Homeobox A9 Transcriptionally Regulates the EphB4 Receptor to Modulate Endothelial Cell Migration and Tube Formation

Thomas Bruhl,* Carmen Urbich,* Diana Aicher, Amparo Acker-Palmer, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—Homeobox genes (Hox) encode for transcription factors, which regulate cell proliferation and migration and play an important role in the development of the cardiovascular system during embryogenesis. In this study, we investigated the role of HoxA9 for endothelial cell migration and angiogenesis in vitro and identified a novel target gene, the EphB4 receptor. Inhibition of HoxA9 expression decreased endothelial cell tube formation and inhibited endothelial cell migration, suggesting that HoxA9 regulates angiogenesis. Because Eph receptor tyrosine kinases importantly contribute to angiogenesis, we examined whether HoxA9 may transcriptionally regulate the expression of EphB4. Downregulation of HoxA9 reduced the expression of EphB4. Chromatin-immunoprecipitation revealed that HoxA9 interacted with the EphB4 promoter, whereas a deletion construct of HoxA9 without DNA-binding motif (Δaa 206-272) did not bind. Consistently, HoxA9 wild-type overexpression activated the EphB4 promoter as determined by reporter gene expression. HoxA9 binds to the EphB4 promoter and stimulates its expression resulting in an increase of endothelial cell migration and tube forming activity. Thus, modulation of EphB4 expression may contribute to the proangiogenic effect of HoxA9 in endothelial cells. (Circ Res. 2004;94:743-751.)

Key Words: homeobox ♦ migration ♦ angiogenesis ♦ Eph receptor ♦ endothelial cells

Homeobox morphoregulatory genes encode for transcription factors, which are characterized by a common 60 amino acid DNA-binding motif, the homeodomain, and are mostly arranged in four unlinked genomic clusters (HoxA through HoxD).1 Homeobox genes exert pleiotropic roles in many cell types and can regulate cell proliferation, differentiation, adhesion, and migration (see review2). They play an important role in organogenesis and the development of the cardiovascular system during embryogenesis as well as vessel remodeling in adults as it occurs during angiogenesis and atherosclerosis.2–4 Endothelial cells express homeobox genes from several clusters such as HoxA, HoxB, and HoxD.2 Migration and proliferation of endothelial cells are crucial for the process of angiogenesis and previous studies showed an important role of homeobox genes for an angiogenic phenotype in endothelial cells.5,6 Functional changes or morphological reorganization of endothelial cells may be regulated by homeobox genes via transcriptional regulation of cell adhesion molecules or matrix proteins.5,7,8 Indeed, HoxD3 has been described as an essential transcription factor for the protein expression of the β3-subunit of the αβ3 integrin and converts endothelial cells from the resting to the angiogenic state.5,9 Moreover, homeobox C6 has been shown to bind to the promoter of neural cell adhesion molecule (N-CAM) and to transcriptionally regulate its expression pattern.10 The liver cell adhesion molecule (L-CAM) as well as matrix proteins are under the control of homeobox transcription factors, eg, osteopontin is regulated by HoxC8 via interaction with Smad1 in the bone morphogenetic protein signaling.11–13

The homeobox A9 gene exhibits three exons resulting in the expression of two different proteins.14,15 Both HoxA9 proteins share a common exon encoding the homeodomain.15 One HoxA9 protein (HA-9A) is exclusively expressed in fetal development and a distinct HoxA9 isoform (HA-9B) is expressed in multiple tissues in the fetal and adult organism and specifically in endothelial cells.15,16 Mice lacking HoxA9 have a disturbed early T-cell development, an increased apoptosis of primitive thymocytes and defects in myeloid, erythroid, and lymphoid hematopoiesis.17,18 Overexpression of HoxA9 in bone marrow cells induces stem cell expansion and contributes to acute myeloid leukemia within 3 to 10 months in mice.19

The Eph receptor family is the largest known subfamily of receptor tyrosine kinases (see review20). Engagement of Eph
receptors by membrane-bound ephrin ligands regulates a variety of processes including embryonic vascular and neuronal development and may also be involved in adult functions such as synaptic plasticity, proliferation of stem cells, and cell migration.\textsuperscript{21,22} Recent reports demonstrated a role for the regulation of the EphA2-receptor by homeobox genes during development.\textsuperscript{23,24} Moreover, ephrinA1 ligand is decreased after transfection with antisense oligonucleotides against HoxB3 and the resulting defect in capillary morphogenesis can be restored by addition of clustered ephrinA1/Fc fusion proteins.\textsuperscript{6} Both, Eph receptors and ephrins are able to signal to the interior of the cell that expresses them, resulting in bidirectional signaling.\textsuperscript{25} Downstream signaling of ephrinB ligands is first regulated by phosphorylation by Src kinases and in a delayed fashion by PDZ-containing proteins including the PTP-BL phosphatase.\textsuperscript{26} Downstream signaling of EphB4 receptor includes the activation of the PI3K-pathway.\textsuperscript{27}

In this study, we show that HoxA9 plays an important role for migration and tube formation of endothelial cells in vitro. Downregulation of HoxA9 by RNA interference (RNAi) or antisense oligonucleotides resulted in a decreased cell migration and tube formation and decreased EphB4 expression. Consistently, overexpression of HoxA9 increased migration and the expression of EphB4. Moreover, HoxA9 binds to and transcriptionally regulates the EphB4 promoter (1365 bp upstream from ATG) of the EphB4 gene. Our findings suggest that HoxA9 is crucial for endothelial cell migration and may exert its function by regulating the expression of EphB4. This mechanism may play an important role for angiogenesis and revascularization after critical ischemia in adults.

Materials and Methods

Antibodies and Reagents

Anti-HoxA9, anti-HoxD9, anti-EphB4, anti-myc antibodies, and protein A/G plus Agarose beads were all purchased from Santa Cruz Biotechnology and rhodamine-conjugated anti-mouse antibody from Dianova. SiRNA-oligonucleotides were from Eurogentec. Sense/antisense oligonucleotides and oligonucleotides for PCR were generated by Sigma-ARK.

Cell Culture

Pooled human umbilical venous endothelial cells (HUVECs) were purchased from CellSystems and cultured in endothelial basal medium (EBM; CellSystems) supplemented with hydrocortisone (1

Figure 1. Homeobox A9 and endothelial cell function. A and B, HUVECs were transfected with HoxA9 antisense oligonucleotides or siRNA for 24 hours, lysed, and HoxA9 and HoxD9 expression was detected by Western blot analysis. Tubulin serves as loading control. Data are mean±SEM (% control). A, *P<0.05 vs sense, n=7. B, *P<0.05 vs scrambled, n=3. C, Endothelial cell migration was detected with a scratched-wound assay 24 hours after transfection. Data are mean±SEM. *P<0.01 vs control (untransfected cells) and vs scrambled; #P<0.05 vs control and vs sense, n=3. D, HUVECs were seeded on matrigel basement membrane matrix 24 hours after transfection. Length of tube-like structures was measured by light microscopy after 48 hours in a blinded fashion. Data are mean±SEM. *P<0.01 vs control (untransfected cells) and vs scrambled; **P<0.01 vs control and versus sense, n=3. E, Representative micrographs are shown. F through H, Apoptosis was detected by annexin-V staining and proliferation was measured by BrdU incorporation 24 hours after transfection. Data are mean±SEM (% control; n=3 to 4).
μg/mL), bovine brain extract (3 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 μg/mL), and 10% fetal calf serum (FCS) until the third passage. After detachment with trypsin, cells (4×10^6 cells) were seeded on matrigel basement membrane matrix 24 hours after transfection. Representative micrographs are shown. Length of tube-like structures was measured by light microscopy after 48 hours in a blinded fashion. Data are mean±SEM; *P<0.05 vs mock; n=5. C and D, HUVECs were transfected in HUVEC (3.5×10^6 cells/6-cm well) using GeneTrans II (Mobitec) according to the instructions of the manufacturer.

RNA Interference and Antisense Experiments

Sense (5′-ACTACTACGTGGACTCGTTC-3′; each 1.5 μg) or antisense (5′-AGCGGCAACGCTCAGCTCATC-3′) oligonucleotides corresponding to the human HoxA9 sequence and sense (5′-GACGGCGACAGGGAGAAGTG-3′) or antisense (5′-CAGTTCTCCTCAGCGTCGTC-3′) oligonucleotides corresponding to the human EphB4 sequence were incubated in 100 μL RPMI medium in the presence of 5 μL lipofectamine (Invitrogen) for 30 minutes at room temperature. HUVECs (3.5×10^6 cells/6-cm well) were washed with RPMI and incubated with 2 μL RPMI before the lipofectamine/oligonucleotide mixture was added. After 5 hours, 3 mL complete EBM was added.

Scrambled siRNA (5′-AGGUGUACAGCAUGCAGG-3′; each 360 pmol) or siRNA (5′-UGCGAGAAGAGAGCGGCC-3′) corresponding to the human EphB4 sequence were transfected in HUVECs (3.5×10^6 cells/6-cm well) using GeneTrans II (Mobitec) according to the instructions of the manufacturer.

Immunoprecipitation and Western Blot Analysis

For immunoprecipitation, cells were lysed in lysis buffer for 10 minutes (20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L PMSF), centrifuged for 15 minutes at 20,000g and the supernatant (500 μg protein) was incubated with 7 μg anti-myc or anti-HoxA9 on a rocking platform. After 12 hours, 25 μL protein A/G agarose beads were added and incubated for 2 hours on a rocking platform. After precipitation, the protein A/G agarose-protein complex was washed with lysis buffer and analyzed by Western blotting.

Migration Assays

Cells were grown on 6-cm wells, which were previously labeled with a traced line. In vitro “scratched” wounds (wide ~14 mm) were created by scraping cell monolayers.29,30 After injury, the cells were washed and cultured in EBM. Endothelial cell migration from the edge of the injured monolayer was quantified by measuring the distance between the wound edges before and after incubation using a computer-assisted microscope (Zeiss) at five distinct positions (every 5 mm).

Figure 2. Homebox A9 regulates endothelial cell migration and tube formation. A and B, HUVECs were transfected with a myc-tagged HoxA9 wild-type construct. Endothelial cell migration was detected in a scratched-wound assay (A) or a modified Boyden chamber (B) 48 hours after transfection. Data are mean±SEM. A, *P<0.01 vs mock, n=4. B, *P<0.05 vs mock, n=5. C and D, HUVECs were transfected in HUVEC (3.5×10^6 cells/6-cm well) using GeneTrans II (Mobitec) according to the instructions of the manufacturer.

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LYSED WITH LYSIS BUFFER (1% TRITON X-100, 0.32 MOL/L SUCROSE, ADDING 1% FORMALDEHYDE TO THE CULTURE MEDIUM. THE FIXED CELLS WERE FIXED WITH 2% PARAFORMALDEHYDE. FOR QUANTIFICATION, CELL NUCLEI WERE STAINED WITH 4,6-DIAMIDINO-PHENYLIDOLE (DAPI). MIGRATING CELLS INTO THE LOWER CHAMBER WERE COUNTERED MANUALLY IN THREE RANDOM MICROSCOPIC FIELDS.

FLOW CYTOMETRY
FOR MEASUREMENT OF PROLIFERATION, WE USED A BRDU FLOW KIT (BD BIOSCIENCES) ACCORDING TO THE INSTRUCTIONS OF THE MANUFACTURER. APOPTOSIS WAS INVESTIGATED BY ANNEXIN V-PE AND 7AAD STAINING (BD BIOSCIENCES). THE CHAMBER WAS WASHED AND FIXED WITH 2% PARAFORMALDEHYDE. FOR QUANTIFICATION, CELL NUCLEI WERE STAINED WITH 4',6-DIAMIDINO-PHENYLIDOLE (DAPI). MIGRATING CELLS INTO THE LOWER CHAMBER WERE COUNTERED MANUALLY IN THREE RANDOM MICROSCOPIC FIELDS.

TUBE FORMATION ASSAY
HUVECs (1 × 10⁴) WERE CULTURED IN A 12-Well PLATE (GREINER) COATED WITH 200 µL MATRIGEL BASEMENT MEMBRANE MATRIX (BD BIOSCIENCES). TUBE LENGTH WAS QUANTIFIED BY MEASURING THE "DISTANCE" OF TUBES WITH A COMPUTER-ASSISTED MICROSCOPE USING THE PROGRAM KS300 3.0 (ZEISS) AS PREVIOUSLY DESCRIBED.

CHROMATIN IMMUNOPRECIPITATION (CHIP)
HUVECs (1 × 10⁶) WERE CROSS-LINKED FOR 10 MINUTES BY DIRECTLY ADDING 1% FORMALDEHYDE TO THE CULTURE MEDIUM. THE FIXED CELLS WERE LYSED WITH LYSIS BUFFER (1% TRITON X-100, 0.32 MOL/L SUCROSE, 5 MMOL/L EDTA, 1 MMOL/L PMSF, 1 µG/M L LEUPEPTIN, 10 MMOL/L TRIS/HCL, pH 8) AND SONIFIED FIVE TIMES FOR 10 SECONDS WITH OUTPUT 5 (BRANSON SONIFIER 450, BRANSON). FOR CHROMATIN IMMUNOPRECIPITATION, CELLLYSATES WERE INCUBATED WITH AN ANTIBODY AGAINST HOX A9 OR MYC (SANTA CRUZ BIOTECHNOLOGY). THE FOLLOWING STEPS WERE PERFORMED ACCORDING TO THE INSTRUCTIONS OF THE MANUFACTURER (UPSTATE, HAMBURG, GERMANY). THE ISOLATED PRECIPITATED DNA WAS AMPLIFIED BY PCR WITH PRIMERS CORRESPONDING TO A ~310 BP FRAGMENT OF THE HUMAN EPHB4 PROMOTER (FORWARD 5'-ATGAATTTATTCGATGGTGAGTCC-3' AND REVERSE 5'-GCCTGAGCCTGCCGCTGGATCC-3'), HUMAN MONOCYTE CHEMOTACTANT PROTEIN-1 (MCP-1) PROMOTER (FORWARD 5' CAGGCTTTGTGCAGAGATGTC-3' AND REVERSE 5' GCCTTTGTCATATATGCAGACAG-3'), OR HUMAN EPHB2 PROMOTER (FORWARD 5'-CCACACTCTCCACAGAGITCCG-3' AND REVERSE 5'-CCTCAGTGCAGCTGTCTATG-3').

LUCIFERASE ASSAY
CELLS WERE COTRANSFECTED WITH HOX A9 WT OR HOX A9 MT AND LUCIFERASE UNDER THE CONTROL OF THE HUMAN EPHB4 PROMOTER (~1028/-7 BP OR ~1508/-7 BP). AFTER 20 HOURS, CELLS WERE LYSED WITH 1× REPORTER LYSIS BUFFER (PROMEGA), AND A SINGLE FREEZE-TWASH CYCLE WAS PERFORMED. THEN, THE CELLS WERE SCRAPPED AND CENTRIFUGED FOR 2 MINUTES AT 2000G. THE FOLLOWING MEASUREMENT OF THE LUCIFERASE ACTIVITY WAS ANALYZED WITH A LUMAT LB9510 LUMINOmeter (BERTHOHL) AS PREVIOUSLY DESCRIBED.

STATISTICAL ANALYSIS
DATA ARE EXPRESSED AS MEAN±SEM FROM AT LEAST THREE INDEPENDENT EXPERIMENTS. STATISTICAL ANALYSIS WAS PERFORMED BY t TEST. ANOVA WAS PERFORMED FOR SERIAL ANALYSES.

RESULTS
HOMEBOX A9 REGULATES ENDOTHELIAL CELL MIGRATION AND TUBE FORMATION
WE FIRST ADDRESSED THE ROLE OF HOX A9, WHICH IS EXPRESSED IN ENDOTHELIAL CELLS, FOR ENDOTHELIAL CELL FUNCTION. TRANSFECTON OF HUVECs WITH HOX A9 ANTISENSE OLIGONUCLEOTIDES AS WELL AS SMALL-INTERFERING RNA (siRNA) EFFICIENTLY SUPPRESSED PROTEIN EXPRESSION OF HOX A9 (Figures 1A AND 1B). AS CONTROL FOR THE SPECIFICITY OF THE ANTISENSE AND siRNA APPROACH, WE DETERMINED THE EXPRESSION OF HOXD9 OR HOXB4, WHICH WERE ESSENTIALLY UNCHANGED (Figure 1A, DATA NOT SHOWN). REDUCED EXPRESSION OF HOX A9 IN ANTISENSE- OR siRNA-TRANSFECTED HUVECs LED TO A SIGNIFICANT DECREASE OF ENDOTHELIAL CELL MIGRATION (Figure 1C). TO ADDITIONALLY ADDRESS THE INVOLVEMENT OF HOX A9 FOR THE PROANGIOGENIC ACTIVITY OF ENDOTHELIAL CELLS, WE DETERMINED THE EFFECT ON TUBE FORMATION IN A MATRIGEL ASSAY. AS SHOWN IN FIGURES 1D AND 1E, HOX A9 ANTISENSE- OR HOX A9 siRNA-TRANSFECTED ENDOTHELIAL CELLS DEMONSTRATED A SIGNIFICANTLY IMPAIRED TUBE FORMING ACTIVITY. IN CONTRAST, TRANSFECION OF HUVECs WITH HOX A9 ANTISENSE OLIGONUCLEOTIDES OR siRNA DID NOT SIGNIFICANTLY INFLUENCE ENDOTHELIAL CELL PROLIFERATION AND APOPTOSIS (FIGURES 1F THROUGH 1H), SUGGESTING THAT HOX A9 IS SPECIFICALLY REQUIRED FOR THE MIGRATORY AND TUBE FORMING ACTIVITY OF ENDOTHELIAL CELLS.

WE THEN ADDRESSED THE HOX A9 GAIN OF FUNCTION EFFECT ON ENDOTHELIAL CELL MIGRATION BY OVEREXPRESSION HOX A9 IN HUVECs. OVEREXPRESSION OF WILD-TYPE HOX A9 (HOX A9 WT) SIGNIFICANTLY INCREASED ENDOTHELIAL CELL MIGRATION AS ASSESSED BY BOTH SCRATCHED-WOUND ASSAYS AND A MODIFIED BOYDEN CHAMBER EXPERIMENTAL SET-UP (Figures 2A THROUGH 2D). THE
expression of HoxA9 wt was controlled by Western blot analysis and immunostaining (Figures 2E and 2F).

**Homeobox A9 Regulates EphB4 Expression**

EphB4 was previously shown to regulate angiogenesis and cell migration. Because Eph receptor tyrosine kinases are potential downstream targets of homeobox proteins, we analyzed the regulation of EphB4 by HoxA9. Downregulation of HoxA9 expression by antisense oligonucleotides or siRNA significantly reduced the expression of EphB4 (Figures 3A and 3B). In contrast, overexpression of HoxA9 wild-type significantly increased the EphB4 protein expression (Figure 3C), suggesting that HoxA9 indeed upregulates expression of EphB4.

To further elucidate whether the downregulation of EphB4 may account for the impaired migratory and tube-forming activity after HoxA9 downregulation, EphB4 expression was blocked by EphB4 antisense oligonucleotides and EphB4 siRNA (Figures 4A and 4B). Basal migration as assessed by the scratched-wound assay was significantly reduced in EphB4 antisense- or siRNA-treated HUVECs (Figures 4C and 4D). Likewise, EphB4 downregulation was associated with a significantly reduced tube formation (Figures 4E and 4F).

In addition, we investigated the effect of HoxA9 and EphB4 downregulation on ephrinB2-induced endothelial cell migration. Transfection of HUVECs with HoxA9 as well as EphB4 siRNA significantly reduced endothelial cell migration in the presence of ephrinB2 ligand (Figure 4G; data not shown).

In order to address the causal role of EphB4 for HoxA9-induced endothelial cell migration, we simultaneously transfected HUVECs with EphB4 siRNA and HoxA9 wild-type construct (Figure 4H). As shown in Figure 4I, the HoxA9-induced migration was significantly reduced in cells transfected with EphB4 siRNA, suggesting that HoxA9-regulated cell migration depends on EphB4 expression.

**Homeobox A9 Binds to the EphB4 Promoter**

Having demonstrated that HoxA9 regulates the expression of EphB4, we investigated the interaction of HoxA9 with the EphB4 promoter using chromatin immunoprecipitation assays. After cross-linking, immunoprecipitates of endogenous HoxA9 were subjected to subsequent PCR using primers...
directed against the EphB4 promoter sequence. The EphB4 promoter interacts with HoxA9 (Figures 5A and 5B). Importantly, the identity of the amplified immunoprecipitated DNA fragment was confirmed by gel elution and subsequent sequencing (data not shown). As a control, we performed PCR reactions with primers specific to the genomic GAPDH DNA (Figure 5B). GAPDH was only amplified, when genomic DNA was used, but not in the HoxA9-immunoprecipitated DNA (Figure 5B). Likewise, HoxA9 did not interact with the promoter of MCP-1 or EphB2 (Figure 5B). The interaction of HoxA9 with the EphB4 promoter was confirmed in immunoprecipitates of overexpressed myc-tagged HoxA9 (Figures 5C and 5D). The binding of EphB4 promoter was specific for the HoxA9 wt protein, because the HoxA9 mt (Δaa 206-272) lacking the DNA binding domain did not bind to the EphB4 promoter (Figure 5D).

Homeobox A9 Transcriptionally Activates the EphB4 Promoter
In order to prove that HoxA9 activates the transcription of EphB4, we performed a luciferase reporter assay. For this purpose, we cloned the luciferase reporter gene under the control of the EphB4 promoter. A construct (−1508/+7 bp) containing three putative HoxA9 binding sites within the EphB4 promoter was transfected together with wild-type HoxA9 or a deletion mutant of HoxA9 (Δaa 206-272) lacking the homeodomain (Figure 6A). HoxA9 wt overexpression significantly increased luciferase activity of the −1508/+7 bp EphB4 promoter construct (Figure 6B). To identify the HoxA9 binding sites within the EphB4 promoter, one of the three putative binding sites at position −617 bp upstream of ATG was first inactivated by site-directed mutagenesis (Figure 6A). Inactivation of this site (−1508mt1/−7 bp) reduced the HoxA9 WT-induced increase in reporter gene expression (Figure 6C). However, the mutated EphB4 promoter was still significantly activated by HoxA9. To further confirm these results, we transfected a shorter construct of the EphB4 promoter (−1028/+7 bp), which completely abolished the luciferase activity after HoxA9 WT cotransfection (Figure 6B). Because we detected only a partial reduction of luciferase activity in cells transfected with −1508mt1/−7 bp, but a complete inactivation when using the deletion construct (−1028/+7 bp), we hypothesized that there are additional key regulatory elements, which are located between −1508 bp and −1028 bp of the EphB4 promoter. Indeed, mutation of the −1365 bp motif upstream of ATG (−1508mt2/−7 bp) completely suppressed reporter gene activity (Figure 6C), suggesting an important regulatory effect of the TAAT motif at position −1365 bp within the EphB4 promoter.

Discussion
Homeobox genes are known to be functionally expressed in the adult organism and play a crucial role for angiogenesis and revascularization in normal tissue repair after injury or critical ischemia. HoxA9 has been shown to play a crucial role in T cell development, hematopoiesis, and stem cell expan-
However, a specific role of HoxA9 for endothelial cell function and angiogenesis has not been explored. Therefore, we investigated whether HoxA9 regulates angiogenesis. HoxA9 ablation in endothelial cells inhibits in vitro sprout formation and endothelial cell migration, whereas cell proliferation and apoptosis are not affected. Of note, we used antisense oligonucleotides in addition to RNAi to suppress HoxA9 expression, which excludes potential confounding effects of double-stranded RNA intermediates. These data indicate that HoxA9 exerts a specific role in endothelial cell migration without influencing other processes such as cell cycle/division.

Our study further identified a novel downstream target of HoxA9, namely the EphB4 receptor tyrosine kinase. HoxA9 downregulation significantly reduced EphB4 protein expression. Moreover, HoxA9-induced endothelial cell migration depends on the expression of EphB4. These data suggest that HoxA9 regulates expression of the EphB4 receptor in endothelial cells and, thereby, modulates the migratory capacity. However, HoxA9 may additionally affect other proteins (eg, integrins), which are involved in angiogenesis signaling. Thus, HoxA9 also regulated the expression of integrin αβ1, whereas integrin αβ2 was not affected (C.U., personal communication, 2003). Direct evidence for an effect of HoxA9 on the EphB4 transcription was provided by chromatin immunoprecipitation assays, which demonstrated that HoxA9 interacted with the EphB4 promoter. Interaction, thereby, was dependent on the DNA-binding domain of HoxA9, which is located at the C-terminus between aa 206 to 263. Moreover, HoxA9 overexpression also activated the expression of a reporter gene, which was driven by the EphB4 promoter. Two sequence motifs (TAAT and TTAT/C) were described as HoxA9 binding sites. Using site-directed mutagenesis and deletion constructs, our data suggest that the HoxA9 motif at position -1365 bp is essential for HoxA9 stimulation of the EphB4 promoter. Similarly, a TAAT-motif was shown to be essential for the regulation of osteopontin by HoxA9. In a study by Shi et al., HoxA9 acted as a transcriptional repressor to suppress TGFβ-stimulated expression of the proangiogenic molecule osteopontin.

![Figure 6. HoxA9 transcriptionally activates the EphB4 promoter. A, -1508/-7 bp fragment of the EphB4 promoter containing 3 putative HoxA9 binding motifs was cloned in a luciferase reporter vector. The -1508/-7 bp fragment of the EphB4 promoter was mutated by site-directed mutagenesis at position -617 bp (-1508mt1/-7 bp) and at position -1365 bp (-1508mt2/-7 bp), respectively, as indicated. A shorter construct of the EphB4 promoter (-1028/-7 bp) was cloned in a luciferase reporter vector. B, HUVECs were cotransfected with the EphB4 luciferase constructs (-1508/-7 bp or -1028/-7 bp) and HoxA9 wt or HoxA9 mt for 20 hours and the activity of luciferase was measured by enzymatic reaction. Data are mean ± SEM (RLU indicates relative light units). *P<0.01 vs mock, n=3. C, HUVECs were cotransfected with different EphB4 luciferase constructs and HoxA9 wild-type or HoxA9 mutant for 20 hours and the luciferase activity was measured. Data are mean ± SEM (% control). *P<0.05 vs mock, #P<0.05 vs HoxA9 wt/-1508/-7 bp and vs mock, **P<0.01 vs HoxA9 wt/-1508/-7 bp, n=3.](image-url)
tin in epithelial cells. This would results in an inhibition rather than activation of the angiogenic response. Because our study clearly supports a proangiogenic effect of HoxA9 and demonstrates that HoxA9 acts as a transcriptional activator, one may speculate that HoxA9 acts via distinct mechanisms in endothelial cells compared with TGFβ-stimulated endothelial cells. Previous studies suggested that HoxA9 form triple complexes with members of the EXD/PBX or MEIS family, which enhances DNA binding affinity and provides selectivity.42,43 However, the suggested consensus sequence A/G-T-G-A-T-C-G surrounding the HoxA9 binding motif (indicated in bold letters) does not resemble the sequence of the motif at position —1365 bp identified in the present study (C-C-A-G-C-T-A-A-T-T). The depletion of EphB4 by siRNA or antisense transfection revealed a similar phenotype as compared with gene ablation of HoxA9. Basal and ephrinB2-stimulated migration and tube-

formation activity was blocked in endothelial cells after reduction of EphB4 expression by siRNA or antisense treatment. Previous studies showed that mice lacking the EphB4 receptor revealed an impaired remodeling of the hierarchically vascular system that consists of large vessels and capillaries.44 Eph receptors, namely EphA2 and EphA3, are also important for adult neovascularization.45,46 Moreover, elevated expression of Eph receptors and/or ephrin ligands is associated with tumors and tumor vascularization (see review47). This is in accordance with the results of the present study, which demonstrate that EphB4 is essential for endothelial cell migration and tube formation, a hallmark of angiogenesis. Furthermore, EphB4 promotes the differentiation of human CD34+ hematopoietic cells and was shown to be essential for the development of the hemangioblast, suggesting that EphB4 may link angiogenesis and hematopoiesis.48,49 Interestingly, HoxA9 also was shown to be crucial for hematopoietic stem cell development and stem cell expansion.19,37 Postnatal vascularization involves circulating bone marrow–derived endothelial progenitor cells, which in part resemble the hemangioblast in embryonal development. Because these endothelial progenitor cells express HoxA9 and EphB4 (C.U., personal communication, 2003), one may speculate that the HoxA9 EphB4 axis may also regulate hemangio- blasts and contribute to progenitor cell-mediated vasculogenesis.

In summary, HoxA9 modulates endothelial cell migration and tube formation in vitro via the expression of the EphB4 receptor. This may contribute to the proangiogenic effect of HoxA9 in endothelial cells.

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