An In Vivo Functional Screen Identifies ST6GalNAc2 Sialyltransferase as a Breast Cancer Metastasis Suppressor

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ABSTRACT
To interrogate the complex mechanisms involved in the later stages of cancer metastasis, we designed a functional in vivo RNA interference (RNAi) screen combined with next-generation sequencing. Using this approach, we identified the sialyltransferase ST6GalNAc2 as a novel breast cancer metastasis suppressor. Mechanistically, ST6GalNAc2 silencing alters the profile of O-glycans on the tumor cell surface, facilitating binding of the soluble lectin galectin-3. This then enhances tumor cell retention and emboli formation at metastatic sites leading to increased metastatic burden, events that can be completely blocked by galectin-3 inhibition. Critically, elevated ST6GALNAC2, but not galectin-3, expression in estrogen receptor–negative breast cancers significantly correlates with reduced frequency of metastatic events and improved survival. These data demonstrate that the prometastatic role of galectin-3 is regulated by its ability to bind to the tumor cell surface and highlight the potential of monitoring ST6GalNAc2 expression to stratify patients with breast cancer for treatment with galectin-3 inhibitors.

SIGNIFICANCE: RNAi screens have the potential to uncover novel mechanisms in metastasis but do not necessarily identify clinically relevant therapeutic targets. Our demonstration that the sialyltransferase ST6GalNAc2 acts as a metastasis suppressor by impairing binding of galectin-3 to the tumor cell surface offers the opportunity to identify patients with breast cancer suitable for treatment with clinically well-tolerated galectin-3 inhibitors. Cancer Discov; 4(3): 304–17. ©2014 AACR.

See related commentary by Ferrer and Reginato, p. 275.
INTRODUCTION

When breast carcinomas remain confined to breast tissue, cure rates exceed 90% (http://seer.cancer.gov/csr/1975_2006/). However, if the cancer disseminates throughout the body, long-term survival decreases depending upon the extent of, and the sites of, colonization. Metastases in visceral organs and the brain are the most life threatening, with 5-year survival rates usually less than 20% (1). There is an urgent need to identify genes that control the different stages of the metastatic process in order to aid in the development of metastatic biomarkers and provide potential targets for the treatment and prevention of metastatic disease (2).

Genetic screens, such as those that exploit RNA interference (RNAi), provide an unbiased approach to the identification of genes associated with a phenotype of interest (3–6). Although cell-based RNAi screens have been highly informative in identifying genes involved in tumor cell survival, migration, and invasion (4, 7–9), these in vitro approaches are largely unsuitable for interrogating the later stages of the metastatic process, in particular tumor cell dissemination, tumor cell extravasation from the circulation, and colonization of secondary sites. More recent RNAi screens performed in animal models have provided important new insights into in vivo tumor biology (3–6); however, there has, to date, been only one published in vivo RNAi...
screen to identify novel determinants of the metastatic process in solid tumors (5). In contrast to this published study, which used a zero-event model, we have developed an in vivo metastasis short hairpin (sh) RNAi screen combined with massive parallel sequencing to identify enrichment of novel determinants involved in the later stages of breast cancer metastasis. Using this functional approach, we have identified the sialyltransferase ST6GalNAc2 as a novel and clinically relevant metastasis suppressor and uncovered the mechanism by which it promotes tumor cell colonization of secondary tissues.

RESULTS
An In Vivo Screen for Genes that Modulate Metastasis

The aim of this study was to develop an in vivo RNAi screen focused on identifying genes involved in the later stages of the metastatic process. A major benefit of undertaking a pooled shRNA screen combined with next-generation sequencing is the ability to determine the change in representation of the different shRNAs in the resulting metastatic tumors. The 4T1 mouse mammary tumor cell line inoculated intravenously into syngeneic BALB/c mice was used for the RNAi screen, as it provides a robust model of tumor cell colonization in an immunocompetent setting. As opposed to a zero-event model, this approach allowed us to identify individual shRNAs enriched in the metastatic tumors due to their ability to confer an advantage to tumor cells in the later stages of the metastatic cascade.

In brief, the screen protocol was as follows (Fig. 1A): mouse mammary 4T1 tumor cells carrying a luciferase expression construct (4T1-Luc) were infected with pools of virally packaged shRNA-coding constructs of the mouse Cancer 1000 shRNA library, encompassing ∼1,000 genes (10). Cells infected with library pools were inoculated via the tail vein into groups of 3 BALB/c mice, and, 21 days later, tumor-bearing lungs were recovered. Genomic DNA (gDNA) was isolated from the tumor-bearing lungs and from the preinoculation shRNA-infected 4T1-Luc cells, PCR amplified, and subject to massively parallel sequencing. To determine the extent of shRNA enrichment in each tumor-bearing lung sample, and thus the contribution of each gene-silencing effect to the metastatic process, shRNA frequency in the lung samples was compared with shRNA-infected, preinoculation 4T1-Luc cells, and the preinoculation 4T1-Luc cells were compared to the original shRNA plasmid preparation.

The sensitivity of pooled RNAi screens is in part determined by the complexity of the shRNA pools used (11). To determine the optimal pool size, uninfected 4T1-Luc cells (Luc+, GFP−) and 4T1-Luc cells infected with control shRNA constructs (Luc+, GFP−) were mixed at defined ratios, and 0.5 × 10^6 cells were inoculated into the tail vein of mice. Twenty-one days later, bioluminescent imaging indicated an equivalent level of lung tumor burden in the different groups (Fig. 1B). By counting the number of superficial GFP+ nodules, it was calculated that the lungs contained a minimum of 500 independent metastatic tumors. Furthermore, the observation that the GFP+ nodules in the 1:20 and 1:50 dilution samples were scattered evenly throughout the lungs suggested that the majority of tumors arose from individual inoculated cells rather than from locally invading/reseeded lung tumors. To generate statistically powered data, we aimed for at least a 10-fold greater number of lung tumors than number of shRNAs in each pool. Consequently, the in vivo screen was performed with 48 pools of the Cancer 1000 library, each containing 48 different shRNA constructs.

On the basis of the criteria described in Methods, 12 hits were identified from the screen (Fig. 1C). To validate this screening strategy, shRNAs targeting four of these genes (denoted in bold in Fig. 1C) were individually retested for their ability to promote tumor-cell colonization of the lung. To mitigate for shRNA off-target effects, cells were also infected with independent targeting shRNAs or non-targeting control (NTC) shRNAs. In each case, targeting shRNAs efficiently silenced gene expression and, when inoculated into mice, the shRNA knockdown cells significantly increased lung tumor burden as monitored by IVIS (in vivo imaging) system imaging (Fig. 2A; Supplementary Figs. S1A and S1B). ST6GalNAc2 as a Novel Metastasis Suppressor in Mouse and Human Breast Cancer Models

Of the hits, ST6GalNAc2 (alpha-N-acetylglalactosamidase alpha-2,6-sialyltransferase 2) was subjected to further investigation. ST6GalNAc2 is a type II transmembrane Golgi-localized enzyme that catalyzes the attachment of sialic acid in an α2-6 linkage to N-acetylgalactosamine (GalNAc) on O-glycans (12). Although it is well recognized that tumor cells display altered cell surface sialylation (13, 14), little is known about how variant sialylation can modulate tumor cell behavior and, in particular, altered metastatic potential in vivo. 4T1-Luc cells infected with shRNAs targeting St6galnac2 displayed an increased lung tumor burden as assessed by in vivo and ex vivo IVIS imaging and by lung weight (Fig. 2A). Histologic and quantitative PCR (qPCR) analysis confirmed the presence of tumors throughout the lungs with no evidence of lung edema (Fig. 2A, right; Supplementary Fig. S2B) and the retained downregulation of St6galnac2 expression in vivo (Supplementary Fig. S2C). Furthermore, St6galnac2 downregulation in 4T1-Luc cells (Fig. 2B) and parental 4T1 cells (Supplementary Fig. S2D) resulted in increased secondary site colonization in a spontaneous metastasis assay while having no effect, at any time point examined, on primary tumor growth in vivo or on cell viability and colony-forming ability in vitro (Supplementary Fig. S2E and S2F). This increased tumor burden following St6galnac2 silencing was not specific to the lung. Three weeks after intrasplenic inoculation, 4T1-Luc cells with downregulated St6galnac2 expression gave rise to a significantly increased tumor burden in the liver as monitored by ex vivo IVIS imaging, liver weight, and histologic analysis (Fig. 2C).

To independently and directly validate the metastasis-suppressive effect of ST6GalNAc2, an alternative gain-of-function approach was taken. St6galnac2 was stably transfected into human MDA-MB-231 breast cancer cells, which have very low endogenous ST6GALNAC2 expression (Supplementary...
An In Vivo Functional RNAi Late-Stage Metastasis Screen

Next, to address the clinical relevance of our in vivo models, ST6GalNAc2 expression was analyzed in the human breast cancer datasets present in the ROCK database (15). In 9 of 10 gene expression datasets examined, significantly lower levels of ST6GalNAc2 expression were associated with estrogen receptor-negative (ER–) breast tumors compared with estrogen receptor-positive (ER+) breast tumors (Fig. 3A). Similarly, ST6GalNAc2 expression was significantly lower in ER– compared with ER+ breast cancer cell lines (Fig. 3A, final). To assess whether ST6GalNAc2 expression affects survival outcome in ER– patients, a meta-analysis was performed across the 10 clinical datasets and in two additional datasets of ER– breast cancers (ref. 16; total of 551 patients). Within these ER– cohorts, higher expression of ST6GalNAc2 significantly correlated with improved survival outcome [overall rate ratios (RR), 0.67; 95% confidence interval (CI), 0.48–0.93; P = 0.017; Fig. 3B].

ST6GalNAc2 Silencing Promotes Galectin-3 Binding and Retention of Tumor Cells in the Lung

To interrogate the mechanisms linking variant sialylation to increased metastasis, we first undertook matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) glycomic analysis of the cell surface O-glycans isolated from cells transfected with siRNA oligonucleotides targeting St6galnac2 (siST6) or NTC siRNA oligonucleotides (siNTC; Supplementary Fig. S4A). Examination of the spectra (Fig. 4A) revealed that St6galnac2 silencing resulted in changes in the relative abundance of the O-glycans, compared with control siNTC-transfected cells, of which the main characteristics were as follows: (i) an increase in unmodified core 1 O-glycan (also known as T antigen; Galβ1-3GalNAc; m/z 534) and (ii) a reduction in the α2-3,α2-6 disialyl core 1 O-glycan (m/z 1,256). Interestingly,

Figure 1. In vivo shRNA screening strategy. A, schematic of high-throughput screening (HTS). B, 4T1-Luc cells infected with control shRNAs (Luc+ , GFP−) were mixed with uninfected 4T1-Luc cells (Luc−; GFP+) at the indicated ratios. A total of 0.5 × 106 cells were injected into the tail veins of BALB/c mice (3 mice/group). On day 21, animals were IVIS imaged (left) and bright field imaging of the lungs (middle) shows equivalent tumor burden in all animals. Right, GFP+ lung metastases (arrowheads). C, table showing the screen hits. As described in Methods, the log ratio (Z score) of tumor to preinoculation 4T1-Luc cells was calculated individually for mouse A, B, and C. Shown in red are Z scores >2, shown in bold are hits that were taken forward for in vivo validation.
Figure 2. Validation of St6GnNAc2 as a metastasis suppressor gene. A, 0.5 × 10^6 4T1-Luc cells infected with NTC shRNA (shNTC-A) or St6gainac2 shRNAs (shST6-A, shST6-B; see Supplementary Fig. S2A for qPCR analysis) were inoculated into the tail veins of BALB/c mice. On day 21, lungs removed at necropsy were subjected to ex vivo IVIS imaging and weighed. Data shown are from 5 or 6 mice per group ±SEM. Student t test was used to generate P values. There was no statistical difference between the shST6-A and shST6-B groups (P = 0.48, IVIS; P = 0.19, lung weight). Middle, ex vivo bioluminescent images of excised lungs. Right, representative histologic sections illustrating the increase in lung tumor burden in the shNTC mice and the absence of detectable lung edema (see Supplementary Fig. S2B). Scale bar, 1 mm. Equivalent results were obtained in three independent experiments; for example, see Fig. 7C, B, 1.5 × 10^6 4T1-Luc cells were inoculated in female BALB/c mice. Primary tumor volume was measured until tumors reached the maximum allowable size (day 39) P = ns (not significant) at all time points. Metastases were quantified as percentage of tumor area in the lung from hematoxylin and eosin (H&E) sections. Data shown are from 7 animals in each group ±SEM. Two-way ANOVA used to generate P values. (continued on following page)

no core 2 glycan structures were detected in either siNTC or siST6 cells; tandem mass spectrometry (MS-MS) analysis (data not shown) revealed that the ions at m/z 983, 1,187, 1,344, and 1,793 corresponded to core 1 O-glycan structures extended with LacNAc residues. This lack of core 2 glycans is consistent with our gene expression profiling, in which 4T1 cells showed negligible expression of the core 2 β1-6 N-acetylgalactosaminyltransferases (C2GnTs) that are required for the addition of GlcNAc in a β1-6 linkage to core 1 (data not shown).

Given the increase in unmodified core 1 O-glycan (T antigen) following St6galnac2 silencing, we next investigated whether loss of St6galnac2 expression resulted in increased binding of galectin-3. Galectin-3 belongs to the family of soluble S-type lectins with β-galactoside binding specificity (17) and has been reported to bind core 1 glycan present on tumor cell surface glycoproteins (18, 19). Little binding of recombinant galectin-3 (GST-GAL3) was observed in the siNTC-treated 4T1-Luc cells, but cell surface binding was strongly enhanced following St6galnac2 downregulation (Fig. 4B). As galectin-3 can promote tumor cellendothelial cell interactions (18–22), the physiologic relevance of ST6GalNAc2-modulated O-glycosylation was then assessed in an in vivo lung retention assay that monitors tumor cell arrest in the vasculature. siST6- and siNTC-transfected 4T1-Luc cells (Supplementary Fig. S4A) or shST6 and shNTC 4T1-Luc cells were labeled with CellTracker red or green dyes and inoculated in a 1:1 ratio into mice via the tail vein, and lungs were harvested at 1 or 24 hours for examination by confocal microscopy. One hour after inoculation, there was no significant difference in the number of cells present in the lungs, but at 24 hours, there was a significant increase in the retention of siST6 (Fig. 5A) or shST6 (Fig. 5B) cells compared with the siNTC and shNTC cells. Importantly, in a rescue experiment, this increased lung retention was abrogated by expression of human ST6GALNAC2 (Fig. 5C and Supplementary Fig. S4B and S4C), directly eliminating off-target effects of the shRNAs.

Next, to address whether increased cell surface galectin-3 promotes the observed siST6-mediated tumor cell seeding in the lungs, 4T1-Luc cells were cotransfected with siRNA oligonucleotides targeting St6galnac2 and Lgal3 (galectin-3, siGAL3; Supplementary Fig. S4D). siGAL3 cotransfection effectively impaired the siST6-mediated increase in tumor cell retention in the lungs, but had no impact on retention of siNTC-transfected cells (Fig. 5D; Supplementary Fig. S4E).

To validate these observations, these assays were extended to three human breast cancer cell lines: MDA-MB-453 and
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**Figure 2.** (Continued) C, $0.5 \times 10^6$ shNTC-A or shST6-A 4T1-Luc cells were inoculated into the spleen of BALB/c mice. On day 21, livers were weighed at necropsy and subjected to ex vivo IVIS imaging. Data shown are from 5 or 6 mice per group $\pm$ SEM. Student $t$ test was used to generate $P$ values. Representative images of liver metastases are shown. Scale bar, 250 $\mu$m. D, $2 \times 10^6$ MDA-MB-231-Luc cells expressing ectopic St6galnac2 (231-ST6) or vector-alone infected (231-Vec) were inoculated into the tail veins of C57BL/6 NOD SCID mice. At 5 weeks, lungs were subjected to ex vivo IVIS imaging at necropsy. Data shown are from 13 to 14 mice per group $\pm$ SEM. Student $t$ test was used to generate $P$ values. Representative histology images are shown. Scale bar, 1 mm. Equivalent results were obtained in two independent experiments.

ZR75.1 cells that express high levels of ST6GALNAC2, and MDA-MB-231 cells that have low-level expression (Supplementary Fig. S3A). siST6 transfection of MDA-MB-453 and ZR75.1 cells resulted in an efficient downregulation of ST6GalNAc2 mRNA and protein expression and significantly increased retention of both tumor cell lines in the lung (Fig. 5E and Supplementary Fig. S5A–S5D). In contrast, siST6 transfection of the low-expressing MDA-MB-231 cells had no impact on tumor cell retention (Fig. 5F and Supplementary Fig. S5D), demonstrating that the phenotype observed with the siST6 oligonucleotides is not due to an off-target effect. As observed for the 4T1-Luc cells, cotransfection of MDA-MB-453 cells with sGAL3 oligonucleotides effectively reversed the enhanced lung retention observed in siST6-transfected cells (Fig. 5G and Supplementary Fig. S5E). Consistent with the hypothesis that reduced ST6GALNAC2 expression promotes galectin-3 binding, in the MDA-MB-231 cells, which have endogenously low-level ST6GALNAC2 expression, sGAL3 transfection alone or ectopic St6galnac2 expression significantly attenuated the retention of cells (Fig. 5H and I and Supplementary Fig. S5F and S5G). Importantly, treatment with a clinically relevant galectin-3 inhibitor, GCS-100 (23, 24), specifically inhibited the retention of control MDA-MB-231 cells in the lung but had no impact on the retention of MDA-MB-231 cells with stable ectopic St6galnac2 expression (Fig. S1 and Supplementary Fig. S5G).

**Inhibition of Galectin-3 Blocks the Increased Metastasis of Cells with Low-Level ST6GalNAc2 Expression**

The data presented indicate a model in which tumor cells expressing low-level ST6GalNAc2 have an increased level of unmodified core 1 O-glycan facilitating increased binding of galectin-3 to the cell surface (Fig. 6A), and increased retention of tumor cells at sites of metastasis (Fig. 5). It has been proposed that firm adhesion to the vasculature at metastatic sites is preceded by low-affinity interactions, and that efficient metastatic colonization is aided by the formation of tumor cell aggregates (25–27). Consequently, we next addressed the role of ST6GalNAc2 expression and galectin-3 binding under dynamic flow conditions. When a mixed suspension of shNTC- and shST6-infected ZR75.1 cells (Supplementary Fig. S5A) were perfused across a monolayer of human umbilical vein endothelial cells (HUVEC), there was a significant increase in the number of shST6 cells firmly adhered to the endothelium (Fig. 6B, top, and Supplementary Movie S1). Importantly, this increased adhesion of shST6 ZR75.1 cells was completely abrogated in the presence of the galectin-3 inhibitor GCS-100 (Fig. 6B, bottom and Supplementary Movie S2). Furthermore, in static adhesion assays, down-regulation of St6galnac2 expression in 4T1-Luc cells resulted in a significant increase in adhesion to both HUVECs and...
immortalized mouse endothelial cell (sEND) monolayers (Fig. 6C), and this adhesion was attenuated either by cotransfection with siGAL3 oligonucleotides or incubation with the galectin-3 inhibitor lactose (Gal\(\beta\)-1,4Glc; refs. 18, 20, 21; Fig. 6D). Galectin-3 has a single S-type lectin domain but is unique in the galectin family by being able to pentamerize once it has bound to a ligand (28), allowing it to mediate homotypic, as well as heterotypic, cell aggregation (refs. 20, 29; Fig. 6A). Analysis of confocal images collected in the lung retention assays (Fig. 5 and Supplementary Figs. S4 and S5) revealed a significant increase in the proportion of siST6- versus siNTC-transfected 4T1-Luc, MDA-MB-453, and ZR75.1 cells forming tumor cell aggregates in the lungs (Supplementary Fig. S6), and this increase in tumor emboli was fully attenuated by cotransfection with siGAL3 (Fig. 6E and F).

**ST6GalNAc2 Expression Determines Responsiveness to Galectin-3 Inhibition**

There have been numerous reports describing a role for galectin-3 in tumor progression and metastasis (17, 30, 31); however, there are conflicting data about the value of galectin-3 as a prognostic factor either in gene expression profiling of human tumor samples or by monitoring protein levels in serum (32–35). In Fig. 3, we show by meta-analysis of different datasets that higher expression of ST6GalNAc2 in ER\(^{-}\) tumors correlates with improved survival outcome. In contrast, galectin-3 (LGALS3) expression in the same datasets demonstrated no significant association with ER status in human breast cancers or cell lines (Fig. 7A) and, more importantly, no association with survival outcome within the ER\(^{-}\) group (Fig. 7B). This is in keeping with our mechanistic studies that indicate it is the level of galectin-3 bound to the cell surface as determined by ST6GalNAc2 activity, rather than galectin-3 expression per se, which underpins the role of galectin-3 in promoting metastasis (Fig. 6A). To test this directly, mice inoculated with shST6- or shNTC-infected 4T1-Luc cells were treated with or without the galectin-3 inhibitor GCS-100 (Fig. 7C). GCS-100 completely blocked the enhanced metastatic colonization observed in cells with downregulated ST6galnac2 expression, but importantly had no effect on the lung tumor burden of mice inoculated with shNTC cells. Together, these data suggest that low expression of ST6GalNAc2 identifies patients with ER\(^{-}\) breast cancer who could be stratified for treatment with a galectin-3 inhibitor.

**DISCUSSION**

Because of the complexity of the metastatic process, the development of experimental approaches for identifying metastatic biomarkers and therapeutic targets is challenging. The

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### Figure 3.

High levels of ST6GALNAC2 expression correlate with increased survival in patients with breast cancer. **A**, correlation of ST6GALNAC2 normalized expression level between ER\(^{+}\) and ER\(^{-}\) breast tumors and cell lines (final) in the Breast Cancer Gene Expression Datasets (ROCK). Student t test was used to generate \(P\) values. **B**, forest plot showing meta-analysis of ST6GALNAC2 expression and survival outcome in the ER\(^{-}\) tumors from the same datasets and in two additional datasets (GSE31519, GSE5327) of ER\(^{-}\) breast cancers (total of 551 patients). See Methods for statistical analysis.

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Overall, 0.67 0.48–0.93
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Objective: The goal of this study was to integrate RNAi technology and massively parallel sequencing in a well-established mouse model to rapidly discover and validate genes involved in breast cancer metastasis. The study described here demonstrates the feasibility of this approach and the identification of ST6GalNAc2 as a novel breast cancer metastasis suppressor gene.

Results: Our results indicate that the sialyltransferase ST6GalNAc2 functions to suppress seeding of tumor cells at secondary sites, a late stage in the metastatic process. Although there are numerous reports describing an altered cell surface O-glycome in tumor cells (13, 14, 36), there have been no published reports of a role for ST6GalNAc2 in the breast cancer metastatic process. At least 20 human sialyltransferases have been identified in the human genome, of which six catalyze the transfer of sialic acid to GalNAc (37, 38). Of these, ST6GalNAc1, 2, and 4 are active on glycoproteins, whereas ST6GalNAc3, 5, and 6 function to transfer sialic acid residues onto gangliosides. To date, the focus on GalNAc sialyltransferases in cancer has been on ST6GalNAc1 and ST6GalNAc5. Expression of ST6GalNAc5 is normally restricted to brain tissue but has been reported to be a specific mediator of breast cancer infiltration across the blood–brain barrier, promoting the formation of brain, but not lung, metastases (39). ST6GalNAc1, which shows increased expression in breast cancers (40), is the major enzyme responsible for the production of the simple mucin-type sialyl-Tn antigen (Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-O-Ser/Thr). Sialyl-Tn is rarely observed in normal tissues but is abundant in a range of cancer types (37), including breast cancer, where expression is associated with poor prognosis (14, 41–44). Given the increased sialyltransferase activity associated with many cancers, it was of interest that ST6GALNAC2 was identified in our screen as a metastasis suppressor gene. Although ST6GalNAc2 is able to sialylate the Tn antigen in vitro, in vivo it preferentially transfers sialic acid to the 6 position of the T antigen/core 1 antigen (Galβ1-3GalNAc) and the sialyl-3T antigen (NeuAcα2-3Galβ1-3GalNAc), to create the sialyl-6T
antigen \([\text{Gal} \beta 1-3(\text{NeuAcO}2-6)\text{GalNAc}]\) and the disialyl-T antigen \([\text{NeuAcO}2-3\text{Gal} \beta 1-3(\text{NeuAcO}2-6)\text{GalNAc}]\), respectively (12).

As demonstrated here, cells in which ST6GalNAc2 expression is downregulated show an increase in the unmodified core 1 antigen and a reduction in the disialyl core 1 antigen.

Unmodified core 1-3(NeuAc\(\beta\)1-6)GalNAc and the disialyl-T antigen are involved in many of the biologic processes associated with tumor progression and metastasis (31). Of these, there are robust data showing that galectin-3 binding to the core 1 antigen on tumor cells promotes increased tumor cell homotypic aggregation and heterotypic adhesion to the endothelium (18–22, 29) and that galectin-3 binding can protect against anoikis-mediated apoptosis (31). Our studies further support such involvement of galectin-3, but in addition have extended the mechanistic understanding of its role. We show that in mouse and human breast cancer cells with high ST6GalNAc2 expression, inhibition of galectin-3 has no effect on binding to the endothelium \textit{in vitro}, aggregation in the vasculature, or tumor burden \textit{in vivo}. Galectin-3 inhibition is effective only if tumor cells have low-level ST6GalNAc2 expression. These data demonstrate that it is the expression of ST6GalNAc2 and not galectin-3 that determines enhanced retention of tumor cells at metastatic sites and that a role for galectin-3 is regulated by the profile of O-linked glycans on the cell surface that facilitate galectin-3 binding.

Clinical trials using galectin-3 inhibitors report a good safety profile (22–24, 45). However, our meta-analysis demonstrates that galectin-3 expression levels do not correlate with clinical outcome or identify patients who would benefit from treatment with a galectin-3 inhibitor. Certainly, further studies examining ST6GalNAc2 expression in ER\(^-\) breast tumors may identify patients who would benefit from treatment with a galectin-3 inhibitor. Certainly, further studies examining ST6GalNAc2 as a potential biomarker for predicting metastases in ER\(^-\) breast cancers are warranted.

**METHODS**

**shRNA Library and In Vivo Screen**

We used a miR-30–based shRNA library in the LMS (LTR-driven miR30 SV40-GFP MSCV-based vector) backbone targeting the Cancer 1000 gene set (10). Forty-eight separate virus batches were produced from plasmid pools each containing 48 individual shRNAs using retroviral-mediated gene transfer with Phoenix packaging cells (G. Nolan, Stanford University, Stanford, CA). 4T1-Luc cells (SibTech)
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**Figure 5. (Continued)** E, siNTC and siST6-transfected MDA-MB-453 cells were stained for ST6GalNAc2 (green) and the Golgi protein β-COP (red). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 20 μm. F, G, and H, 0.5 × 10⁶ siNTC, siST6-, or siGAL3-transfected MDA-MB-453 or MDA-MB-231 cells labeled with CellTracker dyes were inoculated into the tail veins of CD1 mice nu/nu mice and lung retention assessed at 1 and 5 hours as described in A, 1.0 × 10⁶ 231-VEC or 231-ST6 cells labeled with CellTracker dyes were treated for 30 minutes with vehicle (VEH) or 250 μg/mL GCS-100 and inoculated into the tail veins of CD1 mice nu/nu mice that had been pretreated 1 day previously with 250 μg/mL GCS-100. Lung retention was assessed at 1 and 5 hours as described in A, A–L, qPCR analysis of ST6GALNAC2 and LGALS3 mRNAs and representative confocal images are shown in Supplementary Fig. S4 (4T1-Luc cells) and Supplementary Fig. S5 (human cells). ns, not significant; AU, arbitrary unit.

**High-Throughput Sequencing and Data Analysis**

Amplified shRNA sequences were subject to massively parallel sequencing on an Illumina GAIIx, using a procedure similar to that previously described (46, 47). Briefly, after cluster generation and sequencing-by-synthesis on the Illumina GAIIx, raw image data were analyzed using GA pipeline v1.5. Short reads were aligned to the shRNA library reference sequences using a custom software package shALIGN (48), allowing up to two mismatches to the reference sequence. On average, 94% of the short reads aligned to the reference library. Statistical analysis of screen results was performed in R 2.9.0 (http://www.r-project.org/) using the shRNAseq package (48). shRNAs with no predicted target, or with greater than two predicted targets, were removed from the analysis. Total reads per shRNA were log2 transformed and median normalized per pool. Normalized scores from technical replicates were averaged, and the log ratio of tumor to preinoculation 4T1-Luc cells was calculated individually for mouse A, B, and C. Hits were defined as shRNAs that had increased in representation by two SDs (Z score >2) in two or more of the replicate tumor samples compared with the 4T1-Luc sample and had a Z score ≤−1 when comparing the 4T1-Luc sample to the plasmid sample.

**Cell Culture**

All human cell lines were obtained from American Type Culture Collection (ATCC) and short tandem repeat (STR) tested every 4 months using StemElite ID System (Promega). 4T1-Luc cells were cultured in RPMI-1640; ZR75.1, MDA-MB-453, MDA-MB-231 (ATCC), MDA-MB-231-Luc (SibTech Inc.), and sEND immortalized mouse skin endothelial cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM). Culture media were supplemented with 10% fetal calf serum (FCS; Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin. HUVECs (Lonza) were cultured in EGM-2 Media BulletKit (Lonza) and used between passages two and five. Cells were reverse transfected with 50 nmol/L SMARTpool siRNA oligonucleotides (Dharmacon; Supplementary Table S1) using Lipofectamine 2000 (Invitrogen) or Dharmafect4 (Dharmacon), and cell assays were performed 48 to 72 hours later. For shRNA expression, individual shRNAs, selected on the basis of RNAi Codes (ref. 47; Supplementary Table S1), were synthesized as 97 base pair oligonucleotides (Sigma Genosys), PCR-amplified, cloned into the LMS vector, and verified by sequencing. Infected 4T1-Luc cells (see shRNA library methods) were FACSorted for GFP+ cells. Mission particles were purchased from Sigma, pGIPZ shSi76 and shNTC plasmid DNA (V2LHS_6073 and V2LHS_6187).
Figure 6. ST6GalNAc2 expression enhances tumor cells interaction with the vasculature. A, model of tumor-endothelial interactions modulated by ST6GalNAc2 expression levels. See text for details. B, shST6 and shNTC ZR75.1 cells (see Supplementary Fig. S5A for qPCR analysis) were labeled with CellTracker dyes, mixed at a 1:1 ratio, and co-perfused over activated HUVECs under flow conditions (see Methods) in the absence (left and Supplementary Movie S1) and presence (right and Supplementary Movie S2) of the galectin-3 inhibitor GCS-100. Data shown are the mean number of shNTC and shST6 cells that adhered to HUVECs at 1, 5, and 10 minutes. Equivalent results were obtained in three independent experiments. Two-way ANOVA with Bonferroni posttest was used to generate P values. *, P < 0.05; **, P < 0.01. C and D, 4 × 10^4 4T1-Luc cells transfected with siNTC or siST6, with or without siGAL3 cotransfection, in the lung was quantified for the mice shown in Fig. 5D (4T1-Luc cells) and Fig. 5G (MDA-MB-453 cells) at 24 and 5 hours, respectively. Data shown are mean size of tumor cell aggregates/fov for the 4 mice in each group ± SEM. Student t test was used to generate P values. Representative confocal images are shown. Scale bar, 100 μm. ns, not significant; AU, arbitrary unit.
datasets. A forest plot showing meta-analysis of B, BALB/c mice. For orthotopic inoculation, BALB/c mice were injected 10^6 4T1-Luc cells were inoculated into the spleen parenchyma of 6- to 8-week-old CB17 NOD.SCID mice (Harlan; MDA-MB-231 cells). For the experimental liver metastasis assay, 0.5 × 10^6 cells were labeled with CellTracker Green CMFDA dye and incubated for 4 to 6 hours before use. A total of 4 × 10^6 cells were injected into the tail veins of 6- to 8-week-old BALB/c mice (4T1-Luc cells) or CD1 nu/nu mice (human cell lines). For lung retention assays, cells were transfected with siRNA oligonucleotides, labeled 48 hours later with CellTracker Red CMTPX or FITC-dextran, trypsinized, and mixed at a 1:1 ratio. The following reagents were used for immunofluorescent staining: anti-human ST6GalNAc2 (Abcam; #68510), fluorescein isothiocyanate–conjugated secondary antibody (Invitrogen). Antibody, GST-tagged human recombinant galectin-3 (GST-GAL3; R&D Systems) for 4 to 6 hours. For static adhesion assays, monolayers of HUVEC and sEND cells were stimulated with 10 ng/mL TNF-α (R&D Systems) for 4 to 6 hours. For orthotopic inoculation, BALB/c mice were injected with 1.5 × 10^6 4T1-Luc cells, and tumor volumes were measured until they reached maximum allowable size.

For lung retention assays, cells were transfected with siRNA oligonucleotides, labeled 48 hours later with CellTracker Red CMTPX or Green CMFDA dyes (Molecular Probes), trypsinized, and mixed at a 1:1 ratio. Ten images were taken for each lung. Tumor cell colonization of the lung and tumor cell aggregation within the lung was quantified in ImageJ, by converting the red and green images into separate binary images and measuring tumor cell coverage and mean size of tumor cell aggregates per field of view (fmv). All animal work was carried out with UK Home Office approval.

**In Vitro Studies**

The following reagents were used for immunofluorescent staining: anti-human ST6GalNAc2 (Abcam; #68510), fluorescein isothiocyanate–conjugated anti-glutathione S-transferase (anti-GST; Abcam; #6647), in-house-generated rat anti-β-COP monoclonal antibody, GST-tagged human recombinant galectin-3 (GST-GAL3; Novus Biologicals; #N00003958-P01), and Alexa Fluor–conjugated secondary antibodies (Invitrogen).

For static adhesion assays, monolayers of HUVEC and sEND cells were stimulated with 10 ng/mL TNF-α (R&D Systems) for 4 to 6 hours. A total of 4 × 10^6 siRNA-transfected 4T1-Luc cells were labeled with CellTracker Green CMFDA dye and incubated with monolayer cultures of HUVEC or sEND cells in the presence or
absence of 100 mmol/L lactose for 30 minutes at 37°C. The cells were washed with PBS, and the numbers of adherent cells were quantified on a VICTOR X fluorescent plate reader.

For dynamic adhesion to endothelial cells under flow, HUVEC monolayers were grown to confluency on the base of each parallel plate flow chamber and stimulated with 10 ng/mL of TNF-α (R&D Systems) for 4 to 6 hours before use. Single-cell suspensions of siNTC or siST6 ZR75.1 cells were achieved using enzyme-free cell dissociation buffer (Life Technologies) and labeled with 1 μmol/L CellTracker orange or green dyes. Cells were transferred into perfusion media (DMEM plus 10% FCS and 25 mmol/L HEPES), mixed in a 1:1 ratio and 2 × 10^6 cells/mL perfused over HUVECs for 10 minutes at 1.25 dyne/cm². Three fields of view were selected for each flow experiment and recorded using ×10 inverted objective lens (Olympus IX-80 microscope). Images were acquired at three time points in three channels (phase contrast, GFP, and RFP). Dye swaps did not affect cell behavior. For the galectin-3 inhibitor assays, tumor cells and HUVECs were pre-treated with 300 μg/mL GSC-100 for 30 and 10 minutes, respectively, and GSC-100 was present during the flow assay.

O-glycomic Profile Analysis
A total of 2 × 10^6 siNTC- and siST6-transfected 4T1-Luc cells were snap-frozen and treated as described previously (49, 50). Briefly, all samples were subjected to homogenization in an extraction buffer (25 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA and 1% (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, CHAPS) at pH 7.4. After reduction, carboxymethylation, and tryptic digestion, O-glycans were released by reductive elimination. Released O-glycans were then permethylated and purified by C18 Sep-Pak. All permethylated samples were dissolved in 10 μL of methanol, and 1 μL of the dissolved sample was premixed with 1 μL of matrix (20 mg/mL 2,5-dihydroxybenzoic acid in 70% v/v aqueous methanol), spotted onto a target plate (2 × 0.5 μL) and dried under vacuum. Mass spectrometry (MS) data were acquired using a Voyager-DE STR MALDI-TOF (Applied Biosystems). MS-MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. The 4700 Calibration Standard Kit, Calmix (Applied Biosystems), was used as the external calibrant for the MS mode of both instruments and [Glu1] fibrinopeptide B (Sigma) was used as an external calibrant for the MS-MS mode of the MALDI-TOF/TOF instrument. The MS and MS-MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glycobioinformatics tool GlycoWorkBench. Assignments for the selected peaks were based on 12C isotopic composition together with the knowledge of the biosynthetic pathways. Structures were confirmed by data obtained from MS-MS experiments.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism5 software and R version 2.11.1. Two-tailed Student t tests were performed as indicated. Two-way ANOVA with Bonferroni posttest was used to generate P values for dynamic adhesion assays with and without galectin-3 inhibitor (GCS-100). Clinical relevance of variable ST6GALNAC2 expression was assessed using publicly available data. For meta-analysis, the highest quartile of gene expression was used to dichotomize the ER− samples from each study. For each study, we computed individual RR and 95% CI and then calculated an overall RR and 95% CI using the Mantel-Haenszel method. There was no evidence of interstudy heterogeneity as assessed using Cochran’s Q statistic. We assessed whether the overall RR was significantly different from RR = 1 using a one degree-of-freedom score test.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.


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An In Vivo Functional Screen Identifies ST6GalNAc2 Sialyltransferase as a Breast Cancer Metastasis Suppressor

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