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DISSERTATION

Titel der Dissertation

The VEGF-regulated transcription factor HLX controls the expression of guidance cues and negatively regulates sprouting of endothelial cells

Verfasserin

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1. Introduction

1.1 Endothelial cells and the vascular system

The vascular system is a highly branched, tree-like tubular network that reaches every organ and supplies tissues and organs with oxygen, nutrients and signaling molecules and removes waste products (Adams and Eichmann, 2010). The blood vascular system is one of the earliest organs to develop in the embryo (Lohela et al., 2009). It is established through two different tightly regulated processes, vasculogenesis and angiogenesis. During vasculogenesis the first primitive vascular plexus is formed by hemangioblast progenitors differentiating into endothelial cells. The primary plexi are remodeled into a hierarchically organized network of arterioles and arteries, capillaries, and venules and veins in a process termed angiogenesis. Thereby endothelial cells proliferate, migrate and invade tissues (Adams and Alitalo, 2007). Arteries and veins are stabilized by vascular smooth muscle cells, whereas capillaries are covered by pericytes to form mature and quiescent vessels. Those angiogenic remodeling processes are important during embryonic development as well as during physiological and pathological angiogenesis. Physiologic blood vessel formation in the adult is necessary for example during wound healing, in the cycling ovary or the growth of the placenta and pathological angiogenesis occurs in diseases such as cancer, chronic inflammatory diseases and retinopathy (Carmeliet, 2005).

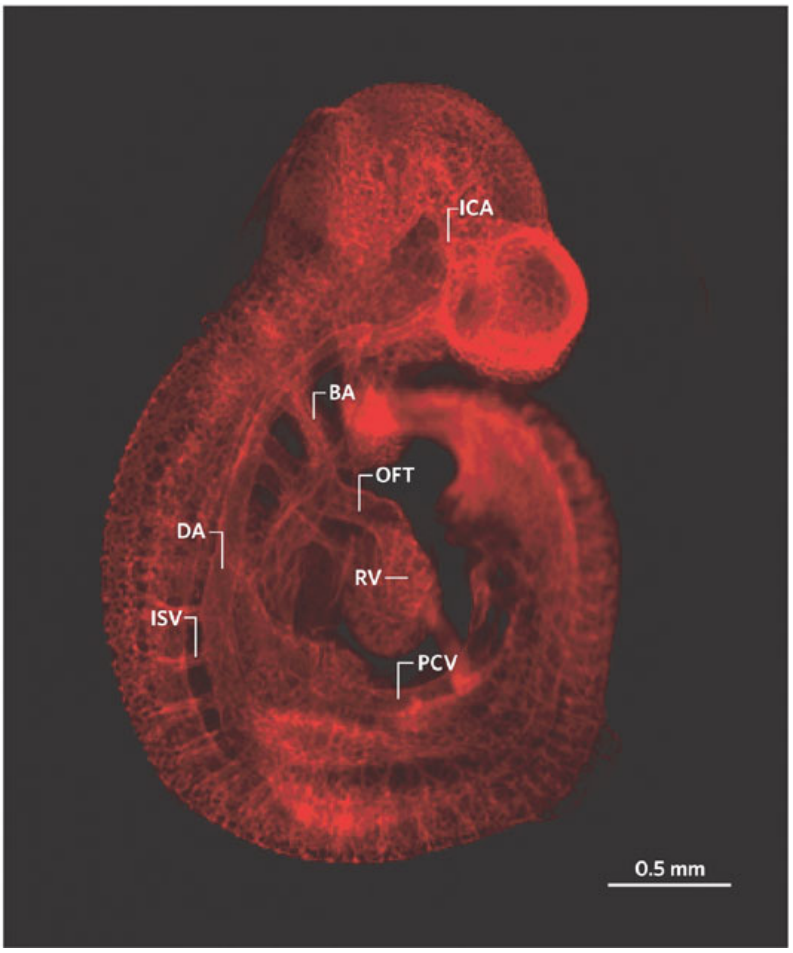

Figure 1. Murine embryonic vasculature
Murine developing vasculature on embryonic day 9.5 was stained for CD31 (PECAM) and analyzed with optical projection tomography. BA, branchial arteries; DA, dorsal aorta; ICA, intercarotid artery; ISV, intersomitic vessels; OFT, outflow tract; PCV, posterior cardinal vein; RV, right ventricle. Reprinted by permission from Macmillan Publishers Ltd: Nature (Coultas et al., 2005), copyright 2005.

1.2 Angiogenic growth of blood vessels

The growth factor VEGF-A is the major trigger of angiogenesis. Binding to its receptor VEGFR2 leads to endothelial cell differentiation, proliferation and sprouting. Some endothelial cells within the capillary wall are selected for sprouting and become motile and invasive and initiate sprouting. These cells are the tip cells which lead the growing sprout. They sense the environment for guidance cues using their continuously searching filopodia. Those specialized cells resemble the axonal growth cone in the nervous system in structure and function to a high degree. The growing sprout is guided by a VEGF-A gradient and by other attractive and repulsive guidance cues in the matrix or on guidepost cells in the tissue. The subsequent stalk cells trail behind the tip cells. They proliferate, form junctions, lay down extracellular

matrix and form a lumen by the fusion of vacuoles. A third group of specialized endothelial cells are the so called phalanx cells that are the most quiescent cells that line the vessels once the new vessel branches have been consolidated. They are already covered by pericytes, have tight junctions and are embedded in a thick basement membrane. The specialized phalanx cells are engaged in optimizing blood flow, tissue perfusion and oxygenation. The expression of platelet-derived growth factor (PDGFB) by the tip cells leads to the recruitment of mural cells, vascular smooth muscle cells and pericytes that express the PDGF receptor ß (Adams and Alitalo, 2007; De Smet et al., 2009).

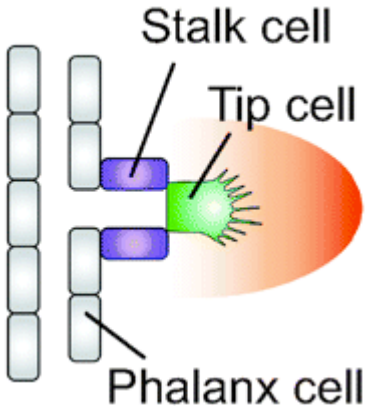

Figure 2. Vascular sprouts are guided by endothelial tip cells.
Tip cell (green) lead the growing sprout and extend their filopodia toward stimuli in the tissue environment (red gradient). Proliferating stalk cells (purple) trail behind, elongating the sprout, while phalanx cells (gray) remain quiescent and form a tight barrier. Reprinted by permission from Wolters Kluwer Health: Arterioscler Thromb Vasc Biol (De Smet et al., 2009), copyright 2009.

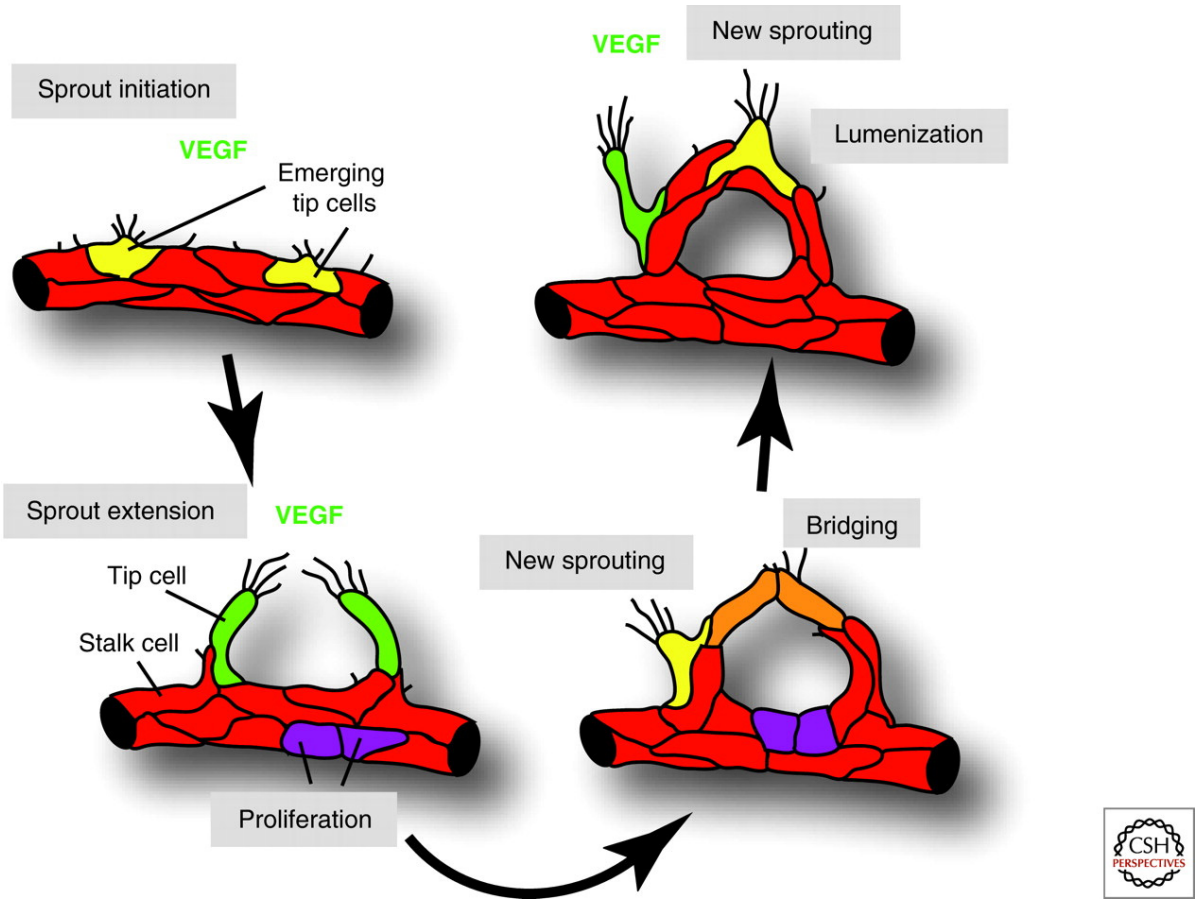

Figure 3. Angiogenic sprouting and blood vessel growth.
Sprouting is initiated upon stimulating signals such as VEGF-A from the tissue-environment. Some endothelial cells (yellow and green) differentiate into tip cells extending filopodia, sensing the tissue surroundings, migrating and invading. The stalk cells (red) trail behind and form the sprout stalk. To form new connections, the tip cells contact other growing sprouts or established vessels. These cell bridges (orange) are altered into perfused vessels with a lumen. Eventually further sprouting initiates elsewhere by tip cells (yellow, green) and additional endothelial cells proliferate (purple). Reprinted by permission from Cold Spring Harb Laboratory Press: (Adams and Eichmann, 2010), copyright 2010.

1.3 Growth factors and inflammatory cytokines in endothelial cells

Vascular endothelial growth factor-A (VEGF-A) is a key molecule for the initiation and direction of sprouting angiogenesis. In hypoxic areas cells express VEGF-A and this leads to the formation of a VEGF-A gradient which serves as a directional and chemoattractive cue for endothelial sprouts. VEGF-A binds to and activates via the receptor tyrosine kinase VEGFR2/KDR/Flk1 the major angiogenic signaling pathway (Eilken and Adams, 2010). In addition, it can also bind VEGFR1, which seems to act as a negative regulator of angiogenesis during development. VEGF-A is part of a large family of angiogenic molecules which includes placental growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D. In addition to angiogenesis, VEGF-A is essential for

chemotaxis and differentiation of endothelial precursor cells and vasculogenesis. Alternative splicing of VEGF-A can generate several different isoforms and those can regulate blood-vessel growth and patterning of the vasculature. The splice variants differ in their matrix binding and co-receptor binding capability. VEGF-A_{121} is freely diffusible, VEGF-A_{165} the predominant angiogenic form and VEGF-A_{189} is tightly matrix-bound. The b-isoform even has anti-angiogenic properties (Adams and Alitalo, 2007; Lohela et al., 2009).

Basic fibroblast growth factor (bFGF) is another important angiogenic growth factor for endothelial cells. bFGF binds and signals mainly through the receptor tyrosine kinase FGF receptor-1. bFGF can trigger basal lamina degradation, migration, proliferation and has effects on morphogenesis and vessel maturation during angiogenic processes (Presta et al., 2005).

In comparison to VEGF-A, epidermal growth factor (EGF) represents a more general growth factor inducing proliferation and survival in many different cell types. EGF and the HER or erbB receptors are prototypes of growth factors and receptor tyrosine kinases and are of wide importance for the development and proliferation of epithelial and many other cell types. In endothelial cells EGF can mediate proliferation and a gene repertoire much smaller than VEGF-A, which is the essential factor for directed endothelial sprouting angiogenesis (Citri and Yarden, 2006; Schweighofer et al., 2009; Sini et al., 2005).

Angiogenesis often takes place in inflammatory surroundings, e.g. during wound healing and in tumors, which are created by immune cells invading the damaged or malignant tissue. In response to cytokines or histamines, endothelial cells are activated to express a large number of inflammatory genes like cytokines, enzymes and adhesion molecules implicated in immune cell recruitment, activation and tissue repair. The cytokine IL-1 signals through the type 1 IL-1 receptor activating the transcription factor nuclear factor-kappaB (NF-κB) and thereby the transcription of proinflammatory genes (Pober and Sessa, 2007; Schweighofer et al., 2009).

1.4 Influence of hypoxia on endothelial cells

A decrease in oxygen tension leads to tissue hypoxia, which attracts angiogenic sprouting into those areas (Germain et al., 2010). Three hypoxia-inducible factor-α

proteins (HIF-1α, HIF-2α and HIF-3α) show oxygen-regulated protein stability and together with HIFß (ARNT) activate HIF target genes in response to hypoxia through binding to hypoxia response elements. These transcription factors induce the expression of hundreds of genes important for the regulation of cell survival, metabolism and angiogenesis. During normoxia prolyl hydroxylases (PHDs) hydroxylate HIF-αs in an oxygen-dependent manner and this induces HIFs to interact with VHL (von Hippel-Lindau) and its degradation through the proteasome. Factor inhibiting HIFs (FIH) also hydroxylates HIF-1α and thereby inhibits the interaction of HIF-1α with its transcriptional coactivator p300 (Fraisl et al., 2009). In the case of hypoxia the hydroxylases cannot hydroxylate the HIF factors, which results in stabilization, increase in concentration and efficient interaction of HIFs with p300. HIF-2α, which is highly expressed in vascular endothelial cells, seems to be the predominant form responsible for modulation of vascular function and angiogenesis. HIF-1α is more broadly expressed throughout most tissues and has a role in mediating the paracrine effects of angiogenesis inducing angiogenic factors such as VEGF-A (Fong, 2009).

Hypoxia upregulates growth factors, provisional extracellular matrix (ECM) components and vascular basement membrane proteins in endothelial cells and therefore regulates mechanical and biological properties of vascular basement membrane and ECM. Among the proteins upregulated by hypoxia are the thrombospondins (TSPs), a family of secreted matricellular proteins that function as adapter proteins to guide ECM synthesis and remodeling. Furthermore, angiopoietin-like 4 (ANGPTL4) is expressed by hypoxic endothelial cells and promotes angiogenesis and lipid metabolism. These cells further induce Type IV collagen, heparin sulfate proteoglycans (HSPGs), and several ECM-modifying enzymes (LOX, LOXL2, PLID1,2 and P4HA1,2) (Germain et al., 2010).

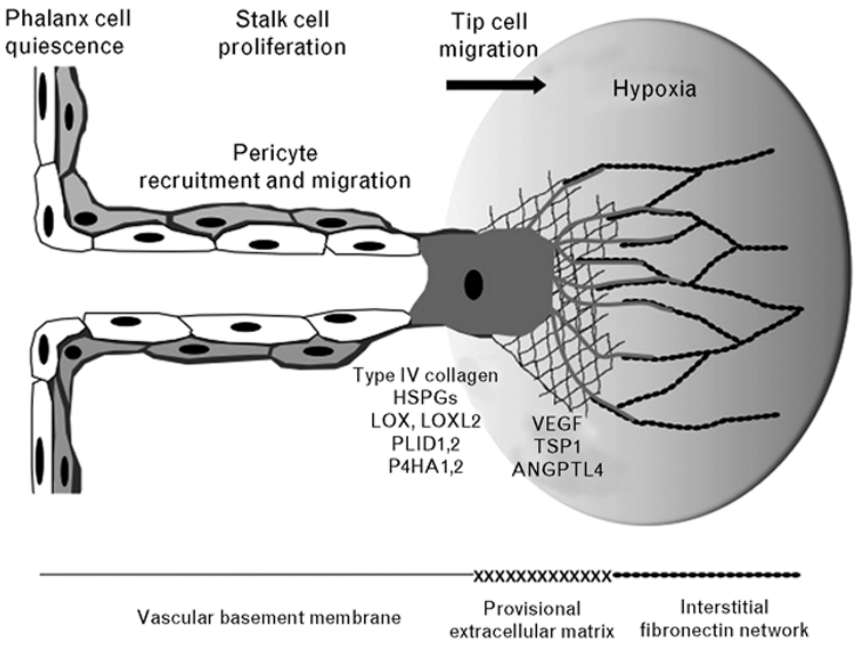

Figure 4. Schematic drawing of a filopodial-extending tip cell (dark gray) migrating toward the hypoxic area (gray gradient)
Tip cells migrate extending their filopodia on an interstitial fibronectin network (black strings) and guide the endothelial sprout. The provisional extracellular matrix is remodeled at the front of the tip cell (gray network) and the basement membrane (black line) is deposited at the back of the tip cell as well as at stalk cells (white) and surrounding pericytes (light gray). Reprinted by permission from Wolters Kluwer Health: Current Opinion in Hematology (Germain et al., 2010), copyright 2010.

1.5 The homeobox transcription factor HLX

HLX (H2.0-like homeobox protein) is an evolutionary highly conserved homeobox transcription factor which was originally detected in the visceral musculature of Drosophila (H2.0) (Barad et al., 1991). The homeodomain is a 60-amino-acid DNA-binding domain showing considerable variations in primary sequence within the different homeobox genes. However, the three-dimensional structure is conserved and consists of three α-helices and an unstructured amino-terminal arm (Abate-Shen, 2002). The largest family of homeobox genes is the HOX family, which consists of four clusters (A-D) and comprises 39 genes. They were discovered to be the mammalin orthologs of homeotic genes of Drosophila. Most homeobox families are unclustered, diverged homeobox genes, where the members are dispersed throughout the genome. Homeobox transcription factors are developmental regulators that are essential for cell proliferation, cell differentiation and migration in many cell types. These properties are responsible for their critical role in regulating pattern formation and organogenesis during embryogenesis. Several members of the

homeobox family have also been implicated in the formation of the embryonic vascular system as well as in vascular remodeling in the adult and have a role in many pathologies including atherosclerosis and tumor angiogenesis (Douville and Wigle, 2007; Gorski and Walsh, 2003).

Human HLX was first isolated from a mitogen-activated human B-lymphocyte cDNA library using probes designed to hybridize homeodomains. HLX is further expressed in bone marrow-derived CD34+ cells and in vitro differentiation results in down-regulation of HLX expression (Deguchi and Kehrl, 1991). Furthermore, in naïve CD4 T lympohcytes HLX interacts with T-bet to promote heritable Th1 gene induction. This genetic interaction initiates optimal induction of interferon-g expression and the Ifng locus then undergoes DNA methylation and stable chromatin remodeling, thereby becoming independent of T-bet activity (Mullen et al., 2002). HLX expression is dispensable for the maintenance of a transcriptional permissive ifng gene, since this signature gene activity of helper T cells becomes epigenetically fixed (Martins et al., 2005). In resting naïve CD4 T cells, HLX furthermore genetically controls IL-4Rα level at the transcriptional level and thereby determines the ration of Th1 and Th2 cell differentiation. Transgenic HLX down-regulates IL4-Rα and IL-4 signaling and enhances Th1 response (Mikhalkevich et al., 2006).

In contrast to CD4 T cells, HLX expression is induced in monokine-activated NK cells with delayed kinetics compared to IFN-γ and ectopic expression of HLX negatively regulated IFN-γ production. This inhibition is at least partly achieved through the accelerated dephosphorylation and proteasomal degradation of STAT4, a key transcription factor for IFN-γ (Becknell et al., 2007).

The homeobox gene HLX is also expressed in the placental vasculature, exhibiting a higher expression in placental microvascular endothelial cells when compared to macrovascular human umbilical vein endothelial cells (HUVEC). The heterogeneity of expression of HLX and other homeobox genes probably reflects differences of function in those endothelial cells. In the placenta, microvascular endothelial cells are important for the formation of the placental vasculature and they vascularize the cotyledons of the placenta, which are essential for maternal-fetal gas and nutrient exchange and play a role in placental disorders (Murthi et al., 2007).

During placental development abnormal throphoblast development is associated with clinically significant pregnancy disorders such as fetal growth restriction and preeclampsia. HLX is expressed in proliferating and migrating human trophoblast cells in the early placenta, showing a nuclear localization in the villous cytotrophoblast cells and the extravillous trophoblasts in the proximal region of the cell column and a cytoplasmic expression in interstitial trophoblasts and in the endovascular trophoblasts. In human fetal growth restriction HLX expression is significantly decreased. Moreover, HLX is a regulator of colony- stimulating-factor-1 dependent trophoblast proliferation and of hepatocyte growth factor/c-met- mediated trophoblast migration (Murthi et al., 2006; Rajaraman et al., 2010; Rajaraman et al., 2007; Rajaraman et al., 2009; Rajaraman et al., 2008).

Furthermore, in endometrial epithelium HLX is expressed in both the proliferative and secretory phase and might be required for the transcriptional control of genes necessary for endometrial cell differentiation and to control epithelial-mesenchymal cell interaction in the endometrium (Quinn et al., 1998).

In mouse development the HLX-/- genotype is lethal around day 15 of embryonic development on a mixed genetic background and the embryos display anemia and severe hypoplasia of the liver and the gut. During murine embryogenesis HLX is most prominently expressed in the visceral mesenchyme of the developing liver, gall bladder and gut and regulates a mesenchymal-epithelial interaction that is required for early aspects of enteric nervous system development (Bates et al., 2006; Hentsch et al., 1996).

1.6 Guidance cues of the endothelial sprout

The angiogenic sprout expresses similar receptors for guidance as the nerve growth cone and responds to similar attractive and repulsive cues (Adams and Eichmann, 2010). Proteins of the ROBO, uncoordinated 5 (UNC5), plexin or neuropilin families as well as of the Eph receptor tyrosine kinase family are expressed on tip cell filopodia and serve as receptors to sense and respond to the corresponding guidance cues. These include proteins of the SLIT, netrin, semaphorin and ephrin families that are either secreted in the tissue environment or are transmembrane proteins on guidepost cells. These ligand-receptor interactions lead either to

attraction or repulsion of the tip cell filopodia and the growing sprout is thus guided to form new connections.

The receptors ROBO4, plexin-D1 and UNC5B are predominately expressed in the vasculature, whereas many of the other isoforms are both expressed on nerve and endothelial cells.

Roundabouts (ROBOs) are single-pass transmembrane receptors for SLITs. ROBO4 -/- mice show that ROBO4 is necessary for blood vessel integrity and they have increased retinal vascular permeability and hypervascularization during oxygen-induced retinopathy. However, whether ROBO4 binds to SLITs during blood vessel regulation is still unclear. Furthermore, expression of ROBO1 in the vasculature and interactions with SLITs have been reported to affect endothelial cell migration.

The netrin receptor UNC5B is vascular-specific and primarily expressed in arterial endothelial cells, sprouting capillaries and tip cells. Binding of netrins leads to repulsion of endothelial sprouts. It was further reported that netrin-4, which is upregulated in endothelial cells by long-term VEGF-A induction, binds to neogenin associated with UNC5B and mediates repulsion. Additional proposed binding partners are repulsive guidance molecule (RGM) for neogenin and fibronectin and leucine rich transmembrane protein 3 (FLRT3) for UNC5B (Adams and Eichmann, 2010).

Another class of guidance molecules are the semaphorins that are characterized by a so-called Sema domain. The class III semaphorins are secreted molecules and bind to neuropilin, which again interacts with the signal transducing plexins. An exception to this rule is SEMA3E that directly binds plexin-D1 and mediates repulsion. Most of these signaling molecules have a preferential repulsive regulatory role on endothelial tip cells. However, neuropilins can also bind VEGF-A and then have an attractive guidance function upon interaction with VEGFR.

Moreover, the Eph receptor tyrosine kinases, transmembrane proteins with a single cytoplasmic kinase domain bind to ephrins, which are cell surface proteins, and thereby regulate different processes in the vasculature. Especially the interaction of ephrin-B2 ligand with the EphB4 receptor is important for the regulation of endothelial

cell migration and angiogenesis leading to different outcomes under diverse circumstances (Adams and Eichmann, 2010).

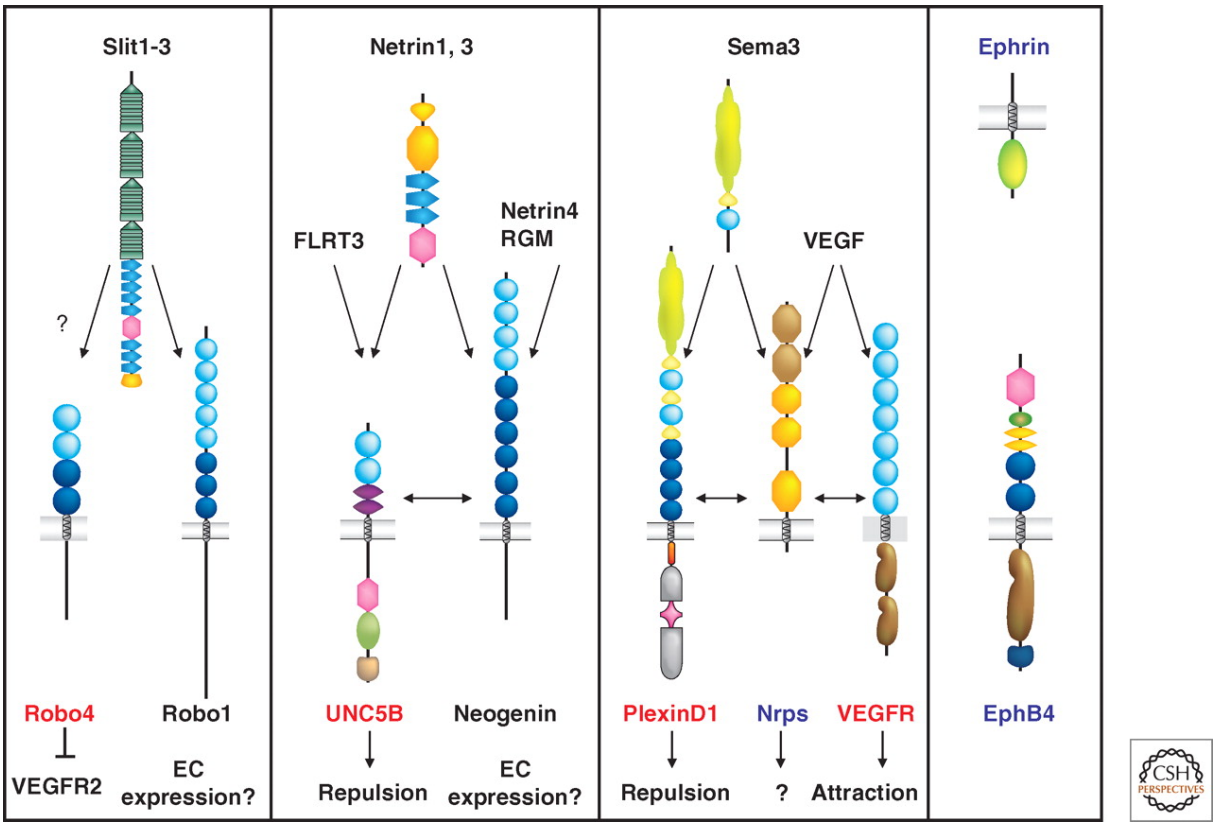

Figure 5. Axon guidance receptor expression in endothelial cells
Schematic representation of the four families of axon guidance cues and their receptors. Predominantly endothelial-expressed receptors are labeled in red, receptors with shared expression in the nervous and the vascular system in blue and molecules with no (known) expression in the vascular system in black. Note that in each axon guidance receptor family, at least one member is expressed in the vasculature. Reprinted by permission from Cold Spring Harb Laboratory Press: Developmental Cell (Adams and Eichmann, 2010), copyright 2010.

1.7 UNC5B

The uncoordinated5 (UNC5) molecule family are transmembrane receptors that contain two immunoglobulin and two thrombospondin-like domains in the extracellular region and a zona occluden 5 domain, a DCC-binding domain and a death domain in the intracellular region. The receptor UNC5B is selectively expressed in the vasculature and strongly expressed in growing capillaries, tip cells and arterial endothelial cells (Larrivee et al., 2009). UNC5B -/- mice show excessive capillary branching and an increase in expression of tip cell filopodia, which indicates a repulsive function in the growing sprout. Upon binding of its ligand netrin-1 the filopodia retract and sprouting is inhibited. Netrins are secreted matrix-binding

proteins, related to the basement-membrane molecule laminin and were first discovered to be guidance molecules for the nerve growth cone (Adams and Eichmann, 2010; Larrivee et al., 2007). Netrins can be either attractive when interacting with the receptors deleted in colorectal cancer (DCC) or repulsive through the UNC5 receptors or UNC5-DCC heterodimers. The cytoplasmic signaling domain of UNC5B contains a death domain which has been shown to induce apoptosis in the absence of the ligand. This effect could explain the hypervascularization seen in UNC5B -/- mice. However, there is only little endothelial cell apoptosis detectable in normal growing sprouts (Lu et al., 2004).

Netrin-4 is another signaling molecule interacting with UNC5B and was shown to negatively regulate angiogenesis by binding to neogenin, which recruits UNC5B (Lejmi et al., 2008). Netrin-4 has also been described to act as a lymphangiogenic factor, but this induction of lymphangiogenesis and enhanced metastasis is independent of neogenin or UNC5B (Larrieu-Lahargue et al., 2010). In neurons repulsive guidance molecule (RGM) binds to neogenin and UNC5B and this interaction leads to repulsion and growth cone collapse. Fibronectin and leucine rich transmembrane protein 3 is also a possible binding partner of UNC5B in xenopus embryos (Karaulanov et al., 2009). It might therefore be supposed that UNC5B in endothelial cells has several so far unknown interaction partners that exert additional influence on vessel guidance and angiogenesis.

1.8 Semaphorin 3G and plexin-A1

Semaphorins are a large family of transmembrane and secreted proteins that contain a highly-conserved Sema domain. The cell-associated semaphorins bind to plexins, whereas the secreted class III semaphorins usually bind to neuropilins and the signal is transduced by the plexins. However, secreted SEMA3E can independently of neuropilins directly bind plexin-D1, which is preferentially expressed in developing blood vessel endothelial cells, and the resulting signal is important for vascular patterning.

Class III semaphorins are secreted by several cell types, including tumor cells, where they act to inhibit tumor growth and angiogenesis. The inhibition of cell motility and migration of tumor as well as endothelial cells is mediated by inducing collapse of the actin cytoskeleton through neuropilins and plexins. SEMA3G is a secreted class III

semaphorin with higher affinity for Neuropilin 2 than Neuropilin 1, which induces signaling through the plexin-A1-4 (Gaur et al., 2009). SEMA3G has recently been found to be expressed primarily in the vasculature (Kutschera et al., 2010).

Plexins are large single-pass transmembrane receptors that regulate cellular organization and migration. The plexin receptor family consists of nine members: four type A plexins (A1,A2, A3 and A4), three type B plexins (B1, B2, B3) and plexins- C1 and D1. The activation of the plexin receptors through the neuropilins upon binding of the class III semaphorins induces actin depolymerisation and cytoskeletal collapse (Gaur et al., 2009). Plexins are characterized by the presence of a split cytoplasmic GTPase-activating protein (GAP) domain on the intracellular part and by a Sema domain and PSI and glycine-proline rich motifs in the extracellular part. By interaction with neuropilin 1 plexin-A1 mediates signal transduction of SEMA3A-D and by interaction with neuropilin 2 it mediates signaling induced by SEMA3B, C, D, F and G.

1.9 HES1 and Notch signaling

The Notch pathway is an evolutionary conserved signaling system which is essential for normal embryonic development, the regulation of tissue homeostasis and the maintenance of stem cells in adults. The Notch receptor is a heterodimeric protein and upon binding of its ligands, Jagged 1,2, Delta-like 1,3 or 4, called DSL ligands, the Notch extracellular domain is transendocytosed into the signal-sending cell. This leads to the cleavage of the remaining transmembrane Notch receptor part by ADAM (a desintegrin and metalloprotease) and γ-secretase, which releases NICD (Notch intracellular domain). NICD directly translocates to the nucleus, interacts with the transcription factor CSL, triggering transcriptional activation of Notch target genes. This interaction removes a corepressor complex containing histone deacetylse and replaces it instead with a transcriptional activation complex including NICD, Mastermind-like and histone acetyltransferases such as p300, which switches on expression of basic helix-loop-helix proteins such as Hairy/Enhancer of split (HES), Hes-related proteins (Hey/HRT/HERP) and Notch-regulated ankyrin repeat protein (Nrarp). The HES and HEY genes are transcriptional repressors of their own expression and additional downstream genes (Phng and Gerhardt, 2009). The HES1 transcriptional repressor plays an important role in the development of the nervous

system, sensory organs (eye, inner ear), pancreas and endocrine cells, as well as lymphocytes (Fischer and Gessler, 2007).

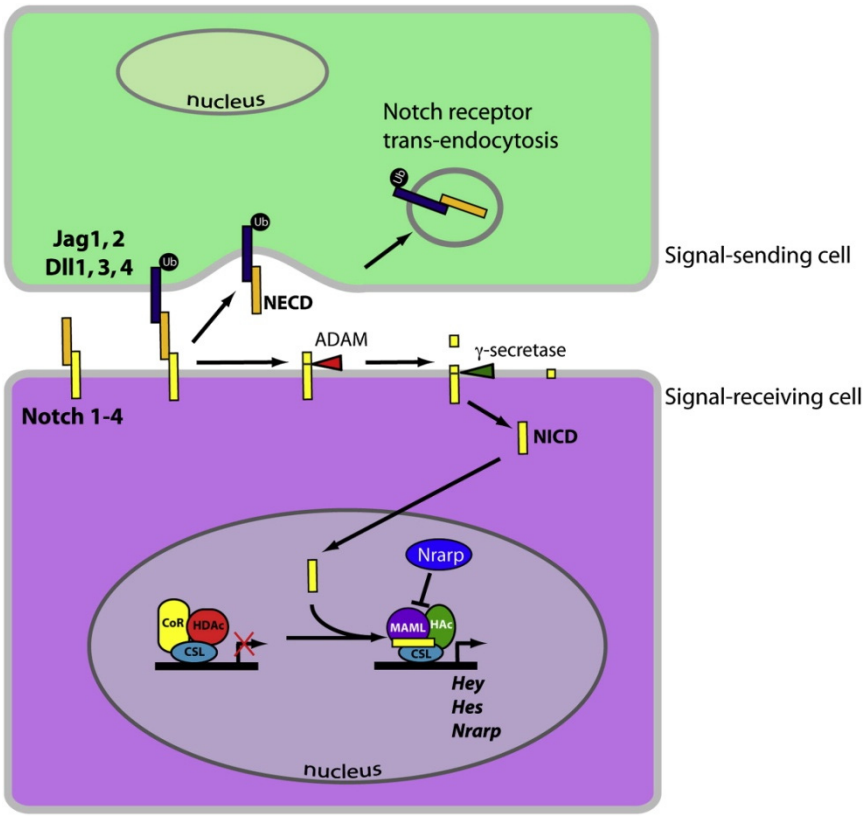

Figure 6. The Notch Signaling Pathway
The Notch receptor consists of an extracellular and a membrane-bound intracellular fragment which interact through noncovalent interactions. The DSL ligand binds to the Notch extracellular domain and dissociates the subunits through endocytosis into the signal-sending cell. The remaining membrane-bound receptor is proteolyzed by ADAM and γ-secretase, which releases the NICD. The NICD translocates to the nucleus to remove HDAc and trigger transcriptional activation of Notch target genes such as Hes and Hey. CSL, CBF, Suppressor of hairless, LAG-1; DSL, Delta, Serrate, LAG-2; HDAc, Histone deacetylase; MAML, Mastermind-like; HAc, Histone acetyltransferase; NECD, Notch extracellular domain; NICD, Notch intracellular domain; ADAM, a disintegrin and metalloprotease; Ub, ubiquitin. Reprinted by permission from Elsevier: Developmental Cell (Phng and Gerhardt, 2009), copyright 2009.

During angiogenesis endothelial cells are exposed to a gradient of VEGF-A_{165} which promotes the formation of tip cells and their extension of filopodia. However, only a fraction of endothelial cells acquires tip cell behavior and others become stalk cells. The Notch pathway, which is important for cell fate determination and differentiation processes, regulates the tip or stalk cell decision in endothelial cells. VEGF-A upregulates the Notch ligand Dll4 and tip cells express highest levels. Dll4 binds and activates Notch in the neighboring stalk cells downregulating VEGF receptor 2 and 3 expression and thereby tip cell phenotype. Jagged1 is most strongly expressed in stalk cells and is a positive regulator of angiogenesis opposing the Dll4-Notch

signaling. Antagonistic activity of Jagged1 requires Fringe-mediated glycosylation of Notch receptors (Adams and Eichmann, 2010).

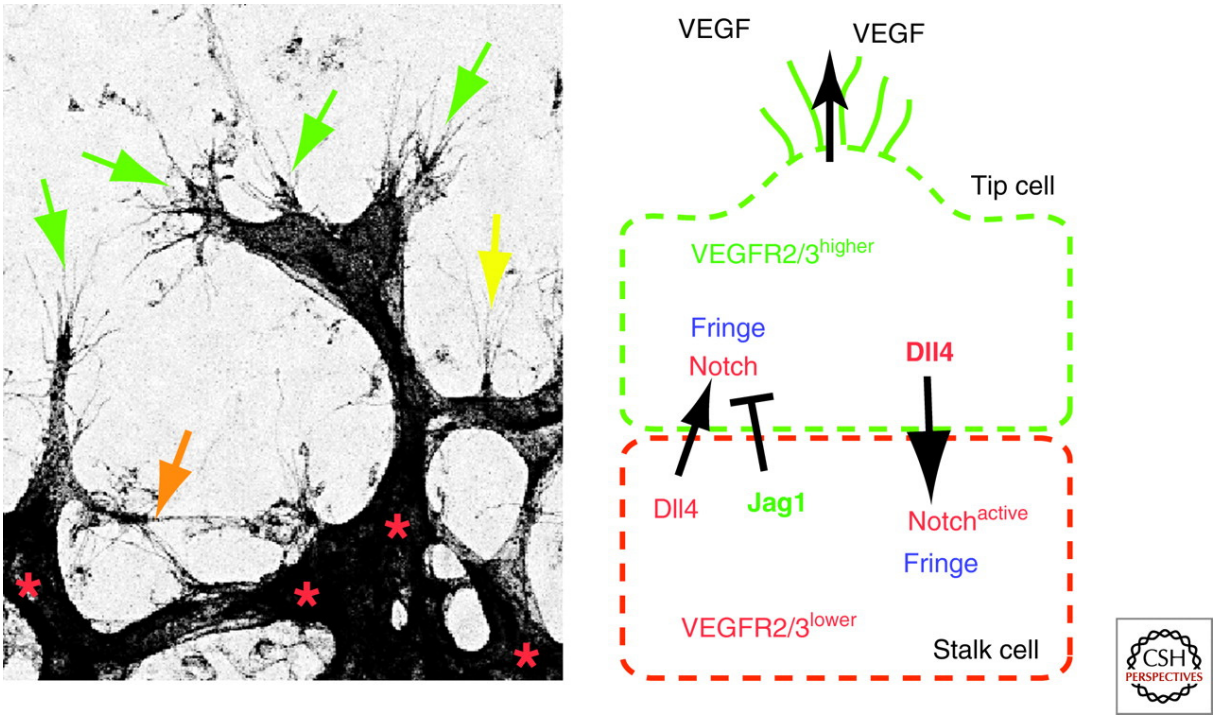

Figure 7. Regulation of tip cell formation
Left: Image of sprouting endothelial cells in the postnatal retina. New sprouting (yellow arrow), established sprouts on distal tip cells (green arrows), endothelial cells forming new connections (orange arrow), and perfused vessels (red asterisks) are highlighted. Right: Scheme showing the selection of a tip cell (green) through Notch signaling. High levels of Dll4 ligand in tip cells activates Notch and dampens VEGF-A signaling by downregulating VEGF receptor expression in adjacent stalk endothelial cells (red). Strong expression of Jagged1 in stalk cells antagonizes Dll4-mediated activation of Notch on neighboring tip endothelial cells. Consequently, tip cells show the strongest response to VEGF-A and grow toward the VEGF-A gradient. The levels of Notch signaling and the antagonistic activity of Jagged1 require Fringe-mediated glycosylation of Notch receptors. Reprinted by permission from Cold Spring Harb Laboratory Press: (Adams and Eichmann, 2010), copyright 2010.

1.10 Aims

The initial aim of this work was to compare the gene expression profiles and signaling pathways induced by the angiogenic growth factor VEGF-A, the inflammatory cytokine IL-1 and the general growth factor EGF. This identified a cluster of genes specifically induced by VEGF-A with potential importance for angiogenic processes. These aspects were covered in the publications (Schweighofer et al., 2009) and (Schweighofer et al., 2007). The major aim was then to select from the VEGF-specific gene cluster a gene mediating an important function for angiogenesis. For this purpose the homeobox transcription factor HLX was

investigated and its biological functions in endothelial cells and in sprouting angiogenesis were defined. The obtained data show that HLX mediates a genetic program to differentially regulate the expression of repulsive guidance cues in normoxia and hypoxia. Details of this study are described in the first authorship publication (Testori et al., 2011) and in the additional data section.

2. Publication (First authorship)

2.1 The VEGF-regulated transcription factor HLX controls the expression of guidance cues and negatively regulates sprouting of endothelial cells.

Julia Testori, Bernhard Schweighofer, Iris Helfrich, Caterina Sturtzel, Karoline Lipnik, Sabine Gesierich, Patrick Nasarre, Renate Hofer-Warbinek, Martin Bilban, Hellmut Augustin and Erhard Hofer. Blood First Edition Paper, prepublished online January 11, 2011; DOI 10.1182/blood-2010-07-293209

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Short title: HLX-mediated regulation of guidance proteins

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Abstract

The HLX gene encoding a diverged homeobox transcription factor has been found to be upregulated by VEGF-A in endothelial cells. We have now investigated the gene repertoire induced by HLX and its potential biological function. HLX strongly increased the transcripts for several repulsive cell guidance proteins including UNC5B, plexin A1 and semaphorin 3G. In addition, genes for transcriptional repressors such as HES1 were upregulated. In line with these findings, adenoviral overexpression of HLX inhibited endothelial cell migration, sprouting and vessel formation *in vitro* and *in vivo*, whereas proliferation was unaffected. This inhibition of sprouting was caused to a significant part by HLX-mediated upregulation of UNC5B as shown by shRNA-mediated downmodulation of the respective mRNA. VEGF-A stimulation of endothelial cells induced elevated levels of HLX over longer time periods resulting in especially high upregulation of UNC5B mRNA as well as an increase in cells displaying UNC5B at their surface. However, induction of HLX was strongly reduced and UNC5B upregulation completely abrogated when cells were exposed to hypoxic conditions. These data suggest that HLX may function to balance attractive with repulsive vessel guidance by upregulating UNC5B and to downmodulate sprouting under normoxic conditions.

Introduction

Vascular endothelial growth factor-A (VEGF-A) is the major trigger of vasculogenesis and angiogenesis during embryogenesis and blood vessel formation in the adult[1,2]. It has also been implicated in pathological angiogenesis in diseases such as cancer, chronic inflammatory disorders and retinopathy.[3] Whereas several peptide products are generated from the VEGF-A gene by differential splicing, the available data suggest that isoform VEGF-A_{165} is the predominant form responsible for the major angiogenic effects.[4]

The gene repertoire induced by VEGF-A mainly via VEGF receptor-2 has been investigated by several groups,[5-7] however the transcription factors upregulated by VEGF-A and how they mediate its specific and unique biological functions remain largely uncharacterized. We have recently identified a group of genes selectively or at least preferentially induced by VEGF-A in endothelial cells, when compared to a more general growth factor such as EGF or inflammatory mediators such as IL-1.[8] Most prominent upregulation in the described manner was found for the transcription factors NR4A2 (nuclear receptor subfamily 4, group A, member 2), EGR3 (early growth response 3), HLX (H2.0-like homeobox 1) and MEF2C (myocyte enhancer factor 2) suggesting their involvement in specific VEGF-A-triggered responses.[5]

In the current study we have focused on the investigation of HLX, which is an evolutionary highly conserved homeobox transcription factor originally detected in the visceral musculature of Drosophila.[9] Furthermore, HLX was shown to be expressed in bone marrow-derived CD34+ cells,[10] lymphocytes[11-13] as well as in endothelial cells.[14] Expression in type 1 helper T lymphocytes seems to play an essential role to establish heritable expression of the γ-IFN gene by epigenetic mechanisms.[11,12] In mouse development, the $HLX^{-/-}$ genotype is lethal around day 15 of embryonic development on a mixed genetic background, the embryos displaying severe hypoplasia of the gut and liver.[15,16] In this context, it has been proposed that, during visceral organogenesis, HLX regulates a mesenchymal-epithelial interaction that is required for early aspects of enteric nervous system development.[16]

There is accumulating evidence that several mechanisms are shared between the development of the nervous and vascular systems.[17] During angiogenesis endothelial tip cells, which lead the growing sprout, sense the environment by dynamically extending filopodia towards attractive signals and retracting them from repulsive

ones. In this regard endothelial sprouts use similar guidance cues as growing nerve fibers do for directional growth and network formation.[17] Guidance molecules with a similar role in endothelial tip cells and axonal growth cones include the semaphorins and plexin receptors as well as the secreted netrins and uncoordinated-5 (UNC5) receptors. However, signals and transcription factors that regulate the expression of these guidance cues have not yet been established.

We have now investigated the genes controlled by the transcription factor HLX in endothelial cells. Our data demonstrate that HLX is a specific regulator of cell guidance molecules such as UNC5B, plexin A1 (PLXNA1) and semaphorin 3G (SEMA3G), that presumably display repulsive functions in vessel guidance and/or may prevent inappropriate sprouting. Furthermore, HLX also induces transcriptional repressors including HES1, SNAI2 and BCOR. Following overexpression of HLX we observed an inhibition of migration of endothelial cells, decreased sprouting and reduced vessel formation. This inhibition of sprouting was in part due to UNC5B upregulated by HLX. Furthermore, we show that VEGF-A can increase UNC5B expression in an HLX-dependent way, however induction of HLX and consecutively UNC5B was strongly reduced or prevented under hypoxic conditions. We propose that HLX serves to counterbalance the effects of attractive guidance cues by upregulating the expression of repulsive guidance molecules such as UNC5B.

Materials and methods

Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously[18] and cultured in EGM-2 medium (Clonetics, Lonza, Walkersville, MD). HUVEC of passage 3 to 5 were used for experiments.

To apply hypoxic conditions the cell culture plates were transferred to a modular incubation chamber (Billups-Rothenberg, Inc., Del Mar, CA). This was inflated with 10% CO_2 compensated with 90% N_2 for 10 min, closed tightly and kept at 37°C for the length of the experiment. Cultures to be analyzed within the first 2 h after VEGF addition were preincubated under hypoxic conditions for 4 h.

HEK293 cells (ATCC No. CRL-1573) were cultured in MEMα medium with 10% NCS (both from Invitrogen, Carlsbad, CA). 293T cells (ATCC No. CRL-11268) were cultured in DMEM with 10% FCS (both from Invitrogen).

VEGF-A_{165} and bFGF were obtained from PromoKine (Heidelberg, Germany) or R&D Systems (Merck, Darmstadt, Germany).

Construction of recombinant adenoviruses and transduction of cells

A cDNA clone of human HLX (IRALp962M1922Q, http://www.imagenes-bio.de/) was obtained from RZPD (Heidelberg, Germany). The coding region together with a Flag-tag sequence at the 3´-end was cloned into the pACCMVplpASR+ expression plasmid[19] (pAC.HLX). The plasmids pAC.HLX and pJM17 (Microbix Biosystems, Toronto, CAN), were cotransfected into HEK293 cells by the calcium phosphate method (Stratagene, LaJolla, CA). Primary adenoviruses generated were subcloned and purified after amplification in HEK293 cells using CsCl-ultracentrifugation[20]. The HLX sequence in the viral preparations was confirmed by sequencing. Viral titre was determined using the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA). An empty control adenovirus[20] was also grown and purified as described above.

The UNC5B encoding adenovirus was a generous gift of Drs. Noriaki Kitamura and Hirofumi Arakawa (National Cancer Center, Tokyo, Japan).

Production of recombinant lentiviruses and transduction of cells

Plasmids (Mission shRNA pLKO.1-puro) containing the corresponding shRNA sequences targeted to HLX and UNC5B and a control shRNA plasmid were obtained

from Sigma-Aldrich. The shRNA plasmids together with two packaging vectors (pMD2.G and psPAX.1) were cotransfected into 293T cells. Supernatants were harvested after 24 and 48 h and used for infection mixed with medium in a ratio of 1:1 or 1:2.

RNA preparation

HUVEC were infected in subconfluent state with control (Ad.con) and HLX encoding adenovirus (Ad.HLX) using multiplicity of infections (MOI) of 20 to 40. Then cells were incubated with RNAlater (Ambion, Austin, TX) for 1 min, lysed with Trizol (Invitrogen, Carlsbad, CA) and RNA was extracted.

Real-time RT-PCR analysis

Total RNA (2 µg) was used to synthesize cDNA with Superscript II Reverse Transcriptase and oligodT primer (both from Invitrogen). mRNA levels were measured using realtime RT-PCR detecting SYBR GreenI in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). Values were normalized to β2-microglobulin mRNA as internal standard. Oligonucleotide primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in Supplemental Table 2.

Affymetrix microarray analysis

cRNA was prepared from total RNA, hybridized to the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) and arrays were scanned according to the manufacturer's protocols (Affymetrix support site, www.affymetrix.com/support/index.affx) as described.[21] RMA (Robust multiarray average) signal extraction and normalization were performed as described (http://www.bioconductor.org/).[22]

Western Blot Analysis

Cell pellets were lysed in Laemmli buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membrane was blocked with 5% skim milk / PBS and 0.1% Tween 20 (PBST) and incubated overnight with the primary antibody at 4 °C. The membrane was washed and incubated with peroxidase-conjugated

secondary antibody for 1 h. The membrane was incubated with ECL Plus reagent (GE Healthcare, Buckinghamshire, UK) and exposed to X-ray film. Bands were quantified in scanned film images by measuring tonality using Adobe Photoshop software.

Antibodies used were: mouse anti-human HLX (Abnova, Taipei, Taiwan), mouse anti-human GAPDH (Chemicon, Billerica, MA, USA), and secondary horseradish peroxidase-conjugated ECL sheep-anti-mouse antibodies (GE Healthcare).

Flow cytometry

Following treatment with accutase (PAA, Pasching, Austria) cells were harvested, fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton-X-100 (Sigma-Aldrich, St.Louis, MO) and blocked with 5% BSA-PBS. Cells were stained with 5 µg/ml of the humanized UNC5B antibody #4 (Genentech, San Francisco, CA) for 1 h at 4°C followed by the secondary antibody Alexa Fluor® 647 goat anti-human IgG (Invitrogen). Binding was assessed using a FACSCalibur (BD Biosciences).

Immunocytochemistry

HUVEC were grown in fibronectin-coated chamber slides. Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA/PBS. To reveal HLX cells were in addition permeabilized with 0.1% TritonX100/PBS. Slides were stained with: affinity-purified rabbit anti-human HLX antibodies[13] raised against a GST-HLX fusion protein (1:500 dilution); anti-human UNC5B antibody #4 (20 µg/ml, Genentech); secondary Alexa Fluor 568 goat anti-rabbit IgG at a dilution of 1:5000 or Alexa Fluor 647 goat anti-human IgG at 1:5000 (Molecular Probes, Invitrogen).

Proliferation assay

HUVEC were infected with adenoviruses using a MOI of 8. The cells were trypsinized on the following day and seeded into 96-well plates (5000 cells/well). Proliferation was determined by measuring the total protein content using the sulforhodamineB (SRB) colorimetric assay.[23] Experiments were performed with 5 wells per data point.

Transwell migration assay

One day after infection $5x10^4$ HUVEC were seeded into the upper well of a gelatinized transwell with a pore size of 8 µm (Corning, Lowell, MA). Following the

addition of 50 ng/ml VEGF-A_{165}/ml to the lower chamber, the transwell was incubated for 4 h at 37°C and the migration of cells towards the lower chamber was determined. Cells were fixed with methanol and stained with 1 µg/ml 4´,6-Diamidin-2-phenylindol (DAPI, Sigma-Aldrich, Taufkirchen, Germany). Cells in the lower chamber were counted in 5 randomly chosen microscopic fields photographed using a Nikon Diaphot TMD microscope and a CCD camera (Kappa GmbH, Gleichen, Germany).

Generation of endothelial cell spheroids

HUVEC were transduced at a MOI of 10 to 20 or infected with 0.5 to 1ml of lentivirus. Cells were then used for the spheroid-based *in vitro* or *in vivo* assays. Cells were used 24 h after infection with the adenoviruses or 48 h after infection with the lentiviruses to generate spheroids. Transduction efficiency of all viruses was regularly controlled.

Spheroids of a defined cell number were generated from non-transduced or transduced cells as described in Korff et al (2004).[24] Briefly, HUVEC were suspended in medium containing 0.25% (wt/vol) methylcellulose and grown in hanging drops overnight. Single spheroid with a defined cell number of approximately 400 (for the *in vitro* assay) or 100 (for the *in vivo* assay) cells/spheroid were obtained.

Spheroid-based *in vitro* angiogenesis assay

HUVEC spheroids were embedded into rat collagen gels as described[25] with the following modifications: Fifty spheroids of 400 cells per spheroid were mixed with 80% Methocel / 20% FCS. 0.1 ml ECGM (basal medium; PromoCell) without or containing VEGF or bFGF (25 ng/ml each) was layered onto the top of the formed gel. After 24 h sprouts were quantified by measuring the total length grown out of each spheroid on pictures taken on the Nikon microscope.

***In vivo* implantation of spheroids**

Spheroids were harvested and carefully suspended in 500 µl Matrigel (growth factor reduced; BD Biosciences) and fibrinogen (2 mg/ml; Calbiochem, Merck, Darmstadt, Germany) containing VEGF plus bFGF (500 ng/ml each). Gel formation was initiated by the addition of thrombin (0.4 U; Calbiochem).

Spheroid suspensions containing about 1000 spheroids of 100 cells each were injected subcutaneously on each side lateral to the abdominal midline region into 4-6

week old SCID mice. Two plugs were implanted per mouse, each experimental group consisted of 8 mice. After 14 days, mice were sacrificed, the harvested plugs were fixed overnight (0.5 g/l calcium acetate, 5 g/l zinc acetate, 5 g/l zinc chloride in 0.1 M Tris, pH 7.4) and analyzed by immunohistochemistry. Animal procedures were carried out in accordance to guidelines of the local committee for animal experimentation (DKFZ Heidelberg and RP Karlsruhe, Germany).

Immunohistochemistry

Zink fixed Matrigel plugs were embedded in paraffin. Plugs were sectioned at 8 µm. Primary antibodies used were: mouse-anti-human CD34 (QBEND10, Novocastra, Newcastle, UK); sheep-anti-human CD31 (Dako, Glostrup, Denmark). Antigen retrieval for anti-human-CD34 stainings was performed by digestion with Proteinase K (Sigma-Aldrich, Taufkirchen, Germany) at 37°C for 15 min. The secondary antibodies donkey-anti-sheep Alexa 488 (Invitrogen), and biotinylated goat-anti-mouse (Zymed, Invitrogen) were detected by streptavidin-peroxidase conjugate (Dako) and DAB substrate chromogen system (Dako).

Quantificationof the human grafted vasculature and statistical analysis

Sections were prepared across the entire implant size and three sections were selected from the beginning, the center and the end. MVD (microvessel density) analysis was performed using immunohistochemistry for human CD31 and CD34. Images of the complete plug area were taken using an Olympus IX50 microscope and assembled by multiple alignment (Cell-P, Olympus). Fluorescent signals of the complete plug matrix were calculated as vessel number per mm^2.

Results

HLX upregulates genes for cell guidance proteins and transcriptional repressors

The gene encoding the homeobox transcription factor HLX has previously been identified by us to be induced by VEGF in endothelial cells suggesting the involvement of HLX in VEGF-mediated transcriptional responses.[5] We therefore investigated the transcriptomic program induced by HLX. An adenoviral expression construct encoding a Flag-tagged HLX cDNA was generated and used to transduce HUVEC in parallel with control adenovirus. mRNA was extracted from cells between 4 and 32 h after infection and subjected to Affymetrix microarray analysis (GEO GSE13054). 53 genes were identified to be induced more than 2.5 fold 16 h after infection (see Supplemental Table 1). This group of genes was analyzed according to the categories surface receptors, secreted proteins, cytoplasmic signaling proteins and transcription factors. Thereby it became apparent that among the strongest induced genes were those for guidance receptors with proposed repulsive functions in the guidance of endothelial sprouts, namely UNC5B and plexin A1. Furthermore, transcripts for a secreted guidance molecule, SEMA3G, and for several transcription factors with repressive function were found to be upregulated. One example is the repressor HES1, which has been described as a direct target gene of the Notch signaling pathway. Table 1 displays the most strongly induced genes, which were confirmed by real-time RT-PCR. The kinetics of the upregulation of mRNAs for UNC5B, PLXNA1, SEMA3G and HES1 as measured by real-time RT-PCR shows that the relative upregulation was most pronounced for UNC5B (Figure 1A). The accumulation of recombinant HLX protein after transduction with Ad.HLX is depicted in Figure 1B.

VEGF most prominently upregulates UNC5B in an HLX-dependent way

To evaluate whether VEGF upregulates genes for repellent guidance molecules, which could be mediated by HLX as a secondary transcriptional response to VEGF, we examined mRNA levels for UNC5B, PLXNA1, SEMA3G and HES1 by real-time RT-PCR. From these preferentially UNC5B mRNA was found to be increasingly

upregulated following VEGF stimulation starting between 2 and 4 h, being 10-fold increased at 16 h and reaching levels several hundred-fold of the starting values between 24 and 56 h (Figure 2A, Supplemental Figure 1A). This upregulation of UNC5B mRNA was reflected in the appearance of a fraction of cells displaying UNC5B at their surface as shown by flow cytometry (Figure 2C) and cytoimmunochemistry (Figure 2D).

In the same samples HLX mRNA already displayed a rapid peak value after 1 hour of stimulation and thereafter stayed 5- to 10-fold elevated up to 56 h. HLX protein was increased starting at 4 h and reaching 10-fold higher levels at later time points as shown by Western blot analysis (Figure 2B). The VEGF induced HLX accumulated in the nuclei (Figure 2D). It therefore seemed possible that VEGF-induced HLX is involved in the upregulation of UNC5B. We further investigated how general the upregulation of HLX by VEGF would be in endothelial cells of different origin. This indicated that VEGF indeed induces HLX in a broad range of different endothelial cell types obtained from various sources including large vessel and microvascular endothelial cells. The induction rates were comparable to those observed with different HUVEC isolates ranging from 5- to 30-fold with a tendency of somewhat lower inducibility in microvascular cells (Supplemental Figure 1B).

Next we tested whether HLX would directly mediate the observed upregulation of UNC5B by VEGF. Indeed, downmodulation of HLX using a respective shRNA expressed from a lentiviral vector reduced the VEGF-induced accumulation of UNC5B mRNA and protein by more than 80% (Figure 2E). This showed that UNC5B upregulation by VEGF is mediated via HLX.

HLX inhibits migration of endothelial cells, but proliferation is unaffected

Considering that HLX appeared to preferentially upregulate UNC5B and other repellent guidance cues and a transcriptional repressor of the Notch pathway, we next investigated to what extent HLX would affect proliferation and/or migration of endothelial cells. When HUVEC were infected with HLX encoding adenoviruses, no significant influence of HLX overexpression on the growth of cells could be detected in comparison to control adenovirus infected or non-infected cells (Figure 3A). However, in a transwell system, migration towards VEGF was inhibited by about 80%

as compared to cells infected with control adenovirus or non-infected cells (Figure 3B).

We have further assessed the potential triggering of apoptosis by HLX overexpression, but no signs of apoptosis were detectable 4 days after infection (Supplemental Figure 2). This suggests that, in endothelial cells, HLX specifically interferes with a migratory mechanism, while it does not affect proliferation and apoptotic signaling pathways.

HLX and UNC5B strongly inhibit sprouting of endothelial cells *in vitro*

To investigate the effect of HLX on angiogenesis and particularly on sprouting of endothelial cells, we first used an *in vitro* angiogenesis assay, which evaluates sprout formation of cells embedded as spheroids in a collagen matrix. In this assay HLX overexpression strongly inhibited sprouting of cells induced by VEGF or bFGF (Figure 4A and B). When decreasing MOI of virus infection were used, the inhibiting effect diminished in a dose-dependent manner (Supplemental Figure 3).

To evaluate whether UNC5B expression could mediate the inhibition of sprouting we first used an adenovirus for overexpression of UNC5B. Indeed, ectopic UNC5B expression was able to strongly reduce sprouting activity (Figure 4C). This indicated that the inhibitory effect of HLX overexpression is at least in part mediated via UNC5B.

Inhibition of sprouting by HLX is mediated to a significant part by UNC5B

To establish to which extent UNC5B would contribute to the inhibition of sprouting observed after HLX overexpression we used lentiviral expression of shRNA for the downmodulation of UNC5B mRNA. On average a 60% downmodulation of the UNC5B mRNA was achieved after VEGF induction at the 56 h time point (Supplemental Figure 4A). The results show that a reduction in the HLX-mediated upregulation of UNC5B led to significantly decreased inhibition of sprouting (Figure 4D). This shows that the inhibitory effect of HLX overexpression is at least to a significant part caused by UNC5B upregulation.

It was further intriguing, that also in the VEGF-induced control samples downmodulation of UNC5B caused a strong increase of sprouting activity as

evidenced by the cumulative sprout length depicted in Figure 4D. This effect was primarily composed of a higher number of sprouts (Supplemental Figure 4B) suggesting that UNC5B upregulated in VEGF-induced endothelial cells reduces sprout initiation.

Upregulation of HLX and UNC5B is strongly reduced under hypoxic conditions

Furthermore, we tested the hypothesis that a potential reason for the upregulation of inhibitory pathways and molecules by VEGF would be the downmodulation of sprouting in a negative feed-back loop when normoxic conditions are restored after neovascularization. Therefore we comparatively evaluated the effects of VEGF on HLX and UNC5B mRNAs under normoxic and hypoxic conditions. The obtained results show that under hypoxia the VEGF-inducible levels of HLX mRNA were strongly reduced, especially at later time points, and the upregulation of UNC5B mRNA was completely prevented (Figure 5A). In contrast hypoxia induced VEGF mRNA as previously shown.[26]

When we performed the spheroid sprouting assay under hypoxic conditions we observed a significant increase of sprouting supporting that hypoxia has an additive effect on VEGF-induced sprouting. Hypoxia- plus VEGF-induced sprouting could be inhibited by overexpression of HLX, however the relative inhibition appeared less pronounced when compared to the inhibition under normoxic conditions (Figure 5B). This suggests that HLX-mediated induction of UNC5B is involved in downmodulation of sprouting under normoxic conditions and hypoxia would facilitate sprouting by reducing UNC5B expression.

Overexpression of HLX inhibits vessel formation *in vivo*

To test whether overexpression of HLX would exert an inhibiting effect on angiogenesis *in vivo* we further employed an *in vivo* angiogenesis assay with human endothelial cells in mice[27]. In this assay, HUVEC spheroids infected with HLX or control adenoviruses were suspended in a mixture of Matrigel and fibrinogen supplemented with growth factors and implanted subcutaneously into SCID mice. Grafted human endothelial cells vascularize the plug and anastomose with the mouse vasculature to give rise to a perfused vascular network (see Supplemental

Figure 5). Plugs were harvested after 14 days and analyzed by immunohistochemistry.

A significant reduction in the density and length of vessels was observed in vascular networks originating from HLX overexpressing HUVEC compared to control cells (Figure 6). The inhibition was similarily pronounced as the inhibition of sprouting *in vitro* supporting that HLX plays a comparable role *in vivo.*

Discussion

Several members of the homeobox family have been implicated in the development of the cardiovascular system as well as in vascular remodeling in the adult and have been proposed to be important to many pathologies including atherosclerosis and tumor angiogenesis.[28-30]

We have recently identified HLX,[5,8] a diverged homeobox gene, so far not known to be involved in vascular remodeling, as being preferentially upregulated in endothelial cells by VEGF-A and to some extent also by another main angiogenic mediator, bFGF.[5,31] HLX has previously been observed to be expressed in HUVEC, in placental endothelial cells and in trophoblasts.[6,14,32,33] However, no precise function and detailed mechanism of action has yet been defined for HLX in the vascular system. Since our experiments neither identified an induction of HLX expression by inflammatory mediators nor by other more general growth factors such as EGF,[5] we proposed that HLX might mediate a specific function of VEGF-A and bFGF related to angiogenesis and/or vascular remodeling.

In the present study, we have undertaken an analysis of downstream target genes of HLX by gene profiling following overexpression in endothelial cells (Supplemental Table 1). It was intriguing that HLX can be a prominent inducer of genes implicated in the negative regulation of the sprouting process, including genes for repulsive guidance cues such as UNC5B, PLXNA1 and SEMA3G as well as a transcriptional repressor, HES1 (Table 1).

UNC5B belongs to the Uncoordinated-5 family of transmembrane receptors which consists of four members. From these UNC5A plays a pivotal role in neurons for axon guidance, whereas UNC5B is largely restricted to the vascular system.[34] It has been demonstrated that upon binding to its soluble ligand Netrin-1, angiogenesis is inhibited.[17,35] A primarily repulsive role of UNC5B and its ligands in vessel formation is further indicated by the finding that disruption of the UNC5B gene in mice leads to increased extensions of tip cell filopodia, excessive vessel branching and abnormal navigation.[34] UNC5B is expressed in growing embryonic vessels and is then downregulated in the quiescent adult vasculature, but reexpressed upon stimulation of sprouting angiogenesis, e.g. in tumor angiogenesis.

PLXNA1 and SEMA3G are members of another complex system regulating axonal and vessel guidance.[36,37] SEMA3G has recently been found to be a primarily

endothelial cell expressed isoform.[38] In general, members of the class 3 semaphorin family bind to neuropilin-1 or neuropilin-2 in a complex with plexins as co-receptors.[37,39] As neuropilins are also coreceptors for various VEGF isoforms these interactions interfere with VEGF receptor signaling and mediate repulsive signaling via the plexin receptors.

The transcriptional repressor HES1 is a hallmark of the activated Notch pathway.[40] This could indicate a link to tip and stalk cell differentiation as it has been shown that tip cells via Dll4 activate the Notch receptor in neighboring cells. Thereby stalk cells are inhibited to sprout and are prevented from becoming additional tip cells.[41,42] Since HLX induces HES1, it is possible that this might support the stalk cell phenotype.

Based on further functional data that, in line with the repulsive and repressive functions of the described molecules, HLX overexpression strongly inhibited sprouting of endothelial cells, we started a systematic analysis to define which of the genes would be most significantly involved in HLX- and VEGF-regulated mechanisms. The obtained data showed that especially UNC5B is intimately linked in expression and function to HLX and VEGF-A. First, from the four genes analyzed by realtime RT-PCR, which included UNC5B, PLXNA1, SEMA3G and HES1, overexpression of HLX upregulated UNC5B mRNA several hundred-fold, which was by far the strongest effect (Figure 1). Second, we observed that in VEGF-A-containing cultures UNC5B mRNA displayed a continuous several hundred-fold upregulation over several days, whereas PLXNA1, SEMA3G and HES1 mRNAs increased only modestly around five-fold (Figure 2A). The VEGF-induction of UNC5B mRNA was also reflected in the appearance of UNC5B protein at the surface of a fraction of the cells (Figure 2C). UNC5B upregulation was preceded by the induction of continuously elevated level of HLX mRNA resulting in over 10-fold nuclear accumulation of HLX protein at 24 h (Figure 2B and 2D). This was in line with the possibility that HLX is involved in upregulating transcription of the UNC5B gene. Importantly, downmodulation of HLX mRNA by shRNA reduced VEGF-inducible UNC5B mRNA and protein by over 80 % (Figure 2E). This directly confirmed that HLX indeed mediates the VEGF-triggered UNC5B induction to a large extent.

Since our data showed that HLX overexpression caused a strong inhibition of migration (Figure 3B) and sprouting of endothelial cells (Figure 4A and B), we first tested the possibility that UNC5B upregulated by HLX might be decisively involved.

Indeed, the obtained data showed that overexpression of UNC5B alone using a recombinant adenovirus caused a strong inhibition of sprouting (Figure 4C). Vice versa, downmodulation of UNC5B in HLX overexpressing cells significantly reduced inhibition of sprouting restoring activity to the level of control virus infected cells (Figure 4D). This confirmed that UNC5B expression by itself can cause significant inhibition of sprouting and at least a significant part of the HLX-mediated inhibition. In this context, it is important to mention that downmodulation of UNC5B not only increased sprouting in HLX suppressed endothelial cells, but also in VEGF-induced control cells reaching 3-fold higher sprouting levels (Figure 4D and Supplemental Figure 4B). This may indicate that HLX-mediated inherent expression of UNC5B in VEGF-induced cells reduces sprouting activity. This is in accordance with previous reports that UNC5B is preferentially expressed in growing vessels and capillaries.[35] Based on these data and considering a primarily repulsive role, one can principally speculate on two non-exclusive potential roles of VEGF induced HLX and UNC5B for sprouting angiogenesis. It might be possible that UNC5B expression is generally needed to counterbalance the effects of attractive guidance cues. This may be necessary to fine-tune sprouting by preventing excessive sprout initiation under VEGF stimulation and/or to give appropriate direction to the growing sprout by reducing branching. This possibility is in line with the finding that downmodulation of UNC5B in VEGF induced cells additively increases the number of sprouts (Supplemental Figure 4B).

Furthermore, it might be possible that UNC5B is an inbuilt inhibitory feed-back mechanism that serves to adapt sprouting activity to physiological needs. Such a possibility could be the modulation of UNC5B expression by a hypoxic gradient. It has been previously shown that hypoxia via the transcription factor HIF1α can lead to the up- and downregulation of several hundred genes.[26] We therefore tested the hypothesis that HLX and UNC5B expression in the presence of VEGF could be modulated by hypoxia. Indeed, we find that under hypoxic conditions VEGF induction of HLX mRNA is strongly reduced and accumulation of UNC5B mRNA is prevented. Furthermore, hypoxia increased sprouting additively to VEGF. It is tempting to speculate that this is due to the reduced capacity of the cells to produce UNC5B under hypoxic conditions. These data strongly suggest that one function of the VEGF/HLX/UNC5B induction axis may be to prevent inappropriate sprouting under normoxic conditions and to adapt sprouting activity to an hypoxic gradient.

We have further used an endothelial spheroid xenografting assay to evaluate whether HLX might similarly function *in vivo* and exert a negative effect on vessel formation. In this assay HUVEC spheroids are implanted in a Matrigel/fibrinogen matrix below the skin of SCID mice. The human cells can normally give rise to a perfused vascular network fused with murine vessels as indicated by the presence of erythrocytes in the human vessel parts (Supplemental Figure 5). Comparable to the *in vitro* assay, HLX expression strongly reduced the capacity of the cells to form vessels supporting that HLX exerts similar inhibitive function *in vivo*.

This work defines for the first time a transcription factor apparently controling a genetic program for the expression of several repulsive molecules. Whereas the data described here show that UNC5B is most closely linked to VEGF induction as well as the inhibitory effects of HLX, it will be further interesting to determine to what extent PLXNA1 and SEMA3G might function in a comparable or rather different way. Considering these newly described properties of HLX, we propose that the factor might be a potent inhibitor of pathologic angiogenesis and could be used as a novel principle to inhibit tumor angiogenesis.

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Authorship contributions

JT did the majority of detailed planning, experimental work and analysis as well as writing of parts of the manuscript, BS, IH, CS, KL, SG, PN, RHW and MB performed experiments and analyzed data. HGA contributed in the design of and conclusions from the vessel formation experiments. EH perceived and designed the overall research work and wrote the majority of the paper.

Disclosure of conflicts of interest

The authors declare no competing financial interests.

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UniGene ID	Entrez Gene	Gene Symbol	Gene Title	Ad.HLX/Ad.con 4h	8h	16h	32h
Surface Receptors							
Hs.585457	219699	UNC5B	unc-5 homolog B (C. elegans)	1.41	2.23	5.71	7.14
Hs.432329	5361	PLXNA1	plexin A1	1.15	2.96	4.61	8.38
Hs.132781	9466	IL27RA	interleukin 27 receptor, alpha	0.91	1.86	4.18	5.53
Secreted proteins							
Hs.9315	56944	OLFML3	olfactomedin-like 3	1.09	1.72	4.62	23.68
Hs.59729	56920	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	1.27	2.28	2.88	4.89
Cytoplasmic (signaling) proteins							
Hs.417050	8900	CCNA1	cyclin A1	0.78	2.15	3.79	2.96
Hs.2128	1847	DUSP5	dual specificity phosphatase 5	1.07	2.38	3.76	3.54
Hs.381167	1992	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	0.93	1.33	3.15	4.25
Transcription factors							
Hs.360174	6591	SNAI2	snail homolog 2 (Drosophila)	1.58	3.87	4.51	6.91
Hs.525704	3725	JUN	Jun oncogene	1.48	1.51	4.06	3.14
Hs.250666	3280	HES1	hairy and enhancer of split 1, (Drosophila)	2.13	3.63	3.49	4.37
Hs.659681	54880	BCOR	BCL6 co-repressor	2.01	2.54	3.45	5.37

Table 1: Selected genes upregulated by HLX in endothelial cells. Total RNA was isolated from HUVEC after infection with HLX encoding (Ad.HLX) and control adenoviruses (Ad.con) for 4, 8, 16 and 32 h. RNA was subjected to microarray analysis using the Affymetrix Human Genome U133 Plus 2.0 Array. Changes in gene expression induced in Ad.HLX relative to Ad.con infected cultures are displayed as fold induction at the given time points. The full set of Affymetrix data is available in the GEO database under GSE13054.

Figure legends

Figure 1. HLX upregulates genes for repellent cell guidance molecules and a transcriptional repressor of the Notch signaling pathway.

(A) Real-time RT-PCR analysis of UNC5B, PLXNA1, SEMA3G and HES1 mRNA: HUVEC were transduced for 4, 8, 16 and 32 h with recombinant adenoviruses encoding HLX (Ad.HLX) or control viruses (Ad.con) using an MOI of 20. Total RNA was isolated and real-time RT-PCR performed as described in the methods section. Values were normalized to ß2-microglobulin mRNA as internal standard. Mean values +/- standard deviation (SD) calculated from triplicate wells are depicted as fold induction of the mRNA levels obtained after infection with Ad.HLX (■) or Ad.con (○) as compared with non-infected cells. Results of one representative experiment of three independently performed are shown.

(B) Western blot analysis of HLX: Total cell lysates were prepared from HUVEC non-transduced (0 h) or tranduced with Ad.HLX (4, 8, 16 and 32 h) or with Ad.con for 32 h using an MOI of 20. Cells were harvested and proteins were separated by SDS-PAGE. Membranes were probed with anti-HLX and anti-GAPDH antibodies.

Figure 2. VEGF upregulates HLX and UNC5B in endothelial cells.

(A) Real-time RT-PCR analysis of UNC5B, PLXNA1, SEMA3G, HES1 and HLX mRNA: HUVEC were cultured to density, starved overnight in EBM-2 medium without supplements and then induced with 100 ng VEGF/ml for 1, 2, 4, 8, 16, 32 and 56 h. Total RNA was isolated and mRNA levels were determined by real-time RT-PCR. All values were normalized to ß2-microglobulin mRNA as internal standard. Results displayed represent the mean of fold induction +/- standard error of the mean (SEM) of the respective mRNA levels calculated from triplicate wells. One representative experiment of three performed is shown.

(B) Western blot analysis of HLX: Total cell lysates were prepared from HUVEC induced with 100 ng/ml VEGF for 4, 6 and 24 h and HUVEC transduced with adenoviruses encoding HLX (Ad.HLX) for 72 h. Cells were harvested and proteins were separated by SDS-PAGE. Membranes were probed with anti-HLX and anti-GAPDH antibodies. The left part displays one exemplary blot, the right part the quantification obtained from three independent experiments.

(C) Flow cytometry analysis of UNC5B surface expression after VEGF induction: HUVEC starved as described above were induced with VEGF-A for 56 h or kept without stimulation. Cells were detached from the plates, stained with UNC5B and Alexa Fluor® 647-labelled goat anti-human IgG antibodies and subjected to flow cytometry. The left part displays representative dot blots of untreated, 56 h VEGF-induced and control samples transduced for 56 h with an UNC5B adenovirus. The right part shows the quantification of three independent experiments performed in triplicates. Mean percentage of positive cells +/- SEM calculated from at least three independent experiments is displayed.

(D) Immunocytochemistry analysis of HLX and UNC5B after VEGF induction: HUVEC were induced for 24 and 48 h, cells were fixed and stained with anti-HLX and anti-UNC5B antibodies. The upper part displays the VEGF induction, the lower part a control experiment using transduction with HLX and UNC5B expressing adenoviruses.

(E) Upregulation of UNC5B by VEGF-A is HLX-dependent: HUVEC transduced with lentiviruses containing shRNA.con and shRNA.HLX for 48 h were starved overnight and then induced with VEGF for 24, 48 or 56 h as indicated. Cells were harvested and UNC5B mRNA and protein analyzed by realtime RT-PCR and flow cytometry, respectively. The left part displays fold induction of UNC5B or HLX mRNA +/- SD in comparison to non-induced control shRNA cells as calculated from one experiment representative of four performed in triplicates. The right part displays percent UNC5B positive cells +/- SD as measured by flow cytometry in a representative experiment performed in triplicates.

Figure 3. HLX does not affect the proliferation of endothelial cells, whereas migration is strongly reduced.

(A) Proliferation assay: HUVEC were infected with HLX encoding adenoviruses (Ad.HLX ▲) or control viruses (Ad.con ■) using an MOI of 8 or remained non-infected (w/o ♦). After 24 h $5x10^3$ cells were seeded per well into 96-well plates. 0, 1, 2 and 3 days after seeding the protein content was measured at OD_{492} using the sulforhodamineB (SRB) assay. The values display the increase in protein content over the indicated time period +/- SD calculated from 5 wells each. One experiment of three with similar results is shown.

(B) Migration assay: HUVEC were transduced with Ad.HLX or Ad.con with a MOI of 8 each or remained non-infected. Three days after infection cells were seeded into a transwell system and the migration of the cells from the upper chamber towards the lower chamber containing medium with 50 ng VEGF/ml or without VEGF was scored 4 h after seeding of the cells. Cells migrated into the lower chamber were visualized by staining with DAPI. Five random microscopic fields per transwell were photographed at a magnification of 10-fold and the stained cells counted. Values display the percentage of migrated cells in the individual samples in comparison to the VEGF-induced migration in samples containing non-infected cells arbitrarily set to 100%. Mean values were calculated from two independent experiments with triplicates each +/- SD. Inhibition of migration by Ad.HLX was significant as calculated by t test (** $P < .005$, *** $P < .001$).

Figure 4. HLX causes strong inhibition of sprouting which is mediated to a significant part by UNC5B.

HUVEC were infected with Ad.HLX, Ad.UNC5B or Ad.con. The following day spheroids of infected and non-infected endothelial cells were generated as described in Materials and Methods. The spheroids were embedded into collagen gels and induced with VEGF or bFGF (25 ng/ml) or cultured without cytokine stimulation. After 24 h the spheroids were fixed and photographic images were taken for quantification. (A) Representative images of spheroids generated from Ad.HLX or Ad.con infected cells (10 MOI) and induced with VEGF (+) or left untreated (-) are displayed. (B) Quantification of inhibition of sprouting by overexpression of HLX: analyses of sprout lengths were performed by measuring the total lengths of sprouts for 10 spheroids each on microscopic images using the ImageJ software (http://www.uhnres.utoronto.ca/facilities/wcif/imagej/). Results are displayed as mean values +/- SEM of the fold induction of sprout lengths observed when compared to non-induced samples infected with control viruses (arbitrarily set to 1). One representative experiment out of four performed is shown. (C) Quantification of inhibition of sprouting by overexpression of UNC5B: Analyses were as described above. One representative experiment out of three is shown. (D) Quantification of increase in sprouting by downmodulation of UNC5B in endothelial cells infected with Ad.con or Ad.HLX: HUVEC were first transduced with lentiviruses for control shRNA or shRNA targeted against UNC5B. After 1 d cells were in addition infected with 15

MOI of Ad.con or Ad.HLX. The following day spheroids were prepared and the next day incorporated into collagen gels in the presence of VEGF-A. Total sprout lengths of 15 spheroids each were measured. The shown values are calculated from four independent experiments and display percent sprout length +/-SEM in comparison to the samples transduced with Ad.con and shRNA.con. *** P < .001, t- test.

Figure 5: Under hypoxic conditions VEGF-mediated upregulation of HLX and UNC5B mRNA is strongly reduced whereas sprouting activity is increased.

(A) Comparison of VEGF-A effects on HLX, UNC5B and VEGF-A mRNA under hypoxic and normoxic conditions: Starved HUVEC were treated with VEGF-A and were kept under normoxic or hypoxic conditions for 4, 8, 16 and 32 h. The sample for the 1 h value was kept under hypoxic conditions already for 4 h before addition of VEGF. Cells were harvested, the RNA isolated and subjected to realtime RT-PCR analysis. One represenative experiment of three performed in triplicates is displayed. The values depict the mean of triplicates +/- SEM.

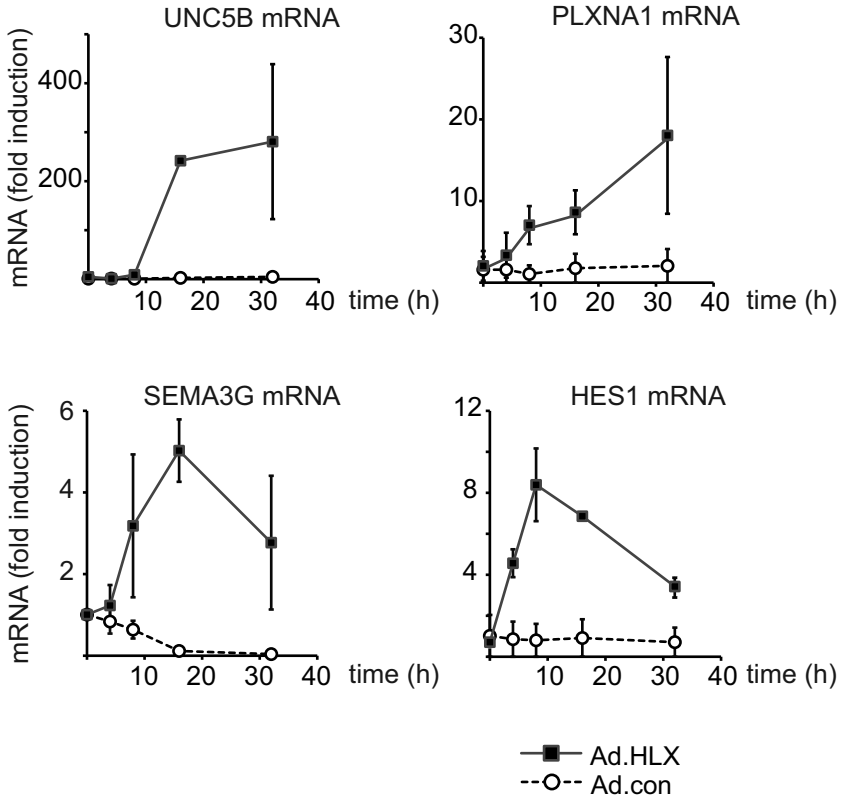
(B) Comparison of sprouting activity under hypoxic and normoxic conditions: HUVEC infected with Ad.con or Ad.HLX (20 MOI each) for 1 day were used in the spheroid sprouting assay. Normoxic or hypoxic conditions were employed immediately after embedding into the collagen gel concomitant with VEGF addition. Results are displayed as fold incuction +/- SEM of total sprout length in comparison to spheroids incubated at normoxic conditions without VEGF. ** P < .005, *** P < .001, t-test.

Figure 6. HLX reduces vessel formation in an endothelial spheroid xenografting assay *in vivo.*

HUVEC were infected with HLX encoding adenoviruses and control viruses with an MOI of 20. The infected cells were used to generate endothelial cell spheroids consisting of about 100 cells/spheroid. Spheroids were suspended in a Matrigel/fibrinogen mixture containing 500 ng/ml VEGF and bFGF. Following addition of thrombin the spheroid suspension was injected subcutaneously into SCID mice (2 plugs/mouse, 8 mice/experimental group). Plugs were harvested after 14 days, fixed and analyzed by immunohistochemistry using CD31 or CD34 or antibodies. (A) Representative images of sections of plugs containing HUVEC infected with Ad.HLX or Ad.con and stained with anti-human CD34 antibodies or anti-human CD31 antibodies plus hematoxylin. (B) Evaluation of vessel formation was performed by

measuring the vessel areas stained for CD34 on 16 images of 8 sections. The microvessel density (MVD) per mm^2 +/- SEM is depicted for plugs containing vessels with Ad.HLX (black bar) and Ad.con (white bar) infected HUVEC. * $P < .05$, t-test.

A


UNC5B mRNA
PLXNA1 mRNA
SEMA3G mRNA
HES1 mRNA
mRNA (fold induction)
time (h)
Ad.HLX
Ad.con

B

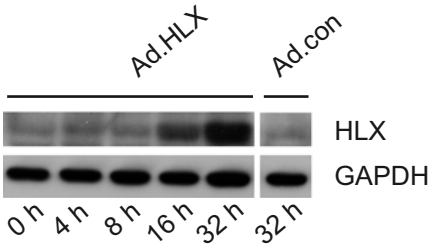
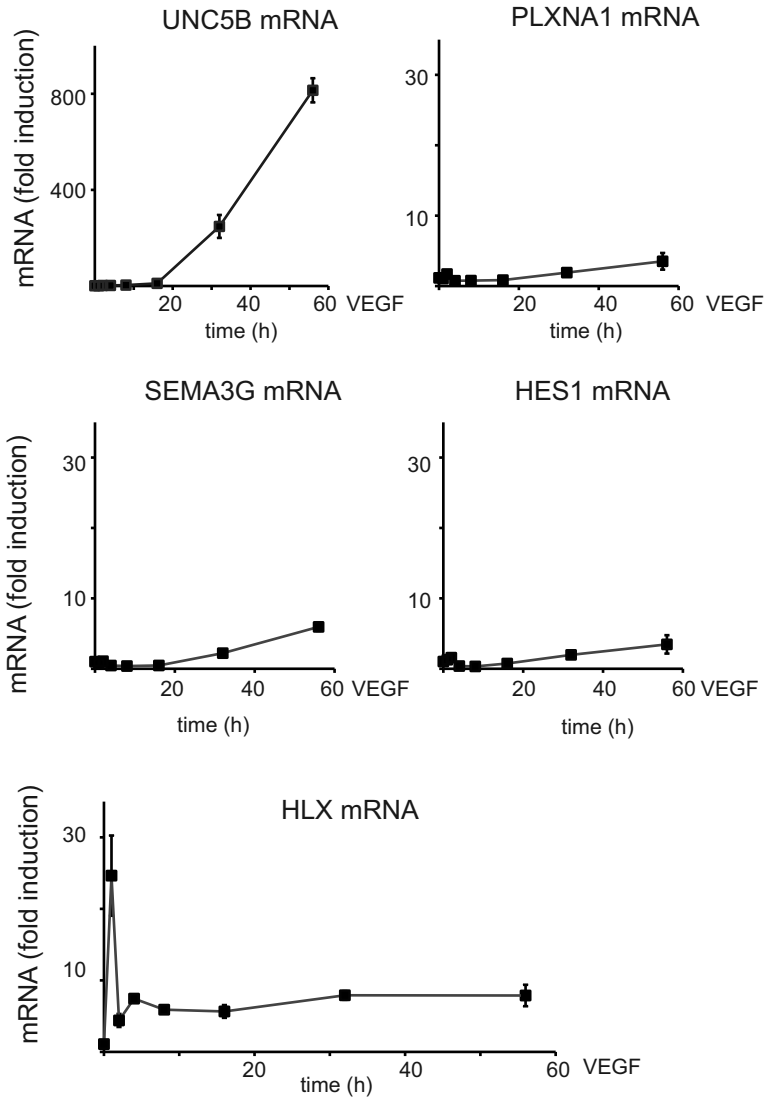
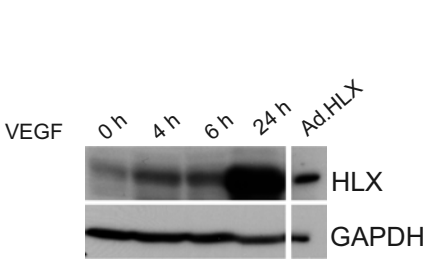

Ad.HLX
Ad.con
HLX
GAPDH
0 h
4 h
8 h
16 h
32 h
32 h

Fig. 1

A


UNC5B mRNA
PLXNA1 mRNA
SEMA3G mRNA
HES1 mRNA
HLX mRNA
mRNA (fold induction)
800
400
30
10
20
40
60
VEGF
time (h)

B


VEGF
0 h
4 h
6 h
24 h
Ad.HLX
HLX
GAPDH

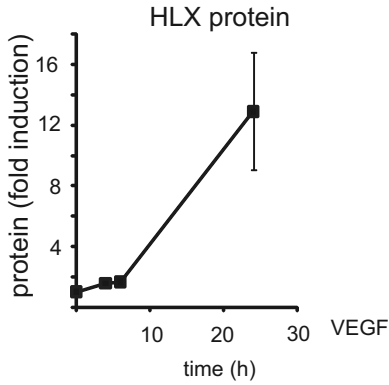
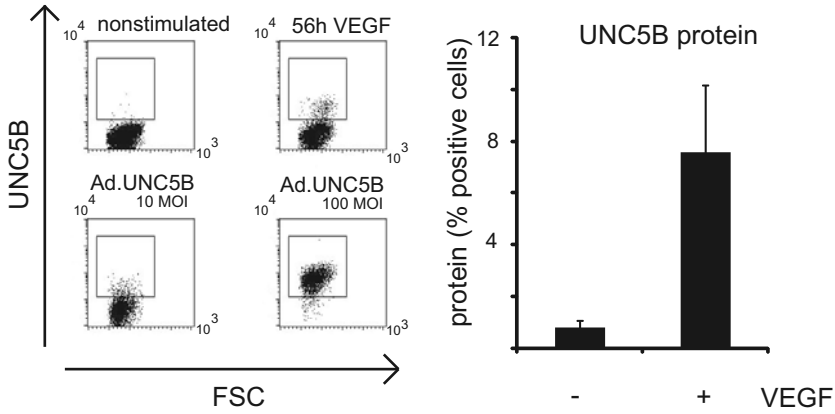

HLX protein
protein (fold induction)
16
12
8
4
10
20
30
VEGF
time (h)

Fig. 2A+B

C


nonstimulated
56h VEGF
Ad.UNC5B 10 MOI
Ad.UNC5B 100 MOI
UNC5B
FSC
UNC5B protein
protein (% positive cells)
12
8
4
-
+
VEGF

D

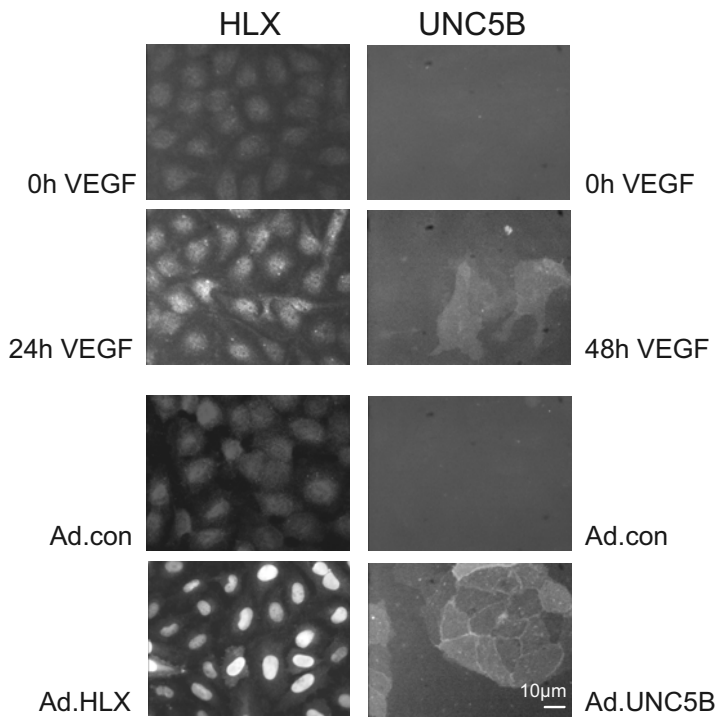

HLX
UNC5B
0h VEGF
0h VEGF
24h VEGF
48h VEGF
Ad.con
Ad.con
Ad.HLX
Ad.UNC5B
10μm

Fig. 2C+D

E

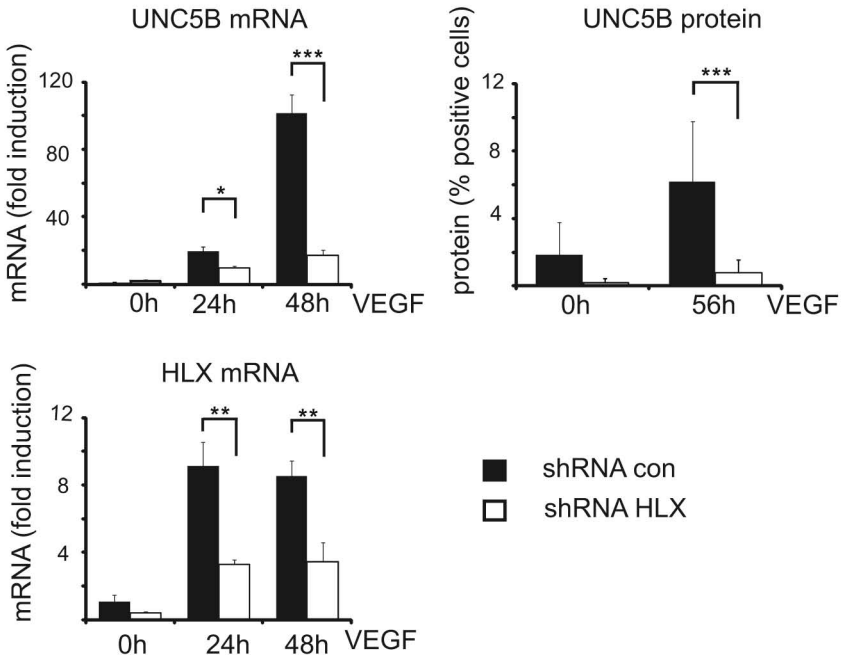
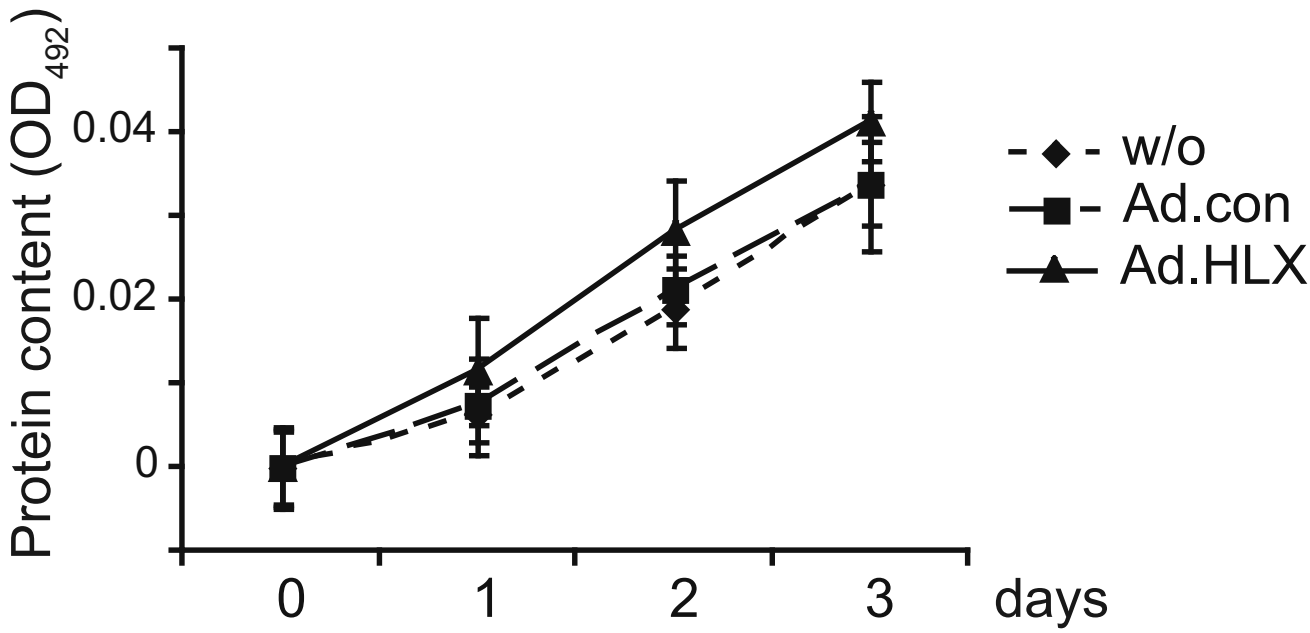

UNC5B mRNA
mRNA (fold induction)
120
80
40
*
***
0h
24h
48h
VEGF
UNC5B protein
protein (% positive cells)
12
8
4
***
0h
56h
VEGF
HLX mRNA
mRNA (fold induction)
12
8
4
**
**
0h
24h
48h
VEGF
shRNA con
shRNA HLX

Fig. 2E

A


Protein content (OD492)
0.04
0.02
0
0
1
2
3
days
w/o
Ad.con
Ad.HLX

B

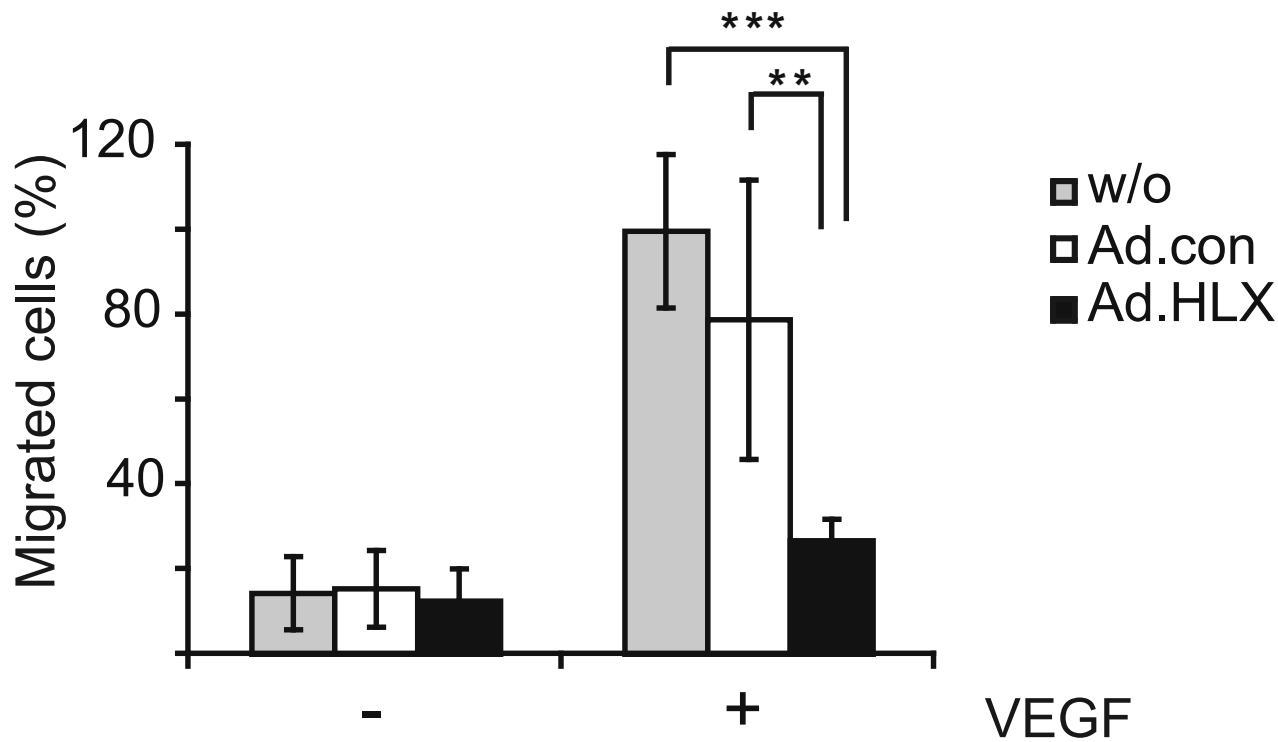
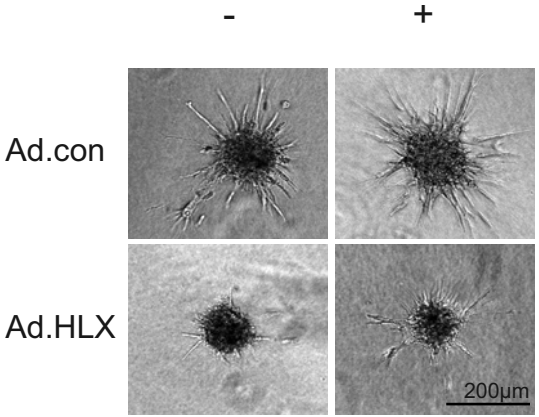

***
**
Migrated cells (%)
120
80
40
-
+
VEGF
w/o
Ad.con
Ad.HLX

Fig. 3

A


-
+
VEGF
Ad.con
Ad.HLX
200μm

B

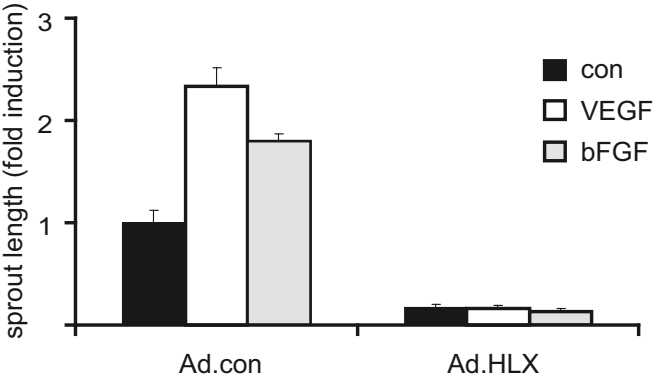
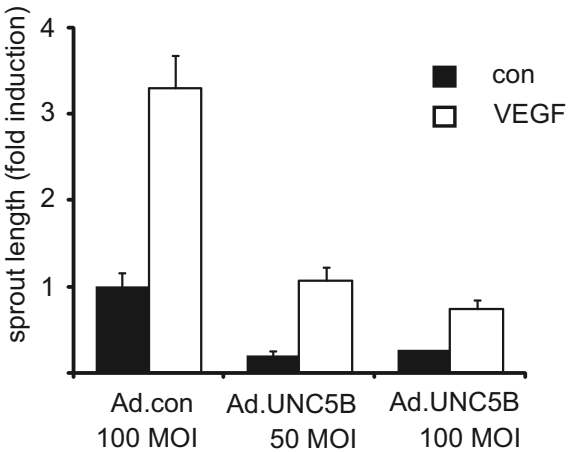

sprout length (fold induction)
3
2
1
con
VEGF
bFGF
Ad.con
Ad.HLX

Fig. 4A+B

C


sprout length (fold induction)
4
3
2
1
con
VEGF
Ad.con
100 MOI
Ad.UNC5B
50 MOI
Ad.UNC5B
100 MOI

D

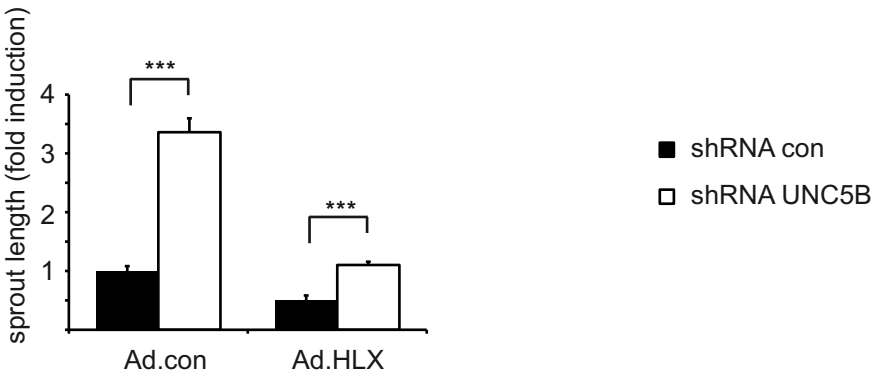
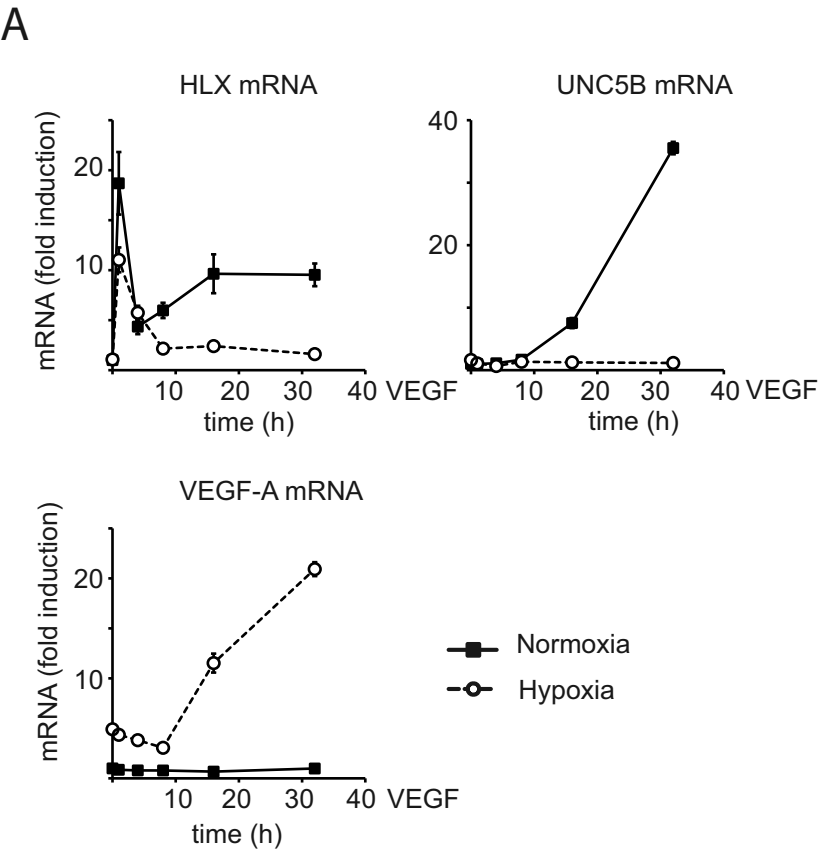

sprout length (fold induction)
4
3
2
1
***
***
Ad.con
Ad.HLX
shRNA con
shRNA UNC5B

Fig. 4C+D


A
HLX mRNA
UNC5B mRNA
mRNA (fold induction)
20
10
40
20
10
20
30
40 VEGF
time (h)
VEGF-A mRNA
Normoxia
Hypoxia

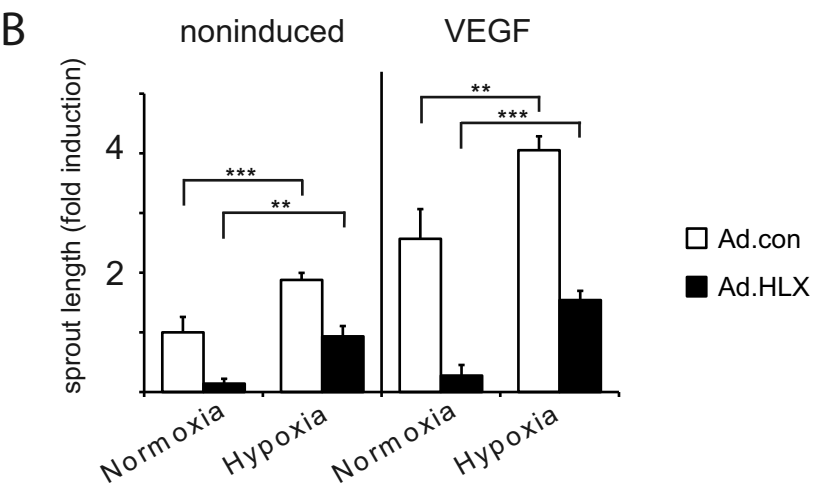

B
noninduced
VEGF
sprout length (fold induction)
4
2
***
**
Normoxia
Hypoxia
Ad.con
Ad.HLX

Fig. 5

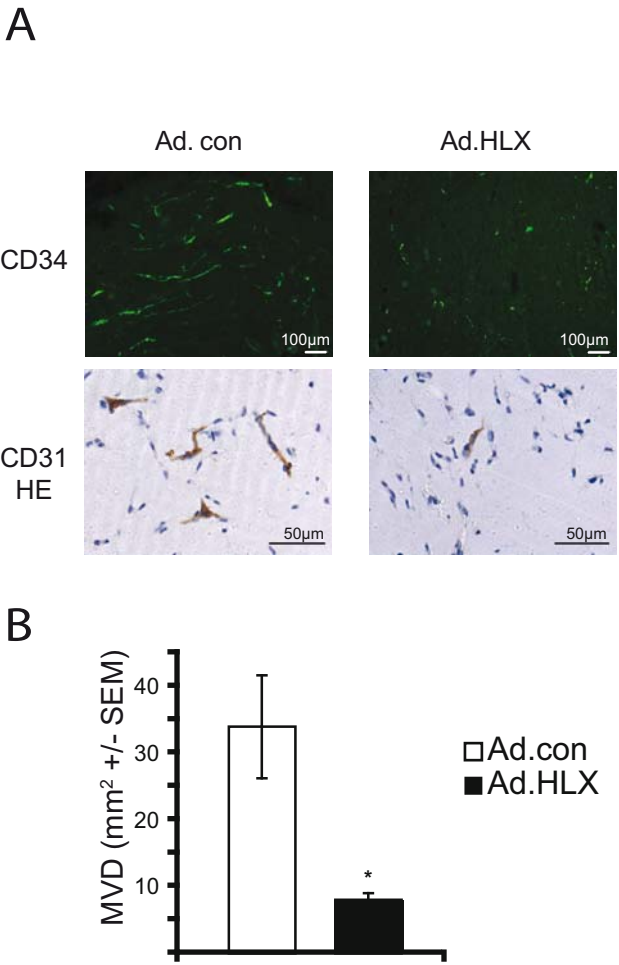

A
Ad. con
Ad.HLX
CD34
CD31
HE
100μm
100μm
50μm
50μm
B
MVD (mm² +/- SEM)
40
30
20
10
*
Ad.con
Ad.HLX

Fig. 6

UniGene ID	Entrez Gene	Gene Symbol	Gene Title	Ad.HLX/Ad.con 4h	8h	16h	32h	Ad.con/non infected 4h	8h	16h	32h
Hs.585457	219699	UNC5B	unc-5 homolog B (C. elegans)	1.41	2.23	5.71	7.14	0.69	1.17	1.21	2.61
Hs.9315	56944	OLFML3	olfactomedin-like 3	1.09	1.72	4.62	23.68	1.00	0.87	1.23	2.33
Hs.432329	5361	PLXNA1	plexin A1	1.15	2.96	4.61	8.38	1.11	1.38	1.22	1.40
Hs.360174	6591	SNAI2	snail homolog 2 (Drosophila)	1.58	3.87	4.51	6.91	1.00	1.37	2.14	2.40
Hs.132781	9466	IL27RA	interleukin 27 receptor, alpha	0.91	1.86	4.18	5.53	1.25	1.17	1.57	1.57
Hs.525704	3725	JUN	Jun oncogene	1.48	1.51	4.06	3.14	1.07	1.84	0.69	0.67
Hs.532626	23093	TTLL5	tubulin tyrosine ligase-like family, member 5	0.92	1.43	3.93	2.17	0.97	1.18	0.40	1.25
Hs.417050	8900	CCNA1	cyclin A1	0.78	2.15	3.79	2.96	1.00	0.85	0.95	0.40
Hs.2128	1847	DUSP5	dual specificity phosphatase 5	1.07	2.38	3.76	3.54	1.19	1.13	1.72	2.49
Hs.593413	7852	CXCR4	chemokine (C-X-C motif) receptor 4	1.22	1.46	3.74	11.61	1.11	0.95	0.16	0.03
Hs.657797	---	---	CDNA FLJ12055 fis, clone HEMBB1002049	0.97	1.29	3.70	2.22	0.93	1.70	0.36	1.12
Hs.356216	54855	FAM46C	family with sequence similarity 46, member C	0.98	1.43	3.70	2.85	1.07	1.24	1.45	1.68
Hs.530443	387763	LOC387763	hypothetical LOC387763	0.94	2.51	3.52	3.15	1.15	1.06	0.92	1.10
Hs.250666	3280	HES1	hairy and enhancer of split 1, (Drosophila)	2.13	3.63	3.49	4.37	1.22	1.28	1.41	1.88
Hs.702013	10687	PNMA2	paraneoplastic antigen MA2	0.94	1.82	3.49	4.41	0.87	0.84	0.41	0.07
Hs.659681	54880	BCOR	BCL6 co-repressor	2.01	2.54	3.45	5.37	1.00	1.50	0.84	1.40
Hs.653792	---	---	Transcribed locus	0.68	1.35	3.30	0.93	0.87	0.71	1.73	3.13
Hs.437075	9586	CREB5	cAMP responsive element binding protein 5	0.88	1.64	3.28	10.81	0.98	1.38	0.81	0.73
Hs.655332	10894	LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	0.97	1.32	3.17	1.55	0.59	0.34	0.45	0.70
Hs.381167	1992	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	0.93	1.33	3.15	4.25	0.94	0.64	0.97	1.44
Hs.654568	3880	KRT19	keratin 19	0.72	1.18	3.05	3.76	0.83	0.55	0.64	0.88
Hs.76152	358	AQP1	aquaporin 1 (Colton blood group)	1.22	3.35	3.03	3.33	0.54	0.24	0.44	0.51
Hs.647068	6009	RHEB	Ras homolog enriched in brain	0.76	1.53	2.96	1.70	0.92	1.14	0.78	1.17
Hs.59729	56920	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	1.27	2.28	2.88	4.89	1.10	0.89	1.08	0.77
Hs.438489	27109	ATP5S	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit s (factor B)	1.02	1.60	2.85	2.80	0.90	1.04	0.44	0.77
Hs.459265	3669	ISG20	interferon stimulated exonuclease gene 20kDa	1.09	1.90	2.83	2.88	0.77	0.71	0.92	1.16
Hs.523852	595	CCND1	cyclin D1	1.04	0.92	2.83	8.14	0.92	1.49	1.41	0.82

Hs.93675	11067	C10orf10	chromosome 10 open reading frame 10	1.45	1.48	2.83	7.98	0.88	0.86	0.27	0.06
Hs.122514	51312	SLC25A37	solute carrier family 25, member 37	1.23	1.20	2.83	2.74	0.83	1.07	0.65	0.92
Hs.657642	---	---	Transcribed locus	1.07	2.73	2.82	2.09	0.92	1.06	0.58	0.77
Hs.535713	727811 /// 9034	CCRL2 /// LOC727811	chemokine (C-C motif) receptor-like 2 /// similar to chemokine (C-C motif) receptor-like 2	0.98	2.13	2.80	3.69	0.83	0.64	1.05	0.95
Hs.654678	---	---	Transcribed locus	0.88	1.11	2.79	2.09	0.87	1.29	0.96	0.64
Hs.459538	220	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	0.84	1.49	2.78	7.81	1.03	1.05	0.80	0.64
Hs.25960	4613	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	1.54	3.75	2.78	10.33	1.02	0.87	0.71	0.43
Hs.592409	---	---	Transcribed locus	0.69	1.23	2.77	4.81	1.12	0.96	1.11	1.83
Hs.96	5366	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	1.45	1.70	2.75	1.53	0.99	1.24	0.73	0.80
Hs.585118	1903	EDG3	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	1.14	2.41	2.73	6.17	0.99	1.05	0.93	0.54
Hs.593413	7852	CXCR4	chemokine (C-X-C motif) receptor 4	1.15	1.73	2.72	3.77	0.97	0.68	0.28	0.08
Hs.494557	195828	ZNF367	zinc finger protein 367	0.75	1.04	2.67	2.64	1.07	0.92	0.36	0.11
Hs.658524	---	---	Transcribed locus	1.43	1.20	2.66	3.03	1.05	2.05	0.82	1.52
Hs.591251	154	ADRB2	adrenergic, beta-2-, receptor, surface	0.94	2.02	2.64	4.45	1.04	0.77	1.21	1.13
Hs.110571	4616	GADD45B	growth arrest and DNA-damage-inducible, beta	1.20	1.68	2.64	4.26	0.77	0.82	0.35	0.19
Hs.593318	---	---	Full-length cDNA clone CS0DI001YP15 of Placenta Cot 25-normalized of Homo sapiens (human)	0.96	1.41	2.63	4.67	1.06	1.05	1.12	1.52
Hs.107125	83483	PLVAP	plasmalemma vesicle associated protein	1.43	1.79	2.63	2.56	0.82	0.91	1.65	2.89
Hs.19985	---	---	Transcribed locus	0.89	1.30	2.62	2.38	0.78	0.62	0.39	0.72
Hs.203637	5357	PLS1	plastin 1 (I isoform)	0.61	1.41	2.59	1.36	1.21	1.02	0.65	0.64
Hs.520819	3638	INSIG1	insulin induced gene 1	0.92	1.78	2.59	2.78	0.92	1.09	2.20	1.11
Hs.659130	---	---	MRNA; cDNA DKFZp564A023 (from clone DKFZp564A023)	1.32	0.93	2.59	0.98	0.92	1.32	0.52	2.09
Hs.491582	5327	PLAT	plasminogen activator, tissue	0.98	1.42	2.57	4.27	1.18	1.07	1.44	1.31
Hs.460	467	ATF3	activating transcription factor 3	1.45	2.70	2.57	4.86	1.00	1.47	1.49	1.65
Hs.42645	9120	SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	1.19	2.39	2.55	2.97	0.98	0.92	0.49	0.88
Hs.59332	200734	SPRED2	sprouty-related, EVH1 domain containing 2	0.80	1.80	2.51	4.25	1.01	1.02	0.76	0.46
Hs.25590	6781	STC1	stanniocalcin 1	1.06	0.94	2.51	1.07	0.92	1.12	1.22	1.46

Table S1. Genes upregulated by HLX in endothelioal cells more than 2-fold. Total RNA was isolated from HUVEC after infection with HLX encoding adenoviruses (Ad.HLX) and control (empty) adenoviruses (Ad.con) for 4, 8, 16 and 32h. Non-infected cells were used as control for virus effects. The RNA was subjected to microarray analysis using the Affymetrix Human Genome U133 Plus 2.0 Array. Gene expression changes for HLX adenovirus relative to control adenovirus infected cultures are displayed as fold induction in the Ad.HLX/Ad.con lane, for control adenovirus infected relative to non-infected cultures in the Ad.con/non-infected lane. The full set of Affymetrix data is available in the GEO database under under GSE13054. All genes induced more than 2.5-fold at the 16 h time point are listed according to decreasing HLX induction values. Genes occuring several times in the original Affymetrix array dataset are only given once using their highest value.

Primer	Sequence (5’ – 3’ direction)
UNC5B-forward	CTG GGA CCT TAT GCC TTC AA
UNC5B-reverse	CGC TTT GGT GGC AAA GTA AT
PlexinA1-forward	TGT CTG GAT TCA GTG GCT CA
PlexinA1-reverse	GGT TCT GCG GTG TTA AAG GA
HES1-forward	ACG CAG TGT CAC CTT CCA G
HES1-reverse	AGA GTC CGG AGG GAA GAG AG
HLX1-forward	CTAGCCAGCCGAACACTTCT
HLX1-reverse	CGTCCTAAGTGCCTTTGAGC
Sema3G-forward	ACGGAGCACAATAGCACCTT
Sema3G-reverse	AGACTCGCTCGTCCGTCTT
VEGF-A-forward	GTGGGCCTTGCTCAGAGCGG
VEGF-A-reverse	AGGCTCCTTCCTCCTGCCCG

Table S2. PCR primers used in Realtime RT-PCR

Gene	shRNA sequence (5’ – 3’ direction)
HLX	CCGGCGCATCTCTAGATCCCATTAACTCGAGTTAATGGGATCTAGAGATGCGTTTTT
UNC5B	CCGGCAGAAGATATGCAACAGCCTACTCGAGTAGGCTGTTGCATATCTTCTGTTTTTG
control	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT

Table S3. shRNA sequences in Mission shRNA pLKO.1-puro

Figure S1. Induction of UNC5B and HLX mRNA in HUVEC and other endothelial cells of different origin

(A) UNC5B is starting to be upregulated at 2 h after VEGF induction. HUVEC were cultured to density, starved overnight in EBM-2 medium without supplements and then induced with 100 ng VEGF/ml. Total RNA was isolated and mRNA levels were determined by real-time RT-PCR. All values were normalized to ß2-microglobulin mRNA as internal standard. Results are displayed for the early time points from 1 to 16 h after VEGF addition and represent the mean of fold induction +/- standard error of the mean (SEM) of the respective mRNA levels calculated from triplicate wells. One representative experiment of three performed is shown.

(B) HLX is induced by VEGF-A in endothelial cells of different origin.

Various endothelial cells isolated from different tissues including Human Aorta Endothelial Cells (HAEC), Human Pulmonary Artery Endothelial Cells (HPAEC), Human Retina Endothelial Cells (HREC), Human Skin Microvascular Endothelial Cells (HSMEC), Human Saphenous Vein Endothelial Cells (HSVEC) and HUVEC as well as Human Smooth Muscle Cells (HSMC) were grown to density, starved overnight and induced with VEGF-A for 1 h. RNA was isolated from the cells and subjected to realtime RT-PCR analysis. Results are displayed as fold induction +/-SEM from one experiment performed in triplicates.

Figure S2. Infection of HUVEC with Ad.HLX does not induce apoptosis

HUVEC seeded in a chamber slide (Nunc, Naperville, IL, USA) were infected with HLX and control adenoviruses with a MOI of 8. 24h later the cells were fixed, permeabilized and stained according to the In Situ Cell Death Detection Kit (TMR red, Roche, Mannheim, Germany). As a positive control one well was treated with 100 U/ml DNase I for 10 min to induce DNA strand breaks. The cells were photographed using a Nikon Diaphot TMD microscope and a CCD camera (Kappa GmbH, Gleichen, Germany).

Figure S3. HLX reduces sprout formation *in vitro* in a dose-dependent manner

HUVEC were infected with HLX and control adenoviruses using a MOI of 10, 3.3, 1 or 0.1. The following day spheroids of infected and noninfected endothelial cells were prepared consisting of about 400 cells/spheroid as described in Materials and Methods. The spheroids were embedded in collagen gels and stimulated with VEGF or bFGF (25 ng/ml) or left without cytokine stimulation. After 24 h the spheroids were fixed with PFA and the length of sprouts was analysed for 10 spheroids per group. The results are displayed as fold induction of sprout length compared to cells infected with control viruses without stimulation ± standard error of the mean (SEM) calculated from triplicate wells.

Figure S 4. Downmodulation of UNC5B mRNA by shRNA increases the number of VEGF-inducible sprouts from endothelial spheroids.

(A) Downmodulation of UNC5B mRNA by shRNA: HUVEC were transduced with lentiviruses expressing shRNA targeted against UNC5B or control shRNA. Then they were starved overnight and induced with VEGF-A for 56 h. RNA was isolated and subjected to realtime RT-PCR. Results are displayed as percent UNC5B mRNA +/- SEM calculated from triplicate wells.

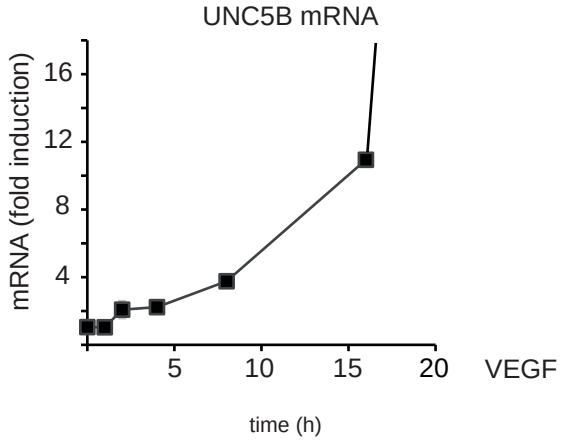
(B) Increased number of sprouts following downmodulation of UNC5B in VEGF-induced spheroids: HUVEC were first transduced with lentiviruses for control shRNA or shRNA targeted against HLX. After 2 d cells were in addition infected with 15 MOI of Ad.con or Ad.HLX. The following day spheroids were prepared and incorporated into collagen gels in the presence of VEGF-A. Sprouting of spheroids was evaluated the next day. The shown values are calculated from four independent experiments and display average sprout number per spheroid +/-SEM in comparison to the samples transduced with Ad.con and shRNA. con.

Figure S5. Perfusion of vessels generated in the *in vivo* endothelial spheroid xenografting assay

Sections obtained from plugs generated with endothelial spheroids infected with control adenovirus. Arrows indicate red blood cells in the luminal areas of vessels containing human endothelial cells.

Zink fixed Matrigel plugs were embedded in paraffin and sectioned at 8 µm, deparaffinized and rehydrated. The primary antibody, mouse-anti-human CD31 (Dako, Glostrup, Denmark) was detected by the following secondary antibody: biotinylated anti-mouse (Vector Laboratories, Burlingame, USA). The incubation with the secondary antibody was followed by the incubation with streptavidin-peroxidase conjugate (Vector Laboratories) and developed using DAB substrate chromogen system (Dako). Afterwards the sections were stained with Hämalaun (Merck) and Eosin.

A


UNC5B mRNA
mRNA (fold induction)
16
12
8
4
5
10
15
20
VEGF
time (h)

B

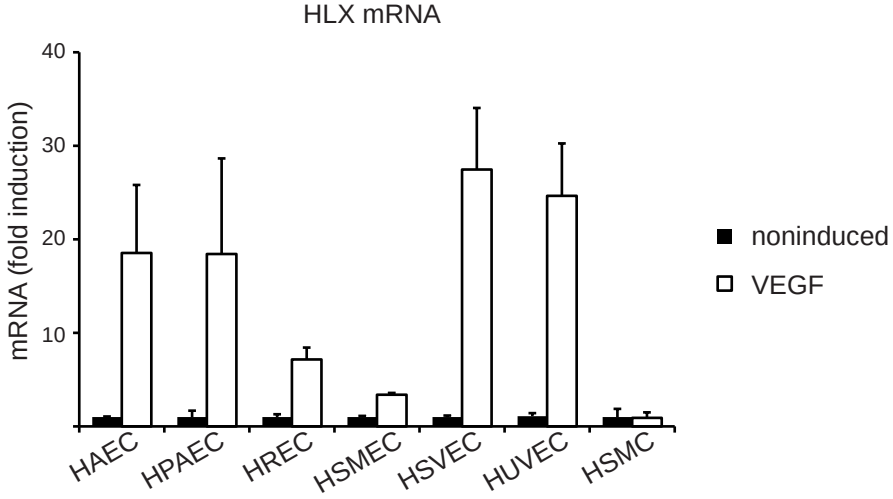

HLX mRNA
mRNA (fold induction)
40
30
20
10
HAEC
HPAEC
HREC
HSMEC
HSVEC
HUVEC
HSMC
noninduced
VEGF

Figure S1

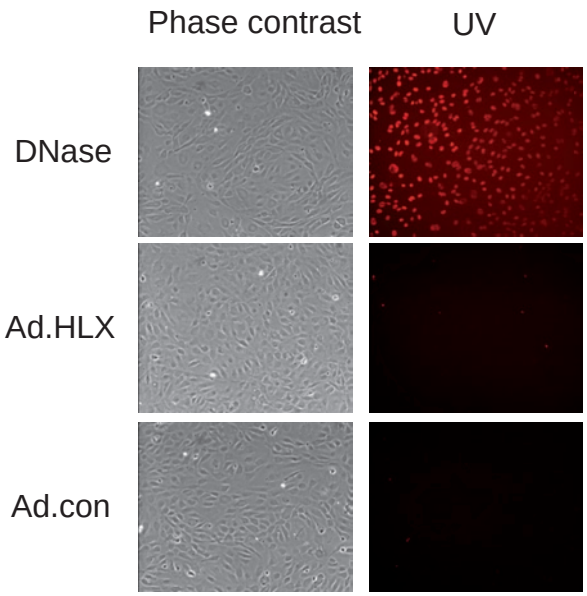


Figure S2

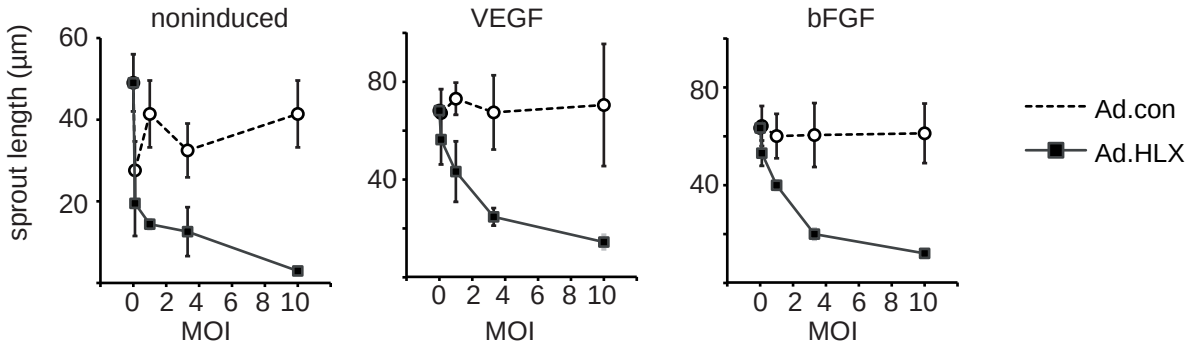
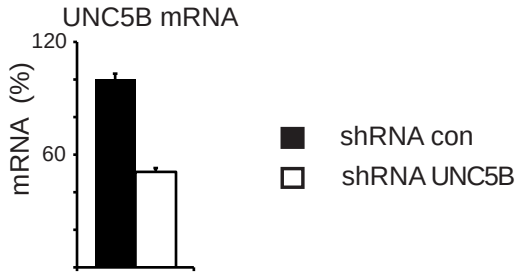

noninduced
VEGF
bFGF
sprout length (μm)
60
40
20
80
40
80
40
0 2 4 6 8 10
MOI
Ad.con
Ad.HLX

Figure S3

A


UNC5B mRNA
120
60
mRNA (%)
shRNA con
shRNA UNC5B

B

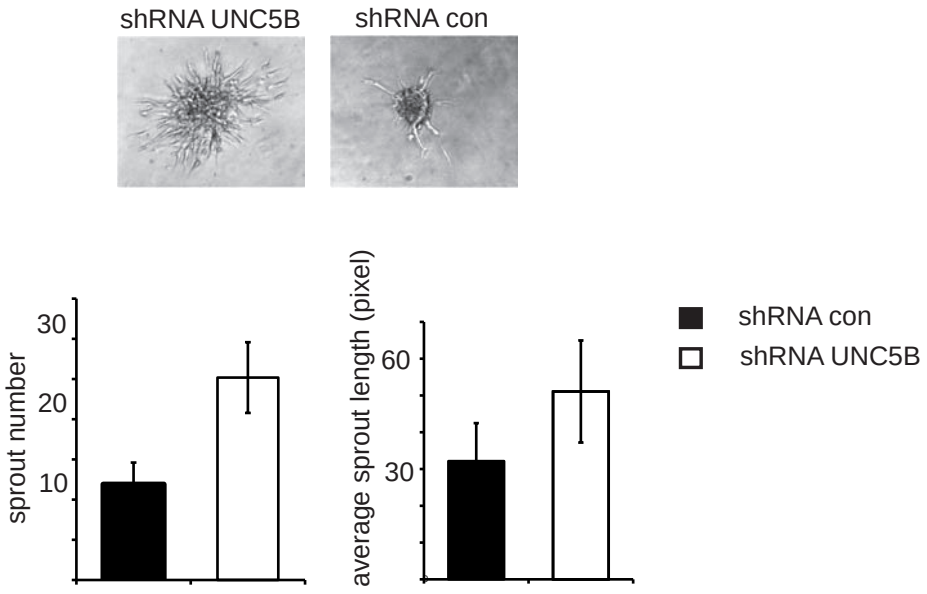
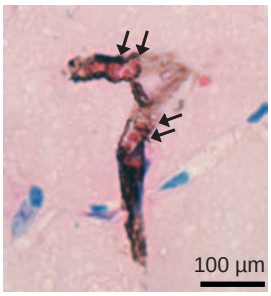

shRNA UNC5B
shRNA con
30
20
10
sprout number
60
30
average sprout length (pixel)
shRNA con
shRNA UNC5B

Figure S4

hCD31 DAB staining
+ HE staining
arrow: red blood cells



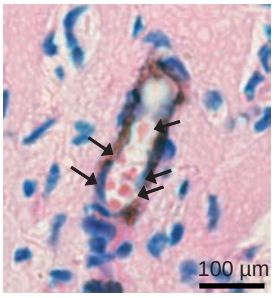


Figure S5

3. Additional data

3.1 Overexpression and downmodulation of HLX inhibits sprouting *in vitro* and vessel formation *in vivo*

Inhibition of sprouting by HLX overexpression is mediated via UNC5B and SEMA3G

To establish which gene products contribute to the inhibition of sprouting following HLX overexpression, we used lentiviral expression of shRNAs for the individual downmodulation of mRNAs for UNC5B, PLNXA1 and SEMA3G. Usually a 60% downmodulation of the respective mRNA was achieved after 56 hours VEGF induction (Figure 8B). The obtained results show that a reduction of the HLX-mediated upregulation of UNC5B as well as of SEMA3G mRNAs led to significantly reduced inhibition of sprouting, whereas reduction in PLNXA1 message had no effect (Figure 8A). This shows that HLX-mediated inhibition of sprouting is at least partly caused by the combined upregulation of UNC5B and SEMA3G. In the control adenovirus samples (Figure 8A) downmodulation of all three mRNAs caused some increase in sprouting, although this was a lot less pronounced for PLNXA1 shRNA when compared to UNC5B and SEMA3G. This indicates that normal low expression of all three genes in endothelial cells can exert a negative effect on sprouting activity and downmodulation of the endogenous expression of UNC5B and SEMA3G and, to a limited extent, also of PLXNA1 can increase the basal sprouting activity.

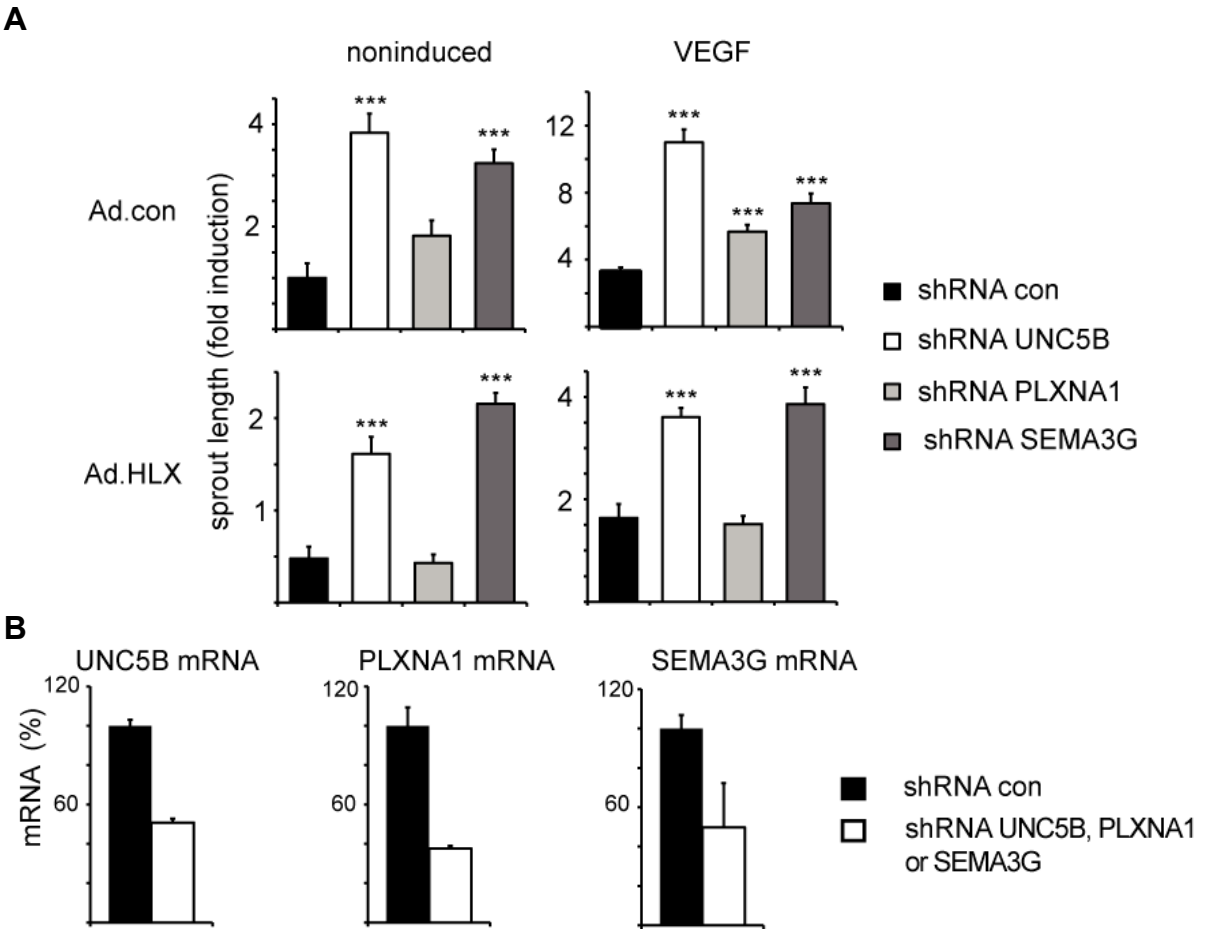


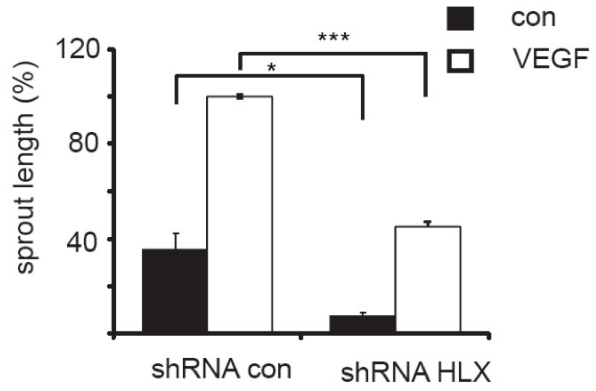
Figure 8. Effects of downmodulation of UNC5B, PLXNA1 and SEMA3G by the respective shRNA.
(A) Inhibition of sprouting by HLX is reduced by downmodulation of UNC5B or SEMA3G. HUVEC were transduced with lentiviruses for control shRNA or shRNA targeted against UNC5B, PLXNA1 or SEMA3G. After 1 d cells were in addition infected with 15 MOI of Ad.con or Ad.HLX. The following day cells were used for the spheroid sprouting assay. Total sprout lengths of 15 spheroids each were measured. Results are displayed as fold induction +/- SEM in comparison to the sprout lengths obtained for non-induced spheroids prepared from control shRNA and control adenovirus transduced cells. One representative experiment of two performed is shown. ***P < .001, *t* test
(B) Downmodulation of UNC5B, PLXNA1 and SEMA3G mRNA by the respective shRNAs. HUVEC were transduced with lentiviruses expressing shRNA targeted against UNC5B, PLXNA1 or SEMA3G for 2 days. Then they were starved overnight and induced with VEGF for 56 h. RNA was isolated and subjected to realtime RT-PCR. Results are displayed as percent UNC5B, PLXNA1 or SEMA3G mRNA +/- SD calculated from triplicate wells.

Reduction of HLX expression by shRNA reduces sprouting

We next asked how the reduction of HLX by lentiviral expression of a corresponding shRNA would affect the sprouting activity. Surprisingly we found that downmodulation of HLX by shRNA also exerted an inhibitive effect on sprouting, although the achieved inhibition seemed less pronounced when compared to overexpression of HLX (Figure 9A). When a dilution series of the shRNA lentivirus

was tested, the inhibition diminished in a dose-dependent way (Figure 9B). This shows that a minimal expression level of HLX is necessary for sprouting, which might be due to the HLX-mediated induction of a so far non-identified protein that would support sprouting. This indicates that overexpression of HLX as well as levels of HLX reduced below a certain threshold can lead to inhibition of sprouting.

A



B

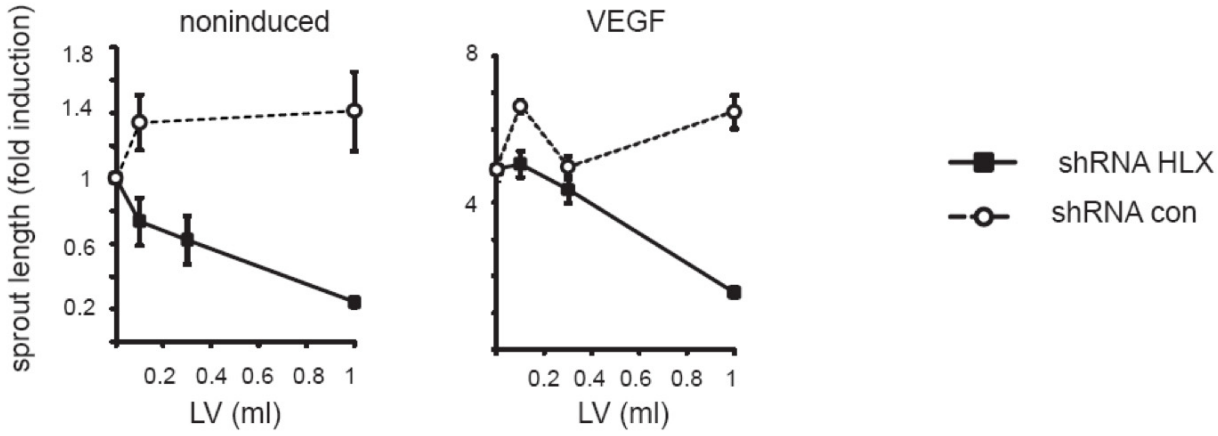


Figure 9. Downmodulation of HLX reduces sprouting.
(A) HUVEC were transduced with lentiviruses for control shRNA or shRNA targeted against HLX. After 2 d cells were used for the spheroid sprouting assay. Total sprout lengths of 15 spheroids each were calculated. The shown values are calculated from four independent experiments and display percent sprout length +/-SEM in comparison to the control shRNA samples stimulated with VEGF.
(B) Downmodulation of HLX using shRNA lentiviruses reduces sprout formation *in vitro* in a dose-dependent manner: HUVEC were transduced using different volumes of lentiviral stock preparations for two days. Then cells were used for spheroid sprouting assay. The results show fold induction of total sprout length +/- SEM as calculated from 15 spheroids for each condition.

Downmodulation of HLX can inhibit vessel formation *in vivo*

To confirm that the inhibitory effect observed on sprouting in vitro would also be reflected in vessel formation *in vivo,* a spheroid xenografting assay was performed. Indeed, in line with the *in vitro* sprouting results a reduction of HLX mRNA by a corresponding shRNA similarly reduced vessel density as well as vessel length *in vivo* supporting the importance of appropriate HLX levels to vessel formation (Figure 10).

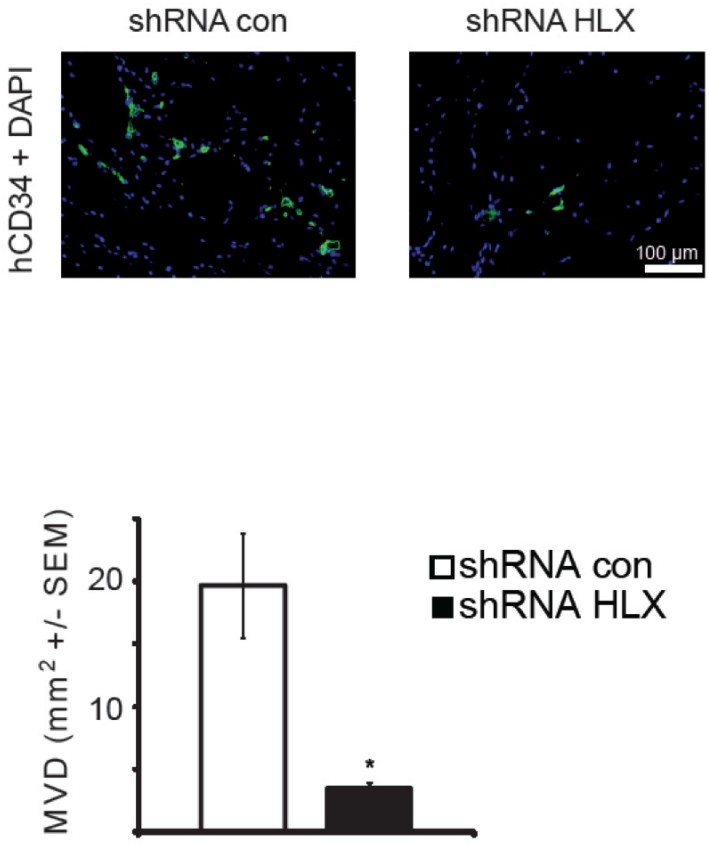


Figure 10. Downmodulation of HLX reduces vessel formation in an endothelial spheroid xenografting assay *in vivo*
HUVEC were transduced with lentiviruses containing control or HLX shRNA. Stably shRNA expressing cells were selected with puromycin for 5 days. The infected cells were used to generate endothelial cell spheroids consisting of about 100 cells/spheroid. Spheroids were suspended in a Matrigel/fibrinogen mixture containing 500 ng/ml VEGF and bFGF. Following addition of thrombin the spheroid suspension was injected subcutaneously into SCID mice (2 plugs/mouse, 8 mice/experimental group). Plugs were harvested, fixed and analyzed by immunohistochemistry using CD34 antibodies after 20 days. MVD per mm2 +/- SEM was calculated from five representative sections of five plugs.

3.2 Effect of HLX overexpression on MDA breast cancer growth

Based on the upregulation of repulsive guidance molecules by HLX and the connected inhibition of vessel growth we predicted that overexpression of HLX throughout the tumor tissue including tumor vessels might lead to inhibition of tumor growth. To evaluate such a potential inhibition we used a human MDA breast cancer model in SCID beige mice. In this model HLX was overexpressed in tumor and endothelial cells by 3 consecutive injections of Ad.HLX into the tumor tissue on days 7, 11 and 15 after injection of tumor cells. This indeed led to a significant inhibition in tumor growth. Following the first injection of Ad.HLX no further increase in tumor volume was observed (Figure 11). This might be due to the expression of HLX in infected endothelial cells of the tumor vessels, which should lead to reduced neovascularization as observed before for the murine spheroid xenografting assay. However, as adenoviruses would equally infect tumor cells, the inhibition of tumor growth could also be a direct or indirect effect of the expression of HLX in tumor cells. This possibility was tested by analyzing MDA breast cancer cells following infection with HLX adenoviruses in cell culture. Whereas no effect on the proliferation of the tumor cells was observed (data not shown), the analysis of guidance molecule expression revealed an increased expression of SEMA3G by the tumor cells overexpressing HLX (Figure 12). UNC5B and PLXNA1 were not expressed in the tumor cells (data not shown). It is therefore possible that increased secretion of SEMA3G into the tumor tissue environment might contribute to diminished endothelial sprouting and vessel growth in the growing tumor, thereby blocking adequate oxygen and nutrient supply resulting in inhibition of tumor growth. However, it remains possible that SEMA3G also exerts direct negative effects on tumor cells *in vivo* (Gaur et al., 2009). Further experiments will be required to establish the exact mode of action of the astonishingly strong effect of HLX on tumor growth.

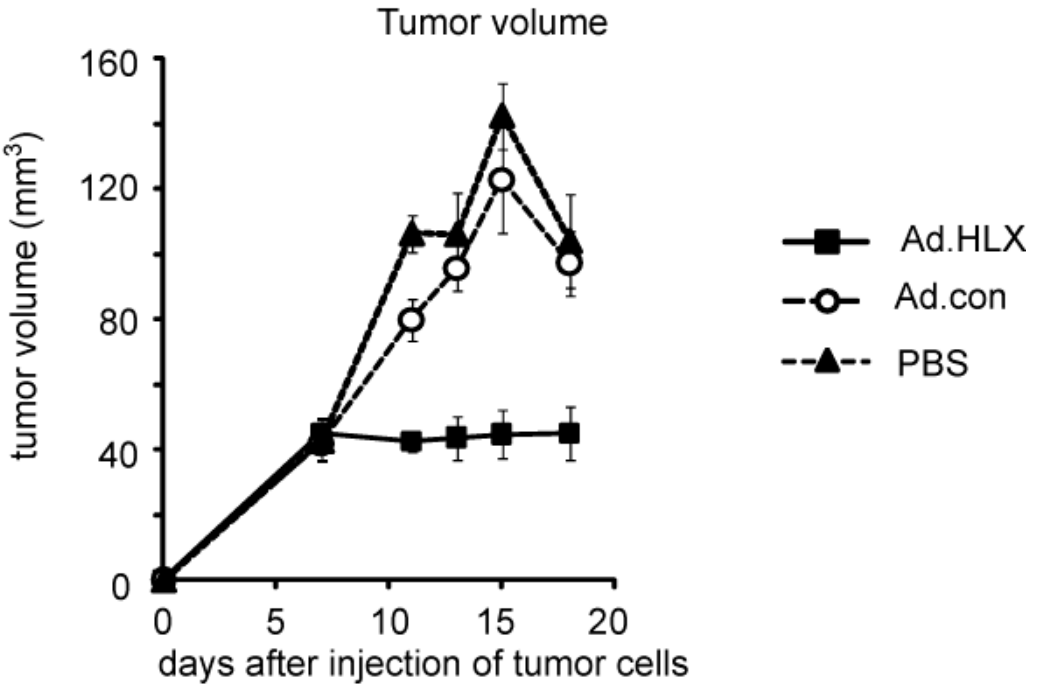


Figure 11. Overexpression of HLX inhibits tumor growth of breast cancer cells
5 x 10^6 MDA-MB-231 cells were injected subcutaneously into the flanks of SCID/beige (C.B-Igh1^b/IcrHsd-Prkdc^{scid}Lyst^{bg}) mice. On day 7, 11 and 15 after the tumor cell application 1x10^8 PFU of Ad.HLX or Ad.con (expressing GFP) or the same volume of PBS was injected into the grown tumor. The tumor volume was established during the whole experiment by measuring the tumor size using a caliper rule. The tumor volume was calculated according to the formula: tumor volume= tumor length x tumor width x tumor width/2. The experiment was terminated on day 18 after tumor cell injection. Results display tumor volume from one representative experiment of two performed with at least 5 mice per group and show tumor volume (mm^3) +/- SEM.

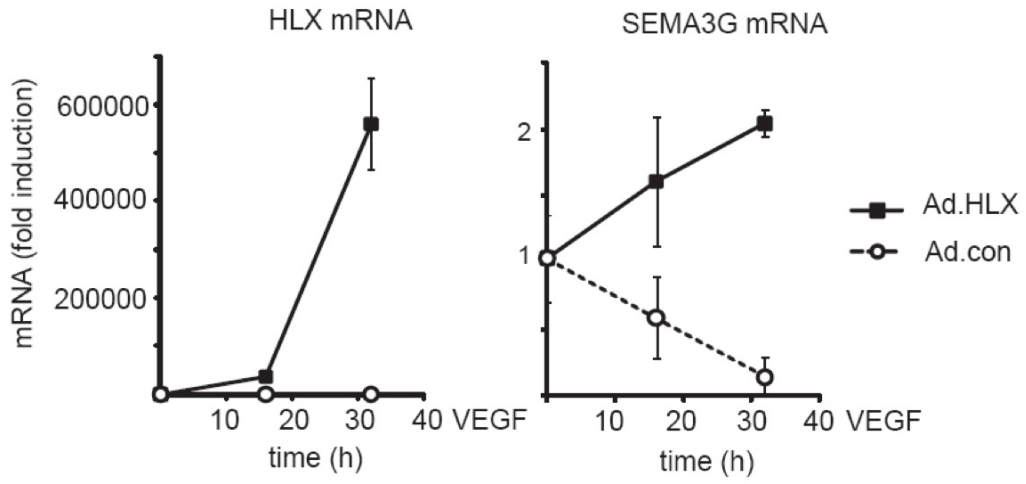


Figure 12. Overexpression of HLX induces SEMA3G mRNA expression in MDA cancer cells
MDA-MB-231 cancer cells were transduced with a MOI of 20 of Ad.HLX and Ad.con (expressing GFP) for 16 and 32h. Cells were harvested, the RNA isolated and subjected to real-time RT-PCR analysis. Values were normalized to ß2-microglobulin mRNA as internal standard.

4. Additional publications (Coauthorship)

4.1 The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation.

Bernhard Schweighofer, Julia Testori, Caterina Sturtzel, Susanne Sattler, Herbert Mayer, Oswald Wagner, Martin Bilban, Erhard Hofer.

Thromb Haemost. 2009;102:544-554.

The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation

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Summary

VEGF-A is the major trigger of vasculogenesis and physiologic angiogenesis. We have investigated to which extent the gene repertoire induced by VEGF-A in endothelial cells is distinct from that of other growth factors and inflammatory cytokines. Genes upregulated in human umbilical vein endothelial cells treated with VEGF, EGF or IL-1 were compared by microarray analysis and clusters characteristic for individual or combinations of inducers were defined. VEGF-A upregulated in comparison to EGF a five-fold larger gene repertoire, which surprisingly overlapped to 60% with the inflammatory repertoire of IL-1. As shown by real-time RT-PCR for selected genes, VEGF-induction was mostly mediated by VEGF receptor-2 and the capacity of VEGF-A to induce genes in common with IL-1 largely depended on activation of the calcineurin/NFAT pathway, since cyclosporin A inhibited this induction. Another angiogenic growth factor, bFGF, did not share a comparable induction of inflammatory genes, but partially induced a small group of genes in common with VEGF-A, which were not regulated by EGF. Thus, the data display that VEGF-A induces a distinct gene repertoire, which, contrasting with other growth factors such as EGF or bFGF, includes an inherent inflammatory component possibly contributing to the cross-regulation of angiogenesis and inflammation as further indicated by the VEGF-mediated induction of leukocyte adhesion. Furthermore, a small group of genes selectively induced by VEGF-A with potential importance for angiogenesis is defined.

Keywords

VEGF-A, endothelial cells, angiogenesis, inflammation, gene repertoire

Thromb Haemost 2009; 102: 544–554

Introduction

Vascular endothelial growth factor-A (VEGF-A) is the primary inducer of vascular development during embryogenesis and physiological blood vessel formation in the adult. It is also a causative factor of pathologic angiogenesis associated with a multitude of diseases including cancer, chronic inflammatory diseases and retinopathy (1, 2). VEGF-A is normally produced in tissues in response to low oxygen tension and forms a chemotactic gradient sensed by endothelial cells in preexisting vessels, which respond by growing sprouts leading to the formation of new capillaries (3, 4). Several peptide products are generated by differential splicing from the VEGF-A gene such as VEGF-A_{121}, VEGF-A_{165} and VEGF-A_{189} or the inhibitory variant VEGF-A_{165b}. However, isoform VEGF-A_{165} seems to be the predominant form responsible for major angiogenic effects (5).

The responses induced by VEGF-A in endothelial cells include promotion of survival, proliferation, migration and invasion of surrounding tissue as well as the formation of the three-dimensional vascular tube. Furthermore, VEGF-A induces vascular leakage and is also known as vascular permeability factor (2). These biological effects of VEGF are thought to be mediated mainly by VEGF receptor-2 (VEGFR-2), whereas VEGF receptor-1 (VEGFR-1) transduces only a weak intracellular signal and was initially proposed to function in endothelial cells primarily as a decoy receptor and negative regulator of angiogenesis (6). In

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line with this proposal VEGF-E, a viral VEGF homologue selectively binding VEGFR-2, can reproduce many of the angiogenic effects of VEGF-A in assays *in vitro* and *in vivo* (7), whereas PlGF, which specifically binds VEGFR-1, is much less potent in this respect (8). However, additional data support a synergy of VEGFR-1 and VEGFR-2 in pathologic angiogenesis (9).

The VEGF receptors share certain regulatory mechanisms with other receptor tyrosine kinases such as the platelet-derived and epidermal growth factor receptors. These include receptor dimerization and phosphorylation of the cytoplasmic domain on tyrosine residues, which serve as docking sites for downstream signal transducers (10, 11). EGF and the HER or erbB receptors are the prototypes and probably most studied of growth factor and receptor tyrosine kinase systems (12) of wide importance for the development and proliferation of epithelial and many other cell types. In endothelial cells EGF was described to mediate proliferation (13). However, due to the more specific and distinct roles of VEGF-A in directing endothelial sprouting and tubulogenesis it is to be anticipated that VEGF transduces in part unique signals and upregulates a unique gene repertoire in endothelial cells in addition to genes involved in survival and proliferation.

In addition to VEGF-A, bFGF has been known for a long time as potent stimulator of angiogenesis *in vitro* and *in vivo*. Its exact role in physiological vessel formation has remained controversial (15), but bFGF production by tumour cells can play an important role in tumour angiogenesis (16). bFGF exerts its activity via the receptor tyrosine kinase FGF receptor-1.

Angiogenesis during wound healing and in tumours takes place in an inflammatory surrounding created by immune cells invading the damaged or malignant tissue. Inflammatory cytokines such as interleukin (IL)-1 induce a response program in endothelial cells including a large number of inflammatory genes like cytokines and adhesion molecules involved in various aspects of immune cell recruitment and tissue repair. Inflammatory cytokine receptors such as the IL-1 receptor transduce signals by recruiting adaptor molecules which link to the inhibitor of kappaB (IκB)-kinase complex leading to the release of nuclear factor-kappaB (NF-κB) subunits for nuclear transfer (17). The strong upregulation of the NF-κB pathway seems to be the major determinant for the inflammatory transcriptional response (18, 19).

To define the genes specifically induced by VEGF-A, which might be involved in specialised functions of the factor not shared by other growth factors and inflammatory cytokines, we have investigated the gene repertoire induced by VEGF-A in endothelial cells in comparison to the repertoire of a more general growth factor, EGF, and an inflammatory cytokine, IL-1. We detect a surprisingly large overlap with the inflammatory repertoire of IL-1 and define a small group of genes specifically regulated by VEGF with the potential to be involved in processes selectively induced by VEGF-A.

Materials and methods

Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (14) and used between passage 3 and 5. In short, HUVEC were cultured in M199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% SCS (HyClone, Logan, UT, USA), 50 µg/ml ECGS (Technoclone, Vienna, Austria), 1 U/ml heparin (Roche Diagnostics GmbH, Mannheim, Germany), 2 mM glutamine, 100 U/ml penicillin and 1 mg/ml streptomycin (BioWhittaker, Verviers, Belgium). Cells were seeded into gelatine-coated six-well cell culture plates at $1x10^6$ cells/well and grown to density for four days without further medium change. Then cells were induced by addition of 100 ng/ml VEGF-A_{165}, VEGF-E, PlGF-1, IL-1 or 50 ng/ml EGF, respectively, for time periods of 30 to 360 min. When indicated cells were preincubated with CsA at 1 µg/ml for 30 minutes (min) before addition of the factors. Recombinant human VEGF-A_{165} was obtained from PromoKine (Heidelberg, Germany) or PeproTech (Rocky Hill, NJ, USA), VEGF-E and PlGF-1 from Reliatech (Braunschweig, Germany), bFGF from PeproTech. IL-1α was purchased from Biosource (Nivelles, Belgium) and EGF from PromoKine. Cyclosporin A (CsA) was kindly provided by the Novartis Research Institute (Vienna, Austria).

RNA preparation

After incubation with the indicated factors, cells were treated with RNAlater (Ambion, Austin, TX, USA), shortly washed with DEPC-treated water and RNA was extracted with Trizol (Invitrogen), according to the instructions of the manufacturer.

Affymetrix microarray hybridisation

Preparation of cRNA, hybridization to the human HG-U133 or HG-U133 Plus 2.0 GeneChip set (Affymetrix, Santa Clara, CA, USA), and scanning of the arrays were carried out according to the manufacturer's protocols (20) as described previously (21). Data were analysed with GeneChip software (MAS 5.0, Affymetrix), and normalised to "Selected Probe Sets" (100 housekeeping genes pre-selected by Affymetrix in a mask file) and a target signal of 2000. Gene expression changes were calculated as the ratio of induced cells to uninduced control cells. Sets of data for two independent experimental series using the HG-U133 and HG-U133 Plus 2.0 GeneChip sets, respectively, have been submitted to NCBI's Gene Expression Omnibus (GEO) (22), accession number GSE10778 and GSE15464. A list of the 59 genes most strongly upregulated by VEGF-A in both experimental series is provided as Supplementary Table 1 (available online at www.thrombosis-online.com).

Real-time RT-PCR analysis

Two µg of total RNA were reverse transcribed into cDNA (SuperscriptTM II RT, Invitrogen) according to instruction of the manufacturer, using oligo-dT primers. Real-time PCR was used to monitor specific gene expression using SybrGreen detection and a Light Cycler instrument (Roche Diagnostics GmbH) according to the manual. As internal standard β2-microglobulin mRNA was used for normalisation. Primers were designed using the program Primer3 (23). Sequences of the oligonucleotide primers used are listed in Supplemental Table 2 (available online at www.thrombosis-online.com). To choose sense and antisense primers annealing to different exons, the genomic organisation of the genes was obtained from the University of California, Santa Cruz Human Genome Browser (24).

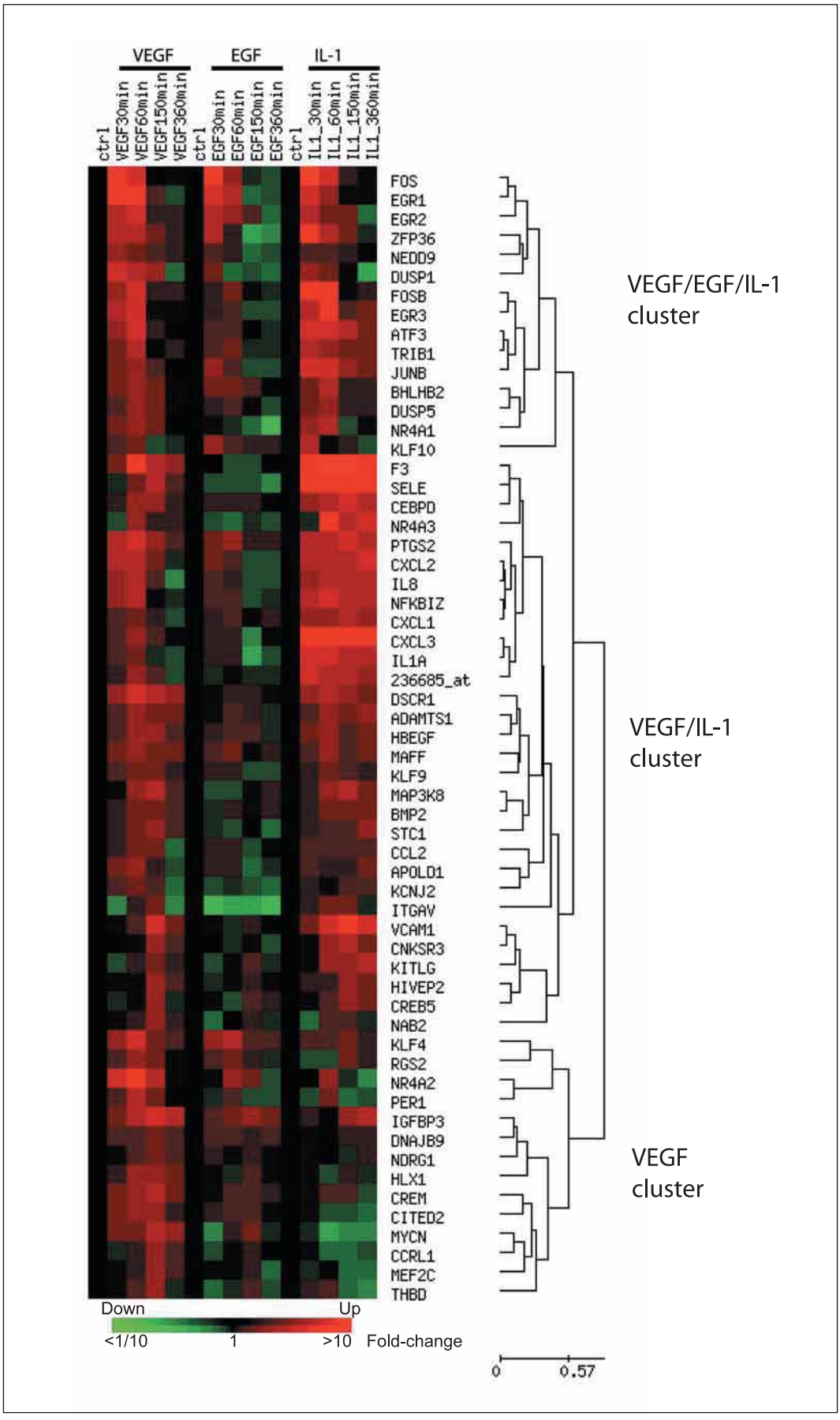

Figure 1: VEGF-A induced genes compared to IL-1α and EGF induction patterns in HUVEC. The hierarchical clustering of all genes induced in HUVEC by VEGF-A, more than three-fold at one of the indicated time points, is displayed as a heatmap in comparison to the IL-1α and the EGF gene expression pattern. Genes were clustered with EPCLUST (http://www.ebi.ac.uk/microarray-srv/EP/) using Pearson's correlation coefficient as distance measure and average linkage. Induction of expression is indicated by shades of red, repression by shades of green indicating approximately 10– to 10^{-1}-fold regulation. To visually separate the induction patterns of the individual factors, the null control value (ctrl) is shown in black before the respective induction.

Clustering analysis

First, genes as represented by Affymetrix "probe sets" showing absolute calls of „absence", difference calls of „no change" as determined by the Affymetrix MAS 5.0 software, less than three-fold upregulation or a signal intensity of less than 60 for all VEGF-induced data points were excluded from further analysis. Then the genes selected for more than three-fold upregulation by VEGF in two independent induction series were subjected to real-time RT-PCR analyses to confirm over three-fold upregulation for at least one time point and genes displaying lower regulation were again abandoned. For the remaining fifty-nine genes cluster analysis was performed using the Expression Profile data CLUSTering and analysis (EPCLUST) program (25) using linear correlation based distance (Pearson, centered) and average linkage (UPGMA).

Flow cytometry

HUVEC stimulated for six hours (h) with VEGF-A_{165}, IL-1 or EGF were harvested following short trypsin treatment, washed and incubated with PBS containing 5% FCS for 15 min. Then 5 µg/ml mouse anti-human VCAM1 antibody (R&D systems, Minneapolis, MN, USA) was added and the cells incubated for 30 min on ice. Samples were washed and incubated for another 30 min with FITC-labeled secondary anti-mouse antibody (Dako, Denmark). After a final washing step, flow cytometry was performed using a FACSCalibur (Becton-Dickinson, San Jose, CA, USA). The number of VCAM1-positive cells was deduced in comparison to uninduced cells.

Adhesion assay

HUVEC induced for 4, 6 or 8 h with VEGF-A_{165}, IL-1 or EGF were incubated with 3,3'-dioctadecyl-oxacarbocyanine perchlorate (DiO)-labeled (Invitrogen) HL-60 cells on a shaker for 30 min (15 min horizontal, 15 min vertical shaking) at 37°C in a 5% CO_2 atmosphere. Non-adherent HL-60 cells were removed by washing with phosphate-buffered saline and adhering HL-60 cells and HUVEC were detached by trypsin treatment. The percentage of DiO-labeled HL-60 cells in the cell mixture was determined by flow cytometry using a FACSCalibur.

Results

A VEGF-A-specific gene signature: clustering of VEGF-induced genes

To delineate genes induced by VEGF-A and involved in VEGF-specific functions during angiogenesis and/or endothelial differentiation we comparatively analysed the gene repertoires upregulated in endothelial cells by VEGF-A, EGF or IL-1. The selection of these factors was based on the assumption that VEGF-A, the main trigger of vasculogenesis and angiogenesis, would induce gene clusters in addition to the gene repertoire upregulated by EGF, a growth factor which promotes proliferation in endothelial cells and is a general component of the growth supplements used for primary endothelial cell cultures (26). The VEGF-induced genes should further be distinguished from the IL-1 response, which should primarily include genes involved in endothelial inflammation.

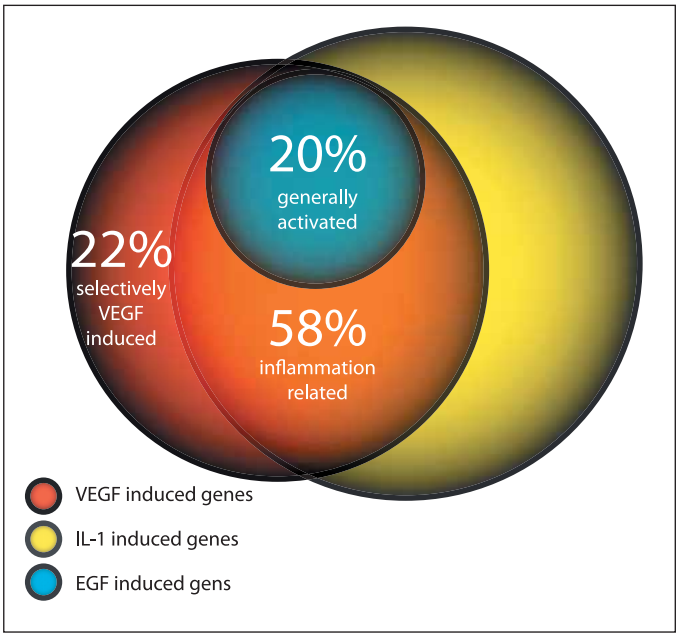

Figure 2: Venn Diagram schematically displaying the percentage of genes in each of the three clusters. Percentage numbers are calculated by comparing the number of genes in the respective groups (VEGF/EGF/IL-1 or generally activated cluster; VEGF/IL-1 or inflammation-related cluster; VEGF-specific cluster) to the total number of VEGF-induced genes. As indicated by the diagram, the number of genes induced by IL-1 is higher than the number of genes induced by VEGF-A. EGF induced a much smaller repertoire than VEGF-A.

Quiescent HUVEC were treated in parallel with VEGF-A or EGF for time periods ranging from 30 to 360 min and total RNA was isolated from the cultures and subjected to Affymetrix microarray analysis. The obtained results were analysed including a separate comparable experiment with IL-1 using identical culture conditions and the same Affymetrix chips (27). Fifty-nine genes were selected to be reproducibly upregulated by VEGF-A more than three-fold as described in *Methods* (see Supplementary Table 1 available online at www.thrombosis-online.com).

The clustering of the gene repertoire induced by VEGF-A more than three-fold is displayed in Figure 1. Two smaller clusters of genes, one regulated similarly by VEGF-A, EGF or IL-1, another preferentially by VEGF-A, and a larger cluster of genes induced by VEGF-A as well as IL-1 can be distinguished. The distribution of the genes in the VEGF-specific, the VEGF/IL-1 and the VEGF/EGF/IL-1 clusters is schematically indicated in the Venn diagram of Figure 2. The regulation of all genes of the VEGF-specific gene cluster and of about half of the genes of the other two clusters were further tested by real-time RT-PCR using RNA from three independent induction experiments and found to closely resemble the results of the microarray analysis (Table 1).

The VEGF-selective gene cluster defines a group of genes induced by VEGF-A, but not by EGF or IL1

Whereas the VEGF/EGF/IL-1 cluster contains to a large extent immediate early genes reaching peak levels between 30 and 60 min of induction such as the transcription factors FOS, JUNB

Group	Relative VEGF induction	At timepoint	Gene symbol	Induction by bFGF	VEGF inducibility in prescence of CsA
SVI (VEGF)	1300	60	NR4A2	– (26)	– (260)
	120	60	EGR3	+ (25)	+ (38)
	13	60	IGFBP3	–	– (3)
	7	60	HLX1	++ (5)	+++ (7)
	2	60	CREM	+++(2)	+
	6	150	PER1	++(3)	n/d
	4	150	MEF2C	+ (2)	++ (3)
	2	150	CCRL1	+	–
	2	150	THBD	+	+
	36	360	NDRG1	+ (9)	++ (23)
	19	360	DNAJB9	n/d	n/d
	5	360	MYCN	++ (3)	+++ (4)
IR (VEGF + IL1)	225	30	FOSB	– (25)	++++ (1240)
	105	30	ATF3	– (2)	+++ (92)
	51	30	CXCL2	–	++ (27)
	30	30	NEDD9	– (3)	++ (17)
	11	30	KCNJ2	– (3)	+ (5)
	9	30	NFKBIZ	–	+++ (7)
	80	60	DSCR1	–	–
	40	60	IL8	– (2)	+++ (34)
	17	60	PTGS2	– (2)	– (4)
	10	60	ITGAV	–	– (2)
	100	150	F3	– (2)	++ (72)
	4	150	CNKSR3	– (2)	+ (2)
	380	360	VCAM1	–	– (60)
	60	360	SELE	–	+++ (70)
	16	360	STC1	– (4)	+ (6)
GA (VEGF+IL1+EGF)	130	30	EGR2	++ (73)	+++ (88)
	35	30	FOS	+++ (35)	++++ (192)
	25	30	EGR1	++++ (60)	++++ (39)
	8	60	BHLHB2	+ (3)	++++ (8)
	4	60	KLF4	++ (3)	n/d

Relative mRNA induction by VEGF is given in -fold of uninduced control. Time points displayed are those when peak levels were observed for the respective mRNA and are given in minutes following induction. For bFGF induction and VEGF inducibility in presence of CsA the percentage of the comparable VEGF value is indicated by: – <25%; + 25–50%; ++ 50–75%; +++ 75–100%; ++++ >>100% of VEGF induction. The values in parantheses display the approximate -fold upregulation observed in comparison to the corresponding untreated control values. Values are given only for at least two-fold or larger changes. SVI, selectively VEGF induced; IR, inflammation related; GA, generally activated; n/d, not determined.

Table 1: Real-time RT-PCR based grouping of VEGF-induced genes displaying bFGF induction and CsA inhibition. Genes were grouped according to inducibility by VEGF-A, IL-1 and EGF. Genes representing individual groups were further sorted in relation to 1) fold induction and 2) the time point when maximum induction is observed. Furthermore, induction by bFGF is shown as percentage of the VEGF-A induction, and the capacity of VEGF-A to induce the genes in the presence of CsA is depicted. Data display the average from at least three independent experiments.

and EGR-1, the VEGF-specific gene cluster comprises rather genes upregulated with peak values between 60 and 360 min and includes transcription factors, intracellular signalling molecules as well as secreted proteins and receptors. In the VEGF-specific group the earliest and most highly VEGF-upregulated genes are the transcription factors NR4A2 (Nurr1) and EGR-3, which could function in the induction of secondary genes of importance for angiogenesis. It further includes genes with more delayed induction kinetics such as the transcription factors HLX1, MEF2C and PER1, the secreted IGFBP3 and the chemokine receptor CCRL1 (Fig. 3A and Table 1).

To establish whether the upregulation of these genes is mediated via VEGFR-2 or VEGFR-1 we used further VEGF-E as a specific trigger of VEGFR-2 and PlGF as a factor binding specifically to VEGFR-1. As shown in Figure 3B, VEGF-E was as potent as VEGF-A in the upregulation of VEGF-cluster genes, whereas PlGF had no significant effects.

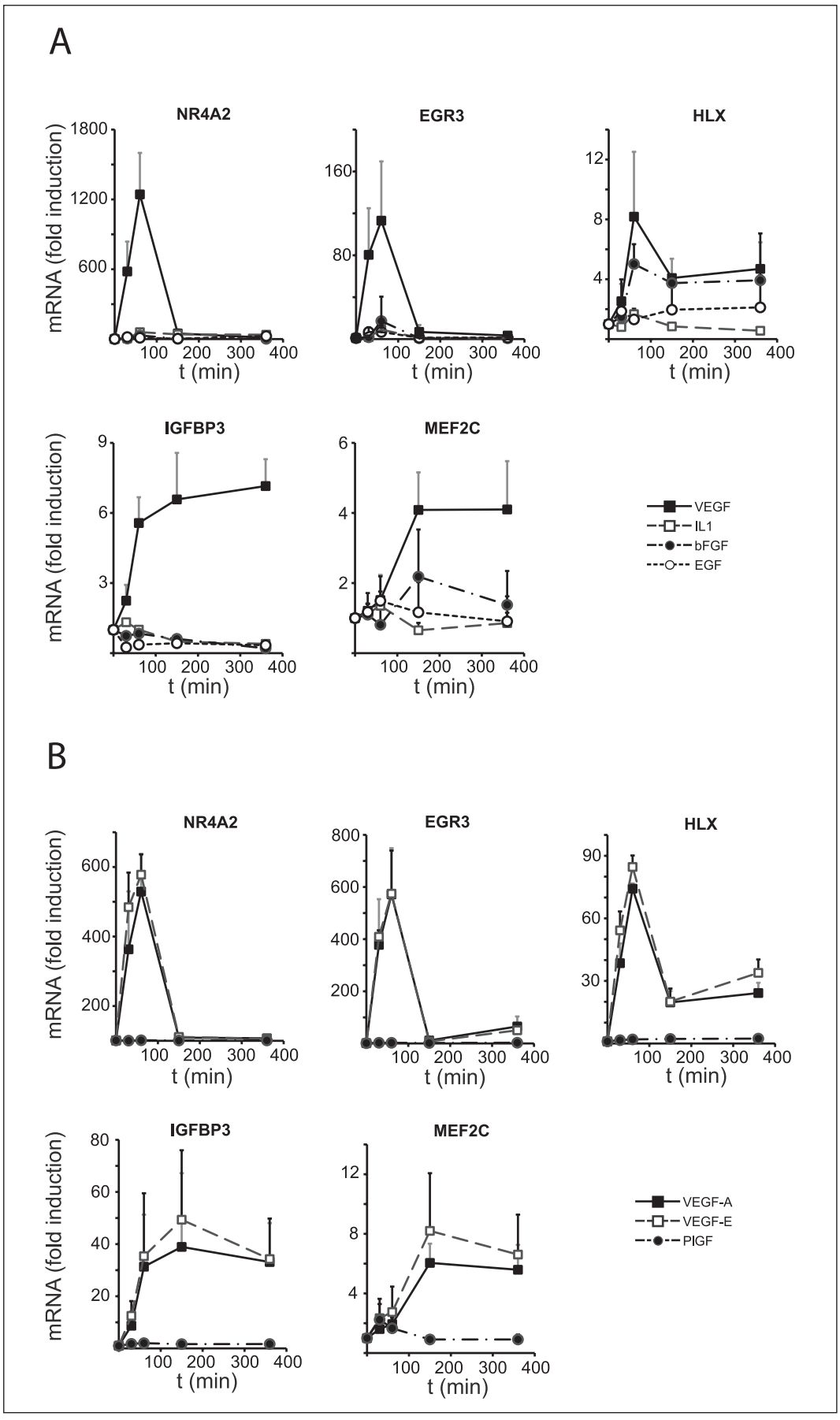

Figure 3: Real-time RT-PCR of selected genes from the VEGF-specific gene cluster. A) Comparison of inducibility by VEGF-A, IL-1, EGF and bFGF. HUVEC were seeded in six-well plates, grown to confluence and stimulated in parallel with VEGF-A (100 ng/ml), IL-1α (100 ng/ml), EGF (50 ng/ml) or bFGF (100 ng/ml) for 0.5, 1, 2.5 and 6 hours. RNA was isolated and analysed by real-time RT-PCR as described in the methods section. All values were normalised to β2-microglobulin. The mean values ± SD calculated from three independent experiments are shown. B) Comparison of inducibility by VEGF-A, VEGF-E and PlGF. HUVEC were stimulated by VEGF-A (100 ng/ml), VEGF-E (100 ng/ml) or PlGF (100 ng/ml) and analysed as described above.

The VEGF/IL-1 gene cluster comprises the majority of VEGF-induced genes

Surprisingly the largest group is the gene cluster upregulated by VEGF-A as well as IL-1, but not by EGF. This cluster contains a heterogeneous group of genes displaying peak values between 30 to 360 min. It comprises a whole group of classical inflammatory genes such as CXCL2, IL8, PTGS2 (COX-2), tissue factor and the adhesion molecules VCAM1 and E-selectin. Whereas VEGF-A induction of IL8, PTGS2 and tissue factor is largely comparable to the induction rate observed for IL-1, induction of VCAM1, E-selectin and CXCL2 by VEGF was approximately 10-fold and 100-fold less pronounced than IL-1 induction, respectively. In addition transcription factors such as ATF3, members of cytoplasmic signalling pathways such as NEDD9 and NFKBIZ and the ion channel KCNJ2 belong to this cluster (Table 1 and Supplementary Fig. 1A available online at www.thrombosis-online.com).

To test for the involvement of VEGFR-2 in the upregulation we evaluated VEGF-E and PlGF effects on selected inflammatory genes. Again VEGF-E could completely or in part reproduce VEGF-A effects, whereas PlGF did not display detectable activity (Supplementary Fig. 1B available online at www.thrombosis-online.com).

To confirm that also the corresponding inflammatory proteins are upregulated in the cells, we have analysed VCAM1 expression by flow cytometry. As shown in Figure 4, VCAM1 expression was significantly induced by VEGF-A on the surface of the cells to about 25% of the level observed following treatment with IL-1. This correlates well with the observed VCAM1 mRNA induction (Supplementary Fig. 1A available online at www.thrombosis-online.com). In contrast, EGF did not induce any detectable VCAM1. Furthermore, we have tested whether VEGF-A induction would also mediate corresponding leukocyte adhesion in an assay using HL60 cells. Indeed, treatment of HUVEC with VEGF-A induced a significant increase in the adhesion of HL60 to the HUVEC monolayer. Adhesion was about 15% of the level obtained with IL-1. Again EGF did not induce increased adhesion.

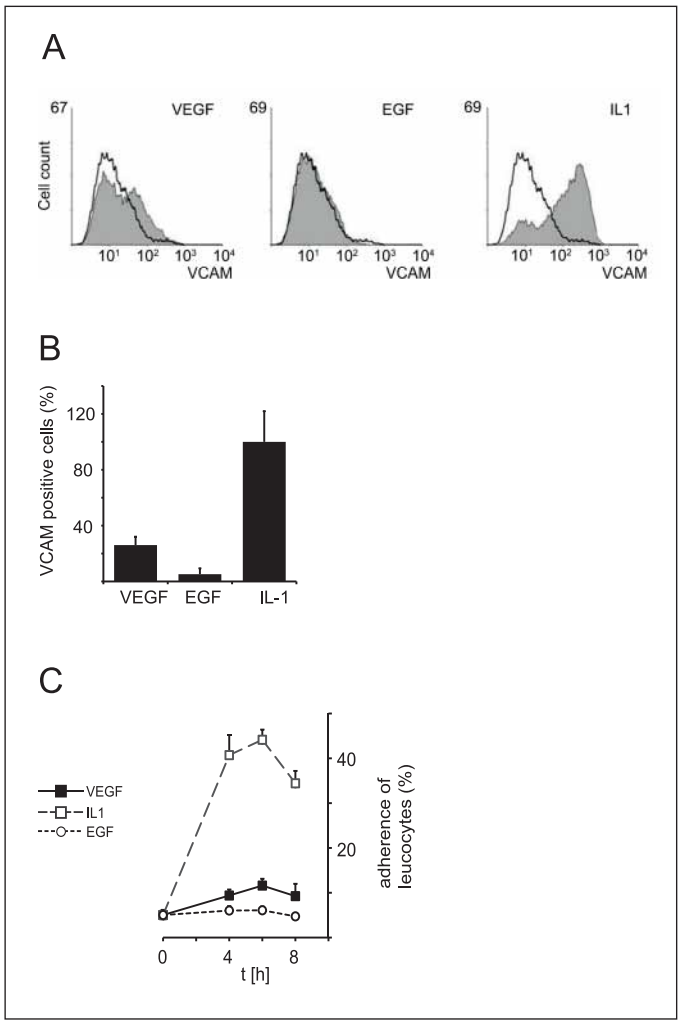

Figure 4: Expression of VCAM1 and adherence of leukocytes to monolayers of VEGF-induced HUVEC. A) Flow cytometry analysis displaying VCAM1 expression on VEGF-A- or IL-1-induced HUVEC. Cells were treated VEGF-A, IL-1 or EGF for 6 hours, harvested and stained with anti-VCAM1 antibodies and corresponding FITC-labelled secondary antibodies. Filled histograms display treated, open histogram uninduced cells. B) Quantification of VCAM1-positive cells. The number of VCAM1 expressing cells was determined from the flow cytometry data by setting the appropriate gate in comparison to uninduced cells. C) Assay displaying adhesion of HL60 cells to VEGF-A- or IL1-induced HUVEC. HUVEC treated for 4, 6 and 8 hours with VEGF, IL-1 or EGF were incubated with DiO-labeled HL60 cells. The number of DiO-labeled adherent HL60 cells were determined following detachment of all cells by flow cytometry and displayed as percentage of total cells. Mean values and standard deviations are calculated from two experiments performed with triplicate wells.

The upregulation of the majority of the VEGF-specific and inflammatory cluster genes depends on the transcription factor NFAT

Since we have previously described that VEGF-A strongly induces a pathway via PLC-γ and calcineurin/NFAT activation (14, 28), which is in accordance with the reports of others (29–31), we have been interested to determine, whether the upregulation of the VEGF/IL-1 cluster is due to NFAT activation and distinguished from the upregulation of the VEGF-specific and the VEGF/EGF/IL-1 cluster. Alternatively, it would also be possible, that similar to the induction by inflammatory cytokines (18), VEGF-A could use NF-κB for the upregulation of the VEGF/IL-1 cluster genes. To test the involvement of calcineurin/NFAT we have used a specific inhibitor of calcineurin, cyclosporin A (CsA) (32). The effects of CsA on the upregulation of selected VEGF cluster genes is displayed in Figure 5 and summarised in Table 1. The data clearly show that the VEGF induction of most of the genes of the VEGF-specific cluster can be inhibited by CsA, which is similar to the inhibition of VEGF/IL-1 cluster genes (Supplementary Fig. 1B available online at www.thrombosis-online.com). In contrast, none of the genes inducible by all three factors could be inhibited, but rather displayed in most cases an enhancement of their VEGF-induction in the presence of CsA (Table 1). This shows that calcineurin/NFAT does not contribute to, but rather negatively regulates the induction of the immediate early genes upregulated by all three factors. However, NFAT is an important component in the induction of the major-

ity of the genes of the VEGF/IL-1 cluster as well as the VEGF-specific cluster. To exclude a significant contribution of NF-κB we in parallel tested a potential cytoplasmic/nuclear translocation of the p65/RelA subunit of NF-κB in reponse to VEGF-A (Supplementary Fig. 2 available online at www.thrombosis-online.com). In line with our previous results displaying absence of IκB degradation following VEGF-A treatment (28), no significant translocation of NF-κB was observed.

bFGF does not upregulate inflammatory VEGF/IL-1 cluster genes, but part of the VEGF cluster genes

In addition to VEGF-A, bFGF is a growth factor with strong capacity to induce angiogenesis *in vitro* and *in vivo*. Although the extent of its participation in physiological angiogenesis is not clarified, it has been described as a major contributor to pathologic tumour angiogenesis. Therefore we have evaluated to which extent bFGF would be able to induce genes of the selected VEGF repertoire by real-time RT-PCR. As summarised in Table 1, bFGF, as expected, upregulated all generally induced genes. It could also induce in part VEGF-specific cluster genes (Fig. 3A); however, it was unable to significantly induce any of the inflammatory VEGF/IL-1 cluster genes (Supplementary Fig. 1 available online at www.thrombosis-online.com). This is in accordance with the possibility of a functional overlap of VEGF-A and bFGF in the induction of specific angiogenesis-related responses.

Discussion

In comparison to other growth factors there are unique responses mediated by VEGF-A signalling, which comprise the triggering of progenitor cell differentiation towards the endothelial cell lineage, directing tip cell filopodia extension (3), formation of a three-dimensional vascular tube, and regulation of vascular permeability (2). Although several forms of VEGF-A have been described to be produced by differential splicing, which may act in concert, it is generally assumed that isoform VEGF-A_{165} is the major form, which seems to be to a large degree responsible for gene regulation important for vasculogenesis and angiogenesis of blood vessels (5). The functions exerted by VEGF-A appear not to be inducible to the same extent and quality by other growth factors and cytokines. It has therefore to be anticipated that the unique properties of VEGF-A are mediated via a distinct capacity to signal and to induce a unique gene signature in comparison to other factors. VEGF-A has been shown to bind to VEGFR-1 as well as VEGFR-2, both receptors being expressed on HUVEC (33). However, whereas binding to VEGFR-2 results in the strong induction of downstream signalling pathways such as PLC-γ and PI3-kinase, VEGFR-1 can only trigger much weaker intracellular signals (8) and little gene regulation (see below). Given the fact that the VEGFR-2 contains 19 tyrosine residues in its cytoplasmic domain, which in part seem to be phosphorylated in a dynamic way dependent on the activity state of the cell (11),

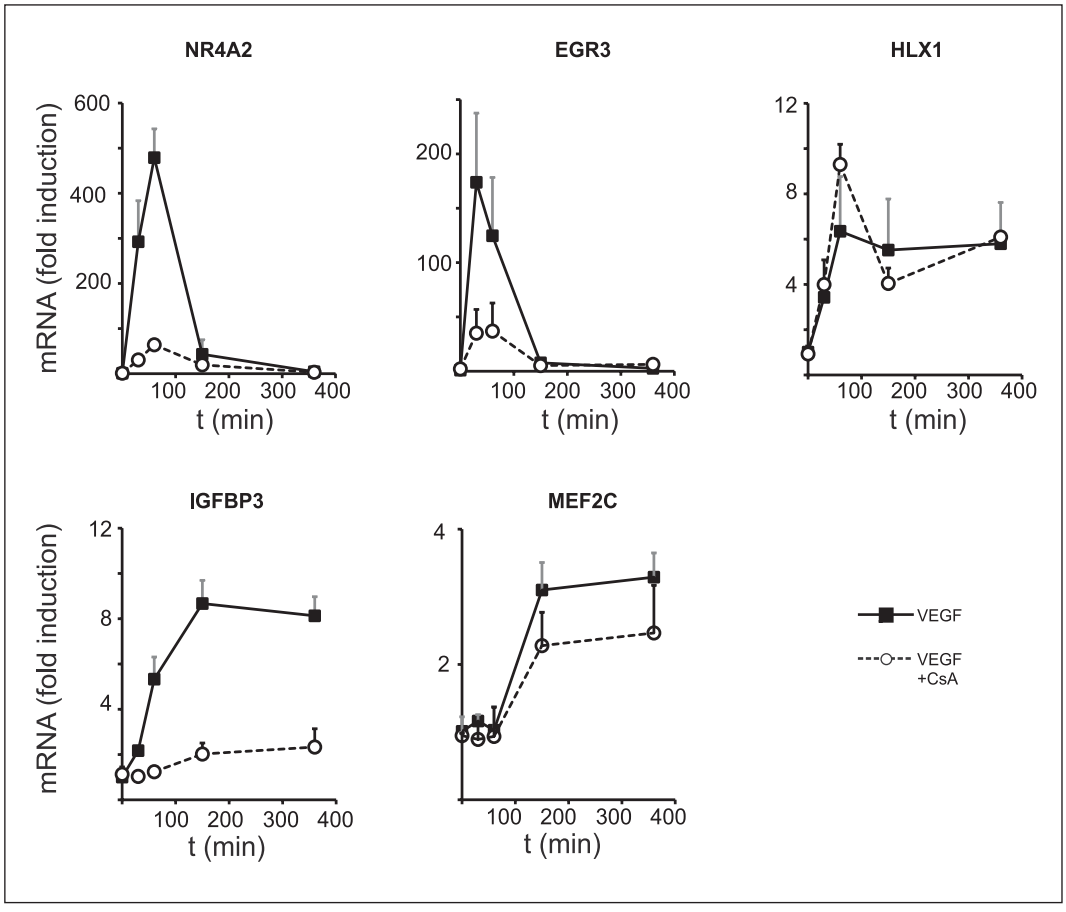

Figure 5: Effect of the calcineurin inhibitor CsA on the VEGF-mediated induction of selected genes of the VEGF-specific cluster. HUVEC were seeded in six-well plates and grown to confluence. Following preincubation with CsA (1 μg/ml) for 30 minutes the cells were induced with VEGF-A (100 ng/ml) for 0.5, 1, 2.5 and 6 hours. Real-time PCR data are obtained and displayed as described for Figure 3.

it is likely the qualitatively and quantitatively differential docking of signal transducers which mediates a unique gene signature and responses important for specific VEGF-A_{165} functions.

Indeed, a group of about 20% of the VEGF-induced genes were selectively induced by VEGF-A and not at all or to a much lower extent by EGF or IL-1. Addressing the question whether the upregulation of these genes was mediated by VEGFR-2 and/or VEGFR-1 we have tested by real-time RT-PCR the regulation of selected genes in response to VEGF-E and PlGF, two factors binding selectively to VEGFR-2 or VEGFR-1, respectively. Clearly, VEGF-E resulted in an upregulation of these genes comparable to VEGF-A, whereas PlGF had little if any effect on gene expression suggesting that VEGFR-2 triggering by itself can mediate the majority of the observed transcriptional response. We propose that these genes regulated by VEGF-A via VEGFR-2 are induced to fulfill a function exerted predominantly by VEGF-A, but not by EGF or IL-1. The most strongly upregulated genes in this group are NR4A2 (Nurr1) and EGR-3. NR4A2 is a member of the NR4A subfamily of orphan nuclear receptors and has, among others, been previously implicated in nerve cell development (35). It could be involved in processes of axon guidance as well as angiogenesis via regulation of neuropilin levels (36), EGR-3 is unique among the EGR family, in that it is preferentially upregulated by VEGF and partially sensitive to NFAT inhibition, whereas EGR-1 and EGR-2 seem to be more generally activated and induced without NFAT contribution. EGR-3 has been previously described as an important regulatory factor in T lymphocytes (37) and certain nerve cells, e.g. to be essential for adaptation to stress and novelty (38). It should be interesting to define the secondary response genes regulated by NR4A2 and EGR-3, as, while this work was in progress, it has been reported that NR4A2 as well as EGR-3 induction are a prerequisite for VEGF-mediated endothelial proliferation, migration and tubulogenesis *in vitro* (39).

Three additional transcription factors selectively induced by VEGF-A are the homeobox gene HLX1, the MADS box factor MEF2C and the period family member PER1. In accordance with the proposed importance of the VEGF cluster genes for angiogenic processes, our recent results support that HLX1 is a specific regulator of the expression of guidance receptors involved in endothelial sprouting (Testori et al., in preparation). MEF2C has been previously shown to be required for vascular development (40) and PER1 extends the list of VEGF-regulated genes, which reported functions in nerve cells (41). Another gene of interest from the VEGF-specific group encoding a secreted protein is IGFBP-3. Although several available reports are conflicting in regard of pro- or antiangiogenic effects of IGFBP-3, two recent reports suggest a role for IGFBP3 in endothelial progenitor cell migration, differentiation and capillary formation (42).

The contribution of inflammatory mediators to angiogenic processes is a debated issue. There is considerable evidence of cross-regulation of inflammation and angiogenesis in many pathologies (43). Increased angiogenesis is frequently observed in chronic inflammatory diseases and excessive angiogenesis and inflammation is the hallmark of malignant tumours. Inflammatory cytokines such as IL-1 as well as VEGF-A may therefore play an important role in the complex link between inflammation and angiogenesis. It was therefore of interest to investigate to which extent the inflammatory signalling cascades and gene signatures overlap with or are distinct from angiogenic VEGF-mediated signalling and function.

Surprisingly, 60% of the VEGF cluster genes are IL-1-regulated genes and are not induced by EGF, indicating that the VEGF-A response in contrast to other growth factors contains a significant inflammatory component. It appears that mainly VEGFR-2 is mediating this property of VEGF-A as VEGF-E, but not PlGF, could mediate induction of selected genes from this group. The inflammatory gene cluster encodes proteins with a typical inflammatory function such as PTGS2/COX-2, which via synthesis of prostaglandins plays a prominent role in inflammatory processes (44), or VCAM1, which mediates leukocyte adhesion (45). As we have previously shown it also includes as one of the most strongly VEGF-upregulated genes the tissue factor gene (14, 28), which as a primary initiator of the extrinsic coagulation pathway is an important component of the repair mechanisms of vascular cells after inflammatory or mechanical injury. Whereas some genes such as PTGS2/COX-2 and tissue

What is known about this topic?

- VEGF-A is the major trigger of angiogenesis and vasculogenesis and VEGF-regulated genes have been described by several authors.
- However, it remains so far undefined which part of the gene repertoire induced by VEGF is specifically induced by VEGF-A165, necessary for angiogenesis and cannot be induced by other growth factors or cytokines and to what degree the VEGF repertoire overlaps with that of the other factors.
- Furthermore, the major key regulatory transcription factors mediating the VEGF-response and angiogenesis remain largely undefined.

What does this paper add?

- We have directly compared in parallel experiments the gene repertoires induced by the "angiogenesis-inducing" growth factor VEGF-A, the "proliferation-inducing" growth factor EGF and the "inflammation-inducing" cytokine IL-1.
- The obtained data define for the first time
  i) a group of genes selectively regulated by VEGF-A which we propose to fulfill specific roles for angiogenesis which can not be contributed by the other factors,
  ii) the data display an unexpected large overlap of the VEGF repertoire with that of inflammatory cytokines (which is in contrast to the EGF-repertoire) suggesting that VEGF has itself some inflammatory capacity,
  iii) define the transcription factor NFAT as a mediator not only of a major part of the inflammatory component of VEGF induction, but also as an important component of the "VEGF-specific" gene induction and
  iv) suggest several additional transcription factors as key mediators of specific VEGF effects such as HLX1 and MEF2C.

factor are upregulated to a similar extent by VEGF-A and IL-1, others such as VCAM1 are much stronger induced by IL-1 than by VEGF-A.

To minimise the possibility that culture conditions using sparse cells would induce the upregulation of inflammatory genes, we have used for induction densely grown HUVEC monolayers for which cell to cell contact should mediate contact inhibition via VE-cadherin (46). Although we can not completely exclude that the cell culture conditions would facilitate the induction of inflammatory genes, nevertheless we believe that the data clearly show the principal capacity of VEGF to induce genes with inflammatory functions. In the case of VCAM1, which is involved in immune cell adhesion during extravasation (47), we have demonstrated that the protein is expressed on the cell surface of endothelial cells following VEGF-A induction. Furthermore, significantly increased leukocyte adhesion can be observed as to be expected for cells with upregulated adhesion molecules such as VCAM1.

Taken together it appears that VEGF-A preferentially induces a significant subfraction of inflammatory genes, each to a specific extent, which may vary from IL-1 induction. This may indicate a role of these inflammatory genes for neovascularisation occurring during repair processes. Given the intrinsic inflammatory component in VEGF-A signalling, it is possible, that VEGF-A by itself is capable to promote inflammation, as it may be necessary for efficient tissue repair and angiogenesis. Furthermore, it may be that inflammatory cytokine signalling may considerably lower the threshold for angiogenic stimulation and vice versa, which could facilitate the initiation and progression of chronic inflammatory diseases.

It needs to be mentioned that whereas many of the genes of the VEGF-specific and of the inflammatory cluster are upregulated with peak values between 30 and 60 min after addition of the factors, some genes such as MEF2C and PER1 of the VEGF-specific cluster or VCAM1 and E-selectin of the inflammatory cluster display more delayed induction kinetics with mRNA peak values between 2 and 6 h. It is likely that the induction of these genes involve secondary gene regulatory events presumably requiring some of the transcription factors induced with immediate early kinetics.

We have previously analysed signalling cascades triggered by VEGF-A and EGF in endothelial cells and have shown that a major difference is that VEGF preferentially activates pathways leading via Ca++/calcineurin and PKC/MEK/ERK to transcription factors such as NFAT and EGR-1, respectively. In contrast, EGF was not capable to induce NFAT at all and upregulated EGR-1 in a PKC-independent way, probably via RAS-mediated MEK/ERK activation (14, 28). Given the strong induction of NFAT by VEGF-A and the inability of EGF to induce NFAT, it is likely that NFAT is a predominant factor endowing VEGF-A with the potency to induce a several fold larger gene repertoire when compared to EGF. The sensitivity of the majority of the VEGF- and VEGF/IL-1 cluster genes to the specific calcineurin inhibitor CsA (32) strongly supports this possibility. Given the short preincubation time of 30 min and the rapid VEGF-mediated induction of a major part of the genes within 1–2 h the CsA-mediated inhibition should be due to the direct inhibition of calcineurin and NFAT activation and the influence of secondary CsA effects as reported after longer incubation periods of 24 h (48) is less likely.

It is well established that inflammatory cytokines such as IL-1 strongly induce the NF-κB pathway, which is essential for the upregulation of most of the inflammatory response genes (18, 19). It is therefore conceivable that the VEGF/IL-1 cluster genes comprise a group of genes with NFAT as well as NF-κB binding sites in their promoters. VEGF-A would then preferentially use NFAT and IL-1 NF-κB for the induction of these genes. Results of a TOUCAN promoter analysis (49) of the genes of the VEGF/IL-1 cluster supported this possibility (B. Schweighofer and E. Hofer, unpublished results).

Another growth factor long known to have a role in repair associated angiogenesis and pathologic tumour angiogenesis is bFGF (15), which in endothelial cells induces signals mainly via the receptor tyrosine kinase FGF receptor-1. Whereas part of the experimental evidence suggests that bFGF induces vascularisation indirectly via upregulation of the VEGF/VEGFR system, other data have shown a direct dependence of tube formation in embryonic explants on bFGF and a synergistic activity of bFGF with VEGF in tumour angiogenesis (15). In our study bFGF was competent to upregulate in part genes from the VEGF cluster, which we propose to fulfill specific roles in angiogenesis.

As shown in Figure 2, about 20% of the VEGF-induced genes were similarly induced by VEGF-A, EGF and IL-1. This group of generally induced genes, which includes for example members of the Fos, Jun and EGR families, are presumably necessary for general responses triggered by all three factors and induced by a wide variety of different growth factors and cytokines. The promoters of these genes are characterized by the presence of a high number of serum response factor (SRF) binding sites (B. Schweighofer and E. Hofer, unpublished observation) suggesting their predominant activation by the MEK/ERK MAP-kinase pathway (50). These immediate early response genes encode mainly transcription factors involved in survival and proliferation responses and/or necessary in part as co-factors to activate secondary transcriptional responses together with the more inducer-specific transcription factors such as NFAT or NF-κB. For example, it has been shown that AP-1 functions by interacting with NFAT as a necessary factor in the upregulation of NFAT-controlled genes such as IL-2 (51) and we have recently shown that EGR-1 similarly can functionally interact with NFAT to induce tissue factor expression by VEGF-A (14).

From the multitude of genes regulated by VEGF-A (30) this work has defined a small group selectively upregulated by VEGF-A, but not by EGF or IL-1. It is intriguing that this group contains several genes previously implicated in nerve cell differentiation and axon guidance which could provide additional examples for the proposed analogy of endothelial with neuron sprouting and guidance mechanisms (52). Based on the hypothesis that the VEGF cluster genes may have a role in specific functions induced by VEGF-A and essential for endothelial differentiation, angiogenic sprouting and/or tubulogenesis, it is possible that these genes will constitute preferential targets to interfere with angiogenesis as it has already been indicated for some examples by the reports of others (39).

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Supplementary Figures and Tables to Schweighofer et al. "The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation" (Thromb Haemost 2009; 102.3)

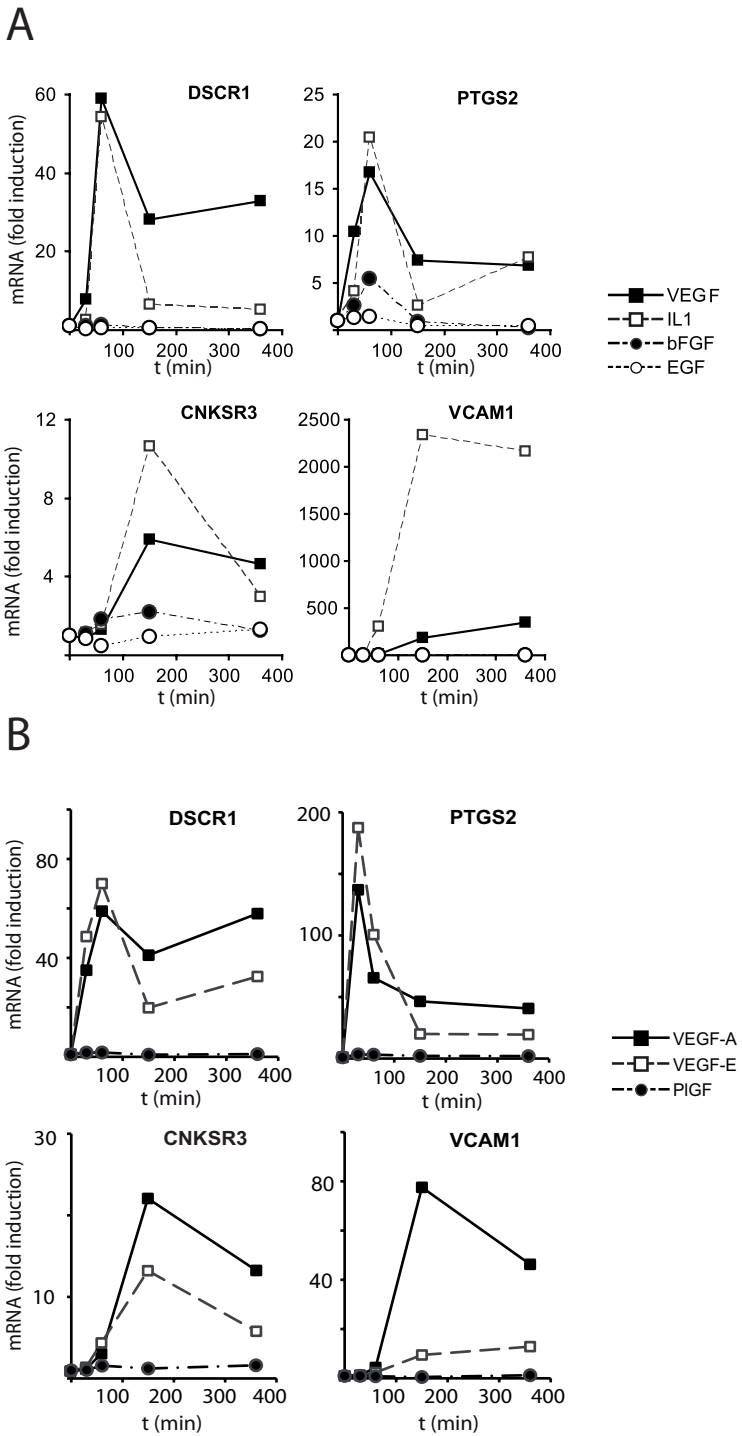
Supplementary Figure 1. ***Induction pattern and CsA sensitivity of selected genes from the VEGF/EGF/IL-1 and VEGF/IL-1 clusters***. A) Induction pattern for VEGF, EGF, IL-1 and bFGF. HUVEC were seeded in 6-well plates, grown to confluence and stimulated in parallel with VEGF (100 ng/ml), IL-1α (100 ng/ml), EGF (50 ng/ml) or bFGF (100 ng/ml) for 0.5, 1, 2.5 and 6 h. RNA was isolated and subjected to realtime RT-PCR analysis as described in the Methods. All values were normalized to β2-microglobulin. One representative experiment of three performed with similar results is shown. B) Induction pattern for VEGF-A, VEGF-E and PlGF. HUVEC were stimulated by VEGF-A (100 ng/ml), VEGF-E (100 ng/ml) or PlGF (100 ng/ml) and analyzed as described above. C) Effects of CsA on induction pattern. HUVEC were seeded in 6-well plates and grown to confluence. The cells were preincubated with CsA (1 µg/ml) for 30 minutes and induced with VEGF (100 ng/ml) for 0.5, 1, 2.5 and 6 h. All values were normalized to β2-microglobulin. One representative experiment of three performed with similar results is shown.

Supplementary Figure 2: ***In contrast to IL-1α VEGF fails to induce significant cytoplasmic-nuclear translocation of NF-κB.*** Immunofluorescence of nuclear accumulation of NF-κB. HUVEC were seeded on LabTek 8-well tissue culture chamber slides (NUNC, Wiesbaden, Germany) coated with 100 µg/ml fibronectin (Becton Dickinson, Heidelberg, Germany) for 1 h. After treatment with VEGF (100 ng/ml) or IL-1 (100 ng/ml) for 20 min the cells were fixed in 4% paraformaldehyde/PBS for 10min. Permeabilization was achieved by addition of PBS containing 0.5% Triton X-100 for 5 minutes at RT, anti-NF-κB p65 rabbit antibodies sc-109 from Santa Cruz Biotechnology (Santa Cruz, CA) were diluted 1:500 in PBS/1% BSA and incubated for 1 h at RT. The cells were washed four times in PBS/1% BSA. Secondary Alexa 488 goat anti-rabbit antibodies from Molecular Probes (Eugene, OR), diluted 1:10000 were used for visualization followed by washing of the cells 5 times in PBS/1%BSA. For nuclear staining, Hoechst (Sigma, St.Luis, MO) diluted 1:1000 was

added in the last washing step. Cover slips were mounted in fluorescent mounting medium (Dako, Glostrup, Denmark) and analyzed on a Nikon Diaphot TMD microscope. Images were taken by a CCD camera (Kappa GmbH, Gleichen, Germany).

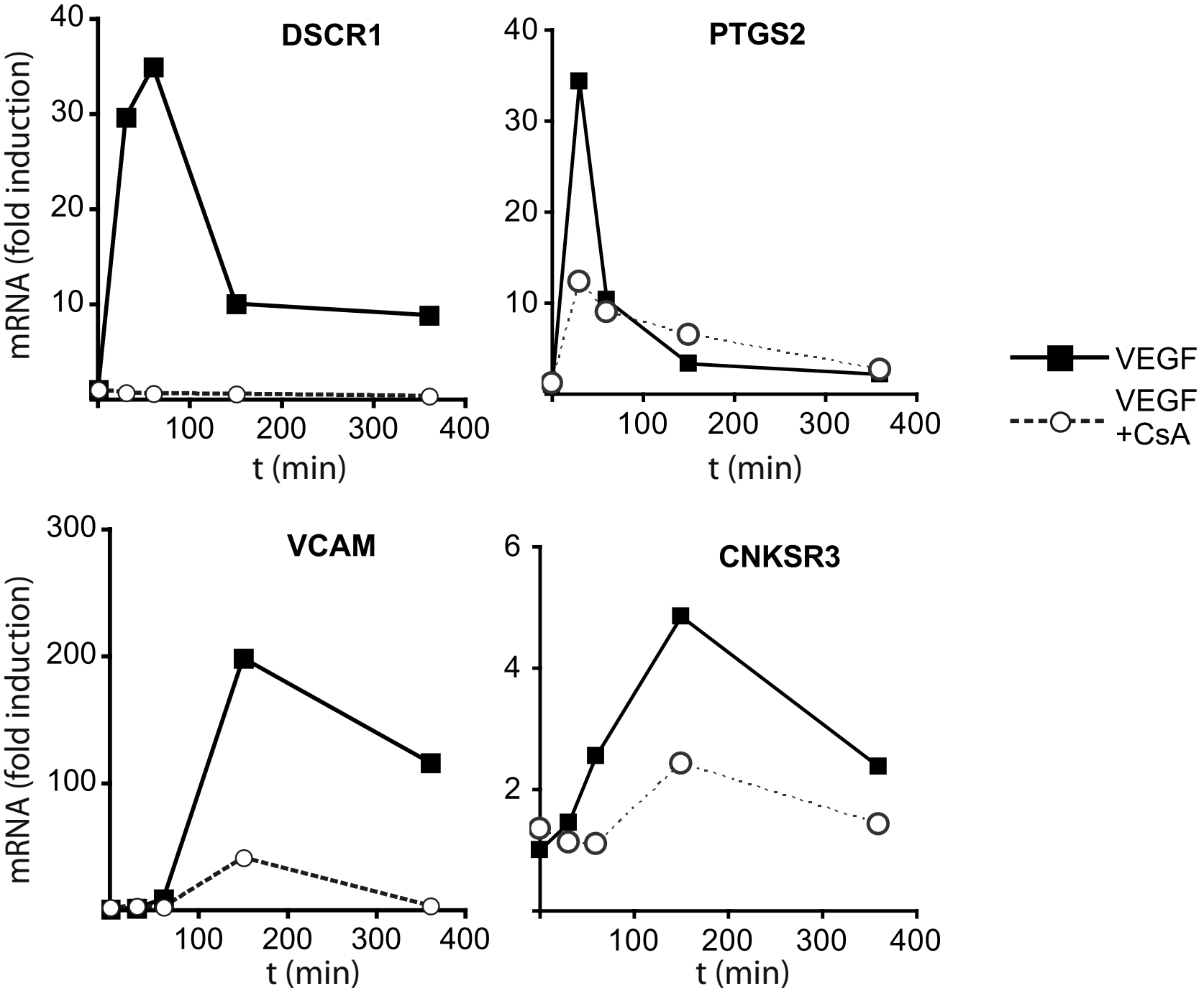
Supplementary Table 1: List of all genes induced by VEGF over three-fold. VEGF induced genes are sorted according to their fold induction value at the 60 min time point and dependent on their classification as *SVI (selectively VEGF induced), IR (inflammation related)* and *GA (generally activated)*. Furthermore, the corresponding fold induction values for EGF and IL-1 is given. The displayed genes were selected according to the following criteria. Firstly, Affymetrix probe sets showing absolute calls of "absence", difference calls of "no change", less than 3-fold upregulation or a signal intensity of less than 60 for all VEGF-induced data points were excluded. Secondly, only genes upregulated in two arrays obtained from two independent experimental induction series were included in the selection. Thirdly, the VEGF induction of few genes could not be verified by realtime RT-PCR and these were again excluded from the selected list.

Supplementary Table 2: Primers used for realtime RT-PCR. Forward and reverse primers as used for realtime RT-PCR are displayed.

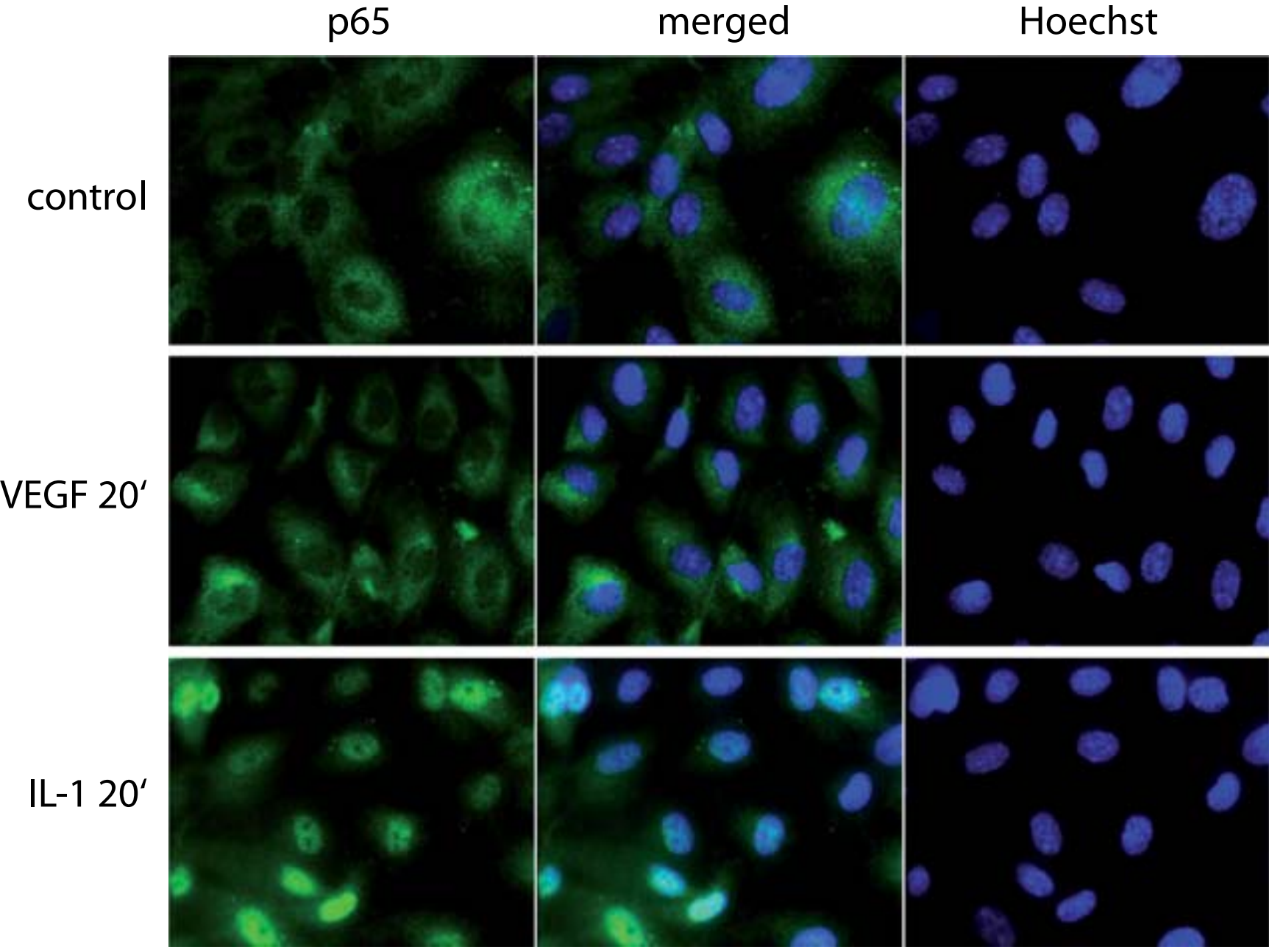


Supplemental Figure 1A and B

C



Supplemental Figure 1C



Supplemental Figure 2

Supplemental Table 1

UniGene ID	Entrez Gene	Gene Symbol	David Gene Name	VEGF 30min	VEGF 60min	VEGF 150min	VEGF 360min	EGF 30min	EGF 60min	EGF 150min	EGF 360min	IL1 30min	IL1 60min	IL1 150min	IL1 360min	Classification
HS.165258	4929	NR4A2	Nuclear receptor subfamily 4, group A, member 2	16.0	36.8	8.6	1.1	1.1	4.3	3.0	0.7	1.0	5.3	0.9	0.4	*SVI*
HS.534313	1960	EGR3	Early growth response 3	6.5	21.1	1.1	1.1	1.4	1.9	0.8	0.8	8.6	24.3	1.9	2.5	*SVI*
HS.445534	5187	PER1	Period homolog 1 (Drosophila)	2.0	8.6	2.3	1.0	0.9	1.7	0.7	0.7	0.8	2.8	0.5	0.8	*SVI*
HS.450230	3486	IGFBP3	Insulin-like growth factor binding protein 3	2.3	8.0	21.1	9.2	2.0	2.6	3.7	2.8	1.2	1.0	4.3	9.8	*SVI*
HS.82071	10370	CITED2	Cbp/p300-interacting transactivator 2	2.8	4.3	3.5	1.4	1.5	1.6	1.7	1.1	1.1	0.7	0.8	0.5	*SVI*
HS.200250	1390	CREM	CAMP responsive element modulator	2.3	3.5	8.0	2.3	1.1	1.7	2.1	1.2	1.1	1.7	1.6	0.7	*SVI*
HS.74870	3142	HLX1	H2.0-like homeobox 1 (Drosophila)	1.3	3.5	3.5	3.2	1.1	0.9	1.6	1.1	1.1	0.7	1.4	0.9	*SVI*
HS.25960	4613	MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived	2.8	2.5	7.5	3.5	0.4	1.3	2.3	0.9	0.9	0.3	0.4	0.4	*SVI*
HS.6790	4189	DNAJB9	Dnaj (hsp40) homolog, subfamily B, member 9	1.7	2.1	3.2	2.0	1.7	1.6	1.4	1.6	1.1	1.0	1.5	1.5	*SVI*
HS.372914	10397	NDRG1	N-myc downstream regulated gene 1	1.1	1.9	3.2	1.9	1.2	1.1	1.3	1.1	1.1	1.0	0.9	1.5	*SVI*
HS.2030	7056	THBD	Thrombomodulin	1.1	1.6	3.7	0.9	0.6	0.9	1.6	0.8	1.4	1.6	0.5	0.3	*SVI*
HS.310512	51554	CCRL1	Chemokine (C-C motif) receptor-like 1	0.9	1.5	3.7	2.0	0.8	1.1	1.4	1.0	1.1	0.6	0.6	0.8	*SVI*
HS.654474	4208	MEF2C	MADS box transcription enhancer factor 2, polypeptide c	1.1	1.3	4.3	1.6	1.1	1.1	1.3	0.6	0.9	1.0	0.5	0.5	*SVI*
HS.62192	2152	F3	Coagulation factor III (thromboplastin, tissue factor)	3.0	29.9	6.5	5.7	1.0	0.7	0.8	1.0	29.9	128.0	73.5	24.3	*IR*
HS.196384	5743	PTGS2	Prostaglandin-endoperoxide synthase 2	8.0	21.1	3.5	1.6	2.8	3.7	1.3	1.2	8.0	7.5	13.9	10.6	*IR*
HS.282326	1827	DSCR1	Down syndrome critical region gene 1	4.3	13.0	8.6	4.9	1.1	1.5	1.2	1.1	4.9	10.6	5.7	2.5	*IR*
HS.460	467	ATF3	Activating transcription factor 3	5.3	12.1	1.2	1.0	2.1	2.1	1.0	0.9	13.9	13.9	8.0	3.0	*IR*
HS.75678	2354	FOSB	Fbj murine osteosarcoma viral oncogene homolog B	4.3	11.3	1.3	1.2	1.2	3.0	1.1	0.9	24.3	36.8	1.1	1.2	*IR*
HS.75765	2920	CXCL2	Chemokine (C-X-C motif) ligand 2	6.5	9.8	2.3	0.9	3.0	1.9	0.8	0.8	10.6	10.6	10.6	13.0	*IR*
HS.319171	64332	NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta isoform A	3.5	8.0	1.1	0.9	1.9	1.9	0.7	0.9	8.0	12.1	8.6	8.6	*IR*
HS.444947	10221	TRIB1	Tribbles homolog 1 (Drosophila)	2.8	7.5	1.0	1.2	2.0	2.8	0.9	0.9	6.5	5.7	3.0	2.5	*IR*
HS.624	3576	IL8	Interleukin 8	3.5	6.5	2.0	0.4	1.6	1.2	0.7	0.7	4.9	7.5	8.6	8.6	*IR*
HS.432453	1326	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.1	5.3	6.5	1.7	0.8	0.7	1.1	1.3	1.5	4.3	7.5	2.6	*IR*
HS.524430	3164	NR4A1	Nuclear receptor subfamily 4, group A, member 1	2.3	5.3	2.6	1.0	1.1	0.9	0.5	0.2	4.0	6.5	0.8	1.1	*IR*
HS.534115	9510	ADAMTS1	Adam metallopeptidase with thrombospondin type 1 motif, 1	1.6	5.3	3.2	2.3	1.1	1.9	1.2	0.9	2.1	4.0	3.0	4.3	*IR*
HS.23388	81575	APOLD1	Apolipoprotein l domain containing 1	2.6	5.3	1.4	0.8	1.5	1.5	0.5	0.9	1.7	1.5	1.5	2.6	*IR*
HS.279522	8013	NR4A3	Nuclear receptor subfamily 4, group A, member 3	0.8	4.3	1.5	1.4	0.8	0.6	0.9	0.8	0.9	24.3	9.2	14.9	*IR*
HS.440829	1052	CEBPD	Ccaat/enhancer binding protein (C/EBP), delta	1.4	4.0	5.3	1.5	1.2	1.4	1.3	1.0	3.5	8.0	12.1	6.1	*IR*
HS.89690	2921	CXCL3	Chemokine (C-X-C motif) ligand 3	1.7	3.7	2.1	1.1	1.3	1.3	0.4	1.1	24.3	42.2	36.8	39.4	*IR*
HS.799	1839	HBEGF	Heparin-binding EGF-like growth factor	1.9	3.5	3.7	1.6	1.4	1.6	1.6	0.9	2.3	4.0	1.6	2.8	*IR*
Hs.436029	---	236685_at	---	1.5	3.5	1.9	0.7	0.9	1.1	0.9	0.9	11.3	9.8	9.2	3.5	*IR*
HS.789	2919	CXCL1	Chemokine (C-X-C motif) ligand 1	2.0	3.2	1.5	0.8	1.3	1.9	1.0	1.2	4.6	5.7	6.1	5.7	*IR*
HS.1547	3759	KCNJ2	Potassium inwardly-rectifying channel, subfamily J, member 2	1.7	3.2	1.6	0.5	0.8	0.9	0.8	0.5	1.4	1.1	1.7	1.6	*IR*
HS.37982	4739	NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	4.0	3.2	1.9	1.5	1.7	1.3	0.5	0.7	1.6	1.6	1.3	1.0	*IR*
HS.150557	687	KLF9	Kruppel-like factor 9	1.7	3.0	2.6	1.2	1.2	1.5	0.7	0.7	1.2	2.8	2.0	1.4	*IR*
HS.1722	3552	IL1A	Interleukin 1, alpha	1.7	3.0	0.9	0.8	1.3	1.4	0.3	0.8	16.0	17.1	8.6	9.2	*IR*
HS.73853	650	BMP2	Bone morphogenetic protein 2	1.2	2.8	2.8	1.7	0.9	1.2	1.1	1.4	1.4	2.6	2.6	2.5	*IR*
HS.25590	6781	STC1	Stanniocalcin 1	1.3	2.8	5.7	2.1	0.9	0.8	1.0	0.6	1.3	2.1	1.7	3.5	*IR*
HS.303649	6347	CCL2	Chemokine (C-C motif) ligand 2	1.4	2.6	3.2	0.6	1.5	1.3	0.8	1.0	1.7	2.1	2.1	2.1	*IR*
HS.89546	6401	SELE	Selectin E (endothelial adhesion molecule 1)	0.9	2.3	7.0	2.1	0.8	0.7	0.8	0.4	24.3	42.2	48.5	39.4	*IR*
HS.109225	7412	VCAM1	Vascular cell adhesion molecule 1	1.1	2.1	12.1	3.2	1.1	0.9	1.3	1.0	3.0	17.1	29.9	17.1	*IR*
HS.1048	4254	KITLG	Kit ligand	0.8	1.4	3.0	1.5	0.8	1.1	1.7	0.9	1.1	3.7	4.0	7.5	*IR*
HS.159223	4665	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	1.1	1.4	4.0	1.2	0.5	1.0	1.4	0.9	0.6	1.9	2.1	0.9	*IR*
HS.436873	3685	ITGAV	Integrin, alpha v (vitronectin receptor, alpha polypeptide, antigen cd51)	0.4	1.2	2.8	0.4	0.1	0.2	0.3	0.1	1.5	3.2	2.5	0.9	*IR*
HS.16064	154043	CNKSR3	CNKSR family member 3	1.0	1.1	4.9	1.6	1.0	0.9	1.1	0.8	1.1	3.7	8.6	2.6	*IR*
HS.510172	3097	HIVEP2	Human immunodeficiency virus type I enhancer binding protein 2	1.1	1.1	4.9	1.6	0.9	1.1	1.3	1.1	1.2	1.2	5.7	3.0	*IR*
HS.437075	9586	CREB5	CAMP responsive element binding protein 5	0.9	1.1	3.5	0.9	1.1	0.8	1.9	1.3	1.1	1.6	4.6	2.8	*IR*
HS.326035	1958	EGR1	Early growth response 1	26.0	22.6	1.9	0.8	18.4	9.8	0.5	0.8	19.7	5.3	1.1	1.1	*GA*
HS.1395	1959	EGR2	Early growth response 2	10.6	21.1	1.2	1.4	7.0	4.6	1.9	0.5	6.5	3.2	2.6	0.6	*GA*
HS.25647	2353	FOS	V-fos FBJ murine osteosarcoma viral oncogene homol.	32.0	13.9	1.0	1.1	42.2	4.3	0.9	0.8	90.5	13.0	1.3	1.1	*GA*
HS.376206	9314	KLF4	Kruppel-like factor 4 (gut)	5.7	12.1	2.8	2.1	4.9	6.1	1.7	1.9	1.7	1.6	2.6	1.5	*GA*
HS.534052	7538	ZFP36	Zinc finger protein 36, C3H type, homolog (Mouse)	10.6	7.5	2.5	1.5	6.1	1.4	0.2	0.4	26.0	6.5	3.2	0.9	*GA*
HS.171695	1843	DUSP1	Dual specificity phosphatase 1	11.3	6.1	4.0	0.6	2.8	0.6	0.7	0.6	5.3	2.5	1.1	0.3	*GA*
HS.171825	8553	BHLHB2	Basic helix-loop-helix domain containing, class B, 2	3.0	5.7	2.3	1.1	2.5	3.2	1.6	1.0	2.8	6.1	1.4	1.5	*GA*
HS.517617	23764	MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog f (Avian)	2.3	4.9	2.8	2.3	2.1	2.8	1.1	1.4	1.9	4.3	1.9	2.5	*GA*
HS.25292	3726	JUNB	Jun B proto-oncogene	3.0	4.6	1.6	1.0	3.5	1.9	0.7	0.8	13.0	13.0	4.9	2.6	*GA*
HS.2128	1847	DUSP5	Dual specificity phosphatase 5	1.6	3.7	2.8	1.1	1.5	2.0	1.1	0.7	2.8	4.6	1.4	1.5	*GA*
HS.78944	5997	RGS2	Regulator of G-protein signalling 2, 24kda	1.6	3.5	2.5	1.1	1.6	2.6	1.2	0.9	0.7	0.7	2.6	1.4	*GA*
HS.435001	7071	KLF10	Kruppel-like factor 10	1.7	3.2	0.7	0.9	3.5	2.1	1.4	1.4	9.2	1.1	1.4	0.7	*GA*

Supplemental Table 2: PCR primers 5'-3'

Primer	Sequence
ATF3-forward	GTGCCGAAACAAGAAGAAGG
ATF3-reverse	TCTGGAGTCCTCCCATTCTG
beta-2-microglobulin-forward	GATGAGTATGCCTGCCGTGTG
beta-2-microglobulin-reverse	CAATCCAAATGCGGCATCT
beta-actin-forward	GTGATGGTGGGCATGGGTCA
beta-actin-reverse	TTAATGTCACGCACGATTTCCC
BHLHB2-forward	GGCATAGCACGGTAGTGGTT
BHLHB2-reverse	TCAGACCTTGGTTTGGTTCC
CCRL1-forward	CTTGGTTGCAGTGGTGCTTA
CCRL1-reverse	AGTGGTCCTGGGTACCCTTC
CNKSR3-forward	GACTGGTGGCATTGTTCCTT
CNKSR3-reverse	GCCACGTTATTGCAAAGTCA
CREM-forward	GCCCTTTCTACCATCTCACG
CREM-reverse	GGACAGCTCCAGACCACCTA
CXCL2-forward	CTCAAGAATGGGCAGAAAGC
CXCL2-reverse	CTTCAGGAACAGCCACCAAT
DNAJB9-forward	AAAATAAGAGCCCGGATGCT
DNAJB9-reverse	TGACTGCTCAAAAGAACTTCCA
DSCR1_E4-forward	TAGCTCCCTGATTGCCTGT
DSCR1_E5-reverse	GGAGAAGGGGTTGCTGAAGT
EGR1-forward	AGCCCTACGAGCACCTGAC
EGR1-reverse	TGGGTTGGTCATGCTCACTA
EGR2-forward	AGTTGGGTCTCCAGGTTGTG
EGR2-reverse	AGCAAAGCTGCTGGGATATG
EGR3-forward	GACAATCTGTACCCCGAGGA
EGR3-reverse	TCCCAAGTAGGTCACGGTCT
EGR4-forward	ATGAGAAGAAACGGCACAGC
EGR4-reverse	GCTCAGAGAGAAGCGAAGGA
F3-forward	CCGAACAGTTAACCGGAAGA
F3-reverse	TCAGTGGGGAGTTCTCCTTC
FOSB-forward	CGTGCTGCATGAAAAACATT
FOSB-reverse	CGCACACACACACATCCATA
HLX1-forward	CTCCAACCTGCAGAGGAAAG
HLX1-reverse	GGTTCTGGAACCACACCTTC
IGFBP3-forward	GCTACAGCATGCAGAGCAAG
IGFBP3-reverse	CTGCTGGTCATGTCCTTGG
IL8-forward	CTCTTGGCAGCCTTCCTGATT
IL8-reverse	TATGCACTGACATCTAAGTTCTTTAGCA
ITGAV-forward	ACCAGAGCCTGCATCAAAAT
ITGAV-reverse	CAAGTTGCATCTGGGGAACT
JunB-forward	CCTTCCACCTCGACGTTTAC
JunB-reverse	TTCCACAGTACGGTGCAGAG
KCNJ2-forward	GGGTCTTGGGAATTCTGGTT
KCNJ2-reverse	TGGGAGCCTTGTGGTTCTAC
KLF4-forward	GCGGCAAAACCTACACAAAG
KLF4-reverse	CCCCGTGTGTTTACGGTAGT
MEF2C-forward	CATAACATGCCACCATCTGC
MEF2C-reverse	CGTGTGTTGTGGGTATCTCG
MYCN-forward	CGCAAAAGCCACCTCTCATTA

MYCN-reverse	TCCAGCAGATGCCACATAAGG
NDRG1-forward	CTCGCTGAGGCCTTCAAGTA
NDRG1-reverse	GGGTGCCATCCAGAGAAGT
NEDD9-forward	CAGAAATTCAGG GAGCTGGA
NEDD9-reverse	GCACGTGGACAAGTTTTCTG
NFKBIZ-forward	ACTCGGAACTTGGAGAACGA
NFKBIZ-reverse	AATACGGTGGAGCTCTCTGC
NR4A2-forward	TTTCTGCCTTCTCCTGCATT
NR4A2-reverse	GTGGCACCAAGTCTTCCAAT
PER1-forward	AGCCTCGGTTTTCTGAGGAC
PER1-reverse	CCAGTCCATCCAGCTCTGA
PTGS-forward	ATCACAGGCTTCCATTGACC
PTGS-reverse	CAGGATACAGCTCCACAGCA
SELE-forward	CGCTGTAAAATCTTGGCACA
SELE-reverse	CTGTGGGCATTCAACATCTG
STC1-forward	AGCTGAATGTGTGCAGCATC
STC1-reverse	ATCACATTCCAGCAGGCTTC
THBD-forward	CGGGTTGTGTGTCTGTTCAC
THBD-reverse	AGCCCTCCATGCATCTCATA
VCAM-forward	AAAAGCGGAGACAGGAGACA
VCAM-reverse	CCCTTCATGTTGGCTTTTCT

4.2 Signals and genes induced by angiogenic growth factors in comparison to inflammatory cytokines in endothelial cells.

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Signals and genes induced by angiogenic growth factors in comparison to inflammatory cytokines in endothelial cells

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Abstract. The evaluation of signaling pathways leading to gene induction by VEGF-A and IL-1 in endothelial cells supports the importance of the NF-κB pathway for the IL-1-induced gene repertoire, whereas VEGF-A is a strong and preferential trigger of signals via PLC-γ. This leads (i) via Ca^{++} to the activation of calcineurin and NFAT and (ii) via PKC and the MEK/ERK MAPK pathway to the upregulation of EGR-1. Part of the VEGF-triggered gene induction depends on a cooperation of the transcription factors NFAT and EGR-1. Gene activation via PLC-γ provides VEGF with the potency to induce a wide spectrum of genes including many also upregulated by IL-1. A gene upregulated by VEGF and IL-1 is the DSCR-1 gene, which encodes an inhibitor of calcineurin. DSCR1 is induced by NFAT or NF-κB and limits Ca^{++} signaling in a negative feed-back loop. Similarly, NAB2, a corepressor of EGR-1, is induced by EGR-1 and limits EGR-1 effects. Adenoviral overexpression of DSCR1 or NAB2 inhibited part of VEGF-induced gene expression and reduced sprouting in angiogenesis models.

1. Introduction

The endothelium-specific growth factors important for vasculogenesis and angiogenesis include five members of the VEGF family and four members of the angiopoietin family [23]. They exert their specific activities through binding to three forms of VEGF receptors and two forms of Tie receptors. VEGF-A signaling mainly via VEGFR-2 (VEGF receptor-2) mediates the major growth and differentiation activities for vascular endothelial and progenitor cells and is the primary factor initiating physiological sprouting angiogenesis [4]. VEGF-C, based on its ability to bind to the lymphatic-specific VEGFR-3, is important for the formation of the lymphatic system [1]. PlGF (placenta growth factor) and VEGF-B, which bind to VEGFR-1, and VEGF-D binding to VEGFR-2 and -3 are the remaining isoforms of the VEGF family. They appear to have more subtile roles in adult vascular remodeling, coronary vascularization or the lymphatic system, respectively. Ang-1 by binding to the Tie-2 receptor and potentially co-activating Tie-1 [19] has a role in vascular remodeling events, probably optimizing the manner in which endothelial cells integrate with supporting cells. Ang-2 on the other hand appears to antagonize Ang-1 action by blocking its binding to Tie-2 [5,23].

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The VEGF and Tie receptors are transmembrane proteins with intrinsic tyrosine kinase activity in their cytoplasmic domains. It is still largely unclear to what extent the multitude of different tyrosines in the cytoplasmic tails (the VEGFR-2 contains 19 tyrosine residues) are differentially phosphorylated, bind different SH-2 domain-containing proteins and thus lead to the activation of receptor-specific intracellular signaling and gene induction pattern. It seems, that in addition to differences in the temporal and spatial expression of the various receptors, a receptor-specific signaling pattern is responsible for the differential effects of the various growth factors and receptors which govern the differentiation, growth, tube formation and maturation aspects involved in new vessel formation.

Endothelial cells express further a number of receptors found on many different cell types, which can either synergize or interfere with signals of the endothelial-specific receptors. Among those are the bFGF receptor and members of the PDGF and EGF receptor families [2,24]. There is further considerable evidence that receptors for inflammatory cytokines play important roles and participate in the cross-regulation of inflammation and angiogenesis in many pathologies [10,18]. Whereas the main angiogenic factors as well as PDGF and EGF mediate their effects via tyrosine kinase receptors, binding of inflammatory cytokines to their receptors generates signals by recruiting cytoplasmic adaptor molecules which link to the IκB-kinase complex. The IL-1 receptor recruits first the adaptor protein MyD88 to the cytoplasmic part of the IL-1 receptor [16] and MyD88 bridges via IRAK and TRAF6 to the protein kinases TAK-1 and NIK and these activate the IκB-kinase complex. When IKK-2 (IκB kinase-2) in the complex is activated it phosphorylates IκB leading to ubiquitinylation and proteasomal degradation of IκB which releases the NFκB subunits for nuclear transfer [6].

In an attempt to define the specificities in signaling and gene induction of the different receptors present on endothelial cells, which contribute to the angiogenic and inflammatory response, we have so far investigated in detail the signal transduction pathways initiating at VEGFR-2 and compared them to the signals of the EGFR and the IL-1 receptor. This was done by directly studying induced signaling pathways and how they interact to trigger the induction of selected genes. Initially, we have used the tissue factor (TF) gene as a model of a gene induced by VEGF and IL-1. Then we have investigated by microarray analyses the complete gene repertoires induced by the same receptors and to what extent they overlap or lead to the induction of distinct genes. Furthermore, we have tested whether the adenoviral overexpression of natural feed-back inhibitors detected in our screens, such as DSCR-1 and NAB2, could be used to inhibit VEGF-triggered gene expression and angiogenesis models.

2. Tissue factor as a model for an IL-1 and VEGF-regulated gene

To determine specificities of VEGF-mediated signaling and gene induction we have investigated the downstream signaling of VEGFR-2 and the gene repertoire induced in comparison to another, non-endothelial specific growth factor receptor, the EGFR, and the inflammatory cytokines TNF-α and IL-1 [11–13]. Our initial data had shown that EGR-1 is an important transcription factor for VEGF-mediated gene induction in endothelial cells [11]. We have then analyzed signals at the MAP kinase level originating from the VEGFR-2 and leading to EGR-1. The obtained results showed that in endothelial cells VEGF-A mainly activates ERK1/2 and p38, but only trace amounts of JNK. The upregulation of EGR-1 by VEGF could be completely blocked by a specific MEK inhibitor, but not by a p38 inhibitor, demonstrating the upregulation of EGR-1 via the MEK/ERK MAP-kinase cascade [13]. Furthermore, EGR-1 upregulation was blocked by a PKC inhibitor suggesting that PKC was involved in VEGF-mediated MEK/ERK and EGR-1 induction. This is in contrast to EGF-induced EGR-1, which cannot be inhibited by PKC inhibitors.

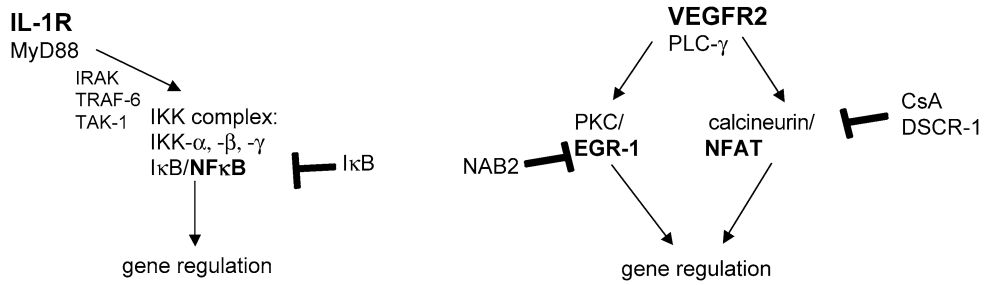

Fig. 1. Signal tranduction by VEGF-A/VEGFR2 in comparison to IL-1/IL-1R. Major characteristic pathways activated by VEGF-A/VEGFR2 and the IL-1 receptor in endothelial cells are shown. VEGF-A couples *via* Tyr1175 to PLC-γ [20,21] activating the Ca^{++}/calcineurin and PKC/MAPK pathways. This leads to the induction of the transcription factors NFAT and EGR-1, which are important for part of the VEGF-induced gene repertoire and the angiogenic/proliferative response [8,11,13, 14,25]. The IL-1 receptor induces genes to a large extent via NFκB [17,22]. A significant fraction of the NF-κB-induced genes appears to be also upregulated by VEGF-A-induced NFAT. VEGF-A does not trigger NF-κB activation to a significant extent [13]. Adenoviral overexpression of natural feed-back inhibitors inhibits gene induction via these pathways, e.g. IκB interferes with NFκB, NAB2 with EGR-1 and DSCR1 with calcineurin/NFAT activation. Alternatively, calcineurin/NFAT induction can be blocked by cyclosporin A (CsA).

The TF gene promoter was used in these studies as we have shown that TF is an example of a gene/protein upregulated not only by inflammatory cytokines [15], but also by VEGF-A in endothelial cells [11]. Therefore TF seems to be a valid model to delineate principal signaling pathways leading to gene activation during the angiogenic and inflammatory activation of endothelial cells. Whereas we had previously shown that NFκB activation is essential for TF gene induction by TNF-α [22] and a specific p65/c-Rel heterodimer regulates the TF promoter [15], we found NFκB not activated by VEGF-A [11,13]. However, the obtained data displayed that VEGF-A is a strong and preferential trigger of signals via PLC-γ, PKC and Ca^{++}. On the one hand PKC-α and PKC-ε are activated and lead to the induction of the MEK/ERK MAP-kinase pathway and consecutively to the upregulation of EGR-1. The Ca^{++} signals, on the other hand, activate calcineurin and the transcription factor NFAT (see Fig. 1). NFAT cooperatively with EGR-1 activates the TF gene in endothelial cells [25]. NFAT has been previously shown to bind to a site closely overlapping with the NFκB binding site in the TF promoter [3]. Based on these data it appeared that full transcriptional response of the TF and potentially other genes in response to VEGF requires EGR-1 and NFAT.

3. VEGF-regulated gene repertoire in comparison to EGF and IL1

In the next step we have investigated this more closely by Affymetrix microarray analysis of the genes induced by VEGF-A in endothelial cells in comparison to the genes induced by EGF and IL-1 (B. Schweighofer and E. Hofer, in preparation). The obtained data show that VEGF-A induces a large spectrum of more than 100 genes. It is a characteristic feature of the VEGF-A-induced gene repertoire that about 40% of these genes are also upregulated by IL-1. In contrast, this fraction of genes cannot be upregulated by EGF. It seems that gene activation via PLC-γ, PKC and calcineurin provides VEGF-A with the competence to regulate many genes in common with IL-1. The EGFR, which can not induce the same genes, does not trigger PLC-γ and NFAT in endothelial cells, but rather induces the MAPK pathway solely via Ras [25]. Based on these data it is possible that VEGFR-2 signaling and gene regulation includes an inflammatory component, which at least in part is caused by the upregulation of a group of genes with NFAT and NF-κB binding sites in their promoters, IL-1 uses NF-κB and VEGF

NFAT for their upregulation. Furthermore, about 10% of the genes are specifically regulated by VEGF and not by EGF or IL-1 and we propose that these might be important for the strong angiogenic properties of the factor. One example of a specifically VEGF and not EGF or IL-1 regulated gene is HLX1, a diverged human homeobox gene, which we have chosen for further analysis (J. Schultes and E. Hofer, in preparation).

4. Inhibition of angiogenesis by modulating these signals/genes

Another aspect of our work deals with the identification of natural feed-back inhibitors of endothelial cell activation, which could be used to inhibit angiogenic gene induction. As a matter of fact the groups of Aird and Gerber [7,14] and our laboratory (B. Schweighofer and E. Hofer, in preparation) find that among the genes most strongly upregulated by VEGF is the DSCR1 gene, which encodes an inhibitor of calcineurin. It is induced by NFAT likely to shut down calcineurin and consecutive NFAT activation in a negative feed-back loop. Another example is NAB2, a specific corepressor of EGR-1, which is induced by EGR-1 and limits EGR-1 effects [8]. As suggested by the cooperative gene regulation observed for EGR-1 and NFAT, adenoviral overexpression of DSCR1 and NAB2 inhibited VEGF-induced expression of genes with potential importance for angiogenesis reflected in reduced sprouting and tubule formation in angiogenesis models [8,14].

In contrast to the positive effects on proliferation and angiogenesis of VEGF-A induced transient expression of EGR-1, sustained adenoviral overexpression of EGR-1 itself led to preferential strong induction of negative feed-back mechanisms including upregulation of transcriptional inhibitors such as NAB2 as well as anti-angiogenic, anti-proliferative and pro-apoptotic genes. EGR-1 expressing adenoviruses applied to endothelial cells inhibited not only angiogenesis in cellular and animal models, but furthermore inhibited tumor growth in a murine fibrosarcoma model [9]. This may serve as an example, that the modulation of natural inhibitive feed-back pathways could be exploited for inhibition of angiogenesis.

5. Conclusions

Whereas NF-κB is the essential transcription factor in the inflammatory response mediated by IL-1, EGR-1 and NFAT are important transcription factors in VEGF-A/VEGFR2-mediated gene regulation. Both factors seem to cooperatively activate transcription of part of the VEGF-induced gene repertoire. EGR-1 is upregulated by VEGF via PLC-γ, PKCα/ε and the MEK/ERK MAP kinase pathway, NFAT is activated via PLC-γ, Ca^{++} and calcineurin. In contrast to VEGFR-2, some tyrosine kinase receptors, such as the EGFR, can not activate NFAT. VEGF via VEGFR-2 is competent to induce a large gene repertoire, 40% of which overlaps with IL1 induced genes. It appears that at least part of the genes induced by VEGF in common with IL-1 are regulated by VEGF via NFAT and by IL-1 via NF-κB. This property of VEGF to induce a large spectrum of genes including genes also regulated by inflammatory mediators and a smaller number of VEGF-specific genes could be important for the strong angiogenic properties of the factor. Modulation of natural inhibitory feed-back loops leading via EGR-1 to NAB2 and via calcineurin/NFAT to DSCR1 by adenoviral overexpression of the respective inhibitors leads to inhibition of in vitro and in vivo angiogenesis models.

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5. Contributions to Publications as Coauthor

5.1 The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation

Bernhard Schweighofer, Julia Testori, Caterina Sturtzel, Susanne Sattler, Herbert Mayer, Oswald Wagner, Martin Bilban, Erhard Hofer.
Thromb Haemost. 2009;102:544-554.

This publication shows the transcription response induced by VEGF-A in comparison to the gene repertoire induced by the growth factor EGF and the inflammatory cytokine IL-1. VEGF-A induced a large repertoire of genes and it overlapped to 60% with the inflammatory repertoire induced by IL-1. About 20% of VEGF-upregulated genes were selectively induced by VEGF including genes such as NR4A2, EGR3, MEF2C and HLX, forming a potential pool of preferentially angiogenesis-related genes. This work further demonstrates that the VEGF-induction of the majority of genes is mediated by VEGF receptor-2 and not VEGF receptor-1. A small cluster of genes was induced by all three factors, VEGF-A, IL-1 and EGF, and constitutes general immediate early response genes presumably activated by a wide range of factors. In addition, we demonstrated that a significant portion of the VEGF-cluster and VEGF-/IL-1-cluster genes were activated through the calcineurin/NFAT pathway. The finding that VEGF is able to induce a large portion of genes with an inflammatory component, was also confirmed by the ability of VEGF to induce leukocyte adhesion.

My contribution to this work was primarily related to a more precise analysis of the VEGF-specific gene cluster leading to the identification and selection of HLX as a potential relevant gene for angiogenesis. It was our goal to select genes which would fulfill the requirement to be inducible by more than one highly angiogenic factor, but not by inflammatory cytokines or growth factors which are not primary inducers of angiogenesis. For this reason I applied secondary selection criteria analyzing the inducibility of the VEGF cluster genes by bFGF, the second major inducer of angiogenesis. Among all genes of the cluster HLX turned out to be similarly induced by VEGF and bFGF, whereas most of the other genes were much

less inducible by bFGF. These data are included in this publication and have led to the further analysis of HLX.

5.2 Signals and genes induced by angiogenic growth factors in comparison to inflammatory cytokines in endothelial cells

Bernhard Schweighofer, Julia Schultes, Jiri Pomyje and Erhard Hofer.
Clin Hemorheol Microcirc. 2007;37:57-62.

This publication summarizes results of several manuscripts on the signaling pathways induced by VEGF-A and IL-1 in endothelial cells. In this regard with the tissue factor gene as a model, it was shown that VEGF-A binding to VEGF receptor-2 leads, via PLC-γ activation, to the induction of calcineurin/NFAT on the one hand, and to induction of PKC and downstream MEK/ERK/EGR-1 on the other hand. Gene upregulation triggered by VEGF is to a significant extent caused by a cooperation of the two induced transcription factors NFAT and EGR-1. Furthermore, within a negative feed-back loop NFAT upregulates DSCR1, an inhibitor of calcineurin, and EGR-1 upregulates its own corepressor NAB2. By adenoviral overexpression of the natural feedback inhibitors DSCR1 and NAB2 VEGF-mediated gene expression and angiogenic sprouting activity could be reduced.

In contrast to VEGF, IL-1 signals primarily through the NF-κB pathway activating NF-κB-responsive promoters. As shown by Affymetrix microarray analysis, VEGF induces an astonishingly large repertoire of inflammatory genes overlapping with the IL-1 repertoire. This is presumably caused by the presence of partially overlapping NFAT and NF-κB binding in these inflammatory genes. VEGF uses NFAT and IL-1 NF-κB for the regulation of these genes.

My contribution to this publication was an analysis of the genes induced by VEGF as well as IL-1.

6. Discussion

Comparison of gene induction by the angiogenic growth factor VEGF-A and the inflammatory cytokine IL-1 in endothelial cells

VEGF-A is the major trigger of vasculogenesis as well as angiogenesis and signaling mainly via VEGF receptor 2 induces a gene repertoire which is important for its specific function during endothelial sprouting and vessel formation (Adams and Alitalo, 2007; Lohela et al., 2009). Endothelial cells further express other signaling receptors that are found widely on other cell types such as the epidermal growth factor (EGF) receptor. EGF has a more general role stimulating proliferation and growth (Citri and Yarden, 2006; Sini et al., 2005). During wound healing and in tumors there is a cross-regulation between inflammation and angiogenesis (Pober and Sessa, 2007). Inflammatory cytokines such as interleukin (IL)-1 induce a response program in endothelial cells upregulating inflammatory genes like cytokines and adhesion molecules important for immune cell recruitment and tissue repair. The signaling pathways leading to gene induction induced by VEGF-A and IL-1 in endothelial cells differ, although they are able to induce many genes in common. The IL-1 receptor recruits the adapter protein MyD88 that binds to the cytoplasmic domain of the IL-1 receptor and attracts the proteins IRAK and TRAF6. Those proteins mediate the signal through TAK-1 and NIK activating the IκB-kinase complex. The IKK-2 (IκB-kinase-2) is activated and phosphorylates IκB, which is subsequently ubiquitinylated and degraded by the proteasome. Thereby the NFκB subunits are released and then translocate to the nucleus activating transcription (Schweighofer et al., 2007).

In contrast to the IL-1 receptor leading to the activation and translocation of NFκB, VEGFR2 has an intrinsic tyrosine kinase activity in its cytoplasmic domain and via SH2 domains attracts PLC-γ. PLC-γ activates the Ca^{++}/calcineurin and PKC/MAPK pathways, which leads to the induction of the transcription factors NFAT and EGR-1. The tissue factor (TF) gene is an example of an IL-1 and VEGF-regulated gene and we could show that TF is upregulated by VEGF through activation of PKC and Ca^{++}. PKC induces the MEK/ERK MAP-kinase pathway leading to upregulation of EGR-1.

Ca^{++} activates calcineurin and the transcription factor NFAT. Both transcription factors cooperatively activate TF transcription. IL-1 on the other hand upregulates TF through NFκB activation (Schweighofer et al., 2007).

To investigate the complete gene repertoire induced by VEGF-A, IL-1 and EGF in endothelial cells we used microarray analysis. We found that VEGF induces a large spectrum of genes, which is five-fold larger in comparison to the repertoire induced by EGF. Moreover, it overlapped to 60% with the inflammatory repertoire of IL-1 (Figure 2 in (Schweighofer et al., 2009)). This shows that the VEGF gene repertoire has a large inflammatory component including genes regulating leukocyte adhesion and coagulation during tissue repair, which is likely important for the crossregulation between angiogenesis and inflammation occurring during repair processes. About 20% of the VEGF-induced genes were to a similar degree induced by EGF as well as IL-1. This cluster of genes comprises to a large extent immediate early genes which reach peak levels between 30 and 60 min and are presumably part of a general response triggered by a wide variety of factors (Table 1 of (Schweighofer et al., 2009)).

Cluster of VEGF-selective genes

Since VEGF exerts a unique response in endothelial cells, it was anticipated that it would also induce a specific gene expression pattern. Indeed, 20% of the VEGF-induced genes were VEGF-specific and were not induced by EGF or IL-1. To define whether the upregulation of these genes is mediated through VEGFR1 or VEGFR2 we used two growth factors, VEGF-E and PlGF, binding selectively to VEGFR2 and VEGFR1, respectively. Only VEGF-E induced the VEGF-specific genes to the same extent as VEGF-A, therefore upregulation of those genes has to be mediated through VEGFR2 (Figure 3 in (Schweighofer et al., 2009)). The two most strongly induced genes of the VEGF-specific cluster are the nuclear receptor NR4A2 (Nurr1) and the transcription factor EGR-3. NR4A2 has been described to be involved in nerve cell development and to regulate neuropilin, which could also be a function in endothelial cells (Hermanson et al., 2006; Zetterstrom et al., 1997). EGR3 was previously shown to be important as a transcriptional regulator in T lymphocytes and nerve cells (Gallitano-Mendel et al., 2007; Safford et al., 2005). This can now be extended to endothelial cells as it was reported that both NR4A2 and EGR3 are engaged in

endothelial proliferation, migration and tubulogenesis mediated by VEGF *in vitro* (Liu et al., 2008).

In addition, the transcription factors HLX, a homeobox gene, MEF2C, a MADS box factor and PER1, a period family member were selectively upregulated by VEGF. MEF2C was previously reported to be required during vascular development (Lin et al., 1998) and PER1 was shown to have a role in nerve cells (Shearman et al., 1997) (Figure 3 in (Schweighofer et al., 2009)). It is intriguing that several of the VEGF-specific genes have been described to be important for nerve cell development and function, which may indicate certain functional similarities of nerve cells with endothelial cells.

In regard to HLX, we found this factor to be induced not only by VEGF, but also by another growth factor important for angiogenesis, and that is bFGF (Presta et al., 2005; Schweighofer et al., 2009). Based on its inducibility by the two major angiogenic factors VEGF-A and bFGF, and the inability of EGF or IL-1 to induce HLX, we proposed that HLX would be an excellent candidate for an interesting gene potentially mediating a specific and important function of VEGF-A and bFGF related to angiogenesis or vascular remodeling.

HLX (H2.0-like homeobox protein) is a diverged homeobox transcription factor, which has so far been shown to be expressed in T lymphocytes, NK cells, placental endothelial cells, trophoblasts and HUVEC (Becknell et al., 2007; Mullen et al., 2002; Murthi et al., 2007; Rajaraman et al., 2010; Rajaraman et al., 2007). However, it has previously not been implicated in angiogenesis or vascular remodeling. Homeobox transcription factors are important regulators during developmental processes including pattern formation and organogenesis and several are involved in the formation of the embryonic vascular system and vascular remodeling in the adult. Moreover, they have been reported to influence many pathologies including atherosclerosis, Alzheimer's disease and tumor angiogenesis (Abate-Shen, 2002; Douville and Wigle, 2007; Gorski and Walsh, 2003).

Upregulation of genes for cell guidance molecules by the homeobox transcription factor HLX

To get initial information on the potential function of HLX in endothelial cells we first investigated downstream target genes of the homeobox transcription factor. To this end we performed microarray analysis of endothelial cells overexpressing HLX (supplemental Table 1 of (Testori et al., 2011)). Interestingly the most strongly upregulated genes included genes for repulsive guidance cues involving UNC5B, PLXNA1 and SEMA3G and a transcriptional repressor HES1 (Table 1 of (Testori et al., 2011)). Some of these genes have been implicated in the guidance of the axonal growth cone and of the endothelial sprout, which have recently been shown to be modulated by the same families of guidance proteins. Similar to axonal growth cones, endothelial sprouts need to navigate through tissue sensing and exploring the environment with their filopodia to establish a stereotyped vascular branching pattern (Adams and Eichmann, 2010).

Uncoordinated-5B (UNC5B) belongs to a family of four transmembrane receptors, UNC5A - D. UNC5B was shown to be selectively expressed in the vasculature, strongly in growing capillaries and preferentially in tip cells compared to stalk cells during sprouting angiogenesis. The knockdown of the gene in mice leads to excessive capillary branching and an increase in expression of tip cell filopodia indicating a repulsive function in the growing endothelial sprout (Adams and Eichmann, 2010; Larrivee et al., 2009). Upon binding of netrin-1, a secreted matrix-binding protein, to UNC5B on tip cells, the searching filopodia retract and thereby capillary branching is negatively regulated and sprouting is inhibited.

Semaphorins and plexins belong to another large protein family important for vessel guidance. Semaphorins can be either transmembrane or secreted proteins and contain the highly-conserved Sema domain. SEMA3G is a member of the secreted class III semaphorins that bind to neuropilins which occur in a complex with plexin-A1-4. The signal is transduced by the plexin molecule (Gaur et al., 2009). SEMA3G has been recently found to be expressed primarily in the vasculature (Kutschera et al., 2010). Generally class III semaphorins have been shown to be expressed by several cell types, including tumor cells, which can lead to inhibition of angiogenesis and tumor growth. Semaphorin signaling has been demonstrated to be important for

vascular patterning by mediating endothelial cell repulsion and restricting blood vessel growth (Adams and Eichmann, 2010).

The transcription repressor HES1 is a direct target gene of the Notch signaling pathway (Fischer and Gessler, 2007; Phng and Gerhardt, 2009). The Notch pathway is an important regulator of cell fate decisions and differentiation of various cell types and was recently defined to regulate the tip versus stalk cell phenotype in endothelial cells. By displaying Dll4, tip cells activate the Notch signaling pathway in neighboring cells, thereby inhibiting their sprouting and inducing their stalk cell phenotype. Disruption of the Notch signaling pathway leads to deregulated angiogenesis involving increased endothelial cell sprouting, ectopic branches and higher sprouting motility (Adams and Eichmann, 2010). Given that HLX upregulates HES1, it can be supposed that this affects the negative regulation of sprouting and inhibition of tip cell formation.

Induction of HLX and UNC5B by VEGF

Following the analysis of the microarray data of genes upregulated by HLX, we further defined the genes that would be most significantly involved in the functions of VEGF-induced HLX. We investigated the HLX-mediated upregulation of UNC5B, PLXNA1, SEMA3G and HES1 by real-time RT-PCR and confirmed that UNC5B was the most strongly upregulated gene with induction levels of several hundred fold (Figure 1 in (Testori et al., 2011)). The guidance receptor was also induced by VEGF starting after a few hours and reaching levels of several hundred fold after several days. In comparison to UNC5B, PLXNA1, SEMA3G and HES1 showed only very low inducibility by VEGF (Figure 2A in (Testori et al., 2011)). In addition, in the case of UNC5B the upregulation of the corresponding protein on the cell surface of endothelial cells could be shown (Figure 2C in (Testori et al., 2011)). The kinetics of UNC5B upregulation seemed to follow the upregulation of HLX protein, which increased over several hours and was more than 10-fold after 24h, showing nuclear accumulation (Figure 2B, 2D in (Testori et al., 2011)). Moreover, the downmodulation of HLX by using shRNA inhibited the VEGF-inducibility of UNC5B significantly over 80% (Figure 2E in (Testori et al., 2011)). This confirms that HLX mediates the VEGF-stimulated induction of UNC5B.

Effect of HLX on endothelial proliferation, migration and sprouting

Considering the fact that the described upregulated proteins exhibit a repressive and repulsive function we investigated whether HLX overexpression would mediate a corresponding biological effect in endothelial cells. Indeed, we observed a strong inhibition of migration (Figure 3B) and sprouting of endothelial cells. (Figure 4A, B). In contrast, the proliferation of cells overexpressing HLX was not affected and the cells showed no signs of apoptosis (Figure 3A, supplemental Figure 2, all in (Testori et al., 2011)).

Contribution of HLX-induced genes to the inhibition of sprouting

To determine which of the HLX-upregulated genes would mediate the observed inhibition of sprouting, we first used overexpression of UNC5B by a recombinant adenovirus. This definitely led to a strong inhibition of sprouting and reduction of sprout length (Figure 4C in (Testori et al., 2011)), which showed that UNC5B has the potential to mediate the inhibitive effects of HLX. Conversely, the downmodulation of UNC5B through a corresponding shRNA in HLX-overexpressing cells was able to reduce the inhibition of sprouting by HLX. Sprouting was restored to the same level as observed for control adenovirus infected cells (Figure 4D in (Testori et al., 2011)). We further tested whether some of the other downstream target genes, SEMA3G or PLXNA1, would also cause part of HLX-induced inhibition of sprouting. The downmodulation with the respective shRNAs showed that in addition to shRNA for UNC5B also SEMA3G shRNA reduced the sprouting inhibition of HLX. However, knockdown of PLXNA1 had no effect (additional Figure 8A). This confirms that the combined upregulation of SEMA3G and UNC5B can cause a significant part of sprouting inhibition observed for HLX. Surprisingly, in control adenovirus infected samples the downmodulation of all three mRNA caused an increase in sprouting, showing about 3-fold higher sprout lengths, but this effect was stronger for UNC5B and SEMA3G than for PLXNA1 (additional Figure 8A, Figure 4D in (Testori et al., 2011)). This indicates that endogenous expression of UNC5B and SEMA3G and to some degree also of PLNXA1 suppresses basal endothelial cell sprouting activity.

It has been reported that UNC5B is selectively expressed in growing capillaries and becomes re-expressed during sprouting angiogenesis (Larrivee et al., 2007). Therefore a potential hypothesis for the function of the VEGF-triggered and HLX-

mediated regulation of UNC5B is that the repulsive UNC5B is necessary to counterbalance the activation of attractive guidance cues to prevent excessive sprouting activity. It is possible that the expression of UNC5B is necessary for the growing sprout to be able to react to repulsive guidance cues to achieve directed sprouting in a tissue environment by reducing branching and preventing excessive sprout initiation. Our findings that the downmodulation of basal and VEGF-induced UNC5B expression increased the number of sprouts and thus sprout initiation, support the above mentioned role for UNC5B (supplemental Figure 4B in (Testori et al., 2011)).

Influence of hypoxia on VEGF induction and endothelial sprouting

A second non-exclusive hypothesis might be that UNC5B is a natural feed-back mechanism that serves to adapt sprouting to physiological needs and inhibits inappropriate sprouting. First we hypothesized that UNC5B expression might be regulated by a hypoxic gradient to inhibit angiogenic sprouting under restored normoxic conditions. Recently it was shown that hypoxia can induce the up- and downregulation of several hundred genes. This is mediated by the transcription factor HIF-1, which is a heterodimeric protein composed of HIF-1α and the constitutively expressed HIF-1β. Hypoxia regulated cytokines/growth factors, receptors, signaling proteins and transcription factors accounted for the largest group of genes (Manalo et al., 2005). Consequently, we analyzed whether hypoxia would influence the VEGF-induced upregulation of HLX and UNC5B. Under hypoxic conditions, the VEGF induction of HLX was indeed strongly reduced and the upregulation of UNC5B by VEGF was completely abrogated (Figure 5A). Moreover, we found that sprouting is increased additively to VEGF induction under hypoxic conditions (Figure 5B, both in (Testori et al., 2011)). The VEGF-induced sprouting under hypoxic conditions could still be inhibited by HLX overexpression, but the relative inhibition was less pronounced when compared to sprouting inhibition under normoxia. This enhanced sprouting activity under hypoxic conditions might be due to the inability to express UNC5B. Furthermore, the HLX-mediated UNC5B induction by VEGF may be important to prevent inappropriate sprouting under restored normoxic conditions and adjust sprouting activity to a hypoxic gradient.

Overexpression of HLX in an *in vivo* endothelial spheroid xenografting assay

In order to assess whether HLX would mediate a similar inhibitory effect on endothelial cells *in vivo* and reduce sprouting and vessel formation, we used an endothelial xenografting assay. HUVEC transfected with HLX were implanted together with a Matrigel/fibrinogen matrix and growth factors below the skin of SCID mice. The human endothelial cells then normally establish a perfused vascular network, which is connected to murine invaded vessels (supplemental Figure 5 in (Testori et al., 2011)). In accordance with the *in vitro* findings, HLX overexpression significantly inhibited vessel formation and led to a strong reduction of vessel density (Figure 6 in (Testori et al., 2011)). This shows that HLX exerts a similar repulsive and inhibitive function on angiogenesis *in vivo*.

Downmodulation of HLX

We further investigated the downmodulation of HLX by shRNA and its effect on sprouting activity. Surprisingly, the reduction of HLX expression also led to an inhibition of sprouting activity, although the sprouting reduction (additional Figure 9A, B) seemed to be less pronounced than the inhibition achieved by overexpression of HLX. The inhibition was reduced in a dilution series of the shRNA in a dose-dependent manner. Consequently, a minimal expression level of HLX seems to be essential for regulated, inducible sprouting activity. This could be caused by the HLX-mediated induction of a so far unidentified protein that would be essential for sprouting. There are candidates in our gene profiling data such as the HLX-induced water channel aquaporin-1, which has been reported to regulate endothelial cell migration and angiogenesis by enhancing lamellipodia dynamics (De Smet et al., 2009). The identification of proteins responsible for the shRNA HLX effect will however need additional experiments.

We then used the endothelial spheroid xenografting assay to assess whether the reduction of HLX expression levels by shRNA would lead to inhibition of vessel formation *in vivo*. Indeed downmodulation of HLX significantly reduced vessel formation and vessel density *in vivo.* This implicates that appropriate HLX expression levels are important for the capability of endothelial cells to sprout in a regulated manner (additional Figure 10).

Overexpression of HLX in an in vivo MDA breast cancer model

Since HLX overexpression led to an inhibition of sprouting and hence angiogenesis, we tested whether this would also have an influence on tumor growth. To this end, we used the MDA-MB-231 breast cancer model. The MDA-MB-231 cells were administered under the skin of SCID beige mice and tumors grown for 7 days. Then HLX encoding adenoviruses were injected into the growing tumors in a way that HLX expression was obtained throughout the tumor mass. This led to a strong reduction of tumor growth and a reduction of tumor volume (additional Figure 11). It is possible that this reduction is caused by infection of endothelial cells, which as described before, should prevent further vessel growth. Alternatively the infection of the tumor cells directly or indirectly might lead to the observed inhibition. Our results in this regard indicate that HLX has no direct effect on MDA-MB-231 proliferation, however, we detected an increase in expression of SEMA3G mRNA by the tumor cells. (additional Figure 12) This is in accordance with recent reports that SEMA3G can be secreted by several cell types including certain tumor types and has an inhibitory effect on tumor growth and angiogenesis (Gaur et al., 2009).

In summary, this study characterized a homeobox transcription factor, specifically induced by VEGF that mediates a genetic program resulting in the expression of several repressive and repulsive molecules including UNC5B, SEMA3G, PLXNA1 and HES1. We could show that HLX most prominently mediates the VEGF-induction of the repulsive guidance receptor UNC5B and that UNC5B is responsible for a significant part of the sprouting inhibition observed for HLX overexpression. In addition, we found a similar influence of HLX-induced SEMA3G on the sprouting inhibition achieved by HLX overexpression. Surprisingly, downmodulation of HLX also inhibited sprouting. This suggests that an appropriate HLX level is important for the appropriate regulation of sprouting angiogenesis. In regard to tumor angiogenesis we showed in a breast cancer model that HLX significantly inhibits tumor growth. Consequently our data propose that the homeobox protein HLX is a strong inhibitor of pathological angiogenesis and this could be exploited as a novel therapeutic principle to inhibit tumor angiogenesis and growth.

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Table of Figures

Abstract

We have evaluated the gene repertoire induced by VEGF-A, the main trigger and key regulator of angiogenesis. Genes regulated by VEGF-A in endothelial cells were compared to genes induced by the growth factor EGF and the inflammatory cytokine IL-1 to define genes specifically regulated by VEGF-A. The HLX gene, encoding a diverged homeobox transcription factor, was one of the genes specifically regulated by VEGF-A. We investigated the gene repertoire induced by HLX and its potential biological function. HLX strongly increased the transcripts for several repulsive cell guidance proteins including UNC5B, plexin A1 and semaphorin 3G. In addition, genes for transcriptional repressors such as HES1 were upregulated. In line with these findings, adenoviral overexpression of HLX inhibited endothelial cell migration, sprouting and vessel formation *in vitro* and *in vivo*, whereas proliferation was unaffected. This inhibition of sprouting was caused to a significant part by HLX-mediated upregulation of UNC5B as shown by shRNA-mediated downmodulation of the respective mRNA. VEGF-A stimulation of endothelial cells induced elevated levels of HLX over longer time periods resulting in especially high upregulation of UNC5B mRNA as well as an increase in cells displaying UNC5B at their surface. However, induction of HLX was strongly reduced and UNC5B upregulation completely abrogated when cells were exposed to hypoxic conditions. These data suggest that HLX may function to balance attractive with repulsive vessel guidance by upregulating UNC5B and to downmodulate sprouting under normoxic conditions.

Zusammenfassung

VEGF-A ist der wichtigste Auslöser und Schlüsselregulator der Angiogenese. Diese Arbeit untersucht das Genrepertoire, das durch VEGF-A in Endothelzellen induziert wird, im Vergleich zu den Genen, welche durch den Wachstumsfaktors EGF und das inflammatorische Zytokin IL-1 hochreguliert werden. Dieser Vergleich ermöglichte die Identifizierung von Genen, die spezifisch durch VEGF-A reguliert werden. Unter diesen Genen war das HLX-Gen, welches für einen divergierten Homeobox-Transkriptionsfaktor kodiert. Im Weiteren haben wir das von HLX induzierte Genrepertoire und die biologische Funktion von HLX untersucht. HLX erhöhte stark die Expression der Transkripte für verschiedene Proteine für abstoßende Zellführung, einschließlich UNC5B, Plexin A1 und Semaphorin 3G. Zusätzlich wurden Gene für transkriptionelle Repressoren wie HES1 positiv reguliert. In Übereinstimmung mit diesem Befund verhinderte die Überexpression von HLX die Migration von Endothelzellen und Wachstum und Neubildung von Blutgefäßen *in vitro* und *in vivo*. Hingegen wurde die Proliferation nicht beeinflusst. Diese Hemmung der Blutgefäßsprossung wurde zu einem Großteil durch die von HLX vermittelte Induktion von UNC5B mRNA verursacht. Dies konnte durch die verminderte Expression der UNC5B mRNA mittels shRNA gezeigt werden. Die Stimulierung von Endothelzellen mit VEGF-A über längere Zeiträume induzierte erhöhte Mengen von HLX, und das führte zu einer besonderen Induktion von UNC5B mRNA und auch zu einer Vermehrung der Zellen, die UNC5B an ihrer Oberfläche exprimieren. Allerdings wurde die Induktion von HLX stark reduziert und die UNC5B Hochregulation komplett blockiert, wenn die Zellen Hypoxie ausgesetzt waren. Diese Daten deuten darauf hin, dass HLX die anziehende mit der abstoßenden Führung der Blutgefäße durch die Induktion von UNC5B ausbalanciert und die Neubildung von Blutgefäßen unter Normoxie verhindert.

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