Homeobox B3 Promotes Capillary Morphogenesis and Angiogenesis

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Abstract. Endothelial cells (EC) express several members of the Homeobox (Hox) gene family, suggesting a role for these morphoregulatory mediators during angiogenesis. We have previously established that Hox D3 is required for expression of integrin αvβ3 and urokinase plasminogen activator (uPA), which contribute to EC adhesion, invasion, and migration during angiogenesis. We now report that the paralogous gene, Hox B3, influences angiogenic behavior in a manner that is distinct from Hox D3. Antisense against Hox B3 impaired capillary morphogenesis of dermal microvascular EC cultured on basement membrane extracellular matrices. Although levels of Hox D3-dependent genes were maintained in these cells, levels of the ephrin A1 ligand were markedly attenuated. Capillary morphogenesis could be restored, however, by addition of recombinant ephrin A1/Fc fusion proteins. To test the impact of Hox B3 on angiogenesis in vivo, we constitutively expressed Hox B3 in the chick chorioallantoic membrane using avian retroviruses that resulted in an increase in vascular density and angiogenesis. Thus, while Hox D3 promotes the invasive or migratory behavior of EC, Hox B3 is required for the subsequent capillary morphogenesis of these new vascular sprouts and, together, these results support the hypothesis that paralogous Hox genes perform complementary functions within a particular tissue type.

Key words: endothelial cells • Hox • neovascularization • extracellular matrix • ephrin

Introduction

The Homeobox (Hox)[1] morphoregulatory genes encode transcription factors that play an essential role in organogenesis during development and, more recently, have been linked to both hormonal and pathologically induced tissue remodeling in adults (for review Boudreau and Bissell, 1998; Stelnicki et al., 1998; Chen and Capecchi, 1999; for review Cillo et al., 1999). Although the identity of many genes whose expression is modulated by binding of Hox proteins to target DNA sequences remains to be firmly established, a growing body of evidence indicates that genes associated with cell–cell and cell–extracellular matrix (ECM) interactions are putative targets of Hox activity (Jones et al., 1992; Li and Gudas, 1996; Boudreau et al., 1997; Lorentz et al., 1997).

To this end, we have been investigating a potential role for homeobox genes during angiogenesis, a process which involves extensive, coordinated changes in cell–cell and cell–ECM interactions. In response to angiogenic stimulators, including the tumor or wound microenvironment, normally quiescent endothelial cells (EC) upregulate expression of proteinases, including matrix metalloproteinases and urokinase plasminogen activator (uPA), which degrade the surrounding basement membrane (BM) ECM (for review see Werb et al., 1999). As EC reenter the cell cycle, they also upregulate expression of adhesion molecules, including αvβ3 integrin, which allows the cells to adhere and migrate into the adjacent stromal matrix environment (Brooks et al., 1994). After this, EC must then resynthesize and deposit a new BM ECM and undergo morphological reorganization into tubular structures complete with a lumen (Stromblad and Cheresh, 1996). Maturation of the newly formed capillaries follows via recruitment of pericytes, which strengthen and stabilize the vascular wall (Hirschi and D'Amore, 1996; Maisonpierre et al., 1997).

We previously identified several class I Homeobox genes expressed in cultured EC and showed that one of these, namely Hox D3, is required for expression of the β3 subunit of the αvβ3 integrin, as well as for expression of the uPA. When constitutively expressed in EC in vivo,

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[1]*Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; BM, basement membrane; CAM, chick chorioallantoic membrane; EC, endothelial cells; ECM, extracellular matrix; HMEC, human microvascular endothelial cells; HMEC-1, human dermal microvascular endothelial cells; Hox, Homeobox; RT, reverse transcriptase; uPA, urokinase plasminogen activator.

Hox D3 produced endothelioma-like structures, consistent with a role for this gene in mediating the invasive and migratory behavior of EC during the early stages of neovascularization (Boudreau et al., 1997). Recent work by others has also shown that EC upregulate the expression of several members of the Hox B cluster or splice variants of Hox A9 in response to a variety of angiogenic cytokines (Belotti et al., 1998; Patel et al., 1999). The role of these genes during angiogenesis, however, remains to be established. Given the complex multistep nature of angiogenesis and the coordinate changes in cell–cell and cell–ECM interactions, it is likely that many of the Hox genes expressed in EC contribute to this process. Having established a role for Hox D3 during the early stages of angiogenesis, we are particularly interested in establishing a role for Hox genes that may act at later stages of angiogenesis, which involve resynthesis of BM ECM and/or acquisition of a tubular three-dimensional capillary morphology.

Evidence gathered from single and compound Hox null mice have suggested that paralogous Hox genes (i.e., those with similar numerical designations, but located on different chromosomes such as Hox D3, Hox B3, and Hox A3) may play complementary, overlapping, or synergistic roles in a particular tissue (Condie and Capecchi, 1994; Manley and Capecchi, 1997). For example, whereas deletion of a single member of Hox 9 paralogous group did not significantly impact postnatal mammary gland development, deletion of Hox A9, B9, and D9 dramatically impaired the normal branching morphogenesis and differentiation of this tissue (Chen and Capecchi, 1999). Therefore, we wished to investigate whether paralogues of Hox D3 also contribute to angiogenic behavior in EC and whether these genes acted at the same or later stages of neovascularization. We had previously observed that the paralogous gene Hox B3 is highly expressed in adult EC (Boudreau et al., 1997) and thus wished to establish whether this gene acted in concert with Hox D3 and/or via distinct mechanisms to influence endothelial cell behavior.

Materials and Methods

Cells, Culture Conditions, and Cytokines

An immortalized human dermal microvascular endothelial cell line (HMEC-1; Ades et al., 1992), was a gift from T. Lawley (Emory University, Atlanta, GA). These cells previously have been shown to maintain many properties of primary dermal microvascular cells in culture, including the ability to undergo capillary morphogenesis when cultured on BM (Matrigel) and maintain expression of a number of endothelial cell surface markers (Xu et al., 1994). Cells were maintained in media MCBD 131 supplemented with 10% FCS, gentimicin, and 1% hydrocortisone (Sigma Chemical Co.) and passaged using calcium- and magnesium-free PBS supplemented with 0.053 mM EDTA. Primary cultures of HMEC were purchased from Clonetics. Recombinant mouse ephrin A1/human IgG fusion proteins, recombinant human VEGF, and basic fibroblast growth factor (bFGF) were purchased from R&D Systems. Recombinant human TNF α and Matrigel were obtained from Collaborative Research. Endothelial cell culture on BM (Matrigel) was performed as previously described (Boudreau et al., 1997). To release cells from Matrigel for protein or mRNA determination, cultures were suspended in PBS without Ca^{2+} or Mg^{2+}, containing 0.5 mM EDTA and incubated on ice for 1 h to allow the Matrigel to disperse. Cells were then pelleted by centrifugation at 500 *g* for 5 min at 4°C. For the ephrin add back experiments, 250 ng/ml recombinant mouse ephrin A1 fused to human IgG was diluted in serum-free MCDB 131 and preclustered for 1 h at room temperature using 25 ng/ml of an antibody against the Fc region of human IgG (Sigma Chemical Co.) as described (Wang and Anderson, 1997). Clustered ephrins were added to cells at the time of plating on Matrigel.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from HMEC-1 using the Qiagen RNEasy kit. For Northern blot analysis, a total of 10 or 20 μg total RNA was electrophoresed through 1% agarose formaldehyde gels as previously described using standard methods (Boudreau et al., 1997). Ribosomal RNA was visualized by staining with 1% ethidium bromide. ^{32}P{dCTP} probes were prepared using the Ambion Decaprime Kit and purified using Sephadex G-25 columns (Boehringer Mannheim Corp.). Blots were probed with 1×10^6 cpm/ml of hybridization buffer (Hybridsol I; Oncor) and exposed to Kodak M5 X-Omat film at −70°C. cDNA for integrin β3 was a gift from David Cheresh (The Scripps Research Institute, La Jolla, CA).

Reverse Transcriptase PCR Measurement of Hox B3 in EC

1 μg of total RNA was reverse transcribed using MMuLV RT for 1 h in total volume of 25 μl. 0.1, 1, 2, and 10 μl of this RT reaction was then amplified for 20, 30, or 35 cycles of 95, 58, and 72°C for 30, 30, and 90 s, respectively, with the following primers: forward primer 5′ cgatgcagaaagcacctactacgac 3′ corresponding to bp 362–387 of human Hox B3, and reverse primer 5′ cgccgaccccgggggggctcttct 3′ corresponding to nucleotides 1,666–1,682 of the published sequence. The expected 1.32-kb PCR product was visualized by electrophoresis on 1% agarose gels containing ethidium bromide. From this analysis, it was determined that amplification of 1 μl of the total 25 μl RT reaction for 30 cycles gave optimal, reproducible results within the linear range for amplification. To normalize for total RNA, 1 μl of the same RT reaction was diluted 1:800 in water and amplified under the same conditions with commercially available primer sets for human GAPDH or β-actin (Stratagene). The 1.32-kb PCR product corresponding to Hox B3 was subsequently ligated into the TOPO II TA cloning vector (Invitrogen Corp.) and the identity confirmed as Hox B3 by dideoxy sequencing using the USB Sequenase 2 kit (Nycomed Amersham, Inc.).

Construction of Hox B3 Sense and Antisense Expression Vectors

The insert containing the entire cDNA encoding human Hox B3 was subcloned into the expression vector PCR 3.1 containing a CMV promoter (Invitrogen Corp.). The orientation was confirmed by restriction digest and the ends were resequenced to confirm the identity and orientation. Clones in the antisense orientation were directly transfected into HMEC-1 using a $CaP0_4$ method and stable transfectants selected in the presence of 50 μg/ml G418. To achieve high levels of translation and expression of Hox B3 in the sense orientation, we introduced a Kozak consensus sequence on the 5′ end by reamplifying the cDNA with the primer 5′ ggaattcggccaccatgcaga 3′. The resulting cDNA was religated into the same PCR 3.1 expression in the sense orientation, stably transfected into HMEC-1, and stable pools were selected as described above. To allow us to distinguish transgene expression from endogenous Hox B3, we have also added a COOH-terminal 6×His epitope tag by deleting the stop codon and cloning into the PCR 3.1 mycHis vector (Invitrogen Corp.). This construct was subsequently cloned into the CK proviral vector described below.

Western Blotting

Total protein was isolated from EC using ice-cold 10 mM Tris/150 mM NaCl in the presence of 10 μg/ml aprotinin, pepstatin, and leupeptin, and 0.02 M PMSF. Protein concentration was determined using a protein assay kit (BioRad). For Western analysis, a total of 20 or 50 μg protein lysates were run on SDS-PAGE and transferred to PVDF membranes (Immobilon; Nycomed Amersham Inc.). Membranes were blocked in 5% milk protein in 10 mM TBS, pH 7.6. Polyclonal antibodies against Hox B3 were purchased from Berkeley Antibody Co. Polyclonal rabbit anti–human antibodies against ephrin A1 were purchased from Santa Cruz Biotechnology (sc-911). For Western Blot analysis, a 1:2,000 or 1:500 dilution was used as indicated, followed by incubation with HRP-conjugated goat anti–rabbit antibodies (Nycomed Amersham Inc.). To reduce nonspecific background, blots were washed in 1 M NaCl followed by several washes in TBS, pH 7.6. Bands were visualized by enhanced chemiluminescence (ECL Plus; Nycomed Amersham Inc.).

cDNA Probes for Ephrin A1 and Ephrin B1

A cDNA probe corresponding to ephrin A1 was isolated using the following primer pairs: forward 5′ ggaacccagacccataggagac 3′′ corresponding to nucleotides 17–38, and the reverse primer 5′ ttcagcctgtccctctttaaga 3′ corresponding to nucleotides 711–732 of the human sequence (GenBank/EMBL/DDBJ accession numbers M57730 and M37476). The resulting 0.7-kb PCR product was ligated into the TOPO cloning vector and the identity confirmed by sequencing as described. The entire 700-bp insert was used for Northern blot analysis. A probe for ephrin B1 was also generated by RT-PCR from 1 μg total RNA for 30 cycles at 95, 58, and 72°C for 30, 30, and 90 s, respectively, using the following primer pairs: forward 5′ ttg gtg agg agg cgc caa gg 3′ corresponding to nucleotides 653–672, and the reverse primer 5′′ ggg cac tca gac ctt gta gta ga 3′ corresponding to nucleotides 1,748–1,726 of the published sequence of human ephrin B1 (GenBank/EMBL/DDBJ accession number NM_004429). The 1,095-bp PCR product was ligated into the TOPO II PCR cloning vector (Invitrogen Corp.) and the identity of the PCR product confirmed as ephrin B1 by dideoxy sequencing as described above.

Immunoprecipitation of Eph A2 Receptor

EphA2 receptor was measured by immunoprecipitation of equal amounts of protein lysates of HMEC-1 using a polyclonal antibody against human Eph A2 (sc-924; Santa Cruz Biotechnology). HMEC-1 were lysed in ice-cold 10 mM Tris-HCl/150 mM NaCl, pH 7.6, in the presence of 2 mM NaF, 2 mM orthovanadate, 10 μg/ml aprotinin, pepstatin, and leupeptin, and 0.02 M PMSF. After determination of protein concentrations, 300 μg of cellular lysate was diluted in RIPA containing 2 mM NaF and 2 mM orthovanadate, and immune complexes precipitated using 25 μl of a 10% wt/vol solution of protein A–Sepharose (Sigma Chemical Co.). Pellets were washed three times in RIPA, followed by two additional washes in PBS, and resuspended in 2× Laemmli sample buffer. After separation on 7.5% SDS-PAGE and transfer to PVDF membranes, blots were probed with antibodies against Eph A2 or antibodies against total phosphotyrosine (clone 4G10; Upstate Biotechnology).

Migration Assays

Endothelial cell migration assays were performed using a modification of the method described by Klemke et al. (1997). In brief, migration assays were performed using tissue culture-treated Transwell chambers of 6.5-mm diam, with 8-μm pores (Costar Corp.). The bottom surfaces of the chambers were coated with 20 μg/ml of bovine fibrinogen (Sigma Chemical Co.) or 1% BSA for controls. Before the assay, wells were rinsed twice with migration buffer (fibroblast basal medium + 0.5% BSA; Clonetics). EC were serum-starved for 18 h and removed from the culture dishes by harvesting with Ca^{2+}- and Mg^{2+}-free PBS containing 0.53 mM EDTA, and resuspended in migration buffer. 50,000 cells were added to each well and the assay was allowed to proceed for 5 h at 37°C. Nonmigratory cells were removed from the upper chambers with a cotton swab and cells that had migrated to the bottom of the membrane were visualized by fixing and staining with Diff-Quick (VWR Scientific Products). The total number of cells that had migrated was determined by counting at least five different fields using a Nikon inverted TMS microscope. Alternatively, quantitative analysis of migration was performed by measuring absorbance of methylene blue stained cells at 600 nm. Each determination represents the average of three individual wells and error bars represent SD.

Construction and Application of Avian Replication-defective Retroviruses Expressing Hox B3 in Chick Chorioallantoic Membrane

To produce replication defective retroviruses encoding Hox B3, the cDNA encoding the entire human Hox B3 sequence was excised with HindIII and PmeI and inserted into the proviral vector CK at the HindIII site and an XbaI site that was blunted with Klenow polymerase. The viral packaging cell line, Q4dh, derived from the QT6 quail fibrosarcoma cells line (Stoker and Bissell, 1988), was maintained in M199 containing 4% FCS, 1% chicken serum, and 1× tryptose phosphate broth. Stable transfectants expressing either human Hox B3 proviral vectors or empty vector (CK) were performed using $CaP0_4$, and pools of stable transfectants producing infectious virus were selected in the presence of 200 μg/ml G418 as described (Stoker and Bissell, 1988; Boudreau et al., 1997). To induce angiogenesis, we grafted 5×10^6 Q4dh cells in a volume of 50 μl of medium M199 onto the chick chorioallantoic membranes (CAMs) of 10-day chick SPAFAS pathogen-free chick embryos as previously described (Boudreau et al., 1997). After 72 h, CAMs were harvested and vascular density, morphology, and immunohistochemical analysis were performed. Angiogenesis was quantitated by counting the number of branch points arising from the tertiary vessels in a 6-mm square area on the underside of the tumors. Measurements were made in 12 samples infected with control virus and 12 samples infected with Hox B3 expressing virus from three separate experiments. Statistical significance was assessed using a paired *t* test.

Tissue Fixation and Immunofluorescence

CAMs were fixed in situ in 4% paraformaldehyde, embedded in OCT medium, and frozen in a dry ice/ethanol bath and stored at −70°C. 7-μm sections of the CAMS were used for immunohistochemistry. After brief acetone fixation, sections were air-dried and subsequently blocked in PBS containing 2% BSA for 1 h, followed by staining with appropriate antibodies. A 1:200 dilution of a monoclonal anti–his-6 antibody (Boehringer Mannheim Corp.) diluted in PBS containing 0.025% Triton X-100, 0.1% BSA, or a 1:200 dilution of a polyclonal rabbit anti–human antibody against von Willebrand factor were used, followed by incubation for 1 h with FITC-conjugated goat anti–mouse (Cappel Laboratories) or Texas red-conjugated goat anti–rabbit secondary antibody (Calbiochem-Novabiochem Corp.).

Results

Expression of Hox B3 in Cultured Endothelial Cells

As the levels of Hox mRNA and protein expression in mammalian cells are normally low, we established conditions to measure Hox B3 using semiquantitative RT-PCR. Using the RT-PCR conditions described in Materials and Methods, we assessed expression levels of Hox B3 in both primary human dermal microvascular cells or the immortalized HMEC-1 cultured under various conditions. Although EC continue to proliferate and form a cobblestone monolayer when plated on untreated tissue culture dishes, culturing on dishes coated with reconstituted BM induces EC to become quiescent and adopt a three-dimensional capillary-like morphology (Kubota et al., 1988; Boudreau et al., 1997). Therefore, we compared expression of Hox B3 in cells cultured in the presence or absence BM (Fig. 1 A). In contrast to our previous observations showing that BM suppressed expression of Hox D3, we observed that Hox B3 was expressed at slightly higher levels in HMEC-1 cultured on BM for 24 h, as compared with cells maintained in the absence of BM (Fig. 1 A). Similar results were observed in primary cultures of dermal microvascular EC (not shown). We also treated EC with the angiogenic cytokine, bFGF, and observed that levels of Hox B3 were not increased by this treatment (Fig. 1 B). This distinct expression profile of Hox B3 suggested that, perhaps, Hox B3 and Hox D3 contributed to different endothelial cell phenotypes.

Suppression of Hox B3 Impairs Capillary Morphogenesis of EC Cultured on BM ECM

Because levels of Hox B3 are maintained when EC are cultured on BM, we reasoned that Hox B3 may contribute to the capillary-like morphogenesis that EC undergo when they are cultured on reconstituted BM. Therefore, we stably transfected an immortalized line of dermal HMEC-1 with CMV-driven plasmids expressing antisense against Hox B3 or with empty plasmid (control). Western blot analysis of stable pools of cells transfected with antisense

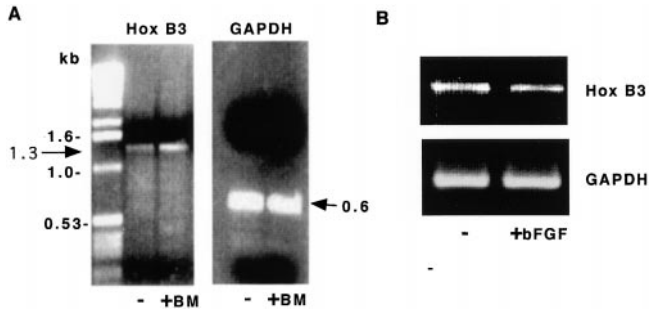

Figure 1. Expression of Hox B3 in HMEC cultured under different conditions. A, Semiquantitative RT-PCR of Hox B3 (left) and GAPDH (right) in HMEC grown in the presence (+) or absence (−) of BM (Matrigel) shows the relative levels of the 1.3-kb transcript encoding Hox B3 in EC under each of these conditions. The 0.6-kb transcript for GAPDH was used to normalize levels of RNA used in this analysis. B, Semiquantitative RT-PCR for Hox B3 in HMEC cultured in the presence or absence bFGF (50 ng/ml) for 24 h.

against Hox B3 displayed a decrease in Hox B3 protein, as compared with control transfected cells (Fig. 2 A). In contrast to controls, we also observed that when cells with reduced levels of Hox B3 were cultured on BM ECM, many formed aggregates and were unable to branch or form capillary-like tubules, indicating that Hox B3 is necessary for BM-dependent capillary morphogenesis (Fig. 2 B). Furthermore, we also observed that BM-induced capillary morphogenesis of EC is not influenced by blocking Hox D3 or its putative target, integrin αvβ3. In addition, we also observed that blocking either Hox D3 or Hox B3 using antisense did not influence expression of the paralogous genes (Boudreau et al., 1997; data not shown). Together, these results indicate that the paralogous Hox B3 and Hox D3 genes have distinct effects on EC behavior.

Impaired Capillary Morphogenesis in the Absence of Hox B3 Correlates with Decreased Levels of Ephrin A1, but Not the EphA2 Receptor in EC

Although many factors have been reported to influence capillary morphogenesis in cultured EC, several recent reports have implicated interactions between ephrin ligands and their corresponding Eph receptors in the morphogenesis of EC cultured on BM and during angiogenesis in vivo (Pandey et al., 1995; Daniel et al., 1996; Adams et al., 1999). In addition, as Hox genes have been linked to regulation of expression of the EphA2 receptor during development, we wished to further explore a potential link between Hox B3 and ephrin or Eph receptor-expression in EC (Chen and Ruley, 1998; Studer et al., 1998). We initially compared the levels of the EphA2 receptor by immunoprecipitation of lysates from control transfected HMEC-1 or HMEC-1 transfected with antisense against Hox B3 (Fig 3 A). Although the levels of the Eph A2 receptor were similar, subsequent blotting revealed that phosphorylation of the EphA2 receptor was dramatically reduced in cells lacking Hox B3 (Fig. 3 A). These findings further suggested that the levels of an ephrin ligand that may bind to and activate phosphorylation of the EphA2 receptor may be reduced in cells lacking Hox B3. Therefore, we performed a Western blot analysis with antibodies against the ephrin A1 ligand and observed that its expression was significantly reduced in EC transfected with antisense against Hox B3 (Fig. 3 A). Furthermore, the reduction of ephrin A1 protein and mRNA (Fig. 3, A and B) appeared to be somewhat selective, since levels of another ephrin ligand, ephrin B1, were not affected (Fig. 3 B). Importantly, the level of integrin β3 (Fig. 3 B) and uPA (not shown), two targets of Hox D3 activity, were unaffected. These results indicate that paralogous Hox genes have distinct target genes within a particular tissue type.

Capillary Morphogenesis Can Be Enhanced by Addition of Ephrin A1 in Cells Expressing Antisense against Hox B3

To determine whether restoring ephrin A1 ligand to EC lacking Hox B3 could help these cells regain a three-dimensional capillary-like morphology, cells were treated with preclustered, recombinant mouse ephrin A1/human Fc fusion proteins. Fig. 4 A shows that addition of clustered mouse ephrin A1/Fc fusion protein was capable of eliciting high levels of phosphorylation of the human EphA2 receptor in both control transfected HMEC-1 and those transfected with antisense against Hox B3. More significantly, addition of recombinant ephrin A1/Fc fusion proteins partially restored the ability of cells lacking Hox

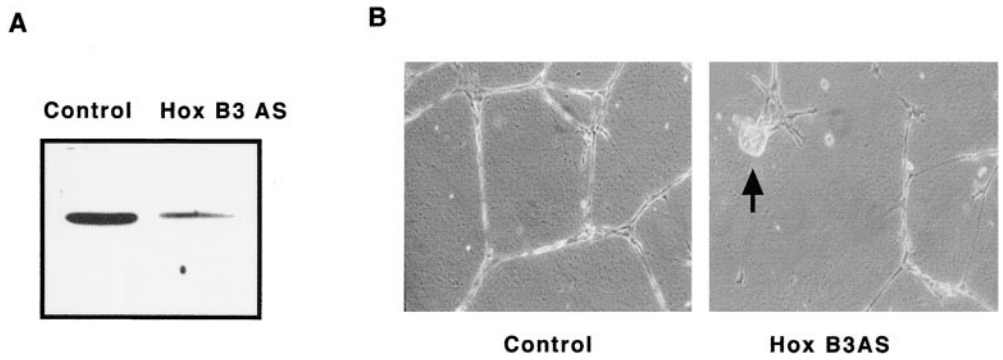

Figure 2. Antisense against Hox B3 impairs BM-induced capillary morphogenesis in EC. A, Western blot for Hox B3 in 50 μg total protein lysates from immortalized HMEC-1 stably transfected with empty vector (control) or plasmid expressing antisense against human Hox B3 (Hox B3 AS). B, Capillary morphogenesis of HMEC-1 after culturing on thick BM (Matrigel) after 18 h. The panel on the left shows normal capillary morphogenesis occurring in HMEC-1 stably transfected with empty vector (Control). The panel on the right shows impaired capillary morphogenesis in HMEC-1 transfected with antisense against Hox B3 (Hox B3 AS). The arrow shows representative groups of cells that remain in clusters and fail to elongate and form contacts with adjacent cells.

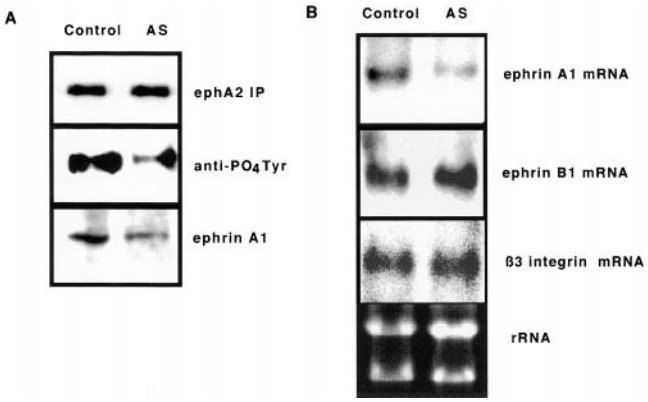

Figure 3. Expression of ephrin A1 is selectively reduced in EC lacking Hox B3. A, The upper panel shows similar amounts of a 120-kD band corresponding to EphA2 recovered by immunoprecipitation from control transfected (Control) HMEC-1 and HMEC-1 transfected with antisense against Hox B3 (AS). The middle panel shows subsequent blotting with an antiphosphotyrosine antibody and reveals a reduced level of phosphorylation of the EphA2 receptor in cells transfected with antisense against Hox B3. The lower panel shows Western blotting with a polyclonal antibody against ephrin A1 (1:500 dilution) performed on 20 μg total EC lysates from HMEC-1 transfected with control plasmid (Control) or antisense against Hox B3 (AS) and reveals a marked reduction in expression of the ~28-kD ephrin A1 protein. B, Northern blot analysis using 10 μg total RNA isolated from control transfected HMEC-1 (Control) or HMEC-1 transfected with antisense against Hox B3 (AS). The upper panel shows reduced levels of ephrin A1 mRNA in AS as compared with control cells. The middle and lower panels show the same blot reprobed with cDNAs corresponding to either ephrin B1 or integrin β3, respectively. The bottom panel shows the corresponding rRNA loading controls.

B3 to elongate and form branching networks resembling the three-dimensional capillary networks produced by untreated control transfected cells (Fig. 4 B). This ephrin A1-enhanced capillary morphogenesis was observed as early as four hours after plating on reconstituted BMs and persisted for at least 18 h. Thus, the Hox B3-dependent expression of ephrin A1 contributes to the normal capillary-like morphogenesis acquired by EC cultured on reconstituted BM.

Overexpression of Hox B3, but Not Hox D3, Results in Increased Levels of Ephrin A1 Expression

To further establish a relationship between Hox B3 and ephrin A1 expression, we transfected HMEC-1 with vectors containing CMV-driven human Hox B3. Western blot analysis of stable pools of cells transfected with Hox B3 showed increased levels of Hox B3 protein, as compared with HMEC-1 transfected with empty vector (control; Fig. 5 A). Cells overexpressing Hox B3 also showed the predicted increase in expression of ephrin A1 protein (Fig. 5 A). Immunoprecipitation with an anti-EphA2 antibody of lysates from HMEC-1 transfected with Hox B3 plasmid or empty vectors indicated that, although levels of the EphA2 receptor were not increased, subsequent blotting with antiphosphotyrosine antibodies revealed an increased level of phosphorylation of the receptor in cells overexpressing Hox B3 and ephrin A1 ligand (Fig. 5 B). HMEC-1 overexpressing Hox B3 also show the expected increase in ephrin A1 mRNA levels (Fig. 5 C), whereas HMEC-1 transfected with the paralogous gene Hox D3 do not upregulate expression of ephrin A1, but do show increased expression of β3 integrin mRNA (Fig. 5 D). Furthermore, we directly compared the ability of EC overexpressing Hox B3 or Hox D3 to migrate into a fibrinogen-rich environment. We observed that, whereas the Hox B3 transfected cells migrated at a rate similar to control transfected cells, Hox D3 transfected EC that express high levels of integrin αvβ3, show a significantly enhanced ability to migrate in this environment (Fig. 5 E). These results again emphasize that Hox B3 and Hox D3 specifically modulate expression of different angiogenic effector molecules in cultured EC, which in turn, contribute to different aspects of the angiogenic process.

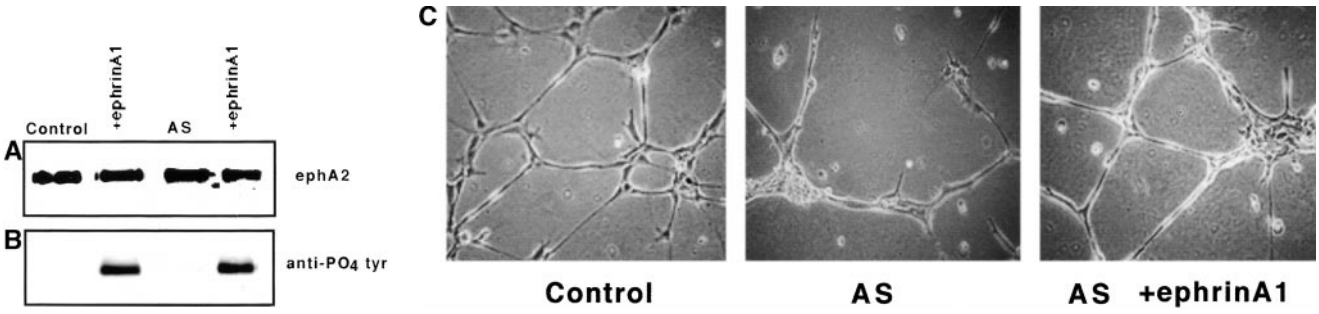

Figure 4. Addition of recombinant ephrin A1/Fc fusion protein helps restore BM-induced capillary morphology in EC lacking Hox B3. A, Immunoprecipitation of EphA2 from 300 μg of protein lysates from HMEC-1 transfected with empty vector (Control) or antisense against Hox B3 (AS) and treated with 250 ng/ml of preclustered ephrin A1 for 20 min. Subsequent blotting with a 1:500 dilution of the polyclonal antibody against EphA2 reveals similar amounts of the 120-kD ephA2 receptor recovered after immunoprecipitation and separation by SDS-PAGE on 7.5% acrylamide gels. B, Duplicate samples from control or Hox B3 antisense transfected cells (AS) with or without the addition of 250 ng/ml of preclustered recombinant ephrin A1 (+ephrinA1) were immunoprecipitated using the anti-EphA2 antibody and separated by SDS-PAGE and blotted with a 1:1,000 dilution of an mAb against phosphotyrosine (anti-PO_4 tyr). Both control and Hox B3 AS transfected cells showed a significant increase in levels of phosphotyrosine in the 120-kD band corresponding to immunoprecipitated EphA2. C, Shows morphology of control transfected HMEC-1 (Control), HMEC-1 transfected with antisense against Hox B3 (AS), or antisense cells treated with 250 ng/ml of clustered ephrin A1 (AS +ephrinA1) 18 h after plating of 0.5×10^6 cells in 60-mm culture dishes coated with 400 μl of BM (Matrigel).

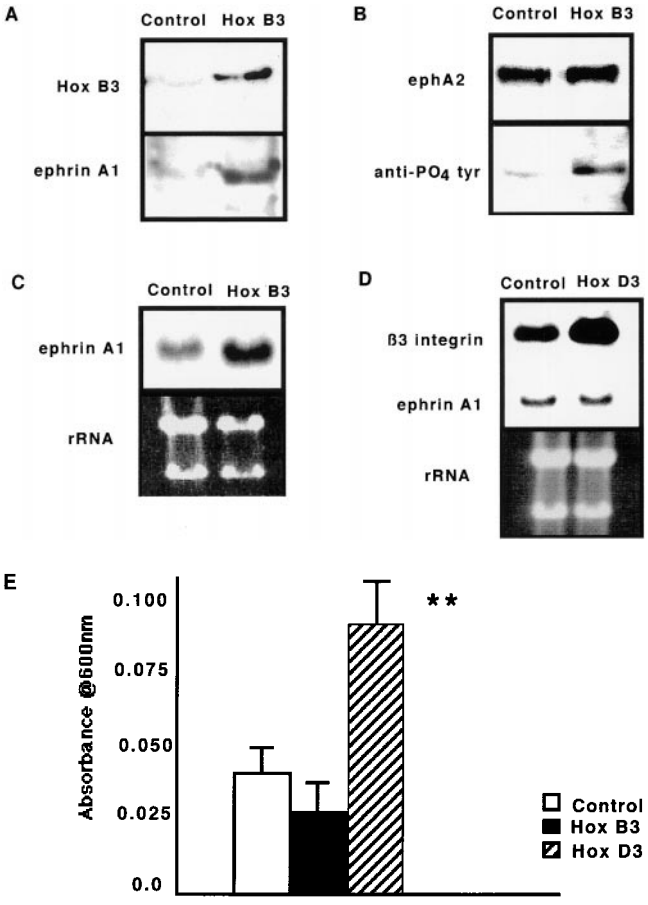

Figure 5. Overexpression of Hox B3, but not Hox D3, enhances expression of ephrin A1 in HMEC-1. A, Comparison of HMEC-1 transfected with empty vector (Control) or CMV-driven Hox B3 expression plasmids (Hox B3). Western blots for expression of Hox B3 (top) and ephrin A1 (bottom) from 40 μg total protein lysates from control transfected HMEC-1 or those overexpressing Hox B3. B, Immunoprecipitation of 300 μg of lysates from control of Hox B3 transfected cells using an ephA2 antibody yields similar levels of the ephA2 receptor (top), whereas subsequent blotting with an antiphosphotyrosine antibody shows an increased degree of phosphorylation of the receptor in cells overexpressing Hox B3 (bottom). C, Northern blot analysis of ephrin A1 mRNA levels (top) from 10 μg total RNA isolated from HMEC-1 transfected with either Hox B3 (Hox B3), or with empty vector (Control). Lower panel shows corresponding ribosomal RNA (rRNA) loading control for each cell type. D, Northern blot analysis for β3 integrin and ephrin A1 mRNA levels in 10 μg total RNA isolated from in HMEC-1 transfected with CMV-driven Hox D3 expression plasmids (Hox D3) or empty vector (Control). Lower panel shows corresponding levels of ribosomal RNA (rRNA) visualized with ethidium bromide. E, The influence of Hox gene expression on EC migration. Migration of HMEC-1 transfected with control plasmid (□), Hox B3 (■), or Hox D3 (▨) was assessed after 5 h in modified Boyden chambers coated with 20 μg/ml fibrinogen. Data are expressed as the mean ± SD ($n = 3$). $^{**}P < 0.05$.

High Levels of Hox B3 Promote Angiogenesis In Vivo

Based on the above results, we predicted that constitutive overexpression of Hox B3 in vivo would yield a phenotype that would be distinct from the hemangiomas that arise in response to constitutive expression of Hox D3. In addition, based on our observations that Hox B3 was required for EC to acquire a tubular three-dimensional capillary morphology, we also reasoned that constitutive expression of Hox B3 in vivo may promote capillary morphogenesis of invasive vascular sprouts induced in response to an angiogenic stimuli. To this end, we induced 10-d-old chick CAMs to undergo angiogenesis by grafting tumorigenic viral packaging cells onto the CAM. The packaging cells were transfected with either empty proviral vector (CK) or retrovirus expressing human Hox B3. After 72 h, we observed that CAMs infected with retrovirus expressing Hox B3 displayed a significant increase in vascular density and capillary branching, as compared with CAMs infected with empty virus (18 ± 6.5 branch points/6 mm^2 vs. 9.33 ± 3.78 branch points/6 mm^2; $n = 12$, $P < 0.05$; Fig. 6). Similar increases in capillary branching/vascular density was observed in at least 24 different embryonic membranes examined from four different experiments. Furthermore, we did not observe any evidence of hemangioma-like structures characteristic of EC constitutively expressing Hox D3 (Boudreau et al., 1997). Thus, both in culture and in vivo, Hox D3 and Hox B3 appear to differentially influence endothelial gene expression and angiogenesis (Table I).

Finally, to demonstrate that the increase in angiogenic activity induced in the CAM was related to the presence of the retrovirally transduced Hox B3, we generated a replication defective retroviral vector expressing Hox B3 fused to a 6×His epitope tag on the COOH terminus. Similar to the results seen with the nontagged Hox B3, infection with the His-tagged Hox B3 also produced increase in endothelial cell density and capillary branches in CAMs after 72 h. Staining of serial 7-μm sections showed that the increased endothelial cell density, indicated by positive staining with von Willebrand factor, colocalized with staining for the His epitope present in the virally expressed Hox B3 (Fig. 7).

Discussion

We have shown that the Homeobox gene, Hox B3, which is expressed by HMEC-1, helps promote capillary morphogenesis in culture and angiogenesis in vivo. Furthermore, Hox B3 influences angiogenesis in a manner that is distinct from the previously described proangiogenic effects of the paralogous gene, Hox D3 (Boudreau et al., 1997). Specifically, whereas Hox D3 upregulated expression of both uPA and integrin αvβ3, which in turn facilitate endothelial cell migration, adhesion, and invasion, Hox B3 induced expression of ephrin A1 and promoted capillary morphogenesis of sprouting EC. Furthermore, this phenotype was reiterated in vivo as Hox B3 promoted branching and angiogenic behavior, in contrast to the hemangioma-like structures generated by Hox D3 (Boudreau et al., 1997). Together, these findings support earlier observations made in mice lacking two or more paralogous Hox genes, which suggested that, although individual Hox genes performed distinct functions in a given tissue type, together, paralogous Hox genes acted in concert to more dramatically influence overall tissue phenotype and morphology (Condie and Capecchi, 1994; Manley and Capecchi, 1997; Chen and Capecchi, 1999).

The requirement for Hox B3 during BM-induced capil-

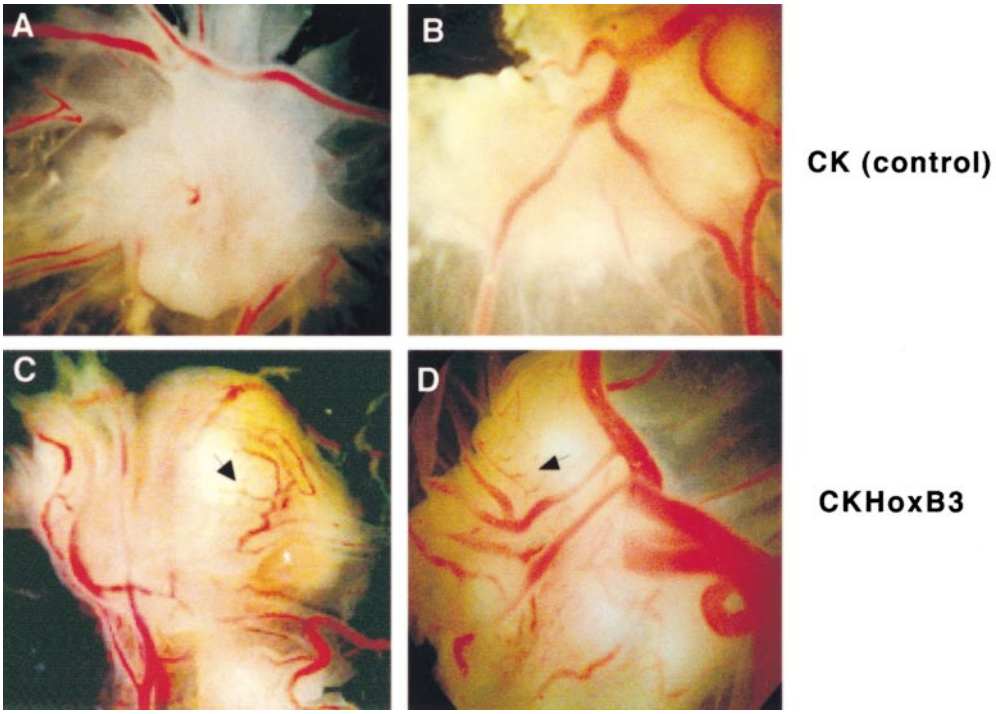

Figure 6. Retrovirally expressed Hox B3 promotes tumor-induced angiogenesis in vivo. 5×10^6 quail fibrosarcoma-derived viral packaging cells transfected with empty vector (CK) or viral vectors expressing Hox B3 were grafted on 10-d-old CAMs. A and B, The resulting small tumors and associated vasculature in the CAMs in response to grafting 5×10^6 quail fibrosarcoma cells producing empty replication-defective retroviruses (CK). C and D, Increased vascular density associated with tumors produced on the CAM in response to addition of 5×10^6 quail fibrosarcoma cells producing replication-defective retroviruses expressing Hox B3 (CKHoxB3). The arrows show areas of angiogenic sprouting from larger vessels observed primarily in CAMs infected with retrovirus expressing Hox B3.

lary morphogenesis appears to stem in large part from regulation of the expression of the ephrin A1 ligand. Although previous studies have linked both Hox A1 and Hox B1 to expression of the ephA2 receptor (Chen and Ruley, 1998; Studer et al., 1998), this is the first report that expression of the ephrin ligands may also be regulated by homeobox genes. It is of interest to note, however, that another ephrin ligand, ephrin B1 (Lerk 2), was identified previously in a screen for retinoic acid-inducible genes (Bouillet et al., 1995), as retinoic acid is amongst the most potent inducers of anterior class I Homeobox genes known (Langston and Gudas, 1994).

Recent work has established morphoregulatory roles for the large family of ephrin ligands and their tyrosine kinase Eph receptors in both neural pathfinding and vascular development, and angiogenesis (for review see Holder and Klein, 1999). In cultured adult EC, the type of ephrin ligand required for BM-induced capillary morphogenesis depends upon the vascular site of origin of the EC. For example, branching morphology in renal microvascular cells requires ephrin B1, whereas capillary morphogenesis of umbilical vein EC depended on the addition of ephrin A1 (Daniel et al., 1996). Our results using EC derived from the dermal microvasculature indicate that ephrin A1 is a potent mediator of capillary morphogenesis in EC derived from this tissue.

Although interaction with, and subsequent phosphorylation of, a corresponding Eph receptor are required for the morphological effects induced by ephrins, it is not clear whether these interactions enhance EC migration or adhesion, or alternatively induce cellular repulsion (and perhaps promote branching of vascular sprouts), as has been observed with cultured neurons (Wang and Anderson, 1997). One recent report also suggested that ephrin B1 may stimulate migration of renal microvascular EC via activation of αvβ3 integrin-mediated attachment (Huynh-Do et al., 1999). However, as αvβ3 is not required for adhesion to BM ECM, and blocking αvβ3 does not influence BM-induced capillary morphogenesis in culture, it is unlikely that the ephrin A1-induced morphological changes we observed arose via activation of αvβ3. It is intriguing, however, to consider the possibility that ephrin A1 may

Table I. Effects of Hox B3 or Hox D3 on EC Behavior

	Hox B3	Hox D3
Exposure to bFGF	No change in Hox B3 expression	Upregulates Hox D3
Culture on BM	Increased Hox B3 expression	Hox D3 expression suppressed
β3 Integrin mRNA levels	Unchanged by overexpression of Hox B3	Upregulated by Hox D3
Ephrin A1 mRNA levels	Upregulated by Hox B3	Unchanged by Hox D3
Migration on fibrinogen	No effect	Enhanced
BM-induced capillary morphogenesis	Hox B3 required	Not required
Overexpression in vivo	Increased angiogenesis	Hemangioma hemorrhagic lesions

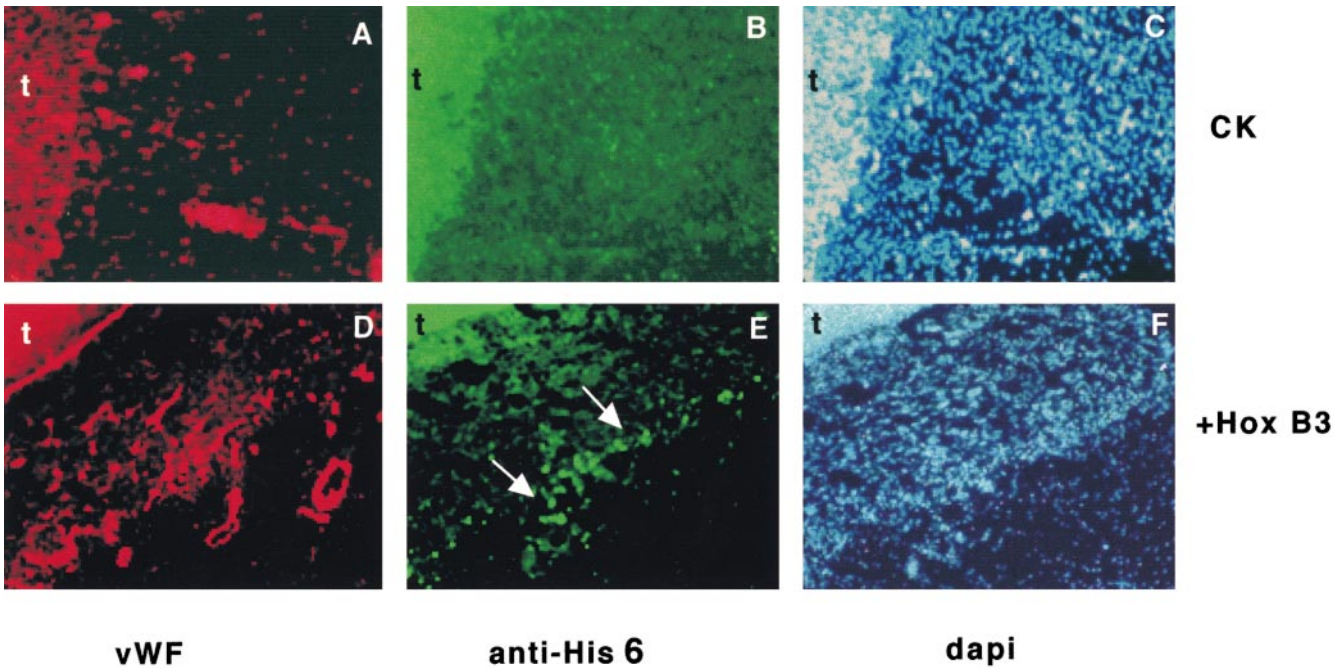

Figure 7. Increased capillary density correlates retrovirally expressed Hox B3. Immunofluorescence staining of serial 7-μM sections of CAMs harvested 72 h after application of fibrosarcoma cells producing empty retrovirus (CK) or retrovirus expressing Hox B3 with a His-6 epitope tag fused to the COOH terminus (+Hox B3). Staining with an antibody against von Willebrand factor (A and D) shows a relative increase in endothelial cell density in membranes exposed to Hox B3. Staining of serial sections with an antibody against the 6×His epitope tag fused to Hox B3 (B and D) reveals positive staining (arrows) in areas associated with increased vascular density. Areas showing strong autofluorescence within the tumor cores are indicated by the letter t. Corresponding DAPI nuclear staining is shown for both tissues (C and E).

activate integrins that mediate EC adhesion to laminin, the major component of BMs required for capillary morphogenesis (Kubota et al., 1988).

Our results suggest that, in addition to ephrin A1, Hox B3 may also induce expression of other genes that promote capillary morphogenesis. For example, although addition of recombinant ephrin A1 helped restore normal capillary-like morphology in many of the EC lacking Hox B3, a small proportion of cells consistently remained in clusters and failed to elongate or form tubules, despite the intense phosphorylation of the ephA2 receptor that followed addition of ephrin A1. Although the incomplete responsiveness to exogenous ephrin A1 may be attributed to heterogeneity amongst the EC population, it is also worth noting that since Hox genes are considered to be master regulatory mediators (Botas, 1993; Duboule, 1998), expression of several, different morphoregulatory genes may be regulated by Hox B3 in EC. Our preliminary results indicate that EC lacking Hox B3 also show decreased expression of laminin β2 (LAMB2; data not shown). A role for this laminin isoform in BM-induced capillary morphogenesis is currently under investigation.

Other potential targets of Hox B3 include the thyroid transcription factor, TTF-1. Studies in HeLa and NIH 3T3 cells showed that TTF-1 expression was specifically activated by Hox B3, but not the paralogous Hox D3 (Guazzi et al., 1994). Whether TTF-1 is also a target of Hox B3 in EC is not known. Studies that examined the effects of Hox B3 during hematopoietic differentiation indicated that Hox B3 can exhibit distinct effects on different cell populations. For example, whereas overexpression of Hox B3 impaired differentiation of B cells, it also promoted expansion of a subset of granulocyte/macrophage cells (Savageau et al., 1997). Thus, the Hox genes may exert tissue-specific influences on gene expression. Indeed, our previous work using Hox D3 expressing retrovirus indicated that upregulation of αvβ3 integrin was restricted to endothelial and blood cells, as infected epithelial and fibroblast cells did not express αvβ3 (Boudreau et al., 1997).

Although compound mutants of the Hox 3 paralogous group have also been generated, the embryonic lethal phenotype makes it impossible to study the contribution of these genes to EC-specific gene expression or angiogenesis in adult organisms (Manley and Capecchi, 1997). Furthermore, as evidence from compound mutants for Hox 9 members indicates, embryonic and adult tissues are differentially effected by the same Hox genes (Chen and Capecchi, 1999) and embryonic and adult angiogenesis may be subject to different modes of regulation (Bader et al., 1998). These genetic models may not help clarify the role of Hox genes in pathologically induced angiogenesis in differentiated adult EC.

Nonetheless, our findings suggest that the multistep process of neovascularization requires participation from at least two paralogous members of the Hox 3 gene family. Whereas Hox D3 is necessary to initiate vascular sprouting and migration in response to angiogenic stimuli such as bFGF, Hox B3 is subsequently required by sprouting EC

to undergo capillary morphogenesis. Whether the combined expression of Hox D3 and Hox B3 will also reveal additional synergistic interactions is currently not known, but is the subject of current investigations in our laboratory.

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