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Engagement of I-Branching β-1, 6-*N*-Acetylglucosaminyltransferase 2 in Breast Cancer Metastasis and TGF-β Signaling

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Abstract

In this study, we have showed that *GCNT2*, a gene-encoding glucosaminyl (*N*-acetyl) transferase 2, I-branching enzyme, is overexpressed in highly metastatic breast cancer cell lines of human and mouse origin and basal-like breast tumor samples. *GCNT2* expression is also significantly correlated to the metastatic phenotype in breast tumor samples. Functional studies showed that ectopic expression of *GCNT2* enhances cell detachment, adhesion to endothelial cells, cell migration and invasion *in vitro*, and lung metastasis of breast cancer cells *in vivo*. Knockdown of *GCNT2* expression decreases cell migration and invasion *in vitro* and lung metastasis *in vivo*. We have further shown the involvement of *GCNT2* in the epithelial-to-mesenchymal transition (EMT). Specifically, the expression of E-cadherin is significantly changed upon *GCNT2* expression at the protein level but not at the RNA level. Moreover, we have shown that *GCNT2* is a direct target of the TGF-β–smad pathway and that change in *GCNT2* expression modulates EMT induced by TGF-β1 treatment. Finally, we have shown that diminution of the glycosyltransferase activity of I-branching β-1, 6-*N*-acetylglucosaminyl transferase 2 (GCNT2) abrogates its cell migration and invasion-promoting function and synergistic effect with TGF-β to induce EMT. Our study for the first time showed that *GCNT2* is a novel gene contributing to breast cancer metastasis with preferential expression in basal-like breast cancer. Moreover, we discovered that involvement of *GCNT2* in EMT and TGF-β signaling, and further glycosylation modification of E-cadherin by GCNT2, are the underlying integrative mechanisms for breast cancer metastasis, implying that blocking TGF-β/*GCNT2* signaling is a promising approach for targeting metastatic breast cancer. *Cancer Res; 71(14); 4846–56. ©2011 AACR.*

Introduction

Metastatic breast cancer is generally considered incurable. The primary reason for this is because gene targets underlying the metastatic process have not been clearly defined, further hindering the development of targeted therapies (1, 2). This is especially true for basal-like breast cancer, which exhibits an aggressive and early pattern of distant metastasis phenotypes (3, 4). In an effort to identify novel genes that play essential roles in breast cancer metastasis, our group conducted a cross-species integrative expression profiling assay, which combines the use of cell models of human and mouse origins and the microarray expression technique. A total of 34 genes, among which 22 genes are upregulated and 12 are downregulated, were identified with marked expression level difference between highly and poorly metastatic cell lines (5). I-branching β-1, 6-*N*-acetylglucosaminyl transferase 2 (GCNT2), a member of the β-1, 6-*N*-acetylglucosaminyltransferase family that plays a critical role in glycosylation, is a novel metastasis-related gene candidate.

Over the past decade, glycosylation alterations of proteins and lipids have been implicated in malignant transformation. For example, increased expression of *MGAT5*, the gene-encoding mannosyl (α-1, 6-)-glycoprotein β-1, 6-*N*-acetyl-glucosaminyltransferase that adds β1, 6 GlcNAc branching to *N*-glycans, is correlated with the progression of invasive malignancies (6, 7). Conversely, suppression of tumor growth and metastasis was observed in Mgat5-deficient mice (8). Mgat5 promotes malignant transformation and invasiveness of cancers by generating β1, 6-poly *N*-acetyllactosamine on *N*-glycans of certain signaling molecules (e.g., epidermal growth factor receptor and Integrin) such that they are retained on the cell surface longer and augment their signals

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for proliferation and invasion of cancer cells (9, 10). On the other hand, MGAT5 is antagonized by MGAT3, another GlcNAc transferase that adds bisecting GlcNAc to *N*-glycans. The presence of bisecting GlcNAc inhibits the formation of β1, 6-*N*-acetyllactosamine by MGAT5. Therefore, MGAT3 acts as a tumor metastasis suppressor (11–13). Moreover, a subset of malignant human breast cancer cell lines was found to have elevated MGAT5 expression. Consistently, the production of *MGAT5*, as detected by lectin L-PHA, was found to be increased in a subset of primary breast cancers and correlated with their invasiveness (14, 15). Furthermore, *GCNT1*, which encodes a core 2 β1, 6-*N*-acetyl-glucosaminyltransferase, is overexpressed in various human cancers, including prostate, colorectal, and pancreatic cancers (16, 17). Specifically, *GCNT1* overexpression enhances the metastatic potential of the testicular germ cell tumor (18). However, as the homologous gene of *GCNT1*, the precise role of *GCNT2* in breast tumorigenesis and metastasis remains elusive.

In this study, we have shown that the expression of *GCNT2* is closely related to basal-like and metastatic phenotypes in both breast cancer cell lines of human and mouse origins and human breast tumor samples. We then intensively studied the functional role of *GCNT2* in typical oncogenic properties including cell proliferation, colony formation, migration and invasion *in vitro* with multiple cell lines, and lung metastasis *in vivo* by using an experimental animal model. We then showed that *GCNT2* plays a role in epithelial-to-mesenchymal transition (EMT). We also found that *GCNT2* is regulated by TGF-β1 and is required for TGF-β1–induced EMT. Finally, we showed that enzymatic activity is required for cell migration, invasion, and the EMT-promoting function of *GCNT2*.

Materials and Methods

Cell lines and cell culture

Human breast cancer cell lines, MDA-MB 231, MDA-MB 435, MDA-MB 361, MCF7, and BT20 were obtained from American Type Culture Collection (ATCC) and were grown by ATCC recommendations. SUM1315, SUM159, and SUM149 were obtained from Dr. Stephen P. Ethier's laboratory and cultured as previously described (19). Three mouse breast cancer cell lines, 67NR, 168FARN, and 4T1 were cultured as previously reported (20). Human and mouse mammary epithelial cell lines HMLE and EpRas were generously provided by Robert A. Weinberg of MIT, and cultured as previously mentioned (21). NMuMG cell was provided by Lindsey D. Mayo at Indiana University, and cultured with Dulbecco's modified Eagle's medium medium with 4.5 g/L glucose, 10 mg/mL insulin, and 10% FBS. All cell lines were authenticated upon receipt by comparing them to the original morphologic and growth characteristics.

Tissue microarray and immunohistochemistry assay

A breast cancer tissue array, 150 cores including 75 cases of normal, reactive, premalignant, and malignant tissues of the breast in duplicates were purchased from Pantomics. Standard procedures were used for the immunohistochemistry (IHC) assay. The GCNT2 antibody was purchased from Sigma (Sigma-Aldrich) and the working dilution is 1:200.

Establishment of stable cell lines

One set of mouse short-hairpin RNAs (shRNA) and the human full-length cDNA for *GCNT2* was purchased from Open Biosystems. Two mutant forms of *GCNT2* were created by using the Stratagene's QuikChange kit based on wild-type *GCNT2*. The Trans-Lentiviral Packaging System and the ViraPower Lentiviral Expression System (Invitrogen) were used to produce shRNA and overexpressing lentiviruses, respectively. shRNA lentiviruses were used to silence *GCNT2* expression in 4T1 and NMuMG cells and overexpressing lentiviruses were introduced into HMLE, EpRas, MCF7, Madin Darby canine kidney (MDCK), and NMuMG cells. Stable cells were generated after 2 weeks of antibiotic selection (22, 23).

Cell proliferation, colony formation, cell invasion, and migration assays

All these assays were done as previously described (23).

Western blotting

A Western blot was done according to regular protocol (5, 22).

Tumorigenesis and metastasis assay

For EpRas cells, 2.5×10^5 cells were injected subcutaneously into the mammary glands of 5-week-old, female NCR Nu/Nu mice (Taconics). For 4T1 cells, 2.5×10^4 cells were injected into the mammary gland of female BALB/C mice (The Jackson Laboratory). Eight to 10 animals per group were used in every experiment. Four weeks later after tumor cell injections, mice were sacrificed and tumors were removed and weighed individually. Lungs were collected and embedded into paraffin blocks. Standard hematoxylin and eosin (H&E) staining of paraffin-embedded tissue was done for histologic examination of metastases. The lung metastasis fields in individual mice were counted as a percentile of the whole lung section area under the parallel dissection scope. All experimental animal protocols were approved by the Institutional Animal Care and Use Committee.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was done by using a student's *t* test for significance. *P* value less than 0.05 was considered significant. The Cochran–Armitage test was used to analyze the statistically significant linear trend between the expression of GCNT2 and metastasis phenotypes.

Results

GCNT2 is overexpressed in highly metastatic breast cancer and its expression correlates with adverse pathologic phenotypes

To confirm *GCNT2* upregulation in metastatic breast cancer cells, we first determined the expression of 3 isoforms of *GCNT2* (24) in a set of human breast cancer cell lines with

different metastatic characteristics. All 3 isoforms of *GCNT2* have a very similar expression pattern. High *GCNT2* expression was shown in most highly metastatic cancer cell lines (all of them are basal-like cells) and low expression of *GCNT2* was seen in most nonmetastatic or low-metastatic cell lines (MDA361 and MCF7 are luminal type and BT20 is basal like; Fig. 1A). However, isoform B and C have significantly low expression levels compared with isoform A (Supplementary Fig. S1). Isoform A of *GCNT2* is then used for the detailed functional study. Moreover, correlation of high expression of *GCNT2* and metastatic phenotype was also confirmed in mouse model cell lines (Fig. 1B). Overall, these results suggest that overexpression of *GCNT2* correlates to metastatic characteristics in breast cancer cell lines.

To investigate the expression pattern of *GCNT2* in clinical breast tumor samples, we first analyzed a breast cancer gene expression dataset obtained from the Netherlands Cancer Institute, which contains 116 basal-like breast tumors and 83 nonbasal-like breast tumors (25). We found that the levels of *GCNT2* expression were significantly higher in basal-like breast tumors than in nonbasal-like breast tumors ($P < 0.0001$; Fig. 1C). We then conducted an IHC to investigate the GCNT2 expression in breast tumors by using a tissue microarray and a pan-isoform GCNT2 antibody. The Cochran–Armitage trend test reveals that there is a statistically significant linear correlation between the expression of GCNT2 and metastasis phenotypes ($P = 0.006$) in breast cancer (Fig. 1D, Supplementary Fig. S2). Furthermore, numerous independent microarray data sets showed that a high level of *GCNT2* expression is strongly correlated with basal-like tumors (26), BRCA1 mutation tumors (27), and estrogen receptor or progesterone receptor-negative tumors (28, 29). Moreover, expression of *GCNT2* increased concomitantly with breast cancer grade (Ref. 30; Supplementary Fig. S3). Overall, our laboratory findings are consistent with the results from data mining, suggesting that upregulation of *GCNT2* expression contributes to adverse pathologic phenotypes and progression of breast cancer.

The effect of *GCNT2* overexpression on oncogenic properties

To determine whether *GCNT2* expression affects regular oncogenic properties, we established GCNT2 A isoform overexpression model cell lines based on HMLE, EpRas, and MCF7 cells using a lentivirus expression system (Fig. 2A, Supplementary Figs. S4 and S5). We compared cell proliferation and colony formation of the HMLE, EpRas, and MCF7 cells carrying either *GCNT2* or control vector. Our results showed that both gene and control vector overexpressing cells originated from all 3 cell lines grew at similar rates and had similar colony number *in vitro* (Fig. 2A and B, Supplementary Figs. S4 and S5). Similar results were also observed in MDCK cells (Supplementary Fig. S6). These results clearly show that the ectopic expression of *GCNT2* has minimal effects on the proliferation and colony formation of mammary epithelial cells.

We then checked the effect of *GCNT2* on cell migration and invasion by using a BD Biocoat migration chamber and matrigel invasion chamber. We found that ectopic expression of *GCNT2* in HMLE, EpRas, and MCF7 cells led to a significant increase in cell migration and invasion (Fig. 2C, Supplementary Figs. S4, S5, and S7). MDCK cells with ectopic expression of *GCNT2* also showed significant increase in cell migration

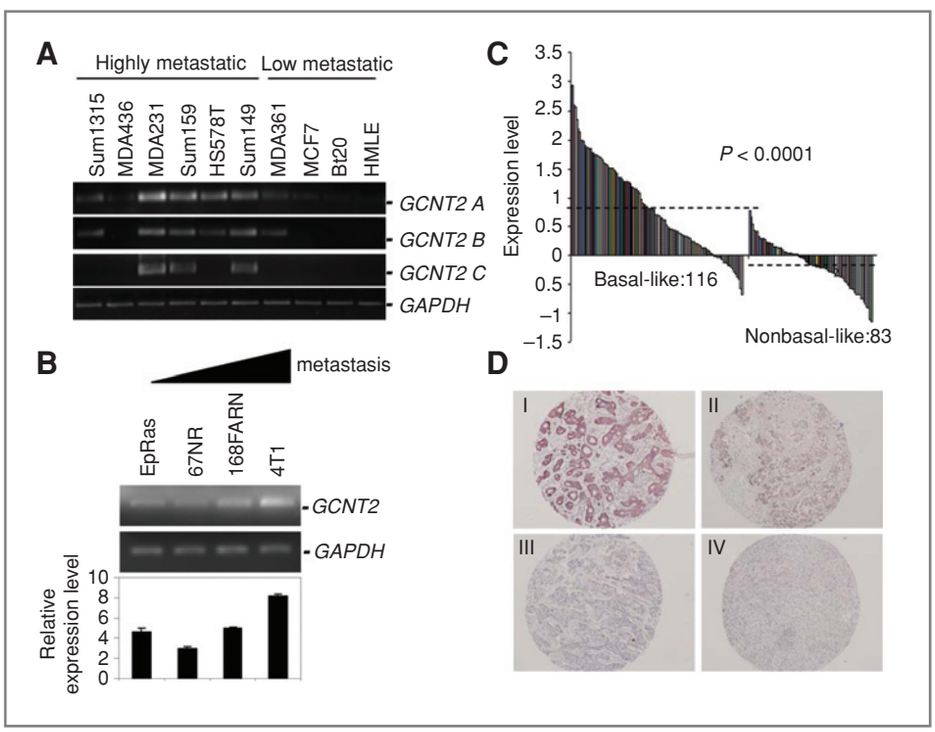

Figure 1. Expression pattern of *GCNT2* in breast cancer. A, the expression pattern of 3 isoforms of *GCNT2* in human breast cancer cell lines. The highly (+) and low (−) metastatic breast cancer cell lines are marked as indicated. B, *GCNT2* RNA expression in a set of mouse cell lines with different metastatic capabilities. The triangular bar indicates the increase in metastatic capability. C, the expression level of *GCNT2* in breast tumor samples (basal-like sample, 116 cases and nonbasal-like sample, 83 cases). Dashed lines indicate the average expression level. D, expression of GCNT2 protein in breast tumors. The panels show representative figures of the IHC assay. I is an example of strong staining. II is intermediate staining. III is weak staining. IV is no staining.

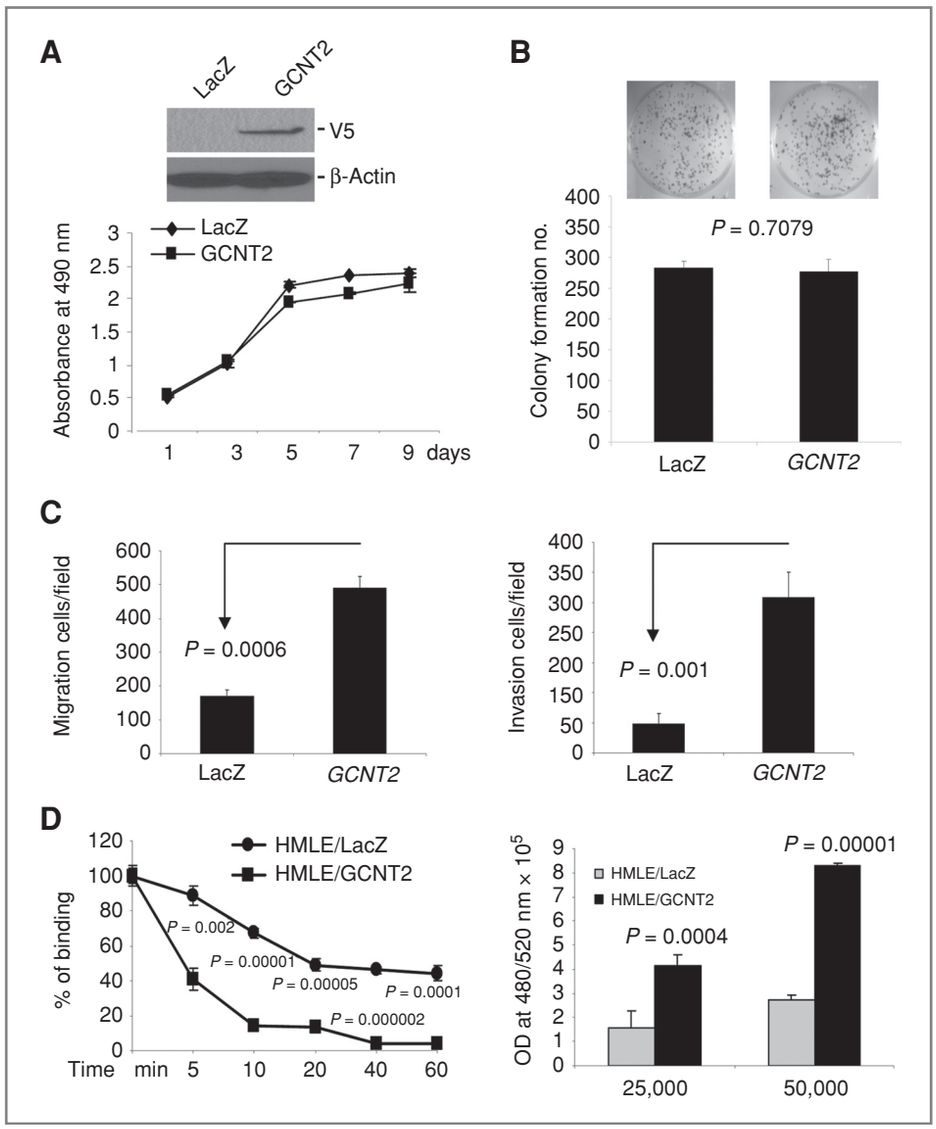

Figure 2. The effect of *GCNT2* on cell proliferation, colony formation, cell migration, invasion, cell detachment, and attachment. A, ectopic expression of *GCNT2* in HMLE cells detected by Western blotting (top). The effect of ectopic expression of *GCNT2* on cell proliferation (low). B, the effect of ectopic expression of *GCNT2* on colony formation in HMLE cells. C, the effect of ectopic expression of *GCNT2* on cell migration (left) and invasion (right) in HMLE cells. D, the effect of *GCNT2* on cell detachment and attachment. The left panel shows that ectopic expression of *GCNT2* led to an increase in detachment of HMLE cells from plates when treated with EDTA (0.02%) at different times. The right panel shows that ectopic expression of *GCNT2* led to an increase of cell adhesion to HUVEC cells. 25,000 and 50,000 HMLE-derived cells were plated on top of the monolayer of HUVEC cells.

(Supplementary Fig. S6). Furthermore, we found that overexpression of *GCNT2* significantly increased the detachment of HMLE cells from the plate ($P < 0.002$; Fig. 2D, left). In addition, the adhesion of HMLE cells to human umbilical vein endothelial cell (HUVEC) was also found significantly increased in cells with *GCNT2* expression (Fig. 2D, right). This result is very similar to what is reported for *Snail* and *MGAT5* genes (31, 32).

The effect of *GCNT2* expression knockdown on oncogenic properties

On the basis of highly metastatic 4T1 cells, which also have high *GCNT2* expression, we established the *GCNT2* A isoform knockdown model cell line. Two clones (4T1/shRNA1 and 4T1/shRNA5) with significant inhibition of *GCNT2* expression, as shown by reverse transcriptase PCR (RT-PCR; there is no specific antibody for A isoform; Fig. 3A), were used to check the effect of *GCNT2* knockdown on cell proliferation and colony formation. We found that 2 clones with *GCNT2* expression knockdown showed a marked decrease in cell proliferation and colony formation compared with nontarget control cells (Fig. 3B and C). We further found that 4T1 cells with *GCNT2* knockdown showed a significant decrease in migration (80%, $P = 0.0001$) and invasion (80%, $P = 0.002$; Fig. 3D and Supplementary Fig. S8).

GCNT2 contributes to distal metastasis *in vivo*

We then injected EpRas cells expressing either control vector or *GCNT2* into the mammary glands of nude mice and examined their lungs for metastasis 4 weeks after injection. Detailed quantification was conducted with H&E staining on the lung sections. In lung sections from 10 mice injected with the EpRas cell expressing control vector, only 3 cases showed a more than 2% metastatic tumor in whole sections. In contrast, the metastatic area made up at an average of 9% of the sections in all 10 lung sections from mice injected with

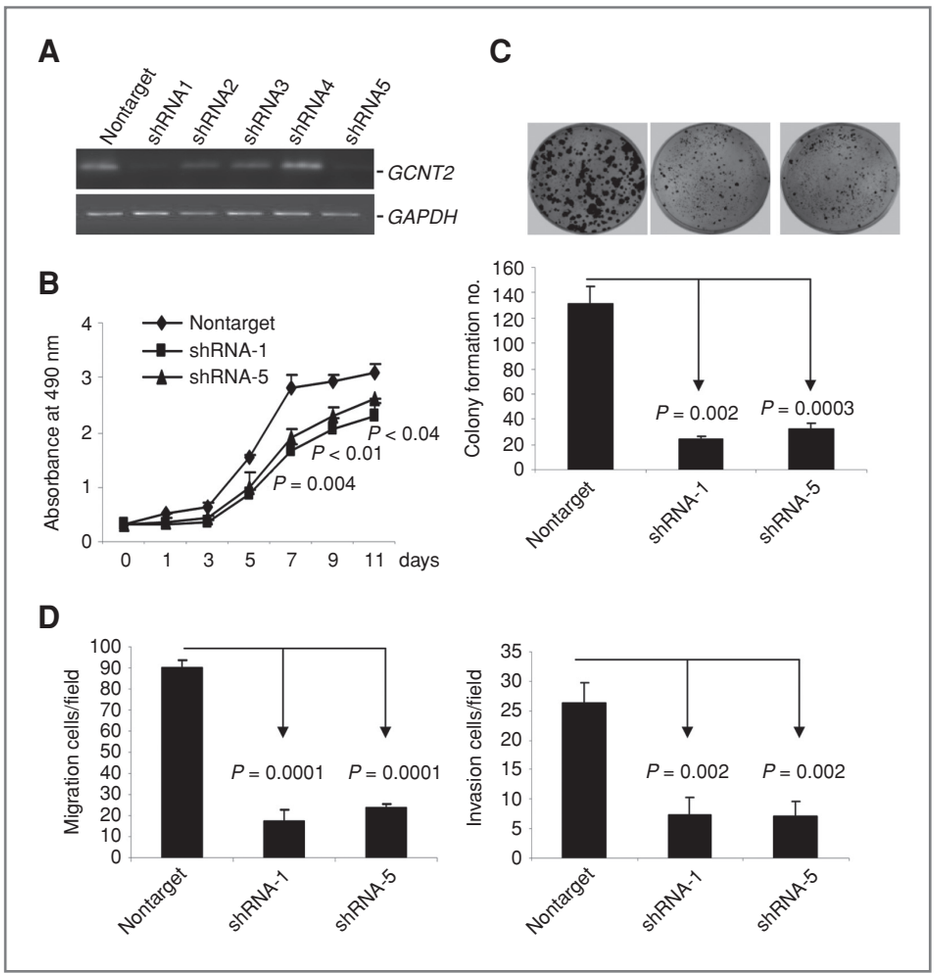

Figure 3. The effect of knockdown of *GCNT2* on cell proliferation, colony formation, cell proliferation, and invasion. A, knockdown of *GCNT2* in 4T1 cells with shRNA. Two clones (shRNA1 and shRNA5) show significant reduction of *GCNT2* expression. B, two clones with significant *GCNT2* expression knockdown show a marked decrease in cell proliferation rates compared with nontarget control by MTT assay. C, two clones with significant *GCNT2* expression knockdown show a marked decrease in colony formation compared with nontarget control. D, two clones with significant *GCNT2* expression knockdown show a marked decrease in migration (left) and invasion (right) compared with the nontarget control.

EpRas cells overexpressing *GCNT2* (Fig. 4A, $P = 0.002$). Moreover, the tumor burden in the control vector group and the *GCNT2* overexpression group had no significant difference during the 4 weeks (Fig. 4B). This data, along with the result of *in vitro* analysis, confirms a role of *GCNT2* as a metastasis-promoting gene.

To further validate the role of *GCNT2* in spontaneous metastasis, 2 clones of 4T1 derivative cells with *GCNT2* knockdown and a nontarget control were injected into the fat pads of BALB/C mice. We observed that 4T1 cells with nontarget control have a similar metastatic propensity as the parental 4T1 cells (30%–40% lung involvement), which showed an average of 37% metastasis tumor in the lung sections. In contrast, the 4T1 cells with *GCNT2* knockdown showed an average of 7.75% and 5% metastatic tumor in the lung section for the shRNA1 clone ($P = 0.01$) and the shRNA5 clone ($P = 0.009$), respectively (Fig. 4C). Moreover, tumor burdens in 4T1 cells with *GCNT2* knockdown also had significant shrinkage compared with nontarget control (Fig. 4D).

GCNT2 is involved in EMT

We next investigated whether *GCNT2* is involved in the EMT process using established model cell lines with *GCNT2* overexpression and knockdown. We first observed that down-regulation of *GCNT2* caused increased levels of epithelial marker E-cadherin and α, β-catenin and decreased levels of mesenchymal markers fibronectin and vimentin in 4T1 cells (Fig. 5A, left and Supplementary Fig. S9). Moreover, down-regulation of *GCNT2* led to a significant cellular morphologic shift from mesenchymal to epithelial in 4T1 cells, which is showed by a more spindle like, scattered distribution in 4T1/vector cells, and a more cobblestone-like appearance in 4T1/shRNA1 cells (Fig. 5B). Conversely, we observed that *GCNT2* overexpression led to reduced levels of epithelial markers E-cadherin and α-catenin, and an increased level of the mesenchymal marker fibronectin in HMLE cells (Fig. 5A, right and Supplementary Fig. S9). No marked morphologic change was shown in this condition. Similarly, we observed significant inhibition of E-cadherin expression, but no other markers, in the MDCK cell without marked morphologic change when *GCNT2* was expressed (Supplementary Fig. S10). These data strongly suggest the involvement of *GCNT2* in the EMT process. However, ectopic expression of *GCNT2* alone is not sufficient to trigger a complete EMT.

We then conducted RT-PCR to investigate E-cadherin expression upon overexpression and siRNA knockdown of

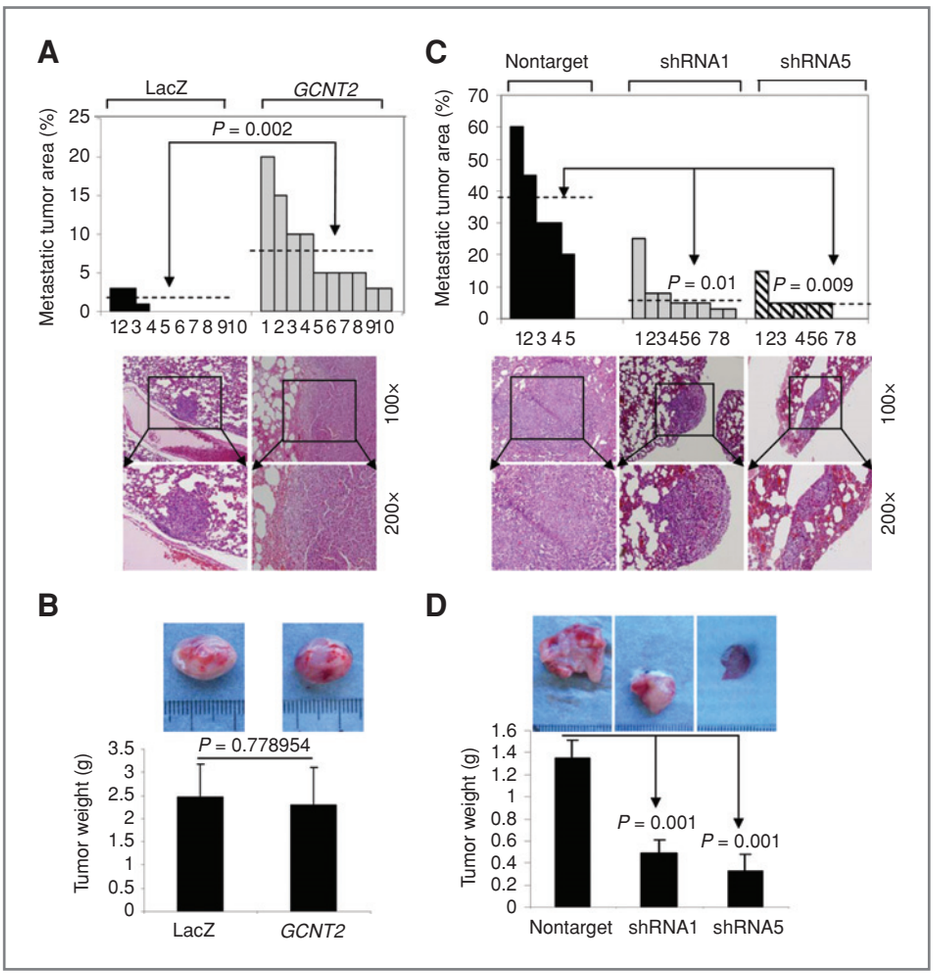

Figure 4. The effect of *GCNT2* on distal metastasis *in vivo*. A and C, quantification of metastasis *in vivo*. A, 2 populations of EpRas cells with the vector (LacZ) or the *GCNT2* gene were used in 10 mice, respectively. C, 4T1 cells with nontarget control and *GCNT2* shRNA1 and shRNA5 were used in 5 and 8 mice, respectively. Representative H&E staining pictures of lung sections from mice injected with genetically modified EpRas or 4T1-derived cells are shown as low panels (magnification, 100× and 200×). B, representative figures of the tumor burdens for EpRas cells with vector control and *GCNT2* expression. No significant difference was observed ($P = 0.778954$). D, representative figures of the tumor burdens for 4T1 cells with nontarget control, shRNA clone1, and shRNA clone5. Significant decrease in tumor weight was observed in *GCNT2* knockdown clones.

GCNT2. We found that neither overexpression of *GCNT2* in HMLE cells nor knockdown of *GCNT2* with shRNA in 4T1 cells changed the expression of E-cadherin at the RNA level (Fig. 5C). In contrast, ectopic expression of the *Snail* gene significantly inhibited expression of E-cadherin at the RNA level. In addition, HMLE cells with and without *GCNT2* overexpression were treated with different doses of proteasome inhibitor lactacystin. We found that the decrease of E-cadherin protein expression caused by *GCNT2* expression was inhibited by the treatment of lactacystin (Fig. 5D). These data, along with the data obtained above, suggest that *GCNT2* modulates the expression of E-cadherin through a posttranslational mechanism, which is completely different from the mechanistic action of transcription factors (33, 34).

GCNT2 is involved in TGF-β signaling and multiple cell signaling pathways

To investigate whether *GCNT2* is involved in TGF-β signaling, we treated the NMuMG cell with TGF-β1 and detected an increase of *GCNT2* gene expression (Fig. 6A). Furthermore, we found that treatment of TGF-β led to the activation of pSmad2 and SB431542 (TβR-II inhibitor) treatment totally erased the activation of pSmad2 in TGF-β–treated and untreated cells (Supplementary Fig. S11A). More importantly, *GCNT2* expression was found concomitantly changed with the activation of pSmad2 (Supplementary Fig. S11B), suggesting *GCNT2* expression is TGF-β/Smad dependent. We next knocked down *GCNT2* expression in NMuMG cells with shRNA technique and produced 3 clones with a significant decrease of *GCNT2* expression (Fig. 6B). Nontarget control cells and shRNA1 clone cells (with most significant *GCNT2* knockdown) were then treated with TGF-β1. We found that TGF-β1–induced significant EMT in nontarget control cells within 5 days, but not in shRNA1 clone cells (Fig. 6C). Furthermore, E-cadherin expression was inhibited in nontarget cells with TGF-β1 treatment. However, E-cadherin expression was unchanged in shRNA1 clone cells with *GCNT2* knockdown (Fig. 6D). Conversely, ectopic expression of wild-type *GCNT2*, along with TGF-β1 treatment, induces significant morphologic change of EMT and corresponding molecular changes, such as decreased E-cadherin expression in NMuMG cells compared with vector control cells (Supplementary Fig. S12). All these results indicate that *GCNT2* is involved in and required for TGF-β1–induced EMT.

In addition, we checked the effect of *GCNT2* on common cell signaling pathways. Our data showed that overexpression

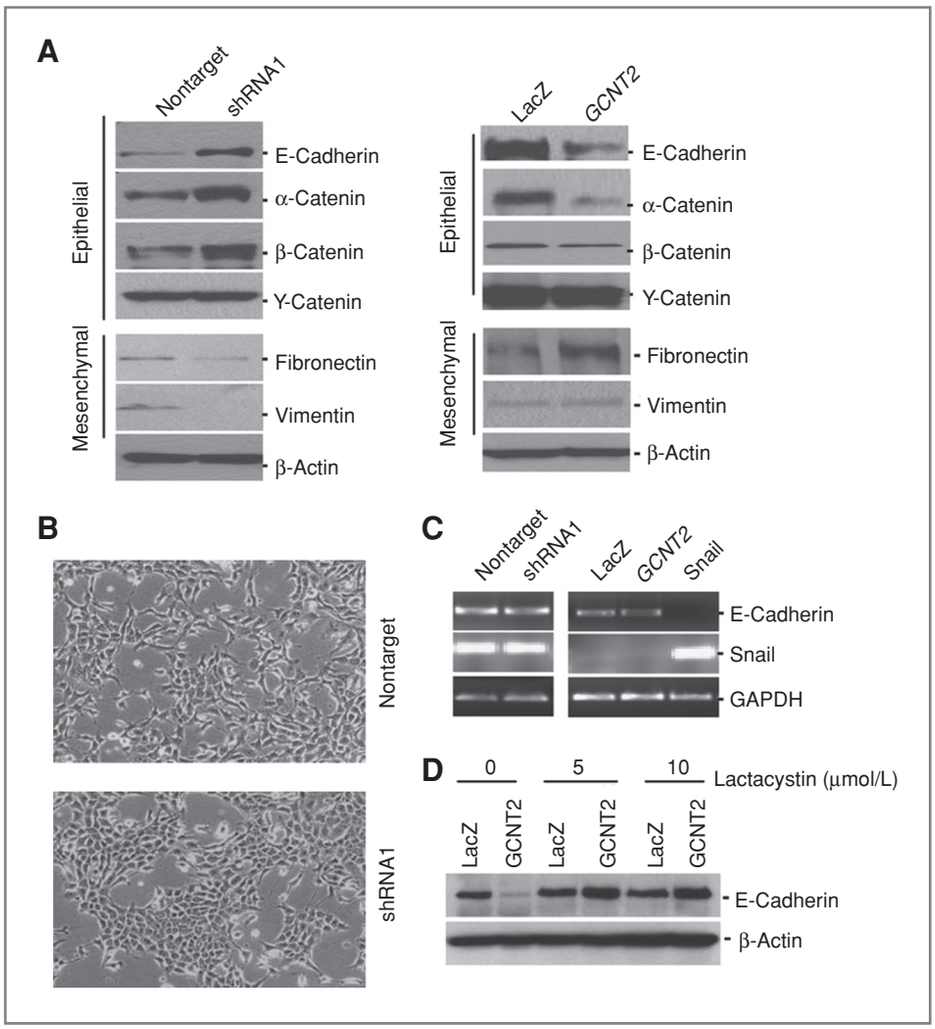

Figure 5. *GCNT2* is actively involved in EMT. A, the effect of *GCNT2* on the EMT program. Left panels show the effect of knockdown of *GCNT2* expression on the EMT program in 4T1 cells. Right panels show the effect of ectopic expression of *GCNT2* on the EMT program in HMLE cells. B, representative pictures show morphologic change after knockdown of *GCNT2* in 4T1 cells. C, the expression of E-cadherin at the RNA level by knockdown of *GCNT2* in 4T1 cells (left) and ectopic expression of *GCNT2* in HMLE cells (right). The *Snail* gene is used as a control. D, the effect of lactacystin on E-cadherin protein level in cells with and without *GCNT2* expression.

of *GCNT2* led to the activation of the ERK and AKT signaling pathway, which is represented by upregulation of pAKT, pSrc, pS6K, and pERK in HMLE and EpRas cells (Supplementary Fig. S13). Conversely, knockdown of *GCNT2* in 4T1 cells led to the inhibition of AKT and ERK signaling.

Glycosyltransferase activity is required for *GCNT2* in promoting cell migration, invasion, and EMT

According to published genetic studies, substitutions of G1049A (Gly350Glu) and G1154A (Arg385His) in exon 3 of *GCNT2* are known to result in an i phenotype (i blood group antigen) and congenital cataracts in patients, which is a result of enzymatic activity elimination of GCNT2 (35). To test whether enzymatic activity is required for the metastasis-promoting function of *GCNT2*, we introduced G1049A and G1154A mutations into the wild-type *GCNT2* and generated corresponding lentiviruses. Stable cell models were then established with this virus to infect the EpRas and NMuMG cells (Fig. 7A). Further studies showed that ectopic expression of mutant *GCNT2* into EpRas cells does not alter cell migration and invasion compared with vector control. These results are markedly different from results obtained with wild-type *GCNT2* expression (Fig. 7B and Supplementary Fig. S14A). Furthermore, ectopic expression of mutant *GCNT2* in cells with wild-type *GCNT2* could not inhibit the promoting effect of wild-type *GCNT2* on cell migration and invasion, suggesting that mutant *GCNT2* does not have a dominant negative effect (Supplementary Fig. S14B). This result is consistent with the genetic evidence showing that patients with I phenotype must have 2 mutant *GCNT2* allele (35). In addition, we found that only the expression of wild-type *GCNT2* but not the mutant *GCNT2* in the *GCNT2* knockdown cells can sensitize the NMuMG cells to TGF-β1 treatment, which is shown by changing of the E-cadherin expression along with the morphologic alterations (Fig. 7C and D). Taken together, these results suggest that the metastasis-promoting effect of *GCNT2* is dependent on its glycosyltransferase activity.

Discussion

Glycosylation is a common posttranslational modification and is involved in a variety of physiologic and pathologic

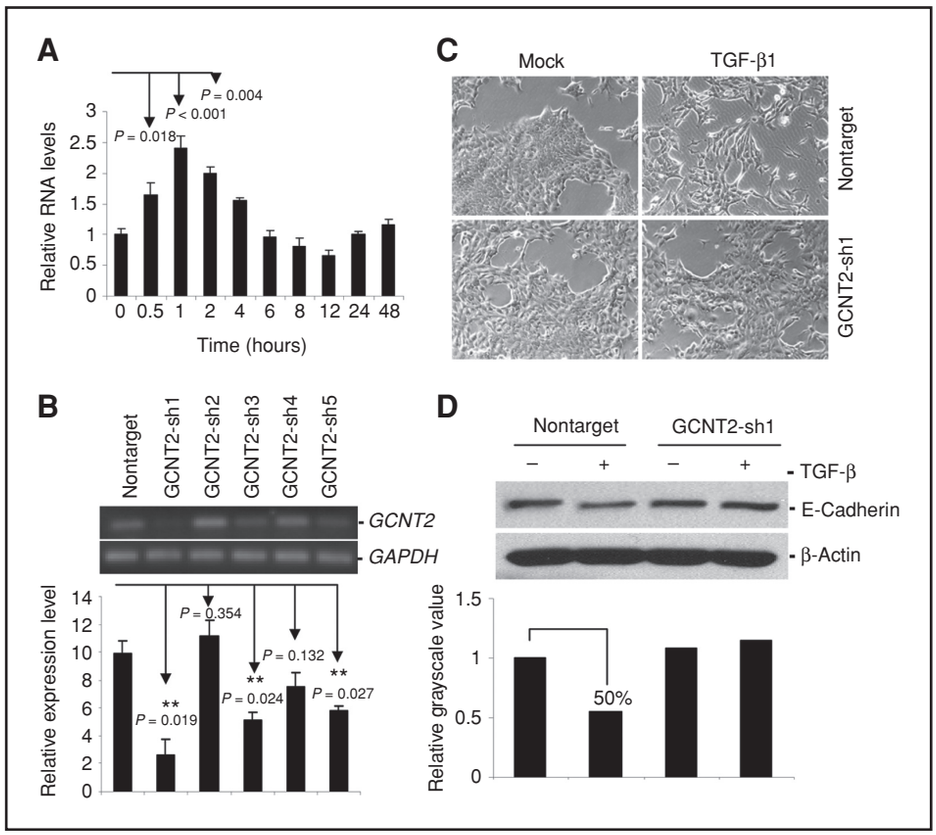

Figure 6. The involvement of *GCNT2* in TGF-β signaling. A, induction of *GCNT2* by TGF-β1. NMuMG cells were treated with 5 ng/mL TGF-β1 for 2 days. *GCNT2* expression is confirmed by quantitative real-time RT-PCR. B, knockdown of *GCNT2* in NMuMG cells with shRNA was confirmed by RT-PCR (top) and real-time RT-PCR (bottom). C, representative pictures show the morphologic change after treatment of TGF-β1 in NMuMG cells with nontarget vector and shRNA1. D, Western blotting results show the effect of *GCNT2* on E-cadherin expression after TGF-β1 treatment in NMuMG cells with nontarget control and shRNA1. The lower panel shows the quantification of the signal intensity.

events (36). Glycosyltransferase is one of the enzymes that catalyze the glycosylation reactions by adding sugar chains to various complex carbohydrates, such as glycoproteins, glycolipids, and proteoglycans. About 170 glycosyltransferases have been cloned (37). However, only a few of them, including *MGAT5*, *MGAT3*, and *GCNT1*, have been associated with cancer metastasis. Our study is the first to identify *GCNT2*, which encodes the enzyme responsible for formation of the blood group I antigen, has a critical biological function in modulating EMT and promoting breast cancer metastasis. Therefore, our study of *GCNT2* in breast cancer metastasis will substantially complement previous studies on other glycosyltransferases and will establish a new paradigm in the metastasis research field.

The biological function of GCNT2 is to branch poly-*N*-lactosamine chains by adding an *N*-acetylglucosaminyl residue via β-1-6 linkage to a galactosyl residue (38). Linear poly-*N*-acetyllactosamines bear the i blood group antigen, whereas the branched molecules bear the I blood group antigen. These i and I antigens are not only present in red blood cells but also on the surface of most human cells (39). Interestingly, I antigen was also found to be present in the soluble glycoproteins in various body fluids including milk, saliva, plasma, urine, and ovarian cyst fluid (39, 40). Specifically, potential roles for GCNT2 in human breast cancers are also suggested by the observation made more than 20 years ago that I antigens in the sera of breast cancer patients were increased and the I antigen levels correlated with the breast carcinoma stages (41–43). The results of our study thus provide direct biological explanation for the long-standing observation and suggest that the overexpression of *GCNT2*, along with breast cancer progression, lead to the abundant expression of protein with I antigen. These proteins with I antigen may be further released into various bodily fluids, including serum. In this regard, direct measurement of the I antigen in body fluids of breast cancer patients may be a promising noninvasive approach to detect metastasis.

The paradox of the TGF-β role in cancer progression has been well documented (44, 45). Recently, TGF-β signaling induced EMT is recognized as an emerging mechanism underlying its metastasis-promoting function (46). In this context, targeting TGF-β signaling has been a promising therapeutic strategy for aggressive cancer including metastatic basal-like breast cancer (47–50). Our current study showed that *GCNT2* is regulated by EMT signaling, such as TGF-β, and enhances EMT and breast cancer metastasis. These results suggest that targeting *GCNT2* will provide an alternative option in targeting TGF-β signaling. However, the regulatory mechanism of TGF-β on *GCNT2* is worthy of further investigation. More importantly, it can be expected that the targets for posttranslational modification by *GCNT2* are not limited to E-cadherin. Many other molecules involved in EMT, cell morphology and cell-to-cell interaction could also be the targets of GCNT2. The changes of these molecules could function together with E-cadherin to contribute to EMT and further cell migration, invasion, and metastasis. Therefore, our study provides an

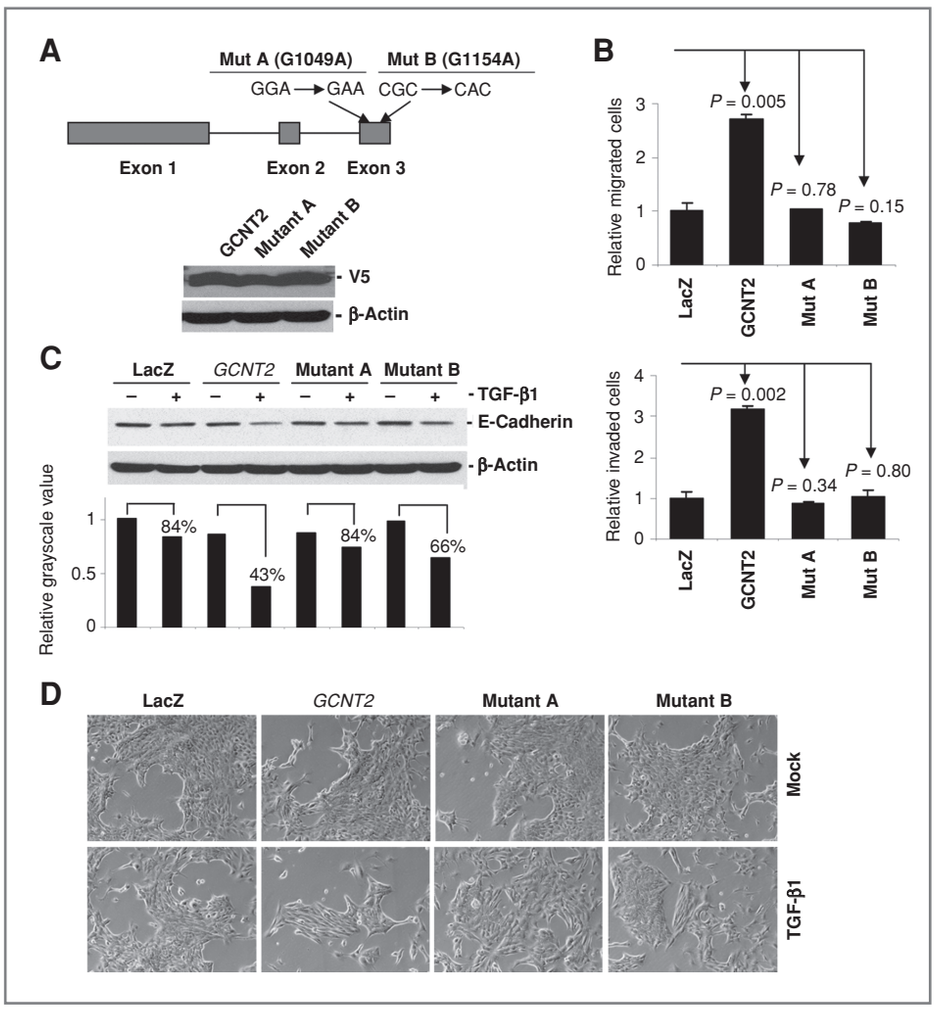

Figure 7. Glycosyltransferase activity is required for cell migration, invasion, and the EMT-promoting function of *GCNT2*. A, the schematic structure of *GCNT2* and the position of the 2 mutations in the molecule (top). The lower panel shows the expression of wild type and 2 mutant forms of *GCNT2* in EpRas cells detected by Western blot with V5 antibody. B, quantitative analysis of the effect of wild type and 2 mutant forms of *GCNT2* on migration and invasion in EpRas cells. C, Western blot shows the expression of E-cadherin in NMuMG cells with *GCNT2* knockdown after expression of LacZ, *GCNT2* wild type and mutant forms. A total of 5 ng/mL TGF-β1 was used for 3 days. The quantitative analysis was done by comparison of the band intensity of TGF-β–treated cells versus the nontreated cells. D, morphologic analysis of the NMuMG *GCNT2* knockdown cells with and without TGF-β1 treatment. Cells with wild type, 2 mutant forms of *GCNT2* and LacZ control were compared.

integrative molecular mechanism that combines the TGF-β signaling and downstream glycosylation modification for breast cancer metastasis. Further in-depth analysis of this mechanism will provide new insight into breast cancer metastasis and will result in novel therapeutic strategies in targeting this disease for which effective therapy is currently unavailable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Eccles SA, Welch DR. Metastasis: recent discoveries and novel treatment strategies. Lancet 2007;369:1742–57.
2. Weigelt B, Wessels LF, Bosma AJ, Glas AM, Nuyten DS, He YD, et al. No common denominator for breast cancer lymph node metastasis. Br J Cancer 2005;93:924–32.
3. Haupt B, Ro JY, Schwartz MR. Basal-like breast carcinoma: a phenotypically distinct entity. Arch Pathol Lab Med 2010;134:130–3.
4. Korsching E, Jeffrey SS, Meinerz W, Decker T, Boecker W, Buerger H. Basal carcinoma of the breast revisited: an old entity with new interpretations. J Clin Pathol 2008;61:553–60.

5. Zhang H, Meng F, Liu G, Zhang B, Zhu J, Wu F, et al. Forkhead transcription factor foxq1 promotes epithelial-mesenchymal transition and breast cancer metastasis. Cancer Res 2011;71:1292–301.
6. Dennis JW, Granovsky M, Warren CE. Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta 1999;1473:21–34.
7. Murata K, Miyoshi E, Kameyama M, Ishikawa O, Kabuto T, Sasaki Y, et al. Expression of N-acetylglucosaminyltransferase V in colorectal cancer correlates with metastasis and poor prognosis. Clin Cancer Res 2000;6:1772–7.
8. Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW. Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat Med 2000;6:306–12.
9. Guo HB, Lee I, Kamar M, Pierce M. N-acetylglucosaminyltransferase V expression levels regulate cadherin-associated homotypic cell-cell adhesion and intracellular signaling pathways. J Biol Chem 2003;278:52412–24.
10. Zhao Y, Li J, Xing Y, Wang J, Lu C, Xin X, et al. N-acetylglucosaminyltransferase V mediates cell migration and invasion of mouse mammary tumor cells 4TO7 via RhoA and Rac1 signaling pathway. Mol Cell Biochem 2008;309:199–208.
11. Iijima J, Zhao Y, Isaji T, Kameyama A, Nakaya S, Wang X, et al. Cell-cell interaction-dependent regulation of N-acetylglucosaminyltransferase III and the bisected N-glycans in GE11 epithelial cells. Involvement of E-cadherin-mediated cell adhesion. J Biol Chem 2006;281: 13038–46.
12. Taniguchi N, Miyoshi E, Ko JH, Ikeda Y, Ihara Y. Implication of N-acetylglucosaminyltransferases III and V in cancer: gene regulation and signaling mechanism. Biochim Biophys Acta 1999;1455: 287–300.
13. Yoshimura M, Nishikawa A, Ihara Y, Taniguchi S, Taniguchi N. Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection. Proc Natl Acad Sci U S A 1995;92:8754–8.
14. Fernandes B, Sagman U, Auger M, Demetrio M, Dennis JW. Beta 1–6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res 1991;51:718–23.
15. Taeda Y, Nose M, Hiraizumi S, Ohuchi N. Expression of L-PHA-binding proteins in breast cancer: reconstitution and molecular characterization of beta 1–6 branched oligosaccharides in three-dimensional cell culture. Breast Cancer Res Treat 1996;38:313–24.
16. Beum PV, Singh J, Burdick M, Hollingsworth MA, Cheng PW. Expression of core 2 beta-1,6-N-acetylglucosaminyltransferase in a human pancreatic cancer cell line results in altered expression of MUC1 tumor-associated epitopes. J Biol Chem 1999;274:24641–8.
17. Shimodaira K, Nakayama J, Nakamura N, Hasebe O, Katsuyama T, Fukuda M. Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. Cancer Res 1997;57:5201–6.
18. Hatakeyama S, Kyan A, Yamamoto H, Okamoto A, Sugiyama N, Suzuki Y, et al. Core 2 N-acetylglucosaminyltransferase-1 expression induces aggressive potential of testicular germ cell tumor. Int J Cancer 2010;127:1052–9.
19. Kuperwasser C, Dessain S, Bierbaum BE, Garnet D, Sperandio K, Gauvin GP, et al. A mouse model of human breast cancer metastasis to human bone. Cancer Res 2005;65:6130–8.
20. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399–405.
21. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, et al. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci U S A 2007;104:10069–74.
22. Zhang H, Chen D, Ringler J, Chen W, Cui QC, Ethier SP, et al. Disulfiram treatment facilitates phosphoinositide 3-kinase inhibition in human breast cancer cells *in vitro* and *in vivo*. Cancer Res 2010;70:3996–4004.
23. Zhang H, Liu G, Dziubinski M, Yang Z, Ethier SP, Wu G. Comprehensive analysis of oncogenic effects of PIK3CA mutations in human mammary epithelial cells. Breast Cancer Res Treat 2008;112:217–27.
24. Inaba N, Hiruma T, Togayachi A, Iwasaki H, Wang XH, Furukawa Y, et al. A novel I-branching beta-1,6-N-acetylglucosaminyltransferase involved in human blood group I antigen expression. Blood 2003;101:2870–6.
25. Kreike B, van Kouwenhove M, Horlings H, Weigelt B, Peterse H, Bartelink H, et al. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Res 2007;9:R65.
26. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, et al. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell 2006;9:121–32.
27. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.
28. Desmedt C, Piette F, Loi S, Wang Y, Lallemand F, Haibe-Kains B, et al. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. Clin Cancer Res 2007;13:3207–14.
29. Ginestier C, Cervera N, Finetti P, Esteyries S, Esterni B, Adelaide J, et al. Prognosis and gene expression profiling of 20q13-amplified breast cancers. Clin Cancer Res 2006;12:4533–44.
30. Lu X, Wang ZC, Iglehart JD, Zhang X, Richardson AL. Predicting features of breast cancer with gene expression patterns. Breast Cancer Res Treat 2008;108:191–201.
31. Haraguchi M, Okubo T, Miyashita Y, Miyamoto Y, Hayashi M, Crotti TN, et al. Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins. J Biol Chem 2008;283:23514–23.
32. Zhang Y, Zhao JH, Zhang XY, Guo HB, Liu F, Chen HL. Relations of the type and branch of surface N-glycans to cell adhesion, migration and integrin expressions. Mol Cell Biochem 2004;260:137–46.
33. Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 2003;116:499–511.
34. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000;2:76–83.
35. Yu LC, Twu YC, Chou ML, Reid ME, Gray AR, Moulds JM, et al. The molecular genetics of the human I locus and molecular background explain the partial association of the adult i phenotype with congenital cataracts. Blood 2003;101:2081–8.
36. Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta 1999;1473:4–8.
37. Narimatsu H. Human glycogene cloning: focus on beta 3-glycosyltransferase and beta 4-glycosyltransferase families. Curr Opin Struct Biol 2006;16:567–75.
38. Bierhuizen MF, Mattei MG, Fukuda M. Expression of the developmental I antigen by a cloned human cDNA encoding a member of a beta-1,6-N-acetylglucosaminyltransferase gene family. Genes Dev 1993;7:468–78.
39. Magnet AD, Fukuda M. Expression of the large I antigen forming beta-1,6-N-acetylglucosaminyltransferase in various tissues of adult mice. Glycobiology 1997;7:285–95.
40. Marsh WL, Nichols ME, Allen FH Jr. Inhibition of anti-I sera by human milk. Vox Sang 1970;18:149–54.
41. Burchell J, Wang D, Taylor-Papadimitriou J. Detection of the tumour-associated antigens recognized by the monoclonal antibodies HMFG-1 and 2 in serum from patients with breast cancer. Int J Cancer 1984;34:763–8.
42. Dube VE, Haid M, Chmiel JS, Anderson B. Serum cold agglutinin and IgM levels in breast carcinoma. Breast Cancer Res Treat 1984;4: 105–8.
43. Dube VE, Kallio P, Chmiel JS, Haid M, Hakim A. An immunosorbent assay for blood group I antigens in breast carcinoma. Clin Immunol Immunopathol 1987;45:196–207.
44. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet 2001;29:117–29.

45. Tian M, Neil JR, Schiemann WP. Transforming growth factor-beta and the hallmarks of cancer. Cell Signal 2010;23:951–62.
46. Taylor MA, Parvani JG, Schiemann WP. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia 2010;15:169–90.
47. Ganapathy V, Ge R, Grazioli A, Xie W, Banach-Petrosky W, Kang Y, et al. Targeting the transforming growth factor-beta pathway inhibits human basal-like breast cancer metastasis. Mol Cancer 2010;9:122.
48. Korpal M, Kang Y. Targeting the transforming growth factor-beta signalling pathway in metastatic cancer. Eur J Cancer 2010;46: 1232–40.
49. Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest 2002;109:1551–9.
50. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. Nat Rev Drug Discov 2004;3:1011–22.