Supporting Information

Hayashida et al. 10.1073/pnas.0912710107

SI Materials and Methods

Cell Culture. Culture conditions used for the maintenance of cell lines have been described previously (1). To generate HOXB9-expressing MCF10A clones, the HOXB9 expression plasmid was nucleofected into MCF10A cells using an Amaxa Biosystems nucleofector device according to the manufacturer's instructions. In brief, 1×10^6 MCF10A cells was harvested, washed with PBS, and resuspended in Cell Line Nucleofector Kit V (Amaxa Biosystems) with plasmid DNAs. Cells were nucleofected with 3 μg of the myc-tagged HOXB9 expression vector, which contains the gene for G418 resistance. After nucleofection, cells were cultured for 2 days before the selection medium containing 250 μg/mL of G418 was applied.

Western Blot Analysis. Expression of the myc-tagged HOXB9 construct was detected using the anti-myc antibody 9E10 (Santa Cruz Biotechnologies). Antibodies against E-cadherin (Zymed); vimentin and β-actin (BD); Smad2, p-Smad2, β-catenin, p-AKT, phospho Y1289 ErbB3, and total ErbB3 (Cell Signaling Technology); phospho Y1068 EGFR (ABCAM); fibronectin (Sigma); total EGFR (Santa Cruz Biotechnologies) and CD31 (ab28364; ABCAM) also were used for Western blot analyses. The Western blot protocols used have been described previously (2).

RNA Analysis. PCR to detect the expression of bFGF, VEGF, TGF-β1, TGF-β2, IL-8, ARG, ERG, NRG-1 and -2, and angiopoietin-like protein 2 was done using the following gene-specific primers:

HOXB9: sense, 5′ CCGGCTACGGGGACAATAA 3′; antisense, 5′ GGTGTAGGGACAGCGCTTTTT 3′
bFGF: sense, 5′ TTCTTCCTGCGCATCCAC 3′; antisense, 5′ CGGTTAGCACACACTCCTTTGAT 3′
TGF-β1: sense, 5′CAGCAACAATTCCTGGCGATA 3′; antisense, 5′AAGGCGAAAGCCCTCAATTT 3′
TGF-β2: sense, 5′ CTGATCCTGCATCTGGTCACG 3′; antisense, 5′TGGGGGACTGGTGAGCTTC 3′
ERG: sense, 5′CTGGGTTTCCATCTTCTACAGG 3′; antisense, 5′ CGTGGATTGTCTTCTGTCTGAACT 3′
ARG: sense, 5′ TACTCGGCTCAGGCCATTA 3′; antisense, 5′ GAAATCTCACTCCCTGAAGACATC 3′
NRG-1: sense, 5′ AAAGAAGGCAGAGGCAAAGGG 3′; antisense, 5′ TTCAATTGGGGAGGCAAGG 3′
NRG-2: sense, 5′ AACCGCAGCCGAGACATT 3′; antisense, 5′ TGCTCACGCTGTTGACGTAA 3′
IL-8: sense, 5′TTGGCAGCCTTCCTGATTT 3′; antisense, 5′ GGGTGGAAAGGTTTGGAGTAT 3′
Angiopoietin-like protein 2: sense, 5′ CACCGACCTCCCGTTAGC 3′; antisense, 5′GGCCACCTTGTGGAAGAGT 3′
VEGF: sense, 5′ AGAAGGAGGAGGGCAGAATC 3′; antisense, 5 TGG CTT GAA GAT GTA CTC GAT CTC 3′
GAPDH: Sense, 5′ ATCATCCCTGCCTCTACTGG 3′; antisense, 5′ TTTCTAGACGGCAGGTCAGGT 3′.

Primers specific for GAPDH were used as controls.

Conditions for semiquantitative amplification of cDNA were 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s, with a final extension cycle of 72°C for 10 min.

RT-PCR analysis was run on a LightCycler 480 Real-Time PCR System using SYBR Green 1 Master Mix (Roche). The following program was run: preincubation for 5 min at 95°C, amplification for 45 cycles (10 s of denaturation at 95°C, 10 s of annealing at 57°C, and a 10-s extension at 72°C), and melt-curve analysis. GAPDH served as an internal control, and LightCycler 480 Relative Quantification Software (Roche) was used for quantification.

Cell Migration and Invasion Assays. In vitro chemomigration and chemoinvasion assays were performed using a 24-well Transwell units with a polyethylene terephthalate membrane with a pore size of 8.0 μm (BD Falcon). For both invasion and migration assays, the lower side was coated with 10 μL of 500 μg/mL of the chemoattractant (type I collagen) and then air-dried. For invasion assays, the upper side of the membrane was coated with 100 μL of reconstituted matrigel (100 μg/mL) and air-dried overnight. The membrane was rehydrated for 3 h with DMEM at 37°C before use in the assays. To carry out the assay, 50,000 cells were resuspended in 500 μL of DMEM containing 0.1% FBS and placed in the upper well of the Transwell chamber. The lower compartment was filled with 600 μL of DMEM containing 0.1% FBS. After incubation for 24 h at 37°C, cells remaining on the upper surface of the filter were mechanically removed, and those migrating to the undersurface were stained with crystal violet and counted under a microscope. Nine fields were counted in each experiment.

Patients, Breast Carcinoma Samples, and HOXB9 Expression Analysis. The breast tumor samples used for analyzing HOXB9 expression by ISH and qPCR have been described previously (3, 4). The tumor samples for qPCR were obtained from 40 patients, 28 of whom had two or more pathological subtypes of breast cancer detectable at diagnosis, and each was accompanied by a patient-matched normal breast tissue sample. In brief, normal, ADH, DCIS, or IDC was laser capture microdissected in triplicate (from consecutive tissue sections). Patient-matched normal breast epithelium was microdissected from normal breast tissue. Malignant epithelial cells were microdissected from representative areas of the tumor.

These samples were analyzed for HOXB9 expression by qRT-PCR using the ABI 7900HT system (Applied Biosystems), as described previously (3). The relative standard curve method was used for linear regression analysis of unknown samples, and data are presented as fold change between samples. The sequences of the HOXB9 PCR primer pairs and fluorogenic MGB probe (5′ to 3′) were CGGTGGCTGTCGTGAAATT, CGAGACAATCACCC CCAAAG, and VIC-TGCTTGTGTTTCGTGATT, respectively.

The human breast carcinoma samples ($n = 20$) used for analyzing HOXB9 expression by ISH have been described previously (4).

In Situ Hybridization. The HOXB9 probe for ISH was generated as follows. The plasmid encoding HOXB9 was cleaved with HincII and XmaI (New England Biolabs). The acquired 286-bp fragment was outside the homeodomain and spanned nucleotides 26–324 of the HOXB9 transcript. This region had 100% homology to HOXB9, as expected, and had little homology with other members of the HOX9 family or other HOX genes (HOXA9, 34%; HOXC9, 35%; HOXD9, 33%; other HOX genes, 0); thus, it was unlikely to detect other HOX transcripts except HOXB9. This fragment was ligated into pBluescript II Phagemid Vector (Stratagene). After linearization of the plasmid, in vitro transcription was carried out, using 11-digoxigenin-UTP (Roche) and 2 units/μL of either T3 or T7 RNA polymerase (Promega) for sense and antisense probes, respectively. Riboprobes were purified by ethanol precipitation, and their size and integrity were checked by gel electrophoresis.

The tissues were dehydrated, washed twice with PBS, and treated with 3% hydrogen peroxide for 15 min. Following treatment with proteinase K (10 μg/mL) for 9 min, the tissues were washed with

PBS and then refixed with 4% paraformaldehyde in PBS for 5 min. The tissues were prehybridized for 1 h at 65°C before hybridizing with sense or antisense digoxigenin-labeled riboprobes overnight at 70°C. After hybridization, the samples were placed in 1% sheep serum for 30 min at room temperature, then incubated with anti-digoxygenin-AP (Roche) antibody for 2 h at room temperature. BM-Purple precipitation (Roche) was used to detect the signal.

Mouse Dorsal Air Sac Assay. The mouse dorsal air sac assay was performed as described previously (5). Both sides of the Plexiglas rings (diameter -14 mm; Millipore) were covered with the MF-Millipore membrane filter of 0.45-μm pore size. The diffusion chamber was filled with HOXB9- expressing MCF10A cells or shHOXB9-infected MDA-MB-231 cells (5×10^6 cells in 0.2 mL of PBS). Vector-MCF10A, shGFP-MDA-MB-231, and PBS alone were used as controls. The chamber containing the cells was implanted into an air sac formed previously in the dorsa of 4-week-old female Friend Virus B-type mice through injection of an appropriate volume of air. On day 5, the implanted chambers were removed from the s.c. fascia of the mice. Angiogenic response was assessed by images captured on a dissecting microscope. The number of newly formed blood vessels > 3 mm long within the area in direct contact with the chamber was recorded.

Tumorigenicity in Mice. Cells expressing activated H-Ras were generated by infecting vector or HOXB9-MCF10A with activated G12V H-Ras–expressing lentivirus containing a GFP cassette. MCF10A xenografts were established by injecting activated H-Ras and activated H-Ras plus HOXB9-expressing cells (5×10^6 in 50 μL of PBS) s.c. into the dorsal flanks of 6-week-old female Swiss nu/nu mice. Each group consisted of four mice. Tumor volumes were measured at regular intervals, and volume was calculated as length × width^2.

To measure the number of microvessels in vector- and HOXB9-positive activated H-Ras tumors, paraffin-embedded tissue sections were immunostained with an anti-CD31 antibody according to manufacturer's instructions and then counterstained with DAPI. Vessel count was assessed by fluorescence microscopy in areas of tumor containing the highest numbers of capillaries and small venules, as described previously (6). Highly vascular areas were first identified by scanning tumor sections, and vessel count was determined in six such areas at 200× magnification.

Knockdown of HOXB9 in MBA-MB-231 Cells. Lentiviruses carrying the HOXB9 target sequences 5′ CCCTTCAATTTGTAGACTCTT 3′ within 3′UTR and 5′ CTCCTCAATCTGAGTGAGAGA 3′ within the coding sequence were obtained from the RNAi Consortium shRNA Library (7), the Broad Institute. To knock down HOXB9 expression in MDA-MB-231 cells, viral constructs were generated by cotransfecting 3 g of lentiviral vector (Plko1), 3 μg of pCMV d8.91, and 3 μg of pHCMV-G into 1×10^6 293T human embryonic kidney cells using FuGENE 6 transfection reagent (Roche Applied Science). Supernatants were collected 48 h after transfection and filtered through a 0.45-μm membrane (Millipore). Then MDA-MB-231 cells were directly infected using 6 μg/mL of polybrene. After 5 days of incubation with growth medium containing 1 μg/mL of puromycin, selected cell pools were used for further experiments.

Chromatin Immunoprecipitation Assay. The HOXB9-expressing vector was constructed using the Virapower Lentiviral gene expression system (Invitrogen) according to the manufacturer's instructions. In brief, HOXB9 cDNA was amplified by PCR and cloned into the plenti6/V5-D-Topo vector (Invitrogen). This construct was transfected into the Lentiviral packaging cell line 293FT together with ViraPower packaging mix (Invitrogen) using Lipofectamine2000 transfection reagent (Invitrogen). Virus-containing culture medium was collected after 48 h and filtered through a 0.45-um syringe filter. The viruses were mixed with 10 ug/mL polybrene (Sigma-Aldrich) and used to infect MCF10A cells. For 48 h after infection, the cells were maintained in medium containing 10 ug/mL of blasticidin. The pLenti6/UbC/V5-GW/lacZ plasmid was used as a control. The expression of HOXB9 protein was detected using the anti-V5 antibody (Invitrogen). Before ChIP analysis, cells were characterized for EMT by evaluating the phenotype and by Western blot analysis for EMT markers, E-cadherin, and vimentin.

Cells at 80% confluence were transduced with either LacZ or HOXB9, crosslinked with 1% formaldehyde for 10 min at room temperature, and quenched with formaldehyde containing 125 mM glycine. The cells were then washed twice with cold PBS and collected into 1 mL of cold lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.0) with protease inhibitors mixture]. The crosslinked chromatin was sonicated to yield DNA fragments of about 400–850 bp. Chromatin solution (400 μL) was diluted 10-fold with dilution buffer [1% Triton X, 150 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl (pH 8.0) with protease inhibitors mixture], and incubated at 4°C overnight with 4 μg of either anti-V5 monoclonal antibody or control IgG. The antibody complexes were incubated with protein G Sepharose beads preabsorbed with 100 μg/mL of BSA and 500 μg/mL of sheared salmon sperm DNA and rotated for 2 h at 4°C. After four washes, the beads were eluted with 1% SDS/100 mM NaHCO3. Following both RNaseA and proteinase K treatment, and reverse crosslinking, DNA was reprecipitated and analyzed by PCR using the primers listed below.

The following primers were used for the detection of promoter regions bound by the HOXB9 protein in ChIP assays:

ARG: sense, 5′ TCGGCTGTGAGATGGTGTAG; antisense, 5′ CCGGAGCTAGTACCTGACAGAAGT
ERG: sense, CTGGACATTAAAGGGCCTTAGA; antisense, 5′ TTGTCGTTTATCTTCTCCCTAGC
NRG-1: sense, 5′ CCCGACCACAACACAAGAGTA; antisense, 5′ TGAAACCTACTCCTTCCCTTGTC
NRG-2: sense, 5′ GGCCTTGGTGATTAATTGGATG; antisense, 5′ TGGTCCAAGGATGAGTGACTTAGG
TGF-β2 region 1: sense, 5′ GGAAAGGGTGGGAGTCCAA; antisense, 5′ TTGCTCCAAACGCCAACC
TGF-β2 region 2: sense, 5′ CTTACTCGCCAAAGTCAGGGTT; antisense, 5′ TGACCAGATGCAGGATCAGA.

1. Kawakubo H, et al. (2004) Expression of the NF-kappaB–responsive gene BTG2 is aberrantly regulated in breast cancer. *Oncogene* 23:8310–8319.
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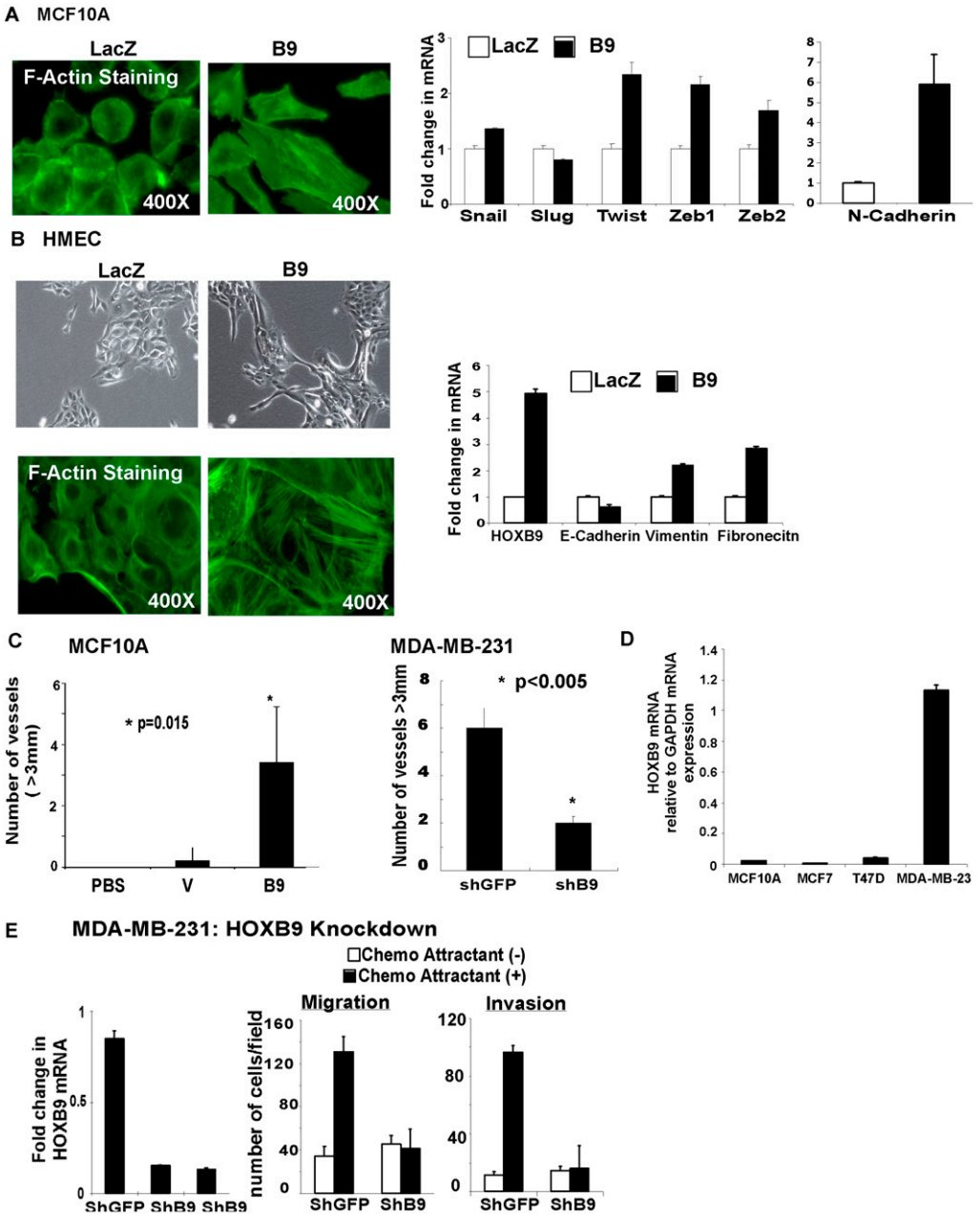

Fig. S1. (*A*) F-actin staining (*Left*) and expression analysis of EMT markers (*Right*), Snail, Slug, Zeb1, Zeb2, Twist, and N-cadherin in MCF10A cells infected with LacZ and HOX9 (B9). (*B*) HMECs were infected with lentiviral constructs expressing LacZ or HOXB9 (B9). (*Left*) Light micrographs and F-actin staining of cells. (*Right*) RNA from infected cells was analyzed by qPCR to detect the expression of HOXB9 and EMT markers, E-cadherin, vimentin, and fibronectin. (*C*) HOXB9 expression Induces angiogenesis in vivo. Angiogenic potential of MCF10A cells expressing vector (V) and HOXB9 (B9) (*Left*), and MDA-MB-231 cells infected with shGFP and shHOXB9 (shB9; *Right*) were assayed using mouse dorsal air sac assays. Assay chambers contained PBS alone or the cells shown. Graphs show the mean number of newly formed vessels larger than 3 mm counted in each animal per experimental group. Each group contained five animals. (*D*) HOXB9 mRNA expression in the cells lines shown was determined by qPCR. (*E*) (*Left*) Quantification of HOXB9 mRNA in MDA-MB-231 cells infected with shGFP- and two different shHOXB9-lentiral constructs that target different regions of HOXB9. (*Right*) Migration and invasion of shGFP- and shHOXB9-infected MDA-MB-231 cells in the presence and absence of chemoattractant. The mean was derived from cell counts of nine fields.

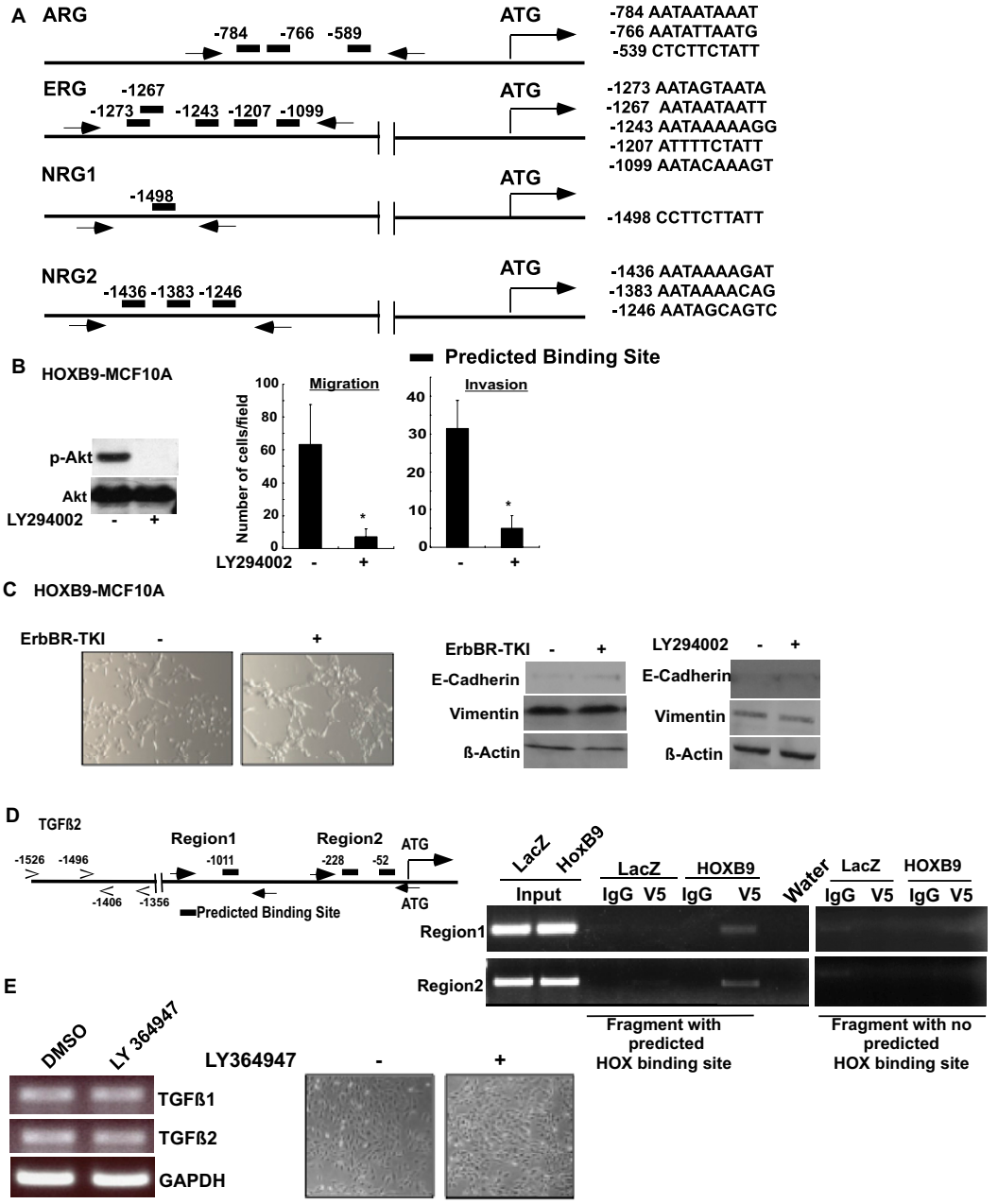

Fig. S2. (*A*) Line diagram representing the putative HOX-binding sites within each promoter. The positions of the primers used for PCR amplification and the sequence of each HOX-binding site are shown. (*B*) Suppression of Akt phosphorylation is associated with abrogation of cell migration. (*Left*) HOXB9-MCF10A cells were treated with LY294002 (10 nM) for 72 h and total proteins were analyzed for phospho-Akt and total Akt expression. (*Right*) HOXB9-MCF10A cells were treated with LY294002 for 72 h, and migration and invasion of the cells were assayed using Transwell units. (*C*) (*Right*) HOXB9-MCF10A cells were treated with 10 μM ErbR-TKI or 10 nM LY294002 for 72 h, and total proteins were analyzed for E-cadherin, vimentin, and β-actin expression. (Left) Phenotype of untreated and ErbBR-TKI–treated cells. (*D*) TGF-β2 is a target of HOXB9. MCF10A cells infected with LacZ and V5-tagged HOXB9 lentiviruses were analyzed by ChIP with anti-V5 and control IgG antibodies. Total lysates were used as controls for input. Precipitated DNA was subjected to PCR using primers spanning the promoter region containing the putative HOX-binding sites. A line diagram representing the putative HOX-binding sites within the TGF-β2 promoter and the positions of the primers used for PCR amplification of the DNA fragments with (closed arrows) and without (arrowheads) HOX sites are shown. (*E*) (*Left*) HOXB9-MCF10A cells were treated with LY364947 for 24 h, and RNA was analyzed for TGF-β1 and TGF-β2 expression. (*Right*) Morphology of HOXB9-MCF10A cells untreated and treated with LY364947 for 72 h.

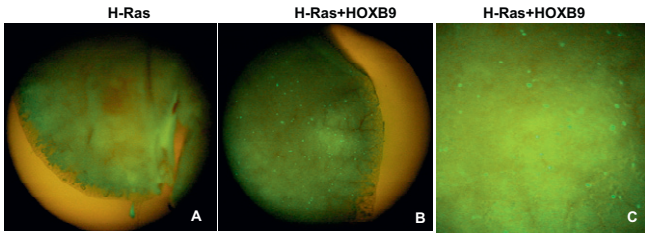

Fig. S3. HOXB9 promotes lung metastasis. (*A* and *B*) Lungs resected from mice 4 weeks after being inoculated s.c. with H-Ras and H-Ras+HOXB9 cells, respectively, were spread on a plastic dish and viewed under a dissecting microscope. (*C*) Higher-magnification image of *B* showing the presence of the several GFP-positive tumor nodules in the H-Ras+HOXB9 tumor-bearing mice.

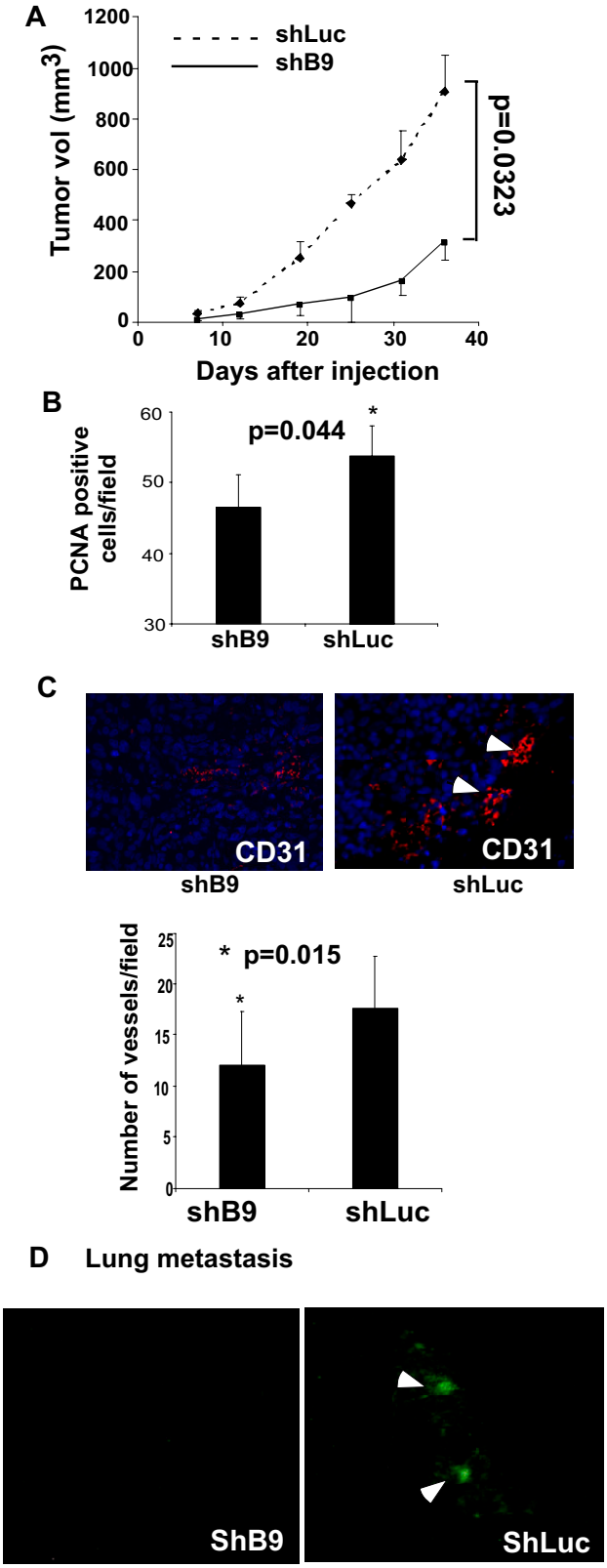

Fig. S4. Knockdown of HOXB9 expression suppresses tumor growth, vascularity, and metastasis to the lung. (*A*) GFP-expressing MDA-MB-231 cells infected with shLuc and shHOXB9 were inoculated s.c. into mice ($n = 4$ for each group); mean change in tumor volume ± SD for each group is shown. (*B*) Tumors from the two groups were stained for PCNA. The mean number of PCNA positive cells ± SD per field is shown ($n = 10$ fields). (*C*) Knockdown of HOXB9 decreases vascularization. Vessels are indicated with arrowheads. Quantification of vessels following CD31 staining is shown (lower panel). (Original magnification 200×) (*D*) Loss of HOXB9 suppresses lung metastasis. The lungs of shLuc-MDA-MB-231 tumor-bearing mice demonstrate micrometastases (50%), whereas animals bearing shHOXB9-MDA-MB-231 tumors had no lung metastasis (0%; $P = .038$ by Fisher's exact test). The GFP-expressing cell clusters in the lung were visualized under a dissecting microscope. The lower panel shows an image of the micrometastatic nodules in the lung. (Original magnification 40×)

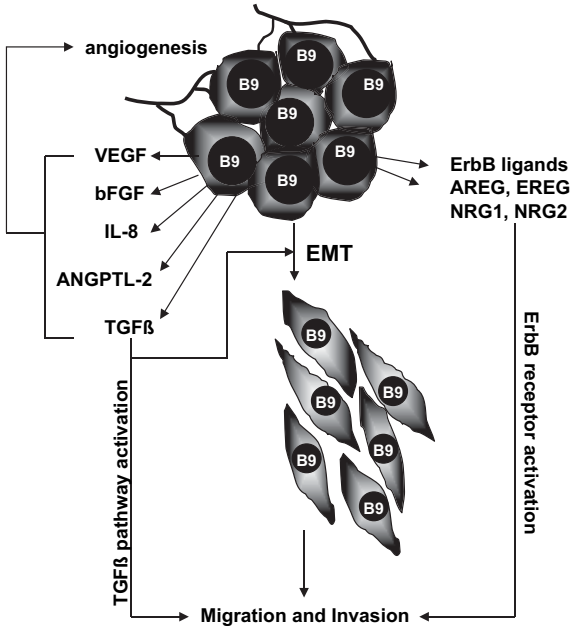

Fig. S5. Model depicting the consequence of tumoral overexpression of HOXB9 on microenvironment and disease progression. HOXB9 induces the expression of angiogenic factors, TGF-β and ErbB ligands, which promote EMT (TGF-β), angiogenesis (VEGF, bFGF, IL-8, ANGPTL-2, TGF-β), and migration and invasion (TGF-β and ErbB ligands) to favor tumor progression.

Table S1. Clinicopathological features of the cohort of 40 breast cancer patients used for quantification of *HOXB9* mRNA

Clinical parameter	Total number of patients (%) (n = 40)	*HOXB9* T > U
Estrogen receptor		
ER+	21 (52.5%)	7 (33%)
ER-	5 (12.5%)	4 (20%)
NA	14 (35%)	4 (28%)
Progesterone receptor		
PR+	20 (50%)	6 (30%)
PR-	6 (15%)	5 (83%)
NA	14 (35%)	6 (43%)
Histological grade		
G1	13 (32.5%)	3 (23%)
G2	16 (40%)	5 (32%)
G3	11 (27.5%)	8 (73%)
Her2		
Positive	6 (15%)	4 (67%)
Negative	16 (40%)	7 (44%)
ND	4 (10%)	2 (50%)
NA	14 (35%)	5 (36%)

ER, estrogen receptor; NA, not available; ND, not determined; PR, progesterone receptor; T, tumor; U, uninvolved epithelial cells.