Sustained Expression of Homeobox D10 Inhibits Angiogenesis

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Homeobox (Hox) genes are master regulatory genes that direct organogenesis and maintain differentiated tissue function. As HoxD3 and HoxB3 promote angiogenesis, we investigated whether endothelial cells use other Hox genes to maintain a mature quiescent phenotype. HoxD10 expression was higher in quiescent as compared to tumor-associated angiogenic endothelium. Microarray analysis of HoxD10-overexpressing endothelial cells revealed a pattern of gene expression consistent with a nonangiogenic phenotype. Moreover, sustained expression of HoxD10 impaired endothelial cell migration and blocked angiogenesis induced by basic fibroblast growth factor and vascular endothelial growth factor in the chick chorioallantoic membrane *in vivo*. HoxD10-overexpressing human endothelial cells also failed to form new vessels when implanted into immunocompromised mice. These results indicate a role for HoxD10 in maintaining a nonangiogenic state in the endothelium. (Am J Pathol 2002, 161:2099–2109)

The development of new blood vessels from pre-existing blood vessels (angiogenesis) is essential for normal tissue repair. However, neovascularization also contributes to the growth and dissemination of solid tumors and thus interfering with tumor angiogenesis presents much promise as a means to limit tumor growth and metastasis.^{1,2} Tumor-derived stimuli induce quiescent endothelial cells (ECs) to re-enter the cell cycle, express extracellular matrix-degrading proteinases, and up-regulate expression of adhesion molecules to allow migration.^{3–5} Vascular sprouts then resynthesize basement membranes (BMs), undergo capillary morphogenesis and withdraw from the cell cycle, and form mature quiescent vessels.

To understand the complex temporal and spatial regulation of expression of proteinases, adhesion molecules, and extracellular matrix molecules during angiogenesis, we have been investigating the role of Homeobox (Hox) master regulatory genes. In addition to their roles in em-

bryogenesis, Hox genes are also expressed in adult cells, including the vascular endothelium, and regulate expression of genes involved in cell-cell, cell-extracellular matrix interactions, and cell proliferation.^{6–11} Previously we showed that HoxD3 induces an angiogenic phenotype and promotes EC migration and invasion via up-regulation of $\alpha v\beta 3$ integrin and urokinase plasminogen activator (uPA) and that HoxB3 contributes to angiogenesis by increasing expression of ephrin A1 that facilitates capillary morphogenesis.^{9,12}

The 40 class I vertebrate Hox genes are clustered in four linkage groups (A to D), on four different chromosomes with both the proangiogenic HoxD3 and HoxB3 being located toward the 3' end of these Hox gene clusters. During embryogenesis, 3' Hox gene expression is followed by the sequential activation of more 5' Hox genes, giving rise to nonoverlapping boundaries of expression.¹³ A similar 3' to 5' wave of Hox gene expression is also observed when adult hematopoietic progenitor cells are induced to differentiate,¹⁴ while maturing cells attenuate expression of 3' Hox genes and begin to express high levels of 5' Hox genes such as HoxA10.^{15,16} Together these observations suggest that 5' and 3' Hox genes control different aspects of cell or tissue phenotype.

As the majority of adult ECs exist in a quiescent state, we reasoned that after angiogenesis maturing capillaries would begin to express 5' Hox genes, which in turn may help to maintain a quiescent, differentiated phenotype. Furthermore, we investigated whether sustaining expression of 5' Hox genes in an angiogenic environment could prevent acquisition of an angiogenic phenotype.

Materials and Methods

Cells, Transfections, and RNA Isolation

Immortalized human dermal microvascular ECs HMEC-1 were a gift from T. Lawley, Emory University, Atlanta, GA.^{17,18} Cells were maintained and cultured on BMs as previously described.⁹ Primary human dermal microvascular ECs were purchased from BioWhittaker (San Diego,

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CA). Recombinant human vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were from R&D Systems (Minneapolis, MN) and Matrigel was obtained from Becton Dickinson (Franklin Lakes, NJ). A polyclonal antibody against HoxD10 (Babco, Richmond, CA) was used at a dilution of 1:100 for Western blotting. A monoclonal antibody against cyclin D1 (sc-246; Santa Cruz Biotechnology, Santa Cruz, CA) was used at dilution of 1:200 for Western blotting. A polyclonal, function blocking antibody against human uPAR (number 399R; American Diagnostica, Greenwich, CT), and used at a dilution of 1:2000 for Western blotting. HMEC-1 cells were transfected using Effectene (Qiagen, Valencia, CA) and pools of stable transfectants selected with 35 μ g/ml of G418 (Life Technologies, Gaithersburg, MD). A total of at least eight different pools of stably transfected cells were used in the various studies. Total RNA isolation and Northern blot analysis was performed as previously described.^{9,12} BrdU-labeling experiments were performed as previously described⁹ on two different pools of HMEC-1 stably transfected with either HoxD10 or control plasmid.

A 1.2-kb cDNA probe for human PAI-1 and a probe for β 4 integrin were purchased from the American Type Culture Collection, Rockville, MD, and probes for MMP-14, RhoC, and α 3 integrin were generated by polymerase chain reaction (PCR) using specific primers (α 3 integrin forward: 5' CAA GTG GCT GCT GTA TCC CAC G 3', reverse: 5' CCA TCC GTG ATG CAC CAG CAC 3'; RhoC forward: 5' TCC TCA TCG TCT TCA GCA AG 3', reverse: 5' GAG GAT GAC ATC AGT GTC CG 3'). PCR products were cloned into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing at the Biomolecular Resource Center, University of California at San Francisco, San Francisco, CA.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Measurement of HoxD10 and Construction of Expression Vectors

One μ g of total RNA was reverse-transcribed using MMLV RT (Life Technologies) and 1/25 of this reaction was linearly amplified for 30 cycles at 95°C, 58°C, and 72°C using *Taq* polymerase (Qiagen) with the following primers: forward, 5' CTGTCATGCTCCAGCTCAACCC 3'; reverse, 5' CTAA-GAAAACGTGAGGTTGGCGGTC 3' corresponding to nucleotides 557 to 578 and 1032 to 1056, respectively, of human HoxD10 (GenBank accession no. X59373). Total RNA was normalized using alternative 18s internal standards at a 2:8 ratio (Ambion, Austin, TX). The entire HoxD10 cDNA was isolated using the forward primer, 5' TCAAAT-TCTTTCCCCAAAATGTCC 3' corresponding to nucleotides 16 to 39 and the reverse primer described above. The 1040-bp product was ligated into the TOPO II TA vector (Invitrogen, San Diego, CA) and the identity confirmed by sequencing using the USB Sequenase2 kit (Amersham-Pharmacia, Piscataway, NJ). HoxD10 expression vectors were constructed by ligating an *Eco*RI fragment into the pcDNA expression vector (Invitrogen). To produce the C-terminal myc fusion protein, the stop codon of HoxD10 was mutated to an *Xba*I site using the primer (5' AATTCGC-

CCTCTAGAAAACGTGA 3') and the resulting cDNA was inserted in-frame into the *Hind*III and *Xba*I sites of PCR 3.1 myc/his vector A (Invitrogen). The sequence of the HoxD10 fusion protein was confirmed using an ABI 3700 DNA sequencer at the Biomolecular Resource Center, University of California at San Francisco. RT-PCRs for HoxD3 and HoxB3 were performed as previously described.^{9,12}

Transwell and Microcarrier Migration Assay

Endothelial cell migration was assessed using a modification of the method described in Myers and colleagues.¹² Briefly, 6.5-mm Transwell chambers with 8- μ m pores were coated with 20 μ g/ml of type I collagen for 2 hours. Wells were rinsed in fibroblast basal medium (Clonetics, San Diego, CA) containing 0.5% bovine serum albumin. Control- or HoxD10-transfected HMEC-1 were serum-starved for 18 hours and 50,000 cells/well were added to each well for a total of 3 hours. At this time nonmigratory cells in the upper chamber were removed with a cotton swab and cells that migrated to the bottom of the membrane were visualized by fixing and staining with Diff-Quick (VWR Scientific Products, West Chester, PA). The total number of migrating cells was determined by counting five fields in each well per experimental condition. Each determination represents the average of eight individual wells and error bars represent the SD.

For three-dimensional migration assays, HMEC-1 were cultured on Cytodex-3 gelatin-coated microcarrier beads (Amersham-Pharmacia) and embedded into three-dimensional fibrin gels with or without 50 ng/ml of recombinant human VEGF or bFGF.¹⁹ Media containing 2% fetal calf serum with or without cytokine was changed every 48 hours. When indicated, 25 μ g/ml of control IgG or function-blocking antibodies against human uPAR (number 399R, American Diagnostica) were added to the gels before clotting and fresh antibody was added to the culture media every 48 hours. Cells were visualized using a Nikon TE300 inverted microscope (Nikon, Melville, NY) and migration assessed by measuring the distance the cell processes radiated from the beads.

Immunoprecipitation of the VEGF Receptor

Total VEGF receptor 2 (KDR) was measured by immunoprecipitation using a polyclonal antibody against human VEGF receptor 2 (Flk-1, Santa Cruz Biotechnology). Control- and HoxD10-transfected HMEC-1 were cultured overnight in MCDB131 media containing 2% fetal calf serum and subsequently treated with 100 ng/ml of recombinant human VEGF (R&D Systems) and lysed 30 minutes later in ice-cold 10 mmol/L Tris-HCl/150 mmol/L NaCl in the presence of 2 mmol/L of NaF and 2 mmol/L of sodium orthovanadate. A total of 300 μ g of cell lysate diluted in RIPA and immune complexes were precipitated using 25 μ l of 10% w/v Protein A-Sepharose. Pellets were washed extensively in phosphate-buffered saline and resuspended in 2 $\times$ Laemmli sample buffer and separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride

Figure 1. Expression of HoxD10 in ECs. **a:** *In situ* hybridization of HoxD10 in human breast tissue. Positive expression (purple/black) is seen in capillaries within breast stromal connective tissue, which is counterstained green. **b:** *In situ* hybridization of HoxD10 in high-grade ductal carcinoma *in situ* (DCIS). HoxD10 is expressed in breast epithelium, but absent in angiogenic microvessels surrounding the lesion (arrows). **c:** *In situ* hybridization of HoxD3 in a serial section of **b**. Strong positive signals (purple/black) are detected in both breast cells and adjacent angiogenic microvessels. Original magnifications: $\times 20$ (**a**); $\times 40$ (**b** and **c**).

membranes. Blots were probed with antibodies against VEGFR2 (Flk-1) or total phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY) and visualized using enhanced chemiluminescence (ECL, Amersham).

In Situ Hybridization

Sense or anti-sense riboprobes against HoxD10 were generated using a 499-bp 3' fragment of HoxD10 subcloned into the Topo II vector. Digoxigenin-labeled probes were prepared using T7 or Sp6 RNA polymerase (Roche, Indianapolis, IN). Seven- μ m sections of paraffin-embedded human breast tissue were hybridized with 800 ng/ml of probe

as previously described.²⁰ A minimum of eight high-grade ductal carcinoma or invasive ductal carcinoma human breast tumor specimens were examined.

DNA Microarray

Total RNA was isolated from 5×10^7 control- or HoxD10-transfected cells using the Atlas pure total RNA kit and Poly A+ mRNA was purified and reverse-transcribed in a reaction mix containing [³²P]-dATP and hybridized to the cell-interaction array membranes according to the manufacturer's instructions (Clontech, Palo Alto, CA). Membranes were exposed to Kodak Biomax MS film (East-

Figure 2. HoxD10 impairs EC migration. **a:** Relative levels of HoxD10 mRNA and protein in control and HoxD10-transfected HMEC-1. **Top:** RT-PCR and the relative amounts of the 499-bp product corresponding to HoxD10 mRNA. The relative RNA loading is shown by the 390-bp band corresponding to 18S ribosomal RNA determined using competitive primers. **Bottom:** Western blotting of control and HoxD10-transfected HMEC-1 lysates probed with a polyclonal antibody against HoxD10. The arrow indicates the position of the HoxD10 protein. **b:** Relative migration of control or HoxD10-transfected HMEC-1 on membranes coated with 10 μ g of collagen. Migration was assessed after 3 hours in the presence of 50 ng/ml of bFGF (▨) or 50 ng/ml VEGF (■) or with no treatment (□). Each panel presents the mean of eight individual determinations and error bars show the SD. **, $P < 0.05$. **c:** Western blot showing levels of the 125-kd VEGF receptor 2 (**top**) and total phosphotyrosine content of the immunoprecipitated VEGF receptor 2 (**bottom**) from control- or HoxD10-transfected HMEC-1 in the presence (+) or absence (-) of 100 ng/ml of VEGF for 30 minutes.

Figure 3. Changes in gene expression and migration in three-dimensional fibrin gels. **a: Far left:** migration of control-transfected HMEC-1 in three-dimensional fibrin gels without cytokine treatment (control). The next panel shows control-transfected HMEC-1 treated with 50 ng of bFGF (bFGF) or after the addition of 50 ng of bFGF and 25 μ g of a function-blocking antibody against uPAR (bFGF+anti-uPAR). **Far right:** Relative migration of HoxD10-transfected HMEC-1 embedded within fibrin gels 48 hours after exposure to bFGF only (bFGF+HoxD10). **b: Top:** Northern blot analysis of uPAR mRNA expression in control- and HoxD10-expressing HMEC-1. **Middle:** Relative loading as visualized by staining of ribosomal RNA (rRNA) with ethidium bromide. **Bottom:** Western blot analysis for uPAR protein expression in whole cell lysates from control- or HoxD10-transfected HMEC-1. **c: Top:** Relative levels of HoxD3 (top) or HoxB3 (middle) in control- or HoxD10-transfected HMEC-1 as determined by RT-PCR. Total mRNA for each sample was determined by RT-PCR using competitive primers against 18s ribosomal RNA in each sample (bottom). **d: Top:** Northern blot analysis of β 3 integrin (top) and ephrin A1 (middle) steady-state mRNA levels in control- and HoxD10-transfected HMEC-1. Total RNA in each sample is visualized by staining of ribosomal RNA with ethidium bromide (bottom). Original magnification, $\times 20$ (a).

man-Kodak, Rochester, NY) and photographed using an Alpha Innotech (San Leandro, CA) 4000 imager and analyzed using AlphaEase db 3.3 software. Microarray analysis was performed on two separate pools of HMEC-1 cells stably transfected with either HoxD10 or control cDNA.

Introduction of HoxD10 and Angiogenesis in the Chick Chorioallantoic Membrane

Pellets containing 0.5% methylcellulose plus 50 ng of recombinant human VEGF or bFGF were placed onto the chick chorioallantoic membrane (CAM) of 10-day-old chick pathogen-free embryos (SPAFAS; Charles River Laboratories, Wilmington, MA). Twenty-five μ g of control (CMV β gal) or HoxD10 expression plasmid in a volume of 50 μ l was applied to the site of angiogenic stimuli 24 hours later (day 11). CAMs were harvested after an additional 48 hours (day 13) and photographed using a Kodak MDS120 camera attached to a Nikon SMX-2T stereomicroscope. Angiogenesis was quantitated by counting branch points arising from tertiary vessels from a minimum of 12 specimens from three separate exper-

iments. Statistical significance was assessed using an unpaired Student's *t*-test.

Transplantation of HMEC-1 in Nude Mice

To determine whether HoxD10 directly impacts the ability of HMEC-1 to form vessels *in vivo*, we adapted the method of Nor and colleagues.²¹ Briefly, 1×10^6 control- or HoxD10-transfected HMEC-1 were mixed with and equal volume of Matrigel (Becton Dickinson) and seeded onto 10-mm PVA sponges (Ivalon, Eudora, KS). Nude mice (nu/nu, Charles River Laboratories) were anesthetized with ketamine and xylazine and sponges were implanted subcutaneously into the left and right flank of each mouse. After 10 days, mice were sacrificed and the implants retrieved, photographed, and fixed in formalin and subsequently embedded in paraffin. Seven- μ m sections were stained with a 1:50 dilution of monoclonal antibody against human CD34 (Neomarkers, Fremont, CA) and color developed using NovaRed (Vector Laboratories, Burlingame, CA). A minimum of six sponges containing either control- or HoxD10-transfected HMEC-1 were analyzed and the number of microvessels per $\times 20$

Figure 4. Microarray analysis of HoxD10-mediated changes in gene expression in ECs. **a:** Radiographical image of cell-interaction microarray hybridized with [³²P]-dATP-labeled mRNA from HoxD10-transfected HMEC-1 (left) or control-transfected HMEC-1 (right). **b:** Northern blot analysis of 10 µg of total RNA harvested from control- or HoxD10-transfected HMEC-1. **Top:** mRNA levels of PAI-1 in control- and HoxD10-transfected cells. The blot was stripped and reprobed for expression of RhoC (**middle**). **Bottom:** Relative loading of RNA as visualized by ethidium bromide staining of ribosomal RNA. **c:** Northern blot analysis of 10 µg of total RNA harvested from control- or HoxD10-transfected HMEC-1 and probed for MMP-14 (**top**). **Bottom:** Relative loading of RNA as visualized by ethidium bromide staining of ribosomal RNA. **d:** Northern blot analysis of β4 integrin mRNA levels in 10 µg of RNA harvested from control- or HoxD10-transfected HMEC-1 (**top**). Relative RNA loading is shown by ethidium bromide staining of ribosomal RNA (**middle**). **Bottom:** Levels of α3 integrin mRNA and relative levels of 18s RNA as determined by RT-PCR in RNA harvested from control- or HoxD10-transfected HMEC-1.

powered field were counted as described²¹ and statistical significance determined using Student's *t*-test.

Tissue Fixation and Immunofluorescence

CAMs were fixed and stained for von Willebrand factor as described.¹² C-myc staining was performed using 5 µg/ml of a monoclonal antibody, clone 9E10 (Oncogene Science, Cambridge, MA) followed by a 1:200 dilution of biotinylated goat anti-mouse IgG (Vector Laboratories) and a 1:100 dilution of streptavidin-fluorescein isothiocyanate (Amersham-Pharmacia).

Results

HoxD10 Is Expressed in Quiescent, Nonangiogenic Endothelial Cells

HoxD10 was identified in an initial screen of primary human ECs using degenerate primers against class I Hox genes.⁹ To evaluate expression in ECs *in vivo* we performed *in situ* hybridization on specimens of human breast tissue. We observed strong expression of HoxD10

in microvessels of connective tissue adjacent to normal breast epithelium (Figure 1a). We also examined expression in angiogenic microvessels that form a characteristic rim around high-grade breast ductal carcinoma *in situ*.^{22,23} Although preinvasive breast cells expressed HoxD10, expression was markedly reduced in the neighboring angiogenic microvessels. (Figure 1b). In contrast, these angiogenic microvessels expressed high levels of the proangiogenic HoxD3 (Figure 1c).

Sustained Expression of HoxD10 Impairs Endothelial Cell Migration

To assess the role of HoxD10 in ECs, we stably transfected HMEC-1 with a CMV-driven human HoxD10 cDNA expression plasmid or control plasmid (CMV-luciferase). The relative levels of expression of HoxD10 mRNA and protein in control- and HoxD10-transfected cells are shown (Figure 2a). We did not observe any significant differences in cell growth rates as determined by BrdU incorporation in the HoxD10- or control-transfected cells ($68.6 \pm 10.25\%$ versus $67.8 \pm 13.9\%$, $n = 8$). We had previously observed that HoxD3, which shows the oppo-

Table 1. Cell-Cell Interaction Microarray Analysis of Changes in Gene Expression by HoxD10

Up-regulated by HoxD10		Suppressed by HoxD10	
Gene	Fold change	Gene	Fold change
PAI-1	(3.9)	MMP14	(2.6)
$\alpha 3$ catenin	(7.5)	ICAM-1	(2.0)
nm23H1	(18.3)	rhoB	(9.0)
Timp-1	(2.6)	rhoC	(16.0)
laminin 37 kd receptor	(30.2)	$\alpha 3$ integrin	(33.1)
		β integrin	(3.4)
		ezrin	(5.4)
		Timp-2	(2.4)
		MTA-1	(2.8)

Genes whose expression is either induced or suppressed at least twofold in HoxD10-transfected HMEC-1 as compared to control-transfected HMEC-1. The relative fold increase or decrease in expression is shown for each gene in parentheses.

site pattern of expression, promoted migration of ECs.⁹ We therefore assessed the influence of HoxD10 on EC migration in modified Boyden chambers coated with type I collagen (Figure 2b). Although control cells showed a significantly increased degree of migration in the presence of either bFGF or VEGF, migration of HoxD10-transfected ECs was not increased in response to these angiogenic cytokines. To determine whether the lack of response to VEGF was because of alterations in expression or activation of VEGF receptors, we immunoprecipitated the VEGFR2 receptor from equal numbers of control- or HoxD10-transfected HMEC-1 stimulated with VEGF. Subsequent immunoblotting with anti-phosphotyrosine antibodies showed no significant difference in activation of this receptor by VEGF in control- or HoxD10-transfected cells (Figure 2c).

Because ECs undergo migration in three-dimensional environments during angiogenesis *in vivo*, we also as-

sessed the impact of HoxD10 on migration in three-dimensional fibrin gels in response to bFGF. Although control-transfected ECs exhibit limited migration in the absence of cytokine, migration into the fibrin gels is markedly increased in response to bFGF (Figure 3a; control, bFGF). Similar to other three-dimensional fibrin gel models, bFGF-induced migration of ECs could be blocked by the addition of a function-blocking antibody against uPAR (Figure 3a, bFGF+anti-uPAR).²⁴ Transfection of ECs with HoxD10 also completely blocked migration into fibrin gels in the presence of bFGF (Figure 3a, bFGF+HoxD10). We therefore investigated whether the inhibitory effects of HoxD10 on migration in these gels was because of a loss of either uPA or uPAR expression. Although the levels of uPA were not affected, we observed a marked suppression of both uPAR mRNA and protein (Figure 3b), consistent with the lack of migration by HoxD10-transfected cells in the three-dimensional fibrin gels.

We also investigated whether HoxD10 influenced the expression of either HoxD3 or HoxB3, which we had previously shown to have proangiogenic effects on HMEC-1.^{9,12} Semiquantitative RT-PCR analysis however showed that levels of HoxD3 and HoxB3 were similar in control- and HoxD10-transfected HMEC-1 (Figure 3c). In addition, Northern blot analysis confirmed that expression of either $\beta 3$ integrin or ephrin A1, downstream targets of either HoxD3 or HoxB3, respectively, were also not altered in cells expressing high levels of HoxD10 (Figure 3d).

HoxD10 Modulates Expression of Genes Required for Migration and Angiogenesis in Endothelial Cells

To further explore whether, in addition to uPAR, HoxD10 influenced expression of other genes involved in cell

Figure 5. Up-regulation of endogenous HoxD10 and related changes in gene expression. **a: Top:** relative levels of HoxD10 and 18s ribosomal RNA in HMEC-1 cultured in the presence (+) or absence (-) of reconstituted BM as determined by RT-PCR. Western blot analysis of HoxD10 protein levels (**middle**) or cyclin D1 levels (**bottom**) in 40 μ g of total cell lysates from HMEC-1 cultured for 24 hours in the absence (-) or presence (+) of reconstituted BM. **b: Top:** Northern blot analysis of $\alpha 3$ integrin (**top band**) and uPAR (**bottom band**) steady-state mRNA levels in 7.5 μ g of total RNA from cells cultured in the absence (-) or presence (+) of reconstituted BM. **Bottom:** Relative RNA loading for each sample as visualized by staining with ethidium bromide. **c:** Histogram shows relative levels of $\alpha 3$ integrin and uPAR mRNA levels in cells cultured in the absence (■) or presence (▨) of reconstituted BM as determined by densitometric analysis and correction for total RNA levels in each sample.

Figure 6. Gene transfer of HoxD10 in the chick CAM. Double-immunofluorescence staining of CAM tissues was performed 48 hours after treatment with VEGF and HoxD10. **a:** Staining for HoxD10/human *c-myc* fusion protein expression in VEGF and HoxD10 cDNA-treated CAM. **b:** Merged image of staining for HoxD10/myc fusion protein (green, shown in **a**) and DAPI nuclear staining (blue). **c:** Merged image showing co-localization (yellow) of the human *c-myc* tagged HoxD10 transgene (green) in vascular ECs stained for von Willebrand factor (red). Additional positive staining for the *c-myc*-tagged HoxD10 is also observed in adjacent fibroblasts. **d:** β -gal staining of CAM tissue treated with VEGF and cDNA encoding the lac z gene. The **arrow** shows the location of a small vessel. Scale bars, 25 μ m (**c** and **d**).

migration and angiogenesis, we screened a cDNA microarray containing 238 genes related to cell-cell and cell-extracellular matrix interactions with RNA from HoxD10- or control-transfected ECs (Figure 4a). After densitometric analysis, we identified several genes whose expression was modulated at least twofold by HoxD10 (Table 1). HoxD10 not only induced expression of several genes but also suppressed expression of a greater number of genes, including several required for cell migration and angiogenesis such as RhoC and MMP-14.^{24–28} Although we did not independently confirm protein expression levels or assess the contribution to migration by each of the genes identified in the microarray analysis, we did confirm that expression of PAI-1 was increased, whereas RhoC, β 4 integrin, and α 3 integrin mRNA and MMP-14 mRNA and protein levels were reduced in HoxD10-transfected ECs as compared to control-transfected ECs (Figure 4, b and c). Because many of these genes work in concert to promote migration and angiogenesis, HoxD10 may globally impair expression of a migratory or angiogenic phenotype.

Because our microarray expression analysis was performed on cells overexpressing HoxD10, we wished to establish a means to modulate levels of endogenous

HoxD10 and determine whether this impacted expression of the same genes in a similar direction. Previous studies have shown that human dermal microvascular ECs become quiescent within 24 hours after culturing on reconstituted BMs.^{9,29} We therefore compared expression of endogenous HoxD10 in either primary microvascular ECs or HMEC-1 cells cultured in the presence or absence of a reconstituted BM. RT-PCR and Western blot analysis showed that expression of HoxD10 mRNA and protein was higher in ECs made quiescent by culturing on a reconstituted BM as compared to ECs cultured in the absence of BM (Figure 5a). In contrast, expression of the cell cycle-related protein, cyclin D1, was markedly down-regulated in cells cultured on reconstituted BM when compared with cells cultured in the absence of BM (Figure 5a). Subsequent Northern blot analysis showed that increased levels of endogenous HoxD10 in quiescent ECs corresponded with a decrease in expression of α 3 integrin and uPAR (Figure 5, b and c). Similar results were also observed for β 4 integrin in that expression was also decreased when endogenous HoxD10 levels were increased by culturing on BM (not shown). Thus overexpressing HoxD10 or increasing levels of endogenous HoxD10 have the same impact on EC gene expression.

Figure 7. Gene transfer of HoxD10 impairs angiogenesis induced by bFGF. **a:** Whole mount showing lack of angiogenesis in 13-day CAM tissue after application of methylcellulose pellets lacking growth factors. **b:** Whole mount of 13-day CAM tissue showing extensive angiogenic branching induced by application of 100 ng of bFGF on day 10. **c:** Corresponding whole mount of 13-day CAM tissue after application of 100 ng of bFGF on day 10 and 25 μ g of control cDNA 24 hours later (day 11). **d:** Whole mount of 13-day CAM tissue 72 hours after application of 100 ng of bFGF (day 10) and the addition of 25 μ g of HoxD10 cDNA 24 hours later (day 11). **e:** Immunofluorescence staining with von Willebrand factor on CAM tissue treated with bFGF and control cDNA. **f:** Immunofluorescence staining with von Willebrand factor on CAM tissue treated with bFGF and HoxD10. Original magnifications: $\times 4$ (**a**, **c**, **d**). Scale bars, 20 μ m (**e**, **f**).

HoxD10 Inhibits Angiogenesis in Vivo

To directly test whether HoxD10 impairs angiogenesis *in vivo*, we first initiated angiogenesis in the CAM with human VEGF. We then directly applied plasmid DNA encoding a *c-myc*-tagged HoxD10 cDNA or CMV-LacZ cDNA (control) to the area containing the VEGF pellets 24 hours later. Immunofluorescence staining for the human *c-myc* epitope tagged HoxD10 (Figure 6a) revealed uptake and expression of the HoxD10 transgene in a variety of cells including fibroblasts and epithelia. Uptake and expression in ECs was confirmed by double-immunofluorescence staining with *c-myc* and the EC-specific marker von Willebrand factor (Figure 6b) and merged images revealed extensive co-localization (Figure 6c) in ECs of the CAM. β -gal staining was performed in tissues treated with CMV β gal (control) DNA and revealed a similar degree of expression and localization expression of this topically applied cDNA (Figure 6d).

Using this protocol, we observed that whereas bFGF induced a strong angiogenic response in the presence of control DNA (Figure 7c), application of HoxD10 cDNA

significantly reduced the angiogenic response to bFGF after 48 hours (Figure 7d). Quantitative analysis of angiogenic branch points revealed that this inhibition was greater than 60%, as compared to control (control, 289 ± 110 versus HoxD10, 106 ± 11.85 ; $P < 0.05$; $n = 12$). Similar results were obtained using VEGF as the stimulus (data not shown). Staining with the EC marker Von Willebrand factor also confirmed the decrease in vascular density in HoxD10-treated CAM as compared to control-treated CAM (Figure 7, e and f).

Because this method of gene delivery into the CAM also introduces HoxD10 into perivascular cells and fibroblasts, we wished to establish whether HoxD10 could directly impair the ability of ECs to migrate independent of any effects on other cell types. We therefore seeded control- or HoxD10-transfected HMEC-1 onto biodegradable PVA sponges and implanted the sponges into nude mice. After 10 days we harvested the sponges and observed that control HMEC-1 readily invaded mouse tissue adjacent to the sponges and formed complete functional vessels containing red blood cells, as determined by staining for the human-specific CD34 cell surface antigen (Figure 8, a and b). In contrast, significantly fewer human-derived vessels were detected in the areas adjacent to the sponges seeded with HoxD10-transfected HMEC-1 (Figure 8, c and d) as determined by counting the number of microvessels/high-powered field (control, 51 ± 24.54 versus HoxD10, 8 ± 4.24 ; $P < 0.05$; $n = 4$). These results indicate that HoxD10 can inhibit angiogenesis by directly modulating gene expression in the endothelium.

Discussion

We show that whereas HoxD10 is abundantly expressed in quiescent ECs, expression decreases in angiogenic vessels in the tumor microenvironment. Furthermore we show that sustained expression of HoxD10 can suppress angiogenesis *in vivo*. Taken together, our results suggests that HoxD10 contributes to maintenance of a quiescent, differentiated phenotype in ECs and that its expression must decrease for angiogenesis to proceed.

Our microarray data also suggests a molecular basis for the lack of EC migration and angiogenesis in the presence of HoxD10. Rather than interfering with the ability to perceive angiogenic stimuli, HoxD10 suppresses expression of genes that directly impact remodeling of the extracellular matrix and cell migration during angiogenesis. Specifically, HoxD10 reduced expression of urokinase plasminogen activator receptor (uPAR), the receptor for uPA that promotes EC migration²⁴ and MMP-14, a membrane-type metalloproteinase that, when complexed with TIMP-2, activates latent MMP-2²⁵ and in turn promotes EC migration and angiogenesis.²⁶ Furthermore, HoxD10 expression reduced the levels of the $\alpha 3$ and $\beta 4$ integrin subunits components of the heterodimeric $\alpha 3\beta 1$ and $\alpha 6\beta 4$ laminin receptors, respectively. Although these receptors mediate adhesion to BM laminin and direct morphogenesis and tissue-specific gene expression, both integrins also promote cell migration and invasion.^{27–30}

Figure 8. HoxD10 blocks angiogenesis of human ECs implanted into nu/nu mice. **a:** CD34 staining of a 7- μ m section of tissue adjacent to implanted PVA sponges seeded with HMEC-1 recovered from a nude mouse after 10 days. **Arrows** indicate the presence of small CD34-positive vessels. **b:** Higher magnification of **a**, showing microvessels consisting of CD34-positive human ECs surrounding red blood cells. **c:** CD34 staining of 7- μ m section of tissue adjacent to PVA sponge (S) seeded with HoxD10-transfected HMEC-1 and recovered from nude mice 10 days after implantation. **d:** Higher magnification of **c** showing little or no positive staining for human CD34-derived microvessels in tissues adjacent to the sponge. Original magnifications: $\times 10$ (**a**, **c**); $\times 40$ (**b**, **d**).

In our *in vivo* CAM studies, HoxD10 was also introduced into fibroblasts of the CAM. Although the net effects of HoxD10 on fibroblast gene expression were not assessed, HoxD10-transfected human fibroblasts do show reduced expression of MMP-14 (N Boudreau, unpublished observations). Thus it is possible that HoxD10 also interferes with fibroblast-mediated remodeling of the perivascular matrix during angiogenesis. However, using HoxD10-transfected HMEC-1 directly implanted into nude mice indicates that HoxD10 can directly influence the ability of ECs to undergo migration during angiogenesis.

Interestingly, many of the genes modulated by HoxD10 promote invasion, migration, and tumor progression in a variety of cells, and raises the possibility that HoxD10 functions as a general inhibitor of cell invasion. In addition to the genes previously mentioned, we observed a reduction in RhoC, which was recently identified as a critical determinant of tumor cell metastasis.³¹ HoxD10 also up-regulated expression of NM-23H1, a tumor suppressor gene that induces BM synthesis and growth ar-

rest in human breast tumor cells.³² Interestingly although both normal human breast epithelial cells and the noninvasive epithelium in ductal carcinoma *in situ* express abundant HoxD10, expression of HoxD10 is markedly diminished in the epithelium of highly invasive ductal carcinoma (N Boudreau, unpublished observations). Normal endometrial tissues also express high levels of HoxD10, while expression is lost in endometrial carcinoma.³³ It is worth noting that the paralogous HoxA10 gene induces growth arrest in myelomonocytic cells and thus might also function in an anti-tumorigenic or anti-angiogenic manner.¹⁶

Our results also provide evidence that 5' and 3' Hox genes have distinct influences on EC behavior and is consistent with the distinct roles predicted for 5' and 3' Hox genes based on their nonoverlapping expression patterns during development and with the increasing expression of 5' Hox genes in differentiating hematopoietic progenitor cells.^{14–16,34} Furthermore, when both 3' and 5' Hox genes are concurrently expressed in tissues,

the 5' Hox genes generally act in a dominant manner to determine phenotype.^{35,36} Our findings, that although HoxD10 did not influence the levels of HoxD3 or HoxB3 in HMEC-1 and yet angiogenesis was impaired by introducing HoxD10 into an angiogenic environment expressing HoxD3, supports the notion of posterior predominance by this or other 5' Hox genes. Whether HoxD10 directly competes with HoxD3 or other angiogenic Hox proteins for similar binding sites within the promoters of target genes is difficult to predict. Like other transcription factor consensus sites, the Hox recognition site (TNAT/C), occurs frequently throughout the genome (eg, 26 putative sites in 3.0 kb of the MMP-14 promoter alone) and thus binding to target promoters is thought to be determined by both chromatin structure and interactions with protein partners such as Pbx-1.^{37–39} Nonetheless, in cases of direct competition by different Hox proteins for binding to similar targets, transcriptional suppression was dominant over activation.⁴⁰ The relatively greater number of genes suppressed rather than activated by HoxD10 in our microarray analysis suggests a dominant suppressor role and further implies that expression or activity of HoxD10 must be reduced for angiogenesis to proceed. It would be of interest to determine how the angiogenic environment overcomes this dominant suppression.

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References

- Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995, 1:27–31
- Conway EM, Collen D, Carmeliet P: Molecular mechanisms of blood vessel growth. *Cardiovasc Res* 2001, 49:507–521
- Brooks PC, Montgomery AMP, Rosenfeld M, Riesfeld RA, Hu T, Klier G, Cheresh DA: Integrin $\alpha v \beta 3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994, 79:1157–1164
- Eliceiri BP, Cheresh DA: The role of alpha v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J Clin Invest* 1999, 103:1227–1230
- Werb Z, Vu TH, Rinkenberger JL, Coussens LM: Matrix-degrading proteases and angiogenesis during development and tumor formation. *APMIS* 1999, 107:11–18
- Botas J: Control of morphogenesis and differentiation by Hox/Hox genes. *Curr Opin Cell Biol* 1993, 5:1015–1022
- Edelman GM, Jones FS: Outside and downstream of the homeobox. *J Biol Chem* 1993, 268:20683–20686
- Boudreau N, Bissell MJ: Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr Opin Cell Biol* 1998, 10:640–646
- Boudreau N, Andrews C, Srebrow A, Ravanpay A, Cheresh DA: Induction of the angiogenic phenotype by Hox D3. *J Cell Biol* 1997, 139:257–264
- Belotti D, Clausse N, Flagiello D, Alami Y, Daukandt M, Deroanne C, Malfoy B, Boncinelli E, Faiella A, Castronovo V: Expression and modulation of homeobox genes from cluster B in endothelial cells. *Lab Invest* 1998, 78:1291–1299
- Patel CV, Sharangpani R, Bandyopadhyay S, DiCorleto PE: Endothelial cells express a novel, tumor necrosis factor-alpha-regulated variant of HOXA9. *J Biol Chem* 1999, 274:1415–1422
- Myers CA, Charboneau A, Boudreau N: Hox B3 promotes capillary morphogenesis and angiogenesis. *J Cell Biol* 2000, 148:343–352
- Duboule D: Vertebrate hox gene regulation: clustering and/or colinearity? *Curr Opin Genet Dev* 1998, 8:514–518
- Van Oostveen JW, Bijl JJ, Walboomers JLM, Meijer CJLM: The role of homeobox genes in normal hematopoiesis and hematological malignancies. *Leukemia* 1999, 13:1675–1690
- Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C, Lawrence HJ, Humphries RK: Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci USA* 1994, 91:12223–12227
- Bromleigh VC, Freedman LP: p21 is a transcriptional target of HoxA10 in differentiating myelomonocytic cells. *Genes Dev* 2000, 14:2581–2586
- Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ: HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992, 99:683–690
- Xu Y, Swerlick RA, Sepp N, Bosse D, Ades EA, Lawley TJ: Characterization of expression and modulation of cell adhesion molecules on an immortalized dermal microvascular endothelial cell line (HMEC-1). *J Invest Dermatol* 1994, 102:833–837
- Nehls V, Drenckhahn D: A novel microcarrier-based in vitro assay for rapid and quantification of three-dimensional cell migration and angiogenesis. *Microvasc Res* 1995, 50:311–322
- Uyeno LA, Newman J, Cheung I, Hunt TK, Young DM, Boudreau N: HoxD3 expression in normal and impaired wound healing. *J Surg Res* 2001, 100:46–56
- Nor JE, Peters MC, Christensen JB, Sutorik MM, Linn S, Khan MK, Addison CL, Mooney DJ, Polverini PJ: Engineering and characterization of functional human microvessels in immunodeficient mice. *Lab Invest* 2001, 81:453–463
- Engels K, Fox SB, Whitehouse RM, Gatter KC, Harris AL: Distinct angiogenic patterns are associated with high-grade in situ ductal carcinomas of the breast. *J Pathol* 1997, 181:207–212
- Guidi AJ, Schnitt SJ, Fische RL, Tognazzi K, Harris JR, Dvorak HF, Brown LF: Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma in situ of the breast. *Cancer* 1997, 80:1945–1953
- Kroon ME, Koolwijk P, van Goor H, Wiedle UH, Collen A, van der Pijlm G, van Hinsbergh VWM: Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. *Am J Pathol* 1999, 154:1731–1742
- Ellerbrook SM, Stack SM: Membrane associated matrix metalloproteinases in metastasis. *Bioessays* 1999, 21:940–949
- Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ: Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell* 1998, 95:365–377
- Kubota Y, Kleinman HK, Martin GR, Lawley TJ: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* 1988, 107:1589–1596
- Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM: Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell* 1997, 91:949–960
- Gianelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V: Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science* 1997, 277:225–228
- Muschler J, Lochter A, Roskelley CD, Yurchenco P, Bissell MJ: Division of labor among the $\alpha 6 \beta 4$ integrin, $\beta 1$ integrins and an E3 laminin receptor to signal morphogenesis and β casein expression in mammary epithelial cells. *Mol Biol Cell* 1999, 10:2817–2828
- Clark EA, Golub TR, Lande RES, Hynes RO: Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 2000, 406:532–535
- Howlett AR, Petersen OW, Steeg PS, Bissell MJ: A novel function for the nm-23H1 gene: overexpression in human breast carcinoma cells leads to the formation of basement membranes and growth arrest. *J Natl Cancer Inst* 1994, 86:1838–1844
- Osborne J, Hu C, Hawley C, Underwood LJ, O'Brien TJ, Baker VV:

- Expression of HOXD10 gene in normal endometrium and endometrial adenocarcinoma. *Soc Gynecol Invest* 1998, 5:277–280
34. Duboule D: The Guidebook to the Homeobox Genes. Edited by Sambrook, Tooze. New York, Oxford Press, 1994
 35. Duboule D, Morata G: Colinearity and functional heirarchy among genes of the homeotic complexes. *Trends Genet* 1994, 10:358–364
 36. Azpiazu N, Morata G: Functional and regulatory interactions between Hox and extradenticle genes. *Genes Dev* 1998, 12:261–273
 37. Shen W-F, Rozenfeld S, Lawrence HJ, Largman C: The Abd-B Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. *J Biol Chem* 1997, 272:8198–8206
 38. Gould AP, Brookman JJ, Strutt DI, White RAH: Targets of homeotic gene control in *Drosophila*. *Nature* 1990, 348:308–311
 39. Johnson A: Transcriptional Regulation. Edited by SL McKnight, KR Yamamoto. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1992, pp 975–1006
 40. Capovilla M, Botas J: Functional dominance among Hox genes: repression dominates activation in the regulation of Dpp. *Development* 1998, 125:4949–4957