RUNX3 Is Frequently Inactivated by Dual Mechanisms of Protein Mislocalization and Promoter Hypermethylation in Breast Cancer

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Abstract

A tumor suppressor function has been attributed to RUNX3, a member of the RUNX family of transcription factors. Here, we examined alterations in the expression of three members, *RUNX1, RUNX2*, and *RUNX3*, and their interacting partner, *CBF-β*, in breast cancer. Among them, RUNX3 was consistently underexpressed in breast cancer cell lines and primary tumors. Fifty percent of the breast cancer cell lines (n = 19) showed hypermethylation at the promoter region and displayed significantly lower levels of RUNX3 mRNA expression ($P < 0.0001$) and protein ($P < 0.001$). In primary Singaporean breast cancers, 9 of 44 specimens showed undetectable levels of RUNX3 by immunohistochemistry. In 35 of 44 tumors, however, low levels of RUNX3 protein were present. Remarkably, in each case, protein was mislocalized to the cytoplasm. In primary tumors, hypermethylation of *RUNX3* was observed in 23 of 44 cases (52%) and was undetectable in matched adjacent normal breast epithelium. Mislocalization of the protein, with or without methylation, seems to account for RUNX3 inactivation in the vast majority of the tumors. In *in vitro* and *in vivo* assays, RUNX3 behaved as a growth suppressor in breast cancer cells. Stable expression of RUNX3 in MDA-MB-231 breast cancer cells led to a more cuboidal phenotype, significantly reduced invasiveness in Matrigel invasion assays, and suppressed tumor formation in immunodeficient mice. This study provides biological and mechanistic insights into RUNX3 as the key member of the family that plays a role in breast cancer. Frequent protein mislocalization and methylation could render *RUNX3* a valuable marker for early detection and risk assessment. (Cancer Res 2006; 66(13): 6512-20)

Introduction

Breast cancer is the most common cancer in women in developed countries, including Singapore. In Singapore, more than 1,000 new cases of breast cancer are diagnosed each year, and ~250 women die from the disease. In the United States, the incidence rate is 111 cases per 100,000 woman-years and a mortality rate of 24 deaths per 100,000 woman-years (1). Identification of the genetic changes involved in the multiple steps of breast cancer from initiation to progression is critical for early detection and prevention.

Transforming growth factor-β (TGF-β) regulates cell proliferation, differentiation, motility, and apoptosis in a variety of different cell types (2, 3). It is the most potent known inhibitor of normal human mammary epithelial cell replication *in vitro* (4). A number of studies provided strong evidence that TGF-β protects against mammary tumor formation (5–7) induced either by a relative lack of TGF-β or inactivation of the TGF-β signaling pathway. Xie et al. (8) showed that the majority of breast cancer cell lines did not respond to TGF-β-induced cell cycle arrest although the TGF-β signaling cascade (involving Smads) was active in these cells. In more than 400 human breast cancer tissues studied, the majority were shown to contain active TGF-β (Smad) signaling pathways, indicating their ability to proliferate within a microenvironment that contains bioactive TGF-β (8). Thus, some other factors seem to be impinging on the TGF-β pathway to cause the relative refractoriness of breast cancer to TGF-β-induced cell cycle arrest or apoptosis. An emerging body of work has provided evidence that RUNX3 is a likely candidate gene (9). RUNX3 was found to be involved in TGF-β-induced tumor suppressor pathway (10). In fact, gastric epithelial cells of Runx3-null mice were resistant to the growth-inhibiting and apoptosis-inducing activity of TGF-β (11).

In 2002, a large body of evidence was presented to support a tumor suppressor role for RUNX3 in gastric cancer (12). Since then, numerous studies have shown loss of RUNX3 expression in many cancer types, usually through allelic loss and epigenetic changes (13–19). Among them, two studies, one on gastric (12) and the other on bladder cancer (20), have reported loss-of-function mutations. Because promoter hypermethylation can cause silencing of a wide range of tumor suppressors concomitantly, discovery of mutations in RUNX3 was particularly important to positively identify the gene involved in the development of these cancers (12, 20). Comparison of the effects in *in vitro* cell growth and xenograft assay in nude mice between wild-type RUNX3 and RUNX3(R122C) identified in gastric cancer suggested that wild-type, but not the mutant form of RUNX3, showed growth inhibitory activity. Furthermore, RUNX3(R122C) bound to DNA and interacted with Smads more weakly than wild-type RUNX3 and attenuated the expression of cyclin/cyclin-dependent kinase inhibitor $p21^{Waf-1}$ (12, 20). RUNX3 induced the proapoptotic gene *Bim* whereas inactivation of RUNX3 attenuated apoptosis mediated

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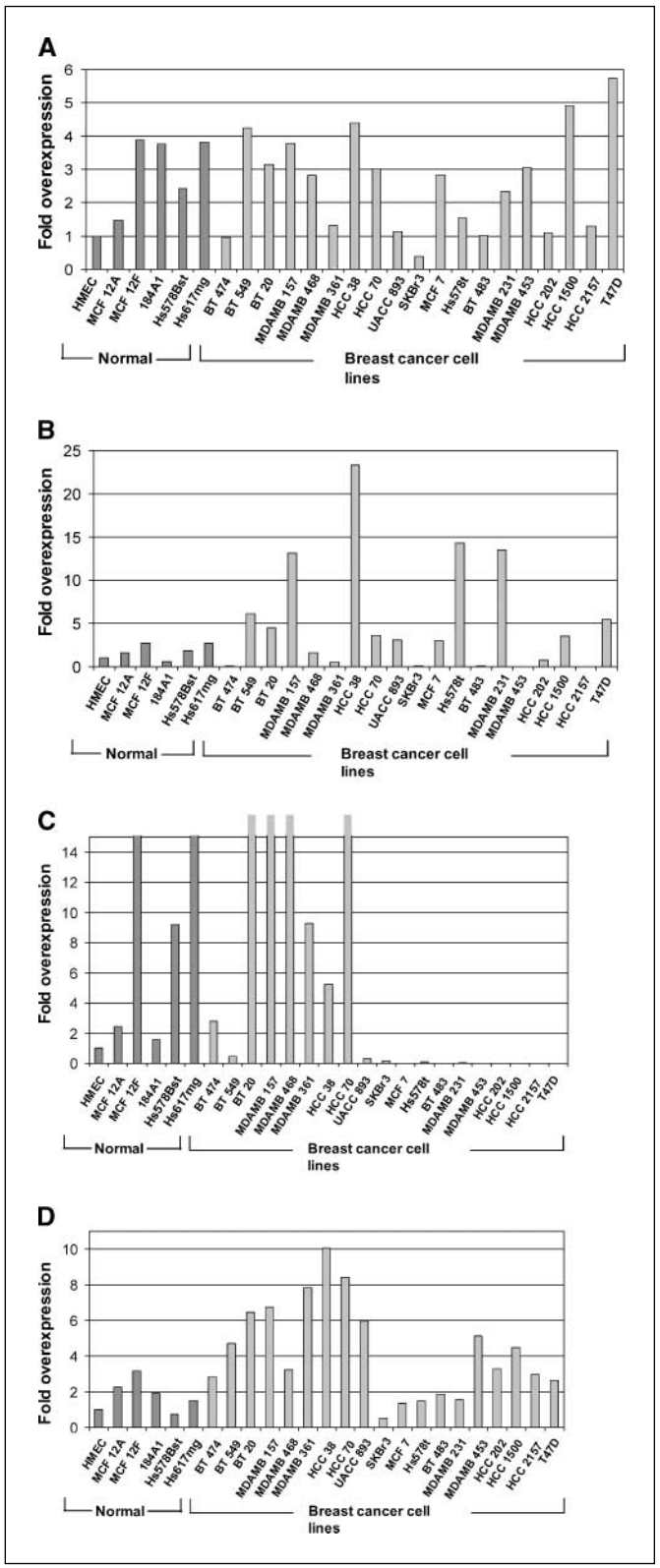


Figure 1. Expression levels of *RUNX* genes in breast cancer cells by real-time RT-PCR analysis. *A, RUNX1*; *B, RUNX2*; *C, RUNX3* expression; *D, CBF-β*. Values were normalized against internal control, *GAPDH*, and expressed as fold overexpression above the value for HMEC. *Dark columns,* normal human mammary epithelial cells strain/cell lines. *Light columns,* breast cancer cell lines. $P = 0.04$; $P = 0.002$, significantly higher levels of expression in breast cancer cells compared with normal cells by two-sample *t* test for *RUNX2* and *CBF-β*, respectively.

by RUNX3 (21). These results suggested that RUNX3 is a strong candidate tumor suppressor gene in gastric cancer.

More recently, another mechanism of inactivation of RUNX3 was recognized. In a substantial fraction of gastric cancer cases in which RUNX3 was expressed, the protein was mislocalized in the cytoplasm as an inactive form. It is possible that disruption of machinery that controls nuclear transport of RUNX3 resulted in the sequestration or aberrant localization of RUNX3 in the cytoplasm. TGF-β was recently identified as one of the signals that control nuclear translocation of RUNX3 in a gastric cancer cell line (22). It is of great interest to examine whether inactivation of RUNX3 by mislocalization occurs in breast cancer as well.

RUNX3 belongs to the RUNX family of transcription factors, which consists of RUNX1, RUNX2, and RUNX3. The three mammalian *RUNX* genes encode a set of closely related DNA binding proteins homologous to Runt, the *Drosophila* pair-rule gene product. The three RUNX proteins bind DNA as heterodimeric complexes with a common partner protein, CBF-β. Yet, each RUNX product has a unique role. *RUNX1* and *RUNX2* are required for hematopoiesis and osteogenesis, respectively, and are genetically altered in leukemia and bone disease (23–27). Interestingly, RUNX2 was implicated in the formation of osteolytic lesions by metastatic breast cancer cells (28, 29). In contrast, *RUNX3* was shown to be involved in neurogenesis (30, 31) and thymopoiesis (32, 33) and functioned as a tumor suppressor of gastric cancer (12, 13). The *CBF-β* gene product seemed to be required for high-affinity DNA binding by its three RUNX cofactors (10, 34). As might be expected from this essential functional role, *Cbf-β* knockout mice succumbed to the earliest manifestation of *Runx* deficiency, which is a failure of definitive hematopoiesis (35). RUNX family members may play a broader role in multistep breast tumorigenesis than currently recognized.

In this study, we report that RUNX3 is frequently underexpressed in breast cancer compared with normal breast epithelium and present evidence for its behavior as a tumor suppressor gene. Loss of RUNX3 function in primary breast cancers was attributed to mislocalized protein expression and promoter hypermethylation. This study contributes to the understanding the function of RUNX3, its family members, and their downstream effector pathways in breast carcinogenesis.

Materials and Methods

Cell lines and finite life span cultures. Nineteen breast cancer cell lines, BT474, BT549, BT20, MDA-MB-157, MDA-MB-468, MDA-MB-361, HCC38, HCC70, UACC893, SKBR3, MCF7, Hs578T, BT483, MDA-MB-231,

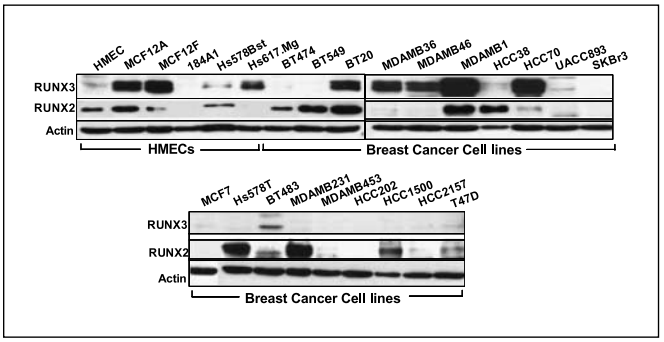


Figure 2. Western blot analysis of RUNX3 and RUNX2 expression in HMECs and breast cancer cell lines. β-Actin served as a loading control.

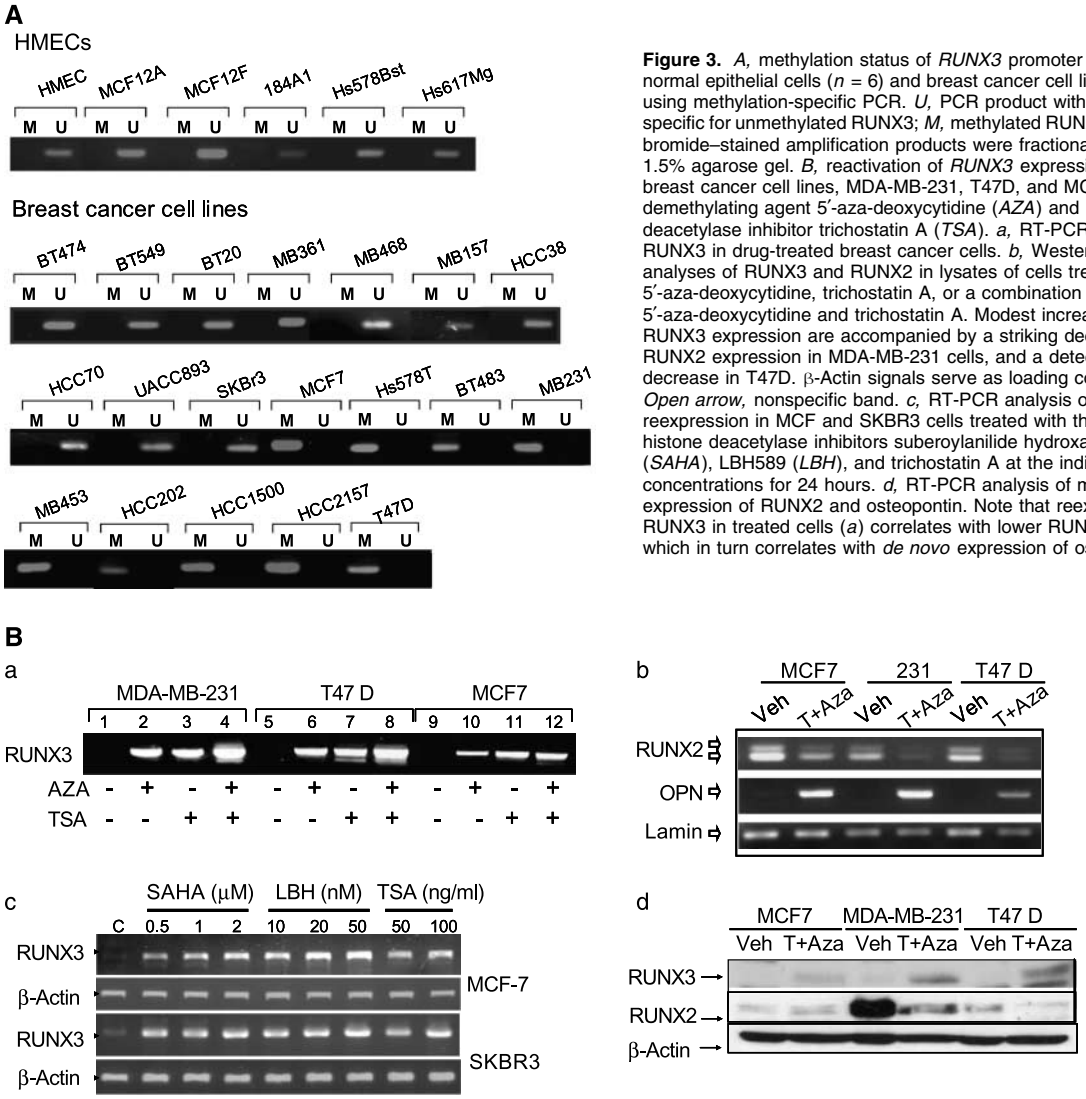


Figure 3. *A,* methylation status of *RUNX3* promoter region in normal epithelial cells ($n = 6$) and breast cancer cell lines ($n = 19$) using methylation-specific PCR. *U,* PCR product with primers specific for unmethylated RUNX3; *M,* methylated RUNX3. Ethidium bromide–stained amplification products were fractionated on a 1.5% agarose gel. *B,* reactivation of *RUNX3* expression in breast cancer cell lines, MDA-MB-231, T47D, and MCF7, with demethylating agent 5′-aza-deoxycytidine (*AZA*) and histone deacetylase inhibitor trichostatin A (*TSA*). *a,* RT-PCR analysis of RUNX3 in drug-treated breast cancer cells. *b,* Western blot analyses of RUNX3 and RUNX2 in lysates of cells treated with 5′-aza-deoxycytidine, trichostatin A, or a combination of 5′-aza-deoxycytidine and trichostatin A. Modest increases in RUNX3 expression are accompanied by a striking decrease in RUNX2 expression in MDA-MB-231 cells, and a detectable decrease in T47D. β-Actin signals serve as loading controls. *Open arrow,* nonspecific band. *c,* RT-PCR analysis of RUNX3 reexpression in MCF and SKBR3 cells treated with the vehicle or histone deacetylase inhibitors suberoylanilide hydroxamic acid (*SAHA*), LBH589 (*LBH*), and trichostatin A at the indicated concentrations for 24 hours. *d,* RT-PCR analysis of mRNA expression of RUNX2 and osteopontin. Note that reexpression of RUNX3 in treated cells (*a*) correlates with lower RUNX2 levels, which in turn correlates with *de novo* expression of osteopontin.

MDA-MB-453, HCC202, HCC1500, HCC2157, and T47D, and immortalized normal breast epithelial cell lines, MCF12A, MCF12F, 184A1, Hs578Bst, and Hs617.Mg, were obtained from American Type Culture Collection (Rockville, MD) whereas finite life span human mammary epithelial cell strain, HMEC, was obtained from Cambrex (East Rutherford, NJ). When indicated, cell lines were treated with 0.75 mmol/L 5-aza-deoxycytidine (36) or with 50 or 100 ng/mL of trichostatin A, 0.5 to 2 μmol/L of suberoylanilide hydroxamic acid (Aton Pharma, Tarrytown, NY), and 10 to 50 of nmol/L of LBH-589 (Novartis, East Hanover, NJ; ref. 37).

Tumors. Primary breast tumors with matching adjacent normal tissue from Singapore patients were obtained from the National University of Singapore-National University Hospital Tissue Repository and the Pathology Department, National University of Singapore, after Institutional Review Board approval.

Reverse transcription-PCR. RNA was extracted from cultured cells using RNeasy Kit from Qiagen (Valencia, CA). cDNAs were synthesized from total RNA using Omniscript reverse transcriptase (Qiagen) with oligo-dT primer according to the manual of the manufacturer. PCR amplification of *RUNX3, RUNX2*, and osteopontin (*OPN*) was done using primers *RUNX3* [Ps-N (Ps-NA, 5′-CGCCACTTGATTCTGGAGGATTTGT-3′; Ps-NB, 5′-TGAAGTGGCTTGTGGTGCTGAGTGA-3′) and Ps-C (Ps-CA, 5′-GAGTTTCACCCTGACCATCACTGTG-3′; Ps-CB, 5′-GCCCATCACTGGTCTTGAAGGTTGT-3′)]; *RUNX2* (R2F, 5′-GCACAGACAGAAGCTTGAT-3′; R2R, 5′-CCCAGTTCTGAAGCACCT-3′), and *OPN* (OPNF, 5′-ATATGATGGCCGAGGTGATA-3′; OPNR, 5′-GACCTCAGAAGATGCACTAT-3′). To confirm the integrity of the prepared RNA, the same cDNAs were subjected to PCR amplification of *Lamin A/C* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with the primer pairs *Lamin* (LamF, 5′-ACCTGCAGGAGCTCAATGAT-3′; LamR, 5′-AACTCCTCACGCACTTTGCT-3′) and *GAPDH* (GAPF, 5′-ACCACAGTCCATGCCATCAC-3′; GAPR, 5′-TCCACCACCCTGTTGCTGTA-3′).

Real-time PCR. Quantitative PCR was carried out using the ABI PRISM 7000 Sequence Detection Systems (Applied Biosystems, Foster City, CA). cDNA was obtained as described above. The ABI TaqMan Gene Expression system was used for analysis in triplicate of RUNX1, RUNX2, RUNX3, and CBF-β, with cDNAs obtained from breast cancer cell lines and normal cell lines/strains. The relative expression of each cell line was compared with that of HMEC with the ABI Prism software version 2.1 using GAPDH as normalization control. At least three independent analyses were done.

Western blot analysis. Western blot was essentially done as described (12) using monoclonal antibodies (mAb) against RUNX3 and RE-6E9 (22) or RUNX2 and 8G5 (MBL, Nagoya, Japan).

DNA extraction. Genomic DNA from cell lines was extracted using the DNeasy Kit (Qiagen, Hilden, Germany). For primary tumors and normal breast tissue, two 4-μm paraffin-embedded sections were first washed with xylene, digested in 100 μL of TNES [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% SDS] containing 40 μg proteinase K for 16 hours at 50°C, heat inactivated at 70°C for 10 minutes, and clarified by centrifugation at 14,000 for 10 minutes. Fifty microliters of the supernatant were used directly as a source of DNA for sodium bisulfite treatment.

Sodium bisulfite treatment of DNA. Tissue and cell line DNAs were treated with sodium bisulfite and analyzed using methylation-specific PCR as described by Yang et al. (36). Samples were aliquoted and stored at −80°C.

Methylation-specific PCR and sequencing. Sodium bisulfite–treated DNA was subjected to PCR using the following primers (12): RUNX3 region between −218 and −69 bp upstream of the translation initiation site of the exon 1 was amplified, methylated DNA primers Rx3-5M (5′-TTACGAGGGGCGGTCGTACGCGGG-3′) and Rx3-3M (5′-AAAACGACC-GACGCGAACGCCTCC-3′), and unmethylated DNA primers Rx3-5U (5′-TTATGAGGGGTGGTTGTATGTGGG-3′) and Rx3-3U (5′-AAAACAACCAA-CACAAACACCTCC-3′). Sequencing of RUNX3 PCR products (−264 to +203) of sodium bisulfite–treated DNA was done as previously described (20).

Immunohistochemistry and immunocytochemistry. Both were done as described in ref. 22 using mAb R3-6E9. In each case, normal breast lobules on the same section as well as sections of normal gastric epithelium analyzed in the same run served as positive controls. Mayer's hematoxylin (Sigma-Aldrich, Singapore) was used as counterstain. For immunofluorescence, cells were fixed and stained with mAb to RUNX3 (R3-6E9) or RUNX2 (8G5) and detected with biotinylated antimouse immunoglobulin G (Vector, Burlingame, CA) followed by fluorescein-avidin D (Vector). The cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) as the nuclear stain.

Retroviral infection. pPal-RUNX3 retrovirus or empty vector was used to infect MDA-MB-231 cells and cells subjected to fluorescence-activated cell sorting to select for green fluorescent protein–positive cells. Independent clones were screened for RUNX3 expression by real-time PCR. Experiments were done using both single and pooled clones.

Cell invasion assays. A single-cell suspension of pooled 231-RUNX3 cells in serum-free medium was added to the upper compartment of Chemicon (Temecula, CA) Cell Invasion Assay chambers. Ten-percent FCS in the bottom chamber served as chemoattractant. After 24 or 72 hours of incubation at 37°C, filters were rinsed, cells on the lower surface were stained (Chemicon, Temecula, CA) for 20 minutes, filters were washed with distilled water and dried, and images were captured at ×200 magnification.

Murine xenografts. Institutional IACUC guidelines were followed for mouse studies. Four- to six-week-old BALB/c nude mice received s.c. injections of pooled 1×10^6 231-RUNX3 or 231-Vec cells. Once detected, tumors were measured every week until they reached 1 cm^3 in volume, whereupon mice were euthanized.

Statistical analysis. The nonparametric Mann-Whitney test or the two-sample *t* test at 5% significance was done following Levene's test for equality of variance with $P < 0.05$ considered statistically significant. The χ^2 association test was done for the presence of an association between RUNX3 methylation and estrogen receptor, progesterone receptor, or lymph node status. The odds ratio for the various factors with its 95% confidence interval was determined. The SPSS version 11.5 statistical software was used.

Results

Alterated gene expression of members of the RUNX family in breast cancer cells. The Runt domain transcription factor family consists of three members, RUNX1, RUNX2, and RUNX3, which share a common NH_2-terminal domain, Runt domain, for DNA binding and heterodimerization with the CBF-β subunit. As a first step to studying alterations in breast cancer, we determined the expression of each of these genes in a panel of 25 breast cell lines. Real-time PCR analysis revealed that there were no significant differences between HMEC (n = 6) and breast cancer cell lines (n = 19) for RUNX1 expression (Fig. 1*A*). For RUNX2, 9 of 19 cell

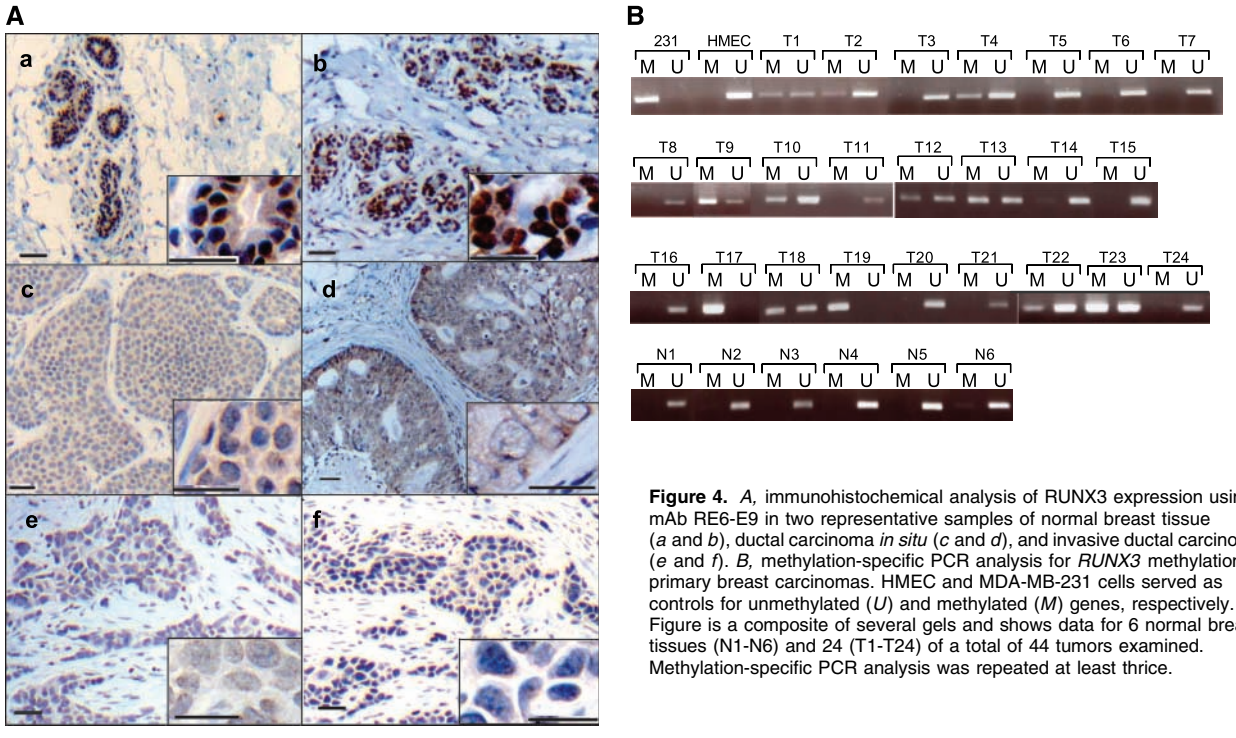


Figure 4. *A,* immunohistochemical analysis of RUNX3 expression using mAb RE6-E9 in two representative samples of normal breast tissue (*a* and *b*), ductal carcinoma *in situ* (*c* and *d*), and invasive ductal carcinoma (*e* and *f*). *B,* methylation-specific PCR analysis for *RUNX3* methylation in primary breast carcinomas. HMEC and MDA-MB-231 cells served as controls for unmethylated (*U*) and methylated (*M*) genes, respectively. Figure is a composite of several gels and shows data for 6 normal breast tissues (N1-N6) and 24 (T1-T24) of a total of 44 tumors examined. Methylation-specific PCR analysis was repeated at least thrice.

Table 1. Frequency of RUNX3 methylation and loss of protein expression in primary breast carcinoma from Singapore Chinese women

Sample	Methylation status of RUNX3/total (%), methylation-specific PCR	Protein expression/total (%)	No. tissues
Normal HMECs	0/6 (0)	Western blot analysis	
		5/6 (83)	
Breast cancer cell lines	M: 9/19 (47)*	0/9 (0)	
	U: 10/19 (53)	7/10 (70)	
Normal breast	U: 20/20 (0)†	Immunohistochemistry	
		Intensity	
		Nuclear staining, 44/44‡	
		Strong	44
Invasive ductal carcinoma	M: 23/44 (52)	Nuclear staining, 0/44	
		None	9
		Weak	35
		Strong	0
Ductal carcinoma *in situ*	nd	Nuclear staining, 0/6	
		None	0
		Weak	6
		Strong	0

NOTE: Scoring system used to assess levels of nuclear or cytoplasmic staining: strong, staining in >80% cells; weak, staining in 1% to 20% of the cells; none, negative for staining. All 23 carcinomas with methylated RUNX3 had negative or weak cytoplasmic staining. Correlation between methylation and loss of protein expression in breast cancer cell lines, $P < 0.001$. nd, not done.

*Each of the nine cell lines carried only methylated alleles of RUNX3. M, methylated; U, unmethylated.

†Tissue from the normal margins of the surgical resection.

‡Normal ducts present within the tumor section were scored for immunohistochemical staining; if absent, tissue adjacent to the tumor was used.

lines showed higher level of expression ($P = 0.04$; Fig. 1*B*). For CBF-β, 13 of 19 cell lines showed higher mRNA expression ($P = 0.002$; Fig. 1*D*) compared with the HMECs. RUNX3 mRNA, on the other hand, was expressed at decreased or undetectable levels (values ranging from 0.5 to 0.005 below that in HMEC) in 12 of 19 breast cancer cell lines compared with immortalized HMECs (Fig. 1*C*). Among the remaining seven cell lines, three showed strikingly higher expression of RUNX3 compared with HMECs: MDA-MB-157 (300×), HCC70 (100×), and MDA-MB-468 (45×). Although anecdotal at this stage of investigation, these three were among five cell lines of African American origin. Loss of expression of RUNX3 was clearly illustrated in Hs578T breast cancer cell line, and not in Hs578Bst, its paired normal breast counterpart.

The cDNA from all 19 breast cancer cell lines was sequenced but no mutations were found: each harbored only wild-type RUNX3 alleles. These results show that two members of the RUNX family and their interacting protein, CBF-β, showed alterations in gene expression, thus highlighting the significance of the RUNX family members in breast cancer.

Next, Western blot analysis was done on the same panel of breast cancer cells to determine whether alterations in mRNA expression of RUNX2 and RUNX3 were accompanied by corresponding changes at the protein level as well. With a few exceptions such as BT474 and HCC38, levels of RUNX3 mRNA expression correlated with levels of RUNX3 protein (Figs. 1*C* and 2). Concordance between RUNX2 mRNA and protein expression was observed in 14 of 19 cell lines (Figs. 1*B* and 2). Interestingly, of 13 cell lines that showed low or undetectable expression of RUNX3, 8 had detectable RUNX2 expression. This inverse correlation between RUNX2 and RUNX3, although not statistically significant, is suggestive of regulatory effect of one RUNX gene on the expression of the other.

Decreased expression of RUNX3 in breast cancer cells correlates with methylation of RUNX3 promoter. Promoter hypermethylation of RUNX3 was previously reported in ~25% of primary breast cancers (18, 19). However, characterization of RUNX3 in breast cancer cell lines that provide valuable model systems has not yet been reported. To determine if decreased expression of RUNX3 correlated with promoter hypermethylation, we did methylation-specific PCR on the panel of breast cancer cell lines (Fig. 3*A*). Methylated alleles of RUNX3 were detected in 9 of 19 breast cancer cell lines whereas the unmethylated *RUNX3* gene was observed in all 6 HMEC lines. Methylation of the RUNX3 promoter region correlated with low expression of RUNX3 mRNA (Fig. 1*C*). In 9 of 19 breast cancer cell lines with RUNX3 promoter methylation (Fig. 3*A*), no detectable or very low RUNX3 expression was observed (Figs. 1*C* and 2). On the other hand, UACC893 and SKBR3 contained unmethylated RUNX3 but nevertheless displayed low levels of RUNX3 mRNA and protein compared with HMECs. These findings suggest that other mechanisms are also involved in the regulation of RUNX3 expression. Collectively, these data showed that RUNX3 expression correlated strongly with RUNX3 promoter hypermethylation ($P < 0.0001$) on analysis of all 25 cell lines/strains. To confirm the data shown in Fig. 3, in 19 cancer cell lines the −264 to +203 bp region relative to the translation initiation site, encompassing a dense CpG island in exon 1 of RUNX3, was sequenced using sodium bisulfite–treated, PCR-amplified DNA. Sequencing data reflected that seen in the methylation-specific

PCR analysis, indicating that methylation is spread evenly across this region (data not shown). Thus, by methylation-specific PCR analysis, sodium bisulfite sequencing, reverse transcription-PCR (RT-PCR), and Western blot analysis, the results showed that low RUNX3 expression in breast cancer cells lines correlated strongly with hypermethylation of the *RUNX3* gene in most cell lines. However, additional, yet undeciphered, mechanisms of silencing of *RUNX3* gene exist in breast cancer cells. Our results, thus far, led us to conclude that for many breast cancer cell lines, promoter hypermethylation could be a major underlying mechanism for loss of expression of RUNX3.

Treatment with 5′-aza-deoxycytidine and/or trichostatin A restores RUNX3 and target gene expression. Definitive evidence for promoter hypermethylation as the cause for loss of expression can be obtained by reversing methylation using well-known demethylating agents. There is ample evidence that histone deacetylase inhibitors cooperate with demethylating agents for more efficient restoration of gene expression through their effects on chromatin (36, 38, 39). We treated three breast cancer cell lines, MDA-MB-231, T47D, and MCF7 cells, with 5′-aza-deoxycytidine, an inhibitor of DNA methyltransferase, and/or with trichostatin A, a histone deacetylase inhibitor. Analyses of cDNA by real-time PCR (data not shown) and RT-PCR [Fig. 3*B*(*a*)] and of protein by Western blotting [Fig. 3*B*(*b*)] showed that RUNX3 expression could be reactivated with either 5′-aza-deoxycytidine or trichostatin A, or a combination of both. The observation that trichostatin A alone reactivated gene expression robustly suggested that chromatin modification by trichostatin A and other histone deacetylase inhibitors might be able to reactivate gene expression in cell lines that are unmethylated and yet do not express the mRNA or protein. To test this premise, MCF7 (positive for methylation) and SKBR3 cells (negative for methylation) were treated with various doses of histone deacetylase inhibitors, trichostatin A, suberoylanilide hydroxamic acid, or LBH-589 (37). In both cell lines, all three histone deacetylase inhibitors were effective in a dose-responsive manner to reactivation of *RUNX3* gene expression [Fig. 3*B*(*c*)]. These data added further strength to the conclusion that in breast cancer cells, both hypermethylation and histone deacetylase inhibition of RUNX3 played a critical role in down-regulation of RUNX3 expression.

Because we had previously observed a trend towards an inverse correlation between RUNX3 and RUNX2 expression, we examined RUNX2 levels in the drug-treated cells. Interestingly, in MDA-MB-231 and T47D, and less so in MCF7, reexpression of RUNX3 correlated with reduced levels of RUNX2 mRNA, as measured by real-time PCR (5-fold with 5′-aza-deoxycytidine plus trichostatin A; data not shown) and RT-PCR [Fig. 3*B*(*d*)], and of RUNX2 protein [Fig. 3*B*(*b*)]. RUNX2 is a known transcriptional regulator controlling osteogenesis (29). *OPN* is a bone stromal cell gene involved in bone differentiation that is responsive to RUNX2 (29). Interestingly, in drug-treated cells, RUNX3 reexpression [Fig. 3*B*(*a,b*)] is accompanied by reduced RUNX2 expression [Fig. 3*B*(*b,d*)] and a corresponding striking increase in the mRNA expression of the RUNX2 target gene *OPN* [Fig. 3*B*(*d*)]. These data suggest that RUNX3 is not only reexpressed on demethylation and histone deacetylase inhibition in breast cancer cells but may also be transcriptionally active and functionally competent.

Loss of RUNX3 protein expression and hypermethylation occurs frequently in primary breast cancer. We examined 44 invasive ductal carcinomas from Singapore for RUNX3 expression by immunohistochemistry using an anti-RUNX3 mAb (10). Representative cases are shown in Fig. 4*A*. Normal breast tissue adjacent to the tumors in the same section from all 44 patients stained positive for RUNX3 and, in each case, the positive staining was found to be nuclear (*a* and *b*). On the other hand, decreased RUNX3 expression was seen in six cases of ductal carcinoma *in situ* within the invasive carcinoma tissues (*c* and *d*). Strikingly, the invasive component of all 44 cases showed negative or weak staining for RUNX3 (*e* and *f*). When weakly positive, the staining was not nuclear but was cytoplasmic (*c-f*). These results strongly suggested that RUNX3 down-regulation and sequestration in the cytoplasm is a frequent event in breast cancer and occurs fairly early in the carcinogenic process.

Previous studies from Korea (18) and United States (19) have reported up to 25% incidence of RUNX3 methylation in primary breast cancer. To determine the frequency of hypermethylated

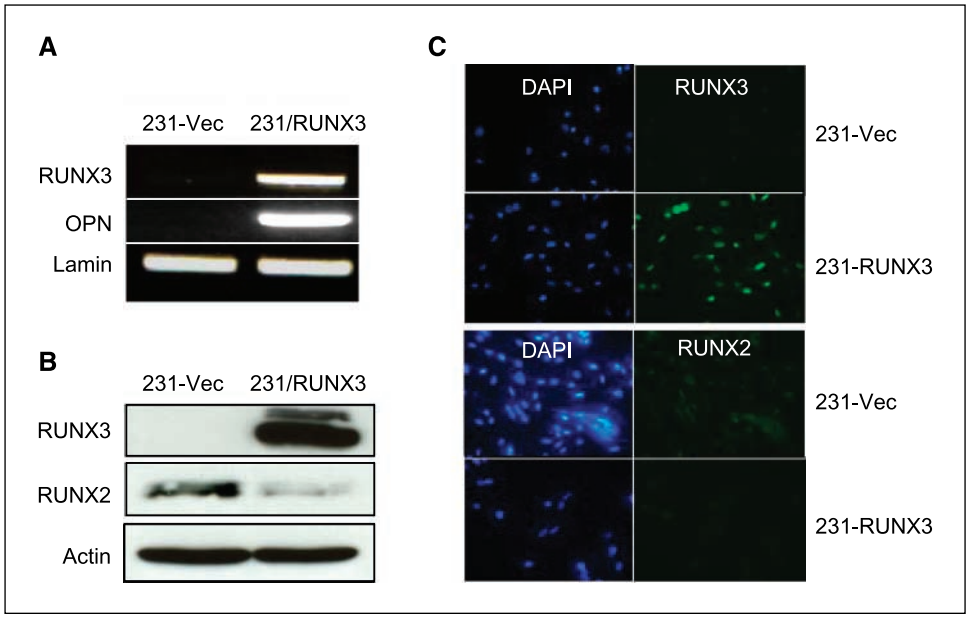
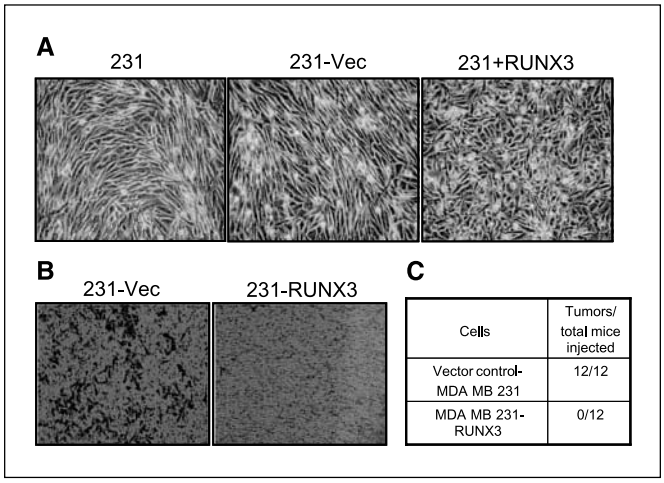


Figure 5. Exogenous RUNX3 expression in MDA-MB-231 cells. *231-RUNX3,* stable pooled clones of RUNX3 retrovirus–infected cells. *231-Vec,* cells stably infected with the retroviral vector. *A,* RT-PCR analysis of *RUNX3* and *OPN*, with *Lamin A/C* as loading control; *B,* Western blot; *C,* immunofluorescence analysis of RUNX3 using mAb RE6-E9 and changes in RUNX2 expression using mAb 8G5 in stably transfected MDA-MB-231 cells. DAPI was used to stain the nuclei.



Cells	Tumors/ total mice injected
Vector control-MDA MB 231	12/12
MDA MB 231-RUNX3	0/12

Figure 6. Morphologic and biological changes caused by expression of RUNX3 in MDA-MB-231 cells. *A,* phase-contrast micrographs of cell cultures show reversion to a more epithelial morphology with RUNX3 expression. *B,* inhibition of invasion of 231-RUNX3 cells in cell invasion chambers. *C,* RUNX3 suppresses tumorigenicity of MDA-MB-231 cells in BALB/c nude mice. Mice received s.c. injections of 1×10^6 cells of 231-Vec or 231-RUNX3 cells on the flank. Tumor size was assessed until the size reached 1 cm^3.

RUNX3 in Singapore breast cancer, we did methylation-specific PCR analysis on DNA from the same 44 primary breast cancers used for immunohistochemistry analysis and their adjacent normal breast tissue. Twenty three of 44 (52%) of the breast cancer DNAs, and none of the adjacent normal tissues, were positive for methylation (Table 1). Representative data are shown in Fig. 4*B*.

Hypermethylation of RUNX3 correlated with undetectable protein expression by immunohistochemistry in 5 cases and with reduced expression in 18 cases. Mislocalized, low-level expression of RUNX3 was observed in nearly 80% of cases. Collectively, protein mislocalization and hypermethylation accounted for loss of tumor suppressor function in nearly all breast cancers, the first observation of such widespread loss of any protein in breast cancer.

RUNX3 has tumor suppressor activity. To determine if RUNX3 behaves as a tumor suppressor gene in breast cancer cells, stable clones of MDA-MB-231 cells (which lack detectable RUNX3 expression) were derived following infection with a RUNX3 retrovirus (231-RUNX3). RUNX3 mRNA and protein expression were verified (Fig. 5*A* and *B*). Unlike previous observations in gastric cancer cells (12, 40), the rate of cell proliferation between parental, 231-Vec, and 231-RUNX3 cells, as determined by growth assays, was not found to be significantly different (data not shown). Interestingly, as seen in 5′-aza-deoxycytidine-treated and trichostatin A–treated cells (Fig. 3*B*), exogenous expression of RUNX3 in the MDA-MB-231 cells was accompanied by reduced endogenous levels of RUNX2 mRNA (data not shown) and protein (Fig. 5*B*) and an increase in *OPN* mRNA levels (Fig. 5*A*). By immunofluorescence analysis, RUNX3 was localized to the nucleus in 231-RUNX3 cells (Fig. 5*C, top*). Again, reduced expression of RUNX2 was observed in 231-RUNX3 cells compared with the 231-Vec control cells (Fig. 5*C, bottom*). Thus, the presence of functional RUNX3 expression was shown in MDA-MB-231 cells stably expressing RUNX3.

Three tests were conducted in cell culture to determine changes in the properties of 231-RUNX3 cells. First, we observed a change in the morphology of the 231-RUNX3 cells to a more cuboidal phenotype compared with parental MDA-MB-231 or 231-vector cells (Fig. 6*A*). Second, in Matrigel assays, unlike parental cells or 231-vector cells, 231-RUNX3 cells did not form any colonies after plating (data not shown). Third, in Matrigel invasion assays, less than ten 231-RUNX3 cells invaded through the membrane between the chambers (Fig. 6*B*). In contrast, large numbers of vector-transfected cells invaded through the membrane and were observed on staining (Fig. 6*B*).

Finally, we tested the ability of RUNX3 to alter tumorigenicity of MDA-MB-231 cells in nude mice. Pooled clones of RUNX3-expressing MDA-MB-231 cells were injected into nude mice. No tumors were observed in 12 mice that were injected with 231-RUNX3 cells whereas all 12 mice injected with the vector control cells, 231-Vec, developed tumors that increased in size during the observation period (Fig. 6*C*).

Collectively, these data provide strong evidence for the growth-suppressive effects of RUNX3 in breast cancer cells both *in vitro* and *in vivo* and suggest that loss of, or reduced, RUNX3 expression seen commonly in primary breast cancer could have functional consequences that promote tumorigenesis.

Discussion

Through a comprehensive analysis of RUNX3 in a large panel of breast cancer cell lines and primary tumors, we have presented several lines of evidence that indicate an important role for RUNX3 in breast cancer. mRNA levels of RUNX family members, *RUNX1, RUNX2*, and *RUNX3*, and their interacting partner *CBF-β* were studied for the first time in breast cancer cell lines. Significantly higher levels of *RUNX-2* and *CBF-β* mRNA were observed in breast cancer cells when compared with normal HMECs. RUNX3 mRNA and protein, on the other hand, were underexpressed in breast cancer cell lines. We show that loss of RUNX3 expression usually occurred by hypermethylation of RUNX3 promoter. Reexpression of RUNX3 in cells carrying methylated *RUNX3* genes using chemical agents or by introducing exogenous RUNX3 resulted in down-regulation of RUNX3 responsive gene, *RUNX2*, and up-regulation of a RUNX2 target, *OPN*. Further, in cell lines with and without hypermethylated promoter sequences, several histone deacetylase inhibitors were highly effective in restoring gene expression, suggesting an important role here for chromatin remodeling agents. Thus, in all likelihood, a functional RUNX3 is induced on demethylation/histone deacetylase inhibition and we can conclude that epigenetic phenomena are responsible for silencing the gene in the majority of breast cancer cell lines. More importantly, these observations were validated in primary cancers. Hypermethylation of RUNX3 was found to be common among young Singapore Chinese breast cancers and also correlated with a loss of expression in half the cancers.

In this study, we found that all the breast cancer tissues ($n = 44$) have weak or no expression of RUNX3 compared with normal tissues. Moreover, when present, the weak staining was in the cytoplasm, not in the nucleus. These data suggest that, as in gastric cancer, RUNX3 in breast cancer tissues was sequestered in the cytoplasm and, possibly, inactive. In contrast, all the corresponding normal tissues in the same patients showed strong RUNX3 expression and, in each case, localized to the nucleus. Hence, besides methylation, cytoplasmic mislocalization could be another important mechanism whereby RUNX3 is inactivated in breast cancer. Nuclear expression of RUNX3 in normal ductal epithelial cells is quite strong and easy to recognize whereas this strong nuclear staining is almost completely absent in cancer cells. This observation would

suggest a potential role of RUNX3 immunohistochemistry for diagnosis of breast cancer. In particular, because nuclear expression of RUNX3 is clearly missing in ductal carcinoma *in situ*, RUNX3 immunohistochemistry might be an excellent tool for diagnosis of early breast cancer. Future studies will address when RUNX3 is altered in the continuum of breast disease progression and whether RUNX3 inactivation in stages as early as benign breast disease could serve as an indicator of high risk.

Loss of expression and loss of activity due to mislocalization in a large proportion of breast cancers suggested a tumor/growth suppressor role for RUNX3 in breast cancer as well. As predicted, RUNX3 displayed tumor-suppressive effects in breast cancer cells in *in vitro* change in morphology, growth in Matrigel, and invasion assays without any change in growth rate, and completely suppressed tumorigenicity in mouse xenograft assays. These results examining the gene, its mRNA, and protein propose a key role for RUNX3 in breast cancer and point to its potential value as a marker that is frequently methylated and mislocalized in breast cancer.

How does RUNX3 behave as a tumor suppressor gene in breast cells? One mechanism could be by behaving as an inducer of apoptosis. In a recent publication, it was shown that, to achieve apoptosis, RUNX3 cooperated with FoxO3a/FKHRL1 to directly activate expression of the proapoptotic gene, *Bim* (21). Another line of evidence (41) showed that RUNX3 formed complexes with receptor-regulated Smads (R-Smads), suggesting that RUNX3 could be a downstream target of the TGF-β pathway. In the context of breast cancer, it was reported (8) that the majority of breast cancer cells were resistant to the growth-suppressive effects of TGF-β although the downstream Smad pathway was active. It is possible that lack of RUNX3 function could blunt the effect of TGF-β despite the presence of an active Smad pathway. As described above, in gastric cancer cells, TGF-β was shown to be an inducer of nuclear translocation of RUNX3 whereas RUNX3 in the cytoplasm was inactive as a tumor suppressor protein (22). Recently, Javed et al. (29) showed that mutations in RUNX2 could impair its intranuclear trafficking in breast cancer cells and the formation of osteolytic lesions in bone *in vivo*. However, the extent of such mislocalization in actual breast tumor samples was not examined.

As members of a family of transcription factors, one question that arises is whether RUNX genes are involved in the regulation of other family members or whether they function independently of each other. It has been recently shown that the expression of RUNX1 is regulated by RUNX3 in human B-lymphoid cell lines (42). RUNX3 inhibition by small interfering RNA in lymphoblastoid cells resulted in increased RUNX1 expression, indicating that continuous expression of physiologic levels of RUNX3 is required to maintain repression (42). Frequent down-regulation of RUNX3, RUNX1, and CBF-β was also observed in gastric cancer (43). In the present study, we observed a trend towards an inverse correlation between RUNX3 expression and that of RUNX2 in breast cancer cell lines. More direct evidence was provided when reactivated expression of RUNX3 in three breast cancer cell lines with 5′-aza-deoxycytidine and trichostatin A resulted in a corresponding decrease in RUNX2 expression (Fig. 3*B*). Additionally, exogenous expression of RUNX3 in MDA-MB-231 cell lines also resulted in a significant decrease in RUNX2 expression (Fig. 5). These data suggest that RUNX3 might regulate the expression of RUNX2 in breast cancer cells, a concept that needs further investigation. Breast cancer often metastasizes to the bone, and there is evidence for RUNX2 involvement in bone metastasis (28, 29). It is tempting to speculate that loss of RUNX3 expression may be one of the pathways that can lead to an increase in RUNX2 expression, which, in turn, promotes progression of bone metastasis. Whether RUNX3 plays a direct role in up-regulation of *OPN* in breast cancer cells remains to be studied. It is well known that transcriptional regulators of the RUNX family play critical roles in normal organ development and, when mutated, lead to genetic diseases and cancer (10). Taken together, our study and those of others indicate that interplay between different RUNX family proteins may be important in regulating the development and differentiation of different cell types. Loss of RUNX3 expression could also lead to abnormal expression of other family members of RUNX and thus lead to pathologic changes.

Two recent studies showed that the frequency of hypermethylated *RUNX3* genes in both Caucasian ($n = 37$) and Korean ($n = 25$) breast cancers was ~25% (18, 19). In Singapore breast cancer among women below the age of 55 years, however, the frequency of methylation was found by us to be significantly higher, at 52%. It is not clear what factors and whether age contributed to this higher frequency. For immediate application, RUNX3 will provide a valuable addition to panels of methylated genes that are being assembled (44) to facilitate early detection of breast cancer DNA in body fluids such as nipple aspirations, fine needle aspirations, and serum.

In summary, we have shown that RUNX3 was expressed at very low levels in breast cancer. RUNX3 promoter hypermethylation and/or protein sequestration in the cytoplasm occurred in nearly all breast cancers, and the dual mechanisms acting together in many cases likely account for loss of function of RUNX3 in breast cancer. If preliminary evidence is confirmed that this loss occurs very early in cancer development, RUNX3 immunohistochemistry on breast biopsies could serve as a sensitive test for distinguishing between benign and malignant lesions. A better understanding on the role of RUNX3 in breast cancer would help us in establishing its function as a tumor suppressor and also gain insight into the biology of breast carcinogenesis.

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