUC1, can exhibit profound degrees of O-glycosylation, and differential regulation of such glycoproteins can significantly alter the overall glycan presentation of cells. Thus, we asked whether increased T-Antigen expression is a result of upregulation of individual glycoproteins or altered glycosyltransferase activity. We isolated cell membrane proteins from T_nonMet, T_Met, and M lines and performed PNA lectin blots. These blots revealed a global increase in T-Antigen expression across many glycoproteins in the increasingly metastatic cells, as opposed to PNA labeling of a specific glycoprotein (Fig. 5A).

In light of this broad distribution in glycosylation, we investigated alterations in the expression and activity of glycosyltransferases. Analysis of gene expression microarrays for 216 glycosyltransferase genes did not demonstrate widespread shifts in expression, nor were there significant changes in the subset of genes that encode transferases specific for generation of the galectin-3 ligands (Supplementary Fig. S11A and S11B). Comparisons between the averages for the primary tumor and metastasis-derived cell lines, however, identified Gcnt3 and St6galnac4 as being under- and overexpressed in the metastatic lines, respectively (Fig. 5B); these alterations were confirmed by qRT-PCR in the representative cell lines (Fig. 5C, top). GCNT3 induces the addition of a β(1-6)GlcNAc to the T-Antigen, whereas St6GalNAc4 adds an α(2-6)NeuAc to the sialyl-T-Antigen (Fig. 5C, bottom). As further elongation of the core 1 disaccharide prevents binding by galectin-3 (Fig. 4B), changes in transferase activity consistent with the expression changes in Gcnt3 and St6galnac4 would promote T-Antigen presentation through prevention of branching and capping of elongation, respectively. Gene expression microarray analysis of the other cell lines of each class revealed similar trends for these and analogous transferases (Supplementary Fig. S12). Consistent with the fact that generation of the T-Antigen occurs in the Golgi before subsequent glycosylation (38), no difference in T-Antigen blotting was visible between whole cell lysates of T_nonMet and M lines (Supplementary Fig. S13A), further confirming that differences in its presentation are due to decreased glycan extension rather than increased production of the disaccharide.

To determine the relevance of the capping and branching transferases to human disease, we again examined copy number of the GCNT3 gene in the human TCGA (The Cancer Genome Atlas) lung cancer datasets. This analysis revealed a decrease in copy number in lung adenocarcinoma compared with normal blood or lung (Fig. 5D, P = 1.44 × 10^{-11}). In addition, we examined gene expression of the relevant glycosyltransferase genes in the four human NSCLC cell lines that exhibited varying degrees of PNA staining. Although minimal differences in expression were observed for the transferases that give rise to the core disaccharide (CIGALT1 and CIGALT2), there were marked variances across the lines for the genes encoding transferases that produce the branched and capped structures (Supplementary Fig. S13B). These data suggest that alterations in glycosyltransferase activity that regulate capping or branching of the T-Antigen may also pertain to the human disease.

**Regulation of Gcnt3 and St6galnac4 Mediates Galectin-3 Adhesion In Vitro and Metastasis In Vivo**

To test the functional role of these transferases on galectin-3 binding and metastasis, we transfected the metastatic cells with Gcnt3 or knocked down St6galnac4 with short hairpins. Transfection of Gcnt3 resulted in decreased T-Antigen presentation and decreased galectin-3 binding, and, in a complementary fashion, knockdown of St6galnac4 yielded a reduction in galectin-3 binding, while having no effect on nonsialylated T-Antigen presentation (as PNA does not recognize sialylated forms of the antigen; Fig. 6A and Supplementary Figs. S14A–S14E, S15A, and S15B). To determine the functional role of St6GalNAc4 activity on metastasis in vivo, we performed experimental metastasis assays with the St6galnac4 knockdown line. Cells expressing a hairpin for the sialyltransferase or a control hairpin against firefly luciferase were injected into the spleens of mice and monitored for liver tumor formation following hematogenous dissemination. We found that mice injected with the 393M1-shSt6galnac4 cells exhibited more than 95% fewer liver metastases than control mice 2 weeks following tumor cell injection (Fig. 6B and C and Supplementary Fig. S16). We observed no differences in proliferation rates between the knockdown and control hairpin cell lines in vitro (Supplementary Fig. S17). Taken together, these data suggest that alterations in glycosyltransferase activity observed in increasingly metastatic cells have a functional impact on the metastatic potential of the tumor cells and support the hypothesis that presentation of the T-Antigen, through the
DISCUSSION

A variety of molecules have been demonstrated to play important roles in colonization of the early metastatic niche through their systemic secretion and by interacting with recruited CD11b+ leukocytes (14–19). The identification of direct interactions between these leukocytes and tumor cells within the blood vessels and colonized tissues suggests a role for a conserved adhesion receptor–ligand axis during metastatic progression. Here, we identify galectin-3 as a novel regulator of lung adenocarcinoma metastasis through its presentation within the metastatic niche and binding to the oncofetal disaccharide known as the T-Antigen (Fig. 7). On the basis of our findings, we propose that early in tumorigenesis, tumors secrete soluble factors, such as IL6, that induce the mobilization of CD11b+galectin-3+ BMDCs into circulation. In addition, galectin-3 is presented on macrophages in the early metastatic niche of tumor-bearing mice. As tumors gain metastatic potential, they increase their presentation of the T-Antigen on their surfaces. In the context of our lung adenocarcinoma mouse model, we show that this elevated presentation does not result from increased O-glycosylation through core 1 glycosyltransferases, but rather is due to the abrogation of core 1 chain extension conferred by down-regulation of GCNT3 and capping of the motif by the α2–6 sialyltransfer and St6galnac4. These changes in transferase activity and T-Antigen presentation result in increased carbohydrate-dependent binding of the tumor cells to galectin-3 and increased metastasis in vivo. As the presence of tumors induces elevated numbers of galectin-3+ myeloid cells in the circulation and metastatic niche, these alterations in transferase activity within the tumor cells may represent an essential mechanism by which metastatic cells interact with these protumorigenic leukocytes at those sites. Furthermore, perturbations in expression of these transferases are correlated with humans with NSCLC (Fig. 5 and Supplementary Fig. S13B), and the T-Antigen is frequently overexpressed on BMDCs into circulation and metastatic niche, these alterations in regulation may extend to many clinical settings.

Figure 5. Metastatic cells downregulate Gcnt3 and upregulate St6galnac4. A, lectin blot of surface proteins. Cell-surface proteins were isolated from representative T nonMet (393T4), T Met (393T5), and M (393M1) lines and run on SDS-PAGE gels. Membranes were blotted for the T-Antigen with PNA. B, gene expression microarray analysis of all glycosyltransferases. The average expression for all primary tumor-derived lines (ordinate) is plotted against the average expression for all metastatic lines (abscissa). Size of points represents the absolute difference in expression between the clonally related T nonMet and M lines (393T5 and 393M1, respectively). The color represents statistical significance of the differences between any two adjacent cell line classes (i.e., T nonMet vs. T Met , T Met vs. N, N vs. M) as determined by the Student t test. The dashed line represents equivalent expression between the metastatic and primary tumor-derived lines. C, qRT-PCR analysis of transferase gene expression. Top, gene expression of Gcnt3 and St6galnac4 in representative T nonMet, T Met , and M cell lines. Bottom, GCNT3 can transfer a GlcNAc to the core GalNAc of the T-Antigen (T-Ag) by a β1→6 linkage. St6galnac4 can transfer a NeuAc to the core GalNAc of the sialyl-T-Antigen. D, GCNT3 exhibits a loss of copy number in human lung adenocarcinomas compared with normal lung tissue or blood (P = 1.44 × 10^{-11}) in the “lung adenocarcinoma” dataset available from the TCGA website (54). P values in C were determined by one-way ANOVA with the Tukey multiple comparison test. * P < 0.05; ** P < 0.01; *** P < 0.001.

prevention of further glycosylation, promotes adhesion of tumor cells to galectin-3 in the metastatic niche.
local microenvironments in a manner that permits colonization (11). Additional studies have suggested that early, or even “pre-,” metastatic niches may exist wherein a protumorigenic microenvironment is generated (12). Such microenvironments harbor inflammatory leukocytes that act to promote metastatic colonization (11, 13, 40). Our findings on the mobilization and recruitment of these inflammatory populations by nonmetastatic primary tumors are consistent with the hypothesis that involvement of prometastatic stromal populations is, at least in part, an early event. Thus, adaptations by the metastatic tumor cells that potentiate interactions with these leukocytes may represent the rate-limiting events permitting colonization. Here, we find that alteration in glycosylation that results in T-Antigen presentation is one such event in that it occurs later in tumor progression and promotes interactions with galectin-3 in the metastatic niche.

A variety of studies have implicated galectin-3 in tumor progression and metastasis (1). The majority of these studies have demonstrated strong clinical correlations with advanced-stage disease or decreased survival with elevated levels of galectin-3 (4, 5). In agreement with these clinical reports, our model also demonstrated elevated serum levels of galectin-3 in mice bearing tumors (Supplementary Fig. S18). Experimental studies have primarily investigated the roles of galectins in modulating the immune system (3) or in interactions with blood vessels. In addition to its reported role in promoting angiogenesis (41), surface expression of galectin-3 on endothelial cells has been suggested to promote the adhesion of breast and prostate cancer cells to the endothelium through carbohydrate interactions (7). Here, we suggest an additional role for galectin-3 in metastasis in that it is presented within the early metastatic niche on recruited metastasis-associated myeloid cells. Thus, galectin-3 may act not only to promote adhesion to endothelium but also to promote interactions between the cancer cells and BMDCs within the tissues or within circulation in a manner that promotes not only extravasation but also colonization.

An abundance of clinical and experimental evidence has suggested a role for IL6 in cancer metastasis (19, 21, 42–44). Tumor-derived soluble factors, including a variety of ECM molecules such as laminins and versican (19), have been reported to act upon macrophages, through Toll-like receptors, to induce the production of IL6. Here, we find that lung tumors themselves produce IL6 independent of the presence of myeloid cells. Our conditioned medium experiments suggest that soluble factors such as IL6 are capable of inducing the rapid mobilization of CD11b+Ly6C− and CD11b+Gr-1+ myeloid cells into circulation, which are thought to promote metastasis. Other molecules traditionally considered to be produced by myeloid cells, such as CCL2,
Sialyltransferases prevent glycan elongation and promote preservation of the T-Antigen, which, in turn, mediates galectin-3 adhesion.

Presentation, which is mediated by aberrant glycosyltransferase activity. Decreased expression of the branching transferases and increased expression of the liver early metastatic niche where it is presented on macrophages. Metastatic cells adhere to galectin-3 and galectin-8 through increased T-Antigen.

Figure 7. T-Antigen (T-Ag) presentation promotes metastasis through interactions with galectins in the metastatic niche. Schematic model of galectin-glycan interactions in lung cancer metastasis. Primary lung tumors have high expression of branching glycosyltransferases such as GCT3 and low expression sialyltransferases such as ST6GalNAc4 that act to promote O-glycan branching and elongation. Both the primary and metastatic tumors secrete tumor-derived soluble factors, such as IL6, which act to mobilize CD11b+galectin-3+ myeloid cells into the peripheral blood. Galectin-3 levels become elevated in the liver early metastatic niche where it is presented on macrophages. Metastatic cells adhere to galectin-3 and galectin-8 through increased T-Antigen presentation, which is mediated by aberrant glycosyltransferase activity. Decreased expression of the branching transferases and increased expression of sialyltransferases prevent glycan elongation and promote preservation of the T-Antigen, which, in turn, mediates galectin-3 adhesion.

METHODS

Cell Lines and Mouse Transplantation Assays

Murine cell lines (a gift from Tyler Jacks and Monte Winslow, Massachusetts Institute of Technology, Cambridge, MA) were described previously (28) and were cultured in DMEM with 10% (vol/vol) FBS and 1% l-glutamine. The cell lines were used within five passages and were tested frequently for consistency in gene expression by qRT-PCR. No additional authentication was performed. All cell lines were tested for pathogens by PCR by the MIT Division of Comparative Medicine during the time period in which they were being used. The
human NSCLC lines were provided by the NCI through the Division of Cancer Treatment and Diagnosis Tumor Repository. The repository authenticates the lines and tests all cell lines for pathogens by PCR before distribution. Experiments were performed within five passages.

All animal procedures were performed in accordance with the MIT Institutional Animal Care and Use Committee under protocol 0211-014-14. Cell injection studies were performed in female B6129SF1/J mice 6 to 10 weeks of age (The Jackson Laboratory; Stock Number 101043). In addition, IL6 experiments were performed in B6.129 × 129SvEv (C57BL/6J) and B6.129S1-Galectin3tm1Poi/J mice (The Jackson Laboratory; Stock Numbers 017996 and 060532) with C57BL/6J mice as controls (The Jackson Laboratory; Stock Number 000664). For CD11b mobilization experiments, 3 × 10^5 cells resuspended in 100 μL of PBS were injected into the subcutaneous region of flanks of mice anesthetized with isoflurane. Tumors and peripheral blood were harvested 5 to 8 weeks following injections. Approximately 1 mL of peripheral blood was harvested by cardiac puncture, while mice were anesthetized with isoflurane, and used for analysis by flow cytometry. Mice were immediately euthanized following recovery of the blood, and their tumors were harvested. Intraspinal transplantation assays were performed as previously described (30). Briefly, 5 × 10^5 cells were resuspended in 100 μL of PBS. Animals were anesthetized with 2.5% isoflurane and administered buprenorphine (150 μg/kg). Fur was removed with surgical clippers, and the incision site was sterilized with Betadine and 70% ethanol. The spleen was exposed through a small incision, and cells were injected into the tip of the spleen with a 27-gauge needle. The cells were allowed to travel through the circulation for two minutes before removal of the spleen. The splenic vessels were then cauterized and the entire spleen was removed. The muscle wall was closed with 5-0 dissolvable sutures, and the skin was closed with 7-mm wound clips (Roboz). Mice were euthanized 2 weeks following injection, and their livers were excised. GFP+ tumors were visualized and quantified using an epifluorescence dissection scope (Nikon). Tissues were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin following paraffin embedding.

**Immunohistochemical and Immunocytochemical Analysis**

Following necropsy, tumors and livers were fixed overnight in 4% paraformaldehyde at 4°C. Tissues used for paraffin embedding were transferred to cassettes and placed in 70% ethanol. Those used for frozen sections were placed in 30% sucrose overnight at 4°C. Tissues were then transferred to optimal cutting temperature (OCT) compound (Tissue-Tek) for 4 hours at room temperature followed by freezing in isopentane (Sigma) placed in a liquid nitrogen bath. Containing of galectin-3 and F4/80 was performed on frozen sections using anti-CD11b (AbD Serotec, 1:1,000), anti-Gr-1 (BioLegend), and anti-ε-tubulin (Cell Signaling Technology, 2125, 1:1,000). Lectin blotting for glycans was performed using PNA-Biotin (Sigma) followed by reaction with peroxidase conjugates (Sigma). Ponceau S solution (Thermo) was used for total protein detection following transfer to PVDF membranes.

For liver homogenate Western blots, mice bearing 393M1 flank tumors or no tumors were perfused by intracardiac injection of 20 mL of PBS. Livers were then excised and 50-mg portions were added to gentleMACS M tubes (Miltenyi Biotech) in 4.5 mL of RIPA (Sigma) with Complete Protease Inhibitors (Roche). Tissue was dissociated using the gentleMACS Octo Dissociator (Miltenyi Biotech) and run on polyacrylamide gels as described above.

**Galectin-3 ELISA**

Peripheral blood was harvested from mice without tumors or bearing contralateral flank tumors of both 802T4 and 393T5 cell lines by cardiac puncture as described above. Blood was collected in serum collection tubes (Capiject) and allowed to clot for 30 minutes before centrifugation and removal of serum. The galectin-3 ELISA Kit (R&D Systems) was used according to the manufacturer’s instructions.

**Cell-Surface Protein Isolation**

Cell-surface proteins were isolated before Western blot analysis using Thermo Scientific kit 89881 following the manufacturer’s instructions. Briefly, cells were washed and incubated with biotinylated antibodies to the surface proteins by a Sulfo-NHS-Biotin conjugation to free amines. The reaction was quenched and cells were harvested and lysed. Lysate was washed through a column containing a neutravidin resin to capture the biotinylated surface proteins. Finally, the disulfide linkage was reduced through incubation with 50 mM/L DTT, and the proteins were captured.

**Flow Cytometric Analysis**

Peripheral blood was drawn by cardiac puncture and transferred to K2 EDTA 5.4-mg Plus Blood Collection Tubes (BD Vacutainer; BD Biosciences). Red blood cells were lysed using red blood cell (RBC) lysis buffer as described above. Cells were fixed in 4% paraformaldehyde and washed before staining. Cells were blocked using anti-mouse CD16/CD32 antibody clone 2.4G2 (BD Biosciences). Antibodies against mouse antigens were as follows: CD11b (M1/70; BD Biosciences), CD115 (AF589; eBiosciences), Mac-2 (M3/8; BioLegend), Ly-6C (HK1.4; BioLegend), and Gr-1 (RB6-8C5; BioLegend). Flow cytometry was performed on an LSR Fortessa (BD Biosciences) and analyzed using FlowJo (Tree Star).

Magnetic separation of CD11b+ cells was performed using CD11b+ MACS microbeads (Miltenyi Biotech). Following collection of peripheral blood by cardiac puncture and lysis of erythrocytes, cells were incubated with the MACS beads following the manufacturer’s instructions. CD11b+ cells were isolated using MACS MS Collection Columns (Miltenyi Biotech). PNA staining was performed by incubating 3 × 10^5 cells in PBS with 10 μL PNA-AlexaFluor 647 (Invitrogen) on ice for 30 minutes. Fluorescent galectin-3 was produced by reacting recombinant murine galectin-3 (R&D Systems) with DyLight 650 NHS Ester (Thermo Fisher). Conjugated protein was purified by fast protein liquid chromatography and concentrated to 0.5 mg/mL using 3,000 MWCO (molecular weight cut-off) Amicon centrifugal filter units (Millipore). Cells were stained by incubating 3 × 10^5 cells in PBS with 10 μL of the fluorescent galectin-3 on ice for 30 minutes.
For galectin-3 binding inhibition experiments on tumor cells, cells were incubated with galectin-3, as described above, in the presence of 20 mmol/L N-Acetyl-D-lactosamine (Carboxyls) or sucrose (Sigma). For competition of galectin-3 presentation on leukocytes, peripheral blood was harvested from mice bearing 393M1 flank tumors, as described above. Cells were incubated with 200 mmol/L β-lactose (Sigma) or sucrose (Sigma) in 2% FBS on ice for 30 minutes. Cells were then fixed, stained, and analyzed by flow cytometry.

Conditioned Media Experiments

Twenty-five milliliters of conditioned medium was harvested from one T150 flask of 393M1 cells following 3 days of culture. This medium (or fresh control medium with serum) was filtered through 0.2-μm filters and concentrated 15-fold using 3,000 MWCO centrifugal filters (Amicon). Medium (200 μL) was injected into the lateral tail vein of mice. Two hours after injections, peripheral blood was harvested from the mice by cardiac puncture, as described above, followed by fixation, staining, and flow cytometric analysis. For experiments involving recombinant galectin-3, murine recombinant galectin-3 (R&D Systems) was supplemented to the medium at 1 ng per injection. For IL6 experiments, murine recombinant IL6 (R&D Systems) was supplemented to the medium at 50 ng per injection.

Luminex Cytokine Profiling

Cytokine profiles of 100 μL cell culture supernatant from control and CM were assessed using an EMD Millipore Milliplex custom magnetic bead panel using the manufacturer’s protocol: IL2, IFNγ, IL10, TNFα, IL17α, IL4, IL5, IL6. Luminex assays were analyzed using Bioplex 200 with Bioplex Manager 6.1.

RNA Isolation and Expression Profiling

Murine gene expression microarray analysis was described previously (28, 30) and is available from NCBI under accession numbers GSE4022 and GSE26874. Human NSCLC expression microarray data were accessed from NCBI accession number GSE32474. RNA was isolated using RNeasy mini kits (Qagen) according to the manufacturer’s instructions. cDNA reactions were performed using the iScript cDNA synthesis kit (Bio-Rad). qPCR reactions were performed using 2 μL of cDNA, 12.5 μL of iQ SYBR Green Supermix (Bio-Rad), and 1 μmol of each primer. Fold change is reported following analysis using the ΔΔCt method, where genes were normalized to the Hprt housekeeping control. Primers were as follows: St6galnac4 forward 5′-CGCTTGGTTGTACCATGATTCTG-3′, St6galnac4 reverse 5′-GAGGGGCGGAGATCTTCTC-3′, Gent3 forward 5′-GCGACAAAGGTTCACCAAAG-3′, Gent3 reverse 5′-ACACAGCGGAGGACCATCAAA-3′ or forward 5′-ATAGGAGGCCATGTTGCCGAGCT-3′ reverse 5′-CTCTTGGTGCAGAGGTTGAT-3′, Hprt forward 5′-GTCACGCGGGAGGCTAAAACG-3′, Hprt reverse 5′-CAACATCAACGATCTCTTAC-3′. Gene expression analysis and visualizations were performed using Spotfire (Tibco) and MATLAB (Mathworks).

Human Copy-Number Analysis

Copy-number determinations of Gcnt3 and IIL6 were performed using Oncomine (S3) based on publicly available data from TCGA (S4).

Hairpins and Plasmids

Knockdown of St6galnac4 was performed using shRNA (5′-TTCGCTCTCTCACACTGTGACATCTGACA-3′) or a control hairpin targeting firefly luciferase in the pGFP-V-RS vector (Origene; TG502032 and TRJ0002). Plasmids were packaged into retroviruses using the Phoenix-Eco system. Viral supernatant was filtered using 0.45-μm syringe filters and cultured with 393M1 cells at no dilution. Transduced cells were selected with puromycin (Invitrogen), and knockdown efficiency was determined by qRT-PCR following 2 weeks of culture. Transfection of Gent3 was performed using Lipofectamine (Invitrogen) according to the manufacturer’s instructions with plasmids purchased from Origene (MC215765). Knockdown of Lgals3 was performed using two shRNAs (Sigma; shGal3-3: 5′-CCGGCGCATGACACCACTGCGTACATCGGTTCATCCGACATGTTGATGTTGACGAGCTGCGGTTTGGTTTG-3′; shGal3-5: 5′-CCGGCGCGCATGCTGACACCACTGCGTACATCGGTTCATCCGACATGTTGATGTTGACGAGCTGCGGTTTGGTTTG-3′) or a control hairpin targeting firefly luciferase (Sigma; SHC007V). In addition, a second hairpin system was used to knockdown St6galnac4 from Sigma (shSt6galnac4-2: CCGGTTACACCTTCACGACTGCGAGATTGCGTTTCTGAGTGAAGTGTACCTTTTT).

In Vitro Proliferation Assays

Cells expressing hairpins against St6galnac4 or control hairpins were seeded into 24-well plates. New wells were trypsinized and counted using a hemocytometer with Trypan Blue exclusion at 24-hour intervals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: N.E. Reticker-Flynn, S.N. Bhatia Development of methodology: N.E. Reticker-Flynn Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.E. Reticker-Flynn, S.N. Bhatia Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.E. Reticker-Flynn, S.N. Bhatia Writing, review, and/or revision of the manuscript: N.E. Reticker-Flynn, S.N. Bhatia Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.E. Reticker-Flynn, S.N. Bhatia Study supervision: S.N. Bhatia

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