**GATA3 Inhibits Breast Cancer Metastasis through the Reversal of Epithelial-Mesenchymal Transition**

Wei Yan, Qing Jackie Cao, Richard B. Arenas, Brooke Bentley, and Rong Shao

*From the Pioneer Valley Life Sciences Institute, University of Massachusetts, Springfield, Massachusetts 01107, the Departments of Pathology and Surgery, Baystate Medical Center, Springfield, Massachusetts 01199, and the Department of Veterinary and Animal Sciences and Molecular and Cellular Biology Program, Morrill Science Center, University of Massachusetts, Amherst, Massachusetts 01003*

GATA3, a transcription factor that regulates T lymphocyte differentiation and maturation, is exclusively expressed in early stage well differentiated breast cancers but not in advanced invasive cancers. However, little is understood regarding its activity and the mechanisms underlying this differential expression in cancers. Here, we employed GATA3-positive, non-invasive (MCF-7) and GATA3-negative, invasive (MDA-MB-231) breast cancer cells to define its role in the transformation between these two distinct phenotypes. Ectopic expression of GATA3 in MDA-MB-231 cells led to a cuboidal-like epithelial phenotype and reduced cell invasive activity. These cells also increased E-cadherin expression but decreased levels of vimentin, N-cadherin, and MMP-9. Further, MDA-MB-231 cells expressing GATA3 grew smaller primary tumors without metastasis compared with larger metastatic tumors derived from control MDA-MB-231 cells in xenografted mice. GATA3 was found to induce E-cadherin expression through binding GATA-like motifs located in the E-cadherin promoter. Blockade of GATA3 using small interfering RNA gene knockdown in MCF-7 cells triggered fibroblastic transformation and cell invasion, resulting in distant metastasis. Studies of human breast cancer showed that GATA3 expression correlated with elevated E-cadherin levels, ER expression, and long disease-free survival. These data suggest that GATA3 drives invasive breast cancer cells to undergo the reversal of epithelial-mesenchymal transition, leading to the suppression of cancer metastasis.

---

*This work was supported, in whole or in part, by National Institutes of Health, NCI, Grant R01 CA120659 (to R. S.). This work was also supported in part by United States Department of Defense Grant W81XWH-06-01-0563. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4. To whom correspondence should be addressed: Pioneer Valley Life Sciences Institute, University of Massachusetts Amherst, Springfield, MA 01107. Fax: 413-794-0857; E-mail: rong.shao@bhs.org.

The abbreviations used are: ER, estrogen receptor; EMT, epithelial-mesenchymal transition; DCIS, ductal carcinoma in situ; IDC, infiltrating ductal carcinoma; WT, wild type; MT, mutated promoter sequence; MMP, matrix metalloproteinase; siRNA, small interfering RNA.

---

GATA3 (GATA-binding protein 3) is a family member of zinc finger transcription factors (GATA1–GATA6) that bind with high affinity to the consensus DNA sites (T/A-GATA-A/G) (1, 2). GATA1, GATA2, and GATA3 are primarily expressed by hematopoietic cells, whereas GATA4, GATA5, and GATA6 are detectable in the cardiovascular system and endodermus-derived tissues, such as lung, liver, intestine, and pancreas (3). Functional studies of GATA3 in the lineage specification of hematopoietic cells have revealed that GATA3 mediates thymocyte maturation and is abundantly expressed by mature T lymphocytes (4, 5). Recently, it also has been found that GATA3 plays an essential role in the morphogenesis of embryonic mammary tissue. In the adult mammary gland, GATA3 acts on ductal epithelium to maintain the differentiation of luminal epithelial cells (6–8); thereof, GATA3 is recognized as a key regulator of mammary tissue development and mammary gland formation.

Over the past decade, considerable attention has been focused on the differential expression profile of GATA3 in different subtypes of human breast cancers. For instance, studies with differential gene expression techniques, including serial analysis of gene expression (available on the NCI, National Institutes of Health, Web site) and gene microarray have shown that GATA3 is highly expressed in estrogen receptor (ER)²-positive, early stage well differentiated breast cancers other than ER-negative, invasive cancers that are associated with worse clinical outcomes (9–15). A multitude of evidence has demonstrated that GATA3 positively regulates ERα expression through its transactivity on the promoter region of the ERα gene (7, 16–18). Reciprocally, ERα has the ability to directly stimulate the transcription of the GATA3 gene when it is translocated to the nucleus, implicating that these two factors constitute a positive cross-regulatory loop. Consistent with these data, there is a study showing a significant correlation between high levels of GATA3 and responsiveness of ER-positive cancer to hormonal therapy (18). Altogether, these data indicate that GATA3 emerges as a strong predictor of breast cancer differentiation, estrogen responsiveness, and favorable clinical outcome.

Accumulating evidence from both animal tumor models and human cancers has established the notion that the progression of an epithelium-derived tumor into an invasive phenotype involves fibroblast-like transformation, the event referred to as epithelial-mesenchymal transition (EMT) (19–23). During this transformation, tumor cells lose epithelial characteristics that include cell apical-basal polarity, membrane-associated adherents, and the cell-cell adhesion protein E-cadherin. Concurrently, they acquire expression of N-cadherin and vimentin, a mesenchymal cell marker, and also transform to obtain a spin-
dile-like phenotype. Through EMT, tumor cells exhibit the invasive ability to detach from their origin, invade host stroma, escape via vasculature and/or lymphatics, and eventually survive and regrow in a distant region, a process termed tumor metastasis. A number of epithelium-originated carcinomas, including breast and skin cancer, have shown that EMT is essential for cancer progression and metastasis (24–26). However, it has not been rigorously explored whether GATA3 can promote invasive cancer cells to undergo epithelial differentiation through the reversal of EMT. Recently, Kouros-Mehr et al. (27) have found that restoration of GATA3 in advanced mammary carcinoma of transgenic animals triggered cancer cell differentiation and subsequently suppressed cancer metastasis. Here, we attempt to define a role of GATA3 in the regulation of EMT by which it inhibits breast cancer development and metastasis.

EXPERIMENTAL PROCEDURES

Generation of Cancer Cell Lines Stably Expressing GATA3—Full-length GATA3 cDNA was subcloned into a retroviral pCMV-neo vector. Fugene 6 (Roche Applied Science) was used to transfect both the pCL 10A1 vector and pCMV-neo GATA3 expression construct into 293T retroviral packaging cells. Forty-eight hours after transfection, the supernatants were harvested and filtered through 0.45-μm pore size filters. Then the virus-containing medium was transferred to infect human MDA-MB-231 and MDA-MB-435 breast cancer cells. Infected cells were selected with 800 μg/ml G418 starting 48 h after infection, and the drug-resistant cell populations were used for subsequent studies. Cells were cultured with 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin/streptomycin.

GATA3 Gene Knockdown—DNA oligonucleotides (19 bp) specifically targeting the N terminus (siRNA1, bp 154–174, 5’-CATCGACGGTGCAAGGCAAC-3’) or C terminus (siRNA2, bp 1015–1035, 5’-TGCCTGTGGGCTCTACTAC-3’) in 64-oligonucleotide templates were subcloned into a retroviral pSUPER-puro vector. Retroviral media were generated as described above, and the resulting medium was used to infect MCF-7 cells to establish the lines stably silencing the GATA3 gene.

Migration Assays—MDA-MB-231 cells (2 × 105) containing control, GATA3 siRNA1, or siRNA2 vector were preincubated with serum-free medium for 12 h and transferred onto transwells (24-well plates) precoated with collagen IV (100 μg/ml). After 4 h of incubation, cells in the top chamber of the transwell membrane were removed using a Q-tip. The membrane-trapped cells were fixed and stained with hematoxylin. Average cell numbers were quantified from five different fields in each sample.

Cell Invasion Assays—MDA-MB-231 cells (2 × 105) preincubated with serum-free medium were transferred onto transwells (24-well plates) loaded with 50 μl of Matrigel (1 mg/ml) (BD Labware). After 18 h of incubation, the gel, including non-migrating cells, was removed, and cells invading the membrane were stained with hematoxylin and quantified.

Cell Aggregation—Cells (2 × 106) were transferred into a 2-ml Eppendorf tube in serum-free medium containing 1 mM CaCl₂ and placed in shaker at 90 rpm at 37 °C. After 1 h of shaking, the cell suspension was transferred to a culture dish for analyzing cell aggregation. Aggregated cell clones formed by more than 10 cells were quantified under a microscope.

Immunocytochemistry—Cells were fixed with 4% paraformaldehyde and blocked with PBS containing 5% goat serum, 1% bovine serum albumin, and 0.05% Nonidet P-40, followed by incubation with an anti-vimentin or E-cadherin antibody (1:500) overnight. Then a goat anti-mouse IgG fluorescein-conjugated secondary antibody (1:1000; Invitrogen) was added for 1 h, and fluorescence was examined under a microscope.

Western Blot Analysis—Cells were collected by scraping and then extracted in a lysis buffer (pH 7.4) containing 0.25 mM HEPES, 14.9 mM NaCl, 10 mM NaF, 2 mM MgCl₂, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin A, and 20 μM leupeptin. The lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting supernatant was collected for 10% SDS-PAGE. Proteins were transferred to a nylon membrane (Invitrogen) and incubated with one of several antisera: mouse anti-GATA3 (1:500; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), anti-vimentin (1:500; Hybridoma Bank, University of Iowa), mouse anti-MMP-9 (1:500; VWR), E-cadherin (1:400) and N-cadherin (1:250; Invitrogen), and actin (1:1000; Sigma). Horseradish peroxidase-labeled goat anti-mouse antiserum (1:10,000; Sigma) and an ECL kit (VWR) were used for detection.

Gelatin Zymography—Cell-conditioned serum-free media were collected for a zymographic analysis. Gelatinolytic activities were assessed under non-reducing conditions using an 8% SDS-polyacrylamide gel with 1 mg/ml gelatin. After washing with 2.5% Triton X-100 twice for 30 min, the gel was incubated in zymography buffer containing 150 mM NaCl, 5 mM CaCl₂, 50 mM Tris-HCl, pH 7.5, overnight at 37 °C. Then the gel was stained with 0.2% Coomassie Brilliant Blue for 4 h and destained with methanol/acetic acid/water for 2 h.

Transient Transfection and Luciferase Activity of E-cadherin Promoter—A fragment of the E-cadherin promoter region (wild type or mutant from −170 to −221 bp) was introduced into a pGL3-promoter vector containing a luciferase reporter gene (Promega, Madison, WI). The resulting pGL3-promoter plasmid was introduced into cells using Lipofectamine as a transfectant (Invitrogen) according to the manufacturer’s instructions. Following a 48-h incubation, the cells were incubated in lysis buffer provided with the Luciferase Report System kit (Promega). The lysates were centrifuged, and the resulting supernatants were tested for luciferase activity.

Chromatin Immunoprecipitation—The E-cadherin promoter region of 997 bp was analyzed for GATA3 binding by chromatin immunoprecipitation and the PCR method. PCR sense and antisense primers of the E-cadherin promoter region of 997 bp were subcloned into a pGL3-promoter vector containing a luciferase reporter gene. Chromatin immunoprecipitations were assessed under non-reducing conditions using an 8% SDS-polyacrylamide gel with 1 mg/ml gelatin. After washing with 2.5% Triton X-100 twice for 30 min, the gel was incubated in zymography buffer containing 150 mM NaCl, 5 mM CaCl₂, 50 mM Tris-HCl, pH 7.5, overnight at 37 °C. Then the gel was stained with 0.2% Coomassie Brilliant Blue for 4 h and destained with methanol/acetic acid/water for 2 h.

Xenograft Tumor Models—This animal study was approved by the Institutional Animal Care and Use Committee of Baystate Medical Center. Four-week-old female SCID/Beige mice...
(Charles River Laboratories) were injected under the fat pad with MDA-MB-231 cells \((1 \times 10^6)\) expressing GATA3 or control vector or MCF-7 cells \((4 \times 10^6)\) carrying GATA3 siRNA or control vector in 0.1 ml of Hanks’ balanced buffer without calcium or magnesium. Primary tumors were measured weekly until the mice appeared moribund. Tumor volume was calculated based on the equation, volume = length \(\times\) width\(^2\) \(\times\) 0.52. Mouse tumor samples and samples from the lung and the liver were processed for paraffin-embedded sectioning followed by hematoxylin and eosin staining.

**Human Tissue Collection**—Normal mammary tissue and cancer specimens were obtained from patients who underwent surgery at Baystate Medical Center between the years 2000 and 2002. All of the samples were collected under a protocol approved by the University of Massachusetts and the Baystate Medical Center Institutional Review Board. Of these, six normal subjects underwent mammary reduction, 18 patients had ductal carcinoma *in situ* (DCIS), and 36 cases were diagnosed with infiltrating ductal carcinoma (IDC). A review of medical records of cancer patients included ER status, distant metastasis, and disease-free survival.

**Immunohistochemistry**—Paraffin-embedded human specimens were cut to 6-\(\mu\)m thickness and processed for the staining of GATA3 and E-cadherin. In brief, the samples were blocked with 3% \(\text{H}_2\text{O}_2\) to block endogenous peroxidase activity for 30 min, followed by incubation with blocking buffer containing 10% goat serum for 1 h. Then mouse anti-GATA3 (1:200; Santa Cruz Biotechnology, Inc.) or anti-E-cadherin (1:2000; Invitrogen) was incubated at room temperature for 2 h, and goat anti-mouse secondary antibody (1:100) conjugated with horseradish peroxidase was added. Finally, diaminobenzidine substrate (Dako Inc.) was introduced for several min, and after washing, methyl green was used for counterstaining.

**Statistics**—Data are expressed as mean \(\pm\) S.E., and differences among groups were determined using one-way analysis of variance. The 0.05 level of probability was used as the criterion of significance. Survival data were analyzed using the method of Kaplan-Meier. Statistical significance was determined by log-ranking analysis and Cox regression models.

**RESULTS**

We began by testing the differential expression of GATA3 in ER-positive, non-invasive breast cancer cell lines T47D and MCF-7 and in ER-negative, invasive cell lines MDA-MB-231 and MDA-MB-435. Consistent with the data reported in a number of previous publications (13, 14), T47D and MCF-7 cells, but not MDA-MB-231 and MDA-MB-435 cells, expressed GATA3 (supplemental Fig. 1). The former two lines exhibited an epithelium-like morphology, whereas the latter were basal fibroblast-like cells (data not shown). These two distinct phenotypes are keenly associated with cell noninvasiveness and invasiveness, respectively. In an attempt to explore a possible functional role of GATA3 in epithelial breast cancers, we created a full-length GATA3 cDNA from MCF-7 cells, which contains 1329 bp with an open reading frame of 443 amino acids with a molecular mass around 48 kDa (Fig. 1A). We then tested whether GATA3 can drive mesenchyme-like breast cancer cells to undergo epithelial transformation when this gene was introduced to GATA3-negative MDA-MB-231 cells. As shown in Fig. 1A, a population of MDA-MB-231 cells expressing GATA3 or vector were used for immunoblotting using anti-GATA3, vimentin, E- and N-cadherin, and actin antibodies. MCF-7 cells were used as an epithelial cell control. B, cells were grown on 24-well plates and analyzed for either morphological alteration by phase-contrast images or immunocytochemistry staining using anti-vimentin and E-cadherin antibodies as described under “Experimental Procedures.” Bars, 25 \(\mu\)m.

![Figure 1. Reversal of EMT in MDA-MB-231 cells ectopically expressing GATA3.](image-url)

A. Cell lysates from MCF-7, MDA-MB-231 cells expressing GATA3 or vector were used for immunoblotting using anti-GATA3, vimentin, E- and N-cadherin, and actin antibodies. MCF-7 cells were used as an epithelial cell control. B, cells were grown on 24-well plates and analyzed for either morphological alteration by phase-contrast images or immunocytochemistry staining using anti-vimentin and E-cadherin antibodies as described under “Experimental Procedures.” Bars, 25 \(\mu\)m.
invasive activities, cell survival was also decreased when these cells overexpressing GATA3 were challenged with an apoptotic milieu developed from the addition of cobalt chloride (CoCl₂), a hypoxia inducer, to cultured medium (supplemental Fig. 4). Taken together, the results from both phenotypic and functional characterization strongly suggest that GATA3 triggers invasive, fibroblast-like breast cancer cells to reverse EMT.

As a transcription factor, GATA3 binds with high affinity to consensus GATA or GATA-like motifs located in a promoter region by which it transactivates gene expression. To test the likelihood that GATA3 functions to up-regulate E-cadherin expression through binding its motifs located in E-cadherin promoter, we determined the transactivity of GATA3 using a luciferase reporter gene assay. In an analysis of a proximal promoter region of E-cadherin from the translation start codon +1 bp to upstream −221 bp, we found that this promoter sequence harbors two GATA-like binding elements (TGACTG) at −182 and −214 bp and one Snail binding motif (CAGGTG) at −197 bp (Fig. 3A). In order to monitor transactivity of GATA3, this E-cadherin promoter spanning from −170 to −221 bp was introduced into the upstream region of a luciferase reporter gene. Transcriptional activity was subsequently evaluated by measuring luciferase reporter gene levels after a transient transfection into MCF-7 cells that express high levels of endogenous GATA3 and MDA-MB-231 cells that express ectopic GATA3 or vector. Due to the lack of Snail in MCF-7 cells (data not shown), transcriptional regulation of E-cadherin was exclusively dependent on the presence of GATA3 in these cells. As expected, as much as 12-fold activity was induced in MCF-7 cells transfected with a reporter gene construct containing E-cadherin wild type (WT) promoter compared with the basal activity in the cells transfected with a construct lacking this promoter (Fig. 3B). However, transfection of control MDA-MB-231 cells with the WT reporter gene exhibited a decrease of the activity as compared with the basal level (Fig. 3B). This inhibition is presumably due to the presence of Snail (Fig. 3C) that functions to block gene transcription (28, 29). Conversely, transfection of MDA-MB-231 cells expressing ectopic GATA3 with the WT reporter gene resulted in increased activity by 6-fold relative to the vector counterpart (Fig. 3B). To validate the inhibitory effect of Snail on E-cadherin gene transcription, we created a mutated promoter sequence (MT) by substituting GG with CC in the Snail-binding element (Fig. 3A). Following transfection of MCF-7 cells with vector control, WT, or MT reporter gene, the activities in both WT- and MT-transfected cells were induced, the magnitude of which were as much as 12-fold the control level (Fig. 3B). The results suggest that E-cadherin is mainly regulated by GATA3 but not Snail, which is absent in MCF-7 cells. In MDA-MB-231 cells, however, the transcriptional activity is largely controlled by Snail. Transfection of control MDA-MB-231 cells with the WT reporter gene displayed a decrease of basal activity, consistent with the above finding (Fig. 3B), whereas MT gene transfection reversed the activity to the basal level (Fig. 3C). Noticeably, in MDA-MB-231 cells ectopically expressing GATA3, transfection of WT reporter gene led to elevated transactivity of GATA3, which was ~8-fold higher than the control (Fig. 3C). Not surprisingly, once the cells were transfected with MT reporter gene, the
GATA3 Inhibits Breast Cancer Metastasis

To strengthen these important findings, we engaged a complementary approach by silencing the GATA3 gene in MCF-7 cells that express a high level of endogenous GATA3 and exhibit a cuboidal-like phenotype (Fig. 1). GATA3 siRNA1 gene knockdown that targets the GATA3 N terminus, unlike siRNA2 that blocks the C terminus, suppressed GATA3 expression by more than 70% relative to a non-sense control (Fig. 5A). In line with the finding that E-cadherin expression was largely dependent on the level of GATA3 (Figs. 1 and 3), E-cadherin expression in GATA3 siRNA1 cells was decreased, and these cells became more fibroblast-like as compared with either control or siRNA2 cells (Fig. 5, A and B). To further determine whether this reduction of E-cadherin expression is also attributable to the attenuated transcriptional activity, as observed earlier (Fig. 3), we utilized the same luciferase reporter gene system in which GATA3 governs gene expression. As demonstrated in Fig. 5C, the reduced expression of GATA3 by siRNA1 gene knockdown led to a significant decrease of the reporter gene activity. In addition, evidence of the direct binding between GATA3 and E-cadherin promoter displayed the reduction of GATA3 binding to the promoter sequence in siRNA1 cells (Fig. 5D). Accordingly, functional analyses showed that siRNA1 gene knockdown inhibited cell aggregation by 60–80% in comparison with control or siRNA2 cells (Fig. 5E), whereas motility of siRNA1 cells was expectedly increased by ~3-fold as compared with its corresponding counterparts (Fig. 5F). Collectively, these findings by means of siRNA gene knockdown provided strong evidence to underscore the pivotal role of GATA3 in the control of EMT.

To monitor effects of GATA3 gene knockdown on tumor development in vivo, we injected MCF-7 cells containing GATA3 siRNA1 and control vector into mice as we did before. Mice began to form primary tumors in mammary tissue from week 5–6 following injection. Six of eight mice injected with siRNA1 MCF-7 cells developed larger tumors, whereas three of eight control mice formed smaller, palpable tumors, and the other mice did not form tumors within a 21-week period (Fig. 6A). We further found that mice injected with siRNA1 cells began to die from week 8 and continued to diminish (seven of eight mice) by the end of 21 weeks, whereas only two animals receiving control cells died during weeks 16–18, and the remaining six mice lived over 21 weeks (Fig. 6B), indicating a significantly shorter survival rate of mice bearing siRNA1 tumor cells compared with control mice. Pathological analysis

activity was strikingly augmented, the increased level of which was equivalent to the activity observed in MCF-7 cells (Fig. 3C). Altogether, the data illustrate a positive and negative regulatory model for E-cadherin in which GATA3 acts as a predominant factor to up-regulate E-cadherin.

To determine if GATA3 also inhibits tumor cell invasiveness in vivo, we employed a tumor xenograft model by injection of MDA-MB-231 cells carrying GATA3 into mammary fat pad tissue of immunocompromised mice. Consistent with the decreased invasive activity observed in vitro earlier (Fig. 2), mice receiving GATA3-expressing MDA-MB-231 cells gradually developed palpable tumors during a 7-week period (Fig. 4A). These GATA3-expressing cells grew tumors as little as 10% of control tumor volume. In order to determine the inhibitory effects of GATA3 on tumor malignancy, these mice were observed for approximately 3 months. During 2–3 months, the volume of these primary tumors was not markedly altered; however, the survival rate of mice bearing control MDA-MB-231 cells rapidly declined (Fig. 4B). In contrast, all of the mice carrying GATA3 tumors survived over 3 months. In further analysis of tumor metastasis, all of six mice receiving vector control cells developed distant tumors in the lung, contrary to the mice bearing GATA3 tumors, in which none of the secondary lung tumors was identified (Fig. 4C). These findings suggest that GATA3 acts as a tumor suppressor to block tumor development and metastasis.

FIGURE 3. GATA3 binds GATA-like motif of E-cadherin promoter and transactivates reporter gene expression. A, the 5′-untranslated region from bp −170 to −221 of E-cadherin was introduced upstream of a luciferase reporter gene, and the resulting plasmid or a control plasmid without the promoter was transfected into MCF-7 cells and MDA-MB-231 cells ectopically expressing GATA3 or vector. GATA3 and Snail binding motifs are underlined. GG of the wild type Snail binding motif (WT) were mutated to CC (MT). B, following a 2-day transfection with vector control or WT, cells were lysed, and luciferase activity was measured. The luciferase activities were normalized to the levels of lysate protein, and these activities in WT-transfected cells were compared with their corresponding vector control activities arranged as unit. n = 4, *, p < 0.05 compared with corresponding controls. C, MDA-MB-231 cells expressing GATA3 or vector were used to test Snail mRNA levels by PCR. MCF-7 and MDA-MB-231 cells carrying GATA3 or vector were transfected with vector, WT, or MT luciferase reporter gene, and the resulting plasmid or a control plasmid without the promoter was transfected into MCF-7 cells and MDA-MB-231 cells ectopically expressing GATA3 or vector. GATA3 and Snail binding motifs are underlined.

APRIL 30, 2010

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 285 • NUMBER 18 • 14046
levels of GATA3 correlated with E-cadherin as well as ER expression (Table 1). Moreover, in the analysis of 5-year disease-free survival, we discovered that GATA3-positive patients exhibited longer disease-free survival than did GATA3-negative patients (Fig. 7D). The average of disease-free survival of these GATA3-expressing cases was 48.9 months compared with 30.5 months of GATA3-negative patients (Fig. 7E). In agreement with this clinical information, only 9.5% (2 of 21) of patients who demonstrated positive GATA3 gave rise to distant organ metastases, such as liver or contralateral tumors, whereas 5.6-fold more (53.4%, 8 of 15) patients whose tissues were GATA3-negative developed distant metastases (Fig. 7F). All of the data indicate that expression levels of GATA3 positively correlate with elevated E-cadherin, ER$^{+}$, and favorable clinical outcomes.

**DISCUSSION**

GATA3 was primarily identified as a transcription factor regulating T-lymphocyte differentiation (30). A plethora of data documented in literature have demonstrated an essential role of GATA3 in the T cell differentiation and maturation, such as up-regulation of interleukin-4 by GATA3, a cytokine specific for Th2 lineage (31–34). In concert with this cell differentiation property, our current study found that GATA3 acquired by invasive, basal-like breast cancer cells promotes the cells to undergo an epithelium-like differentiation, the reversal of EMT, thereby inhibiting cancer metastasis. Further, GATA3-induced E-cadherin gene expression emerges as the key molecular mechanism underlying the blockade of tumor metastasis.

It is emerging that GATA3 may serve as a non-invasive breast cancer biomarker based on a large body of clinical evidence that ER-positive, well differentiated epithelial breast cancer, in contrast to ER-negative, invasive breast cancer, expresses a noticeably high level of GATA3 (9, 12, 13, 35). Here, we analyzed not only these distinct expression patterns in breast cancer but also the relationship between its expression levels and clinical outcomes, including cancer metastasis and disease-free survival. Indeed, we found that GATA3-positive cancer patients displayed a better prognosis than GATA3-negative patients. Furthermore, we identified the positive correlation between the expression levels of GATA3 and E-cadherin, a tumor suppressor that predicts a favorable prognosis for cancer patients. In context with the studies of cultured cells and animal models,
the human data have presented critical evidence to support the notion that GATA3 could represent a powerful tool for breast cancer diagnosis and prognosis.

EMT is associated with normal embryonic development in addition to cancer metastasis (35). When human embryonic stem cells in culture were treated with an anti-E-cadherin neutralizing antibody that disrupts cell-cell tight contacts, these cells not only underwent morphological transformation displaying a mesenchymal phenotype but also increased expression of N-cadherin, vimentin, Snail, Slug, MMP-2, and MMP-9. In addition, these cells acquired elevated cellular motility and invasiveness (35, 36). All of the events demonstrate a typical process of EMT once E-cadherin function is impaired. A fundamental difference of EMT between the normal embryonic and tumorigenic processes is that the latter involve genetically abnormal cells that are not regulated by normal growth-regulatory signals. A wealth of evidence in cancer research has demonstrated that the loss of E-cadherin, which normally renders epithelial tumor cells immobile, is a prerequisite and rate-limiting for EMT in tumor metastasis (35, 37–40). Apart from the genetic alteration, epigenetic modification of the E-cadherin gene also plays a role in malignant tumor transformation. For example, the status of methylation or hypermethylation of E-cadherin gene was found to be intimately associated with EMT and metastatic stages (41, 42). In the present study, we found that MCF-7 cells with defective E-cadherin by GATA3 gene knockdown displayed increased cell invasiveness and developed distant tumor metastasis. Congruent with this finding, a complementary study by the introduction of GATA3 to MDA-MB-231 cells as well as MDA-MB-435 cells (data not shown) showed the elevated expression of E-cadherin, resulting in the reversal of EMT. This reversible event was not solely observed in the suppression of metastasis; rather, it was also noted in the homing of cancer cells to ectopic regions where disseminated mesenchyme-like cancer cells fell back to a more differentiated epithelial cell state, although the molecular base and pathologic purpose underlying this transformation in the metastatic scenario are currently unclear (43–45). Nevertheless, our data strengthen the notion that the reversal of EMT is primarily dependent on the expression of E-cadherin, highlighting the plasticity of malignant cancer cells. In the animal models, we also interestingly found that GATA3 inhibited...
primary tumor growth, suggesting that it may regulate other tumor suppressor genes and/or oncogenes in addition to E-cadherin, all of which participate in the inhibition of tumor initiation and development.

Recently, Kouros-Mehr et al. (27) have suggested that a small population of breast cancer stem cells lacking GATA3 may contribute to cancer dissemination and malignancy. Indeed, an intimate link between EMT and cancer stem cells has been gradually uncovered. EMT confers many of the properties of the neoplastic stem cell state and also contributes to the generation of cancer stem cells (43, 46). However, the question of whether deficiency of GATA3 in breast cancer stem cells predisposes the cells toward EMT and metastasis deserves further investigation.

Significant progress was made earlier in the characterization of different binding affinities of GATA proteins with consensus GATA or GATA-like motifs (1, 2). GATA1, GATA2, and GATA3 were found to recognize and bind a series of GATA and GATA-like sequences with distinct binding affinities, in which GATA1 and GATA3 demonstrate the highest binding affinity with the T-GATT-G motif (1, 2). In the present study, we focused on a proximal promoter region from the translation initiation site to 221 bp upstream in which two T-GATT-G sequences reside. Transactivity of GATA3 in MDA-MB-231 cells ectopically expressing GATA3 was induced even in the presence of endogenous Snail that exerts an inhibitory impact in E-cadherin expression (28, 29, 47). Moreover, once the Snail
binding motif was mutated, the transactivity of GATA3 was dramatically augmented to the level observed in MCF-7 cells, which express GATA3 but not Snail. Our data revealed a dual regulatory mode in which GATA3 acts as a predominant factor to induce E-cadherin.

There is abundant evidence revealing a keen relationship between functional properties and structural specificity of GATA3 that contains zinc finger 1 (ZnF1) and zinc finger 2 (ZnF2) domains (5, 48, 49). ZnF1 is essential for the stability and specificity of DNA binding, whereas the ZnF2 is required for the recognition of the consensus GATA-binding elements. Either partial deletion or mutation of the ZnF2 domain can fully ablate the abilities to bind to DNA and transactivate gene expression (48, 50). A number of ZnF2-mutated GATA3 genes, such as base insertion, missense, non-sense, and deletion, were identified in primary breast cancer and breast cancer lines, including MCF-7 cells (50). In agreement with these data, we found that MCF-7 cells contained a truncated form with a molecular mass around 31 kDa (data not shown), which is presumably non-functional because of the deletion of ZnF2, as reported previously (50). It should be quite interesting to explore in the future whether primary human breast cancers contain both wild type and mutated GATA3 and, if they do, whether some or all of the different mutated GATA3s are asso-

**TABLE 1**

| E-cadherin (n = 36) | GATA3 | + | − | p value |
|---------------------|-------|---|---|--------|
| High                | 18    | 5 |   | <0.01  |
| Low                 | 3     | 10|   |        |

| ER* (n = 25) | + | − | p value |
|--------------|---|---|--------|
| 12           | 1 |   | 0.001  |
| 1            | 11|   |        |

* In 36 IDC cases, ER status of 11 cases was not available.

**FIGURE 7.** Expression of GATA3 in human breast normal and cancer tissue and its relationship with clinical outcomes. A–C, specimens from six normal subjects, 18 DCIS, and 36 IDC patients were processed for immunohistochemistry of GATA3 and E-cadherin as described under “Experimental Procedures.” Representatives of both GATA3 and E-cadherin staining in normal tissue (A), DCIS (B), and IDC (C) are shown. In IDC, GATA3 was evaluated based on its positive and negative expression, and E-cadherin was determined on low and high expression levels (C). Bars, 50 μm. D–F, clinical data of a 5-year follow up from 36 IDC patients were collected to analyze disease-free survival (D), average survival months (*, p < 0.05) (E), and distant metastasis that did not have any anatomic connection to the primary tumors (F) between the GATA3-positive and -negative group.
GATA3 Inhibits Breast Cancer Metastasis

1. Ko, L. J., and Engel, J. D. (1993) *Mol. Cell. Biol.* 13, 4011–4022
2. Merika, M., and Orkin, S. H. (1993) *Mol. Cell. Biol.* 13, 3999–4010
3. Patient, R. K., and McGhee, J. D. (2002) *Curr. Opin. Genet. Dev.* 12, 416–422
4. Ranganath, S., and Murphy, K. M. (2001) *Mol. Cell. Biol.* 21, 2716–2725
5. Yang, Z., Gu, L., Romeo, P. H., Bories, D., Motohashi, H., Yamamoto, M., Chinnaiyan, A. M., and Kleer, C. G. (2005) *Cancer Res.* 65, 11259–11264
6. Garcia-Clossas, M., Troester, M. A., Qi, Y., Langerød, A., Yeager, M., Lisowski, J., Brinton, L., Welch, R., Peplonska, B., Gerhard, D. S., Gram, I. T., Kristensen, V., Børresen-Dale, A. L., Chanock, S., and Perou, C. M. (2007) *Cancer Epidemiol. Biomarkers Prev.* 16, 2269–2275
7. Bertucci, F., Van Hulst, S., Bernard, K., Lorioïd, B., Granjeaud, S., Taggett, R., Starkey, M., Nguyen, C., Jordan, B., and Birnbaum, D. (1999) *Oncogene* 18, 3905–3912
8. Bertucci, F., Houlgatte, R., Benzaïne, A., Granjeaud, S., Adélaïde, J., Taggett, R., Lorioïd, B., Jacquesier, J., Viens, P., Jordan, B., Birnbaum, D., and Nguyen, C. (2000) *Hum. Mol. Genet.* 9, 2891–2899
9. Hoch, R. V., Thompson, D. A., Baker, R. J., and Weigel, R. J. (1999) *Int. J. Cancer* 84, 122–128
10. Yang, G. P., Ross, D. T., Kuang, W. W., Brown, P. O., and Weigel, R. J. (1999) *Nucleic Acids Res.* 27, 1517–1523
11. Sordet, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., and Børresen-Dale, A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 10869–10874
12. Voduc, D., Cheung, M., and Nielsen, T. (2008) *Cancer Epidemiol. Biomarkers Prev.* 17, 365–373
13. Eckehoute, J., Keeton, E. K., Lupien, M., Krum, S. A., Carroll, J. S., and Brown, M. (2007) *Cancer Res.* 67, 6477–6483
14. Parikh, P., Palazzo, J. P., Rose, L. J., Daskalakis, C., and Weigel, R. J. (2005) *J. Am. Coll. Surg.* 200, 705–710
15. Yan, W., and Shao, R. (2006) *J. Biol. Chem.* 281, 19700–19708
16. Boyer, B., Vallès, A. M., and Thiery, J. P. (1996) *Acta Anatónica* 156, 227–239
17. Birchmeier, C., Birchmeier, W., and Brand-Saberi, B. (1996) *Acta Anatónica* 156, 217–226
18. Savagner, P. (2001) *BioEssays* 23, 912–923
19. Thiery, J. P. (2002) *Nat. Rev. Cancer* 2, 442–454

associated with loss of its activity. Nonetheless, our present study indicates that the wild type GATA3 expressed by MCF-7 cells acts as a transcription factor to transactivate E-cadherin gene expression once engineered to MDA-MB-231 cells.

Altogether, we have presented evidence to unveil an association of GATA3 with the reversal of EMT in the inhibition of tumor metastasis and also define molecular mechanisms for the phenomenon reported previously that GATA3 is differentially overexpressed in well differentiated, epithelium-like breast cancers but not in invasive cancers. Our findings appear to hold a number of implications for breast cancer diagnosis, prognosis, and therapeutic strategies designed to target different subtypes of breast cancer.

REFERENCES