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HOXA9 regulates BRCA1 expression to modulate human breast tumor phenotype

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Breast cancer 1, early onset (BRCA1) expression is often reduced in sporadic breast tumors, even in the absence of BRCA1 genetic modifications, but the molecular basis for this is unknown. In this study, we identified homeobox A9 (HOXA9) as a gene frequently downregulated in human breast cancers and tumor cell lines and noted that reduced HOXA9 transcript levels associated with tumor aggression, metastasis, and patient mortality. Experiments revealed that loss of HOXA9 promoted mammary epithelial cell growth and survival and perturbed tissue morphogenesis. Restoring HOXA9 expression repressed growth and survival and inhibited the malignant phenotype of breast cancer cells in culture and in a xenograft mouse model. Molecular studies showed that HOXA9 restricted breast tumor behavior by directly modulating the expression of BRCA1. Indeed, ectopic expression of wild-type BRCA1 phenocopied the tumor suppressor function of HOXA9, and reducing BRCA1 levels or function inhibited the antitumor activity of HOXA9. Consistently, HOXA9 expression correlated with BRCA1 in clinical specimens and with tumor aggression in patients lacking estrogen receptor/progesterone receptor expression in their breast tissue. These findings indicate that HOXA9 restricts breast tumor aggression by modulating expression of the tumor suppressor gene BRCA1, which we believe provides an explanation for the loss of BRCA1 expression in sporadic breast tumors in the absence of BRCA1 genetic modifications.

Introduction

Developmental regulators that specify tissue morphogenesis are often functionally subverted in adult tissues to promote cancer (1, 2). In particular, the homeobox (HOX) gene family of morphogenic regulators is critical for the establishment of embryonic patterning during embryogenesis and for the maintenance of tissue differentiation in the adult organism (3). HOX genes are transcription factors that regulate the expression of multiple genes that influence cell growth and viability and that mediate stromal-epithelial interactions to drive tissue-specific differentiation (2). HOX expression is frequently perturbed in tumors (3), in which they can act as oncogenes by promoting cell growth and invasion (4, 5) or as tumor suppressors (TSs), because they can alter cell survival and morphogenesis (6–9). HOX genes are especially important in the mammary gland, which undergoes repeated rounds of developmental cycles in the adult organism (10, 11). In the breast, HOX genes have been implicated in the control of embryonic development, branching morphogenesis, and hormonally controlled differentiation (10, 12), and HOX genes are frequently lost or overexpressed in breast tumors (12). Nevertheless, the molecular mechanisms whereby HOX genes regulate mammary development and might modulate breast cancer remain poorly defined.

Women with hereditary mutations in BRCA1 are predisposed to develop breast and ovarian cancers (13). BRCA1 maintains genome integrity through its ubiquitin ligase activity (14) and regulates transcription to modulate the cellular stress response (15, 16). BRCA1 has an established role as a regulator of mammary epithelial cell (MEC) growth, survival, morphogenesis, and transformation (17–20). Moreover, loss of BRCA1 expression and/or function is associated with increased breast tumor aggression, enhanced cancer metastasis, and a poor clinical prognosis (21). There is also a clinical association between familial BRCA1 tumors and an aggressive, triple-negative, basal-like breast cancer phenotype (22). Interestingly, many sporadic breast cancers show decreased BRCA1 expression and display a “BRCA1-like” phenotype, despite the absence of genetic deletions, methylation, or haploinsufficiency (23, 24). The molecular mechanisms leading to reduced BRCA1 expression and/or function in this group of aggressive, sporadic breast cancers remain unclear (20, 25). Studies designed to elucidate molecular modifiers of BRCA1 have identified negative regulators, which in some cases are overexpressed during breast tumorigenesis (26–29, 30, 31). Conversely, the identification of transcription factors that positively and directly regulate BRCA1 expression and whose expression might be concurrently lost during malignant transformation has proven elusive.

BRCA1 expression and MEC proliferation are functionally linked, suggesting BRCA1 could regulate mammary gland development and homeostasis and inhibit tumorigenesis by restricting MEC growth (32–34). BRCA1 also modulates mammary gland

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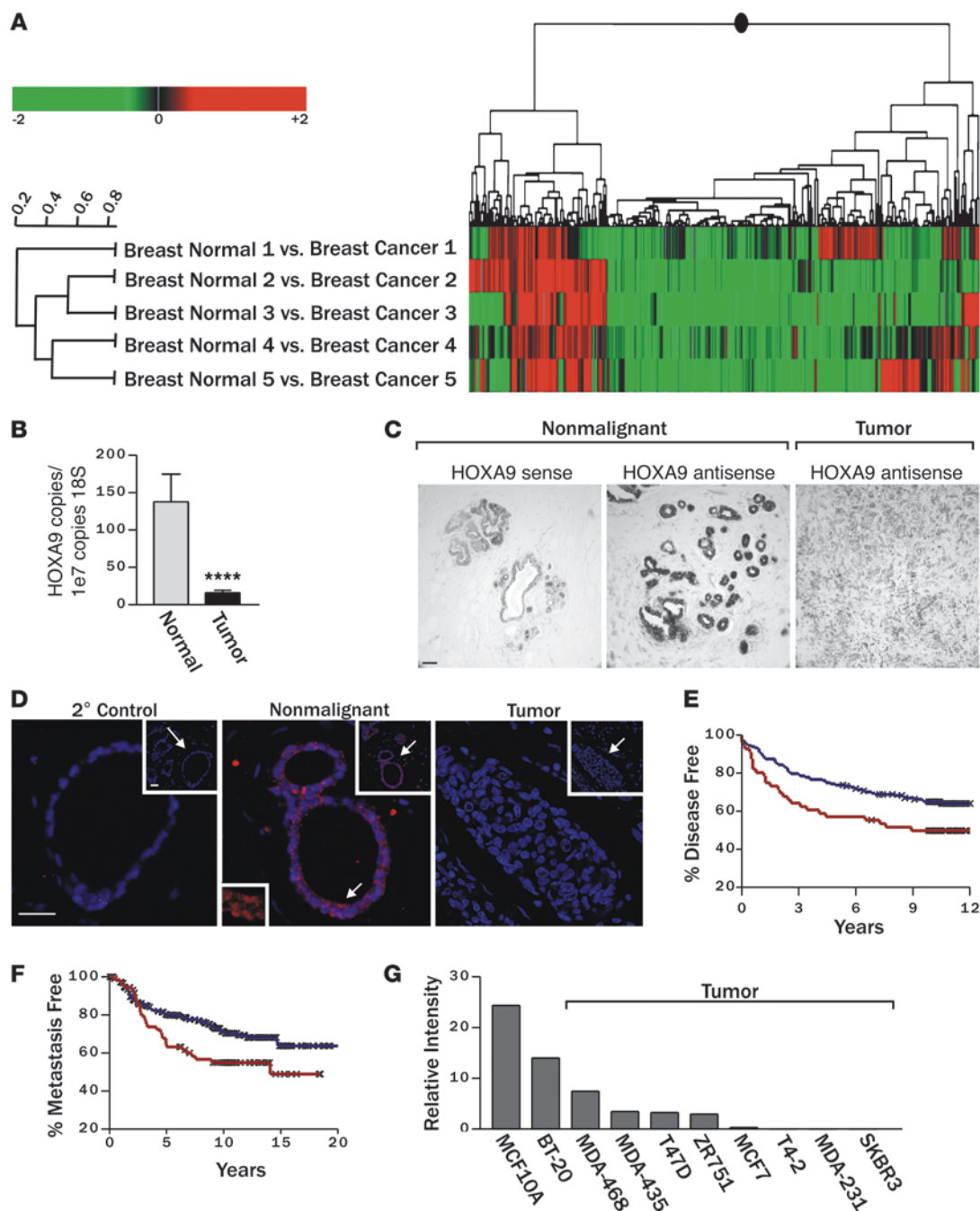


Figure 1

Breast malignancy is associated with reduced *HOXA9* expression. **(A)** Cluster diagram of Affymetrix microarray data using Rosetta Resolver to compare gene expression profiles of matched normal mammary tissue and adjacent primary breast cancers, revealing significantly lower *HOXA9* transcript levels in 4 out of 5 expression sets analyzed ($P \leq 0.01$). **(B)** Levels of *HOXA9* mRNA (by quantitative RT-PCR) in primary human mammary tumors ($n = 47$) compared with normal breast tissue ($n = 16$). **** $P = 0.00035$. **(C)** In situ hybridization using a *HOXA9* probe on nonmalignant ($n = 4$) or malignant ($n = 6$) mammary epithelial tissue. Scale bar: 100 μm . **(D)** Immunofluorescent staining for *HOXA9* (red) and nuclei (blue) demonstrates robust cytoplasmic and nuclear localized *HOXA9* protein in the epithelium of nonmalignant human breast tissue and reduced levels in breast tumors. Top right insets (original magnification, $\times 20$) show a broader view of the breast tissue, with arrows indicating regions blown up in the main images. The arrow in the center main image corresponds with the bottom left inset, which shows a view of *HOXA9* staining (original magnification, $\times 30$). Scale bar: 50 μm . The 2° control shows no nonspecific staining. **(E)** Breast cancer patients whose tumors expressed the lowest *HOXA9* level (lowest expression quartile; red line) experienced significantly reduced disease-free survival compared with all other patients in the study (blue line). An "X" is used to denote each censored sample. $P = 0.025$. **(F)** Patients with the lowest *HOXA9* levels in their tumors (lowest expression quartile; red line) also had significantly increased metastasis as a first event when compared with all other patients (blue line). An "X" is used to denote each censored sample. $P = 0.02$. **(G)** Bar graph demonstrating relative *HOXA9* gene expression levels in nonmalignant and malignant MEC lines.

**Table 1**

Multivariate analysis for relapse-free and overall survival, comparing *HOXA9* transcript expression and clinical characteristics in breast cancer patients

Variable	Death		Relapse	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
ER positive				
<i>HOXA9</i>	1.185 (0.91–1.541)	0.208	1.227 (1.045–1.439)	0.012
Age (per 10-yr increment)	0.615 (0.341–1.109)	0.106	0.527 (0.331–0.842)	0.007
Tumor size (per cm)	1.174 (0.832–1.658)	0.361	1.177 (0.896–1.546)	0.242
Tumor grade				
Grade 2 vs. grade 1	3.38 (1.123–10.172)	0.03	2.185 (1.075–4.445)	0.031
Grade 3 vs. grade 1	6.215 (2.063–18.718)	0.001	3.254 (1.559–6.792)	0.002
Positive vs. negative LN status	2.366 (0.995–5.628)	0.051	2.061 (1.011–4.201)	0.046
Chemotherapy vs. no chemotherapy	0.341 (0.137–0.844)	0.02	0.385 (0.185–0.802)	0.011
Hormonal treatment vs. no treatment	0.753 (0.239–2.373)	0.628	0.662 (0.265–1.656)	0.378
Mastectomy vs. breast-conserving therapy	1.283 (0.678–2.428)	0.444	1.377 (0.821–2.312)	0.225
Molecular subtype				
Normal-like and luminal B vs. luminal A	1.734 (0.804–3.74)	0.161	1.516 (0.843–2.726)	0.165
Basal and ERBB2+ vs. luminal A	2.309 (0.883–6.043)	0.088	1.608 (0.754–3.432)	0.219
ER negative				
<i>HOXA9</i>	0.394 (0.181–0.857)	0.019	0.433 (0.187–1.002)	0.05
Age (per 10-yr increment)	0.59 (0.338–1.027)	0.062	0.552 (0.314–0.973)	0.04
Tumor size (per cm)	1.58 (1.037–2.409)	0.033	1.49 (0.958–2.319)	0.077
Tumor grade (poorly vs. well differentiated)	0.923 (0.365–2.331)	0.865	1.053 (0.382–2.902)	0.921
Positive vs. negative LN status	0.64 (0.209–1.954)	0.433	0.566 (0.196–1.631)	0.291
Chemotherapy vs. no chemotherapy	1.596 (0.513–4.962)	0.419	1.53 (0.54–4.335)	0.423
Hormonal treatment vs. no treatment	0.423 (0.083–2.154)	0.3	0.436 (0.089–2.149)	0.308
Mastectomy vs. breast-conserving therapy	2.1 (0.926–4.759)	0.076	2.187 (0.925–5.171)	0.075

The analysis included the 226 ER-positive and the 69 ER-negative breast cancer patients in the Netherlands Cancer Institute data set. *HOXA9* transcript expression, age, and tumor size were modeled as continuous variables. The molecular subtypes of breast cancer are not included in the model for the ER-negative cancers, as none of the tumors is categorized as the luminal A subtype, plus there is no event of death or relapse for tumors categorized as the normal-like or luminal B subtype. Statistically significant *P* values are shown in bold.

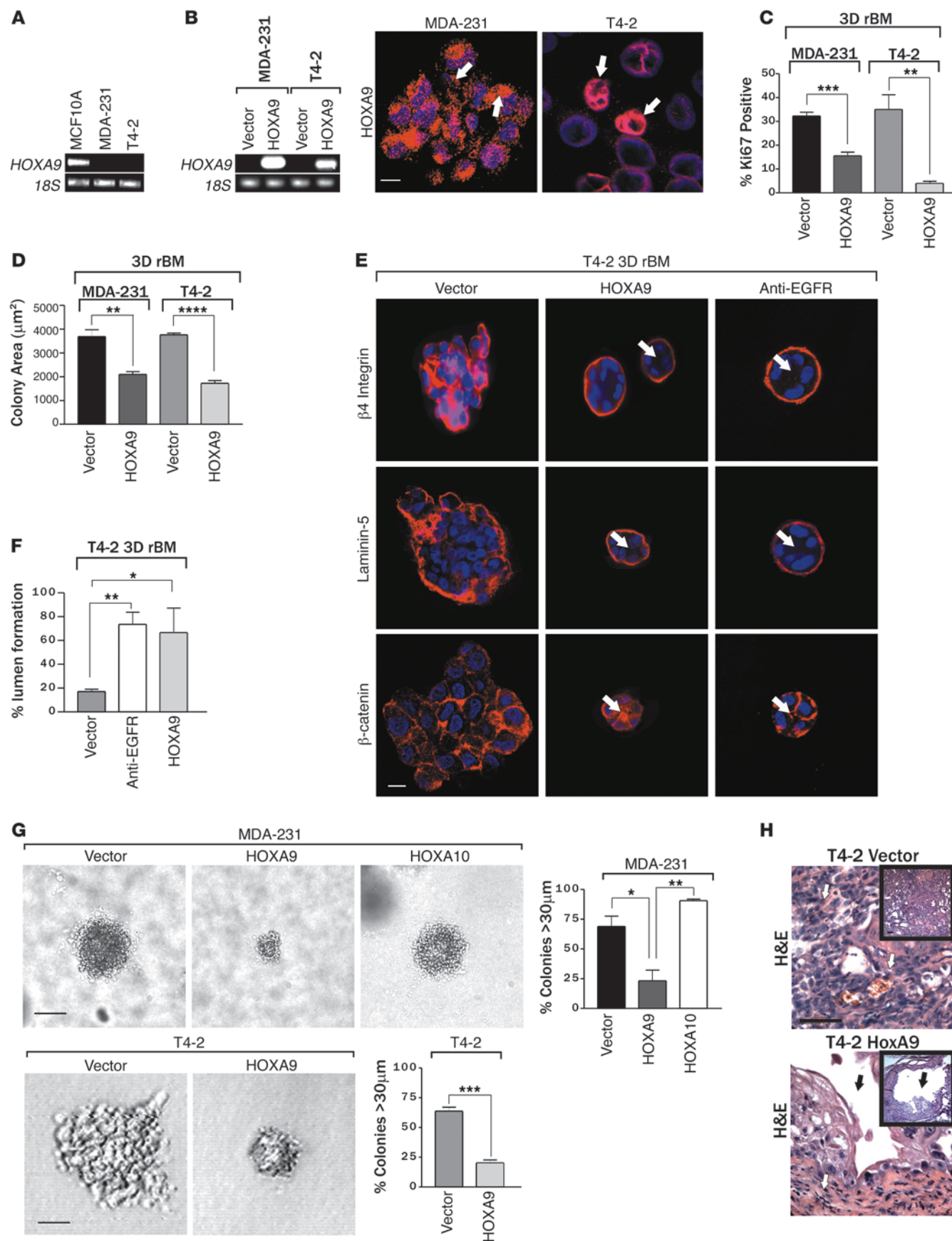
differentiation, and *BRCA1* expression is repressed following reconstituted basement membrane-induced (rBM-induced) acinar morphogenesis (19, 33–36). *BRCA1* expression increases during embryonic mammary gland development and spikes prior to acquisition of acini polarity and pregnancy-associated lactation, consistent with the idea that *BRCA1* expression is functionally linked to breast tissue differentiation (27, 32, 37, 38). If true, *BRCA1* could restrict MEC growth and survival and regulate genome integrity by cooperating with molecules such as homeobox genes, which regulate tissue differentiation.

We identified *HOXA9*, a homeobox gene previously implicated in breast tissue differentiation (39), as a gene whose levels were reduced in breast tumors and whose reexpression promoted breast tumor morphogenesis. Although, paradoxically, *HOXA9* has been characterized as a leukemic oncogene (4) and angiogenesis promoter (40), *HOXA9* also plays an important role in skeletal (41), urogenital tract (42), kidney (43), and mammary gland development (39), and *HOXA9* expression can be regulated by microRNAs that have been implicated in tissue differentiation (44). We found that *HOXA9* promotes breast tumor cell differentiation and inhibits cancer progression by directly regulating expression of the TS gene *BRCA1*. Because *HOXA9* is frequently methylated in breast tumors, the findings offer an alternate explanation for why *BRCA1*

expression is frequently lost in sporadic human breast tumors, even in the absence of genetic modification (45). They further suggest that homeobox genes could regulate tissue development and restrict tumorigenesis by modulating TS genes.

Results

Breast malignancy is associated with reduced HOXA9 expression. Expression profiling is a useful tool to identify gene expression signatures associated with patient prognosis (46, 47), treatment responsiveness (48–50), and risk of tumor metastasis (46, 51–53). However, because sporadic mammary tumors likely arise through input from multiple cooperating yet poorly penetrating genetic, epigenetic, and microenvironmental factors, gene profiling has proven more challenging when applied to identify breast TSs (54, 55). Therefore, to increase the probability of identifying a low abundance breast TS gene using this approach, we selected 5 paired sets of tumor tissue with similar phenotype. Our objective was to discover genes that have lost expression in the tumor tissues when compared with their patient-matched “normal” tissue. Because of the compelling link between developmental regulators and tumor aggression, we focused on identifying misexpressed developmental regulators (1, 56, 57). Moreover, given the paucity of information on basal-like tumors and their recognized aggressive nature



**Figure 2**

HOXA9 modulates the growth and survival of breast cancer cells. (A) Semiquantitative PCR gel, indicating *HOXA9* mRNA levels expressed in human nonmalignant (MCF10A), metastatic (MDA-231), and transformed (T4-2) MECs. 18S rRNA was used as a control. (B) Semiquantitative PCR gel showing transgenic *HOXA9* mRNA levels in MDA-231 and T4-2 cell lines. Immunofluorescence images of nuclei (blue) and FLAG-tagged HOXA9 (red) in MDA-231 and T4-2 cells. Arrows indicate localization of Flag-tagged HOXA9-positive cells. Scale bar: 10 μ m. (C) Proliferation in MDA 231 and T4-2 cells following HOXA9 reexpression. $**P = 0.0025$, $***P = 0.0003$. (D) Cross-sectional area of MDA-231 and T4-2 breast tumor colonies in rBM expressing either the vector or HOXA9 transgene. $****P = 0.0001$, $**P = 0.0068$. (E) Immunofluorescence images of β_4 integrin (red), Laminin-5 (red), β -catenin (red), and nuclei (blue) in T4-2 colonies expressing the vector or HOXA9 or phenotypically reverted acini (anti-EGFR) by inhibiting EGFR activity using tyrphostin. Arrows indicate cleared lumen. Scale bar: 10 μ m. (F) Lumens observed in rBM-generated T4-2 colonies expressing the vector, HOXA9, or anti-EGFR phenotypically reverted acini. $*P = 0.0188$, $**P = 0.0076$. (G) The percentage of tumor colonies greater than 30 μ m in diameter, and phase contrast images of tumor colonies embedded within soft agar. Scale bar: 50 μ m (top panel); 20 μ m (bottom panel). $*P = 0.0221$, $**P = 0.018$, $***P = 0.0005$. (H) High- and low-magnification phase images of H&E-stained tissue sections of control tumor (T4-2 vector) and HOXA9 reexpressing tumor (T4-2 HOXA9) xenografts. Vascularized regions of T4-2 control tumors are indicated with white arrows, and cystic regions of T4-2 HOXA9 reexpressing tumors are indicated with black arrows. Original magnification, $\times 40$; $\times 10$ (insets). Scale bar: 100 μ m.

in younger patients, we chose samples from individuals whose ages ranged from 44 to 54 years and whose tumors were at least 1.5 cm in diameter (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI39534DS1), estrogen receptor/progesterone receptor (ER/PR) negative, and of uniformly high nuclear and histological grade.

Analysis of gene expression, using Rosetta Resolver 2D agglomerative clustering, showed significant differential expression, when comparing normal adjacent and invasive tumor tissue and identified 40 transcripts whose expression was elevated in the tumors and 115 transcripts with reduced gene expression ($P \leq 0.01$; Figure 1A and Supplemental Table 2). Among the genes with reduced expression were 2 developmental HOX genes: *HOXA4* (mean 3.1-fold reduction) and *HOXA9* (mean 4.4-fold reduction). Findings from the *hoxa9-hoxb9-hoxd9* triple knockout mouse suggest that HoxA9 regulates mammary gland differentiation (39), leukemia studies implicate HoxA9 in oncogenesis (4), and in a small cohort of breast cancer patient samples HoxA9 was silenced via methylation (45); therefore, we selected HoxA9 for further study.

Quantitative RT-PCR verified that *HOXA9* mRNA levels were significantly reduced in the normal compared with tumor-matched clinical samples (M.A. Unger, unpublished observations) and that HOXA9 levels were significantly reduced (~75%) in an expanded clinical cohort of ER/PR-positive and -negative, invasive, primary ductal breast carcinomas ($n = 47$), when compared with levels of transcript expressed in normal human breast ($n = 16$; Figure 1B and Supplemental Figure 1). In situ and immunohistochemical analysis confirmed that *HOXA9* mRNA and HOXA9 protein were expressed in the normal breast epithelium and that levels were greatly reduced in mammary tumors (Figure 1, C and D). To explore the clinical relationship between HOXA9 levels and breast cancer clinical histopathology, we used

the Oncomine Cancer Profiling Database (<http://www.oncomine.org>) to survey a large number of breast cancers from multiple independent studies. We found that low levels of HOXA9 expression correlate with features of aggressive disease, such as large- or high-grade tumors, late-stage disease, lymph node involvement, and distant metastasis (Supplemental Table 3). This approach not only validated our original observation that HOXA9 levels were reduced in breast cancers when compared with normal breast tissue, but they also indicated that reduced HOXA9 levels more significantly associate with tumor aggression in ER/PR-negative tumors.

To follow-up on this intriguing observation, we next analyzed publicly available gene expression data sets to look for potential associations between HOXA9 and breast cancer patient clinical outcome. First, we assessed the relationship between *HOXA9* mRNA levels and relapse-free survival in 2 patient data sets that included a cohort of 227 patients, with available clinical follow-up information, and a second independent set of data from 295 patients (47, 58). We found that those patients whose tumors expressed the lowest *HOXA9* levels (lowest quartile) experienced a significantly reduced relapse-free survival, regardless of ER/PR status ($P = 0.025$; Figure 1E). To determine whether low HOXA9 levels could predict distant metastasis, we also assessed the data sets from patients with early-stage breast cancer, all of whom had no evidence of distant metastasis at the time of tumor collection, for a relationship between tumor aggression and HOXA9 levels (47). Upon examination, we noted that the group of patients whose primary tumors expressed *HOXA9* in the lowest quartile developed significantly more distant metastasis as a first adverse event, when compared with all other patients in the study ($P = 0.02$; Figure 1F). A multivariate Cox proportional-hazards analysis revealed that HOXA9 significantly predicts death or disease relapse, independent of standard clinicopathological variables of breast cancers in ER-negative tumors (Table 1). In contrast, the correlation between HOXA9 expression levels and clinical outcomes was less prominent in ER-positive tumors (Table 1). These data show that HOXA9 is significantly downregulated in breast cancer and that its loss correlates with increased disease aggression.

HOXA9 modulates the growth and survival of breast cancer cells. To explore the functional relevance of HOXA9 loss to breast cancer, we examined the effect of HOXA9 reexpression on tumor cell growth, survival, and tissue differentiation. We observed that *HOXA9* mRNA was abundant in the MCF10A nonmalignant human MEC line (Figure 1G and Figure 2A), reduced in the noninvasive, ER-positive breast cancer cell lines T47-D and MCF-7 (Figure 1G), and virtually nondetectable in the ER-negative, basal-like breast cancer cell lines MDA-MB-231 (MDA-231) and HMT-3522 T4-2 (T4-2) (Figure 1G and Figure 2A) (59). We then created multiple pooled clonal populations of MDA-231 and T4-2 breast cancer cells expressing either HA- or FLAG-tagged wild-type HOXA9. Transgene expression was confirmed at the mRNA and protein

Table 2

Physical attributes of excised breast tumor cell xenografts

Xenograft description	Cell mass (mg)	Lesion	Cystic
T4-2 vector	186 \pm 46.7 ^A	10/10 ^A	0/10
T4-2 HOXA9	82 \pm 32.3	2/10	2/10

^A $P < 0.05$.

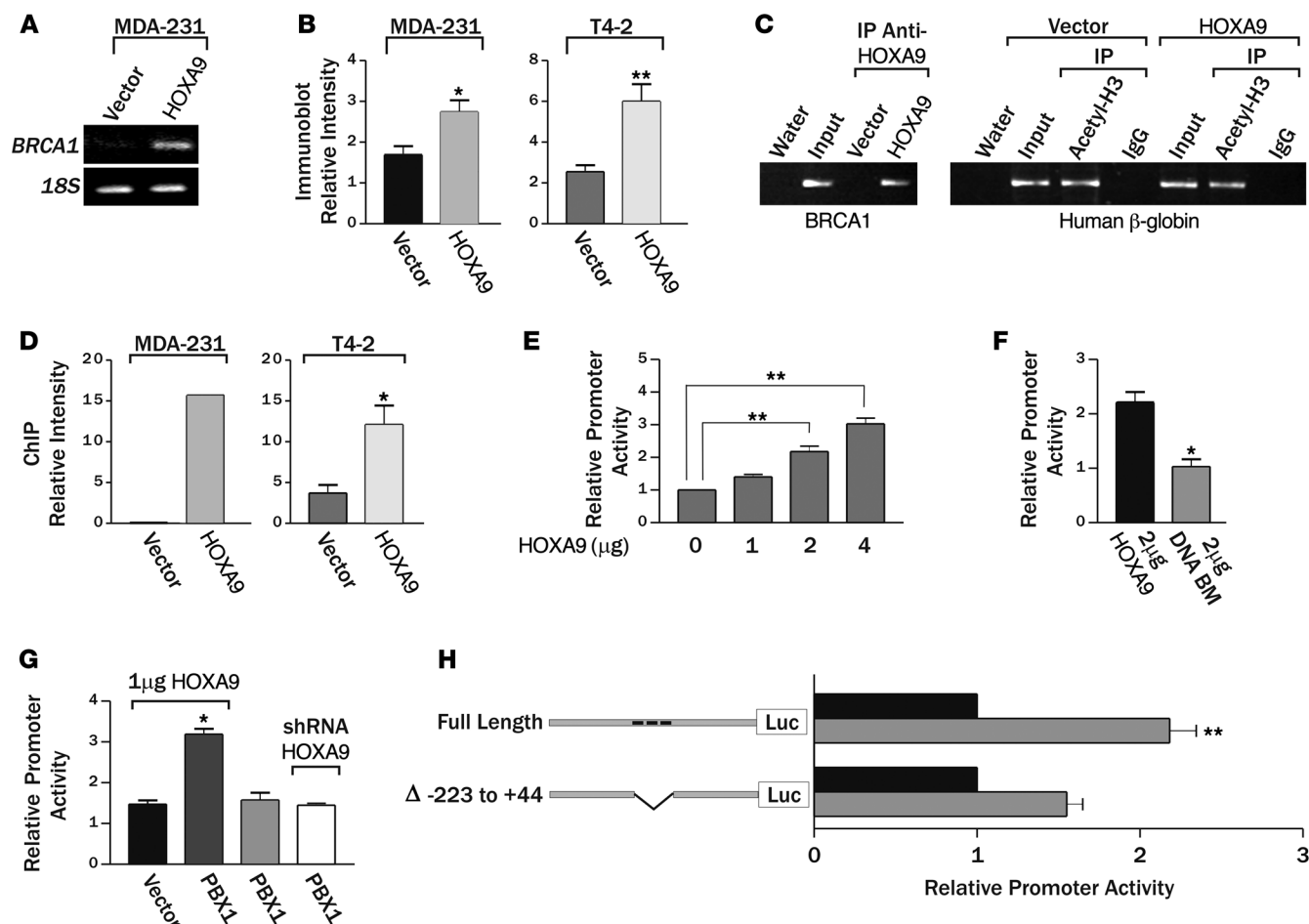


Figure 3

HOXA9 regulates BRCA1 expression. (A) Semiquantitative PCR gel, indicating increased *BRCA1* expression with the reexpression of HOXA9 in MDA-231 cells. (B) Bar graph quantifying immunoblot data from multiple experiments, showing increased BRCA1 protein in MDA-231 or T4-2 breast tumor cells reexpressing HOXA9. $*P = 0.0457$, $**P = 0.0028$. (C) Representative gel of ChIP studies in breast cancer cells, revealing coprecipitation of HOXA9 with the BRCA1 promoter and acetylated acetyl-H3-histone with the β -globin promoter. (D) Bar graphs quantifying ChIP experiments in MDA-231 ($n = 2$) and T4-2 cells ($*P = 0.0178$; $n = 4$). (E) Luciferase reporter analysis, showing a dose-dependent increase in BRCA1 promoter activity in response to addition of wild-type HOXA9. $**P = 0.001$. (F) Luciferase reporter analysis, displaying loss of BRCA1 promoter activity upon addition of HOXA9 containing an N255T (DNA BM) mutation in the conserved DNA binding domain. $*P = 0.03$ (G) Luciferase reporter analysis, indicating enhanced HOXA9-mediated BRCA1 promoter activity upon addition of PBX1 cofactor (2 μ g), compared with Pbx1 alone or Pbx1 cotransfected with a shRNA reducing HOXA9 expression. $*P = 0.0259$. (H) Luciferase reporter analysis, showing a diminished responsiveness of a BRCA1 promoter construct containing a deletion in residues -223 to +44, which contains 3 putative Hox binding sites (gray bar). Data are normalized to matched vector control (black bars). Negative numbers refer to basepairs upstream of the BRCA1 transcription start site. $**P = 0.02$.

level (Figure 2B and Supplemental Figure 2), and the expressed protein was found to localize in both the nucleus and cytoplasm of the infected cells (Figure 2B; see white arrows), in a manner similar to endogenous HOXA9 protein expression (Figure 1, C and D). For easy visualization and manipulation, the HOXA9 transgene was expressed bi-cistronically with EGFP under the control of a tetracycline-repressible promoter (Supplemental Figure 3).

Although HOXA9 reexpression had only a marginal effect on breast tumor cell growth when the cells were propagated on tissue culture plastic (Supplemental Figure 4), we noted that upon embedment within rBM, both MDA-231 and T4-2 breast cancer cells reexpressing HOXA9 grew much slower than their respective vector-only controls, as quantified by significantly reduced Ki-67 levels (Figure 2C), and both cell lines had a decreased colony size

at day 10 (Figure 2D). In fact, reexpression of HOXA9 reverted the malignant phenotype of both of the tumor cell lines toward that of a smaller, more uniform, less invasive, and more cohesive non-malignant colony, similar to that reported previously when epidermal growth factor receptor signaling was inhibited in these cell lines (Figure 2E; see white arrows) (60, 61). HOXA9 reexpression had a particularly pronounced effect on the tissue morphology of the T4-2 rBM colonies, such that the HOXA9-reexpressing tumor colonies reassembled adherens junctions, as indicated by a relocalization of β -catenin to sites of cell-cell interaction, and acquired apical-basal polarity, as revealed by basal relocalization of $(\alpha 6)\beta 4$ integrin and deposition of an endogenous laminin-5 BM around the periphery of the acini (Figure 2E and Supplemental Figure 5). We also noted that HOXA9 reexpression exerted a substantial



effect on cell viability, as revealed by the appearance of lumens within the acini (Figure 2, E and F). Soft agar assays confirmed that HOXA9 but not HOXA10 reexpression, another member of the HOXA cluster (2), influenced tumor cell survival, as shown by a significant inhibition of anchorage-independent growth and survival in both the HMT-3522 T4-2 and MDA-MB-231 tumor cells (Figure 2G, Supplemental Figure 6, and Supplemental Figure 7).

To address the functional relevance of our cell culture observations to breast cancer *in vivo*, we conducted xenograft studies using BalbC *nu/nu* mice. T4-2 breast cancer cells reexpressing HOXA9 (to levels comparable to that detected in the nonmalignant HMT-3522 S-1 MECs; data not shown) failed to grow and survive when injected into the rear flanks of the BalbC *nu/nu* mice (Figure 2H and Table 2). Thus, while vector control T4-2 MECs grew continuously and rapidly to form large, highly angiogenic tumors that were densely populated with actively dividing cancer cells (10 out of 10 lesions), the lesions formed by the T4-2 MECs expressing HOXA9 either completely regressed within 56 days (8 out of 10 lesions) or were highly cystic, avascular, and fibrotic (2 out of 10 lesions) (Figure 2H and Table 2). These data in conjunction with our *in vitro* data suggest that HOXA9 restricts tumorigenic behavior of breast cancer cells by inhibiting cell growth, survival, and invasion and by promoting tissue morphogenesis.

HOXA9 directly regulates BRCA1 expression. Hox genes are transcriptional regulators that exert their effect on cell and tissue phenotype indirectly by modulating gene expression (2). To identify putative HOXA9 targets critical for breast tumor suppression, we defined the global transcriptional profile of MDA-231 breast cancer cells before and after tetracycline-dependent HOXA9 expression (Supplemental Table 4). Among the genes upregulated after HOXA9 reexpression, we observed that the level of the breast cancer susceptibility gene *BRCA1* was dramatically increased and confirmed this by semiquantitative RT-PCR (Figure 3A). Because *BRCA1* is a TS gene that regulates MEC growth, survival, and invasion and can modulate rBM morphogenesis, we next explored the functional relationship between HOXA9 and *BRCA1* regulation (19, 32–34, 38, 62). Immunoblot analysis demonstrated that HOXA9 reexpression consistently elevated *BRCA1* protein levels in MDA-231 and T4-2 breast cancer cells (Figure 3B). These results suggested that HOXA9 could repress breast tumor behavior by regulating expression of the TS gene *BRCA1*.

BRCA1 can be induced by multiple stimuli that might each be independently regulated by HOXA9 (27). While a number of negative regulators of *BRCA1* transcription have been reported, identification of factors that directly upregulate *BRCA1* expression has proven elusive (26–31). Because HOXA9 is a transcription factor, we reasoned there was a strong probability HOXA9 was regulating *BRCA1* levels by directly inducing *BRCA1* gene expression. Computer-assisted analysis confirmed that there were indeed several putative HOX consensus binding sites in the *BRCA1* promoter. To definitively test whether HOXA9 could directly modulate *BRCA1* expression, we conducted *BRCA1* promoter ChIP studies using HA-tagged, exogenously expressed HOXA9 as the bait. Whereas acetyl H3 histone easily and repeatedly coprecipitated the β -globin promoter from both vector control- and HOXA9-expressing cell lines, we could only amplify *BRCA1* promoter product above background from the breast tumor cells reexpressing the exogenous HA-tagged HOXA9 (Figure 3, C and D). Reporter assays, using regions of the *BRCA1* 5' promoter region containing HOXA9 consensus binding sites, confirmed basal luciferase activity could be significantly enhanced after cotransfection with

increasing amounts of a wild-type HOXA9 but not with a HOXA9 expression plasmid containing an N255T mutation (DNA BM) in the conserved DNA binding domain (Figure 3, E and F) (63). Furthermore, HOXA9-dependent *BRCA1* reporter induction could be significantly enhanced by addition of the HOX gene cofactor PBX1 (Figure 3G) (64). In contrast, no increase in reporter activity could be induced by PBX1 alone, PBX1 cotransfected with an shRNA knocking down HOXA9 expression, or HOXA9 cotransfection with *BRCA1* luciferase promoter constructs lacking residues –223 to +44, wherein reside putative HOXA9 consensus binding sites (Genbank U37574; Figure 3, G and H). Interestingly, site-directed mutagenesis of individual HOXA9 consensus binding sites did not significantly compromise *BRCA1* promoter activity, suggesting cooperative release of tandem HOX consensus binding sites might be necessary to ablate HOXA9-dependent control of *BRCA1* gene expression (Supplemental Figure 8). These observations are consistent with previous reports of promoter site cooperation and redundancy in other HOX-regulated genes (65–67). The findings indicate that DNA binding of HOXA9 directly regulates *BRCA1* expression in breast cells.

HOXA9 regulates nonmalignant MEC growth by modulating BRCA1 expression. We identified 2 independent shRNA lentiviral clones that could substantially reduce HOXA9 protein (P.M. Gilbert, unpublished observations) and *HOXA9* mRNA levels in nonmalignant MCF-10A MECs (Supplemental Figure 9) to implicate HOXA9 as a TS. Loss of HOXA9 expression disrupted rBM morphogenesis, as revealed by the formation of larger invasive colonies (Figure 4B), as indicated by lack of β -catenin at sites of cell-cell adherens junctions (Figure 4A, top), loss of basally polarized ($\alpha 6$) $\beta 4$ integrin (Figure 4A, middle), and a discontinuous endogenous laminin-5 basement membrane (Figure 4A, bottom). In addition, MCF-10A MECs with reduced HOXA9 levels exhibited an enhanced survival phenotype, as evidenced by a failure of these structures to clear their lumens (Figure 4A, see arrows; quantified in Figure 4C), and robust anchorage-independent growth and survival (Figure 4, E and F). Consistent with the notion that HOXA9 mediates these TS effects on MEC behavior by regulating *BRCA1* levels, the nonmalignant MECs in which HOXA9 had been knocked down using shRNA showed a greatly blunted induction of *BRCA1* transcription in response to an exogenous stress of 5 Gray irradiation (Figure 4D) (68).

To determine whether reducing *BRCA1* in nontransformed MECs could similarly promote malignant behavior in these MECs, we identified 2 shRNA clones and demonstrated efficient knock down of *BRCA1* protein levels (P.M. Gilbert, unpublished observations). Consistent with prior data implicating *BRCA1* in breast differentiation (19, 32–34, 38), reducing *BRCA1* in the MCF-10A MECs increased cell proliferation in response to rBM cues (inferred by elevated colony size; Figure 4, G and H), enhanced their survival as revealed by luminal filling in the rBM colonies (Figure 4G, see arrow; quantified in Figure 4I), and disrupted tissue morphogenesis (evidenced by loss of cell-cell localized β -catenin; Figure 4G). Likewise, compromising *BRCA1* function in the MCF-10A cells by expressing the *BRCA1* Δ exon11b mutant (Supplemental Figure 10) promoted MEC growth and survival in a 3D rBM and perturbed tissue morphogenesis (Figure 4, J–L). These observations illustrate the importance of HOXA9 and *BRCA1* expression to MEC growth and survival and rBM-induced tissue morphogenesis.

HOXA9 regulates BRCA1 to repress the malignant behavior of MECs. We next manipulated the expression and function of *BRCA1* and HOXA9 in breast cancer cells and assayed for effects on tumor

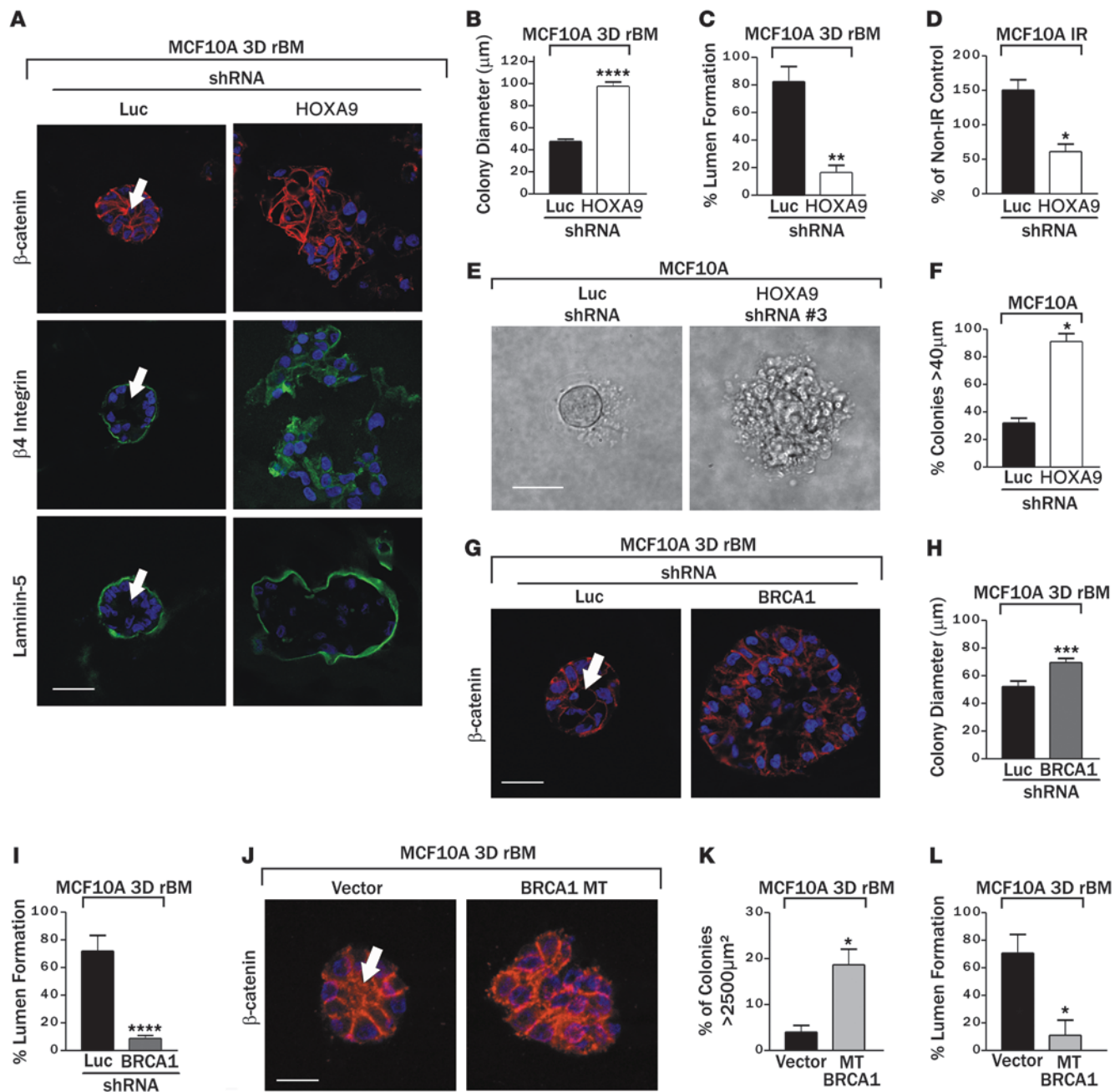


Figure 4

HOXA9 regulates nonmalignant MEC growth by modulating BRCA1 expression. (A) Immunofluorescence images of β -catenin (red), β_4 integrin (green), Laminin-5 (green), and nuclei (blue) in MCF-10A colonies expressing either luciferase control shRNA or shRNA-HOXA9 clone 3. Arrows indicate cleared lumen. Scale bar: 50 μ m. (B) Colony size of MCF-10A cells cultured within a rBM and expressing reduced HOXA9 levels. **** P = 0.0001. (C) Lumens observed in MCF-10A colonies expressing luciferase control shRNA as compared with those with shRNA-mediated HOXA9 knockdown. ** P = 0.0010. (D) The response (BRCA1 protein levels) of MCF-10A cells expressing reduced HOXA9 levels to 5 Gray irradiation compared with nonirradiated samples. * P < 0.001. (E and F) Phase images of tumor colonies embedded within soft agar (E) and the percentage of colonies greater than 40 μ m in diameter (F). Scale bar: 50 μ m. * P = 0.001. (G) Immunofluorescence images of β -catenin (red) and nuclei (blue) in MCF-10A colonies, expressing either luciferase control shRNA or shRNA-BRCA1 clone 5. The arrow indicates cleared lumen. Scale bar: 50 μ m. (H) Colony size of MCF-10A cells cultured within a rBM and expressing reduced BRCA1 levels. *** P = 0.0024. (I) Quantification of lumens observed in MCF-10A colonies expressing luciferase control shRNA as compared with those with shRNA-mediated BRCA1 knockdown. **** P = 0.0003. (J) Immunofluorescence images of β -catenin (red) and nuclei (blue) in MCF-10A colonies expressing vector or mutant BRCA1 (BRCA1 MT). The arrow indicates cleared lumen. Scale bar: 10 μ m. (K) Quantification of cross-sectional area of MCF-10A colonies in cells cultured within a rBM and coexpressing mutant BRCA1. * P = 0.05. (L) Quantification of lumens observed in MCF-10A colonies expressing vector control as compared with those expressing a mutant BRCA1. * P = 0.05.

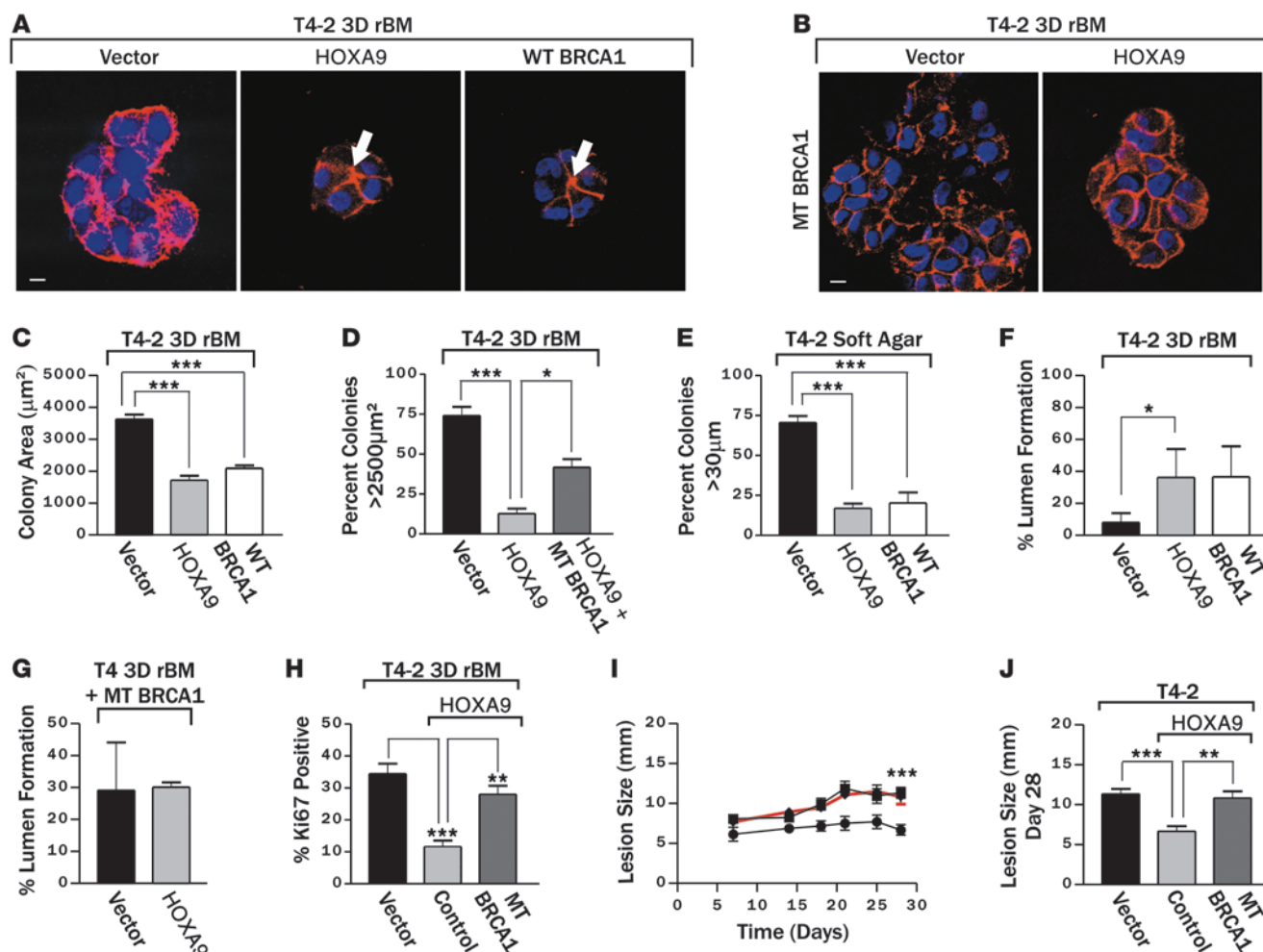


Figure 5

HOXA9 regulates BRCA1 to repress the malignant behavior of MECs. (A) Immunofluorescence images of β -catenin (red) and nuclei (blue) in T4-2 colonies reexpressing HOXA9 or BRCA1. Arrows indicate cleared lumens. Scale bar: 10 μ m. (B) Immunofluorescence images of β -catenin (red) and nuclei (blue) in T4-2 colonies reexpressing HOXA9 alone or with a mutant BRCA1. Scale bar: 10 μ m. (C) Quantification of colony size of T4-2 cells reexpressing HOXA9 or BRCA1. *** P = 0.001. (D) Quantification of cross-sectional area of T4-2 colonies formed by cells reexpressing HOXA9 alone or with a mutant BRCA1. * P = 0.05, *** P = 0.001. (E) Quantification of anchorage-independent growth and survival in T4-2 cells reexpressing either HOXA9 or BRCA1. *** P = 0.001. (F) Quantification of the percentage of T4-2 colonies that formed lumens following the reexpression of HOXA9 or BRCA1. * P = 0.0263. (G) Quantification of lumen formation in rBM T4-2 colonies with reexpressed HOXA9, when BRCA1 function has been compromised through coexpression of a mutant BRCA1. (H) Quantification of proliferation in T4-2 cells following HOXA9 reexpression with a mutant BRCA1. ** P = 0.0025, *** P = 0.0003. (I) The time course of the progressive increase in xenograft size (5–30 days). Reexpression of HOXA9 in T4-2 tumor cells significantly reduced the rate of lesion expansion (filled circles) compared with the T4-2 vector controls (filled squares), which could be restored to that of T4-2 breast tumor cells if coexpressed with a mutant BRCA1 (filled triangles with red line). *** P = 0.001. (J) Lesion size (28 days) in each experimental group. ** P = 0.01, *** P = 0.001.

cell behavior in culture and in vivo. Consistent with a functional link between HOXA9 and BRCA1, increasing the levels of wild-type BRCA1 in breast tumor cells significantly reduced their rBM-dependent growth and normalized their survival behavior, such that they assembled colonies with cleared lumens (Figure 5A, see arrows; quantified in Figure 5F) that were similar in size and morphology to those formed by tumor cells reexpressing HOXA9 (compare in Figure 5, A, C, and F). In addition, rBM-dependent tumor colonies expressing higher wild-type BRCA1 were no longer invasive and had cell-cell localized β -catenin (Figure 5A), indicating that, analogous to HOXA9, BRCA1 also reverted the

malignant phenotype of breast cells toward a normal polarized, growth-arrested acini structure (see Figure 2). Consistent with its TS function, ectopic expression of wild-type BRCA1 reduced the anchorage-independent growth and survival of breast cancer cells (Figure 5E). These data show how BRCA1 can phenocopy the TS effects of HOXA9 and can repress the growth, survival, and invasive behavior of breast tumor cells in rBM.

We compromised BRCA1 function in the T4-2 tumor cells reexpressing HOXA9 through coexpression of the Δ exon 11b BRCA1 mutant and then assayed for effects on rBM growth, survival, and colony morphology to explore the functional relationship between

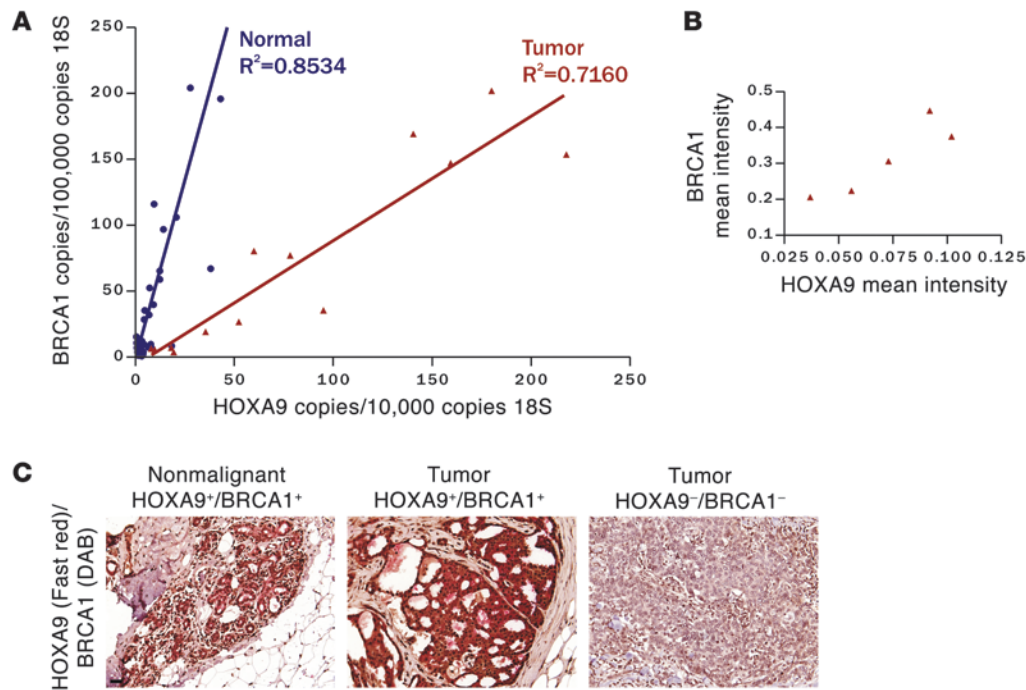


Figure 6

Clinical correlation between HOXA9 and BRCA1 expression. **(A)** Line graph illustrating that significant correlations exist between *HOXA9* and *BRCA1* mRNA levels expressed in a cohort of normal (shown in blue; $P \leq 0.0001$; $r^2 = 0.8534$) and tumorigenic (shown in red; $P \leq 0.0001$; $r^2 = 0.7160$) human breast tissue specimens ($n = 53$). **(B)** Graph illustrating the relationship between HOXA9 and BRCA1 protein levels in human ER-/PR-/ErbB2⁻ breast samples. **(C)** Immunohistochemistry showing colocalized expression of HOXA9 and BRCA1 protein in the epithelium of normal human breast tissue and HOXA9⁺/BRCA1⁺ tumor samples, as compared with HOXA9⁻/BRCA1⁻ tumor samples. (HOXA9 was “stained” with Fast Red, and BRCA1 was “stained” with DAB). Scale bar: 100 μ m.

HOXA9 and BRCA1 expression and MEC phenotype. Disrupting BRCA1 function antagonized the ability of HOXA9 to repress the malignant behavior of the T4-2 tumor cells (Figure 5B). Thus, tumor cells simultaneously expressing HOXA9 and the Δ exon 11b BRCA1 mutant failed to phenotypically revert when grown within rBM and instead formed continuously growing, invasive, and nonpolarized colonies that lacked cell-cell localized β -catenin and cleared lumens (Figure 5, B, D, G, and H). These studies demonstrated that the ability of HOXA9 to repress the malignant behavior of MECs is functionally dependent upon BRCA1.

To address the *in vivo* relevance of a functional link between HOXA9 and BRCA1, we conducted xenograft studies using BalbC *nu/nu* mice injected with human breast cancer cells, with and without HOXA9 and a functional BRCA1, and assayed tumor growth (as indicated by lesion size). Reexpression of HOXA9 in T4-2 breast cancer cells significantly reduced lesion growth rate as compared with T4-2 vector controls (Figure 5, I and J). In contrast, the HOXA9 expressing T4-2 MECs in which BRCA1 function was simultaneously compromised, through coexpression of the Δ exon11b BRCA1 mutant, grew robustly and had growth rates that were comparable to that observed in the vector control T4-2 MECs. T4-2 HOXA9 tumor cells in which BRCA1 function was compromised developed lesions that were, on average, comparable in size and morphology to those formed by the wild-type tumors (28 days; Figure 5, I and J). These data demonstrate that HOXA9 restricts the growth and survival of human breast cancer cells by regulating BRCA1 expression both in culture and *in vivo*.

Clinical correlation between HOXA9 and BRCA1 expression. To address the clinical relevance of a functional link between HOXA9 and BRCA1, we examined mRNA and protein expression and colocalization of HOXA9 and BRCA1 in cohorts of clinically diverse (ER/PR-positive and -negative) human breast cancers and normal tissue ($n = 56$). Quantitative RT-PCR data showed that HOXA9 correlates significantly with BRCA1 in normal ($r^2 = 0.8534$; Figure 6A, blue line) and transformed breast tissue (ER/PR positive and negative; $r^2 = 0.7160$; Figure 6A, red line). Quantitative immunohistochemistry of a panel of 12 triple-negative breast tumors revealed a strong positive association between nuclear HOXA9 and BRCA1 (Figure 6B and Table 3). We also noted that the epithelium of the normal breast coexpressed appreciable levels of cytoplasmic and nuclear HOXA9 and BRCA1 protein (Figure 6C, left), as did the more differentiated, triple-negative breast tumor tissues (Figure 6C, middle). In contrast, less differentiated triple-negative tumors showed complete loss of both proteins (Figure 6C, right). Interestingly, we observed that a loss in HOXA9 expression was associated with a greater reduction in *BRCA1* mRNA in the breast tumor samples as compared with normal tissue (Figure 6A, compare normal to tumor). These clinical findings, together with our experimental data, are consistent with the notion that HOXA9 modulates BRCA1 expression by cooperating with multiple negative and positive regulatory factors, differentially expressed in human breast tumors, whose expression correlates with loss of ER/PR expression and tumor aggression (20, 27).



Discussion

To identify developmental regulators misexpressed during malignant transformation of the breast, we used global expression profiling of microdissected breast tumors and their adjacent normal tissue. We identified the homeobox gene *HOXA9*, previously implicated in mammary development (12, 39), as a gene whose levels were significantly downregulated. We confirmed the microarray observation using RT-PCR, in situ hybridization, and immunohistochemistry and showed that *HOXA9* was highly expressed in the luminal epithelium of the normal breast and that its expression was frequently decreased in a high proportion of invasive human breast tumors and breast cancer cell lines (Figure 1, B and G). Bioinformatics analysis confirmed these findings and also indicated that *HOXA9* loss significantly correlated with features of aggressive disease in ER/PR-negative breast tumors, including large- and high-grade tumors, late-stage disease, lymph node involvement, distant metastasis, and reduced patient survival (Figure 1, E and F, Table 1, and Supplemental Table 3). Using 2 ER/PR/Erbb2-negative, basal-like breast cancer cell lines, we then showed that reexpressing *HOXA9* (but not another member of the *HOXA* cluster, *HOXA10*) inhibited tumor cell growth and survival and promoted acini morphogenesis in culture and restricted tumorigenesis in vivo. Furthermore, we showed that reducing levels of *HOXA9* in nonmalignant MECs enhanced growth and survival and perturbed acini morphogenesis (Figures 2 and 4). Although investigators have previously reported that *HOXA9* is methylated and that its expression is reduced in breast, lung, and ovarian cancers (45, 69, 70), this is the first study to our knowledge to assess the clinical relationship of *HOXA9* loss to solid tumors, to analyze the functional relevance of *HOXA9* to the malignant behavior of MECs in culture, in vivo and in clinical specimens, and to identify a molecular mechanism for these effects. In this respect, we found that *HOXA9* restricts the aggressive and malignant behavior of MECs by directly modulating *BRCA1*, implying that *HOXA9* reduces risk to malignancy and controls tumor aggressiveness by controlling levels of an established mammary gland TS gene. These findings are consistent with the notion that developmental regulators, such as the *HOX* family of transcription factors, influence adult tissue homeostasis by regulating the expression and/or activity of key TS genes that regulate cell growth and survival and morphogenesis (12).

Homeobox genes regulate embryonic development and tissue patterning, and their expression is frequently perturbed and often aberrantly increased in tumors (2, 3, 12, 71). Until recently, the prevailing dogma has been that inappropriate expression of homeobox genes promotes tumor progression. Consistently, the homeobox genes that are highly expressed during early embryogenesis, that promote cell proliferation and survival, and that induce migration are those that are most often over expressed in transformed cells and tissues (6, 8, 72, 73). These are the homeobox genes that have been implicated in altered growth receptor signaling, deregulated cell cycle control, and the elevated growth and apoptosis resistance of cancer cells and that appear to regulate tumor invasion and metastasis and promote angiogenesis (40, 72, 74–76). For instance, the homeobox gene *Six1* is highly expressed during early mammary gland development, where it drives epithelial cell proliferation by modulating levels of the cell cycle gene cyclin A1 (77). Although *Six1* levels are downregulated and barely detectable in the differentiated adult mammary gland, *Six1* is frequently overexpressed in aggressive breast tumors, in which it pro-

Table 3

Relationship between *HOXA9* and *BRCA1* nuclear protein levels in human ER-/PR-/Erbb2- breast samples

Case no.	Nuclear <i>HOXA9</i>	Nuclear <i>BRCA1</i>
4309	+	+++
9667	+	++
6073	+	+
11947	+	+
462	++	–
6697	+	–
8147	–	++
8715	–	+
8954	–	+
6912	–	+
9533	–	–
2440	–	–

Expression is relative between samples. –, no detectable expression; +, low expression; ++, moderate expression; +++, high expression.

motes cell growth and survival, enhances genomic instability, and promotes tumor metastasis (78). Similarly, enforced expression of the early embryonic homeobox gene *Msx1*, which is also often elevated in tumors, promotes the proliferation of undifferentiated stem cells, blocks the terminal differentiation of myoblasts, and downregulates expression of the myogenic differentiation factor MyoD1 to induce their malignant transformation (79–81).

It is now appreciated that homeobox genes of the ANT-C/BX-C type that control rostral-caudal patterning during embryogenesis and are abundantly expressed in differentiated tissues can repress malignancy and may function as “tumor modulators” (9, 71, 82–84). Data that support this concept exist, although, unfortunately, many findings linking homeobox gene loss with tumorigenesis are largely circumstantial (45, 69, 70, 79). At present, few methodical studies exist that clarify molecular mechanisms whereby reduced homeobox levels could restrict tumor progression and/or metastasis (9, 84). In this article, we present evidence that one of the posteriorly expressed homeobox genes, *HOXA9*, is necessary for normal MEC growth and survival and acini morphogenesis, a finding that is consistent with a previous article implicating *HOXA9* in mammary gland differentiation in the mouse (Figures 2 and 4) (39). Our findings clarify these earlier observations and indicate that *HOXA9* inhibits cell growth and survival and promotes morphogenesis in normal and transformed MECs by directly regulating expression of the TS gene *BRCA1* (Figures 3 and 5). Our observations are consistent with and extend prior studies showing that *PITX1*, which is frequently downregulated in prostate, bladder, and colon cancers (6), could function as a TS by inhibiting oncogenic Ras signaling and indicating that *HOXA5*, which is lost in greater than 60% of mammary tumors and breast cancer cell lines, may restrict breast cancer by regulating levels of the TS p53 to alter inappropriate MEC survival (8, 73). Distinct from these reports, we could show that *HOXA9* not only modulates *BRCA1* levels but also directly binds to and regulates *BRCA1* transcription and that *HOXA9*-dependent *BRCA1* induction is markedly enhanced during tissue remodeling and following exposure of breast cells to an exogenous stress, consistent with a role for *BRCA1* in cell cycle regulation and the DNA damage response (85, 86). Intriguingly, *HoxC8* was shown to bind directly to and regulate the mammalian



homolog of lethal giant larvae TS gene; however, to date no functional data exist to clarify the relevance of this relationship (7). In the present studies, we not only showed that HOXA9 directly regulates BRCA1 transcription, but we demonstrated that HOXA9-dependent BRCA1 expression is critical for the growth, survival, and morphogenesis activity of HOXA9 in culture and for its TS-like activity in vivo (Figures 4 and 5). We also provided additional evidence that this relationship has clinical relevance (Figures 5 and 6). Indeed, our findings argue that loss of HOXA9 is a critical determinant of tumor aggression, especially in ER/PR-negative breast tumors, which likely harbor additional modifications that negatively modulate BRCA1 levels. Our findings emphasize the importance of examining the role of homeobox family members as critical regulators of normal tissue differentiation and homeostasis and illustrate the potential permissive role of HOXA9 loss in tumor progression and aggressiveness and as a potential regulator of treatment responsiveness.

In contrast to our observation that HOXA9 restricts the growth, survival, and malignant behavior of breast epithelial cells, it is well known that HOXA9 plays an essential role in normal myeloid lineage development, because it promotes expansion of the stem cell pool and inhibits differentiation (87). Consistently, increased expression or activation of HOXA9 in myeloid stem cells is causally linked to acute myeloid leukemia (88), and enforced expression of HOXA9 in myeloid cells, due to chromosome translocation or overexpression of its regulator MLL, drives transformation (4). Furthermore, elevated HOXA9 expression induces angiogenesis by regulating growth, migration, and invasion of endothelial cells (40). Interestingly, neither myeloid cells nor human endothelial cells ectopically overexpressing HOXA9 upregulate *BRCA1* transcript levels, thereby offering one likely explanation for the strikingly different phenotypic consequences of HOXA9 expression between MECs versus lymphocytes and endothelial cells (P.M. Gilbert, unpublished observations) (89, 90). This observation is consistent with previous studies indicating that homeobox target genes are cell and tissue specific. The data are also in accord with results showing that the expression profile and gene targets of each HOX factor depend upon the complement of cofactors present in each cell and the extracellular microenvironment the cell resides within (91). Thus, while HOXA5 can induce p53 expression in MECs, sustained HOXA5 failed to modulate p53 in endothelial cells and instead induced Thrombospondin-2 (8, 92). Such findings serve to illustrate the urgency of conducting comparative functional studies of homeobox gene regulation in different tissues and stress the relevance of tissue context as a key regulator of cellular behavior. The work also underscores the importance of considering tissue-specific gene regulation in order to understand cancer pathogenesis as well as to identify tissue-specific treatments.

BRCA1 is either lost or mutated in many cases of familial breast cancer (27, 93, 94). Nevertheless, BRCA1 expression is also frequently reduced in sporadic breast cancers, and gene methylation and silencing only account for a subset of these sporadic tumors (27, 93). This means that other parameters and factors that are altered during breast carcinogenesis likely exist to regulate tissue-specific levels of BRCA1 (20, 27). Indeed, the BRCA1 promoter is a highly complex, bidirectional transcriptional unit, with multiple binding motifs, and it is subject to dynamic interactions between its promoter and terminator regions. Its activity can be modulated by multiple generic and tissue-specific factors, including 53BP1,

E2F proteins, and GABP- α/β , and conditions, including stress, hypoxia, growth factors, and estrogens (95, 96). However, despite these findings, very few factors have been shown to bind to and directly modulate BRCA1 expression and, of these, most have been negative regulators. Furthermore, there is a paucity of evidence to link these BRCA1 regulators to defined BRCA1-dependent phenotypes. Metastasis-associated tumor antigen 1 (MTA1) has been implicated in the transcriptional repression of BRCA1 and in abnormal centrosome number and chromosomal instability (30), and E2F4 and the pocket proteins p130/p107 bind the BRCA1 promoter and repress its basal transcription, thereby regulating cell growth (97). In these studies, we demonstrate that HOXA9 directly and positively modulates BRCA1 transcription, thereby restricting the abnormal growth, survival, and stress response of breast cancer cells and nonmalignant MECs in culture and in vivo. This relationship has clinical relevance because loss of HOXA9 significantly predicts tumor aggression in ER/PR-negative cancers. In this respect, HOXA9 correlated significantly with *BRCA1* mRNA and BRCA1 protein levels in breast tissues, in which we observed that reduced HOXA9 expression was associated with a more profound reduction of BRCA1 in tumor cells and that loss of HOXA9 and BRCA1 was strongly associated with a less differentiated phenotype in triple-negative breast tumors. Because basal-like tumors also frequently express “BRCA1 repressors,” these results argue that HOXA9 likely cooperates with other intrinsic or acquired factors to modulate BRCA1 expression in breast cancers. The fact that expression profiling did not reveal reduced *BRCA1* transcripts in the 4 primary tumors with reduced HOXA9 expression is not surprising (Supplemental Table 2). In general, expression levels of *BRCA1* are below the detection sensitivity of Affymetrix arrays, and thus transcript level changes would not normally be noted. Instead, BRCA1 expression is tightly linked to cell cycling and the presence of damaged DNA (98), and we observed a robust induction of BRCA1 in response to HOXA9 most predominantly during tissue remodeling (J.K. Mouw, unpublished observations) or after exposure to an exogenous stress (Figure 4D). Thus, because the HOXA9 promoter is frequently methylated and HOXA9 levels are often reduced in invasive breast tumors, and we showed that HOXA9 is often lost in breast tumors, our data offer an attractive explanation for why BRCA1 expression could be so frequently lost in sporadic human breast tumors, even in the absence of genetic aberrations, promoter methylation, or haploinsufficiency.

Intriguingly, not only did we find that HOXA9-dependent BRCA1 expression restricts tumor progression by inhibiting MEC growth and survival, but we also observed that elevated HOXA9 and BRCA1 levels restored cell-cell adhesions and normalized acini morphogenesis (Figures 2 and 5). These findings are consistent with previous reports that showed that loss of BRCA1 compromises the ability of nonmalignant MCF10A MECs to undergo morphogenesis into polarized acinar structures in a 3D rBM assay (19, 35, 36) and are consistent with data indicating that BRCA1 is critical for lumen formation in primary murine MECs (38). Indeed, during mammary gland remodeling, BRCA1 levels peak prior to tight junction assembly and tissue-specific differentiation, and they decline to barely detectable levels during lactation (27, 37, 38). Consistently, recent studies in which BRCA1 was overexpressed in the epithelium of the murine breast showed that there was a moderate increase in lobular alveolar differentiation in the mammary gland, consistent with accelerated development, and these mice also showed resistance to mutagen-induced mammary neoplasia. In contrast, age-matched



mice expressing a mutated BRCA1 morphologically resembled animals at mid-pregnancy, consistent with increased proliferation and secondary branching, and these mice showed enhanced DMBA-induced transformation (99). Our findings raise the intriguing possibility that some HOX genes, particularly those expressed late during development and those that are expressed in differentiated tissues such as HOXA9, might regulate growth, survival, and invasion to modulate body plan patterning during development, by regulating levels of TSs that control these orchestrated processes. In this respect, HOXA9 plays an important role in skeletal (41), urogenital tract (42), kidney (43), and mammary gland development (39), and HOXA9 expression can be regulated by microRNAs that have been implicated in tissue differentiation (44).

Methods

Substrates, antibodies, and pharmacological reagents. The materials used were as follows: Commercial EHS matrix (Matrigel; Collaborative Research) for the rBM assays; Vitrogen (Vitrogen 100, Inamed Biomaterials; bovine skin collagen I), 3 mg/ml, for coating culture dishes; and Collagen AC-5, 0.5% (ICN Biomedical Inc.) for morphogenesis assays. Primary antibodies used were as follows: actin, clone AC-40 (Sigma-Aldrich); BRCA1, clone Ab-1 (Oncogene); β -Catenin, clone 14 (BD Biosciences – Transduction Laboratories); β_4 integrin, clone 3E1 (Invitrogen); FLAG, clone M2 (Sigma-Aldrich); HA.11, clone 16B12 (Covance Research Products); HA, clone Y-11 (Santa Cruz Biotechnologies Inc.); acetyl H3 histone, rabbit polyclonal (Upstate); Ki-67 clone 35 (BD Biosciences – Transduction Laboratories); HOXA9 (N-20), goat polyclonal (Santa Cruz Biotechnologies Inc.); HOXA9, rabbit polyclonal (a gift from T. Nakamura, Japanese Foundation for Cancer Research, Tokyo, Japan); HOXA10 rabbit polyclonal (Abcam); and laminin 5 (a gift from M.P. Marinkovich, Stanford University School of Medicine, Stanford, California, USA) (100). Secondary antibodies used were Alexa Fluor 488- and 555-conjugated polyclonal anti-rabbit and anti-mouse IgGs (Molecular Probes) and HRP-conjugated polyclonal rabbit and anti-mouse IgGs (Amersham Pharmacia). Pharmaceutical reagents included Tyrphostin AG 1478 (100 μ M; DMSO) (Calbiochem); *p*-Nitrophenyl phosphate disodium salt hexahydrate (Fisher Scientific); and D-Luciferin, potassium salt (Biotium).

cDNA, lentiviral and retroviral, and shRNA constructs and vectors. Please refer to the Supplemental Methods section for a detailed description of constructs used.

Cell culture. The HMT-3522 S-1 and T4-2 MECs were grown and manipulated in 2D and 3D, and the T4-2s were phenotypically reverted exactly as described previously (60, 101). MDA-MB-231, MCF-7, and MCF10A cells were cultured according to manufacturer's recommendations (ATCC) and grown in 3D as described previously (102). BT-20, MDA-MB-468, MDA-MB-435, T47D, ZR751, and SK-BR-3 cells were cultured according to manufacturer's recommendations (ATCC).

Retroviral and lentiviral infections. Amphotrophic retrovirus was produced (103), and retroviral supernatant was harvested and used directly to spin infected cells, followed by antibiotic-induced selection with puromycin (0.5 μ g/ml media) or neomycin (100 μ g/ml) 72 hours after infection (104). Lentiviral particles were produced, harvested, and used to infect target cells as previously described (105).

Soft agar assay. Anchorage-independent growth was assessed using a soft agar assay (60). In brief, 25,000 cells in 1.5 ml 0.35% agarose containing 1X growth media were overlaid with 1.5 ml 0.5% agarose containing 1X growth media, and colonies larger than 30 μ m in diameter were scored positive after 14 days.

In vivo studies. All experiments were performed in accordance with the guidelines of Laboratory Animal Research at the University of Pennsylvania. Four-week-old BalbC *nu/nu* mice were subcutaneously injected in the rear flanks (5×10^6 cells/injection, together with Matrigel), and palpable lesions were

detected, measured, and monitored biweekly for 56 days (Instant read-out digital calipers; Electron Microscopy Sciences). At experiment termination, mice were sacrificed, and lesions were dissected, measured, macroscopically analyzed, fixed in 4% paraformaldehyde, and paraffin embedded, and H&E sections were evaluated for histopathological evidence of tumor phenotype.

Immunofluorescence. Immunofluorescence analysis of cells grown in 2D, 3D, and paraffin-embedded tissues was performed as previously described (101, 102).

Proliferation. Cell proliferation was measured by calculating the percentage of Ki-67-labeled nuclei and quantified as previously described (106).

Immunoblotting. Equal amounts of cell protein lysate (Laemmli; BCA; Pierce) were separated on reducing SDS-PAGE gels, transferred to nitrocellulose or PVDF membrane, and probed with primary antibody. Bands were visualized and quantified using a Fujifilm Gel Documentation system, in conjunction with HRP-conjugated secondary antibodies and ECL-Plus (Amersham Pharmacia). For the irradiation response of MCF-10A cells, cells were irradiated (5 Gray) and lysed 24 hours later.

ChIP. ChIP assays were performed according to the manufacturer's directions (Upstate). In brief, proteins were cross-linked to chromatin (formaldehyde; 1%), cells were lysed, and the chromatin was sheared (sonication; Misonix Ultrasonic). HA-tagged HOXA9/DNA fragments were immunoprecipitated (overnight; at 4°C) using polyclonal anti-HA (clone Y-11) or polyclonal anti-HOXA9, with polyclonal anti-acetyl H3 histone serving as a positive control and isotype-matched IgG serving as a negative control. Protein/DNA complexes were captured (Protein A agarose beads), washed (6–10 times), and eluted from beads, and cross-links were reversed (NaCl and phenol/chloroform extraction). DNA was ethanol precipitated and used directly for PCR reactions. To amplify a human BRCA1 promoter fragment from anti-HOXA9 ChIP experiments we used the following primers: forward, 5'-GATGGGACCTTGTGGAAGAA-3', and reverse, 5'-ACGACCAAACCAACCAAT-3'. To amplify the human β -globin gene (107) from anti-acetyl H3 histone ChIP experiments we used the following primers: forward, 5'-ATCTTCTCTCCACAGCTCCT-3', and reverse, 5'-TTTGCAGCCTCACCTTCTTT-3'. Bar graph data is normalized to an IgG control ChIP.

BRCA1 reporter assay. Luciferase *BRCA1* gene reporter assays were conducted in 293 cells by transient transfection and normalizing transfection efficiency, by quantifying SEAP expression using a MRX microplate reader (Dynex Technologies) 36 hours after transfection, as previously described (108). Forty-eight hours after transfection, cells were lysed (25 mM glycylglycine, 2 mM EGTA, pH 8.0, 1% Triton X-100, 1 mM DTT, pH 7.8), aliquots of lysate were diluted (1:5) in assay buffer (25 mM glycylglycine, 2 mM EGTA, pH 8.0, 10 mM MgSO₄, 2.2 mM ATP, 0.275 mM Acetyl CoA, 1 mM DTT, pH 7.8), transferred to a Microfluor plate (Thomas Scientific), and mixed with equal quantity of luciferin buffer (25 mM glycylglycine, 2 mM EGTA, pH 8.0, 10 mM MgSO₄, 1 mM DTT, 0.55 mM luciferin, pH 7.8). Light emission from the reaction was detected using a Microtiter plate luminometer (Dynex Technologies) in conjunction with Revelation software. Experiments were quantified as the fold change over appropriate control conditions.

Morphometric analysis. Colony size and morphology in 3D rBM was assessed at indicated times, essentially as previously described (101, 102). Briefly, cell-cell integrity within a 3D MEC acinus was considered intact when β -catenin staining was localized and continuous around the periphery of all cells. A 3D MEC acinus was considered properly polarized when β_4 integrin and laminin were expressed continuously and exclusively around the basal surface of the acinar structure.

Expression profiling. Please see the Supplemental Methods section for a detailed description of the expression profiling.

Semiquantitative PCR. Purified total RNA (2.0 μ g) was reverse transcribed, using random primers (Amersham Biosciences), and resultant samples were serially diluted 1:10, 1:100, and 1:1,000 for subsequent PCR reactions.



An initial PCR was performed to amplify the 18S rRNA subunit, together with a standard curve to determine cDNA copy number for each sample. Primer sequences were as follows: 18S rRNA, forward, 5'-CGGCTACCA-CATCCAAGGAA-3', and 18S rRNA, reverse, 5'-GCTGGAATTACCGCG-GCT-3'. Corrected cDNA concentrations were calculated and a second PCR reaction was performed in which equal amounts of cDNA were added to primers specific for HOXA9. The primer sequences used to amplify HOXA9 cDNA were 5'-GCTTGTGGTTCTCTCCAGT-3' and 5'-CCAGGGTCTG-GTGTTTTGTA-3'. These primers cross the exon 1–2 boundary and thus should not amplify contaminating genomic DNA. The primer sequences used to amplify BRCA1 cDNA were 5'-GGAATAACCAACGGAGCA-3' and 5'-TAGGTTTCTGCTGTGCTGA-3'. The primer sequences used to amplify HOXA10 cDNA were 5'-TATCCCAACAATGTCATGCTC-3' and 5'-GTCGCTGGAGATTCATCAGGA-3'.

Quantitative real-time PCR. Total RNA was reverse transcribed using random primers (Amersham Biosciences), and 18S rRNA primers were used to control for cDNA concentration in a separate PCR reaction for each sample (see above for sequences). Primers used to amplify HOXA9 exon 2, using the LightCycler apparatus (Roche Diagnostics), are listed above. LightCycler Fast Start DNA Master SYBR Green Mix (Roche) was added to each PCR reaction along with cDNA and 1 pmol primer in a total volume of 10 μ l. The primers and conditions used to amplify the BRCA1 cDNA junction between exon 12 and 13 were previously described (109).

In situ hybridization. Sense and antisense riboprobes against HOXA9 were generated as previously described (83). Digoxigenin-labeled probes were prepared using T7 or SP6 RNA polymerase (Roche). Paraffin-embedded human breast tissue was hybridized with 800 ng/ml of probe as previously described (83). Six normal and four invasive ductal carcinoma human breast samples were examined.

Immunohistochemistry. Formalin-fixed, paraffin-embedded human breast tissue sections, lacking any patient-identifying information, were obtained with IRB approval from the University of Pennsylvania and the Huntsman Cancer Institute Tissue Resource and Acquisition Core Facility with patient consent. Sections were deparaffinized and rehydrated through 3 concentrations of alcohol and incubated in 3% H₂O₂ for 15 minutes to block endogenous peroxidase. Antigen retrieval was carried out in 0.1 M citrate buffer, pH 6.0, at 95°C for 20 minutes, followed by 20 minutes at room temperature. Nonspecific binding was blocked using PBS containing 1% BSA and 5% goat serum for 30 minutes. Sections were probed with anti-HOXA9 (1:200) and anti-BRCA1 antibodies. Biotinylated secondary

antibody and ABC reagent were used as directed (Vector Laboratories). Fast red (HOXA9) and DAB (BRCA1) were used as chromogens (Vector Laboratories). Sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Please refer to the supplemental method section for information regarding multispectral image acquisition and analysis. Six normal and four invasive ductal carcinoma samples were examined.

Bioinformatics analysis. Please refer to the Supplemental Methods section for a detailed description of the bioinformatics analysis.

Statistics. We used InStat software (Graphpad) to conduct the statistical analysis of our data. Unless otherwise stated, 2-tailed Student *t*-tests were used for simple significance testing, and 2-tailed Pearson tests were used for correlation analysis (mean \pm SEM of 3–5 independent experiments). *P* values of less than 0.05 were considered to be significant. Unless otherwise noted, *n* = 3.

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