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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

The Regulation of SOX7 and Its Tumor Suppressive Role in Breast Cancer

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Both epigenetic silencing and genetic deletion of tumor suppressors contribute to the development and progression of breast cancer. SOX7 is a transcription factor important to development, and its down-regulation has been reported in tumor tissues and cell lines of prostate, colon, and lung cancers. However, the regulation of SOX7 expression and its functional role in breast cancer have not been reported. The current study demonstrates that SOX7 mRNA and protein expression are down-regulated in breast cancer tissues and cell lines compared with adjacent normal tissues and nontumorigenic cells, respectively. The *SOX7* promoter is hypermethylated in breast cancer cell lines compared with non-tumorigenic cells, and the inhibition of DNA methylation increases SOX7 mRNA levels. With shRNA-mediated *SOX7* silencing, nontumorigenic immortal breast cells display increased proliferation, migration, and invasion and form structures that resemble that of breast cancer cells in a three-dimensional culture system. Conversely, ectopic SOX7 expression inhibits proliferation, migration, and invasion of breast cancer cells *in vitro* and tumor growth *in vivo*. Importantly, we discovered that *SOX7* transcript levels positively correlated with clinical outcome of 674 breast cancer patients. Overall, our data suggest that *SOX7* acts as a tumor suppressor in breast cancer. *SOX7* expression is likely regulated by multiple mechanisms and potentially serves as a prognostic marker for breast cancer patients. *(Am J Pathol 2013, 183: 1645–1653; http://dx.doi.org/10.1016/j.ajpath.2013.07.025)*

The Sex-determining region Y-box (SOX) family comprises >20 transcription factors that are divided into subfamilies A through H based on their conserved structural motifs. The founding member of the SOX family, Sex-determining region Y (Sry), is the premiere sex-determining factor in male development.[1,2] SOX proteins share homologic features with Sry both within and outside their high-mobility group domain that recognizes and binds DNA at the SOX consensus site of 5′-ATATCAAAT-3′.[3]

Historically appreciated for their role in development, various SOX proteins are becoming increasingly recognized as important players in the genesis and progression of human cancers.[4] SOX7, a member of subgroup F along with SOX17 and SOX18, has been reported to regulate hematopoiesis and cardiogenesis.[5–8] As a developmental regulator, SOX7 has more recently been demonstrated to regulate vascular endothelial cadherin expression during hematopoietic development, which implicates its role in human cancers.[9] Indeed, SOX7 down-regulation has been observed in tumors of the colon, prostate, and lung.[10–13] Furthermore, SOX7 overexpression suppressed cell proliferation and colony formation in prostate and colon cancer cell lines and induced apoptosis in colon cancer cells.[10,12] Consistently, *SOX7* silencing has been attributed to its hypermethylation in tumors, and this effect correlated with poor prognosis in

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myelodysplastic syndrome patients.[14] The p38 mitogen-activated protein kinase signaling pathway has also been reported to regulate SOX7. Mitogen-activated protein kinase signaling—regulated transcription factors c-fos and c-jun up-regulated *SOX7* promoter activity in a reporter assay.[15] Although the precise molecular mechanism by which SOX7 exerts its tumor suppressive effects has yet to be definitively determined, SOX7 has been reported to interact with β-catenin and inhibit cell proliferation mediated by WNT signaling pathways.[8,10,16]

The *SOX7* gene is located on the p arm of human chromosome 8 at a locus frequently lost in breast tumors.[17] In addition, significant down-regulation of the SOX7 mRNA was reported in a group of nine primary breast cancers.[16] In this study, we investigated the mechanisms of *SOX7* down-regulation and demonstrated its tumor suppressive role in breast cancer and positive correlation with favorable clinical outcome of breast cancer patients.

Materials and Methods

Antibodies and DNA Vectors for Gene Expression and Knockdown

The antibodies, their catalog numbers, and the vendors used in this study are SOX7 (AF2766; R&D Systems, Minneapolis, MN), Ezh2 (AC22; Cell Signaling Technology, Beverly, MA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10R-G109A; Fitzgerald Industries International, Acton, MA), β-actin (MAB1501; Chemicon International, Temecula, CA), DNA methyltransferase 1 (DNMT1) (5032; Cell Signaling Technology), and Ki-67 (sp6; NeoMarkers Inc., Fremont, CA).

We used our previously described lentiviral vector pSL5 with a puromycin selection marker to express any inserted cDNA under the control of the chicken β-actin promoter.[18,19] For inducible *SOX7* expression, we generated the pTetOn-7 vector using a minimal cytomegalovirus promoter to drive cDNA expression with six copies of tetracycline-response element upstream of the promoter. The chicken β-actin promoter drives expression of the advanced reverse tetracycline response element (Clontech Laboratories, Inc., Mountain View, CA) and a puromycin selection gene. shRNAs were designed based on previously published methods.[20–22] The target sequences included a scrambled control (5′-GGGACTACTCTATTACGTCATT-3′), human *SOX7* (5′-GGAATGTTCACTGACGTCTT-3′), human *EZH2* (5′-GGTGATCACAGGATAGGTATT-3′), and human *DNMT1* (5′-GGATGAGAAGAGACGTAGAGTT-3′).

Cell Culture, Lentiviral Production, and Infection

Human mammary epithelial cells (HMECs), nontumorigenic MCF-10A, and tumorigenic MCF-7, MDA-MB-231, SK-BR-3, ZR-75-1, BT-474, BT-549, CAMA-1, HS-578T, and SUM159PT breast cells were cultured according to the protocol of the ATCC (Manassas, VA) or cited literature. Immortalized, nontumorigenic 184B5 breast cells were generated and cultured as previously described.[23,24]

Lentivirus was produced by transfecting 293FT cells with a plasmid encoding the lentivirus and three packaging plasmids (pMDLg/pRRE, pVSV-G, and pRSV-REV) using the calcium phosphate precipitation method.[20,25] Cells were infected with concentrated lentivirus and incubated 6 hours with 8 μg/mL of hexadimethrine bromide (Polybrene) before reverting to normal medium. Cells were subjected to antibiotic selection for at least 48 hours after infection before further studies.

Proliferation, Migration, Invasion, and Three-Dimensional Matrigel Assays

We performed WST-1, migration, invasion, and three-dimensional Matrigel assays as previously described.[26] In these assays, cells were infected by lentivirus expressing either shRNAs or cDNAs. For inducible *SOX7* expression, we first generated clonal MDA-MB-231 cell lines stably expressing advanced reverse tetracycline response element and then infected them with pTetOn-7 lentivirus carrying Dox-inducible *SOX7* cDNA.

Xenograft Study

We performed the xenograft study in athymic nude mice according to a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest Health Sciences. MDA-MB-231 cells (4×10^6) carrying Dox-inducible *SOX7* were injected subcutaneously into the mammary fat pads of 8- to 10-week-old female athymic nude mice at two sites per mouse. Mice were supplied with either 2.0 μg/mL of Dox in their drinking water or normal water ($n = 5$ mice per group). Tumors were measured twice weekly using a Vernier caliper, and tumor volumes were calculated using the following formula: $V = 0.5$ (Length $\times$ Width).[27]

Four weeks after implantation, all mice were sacrificed, and xenografts were isolated, weighed, and analyzed by Western blot analysis.

Methylation Analysis

Patient samples were acquired from the Advanced Human Tissue Bank at Wake Forest Health Sciences and handled according to a protocol approved by the institutional review board of Wake Forest Health Sciences. Genomic DNA (gDNA) from human tissues and cell lines was isolated and converted using the EZ DNA Methylation Kit (D5001; Zymo Research, Irvine, CA). The methylation status of the *SOX7* promoter in these gDNA samples was determined by methylation-specific PCR according to a published procedure.[10] PCR was performed using Taq Polymerase (Zymo Research) under the following parameters: 95°C for 10 minutes; 35 cycles of 95°C for 30 seconds, 54°C or 58°C

for 30 seconds, and 72°C for 30 seconds; and 72°C for 7 minutes.

To study the effects of methyltransferase inhibition on SOX7 mRNA levels in MCF-7 and MDA-MB-231 cells, we treated cells with 1 μmol/L 5-aza-2′-deoxycytidine for 72 hours. Other drugs used to determine the effects of inhibiting histone deacetylase or histone methyltransferase activity included 400 nmol/L Trichostatin A (TSA) for 24 hours and 3 μmol/L BIX01294 for 72 hours, respectively.

RT-qPCR Analysis

Total cellular RNA was extracted using the TRIzol protocol (Invitrogen) and subsequently analyzed by quantitative RT-PCR (RT-qPCR). SOX7 mRNA levels were determined using 1 μg of RNA that was incubated with 0.5 μg/μL of oligo dT primer (Promega Corp., Madison, WI) at 70°C for 5 minutes. Meanwhile, we prepared the reverse transcription mix and left it at room temperature until the annealing step was complete. The reverse transcription mix was immediately added and incubated at 40°C for 1 hour: 5 μL of 5× Moloney murine leukemia virus buffer, 5 μL of 10 mmol/L deoxyribonucleotide triphosphate, 0.6 μL of ribonuclease inhibitor (RNasin; Promega Corp.), 1 μL of reverse transcriptase, and 13.4 μL of nuclease-free water. Quantitative PCR analysis using Taqman gene expression arrays was then performed for SOX7 expression, and data were normalized to GAPDH levels and determined by the ΔΔCT method (Applied Biosystems Inc., Foster City, CA).[28] All analyses were performed using the ABI7000 Sequence Detection System (Applied Biosystems).

Half-Life of SOX7 Study

Experiments were performed as described previously.[29] To test the effects of various mutations on SOX7 protein stability, we infected MDA-MB-231 cells with lentivirus expressing the pSL5 vector or pSL5/SOX7. Cells were treated with 60 μg/mL of cycloheximide for the indicated time points. Lysates were collected and analyzed by Western blot.

Statistical Analysis

Data in RT-qPCR, invasion, WST-1, and xenograft assays are indicated as means ± SD. Comparisons between two groups for a single parameter were performed using Student's *t*-test. All analyses were performed using commercially available software (SigmaPlot version 11.0; Systat Software Inc., San Jose, CA; and Microsoft Excel 2010; Microsoft, Redmond, WA). A difference was considered statistically significant at $P < 0.05$.

Results

Down-Regulation of SOX7 in Breast Cancer

Previous reports suggested reduced SOX7 expression in prostate, lung, and colon cancers.[10–13] To determine its expression in breast cancer, we first evaluated SOX7 mRNA and protein levels in a panel of breast cell lines. Using Western blot analysis and RT-qPCR, we found that both SOX7 protein and mRNA were down-regulated in nearly all breast cancer cell lines compared with normal finite-lifespan HMECs and nontumorigenic, immortal MCF-10A and 184B5[23,24] breast cell lines ($P < 0.05$) (Figure 1, A and B). Previous studies suggested that *SOX7* promoter methylation contributes to its reduced expression.[10,12,14] We also observed DNMT1 up-regulation in several breast cancer cell lines relative to normal or nontumorigenic breast cells (Figure 1A).

We next analyzed SOX7 mRNA levels in human breast tumors from the GSE10780[30] data set. *SOX7* transcript levels were markedly decreased in 42 invasive ductal carcinoma tissues compared with 143 normal tumor-adjacent tissues from 90 patients ($P = 1.64 \times 10^{-16}$) (Figure 1C). These data suggest a consistent and frequent down-regulation of SOX7 expression in human breast cancer cell lines and tumors.

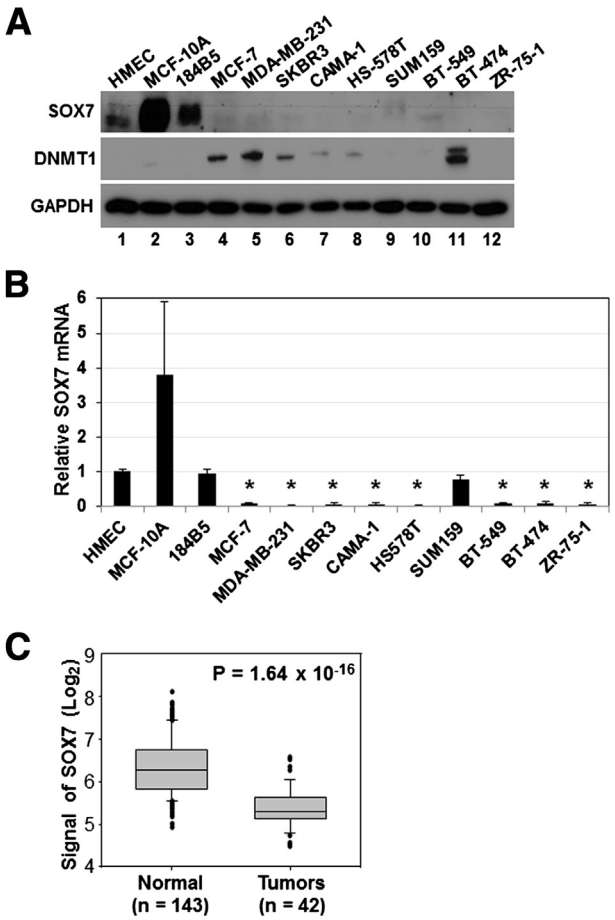

Figure 1 SOX7 is down-regulated in breast cancer. **A:** Western blot analyses of SOX7, DNMT1, and GAPDH expression in normal, nontumorigenic, and tumorigenic breast cell lines. **B:** RT-qPCR analyses of SOX7 mRNA levels in breast cell lines. Data represent the means ± SD. **C:** Analyses of *SOX7* transcript levels in samples from invasive ductal carcinomas ($n = 42$) and normal adjacent tissues ($n = 143$) of patients from the GSE10780 data set. *$P < 0.05$, compared with HMECs.

Regulation of SOX7 in Breast Cancer

Previous studies suggest that promoter methylation is an important mechanism of *SOX7* down-regulation in prostate and colon cancers.[10–12,14] Thus, we tested the methylation status of the *SOX7* promoter in breast cancer using methylation-specific PCR as described previously.[10] Although normal or nontumorigenic HMECs, MCF-10A cells, and 184B5 breast cells[24] did not have detectable methylation, we observed *SOX7* promoter methylation in six of nine breast cancer cell lines (Figure 2A). These data suggest that methylation is a common mechanism of *SOX7* silencing in breast cancer cell lines.

To determine whether DNA methylation plays a role in reduced SOX7 expression in breast cancer, we treated MCF-7 and MDA-MB-231 cells with 5-aza-2′-deoxycytidine, a DNMT inhibitor. As shown in Figure 2B, this treatment led to a significant increase of *SOX7* transcript levels in both cell lines ($P < 0.05$). To further investigate the role of DNA methylation in *SOX7* down-regulation, we infected MCF-7 and MDA-MB-231 cells with lentivirus expressing an shRNA specific to DNMT1 or an scrambled control (shCont) and analyzed SOX7 mRNA levels at 1 and 2 weeks after infection. SOX7 mRNA levels were significantly increased in MDA-MB-231, but not MCF-7 cells, 1 week after infection; however, both cell lines had significantly elevated SOX7 mRNA levels after 2 weeks (Figure 2C). DNMT1 knockdown was validated by RT-qPCR analyses (Figure 2C).

To determine whether *SOX7* promoter methylation occurs in the tumors of human breast cancer patients, we analyzed the gDNA from 27 frozen breast tumor tissues and their matched adjacent normal tissues. Among these samples, we clearly observed *SOX7* promoter methylation in five tumor samples (19%) (samples 1, 3, 4, 5, and 23) (Figure 2D and Supplemental Figure S1A). Interestingly, we also detected weak signal of the methylation in two tumor-adjacent normal samples (patients 1 and 26). To examine whether this standard PCR condition was too stringent, thus causing the relatively low detection of *SOX7* promoter methylation compared with previous reports in other cancers,[10,12,14] we reduced the annealing temperature from 58°C to 54°C and repeated the methylation-specific PCR for samples 6, 8, 9, 16, and 17. With this reduced stringency, we found that most normal samples, as well as MCF-10A cells, became positive in the PCR using the methylation-specific primers

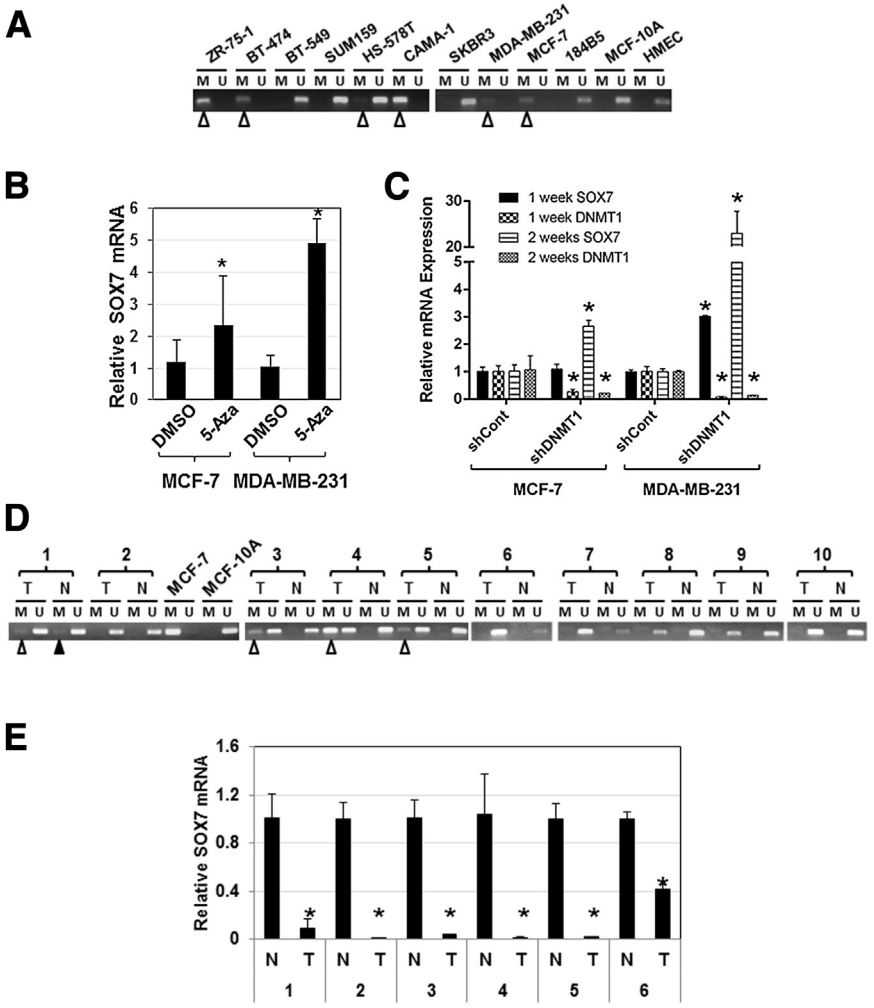

Figure 2 The *SOX7* promoter is hypermethylated in human breast cancer cells and tumors. **A:** gDNA from breast cell lines was analyzed by methylation-specific PCR using primers specific for the unmethylated (U) or methylated (M) *SOX7* promoter. Tumorigenic breast cell lines with *SOX7* promoter methylation are indicated by open **arrowheads**. **B:** RT-qPCR analyses of MCF-7 and MDA-MB-231 cells treated with dimethyl sulfoxide (DMSO) or 1 μmol/L 5-aza-2′-deoxycytidine (5-Aza) for 72 hours. Data represent the means ± SD from two experiments. **C:** RT-qPCR analyses of MCF-7 and MDA-MB-231 cells infected with lentivirus expressing either a DNMT1-specific shRNA or shCont. Data represent the means ± SD. **D:** Methylation-specific PCR of gDNA from human breast tumor (T) samples or their matched adjacent normal (N) tissues as described in **A**. Patients are deidentified as numbers 1 through 10, with MCF-10A and MCF-7 indicated as negative and positive controls, respectively. Samples with *SOX7* promoter methylation in tumor and tumor-adjacent normal samples are indicated by open and closed **arrowheads**, respectively. **E:** RT-qPCR analysis of SOX7 levels in six patient samples from **D**. Data represent the means ± SD. $^*P < 0.05$.

(Supplemental Figure S1B), suggesting that using 54°C as the annealing temperature could produce false-positive results.

To determine whether the methylation status of the *SOX7* promoter correlated with its transcription in human tumors, we compared SOX7 mRNA levels of six tumor samples with their matched normal tissue. We observed SOX7 mRNA down-regulation in all tumor samples regardless of their promoter methylation status (Figure 2E).

The relatively infrequent promoter methylation of *SOX7* compared with that in prostate[10] and colon[12] cancer and its consistently low mRNA expression suggest that additional epigenetic mechanisms or gene deletion is involved in *SOX7* down-regulation in human breast tumors. To determine whether histone deacetylation contributes to inhibiting SOX7 expression in breast cancer cells, we treated MCF-7 and MDA-MB-231 cells with 400 nmol/L TSA, an inhibitor of histone deacetylases, and analyzed *SOX7* transcript levels after 24 hours. Interestingly, TSA significantly increased *SOX7* transcript levels in MCF-7 cells but had an opposite effect on MDA-MB-231 cells ($P < 0.05$) (Supplemental Figure S2A). To determine whether histone methylation contributes to *SOX7* silencing, we studied the effects of inhibiting the methylation of histone H3 lysine 9 and histone H3 lysine 27, two well-characterized marks of gene repression, on *SOX7* transcription. The treatment of BIX01294, an inhibitor of methyltransferases mediating histone H3 lysine 9 methylation, did not affect *SOX7* transcription in MCF-7 cells but modestly decreased that in MDA-MB-231 cells (Supplemental Figure S2A). In addition, we used two shRNAs (shEZH2-1 and shEZH2-2) to silence EZH2, a methyltransferase catalyzing histone H3 lysine 27 methylation. Surprisingly, *EZH2* knockdown in both MCF-7 and MDA-MB-231 cells apparently further reduced SOX7 mRNA levels, instead of increasing it (Supplemental Figure S2B), without causing any detectable change in SOX7 protein levels (Supplemental Figure S2C).

We further explored SOX7 ubiquitination in mammary cells. To determine whether protein ubiquitination contributes to SOX7 degradation, we scanned the SOX7 amino acid sequence for potential ubiquitination sites using the CKSAAP_UbSite Prediction software[31] and found three potential ubiquitination lysines (39, 148, and 159). However, replacing these lysine residues with arginines did not extend the half-life of ectopically expressed SOX7 proteins in MDA-MB-231 cells (Supplemental Figure S3A). Consistently, treatment of MDA-MB-231 cells with two different proteasome inhibitors, MG-132 and $AdaAhx_3L_3VS$,[32] did not lead to detectable SOX7 accumulation (Supplemental Figure S3B), suggesting that proteasome-mediated degradation is unlikely a major mechanism of SOX7 down-regulation.

Effects of Manipulated SOX7 Expression in Breast Cell Lines

To study the effects of SOX7 depletion in nontumorigenic immortal breast cell lines, we stably infected MCF-10A and 184B5 cells with lentivirus expressing shCont or a SOX7-specific shRNA (shSOX7) and generated clonal cells from each. SOX7 knockdown was validated by Western blot analysis (Figure 3A). We assessed the cell proliferation of the 184B5 clones using WST-1 assays and observed a significantly increased mean proliferation rate in these

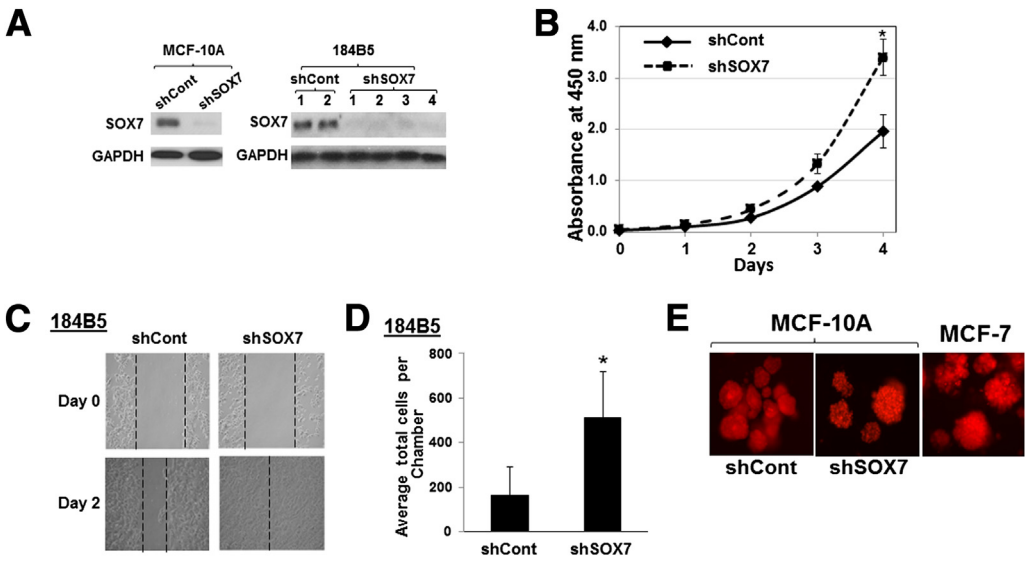

Figure 3 Effects of SOX7 knockdown on mammary cells. **A**: Western blot analyses of SOX7 in MCF-10A and 184B5 cells after infection with lentivirus expressing SOX7-specific shRNA (shSOX7) or shCont. **B**: WST-1 proliferation assays of 184B5 shCont and shSOX7 clones. Data represent the mean proliferation of four shSOX7 clones and two shCont clones. **C**: Representative images of a wound-healing assay of 184B5 cells expressing shCont and shSOX7 at the time of the scratch and 2 days after. The dashed lines mark the edges. **D**: Boyden chamber invasion assays of 184B5 cells expressing shCont and shSOX7 after 48 hours of incubation. Data were derived from two individual experiments, and each had triplicate samples. **E**: Effects of SOX7 silencing on the morphologic features of MCF-10A cells in three-dimensional Matrigel. MCF-7 cells were used as a control. $^*P < 0.05$.

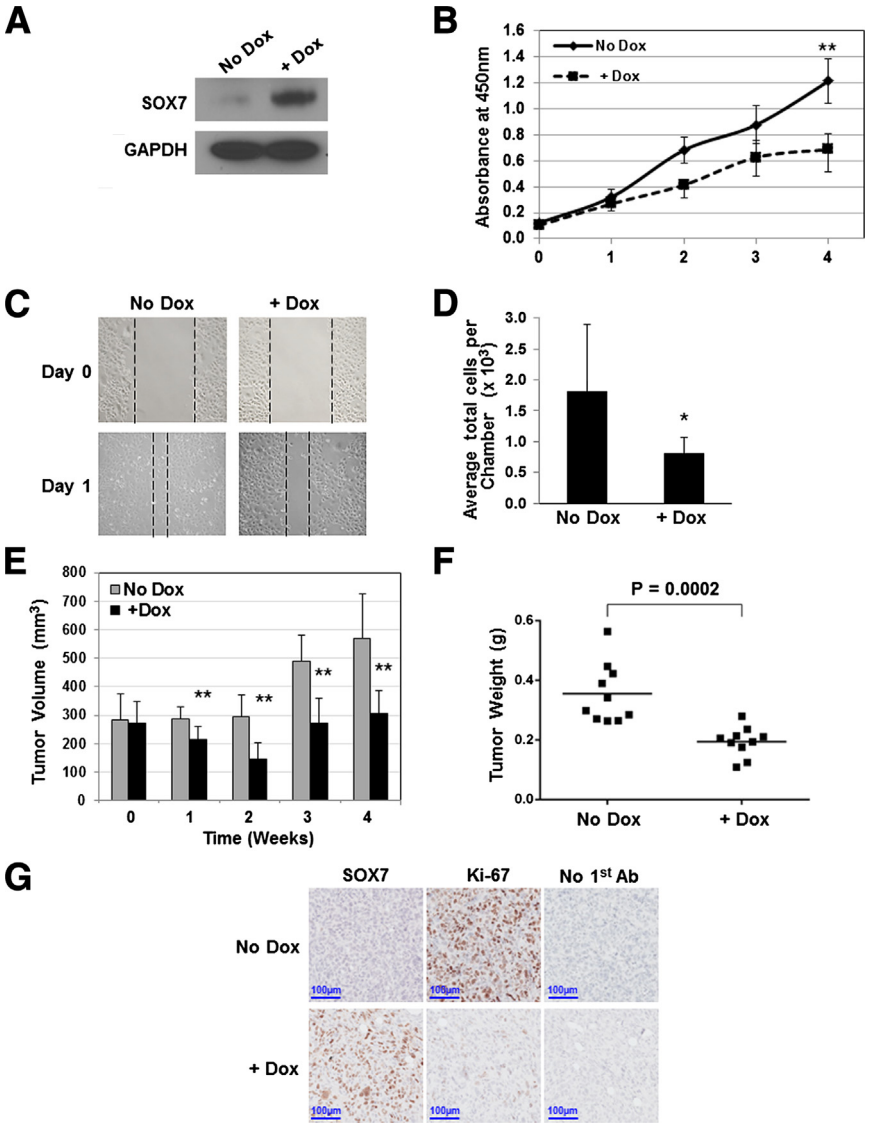

Figure 4 Effects of ectopic SOX7 expression on breast cancer cells. **A**: Western blot analyses of doxycycline (Dox)—inducible SOX7 in MDA-MB-231 cells cultured in the absence and presence of 1.0 μg/mL of Dox for 24 hours. **B**: WST-1 proliferation assays of MDA-MB-231 cells with Dox-inducible SOX7. Data were derived from triplicate samples. **C**: Representative images of wound-healing assays of MDA-MB-231 cells with Dox-inducible SOX7. Images were taken at the time of the scratch and after 24 hours. The dashed lines mark the edges. **D**: Boyden chamber invasion assays of MDA-MB-231 cells with Dox-inducible SOX7 incubated for 48 hours. Data were derived from two individual experiments with triplicate samples in each. **E**: Weekly tumor volumes of mouse xenografts derived from MDA-MB-231 cells with Dox-inducible SOX7. The mice received either regular drinking water or water with 2.0 mg/mL of Dox. **F**: Xenograft tumor weights described in **E** at 4 weeks. **G**: Immunohistochemical staining to detect SOX7 expression and Ki-67 in xenografts. No 1st Ab indicates the control without any primary antibody. Scale bar = 100 μm. $*P < 0.05$, $**P < 0.01$.

SOX7-depleted clones compared with that of the cells that expressed shCont ($P < 0.05$) (Figure 3B). Similar results were observed in MCF-10A cells (data not shown). Furthermore, SOX7 depletion in 184B5 cells enhanced their migration in wound-healing assays (Figure 3C) and increased their invasiveness in Boyden chamber assays ($P < 0.01$) (Figure 3D) compared with the control cells. SOX7 knockdown was routinely validated in the cells used in these assays.

MCF-10A cells form polarized acinar structures when cultured in a three-dimensional Matrigel system, but this ability is lost when the cells are transformed by proliferative genes, such as *ERBB2* and *YY1*.[19,26] To study whether SOX7 depletion altered the morphologic features of MCF-10A cells, we cultured MCF-10A cells that expressed shCont and shSOX7 in the three-dimensional Matrigel system.[26] Although the cells with shCont generated well-shaped acini, SOX7 silencing by shSOX7 caused the MCF-10A cells to form irregular clusters (Figure 3E), suggesting that SOX7 depletion caused a loss of polarity in this three-dimensional culture system that resembles the morphologic features of tumorigenic MCF-7 cells.

To study the effect of ectopic SOX7 expression on breast cancer cells, we generated doxycycline-inducible SOX7-expressing MDA-MB-231 cells and verified SOX7 induction (Figure 4A). Ectopic SOX7 significantly reduced cell proliferation in WST-1 proliferation assays ($P < 0.01$) (Figure 4B), inhibited cell migration in wound-healing assays (Figure 4C), and reduced cell invasion in Boyden chamber assays ($P < 0.05$) (Figure 4D). To investigate the effects of ectopic SOX7 expression on breast tumor growth *in vivo*, inducible SOX7-expressing MDA-MB-231 cells were subcutaneously implanted into mammary fat pads of athymic nude mice at two sites per mouse. Mice received either normal drinking water or water containing 2.0 mg/mL of doxycycline. Tumor volumes were significantly decreased in doxycycline-treated mice throughout the study ($P < 0.01$) (Figure 4E). Tumors from doxycycline-treated

mice weighed significantly less than the control mice ($P < 0.01$) (Figure 4F). We previously demonstrated that doxycycline treatment alone did not affect the xenograft tumor growth in athymic nude mice.[19] Immunohistochemical studies found that the mice supplied with regular water were SOX7 negative in their xenograft tumors, whereas the mice with doxycycline-containing water had positive SOX7 staining (Figure 4G). In addition, Ki-67 staining was markedly stronger in these SOX7-negative tumors (no doxycycline) than the tumors expressing induced SOX7 (with doxycycline) (Figure 4G). These results suggest that ectopic SOX7 reduced cell proliferation in these xenograft tumors, consistent with the *in vitro* data in Figure 4B. The doxycycline-induced SOX7 expression in these tumors was also verified by Western blot analyses (Supplemental Figure S4).

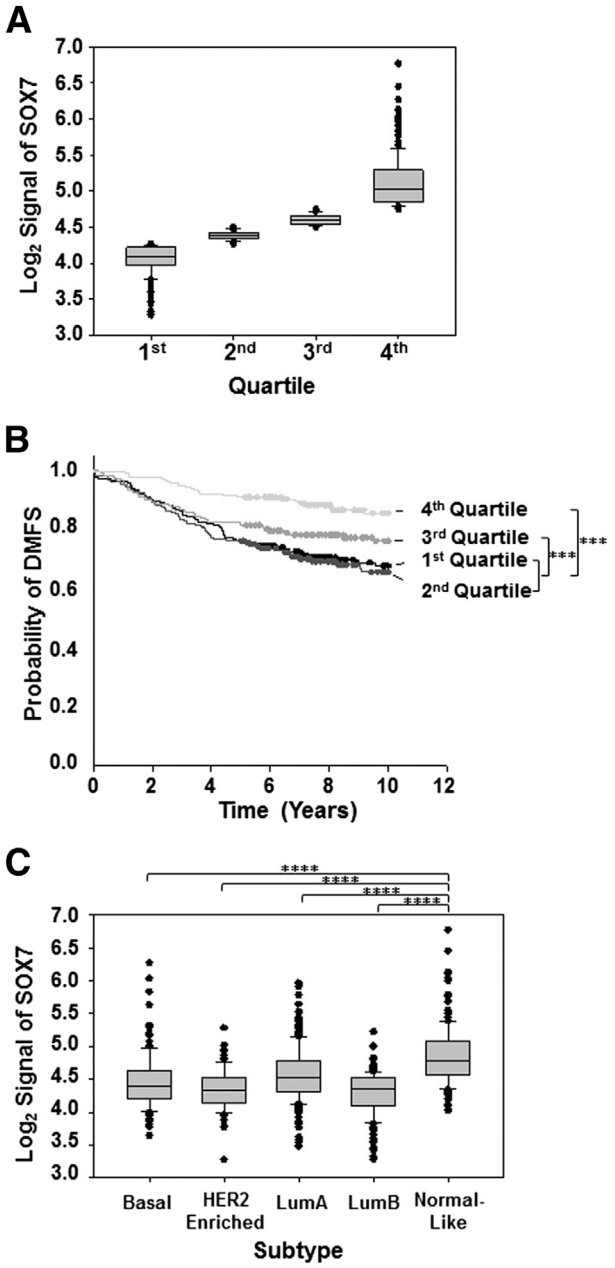

Figure 5 SOX7 expression correlates with clinical outcomes in breast cancer patients. **A:** Breast cancer patients ($n = 759$) from an international metacohort (BrCa759) were grouped into four quartiles based on their *SOX7* transcript levels within their tumors. Shaded regions define the interquartile ranges. The midline of each rectangle marks the median value. T-bars extending from the interquartile range mark the fifth and 95th percentiles; outliers are indicated by filled circles. **B:** Correlation between SOX7 expression and the distant metastasis-free survival (DMFS) by Kaplan-Meier analysis. **C:** Relative SOX7 mRNA levels among different breast cancer subtypes (basal, HER2-enriched, LumA and LumB) when compared with normal-like subtype with favorable prognosis. ***$P < 0.001$, ****$P < 0.0001$. LumA, luminal A; LumB, luminal B.

SOX7 Expression Correlates with Clinical Outcome in Breast Cancer Patients

In vitro and *in vivo* results strongly suggested a tumor suppressive function of SOX7 in breast cancer. To examine the clinical relevance of this discovery, we analyzed SOX7 expression in an international metacohort (BrCa759) that consisted of six microarrays with data from 759 breast cancer patients.[33] The SOX7 intensity detected by the two probes 224013_s_at and 228698_at in these arrays was highly correlated (Pearson correlation coefficient = 0.69) (Supplemental Figure S5); thus, we used their mean intensity as a measure of SOX7 mRNA levels in these patients. Because 674 patients in this metacohort had at least ≥5 years of follow-up after operations, we grouped them into four quartiles based on their *SOX7* transcript levels (Figure 5A). Patients in quartiles 1 and 2 with lower SOX7 expression had a significantly decreased distant metastasis–free survival compared with the patients in quartiles 3 and 4 with higher SOX7 expression (Figure 5B), suggesting that SOX7 down-regulation correlated with poor clinical outcomes of breast cancer patients. We also compared the relative SOX7 transcript levels for five different breast cancer subtypes among all 759 patients. As shown in Figure 5C, SOX7 mRNA levels were significantly decreased in patients of more aggressive breast cancer subtypes (eg, basal, Her2 positive, LumA and LumB) when compared with those with more indolent disease (ie, normal-like; $P < 0.0001$) (Figure 5C).

Discussion

We discovered SOX7 down-regulation in breast cancer cell lines and tumors and explored the mechanisms underlying its reduced expression. Functional studies suggested a tumor suppressive role of SOX7 in breast cancer both *in vitro* and *in vivo*. Importantly, we revealed a positive correlation between SOX7 expression and clinical outcomes of breast cancer patients. On the basis of these data, we conclude that SOX7 acts as a tumor suppressor in breast cancer pathogenesis.

A karyotype analysis indicated that MCF-10A cells contain an additional chromosome 8 derivative with structural abnormalities in these cells, which may at least partially

contribute to their high SOX7 expression.[34] Nonetheless, SOX7 expression in breast cancer cell lines remained markedly reduced when compared with two other non-tumorigenic normal finite or immortalized breast cells (Figure 1, A and B). Promoter methylation has been suggested as a major mechanism of SOX7 down-regulation in human cancers.[10,12,14] Consistently, we detected *SOX7* promoter methylation in six of nine breast cancer cell lines. However, although we clearly detected *SOX7* promoter methylation in some human breast cancer samples, the frequency (5 of 27) was relatively low compared with that in prostate and colon cancers.[10,12,14] We predict that several reasons may contribute to these observations. First, breast cancers have high heterogeneity, and *SOX7* promoter methylation may occur in some tumor cells or at specific stages of tumor progression. Thus, the tumor sections used for gDNA extraction could have various relative portions with *SOX7* promoter methylation. Second, the amplified region of the methylation-specific PCR contains multiple cytosine residues that may be differentially, but not necessarily simultaneously, methylated. Thus, the PCR primer set that was designed to detect the completely unmethylated or methylated sequences, respectively, would not be able to generate signals from the samples with partially methylated *SOX7* promoter. Future studies using DNA methylation sequencing should more conclusively determine the methylation status of the *SOX7* promoter in breast cancer. Third, the down-regulation of SOX7 in breast cancer is likely mediated by additional mechanisms to promoter methylation.

Pharmacologic and shRNA-mediated inhibition of DNMT1 restored SOX7 mRNA but not protein levels in multiple breast cancer cell lines (Figure 2B and data not shown). These results suggest that additional regulatory mechanisms are involved in reducing SOX7 in breast cancer. Further studies excluded the contribution of histone deacetylation, histone H3 lysine 9 and histone H3 lysine 27 methylation, and protein ubiquitination to SOX7 down-regulation. Unexpectedly, EZH2 knockdown apparently further reduced SOX7 mRNA levels, which is likely an indirect effect on SOX7 expression. At this point, we cannot exclude the possibility that other histone modifications, including methylation at other residues, are involved in inhibiting SOX7 expression. Furthermore, regulation of SOX7 mRNA stability or translation rate, such as by miRNAs, can also play a role in this process, and these hypotheses deserve further investigation.

The *SOX7* gene is located on the p arm of chromosome 8 that is frequently deleted in cancers,[17] which may at least partially contribute to SOX7 down-regulation in breast cancer. On the basis of currently available literature, *SOX7* homozygous deletion in cancers is rare. In the current study, the PCR amplification of the *SOX7* promoter in breast cancer samples always revealed positive detection for either the methylated or unmethylated form. However, we cannot conclude that the tumor samples in this study did not contain any *SOX7* homozygous deletion, although the *SOX7* gene is only approximately 7.7 kb in length. This is because stromal or other nonneoplastic cells could contribute to the positive PCR reading when the unmethylated primers were used. In addition, PCR amplification would not be able to detect any minor mutation or deletion of the *SOX7* gene outside the primer binding regions that could genetically diminish SOX7 expression.

Although Guo et al[10] demonstrated *SOX7* promoter methylation in prostate cancer xenografts, to our knowledge we are the first to demonstrate tumor suppressive effects of SOX7 *in vivo* for any cancer. The precise molecular mechanism by which SOX7 antagonizes tumor growth remains unclear, in part because of its understudied transcriptional targets. In murine F9 embryonal carcinoma cells, SOX7 activates transcription of the basement membrane component laminin-α1 and competes with GATA-4 to activate fibroblast growth factor 3.[35,36] However, these genes are not antiproliferative; thus, SOX7-mediated transcription in cancers warrants further study. Multiple reports indicate that SOX7 interacts with and inhibits β-catenin in colorectal cancers with frequently altered WNT signaling,[10,12,37] indicating that the tumor suppressive function of SOX7 may be, at least in part, mediated by its activities independent of its DNA-binding ability.

Collectively, data suggest that SOX7 down-regulation is likely regulated by multiple gene expression mechanisms in breast cancer. SOX7 acts as a tumor suppressor and has a great potential to serve as a prognostic marker for breast cancer patients. Future efforts should endeavor to elucidate mechanisms behind SOX7-mediated tumor suppression, including its transcriptional targets and its tumor-specific down-regulation.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.07.025*.

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