The Transcription Factor HOXC9 Regulates Endothelial Cell Quiescence and Vascular Morphogenesis in Zebrafish via Inhibition of Interleukin 8

Sandra J. Stoll, Susanne Bartsch, Hellmut G. Augustin, Jens Kroll

Rationale: The transcription factor HOXC9 belongs to the homeobox gene family acting as developmental morphogen in several species. HOXC9 is expressed in different vascular beds in vivo. Yet vascular functions of HOXC9 have not been studied.

Objective: This study was aimed at characterizing HOXC9 functions in human vascular endothelial cells and in zebrafish vascular development.

Methods and Results: HOXC9 was abundantly expressed in resting human umbilical vein endothelial cells and was downregulated by hypoxia. Overexpression of HOXC9 inhibited endothelial cell proliferation, migration, and tube formation in vitro. Expression profiling and chromatin immunoprecipitation experiments in human umbilical vein endothelial cells identified interleukin 8 as the major HOXC9 target and demonstrated the direct binding of HOXC9 to the interleukin 8 promoter. HOXC9 overexpression led to reduced endothelial interleukin 8 production, whereas HOXC9 silencing increased interleukin 8. The antimigratory and antiangiogenic effect of HOXC9 overexpression could be rescued by external interleukin 8 administration. Corresponding to the cellular experiments, endothelial-specific overexpression of HOXC9 and morpholino-based interleukin 8 loss-of-function experiments inhibited zebrafish vascular development.

Conclusion: The data identify HOXC9 as an endothelial cell active transcriptional repressor promoting the resting, antiangiogenic endothelial cell phenotype in an interleukin 8–dependent manner. (Circ Res. 2011;108: 1367-1377.)

Key Words: HOXC9 ■ interleukin 8 ■ vascular morphogenesis ■ zebrafish
eration, and angiogenic activation in tumor angiogenesis.\(^3,4\) For example, members of the paralogous group 3—eg, HOXA3,\(^5\) HOXB3,\(^6\) and HOXD3\(^7\)—regulate angiogenic functions such as tube formation, capillary morphogenesis, and endothelial cell proliferation, suggesting that paralogous group 3 may positively control angiogenic functions. In turn, members of the paralogous group 5—eg, HOXA5,\(^8\) HOXB5,\(^9\) and also HOXD10\(^10\)—appear to be negative regulators or modifiers of angiogenesis controlling the expression and interaction of important angiogenic molecules, such as angiopoietin-2 and VEGF receptor-2. Last, members of the paralogous group 9—eg, HOXA9 and HOXC9—have also been studied in the vasculature. Silencing of HOXA9 expression inhibited endothelial cell migration and tube formation. HoxA9 activates expression of the receptor tyrosine kinase EphB4 and binds the eNOS and VEGF receptor-2 promoter in vitro.\(^11,12\) HOXC9 is a differentially expressed gene in fetal human smooth muscle cells. It has been suggested that HOXC9 may play a role in specifying the fetal smooth muscle cell phenotype.\(^13\) Recently, HOXC9 has also been shown to be abundantly expressed in CD133 positive cells,\(^14\) in human bone marrow–derived mesenchymal stem cells,\(^15\) and in hindlimb blood vessels.\(^16\) Yet vascular functions of HOXC9 are not established. Together, the data highlight HOXC9 as a very attractive candidate and potential major regulator of endothelial function and angiogenesis.

Interleukin 8 (IL-8; CXCL8) is a chemokine regulating the release of granular enzymes from neutrophils.\(^17\) Besides its function as an inflammation- and infection-regulating cytokine, IL-8 is also involved in the regulation of angiogenesis, tumor progression, and tissue remodeling.\(^18,19\) Human IL-8 binds to the 2 G-protein coupled receptors CXCR1 and CXCR2 and induces angiogenesis in vitro (eg, by regulating chemotaxis of human umbilical vein endothelial cells [HUVECs]) and in vivo (eg, by activating angiogenesis in the
corneal pocket assay). Gene-targeting studies in mice of IL-8 and its receptors CXCR1 and CXCR2 are hampered by the limited cross-species homology of the corresponding genes. A closely related homolog of human IL-8 does not exist in the mouse genome. Correspondingly, gene-targeting studies of the mouse IL-8 receptor (mIL-8Rh; the homolog of human CXCR2) have shown that mIL-8Rh is dispensable during mouse development. Yet, mIL-8Rh–deficient mice display defects in neutrophil migration and delayed wound healing. Interestingly, zebrafish express homologs for human IL-8 and its receptors CXCR1 and CXCR2, which highlights the zebrafish as an attractive model to study IL-8 and its receptors in vivo. Zebrafish CXCL8 (IL-8) is expressed in leukocytes and in intestinal epithelial cells, and its expression is controlled by proinflammatory stimuli. CXCR1 and CXCR2 are robustly expressed in the intermediate cell mass, where vascular and primitive hematopoietic tissues arise, and within the developing gut and head; yet functional gene-silencing experiments in zebrafish have not been reported.

Using a combination of several cellular and in vivo experiments, the experiments of this study have identified HOXC9 as a major negative regulator of angiogenesis in vitro and of vascular development in zebrafish by repressing IL-8 functions. In HUVEC, HOXC9 is highly upregulated in resting endothelial cells. It is downregulated during hypoxic episodes, and HOXC9 represses angiogenic activation of endothelial cells. In zebrafish, HOXC9 overexpression inhibits vascular development, and expression silencing of its target gene IL-8 recapitulates a similar vascular phenotype. Therefore, the data identify HOXC9 as an endothelial cell active transcription factor promoting the resting, antiangiogenic endothelial cell phenotype in an IL-8–dependent manner.

Methods

Embryos of AB wildtype and the tgf/fil1:EGFP) line were raised and staged as described. Embryos were kept in E3 solution at 28.5°C with or without 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO) to suppress pigmentation and staged according to
somite number or hours postfertilization (hpf). HUVECs were cultured in endothelial cell growth medium EGM-2 (PromoCell, Heidelburg, Germany) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. The following antibodies were used for this study: mouse anti-HOXC9[2] (ab50839) (Abcam, Cambridge, UK), goat anti-HOXC9[1] (G-14) (sc-82914, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-chloramphenicol acetyltransferase gene (CAT) (ab50151) (Abcam), goat anti-IL-8 (ab10769) (Abcam), rabbit anti-NFκB p65 (sc-109, Santa Cruz Biotechnologies), mouse anti-RNA Polymerase II, clone CTD4H8 (EZ-ChIP Chromatin Immunoprecipitation Kit, Upstate, Temecula, CA), normal mouse IgG (EZ-ChIP Chromatin Immunoprecipitation Kit, Upstate), goat anti-actin (I-19) (Santa Cruz Biotechnologies), mouse anti-RNA Polymerase II, clone CTD4H8 (EZ-ChIP Chromatin Immunoprecipitation Kit, Upstate), goat anti-actin (I-19) (Santa Cruz Biotechnology), rabbit anti-GFP (A-11122) (Invitrogen, Carlsbad, CA), Alexa-546 coupled secondary antibodies (molecular probes), and HRP-conjugated antibodies (DAKO, Carpinetia, CA). VEGF was purchased from R&D Systems, and IL-8 (human, recombinant) was purchased from PeproTech (London, UK). Desferrioxamine mesylate and Evans blue were purchased from Sigma-Aldrich.

For details regarding siRNA transfection and viral transduction of HUVECs, hypoxic treatment of HUVECs, Western blot analysis, in vitro angiogenesis assays,28 measurement of secreted IL-8 via EASIA, chromatin immunoprecipitation, reporter gene assay, apoptosis assay, reverse transcription polymerase chain reaction (RT-PCR) protocols, DNA-, morpholino-, and intracardiac injections,29 flow cytometry, whole mount antibody staining, and immunohistochemistry,30 statistical analysis and quantification, and microscopy and analysis, see the Online Supplemental Data at http://circres.ahajournals.org.

Results
HOXC9 Is Abundantly Expressed in Resting Endothelial Cells, Is Downregulated by Hypoxia and Inhibits Endothelial Cell Proliferation, Migration, and Tube Formation in Vitro
HOXC9 has been reported to be expressed in fetal human smooth muscle cells,13 in CD133 positive cells,14 in human bone marrow–derived mesenchymal stem13 cells, and in hind limb blood vessels.16 Yet it remained unclear whether HOXC9 is expressed in endothelial cells, how HOXC9 expression is regulated in endothelial cells, and whether HOXC9 may exert critical roles in regulating endothelial functions and angiogenesis. First, we analyzed expression of
HOXC9 in HUVECs and identified a strong nuclear signal in these cells (Figure 1A). The amount of the HOXC9 protein, however, was strongly dependent on endothelial cell monolayer density. In sparse culture (50% confluence), HOXC9 protein was only weakly expressed (Figure 1B and 1D). In contrast, confluent and superconfluent HUVEC monolayers expressed strongly upregulated levels of HOXC9. Most abundant expression of HOXC9 was observed in HUVEC spheroids, which represents a 3-dimensional intact quiescent endothelial cell culture in vitro, probably reflecting the best in vitro representation of the quiescent endothelium as it occurs in vivo31 (Figure 1B and 1D and Online Figure I). In contrast, angiogenic activation of endothelial cells using hypoxic conditions strongly inhibited HOXC9 expression in endothelial cells. In fact, HOXC9 expression was downregulated close to the detection limit within 24 hours after hypoxic treatment (Figure 1C and 1E and Online Figure I). This process was fully reversible, as evidenced by rising levels of HOXC9 expression following hypoxia withdrawal (Figure 1C and 1E). Similar data were observed under the hypoxic mimicry desferrioxamine mesylate, revealing reduced levels of HOXC9 in endothelial cells (Online Figures I and II). In contrast, angiogenic activation of endothelial cells using hypoxic conditions strongly inhibited HOXC9 expression in endothelial cells. In fact, HOXC9 expression was downregulated close to the detection limit within 24 hours after hypoxic treatment (Figure 1C and 1E). This process was fully reversible, as evidenced by rising levels of HOXC9 expression following hypoxia withdrawal (Figure 1C and 1E). Similar data were observed under the hypoxic mimicry desferrioxamine mesylate, revealing reduced levels of HOXC9 in endothelial cells (Online Figures I and II). Together, the data identify HOXC9 as a cell density–regulated protein that is downregulated in active endothelial cells and on exposure to hypoxic environments. The prominent expression of HOXC9 in quiescent resting endothelial cells consequently suggested a role of HOXC9 in controlling the transition from the resting to the activated endothelial cell phenotype.

In order to examine the role of HOXC9 in controlling the quiescent endothelial cell phenotype, we performed a series of HOXC9 gain-of-function experiments. Adenoviral-driven expression of HOXC9 in HUVECs (Figure 2A, 2B, 2B’ and Online Figure I) strongly inhibited baseline but not VEGF-driven endothelial cell migration in the modified Boyden chamber assay (Figure 2C). Furthermore, tube formation and endothelial cell proliferation in vitro were similarly inhibited in HOXC9 overexpressing HUVECs (Figure 2D and 2E). To exclude unspecific effects of adenoviral HOXC9-driven expression on cell viability, equal numbers of GFP- or HOXC9-transduced HUVECs were seeded, and cell numbers were determined at different time points. Although GFP-transduced HUVECs grew rapidly, growth of HOXC9-transduced HUVECs was inhibited, but no signs of apoptosis were observed (Figure 2F and 2G). In summary, the data demonstrate that HOXC9 expression exerts a negative growth regulatory effect on endothelial cells and blocks functions related to angiogenesis.

**HOXC9 Regulates Endothelial Quiescence and Capillary-like Tube Formation by Regulating Endothelial Cell IL-8**

In order to identify HOXC9 target genes in endothelial cells, we performed a transcriptome analysis of HOXC9-transduced HUVECs. This screen identified IL-8 as a negatively regulated angiogenic gene yielding a 7.6-fold reduction of IL-8 mRNA expression following adenoviral HOXC9 transduction; yet other important angiogenic molecules were
Changes in IL-8 mRNA expression were further validated by quantitating IL-8 protein in the supernatant of the different cell populations. EASIA quantitation revealed significantly reduced levels of IL-8 in the supernatants of HOXC9 overexpressing HUVECs (Figure 3A and Online Figure IV). Correspondingly, the amount of IL-8 protein was also reduced in the lysate of HOXC9 overexpressing HUVECs (Figure 3A, box). In contrast, siRNA-based silencing of HOXC9 resulted in increased levels of IL-8 protein in cell lysates as well as in the supernatant (Figure 3B, 3D, and 3E and Online Figures I and IV). Interestingly, 56 hours after HOXC9 siRNA transfection, IL-8 levels were not any longer increased (Figure 3B and Online Figure IV). This reflects the increased expression of HOXC9 in these settings because it has been observed in confluent and superconfluent endothelial cells (Figure 1B and 1D). Finally, we examined IL-8 levels in HUVEC supernatants grown in sparse culture (low confluence) or under confluent conditions (high confluence), because a reduced expression of HOXC9 in sparsely cultured cells (Figure 1B and 1D) would imply an enhanced secretion of IL-8, and vice versa. Indeed, secretion of IL-8 was strongly reduced in confluent endothelial cells (Figure 3C and Online Figure IV), indicating that high expression of HOXC9 in confluent endothelial cells inhibits IL-8 secretion. Moreover, the data also showed that sparse endothelial cells, e.g., angiogenic endothelial cells, express high amounts of IL-8 (Figure 3C and Online Figure IV). Next, we examined whether the functional consequences of the negative effects of HOXC9 on endothelial cell IL-8 production could be rescued by external IL-8 administration. Endothelial cell migration, endothelial cell proliferation, and Matrigel tube–forming assays with HOXC9 expressing HUVECs were performed toward this end (Figure 4). Exogenous IL-8 was able to completely reverse the negative effect of HOXC9 on endothelial cell migration and Matrigel tube formation, confirming IL-8 as the major target of HOXC9-mediated control of angiogenic endothelial cell invasive migration (Figure 4A and 4B). Yet, endothelial cell proliferation could not be rescued by exogenous IL-8 administration (data not shown), suggesting the involvement of other, yet unidentified HOXC9 downstream targets in the control of endothelial cell proliferation.

To further validate IL-8 as an HOXC9 downstream target, we performed interleukin-8 reporter assays using different interleukin-8 reporter constructs corresponding to the first −1481, −546, −272, −185, −98, or −8 base pairs (bp) of the IL-8 promoter driving expression of CAT (Figure 5A and 5B). Coexpression of the single interleukin-8 reporter constructs together with HOXC9 showed a transcriptional repression of interleukin-8 by HOXC9 as evidenced by reduced expression of CAT in HeLa cells as shown in Figure 5B. Cherry-transduced HeLa cells served as controls and were normalized to 100% (n=3).
expression of CAT (Figure 5A and 5B). Interestingly, HOXC9 inhibited CAT expression in all reporter constructs except for the shortest construct (−8 bp), which did not show a significant expression under control conditions. This suggested a binding site for HOXC9 within the first 98 bp of the interleukin-8 promoter. Indeed, sequence analysis of this interleukin-8 promoter region identified a repressor consensus sequence for HOX proteins (Online Figure V). Last, we performed chromatin-immunoprecipitation assays to examine whether HOXC9 binds directly to the IL-8 promoter. Chromatin immunoprecipitates of HOXC9 (Figure 5C) were subsequently analyzed by genomic PCR using specific IL-8 primers amplifying parts of the promoter region of IL-8. Indeed, we identified an IL-8 promoter PCR signal in HOXC9 immunoprecipitates demonstrating a direct interaction of HOXC9 with the IL-8 promoter (Figure 5D). A similar approach for NFκB, which also binds directly to the IL-8 promoter, was run in parallel as a positive control. Furthermore, an internal assay control using an RNA polymerase II antibody for chromatin immunoprecipitation assays to examine whether HOXC9 binds directly to the IL-8 promoter. Chromatin immunoprecipitates of HOXC9 (Figure 5C) were subsequently analyzed by genomic PCR using specific IL-8 primers amplifying parts of the promoter region of IL-8. Indeed, we identified an IL-8 promoter PCR signal in HOXC9 immunoprecipitates demonstrating a direct interaction of HOXC9 with the IL-8 promoter (Figure 5D). A similar approach for NFκB, which also binds directly to the IL-8 promoter, was run in parallel as a positive control. Furthermore, an internal assay control using an RNA polymerase II antibody for chromatin immunoprecipitation and a genomic PCR protocol for GAPDH were also included (Figure 5E). No genomic PCR signal for IL-8 was identified when using a control antibody instead of the HOXC9 and NFκB antibodies for immunoprecipitations (Figure 5D). In summary, the experiments show direct binding of HOXC9 to the IL-8 promoter and identified HOXC9 as a major transcriptional repressor of IL-8.

HOXC9 Regulates Vascular Morphogenesis in Zebrafish in an IL-8–Dependent Manner

The cellular experiments had identified an important role of HOXC9 in regulating angiogenesis-related endothelial cell functions by regulating endothelial cell IL-8 production. In order to validate these data in vivo, we performed HOXC9 gain-of-function experiments in zebrafish aimed at studying HOXC9 during vascular development. HOXC9 was ubiquitously expressed during early embryonic zebrafish development. It was strongly expressed posterior in the neural tube and in the cardinal vein 24 hours postfertilization (hpf) (Online Figure VI). To study the consequences of vascular HOXC9 overexpression, we pursued endothelial cell–specific HOXC9 overexpression experiments using the flk1 promotor to selectively target endothelial cells (Figure 6A and 6B). Injection of the flk1:HOXC9 DNA construct into 1 cell stage of tg(fli1:EGFP) fish embryos resulted in increased endothelial cell HOXC9 expression as evidenced by fluorescence-activated cell sorter analysis and RT-PCR of isolated endothelial cells (GFP+) from 48-hpf tg(fli1:EGFP) fish embryos. GFP− indicates expression of HOXC9 in GFP negative, nonendothelial cells (eg, neural cells). D, Quantification of 48-hpf tg(fli1:EGFP) fish embryos showing a disturbed trunk vasculature. Black scale bar: 500 μm. White scale bar: 50 μm.
defects seen in endothelial cell HOXC9 overexpressing zebrafish embryos. Silencing of IL-8 expression in zebrafish using 2 independent splice-blocking morpholinos (IL8-SB1-Mo and IL8-SB2-Mo) indeed reproduced the vascular phenotype of endothelial cell HOXC9 gain-of-function experiments in 48 hpf \(tg(fli1:EGFP)\) fish embryos (Figure 7A, 7A/H11032, 7B, 7B/H11032, 7C, 7C/H11032, 7E, 7E/H11032, 7F, and 7H). Several ISVs did not form correctly, and the DLAV was partially interrupted in the \(tg(fli1:EGFP)\) morphants. To validate the specificity of the IL-8 morpholino experiments, we performed rescue experiments using recombinant IL-8 protein. The IL8-SB1-Mo was injected into the 1-cell stage, followed by cardiac injection of 25 pg IL-8 at 24 hpf. The vasculature of \(tg(fli1:EGFP)\) morphants was analyzed at 48 hpf. E, E', Functionality of the splice-blocking morpholinos IL8-SB1-Mo and IL8-SB2-Mo. RT-PCR of Mo control (4 ng), IL8-SB1-Mo, and IL8-SB2-Mo injected fish embryos at 24 hpf. F, Quantification of vascular defects in 48 hpf \(tg(fli1:EGFP)\) IL-8 morphants and rescue experiments of IL-8 morphants using recombinant IL-8. Black scale bars: 500 \(\mu m\) (A through D), white scale bars: 50 \(\mu m\). G, G', Uniform vascular distribution of Evans blue (arrows) after cardiac injection in 24 hpf fish embryos showing the feasibility of cardiac recombinant IL-8 injection. Black scale bars: 200 \(\mu m\). H, Expression of IL-8 in \(tg(fli1:EGFP)\) zebrafish endothelial cells at 48 hpf as shown by RT-PCR of GFP (GFP+) positive sorted cells. Wt indicates wild type; Morph, morphant.

cardiac injection of IL-8 was able to rescue the IL-8 morpholino-driven vascular phenotypes (Figure 7D, 7D', 7F). To causally link the phenotype of endothelial cell HOXC9 overexpression to downregulated IL-8 production, we next examined whether intravascular IL-8 protein injection in zebrafish embryos would be able to rescue the vascular defects of zebrafish embryos overexpressing HOXC9 in endothelial cells. The flk1:HOXC9 DNA construct was toward this end injected into the 1-cell stage of \(tg(fli1:EGFP)\) fish embryos, followed by cardiac IL-8 injection at 24 hpf. Visualization of the vasculature of 48 hpf \(tg(fli1:EGFP)\) fish embryos showed normally developed ISV and DLAV in the majority of fish embryos, indicating that external administration of IL-8 was indeed able to rescue the negative effect of HOXC9 on the formation of the vasculature (Figure 8A, 8A', 8B, 8B', 8C, 8C', and 8D). Last, we studied whether the silencing of HOXC9 expression in zebrafish embryos would
conversely lead to upregulated IL-8 expression as it had been observed in the cellular experiments (Figure 3B). Indeed, silencing of HOXC9 expression in zebrafish embryos using the translational blocking HOXC9 morpholino HOXC9-TB-Mo (Figure 8F) led to increased expression of IL-8 in zebrafish morphants at 48 hpf (Figure 8E). Interestingly, this did not result in excessive angiogenesis in the HOXC9 morphants (data not shown), indicating that IL-8 on its own was not able to induce ectopic angiogenesis in zebrafish. In conclusion, the data validate HOXC9 as an essential negative regulator of vascular development in zebrafish driven by IL-8.

**Discussion**

The homeobox gene HOXC9 was identified in this study as an endothelial cell active transcriptional repressor promoting the resting, antiangiogenic endothelial cell phenotype that negatively regulates vascular morphogenesis in an IL-8–dependent manner. This conclusion is based on the following findings showing (i) abundant expression of HOXC9 in resting endothelial cells and downregulation of HOXC9 under hypoxia; (ii) inhibition of angiogenesis-related functions in cultured endothelial cells by HOXC9; (iii) direct binding of HOXC9 to the IL-8 promoter; (iv) rescue of HOXC9 inhibitory functions in vitro by exogenous IL-8; (v) vascular developmental defects in HOXC9 gain-of-function experiments in zebrafish; and (vi) rescue of HOXC9-driven vascular defects in zebrafish by IL-8 injection. The data identified HOXC9 as a major antiangiogenic transcription factor silencing IL-8 expression and inhibiting angiogenesis in vitro and in vivo (Online Figure VIII).

Angiogenesis is regulated by a number of angiogenic factors. Among them, VEGF stands out as the primary upstream inducer of the angiogenic cascade. HOXC9 acts as an antiangiogenic transcription factor by repressing IL-8 expression. Yet this does not affect the expression of VEGF and other known angiogenic factors (Online Figure III). Moreover, HOXC9 does not interfere with VEGF-dependent signaling in endothelial cells. This conclusion is supported by the reduced HOXC9-mediated baseline endothelial cell migration, proliferation, and tube formation and at the same time maintained responsiveness to VEGF-driven angiogenesis. Thus, HOXC9 does not regulate VEGF-driven vascular development, but rather mediates vessel development, endothelial cell quiescence, and pathological angiogenesis independently of VEGF.

The identified HOXC9 target IL-8 is a well-established positive regulator of angiogenesis. Yet mechanistically, the role of IL-8 in regulating angiogenesis is poorly understood. This is largely due to the lack of appropriate murine genetic models, which results from the fact that the mouse genome harbors no closely related homolog to human IL-8. To circumvent these limitations, we used zebrafish as an in vivo model to study the role of IL-8 during developmental angiogenesis and to establish in gain-of-function and loss-of-function experiments the cause and consequence relationship between endothelial HOXC9 expression and IL-8–mediated regulation of angiogenesis. IL-8 and its receptors CXCR1 and
CXCR2 have only recently been described in zebrafish.\(^{24}\) Morpholino-based gene-silencing studies for IL-8 inhibited vascular development with a largely complementary phenotype resulting from the endothelial cell restricted overexpression of HOXC9. Most definitely, exogenous IL-8 protein was capable of rescuing the angiogenesis defects of HOXC9 overexpressing zebrafish. Thus, the experiments do not just further characterize IL-8 as a regulator of angiogenesis, but more importantly establish a HOXC9/IL-8 axis as critical mediators controlling the switch from the quiescent to the activated endothelial cell phenotype.

IL-8 is only faintly expressed and secreted by quiescent, nonstimulated endothelial cells. Yet on cytokine stimulation, such as by tumor necrosis factor or interleukin-1, IL-8 is transcriptionally activated by AP-1 and NF-κB.\(^{36}\) This implies that expression of IL-8 must be well regulated to prevent an uncontrolled activation. Transcriptional repression of IL-8 can be accomplished by 3 different mechanisms. First, NF-κB repression factor NRF binds to the IL-8 promoter and represses its activation; second, binding of the transcription factor octamer-1 to the IL-8 promoter and third, by deacetylation of histone proteins driven by histone deacetylase-1.\(^{36}\) Our data have now identified a novel mechanism showing that IL-8 repression can also be induced by direct HOXC9 binding to the IL-8 promoter in endothelial cells. This highlights HOXC9 as a major transcriptional repressor of IL-8 in endothelial cells.

In summary, the data of this study have identified a novel transcriptional mechanism of endothelial cell control during vascular development. Because IL-8 is an important regulator of pathological angiogenesis, such as in tumor angiogenesis and in inflammation, and HOXC9 selectively represses angiogenic activation by IL-8, this raises the interesting question of whether a specific activation of HOXC9 may cause inhibition of pathological angiogenesis in vivo. Furthermore, because HOXC9 acts independently of VEGF, HOXC9 repression leading to an increased IL-8 expression may be a contributing mechanism of anti-VEGF resistance during antiangiogenic therapies.\(^{37}\)

**Acknowledgments**

The authors are grateful to Dr Heinz-Georg Belting, University of Basel for providing the zebrafish flk1 promotor construct, Katrin Bennewitz and Melanie Grassl, Heidelberg University, Medical Faculty Mannheim for technical assistance in fluorescence-activated cell sorting of endothelial cells and Dr. Carmen Urbich and Jes-Niels Böckel, University of Frankfurt, for technical help in performing the chromatin immunoprecipitation assays.

**Sources of Funding**

This work was supported by grants from Deutsche Forschungsgemeinschaft (KR1887/4-3, KR1887/5-1, SFB/TR23, project Z5 [to J.K.] and SFB/TR23, project A3 [H.G.A.]).

**Disclosures**

None.

**References**

protein kleip controls endothelial migration and sprouting angiogenesis. Circ Res. 2007;100:1155–1163.


Novelty and Significance

What Is Known?

• The vascular system is the first organ formed during embryonic development.
• Transcription factors regulate several processes important for vascular development, such as endothelial cell formation, differentiation, vessel maturation and remodeling, arteriovenous differentiation, and lymphatic patterning.
• HOX genes act as essential transcriptional regulators for spatial body development.
• HOXC9 is expressed in different vascular beds in vivo.

What New Information Does This Article Contribute?

• HOXC9 is abundantly expressed in resting endothelial cells and is downregulated by hypoxia.
• HOXC9 inhibits endothelial cell proliferation, migration, and tube formation in vitro in an interleukin 8–dependent manner.
• HOXC9 binds directly to the interleukin 8 promoter and acts as a transcriptional repressor of interleukin 8.
• In zebrafish, endothelial-specific overexpression of HOXC9 or the loss of interleukin 8 inhibits vascular morphogenesis.

• Vascular developmental defects mediated by HOXC9 can be rescued by interleukin 8 in zebrafish.

HOX genes are transcription factors regulating important processes of morphogenesis and body development. Recent studies have shown that HOX genes are major regulators for physiological and pathological processes and initial reports have also suggested that some HOX genes regulate various vascular processes. Yet targets and mechanisms are mostly unknown. We show here that HOXC9 acts as a transcriptional repressor of interleukin 8 that keeps mature endothelial cells in a quiescent, antiangiogenic state. Abundant expression of HOXC9 leads to reduced endothelial cell migration, proliferation, and tube formation and inhibits vascular development in zebrafish. These HOXC9-driven negative vascular effects can be rescued by interleukin 8 in vitro and in vivo. Furthermore, loss-of-function experiments for interleukin 8 in zebrafish demonstrated the same vascular phenotype as did the endothelial-specific HOXC9 overexpression. Thus, we describe for the first time an essential developmental vascular function of interleukin 8 in vivo, and we show that HOXC9 acts as a major transcriptional repressor for interleukin 8.
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Circ Res. 2011;108:1367-1377; originally published online April 14, 2011; doi: 10.1161/CIRCRESAHA.111.244095
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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The transcription factor HOXC9 regulates endothelial cell quiescence and vascular morphogenesis in zebrafish via inhibition of interleukin 8

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Subject code: [97, 129]

Running title: Regulation of endothelial quiescence by HOXC9

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Methods

Zebrafish lines, cell lines, antibodies and reagents
Embryos of AB wildtype and the \(tg(fli1:EGFP)\) line were raised and staged as described. Embryos were kept in E3 solution at 28.5°C with or without 0.003% 1-phenyl-2-thiourea (Sigma) to suppress pigmentation and staged according to somite number or hours post-fertilization (hpf). Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial cell growth medium EGM-2 (PromoCell) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. We used following antibodies for this study: mouse anti-HOXC9 [2] (ab50839) (Abcam), goat anti-HOXC9 [1] (G-14) (Santa Cruz Biotechnologies, sc-82914), goat anti-IL-8 (ab10769) (Abcam), rabbit anti-NF\(\kappa\)B p65 (Santa Cruz Biotechnologies, sc-109), mouse anti-RNA Polymerase II, clone CTD4H8 (Upstate, EZ-ChIP Chromatin Immunoprecipitation Kit), normal mouse IgG (Upstate, EZ-ChIP Chromatin Immunoprecipitation Kit), goat anti-Actin (I-19) (Santa Cruz Biotechnology), rabbit anti-GFP (A-11122) (Invitrogen), Alexa-546 coupled secondary antibodies (molecular probes) and HRP-conjugated antibodies (DAKO). Vascular endothelial growth factor (VEGF) was purchased from R&D Systems; IL-8 (human, recombinant) was purchased from PeproTech. Desferrioxamine mesylate (DFX) and Evans blue were purchased from Sigma-Aldrich.

Transfection and viral transduction of HUVEC
Small interfering RNAs for HOXC9 (ID3678) and a validated non-targeting negative control 1 siRNA were synthesized by Ambion. HUVEC were transfected with the indicated siRNA (final concentration: 200nmol/L) using Oligofectamine (Invitrogen) Plasmids were transfected using Lipofectamine (Invitrogen) according to the protocol of the manufacturer. The transfection medium was replaced after 4h by ECGM containing 10% FCS and the cells were incubated for another 48h. Adenoviruses were produced according to the ViraPower Adenovirus Expression Systems protocol (Invitrogen). The full length human HOXC9, GFP or Cherry sequences were cloned into the adenovector and for the transduction a multiplicity of infection (MOI) of 35 was used.

Hypoxic treatment of HUVEC
Cells were seeded on a 6 well plate (Greiner bio-one) and treated with a final concentration of 75µM DFX (chemical hypoxia) or transferred into a hypoxic chamber (BD GasPak™ EZ, Anaerobe Gas Generating Pouch System with Indicator; BD Bioscience) for 8h or 24h. After 24h hypoxia, cells were lysed or released from the chamber and grown in ECGM containing 10% FCS for additional 8h or 24h. Western blot lysates were made at the indicated time points. Control cells were lysed at the beginning (0h) and at the end (48h) of the experiment.

Western blot analysis
Cells were washed with PBS and lysed in buffer (150mmol/L NaCl, 50mmol/L Tris-HCl, pH 7.4, 1% NP40, 10mmol/L EDTA, 10% glycerol, and protease inhibitors). Zebrafish embryos were lysed in the same buffer, followed by homogenization with a syringe. The protein lysates were boiled, and separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with the indicated antibodies, followed by incubation with Western blot detection reagent (Perbio Science). Western blot signals were quantified using Gel-Pro Analyzer 6.0, INTAS and normalized to its respective loading controls.

In vitro angiogenesis assay, apoptosis assay and rescue experiments using recombinant IL-8
HUVEC were transduced with adenovirus and incubated for 48h before the in vitro assays were performed. Endothelial migration and proliferation were done as described. The tube formation assay 25,000 HUVEC were seeded in a 48 well plate filled with Matrigel Basement Membrane Matrix (BD Biosciences). The cumulative tube length was measured under a microscope (Olympus IX50) and with cell^P software. To analyze cell viability, a cell growth curve was generated. HUVEC were seeded in a 6 well plate and counted at the
indicated time points. For each time point three wells were counted and the average was calculated. To investigate on cell apoptosis, caspase 3 and 7 activity was measured using the Promega Caspase-Glo® 3/7 Assay System according to the manufacturer’s protocol. As a positive control cells were stimulated with 250 nM staurosporin for 2h at 37°C. For the rescue experiments HUVEC were transduced with HOXC9 and 24h later different concentrations of IL-8 was added. The assays were performed after additional 24h incubation in presence of IL-8.

**Microarray analysis**
HUVEC were transduced with GFP or HOXC9 adenoviruses followed by RNA isolation 24h later. Successful HOXC9 transduction/ expression in HUVEC was confirmed by RT-PCR and by Western blot analysis. The RNA samples were analyzed at the Microarray core facility of the German Cancer Research Center Heidelberg (DKFZ) using Illumina Human Sentrix-6 BeadChip arrays (Illumina, Inc.). The microarray analysis was performed in biological triplicates.

**Measurement of secreted IL-8 via EASIA**
HUVEC were transfected with siRNA or transduced with adenovirus. After 24h cells were washed with PBS and fresh medium was added. Supernatants of the cells were collected at the indicated time points and an EASIA assay was performed according to the manufacturer’s protocol (BioSource IL-8 EASIA Kit, BioSource Europe S.A.). For testing the effect of cell confluency on interleukin 8 secretion, 8x10^4 or 25x10^4 HUVEC were seeded per 6-well. 24h later HUVEC were washed with PBS and fresh medium was added. Supernatants of cells were collected at the indicated time points and number of cells was counted. After measuring the IL-8 content in the supernatants, the amount of IL-8 per 10^4 cells was calculated.

**Chromatin Immunoprecipitation (ChiP)**
The assay was performed according to the manufacturer’s protocol (EZ-ChIP chromatin immunoprecipitation kit, Millipore). In brief, 2x10^6 HUVEC were seeded onto a 10cm tissue culture dish, one day later cross-linked by 1% PFA for 5 min at room temperature and followed by fragmentation of genomic DNA using a sonification apparatus (Bandelin Sonopuls HD 2070, 5 times for 10sec with 10% power). Cell lysates were used for chromatin immunoprecipitation using NFkB, HOXC9, RNA Polymerase II and control antibodies. The protein G agarose-antibody/chromatin complexes were washed and the antibody/chromatin complexes were subsequently eluted. The cross linked protein/DNA complexes were detached at 65°C for 4h followed by purification of the genomic DNA. The following genomic PCR primers amplifying the IL-8 (length 113 bp; located 953bp upstream from the ATG) and GAPDH (length 166 bp) promoters were used:

*IL-8 promoter sense:* 5'-GGAATGTGCTGTCTCTTCTTTTCTG-3'  
*IL-8 promoter antisense:* 5'-GACATTAATTCCAGTGAGGC-3'  
*GAPDH promoter sense:* 5'-TACTAGCCTTTTACGGGCG-3'  
*GAPDH promoter antisense:* 5'-TCGAACAGGAGGAGCGAAGCGA-3'

PCR program (for IL-8): 94°C for 3min (94°C for 45s, 48°C for 45s, 72°C for 30sec) x 40, 72°C for 10min; PCR program (for GAPDH): 94°C for 3min (94°C for 45s, 59°C for 45s, 72°C for 30sec) x 36, 72°C for 10min

**Chloramphenicol acetyltransferase (CAT) reporter assays**
350.000 HeLa cells cultured in DMEM/10%FCS and antibiotics were seeded per 6-well plate and 24h later transduced with AdCherry or AdHOXC9. After another 24h, HeLa cells were transfected with the different interleukin 8 reporter constructs (4µg plasmid per well for constructs -8, -98, -185 and -272 and 6µg plasmid per well for construct -1481 plus 6µl
lipofectamine, Invitrogen for each conditions) which contained different parts of the interleukin 8 promoter driving the expression of chloramphenicol acetyltransferase (CAT). 24h later after transfection HeLa cells were lysed and CAT expression was analyzed by Western blot.

RT-PCR
Total RNA was isolated from zebrafish embryos using the RNeasy Mini-Kit (Qiagen) following the manufacturer’s protocol. First-strand cDNA was generated from normalized RNA amounts using random hexamer primers and the Superscript II kit (Invitrogen). RT-PCR was performed with specific primer pairs: zebrafish actin (413bp fragment, forward: CTTGCCGTATCCAGCAGAC, reverse: GCGCCATTACACAGCAGAA; Program: 94° C for 3min (94° C for 45s, 55° C for 45s, 72° C for 1min) x 35, 72° C for 10min), zebrafish IL-8 (310bp fragment; forward: GACCAGCAAAATCATTTCTAG, reverse: GGGAAGACAGATTGTGTGTG; Program: 94° C for 3min (94° C for 45s, 50° C for 45s, 72° C for 30sec) x 40, 72° C for 10min), zebrafish HOXC9 (477bp fragment, forward: ATGAGAGCAGGATGTTCTG, reverse: ATTCAGCCTTGTGTCTTTTG; Program: 94° C for 3min (94° C for 45s, 55° C for 45s, 72° C for 30sec) x 35, 72° C for 10min).

Injections of DNA, morpholinos and intracardiac injections of IL-8 and Evans blue
For DNA injections, we cloned full length zebrafish HOXC9 into pflk1MCS_tol2-1, which was a kind gift of Dr. Heinz-Georg Belting, Biozentrum Basel. The flk1:HOXC9 vector was linearized with NotI, purified via agarose gel extraction using the Nucleospin Extract II kit (Macherey-Nagel) and dissolved in 0,1M KCl reaching a final concentration of 60ng/µl. The linearized empty flk1 vector served as a control. Morpholinos were diluted in 0,1M KCl to concentrations of 0.5–12 µg/µl. One nanoliter of each dilution was injected through the chorion of 1-cell or 2-cell stage embryos. The following translational/splicing-blocking (TB/SB) antisense morpholinos (Gene Tools) were used:

IL-8-SB1-Mo: 5’-GTATTAGTTTGAATACATCGTA-3’ (targeting exon2-intron2 junction)
IL-8-SB2-Mo: 5’-GTTACATTGGAATACATCGTA-3’ (targeting intron2-exon3 junction)
HOXC9-TB-Mo: 5’-GTTTCCCTTCTTTCATGCATC-3’ (1 base 5’ of ATG)

Standard Control-Mo (Co-Mo)

For in vivo rescue experiments DNA constructs and morpholinos were injected as described above. At the 24hpf stage, one nanoliter IL-8 (25ng/µl) was injected into the heart of the anesthetized embryo to enter the blood circulation. To validate this protocol, one nanoliter Evans Blue (1 %) was injected into the heart and distribution in the circulatory system was observed under the microscope.

Flow cytometry
To analyze expression of HOXC9 in EGFP positive cells, tg(fli1:EGFP) embryos were injected at the 1 cell stage with flk1:HOXC9 (Flk-HOXC9) or flk1-control DNA constructs (Flk-Control). At 48hpf anesthetized embryos were dechorinated, incubated 15min in modified Ringer solution (116mM NaCl, 2.9 mM KCl, 5mM HEPES, pH 7.2), deyolked, washed in PBS and lysed in 0.25% trypsin for 15-20min. The reaction was stopped with PBS containing 10% FCS and 2mM CaCl₂, cells were centrifuged and resuspended in buffer (0,5% FCS, 1mM EDTA in PBS). EGFP positive cells were sorted using a FACSscan flow cytometer running CellQuest software (Becton Dickinson). EGFP positive and negative cells were then used for RT-PCR.

Whole mount antibody staining and immunohistochemistry
For whole mount antibody stainings tg(fli1:EGFP) embryos were fixed 2h in 4% PFA/PBS, washed, dehydrated in methanol and stored at -20°C. Embryos were rehydrated,
permeabilized with proteinase K (Macherey-Nagel) and fixed again with 4% PFA/PBS. After blocking in 1% BSA plus 2% serum in PBST, embryos were incubated with an anti-GFP antibody at 4°C overnight. On the following day embryos were washed for 6h and the secondary antibody was added at 4°C overnight. The color reaction was developed using the Vectastain ABC kit with horseradish peroxidase and DAB as a chromogen. For immunostaining of zebrafish sections tg(fli1:EGFP) embryos were fixed overnight at 4°C in 4% PFA/PBS, washed, kept in 18% sucrose/PBS overnight at 4°C, embedded in Tissue-Tek (Sakura Finetek Europe) and stored at -80°C. 10 µm sections were made using a microtome (Leica CM-1900). Sections were washed, re-fixed, blocked in 3% BSA/PBS and incubated overnight at 4°C with the HOXC9 antibody (Abcam) and subsequently with Alexa-546 coupled secondary antibody. For HUVEC stainings, cells were seeded in 24 well plates, fixed with 4% PFA/PBS, permeabilized with 0.3% Triton-X-100 and stained with an HOXC9 antibody (Abcam) and an Alexa-546 coupled secondary antibody.

Statistical analysis and quantification
Results are expressed as mean ±SD. Comparisons between groups were analyzed by Student’s t-test (two-sided). P values <0.05 were considered as statistically significant. For quantification of zebrafish, tg(fli1:EGFP) embryos were stained using an anti-GFP antibody with a DAB-based protocol and analyzed under the microscope. Embryos with vascular defects in the ISVs and DLAVs were counted and the percentage of embryos with defects per group was calculated. The number of counted embryos is shown in the graphs.

Microscopy and analysis
For in vivo imaging, EGFP-expressing tg(fli1:EGFP) embryos were manually dechorionated, anesthetized with 0.003% tricaine and embedded in 1% low-temperature melting agarose (Roth). Fluorescence was analyzed using the confocal microscope DMIRE2 with Leica TCS SP2 True Confocal Scanner (Leica Microsystems). Stacks were recorded as indicated in the overview image. Overview pictures and sections of embryo as well as HUVEC were analyzed with the fluorescence microscope DMI 6000 B (Leica).

References
Online Figures
Online Figure I

Online Figure I: HOXC9 antibody stainings of HUVEC. HOXC9 staining is shown in red and DAPI staining in blue. (A, A') High HOXC9 expression in confluent HUVEC and low HOXC9 expression in sparsely cultured HUVEC. (B, B', C, C') Reduced HOXC9 expression in HUVEC cultured under hypoxic conditions or under DFX for 24h. (D, D') Increased HOXC9 expression in HUVEC transduced with an AdHOXC9 virus. Note the shorter exposure time of the AdHOXC9 transduced cells. (E, E') Decreased HOXC9 expression in HUVEC transfected with a HOXC9-siRNA. (F) Background control of HUVEC stained with second antibody only. Note that there is no staining in the nucleus, while there is a weak background signal in the cytoplasm. Values in parantheses indicate microscope recording times. Scale bar: 50µm.
Online Figure II: (A) Chemical hypoxia (DFX) decreased the amount of the HOXC9 protein in endothelial cells which can be reverted after DFX withdrawal and recovery in normal growth medium. (B) The lower panel represents a quantification (n=3).
### Online Figure III

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**Online Figure III:** Selected data from the microarray analysis analyzing HOXC9 overexpressing HUVEC (n=3). Several known and important angiogenic molecules are not regulated by HOXC9. Note -7.6 fold reduction of interleukin 8 expression in HOXC9 transduced HUVEC.
Online Figure IV: HOXC9 inhibited secretion of IL-8 in endothelial cells. (A-C) Adenoviral overexpression of HOXC9 in HUVEC decreased IL-8 secretion in supernatants as determined by EASIA. Three single experiments are shown. (D-F) SiRNA based expression silencing of HOXC9 in HUVEC increased IL-8 secretion in supernatants after 32h and 48h. IL-8 levels in HUVEC supernatants returned to baseline levels after 56h reflecting increased HOXC9 expression in the increasingly confluent endothelial cell monolayer. Three single experiments are shown. (G-I) HUVEC cultured under high confluency showed decreased IL-8 secretion as compared to HUVEC cultured under sparse conditions. Three single experiments are shown.
Online Figure V:

Sequence of the first -100bp of the interleukin 8 promotor (black colour) and the first 44bp of the IL-8 gene (UTR in blue colour) followed by the chloramphenicol acetyltransferase gene (Mukaida et al., 1990). A putative HOXC9 binding site is located between -55bp and -61bp (red colour). This sequence was identified as a repressor consensus sequence for HOX genes (Svingen and Tonissen, 2006).
Online Figure VI: Expression of the HOXC9 protein during zebrafish embryogenesis. (A) Ubiquitous distribution of maternal HOXC9 protein at the 256-cell stage. (B) At 60% epiboly stage, HOXC9 protein is expressed ubiquitously (dorsal to the right). (C, D) At the tailbud and 14-somite stage, HOXC9 protein expression becomes restricted to the posterior part of the embryo (anterior to the left). (E) HOXC9 expression at 24hpf in the neural tube (NT) and in the posterior cardinal vein (CV) (anterior to the left). (A'-E') Whole mount antibody control stainings lacking the primary HOXC9 antibody. (F-J) Stainings of cross-sections for 48hpf old *tg(fli1:EGFP)* embryos reveals vascular expression of HOXC9 in the posterior cardinal vein. (F) Bright field image. (G) EGFP expression in the vasculature in *tg(fli1:EGFP)* embryos, e.g. in the dorsal aorta, cardinal vein, ISV and DLAV. (H) HOXC9 expression in the neural tube and in the posterior cardinal vein. (I) Merge of G and H. (J) Confocal image of a 48hpf *tg(fli1:EGFP)* embryo confirming the co-localization of the EGFP signal and the HOXC9 antibody staining (red colour). Scale bars: 100µm (A through E, A' through E'); 25µm (F through J).
Online Figure VII: FACS-based purification of EGFP-positive endothelial cells from 48hpf old \textit{tg(fli1:EGFP)} fish embryos. 48hpf old \textit{tg(fli1:EGFP)} fish embryos were dissociated by trypsin followed by FACS sorting. (A, A') The dissociated embryonic fish cells were sorted for EGFP-positive (GFP+) and EGFP-negative (GFP-) cells from Flk-Control (A) and Flk-HOXC9 (A') injected fish embryos. (B-C') Sorted EGFP-negative (B, B') and EGFP-positive (C, C') cells that were further used for RT-PCR were re-analyzed for EGFP expression showing 98.6% to 100% purity.
Online Figure VIII: Proposed model for HOXC9’s function in endothelial cells promoting a resting, anti-angiogenic endothelial cell phenotype. Quiescent endothelial cells express high amounts of HOXC9 resulting in a repression of IL-8. Upon angiogenic activation of endothelial cells, such as hypoxia, endothelial cells become activated. Hypoxia suppresses HOXC9 expression thereby inducing IL-8 expression promoting an angiogenic activation.