Modulation of Angiogenesis by Laminins and Heparan Sulfate

LARS JAKOBSSON
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Abstract

Blood vessels transport blood with essential nutrients and oxygen to the cells in our body. In a healthy adult, formation of new vessels (angiogenesis) occurs only in case of tissue repair and growth. Physiological angiogenesis requires precise regulation of multiple signaling components, a process which is deregulated in a number of pathological conditions, such as cancer. This thesis is focused on the role of laminins, heparan sulfate proteoglycans (HSPGs) and vascular endothelial growth factor (VEGF)-A in regulation of vascular development and angiogenesis. As a model, we have used embryonic stem cells that differentiate to form blood vessels in a manner faithfully recapitulating the in vivo processes.

We show that the basement membrane (BM) protein laminin-111 promotes maturation of endothelial cells in the presence of fibroblast growth factor-2, a known endothelial cell mitogen. However, embryonic stem cells are able to differentiate into endothelial cells also in the absence of laminin deposition in the vascular BM. Sprouting angiogenesis, induced by VEGF-A, is also not strictly dependent on laminin deposition. On the other hand, in the absence of laminins, vessels are enlarged. These data suggest an important role for laminins in regulation of the vessel diameter.

We also show that HSPGs serve as coreceptors for VEGF-A to regulate vascular development. The mode of presentation of HSPGs, in cis (on the endothelial cell) or in trans (on an adjacent cell such as pericytes), is critical in regulation of VEGF receptor-2 activation and stimulation of vascular development. Binding of VEGF-A to HSPGs in trans leads to accumulation of activated VEGFR-2 in endothelial cells and to prolonged signaling. This demonstrates a potential role for HSPGs in regulation of receptor trafficking and signaling kinetics, with possible implications also for other HS-binding ligand/receptor systems.

Keywords: Embryonic stem, heparan sulfate, laminin, basement membrane, VEGFR-2, VEGF, endothelial cell, signaling, angiogenesis, vasculogenesis, embryoid body

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No man is an island
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Abbreviations

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<tr>
<td>BM</td>
<td>Basement membrane</td>
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<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia responsive element</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>LM</td>
<td>Laminin</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulphotransferase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet-endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PlGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VPF</td>
<td>Vascular permeability factor</td>
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</table>
Introduction

All species within the animal kingdom depend on oxygen for development and survival. In vertebrates and higher organisms, the delivery of oxygen and nutrients is mediated by the circulatory system, with the heart pumping blood to and from the tissues via the blood vessels. Also, cells require oxygen and nutrients to divide; hence blood vessel formation (angiogenesis) is a prerequisite for tissue expansion. In a variety of pathologies such as cancer, diabetic retinopathy and rheumatoid arthritis, vessel growth contributes to the progression of the disease. In other conditions, for instance in diabetic wounds, the situation is the opposite with insufficient circulation. The data presented in this thesis aim to increase our understanding of the regulation of angiogenesis.

The vascular system

Functional anatomy and histology

The blood vascular system, first described by William Harvey 1628 (Harvey, 1628), carries the blood from the heart via the arteries, arterioles, capillaries and back through the venules and veins. The essential exchange of gases, nutrients, antibodies and metabolic waste products between tissue and blood takes place in the capillaries. In addition, this is the site for extravasation of leukocytes to inflamed or infected tissue. As diffusion of oxygen is limited to 100-200 µm, there is a need of a capillary in close vicinity to every cell.

The capillaries are 7-30 µm in diameter and consist of an inner lining of approximately 1-4 endothelial cells (ECs)/diameter resting on a basement membrane (BM). This BM is shared by mesenchymal smooth muscle cells, denoted pericytes (Fig. 1). The pericytes do not completely cover the capillaries but cling on to them like a hand grabbing a racket. These specialized cells and their interaction with the ECs are of great importance for the function of the vessel (Hellström et al., 2001; Leveen et al., 1994; Soriano, 1994).

Larger vessels serve to regulate blood pressure and to transport the blood to the capillaries. Since these vessels are exposed to high pressure they are surrounded by layers of elastic tissue, continuous smooth muscle cells and
connective tissue. ECs fulfill different tasks depending on their anatomical position. In some tissues, where there are needs for a fast interchange between blood and tissues (for example in kidney glomeruli and intestine), small gaps, fenestrae, are interrupting the plasma membrane of the ECs. The fenestrae will allow macromolecules to pass without crossing the lipid bilayer. In muscles and connective tissue, on the other hand, capillaries lack fenestrae. However, to prevent free diffusion of liquid and lipids, ECs have intercellular connections denoted tight junctions. In addition, they connect via adherens junctions of which the vascular endothelial calcium dependent cell adhesion molecule (VE-cadherin) is the main protein (for a review see Dejana, 2004). Regardless of their organ location, ECs share most properties such as protein expression and location within the vessel.

Vasculogenesis

During gastrulation in mammals, both embryonic and extraembryonic mesodermal cells are formed. Vasculogenesis is the *de novo* formation of vascular structures within the mesoderm (Risau and Flamme, 1995). The cardiovascular system is the first organ to develop. In the mouse embryo, this process is initiated at embryonic day 7 (E7) in the primitive streak by the formation of clusters of pluripotent cells, hemangioblasts (Choi et al., 1998). It has been suggested that a fraction of the hemangioblasts stay within the embryo proper whereas some migrate to the yolk sac, where they differentiate into hematopoietic precursor cells (primitive erythroid cells) and endothelial precursor cells, denoted angioblasts (Fig. 2). Together these cells form multi-cellular structures called blood islands. The angioblasts differentiate into ECs that make up a primitive vascular network which later on reorganizes through sprouting angiogenesis. In the embryo proper, blood islands are lacking. Here, hemangioblasts are found as individual cells in close vi-

![Figure 1. Cross section of a capillary. ECs are surrounded by pericytes and rest on a BM.](image)
cinity of the floor of the developing aorta (for review see Wilting et al., 2003). At E8.5, certain hemangioblasts have become angioblasts that mi-

grate to outline the future dorsal aorta and the cardinal veins. The hemangioblasts have also been suggested to have the capacity to differentiate into supportive smooth muscle cells (Yamashita et al., 2000). The cardinal vein and the dorsal aorta are formed through maturation and connection of the ECs, without involvement of sprouting angiogenesis. The smaller branches that connect the arterial and venous tubes, such as the intersomitic vessels, are in contrast generated through sprouting angiogenesis. However, not all EC precursors seem to originate from the hemangioblast, but are established as individual angioblasts that can not differentiate into hematopoietic cells. Nevertheless, it is difficult to exclude the possibility that all EC and hematopoietic cells stem from a common precursor that might be hard to detect, due to a short life span.

Angiogenesis

-In health

Angiogenesis occurs in two fundamentally different ways, either by sprouting angiogenesis or by intussusceptive growth (Burri and Djonov, 2002). Developmental sprouting angiogenesis has been very well characterized, mainly by live imaging in the zebra fish (Lawson and Weinstein, 2002) and by studies of the developing retina (Uemura et al., 2006). In sprouting angiogenesis, ECs that are integrated in the vessel wall break loose and start to migrate upon a stimulus of growth factors, dominated by vascular endothelial growth factor (VEGF) (Habeck et al., 2002; Leung et al., 1989; Tischer et al., 1989) (first described as vascular permeability factor; VPF (Keck et al., 1989; Senger et al., 1983)). The migrating ECs organize into a solid cord

Figure 2. Schematic outline of the vasculogenic process.
which subsequently opens up to form a lumenized vessel. The sequential steps of lumen formation are not completely clarified. However, developing intersegmental vessels in zebra fish, display fusion of intracellular vacuoles which connects to vacuoles of adjacent ECs to form a continuous lumen (Kamei et al., 2006). As the tip cell encounters another sprout or vessel it connects and circulation is established. Further maturation involves recruitment of smooth muscle cells and/or pericytes to the vessels. Dependent on the tissue, platelet derived growth factor (PDGF) -BB and its tyrosine kinase receptor PDGF receptor β, are main regulators of this process (Hellström et al., 1999; Lindahl et al., 1997). For example, in the retina, protruding endothelial tip cells produce the majority of PDGF-BB which in turn attracts pericytes by activation of PDGF receptor β (Gerhardt et al., 2003; Hammes et al., 2002).

Mature ECs are completely enclosed by layers of interconnected extracellular matrix proteins (Fig. 1 and 6). This envelope is degraded by various proteases including matrix metalloproteases (MMPs) which are released for example during inflammation and as a result of VEGF-receptor (VEGFR) activation (Lamoreaux et al., 1998; Pettersson et al., 2000). This degradation is considered vital for the initiation if angiogenesis. As the ECs and the surrounding pericytes loose their connections to the BM, the ECs start to migrate towards the source of VEGF. Most information about sprouting angiogenesis in the adult has been retrieved by analysis of fixed tissue. This represents a snapshot of a given state of the dynamic angiogenic process. It is therefore difficult to draw clear conclusions about the order of events, since “before and after” can not be distinguished. Microscopic techniques are constantly improved, providing tools for high resolution live imaging. Maybe such studies will unravel the plasticity of the vascular bed in adult and pathological tissue.

Intussusceptive angiogenesis is characterized by longitudinal splitting of capillaries by insertion of tissue pillars resulting in two new vessels parallel to the mother vessel (Burri and Djonov, 2002). In contrast to sprouting angiogenesis, blood flow is maintained throughout the process. Intussusception has not been as extensively studied as developmental sprouting. Few examples of live imaging to visualize this process have been presented. Although sprouting and intussusception are considered two separate processes it is possible that they may combine to facilitate angiogenesis. When sprouts are induced, the EC start to migrate, in turn generating a lateral force to its mother vessel. By doing so, the vessel might be flattened, thereby facilitating the creation of lateral pillars. Again, careful live imaging analyses might resolve such a possibility in the future.

Angiogenesis is mostly induced by cells exposed to low oxygen pressure, denoted hypoxia. Nucleated cells deprived of oxygen will upregulate the transcription factor hypoxia inducible factor-1α (HIF-1α). HIF-1α in turn binds HIF-1β, allowing translocation of the complex into the nucleus. The
HIF-1α/1β complex binds to a specific oligonucleotide stretch, the hypoxia responsive element (HRE). Many genes involved in angiogenesis (for example vegfa and vegfr1) harbour the HRE sequence in their promoter region, and are thereby induced by hypoxia (for a review see Pugh and Ratcliffe, 2003). VEGF enhances the formation of vessel-structures by its specific mitogenic and migratory effects on ECs and by increasing vascular permeability (see Ferrara, 1999 for a review). Several other positive regulators of angiogenesis exist, such as fibroblast growth factors 1 and 2 (FGF) (Shing et al., 1984), transforming growth factors alpha and beta, hepatocyte growth factor, tumor necrosis factor alpha, angiogenin, interleukin-8, etc. (Folkman and Shing, 1992; Tonini et al., 2003).

Although formation of blood vessels in the adult is not abundant, it is a necessity for life and reproduction. All types of tissue expansion like muscle growth, wound healing, endometrial growth and ovulation is accompanied by angiogenesis.

-in disease

Angiogenesis is a critical aspect of a number of pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, and cancer. The formation of new blood vessels is crucial for the progression of most growing tumors. Without vascularization the tumor is dependant on diffusion for the exchange of nutrients and oxygen. In such a poor environment cell division is suppressed. This knowledge has generated a great interest in the possibility to directly target tumor angiogenesis, thereby preventing tumor growth. Also, tumor vessels are known to undergo constant remodeling, a property that would make them susceptible to anti-angiogenic treatment. Research has been intense, trying to find inhibitors of angiogenesis. So far, three drugs that target VEGF function have been approved by the federal drug administration in the USA to be used clinically; 1. Bevacizumab (Avastin™; a VEGF neutralizing antibody). Treatment with bevacizumab in combination with chemotherapy resulted in about four months’ prolonged survival in patients suffering from colon cancer. 2. Pegaptanib, (Macugen™; an oligonucleotide that binds VEGF) is used for treatment of wet age-related macular degeneration (AMD), by intravitreal injections (Gragoudas et al., 2004). 3. Ranibizumab (Lucentis™; an antibody fragment that binds all VEGF isoforms) is another molecule used for treatment of wet AMD. Treatments with these substances have generated side effects that can be linked to the interruption of VEGF-signaling. For a review on VEGF-inhibition in clinical trials see (Jain et al., 2006).

Several molecules with anti-angiogenic properties have been identified in human tissue. These are denoted endogenous inhibitors, and most of them are proteolytic fragments of extracellular matrix (ECM) proteins or plasma proteins, exemplified by endostatin (a fragment of collagen XVIII), endorepellin (the C-terminal part of perlecan), angiostatin (a fragment of plasmino-
gen) and tumstatin (a fragment of the collagen IVα3-chain) (for a review see Bix and Iozzo, 2005). Several other inhibitors have been very promising both in vitro and in animal models, however only a few have had any effect in the clinic. There are a number of plausible reasons for this discrepancy between murine and human studies. Most preclinical studies have been performed on immuno-compromised mice using ectopic and fast growing tumors. These differ from most human tumors in many ways. Also, some tumors gain resistance to hypoxia, with blood vessel independence as a consequence. In the majority of clinical trials, the patients have been suffering from a late stage terminal cancer which could not be cured by conventional therapy. Anti-angiogenic treatment in combination with conventional chemotherapy has therefore been initiated as a last attempt to cure the patient. It is possible that the potential of anti-angiogenic substances are greater at an earlier stage of disease progression. In spite of the intense research in the vasculo- and angiogenesis field, many questions remain unanswered concerning the treatment with anti-angiogenic substances. It is therefore of great interest to develop new models for such studies, in which genetic, as well as other manipulations easily can be made.

Molecular interplay in angiogenesis

The VEGF signaling system

The family of VEGFs and their receptors are major players in angiogenesis (for reviews see Ferrara, 2002 and Olsson et al., 2006). The receptors, VEGFR-1, -2, and -3 are transmembrane tyrosine kinases that dimerize and become activated upon ligand-binding. VEGFR-1, -2 and -3 are primarily expressed on hematopoietic cells, blood ECs and lymphatic ECs, respectively. All three receptors are needed for vascular development, since deletion of any of the receptors in mouse leads to vascular abnormalities and death at E8.5-10.5 (Dumont et al., 1998; Fong et al., 1995; Shalaby et al., 1995). In nonvertebrates, only one VEGFR-related protein is known, indicating diversification of the receptors through gene duplications during evolution of early vertebrates (Shibuya, 2002). The VEGF receptors bind six different ligands, VEGF-A, -B, -C, -D, placental growth factor (PIGF) and a virus encoded VEGF-E, in a distinct pattern (reviewed by Cross et al., 2003 and Olsson et al., 2006) (Fig. 3).

VEGF receptors

VEGFR-1 (also denoted fms-like tyrosine kinase-1 (Flt-1) in mouse) is mainly expressed on blood vascular endothelium, macrophages/monocytes
and hematopoietic stem cells and exists in two forms: 1. The full length protein with a ligand-binding extracellular domain, a transmembrane domain and an intracellular kinase domain. 2. A secreted protein, composed of only the extracellular domain, encoded by an alternatively spliced variant (Kendall and Thomas, 1993). Several different functions for this receptor have been described. The full length form mediates monocyte recruitment to inflamed tissues, a process dependent on receptor activation (known to be much weaker than VEGFR-2). The soluble receptor acts as a suppressor of full length VEGFR-1 and -2 by competing for ligand-binding. These findings are evident from phenotypes with either deleted full length receptor, resulting in death at E8.5-9 due to excessive EC growth (Fong et al., 1995), or deleted tyrosine kinase domain, with reduced macrophage migration as the only reported phenotypic alteration (Hiratsuka et al., 1998). However, tumors grew slower in mice with deleted VEGFR-1 tyrosine kinase domain, maybe due to decreased monocyte/macrophage infiltration (Hiratsuka et al., 2001).

VEGFR-2 (also denoted Flk-1 (fetal liver kinase-1) in mouse and kinase domain region (KDR) in humans) is primarily expressed by EC precursors and blood ECs, but also by neuronal stem cells (Zachary, 2005). Deletion of the receptor in mouse is lethal at E8.5-9.5 with defective vascularization, illustrating its requirement for vascular development (Shalaby et al., 1995). The receptor was initially considered vital for angioblast commitment, but

Figure 3. The VEGFs bind the VEGFRs in an overlapping pattern. Arrows points at binding partners. Dashed lines indicate that proteolytic processing is required for binding. Neuropilin-1, -2 and HSPGs are coreceptors for the VEGFs.
studies in zebra fish and differentiation of embryonic stem cells (ESCs) and caref
ful analysis of embryos lacking the receptor have suggested the opposite (Chil
ds et al., 2002; Schuh et al., 1999). It seems rather that the receptor is vital for migration, remodeling and survival of the EC progenitors. An important role for VEGFR-2 is to regulate permeability of the adult endothelium. This leads to leakage of plasma proteins such as fibrinogen into the surrounding tissue which in turn has been suggested to promote angiogenesis and tumor growth (Dvorak et al., 1987).

The VEGFR-2 extracellular domain has seven immunoglobulin-like stretches of which the second and third compose the ligand-binding sites. In addition, the extracellular part may interact with heparan sulfate proteoglycans (HSPGs) and possibly with neuropilins that both function as coreceptors for the VEGFs (discussed below) (Dougher et al., 1997). The intracellular part consists of a tyrosine kinase domain interrupted by a 70 amino-acid stretch. Within and around this kinase domain, 18 tyrosines are dispersed that may be phosphorylated upon dimerization of the receptor (the downstream effectors will be discussed in detail below).

VEGFR-3 (alternatively denoted fms-like tyrosine kinase-4 (Flt-4) in mouse) is predominantly expressed on embryonic blood vasculature, lymphatic ECs, monocytes and macrophages. Mice with deleted VEGFR-3 show vessel malformations and die at E9.5-10.5 due to cardiac failure (Dumont et al., 1998). The basic structure of VEGFR-3 is similar to VEGFR-1 and -2, apart from the substitution of the fifth immunoglobulin-like domain by a disulfide bridge.

**VEGF-A**
The VEGFs are glycoproteins usually secreted as anti-parallel homo-dimers linked by cysteine bridges. VEGF-A is transcribed into one pre-mRNA which

![Figure 4. The major VEGF-A splice variants. Numbers indicate exons. Exon 3 encodes the VEGFR-1 binding site and exon 4 the VEGFR-2 binding site.](image-url)
gives rise to at least six different human splice variants: 121, 145, 165, 183, 189 and 206, corresponding to the number of amino acids (Fig. 4) (reviewed by Robinson and Stringer, 2001). The murine forms are one amino acid shorter; VEGF-A120 etc. All isoforms are capable of binding both VEGFR-1 and -2, with distinct affinities. In general, VEGF-A165 seems to be the overall dominating form. However, it is not clear to what extent the respective isoforms contribute to certain types of angiogenesis, i.e. pathological or developmental (Ng et al., 2001). The great importance of VEGF-A for vascular development was demonstrated in studies by Carmeliet et al. and Ferrara et al. 1996, where mice lacking only one vegf-a allele were able to form the dorsal aorta but showed abnormal vasculature and died at E11-12 (Carmeliet et al., 1996; Ferrara et al., 1996). Also, mice with homozygote deletion, generated by tetraploid aggregation, were even more severely affected with death at E9.5-10.5 (Carmeliet et al., 1996). The isoforms differ in their ability to bind heparin/heparan sulfate and neuropilin-1 and -2 with the longer forms displaying the highest affinity to these molecules (Gluzman-Poltorak et al., 2000; Jakobsson et al., 2006; Mamluk et al., 2002; Soker et al., 1998). Thus, VEGF-A121, that lacks the heparin/heparan sulfate binding domain, can travel further in tissue than VEGF-A189 since it does not stick to the extracellular matrix (ECM) (Park et al., 1993). However, VEGF-A121 and -A165 are equally potent in inducing VEGFR-2 activation, at least in porcine aortic ECs over-expressing the receptor (unpublished data). Previously, VEGF-A121 has been suggested to be a poor activator of the receptor even in acute stimulations, which may be due to sub-optimal preparation of the different isoforms. Although equally potent in simplified in vitro models, large differences between the isoforms have been recorded in vivo. Mice genetically altered to express only VEGF-A120 display internal bleedings and deregulated patterning of the vasculature in the retina (table 1, p19-20). A combination of factors such as inability to shape gradients, or altered signaling properties due to lack of binding to HSPGs and neuropilin-1 and -2, might be reasons for these defects (Gerhardt et al., 2003; Jakobsson et al., 2006; Ruhrberg et al., 2002).

Receptor activation and signal transduction

Ligand-binding to the second and third extracellular IgG-loops (out of seven) in VEGFR-2 induces dimerization of receptors leading to transphosphorylation of at least 8 tyrosines in the intracellular domain. Receptor activation is initiated by phosphorylation of tyrosines 1054, 1059 in the catalytic domain followed by phosphorylation of Y951 (Matsumoto et al., 2005), Y1175 and Y1214 (Takahashi et al., 2001) (Fig. 5). Each phosphorylated tyrosine constitutes a specific binding site for one or several downstream effectors. Knock-in mutation of tyrosine 1173 (1175 in human) to phenylalanine in mouse results in lethality at E9, which is similar to the effect of VEGFR-2 deletion itself, whereas exchange of Y1212 is of no consequence
The phosphorylation of Y1173/1175 and the accompanying downstream signaling may therefore be considered the prime pathway of VEGFR signaling during developmental angiogenesis. Phosphorylated tyrosine 1175 binds and activates several effector molecules such as, phospholipase C-γ (PLC-γ) (Takahashi et al., 2001), Shb (Holmquist et al., 2004) and Sck (Warner et al., 2000). Out of these, PLC-γ seems to be the main regulator of EC proliferation by activation of protein kinase C and mitogen-activated protein kinase (extracellular signal–regulated kinase 1 and 2 (ERK1/2)). Furthermore, phosphorylation of Y951 recruits VEGFR-associated protein (VRAP)/T-cell-specific adapter/Lad (Wu et al., 2000) involved in EC movement (Matsumoto et al., 2005). Other classical signal transduction pathways involving ERK, focal adhesion kinase (FAK), phosphoinositide 3 kinase (PI3K), and Src are also activated downstream of VEGFR-2. There is however growing evidence indicating that the cellular context dictates which pathway will be activated.

Figure 5. The intracellular part of VEGFR-2 and its major phosphorylation sites. The outcome of each signaling cascade is indicated in the blue boxes. DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HSP27, heat-shock protein-27; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol 3’kinase; PKC, protein kinase C; PLCγ, phospholipase C-γ; Shb, SH2 and β-cells; TSAd, T-cell-specific adaptor. Adapted by permission from Macmillan Publishers Ltd: (Olsson et al., 2006), copyright 2006.
**Receptor inactivation and degradation**

Even though activation of VEGFRs has been extensively studied, receptor down regulation and inactivation has received less attention. Naturally, this process is equally important since a continuously activated receptor would be unable to sense new signals. Internalization of RTKs other than the VEGFRs, like epidermal growth factor receptor (EGFR), is in contrast very well characterized and is known to require intrinsic receptor kinase activity. In brief, ligand-induced receptor activation leads to coated pit formation that facilitates the endocytosis of the complex. Depending on the state of ubiquitination, primarily mediated by c-Cbl E3 ubiquitin ligase in a phosphotyrosine specific manner (Duval et al., 2003), VEGFRs are either sorted to early endosomes, or alternatively to lysosomes for degradation. In the early endosome, the signaling complex is at least in part intact with persistent activity (Lampugnani et al., 2006). Several reports suggest that signals from this compartment engage other pathways than those activated from the plasma membrane. Although it is likely that the basic mechanisms are shared by most RTKs, there are differences in the precise regulation; for example also inactive PDGFRs can be sorted to lysosomes (Wiley and Burke, 2001). The receptor activation can moreover be modulated or turned off by intracellular phosphatases (for a review see Kappert et al., 2005). The importance of phosphatases in the regulation of VEGFR signaling is however largely unknown.

**VEGF coreceptors**

VEGF is the key activator of VEGFR-2 but the outcome of this activation is affected by the cellular context and the presence of coreceptors.

Neuropilin-1 is a transmembrane glycoprotein that function as a coreceptor for VEGFR-2 (Soker et al., 2002; Soker et al., 1998). Embryos deficient in neuropilin-1 display reduced neuronal vascularization and development as well as disorganized yolk sac vascularization (Kawasaki et al., 1999; Kitsukawa et al., 1997). Ectopic overexpression of neuropilin-1 instead results in excessive angiogenesis and malformed hearts, in addition to abnormal axonal development (Kitsukawa et al., 1995).

HSPGs, which also function as coreceptors, will be discussed in detail below.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>vegfr-2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Normal.</td>
<td>(Shalaby et al., 1995)</td>
</tr>
<tr>
<td>Genotype</td>
<td>Stage</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>vegfr-2−/−</td>
<td>† E8.5–9.5</td>
<td>Defective blood-island formation and vasculogenesis.</td>
</tr>
<tr>
<td>vegfr-3−/−</td>
<td>† E9.5-10.5</td>
<td>Vasculogenesis and angiogenesis occurred, large vessels abnormally organized with defective lumens, fluid accumulation in the pericardial cavity and cardiovascular failure.</td>
</tr>
<tr>
<td>vegfa+/−</td>
<td>† E11–12</td>
<td>Defective vascular development.</td>
</tr>
<tr>
<td>vegfa−/−</td>
<td>† E9.5–10.5</td>
<td>Generated by aggregation of embryonic stem cells with tetraploid embryos, more severe defects in vascular development than heterozygote.</td>
</tr>
<tr>
<td>vegfa164/164</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>vegfa120/120</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>pdgfb−/−</td>
<td>Perinatal death, kidney defects and late stage-hemorrhages.</td>
<td>(Leveen et al., 1994)</td>
</tr>
<tr>
<td>pdgfrβ−/−</td>
<td>Lethal shortly before birth, hemorrhagic etc.</td>
<td>(Soriano, 1994)</td>
</tr>
<tr>
<td>LM-α5-null</td>
<td>† E14-17.5</td>
<td>With severe brain and limb defects and enlarged and disorganized placental vessels</td>
</tr>
<tr>
<td>LM-α4-null</td>
<td>Viable and fertile, but exhibit prenatal vascular bleedings, a phenotype that is gradually lost during development.</td>
<td>(Thybo et al., 2002)</td>
</tr>
<tr>
<td>LM-α1-null</td>
<td>† E7</td>
<td></td>
</tr>
<tr>
<td>LM-β1-null</td>
<td>† E ~6</td>
<td></td>
</tr>
<tr>
<td>LM-γ1-null</td>
<td>† E5.5</td>
<td></td>
</tr>
<tr>
<td>fibronectin-null</td>
<td>† E11; defective notochord and somite formation as well as vascular malfunctions</td>
<td>(George et al., 1997; George et al., 1993)</td>
</tr>
</tbody>
</table>
collagen IV-null | † E11; normal composition of the BM until E9.5, a mild vascular phenotype with irregular protrusion of capillaries into the neural layer | (Poschl et al., 2004)
Jagged-1-null | † E11; lack of obvious large vessels in the yolk sac, failure to remodel the primary plexus in the yolk sac, less intricate network and a reduced diameter of vessels in the head | (Xue et al., 1999)

### Fibroblast growth factor receptor-1 and its ligands

The fibroblast growth factor (FGF)-2 is a member of a family of 23 heparin-binding proteins (Yamashita et al., 2000). It is synthesized as a monomer but binds in a dimeric form, in complex with heparan sulfate, to the third IgG-loop (out of three) of FGFR-1 (McKeehan and Kan, 1994). The binding mediates dimerization of the receptor, and thereby cross phosphorylation of tyrosine residues in the receptor intracellular domain. This in turn leads to conformational changes and binding of signaling proteins resulting in various cellular responses. Mice deficient in FGFR-1 die between E7.5 and E9.5 with reduced yolk sac mesoderm and reduced numbers of blood islands (Yamaguchi et al., 1994).

### The basement membrane

BMIs are specialized ECM structures, 50-100 nm in thickness, that function not only as structural support and a barrier between different tissues, but also in mediation of signals affecting cellular behavior. The BM is a large and complex meshwork of interconnected proteins, such as type IV collagen, laminins (LMs), HSPGs (such as perlecan and agrin), nidogen/entactin and fibronectin (see Fig. 6). Both LMs and collagen IV have the ability to polymerize, and hence make up two separate sheets suggested to connect via the binding to nidogen (Smyth et al., 1999; Timpl and Brown, 1996). In addition to interactions between these molecules they also bind a variety of cell surface molecules, thereby influencing attachment, differentiation, migration, polarization or proliferation, depending on cell type and BM composition (Timpl and Brown, 1996). The heterogeneity of BMs in tissue and during development is mostly distinguished by differences in LM isoforms present.

Although the vascular BMs have been structurally well characterized, there is much to learn about their function in developmental and adult angiogenesis. Blood capillaries are surrounded by a specialized BM, composed of collagen IV, LM-411, LM-511, perlecan, nidogen/entactin and fibronectin, which are deposited by ECs in conjunction with vessel formation. Study III
of this thesis asked at what stage this occurs and which components that may influence EC differentiation, organization and stability. Numerous studies have shown that proteins and proteolytic fragments of the BM can stimulate or inhibit the processes of both normal and pathological angiogenesis (Nyberg et al., 2005). This is rather expected since some of the main LM receptors (the family of integrins) are implicated in a variety of signaling pathways. For example, undifferentiated, pluripotent embryonic stem cells can be directed into different cell lineages depending on which matrix they are cultured on (Yamashita et al., 2000). In the following section each family of BM proteins will be described in more detail.

**Laminins and the assembly of the BM**

The laminins are a family of heterotrimeric glycoproteins secreted by many cell types to become a main constituent of the BM. In mammals, there are 16 laminins (including major splice variants) known so far, each of them consisting of a particular combination of α-, β- and γ-chains (Aumailley et al., 2005) (Fig. 7).

The chains are joined in a trimeric α-helical coiled-coil structure in the C-terminal tail (the long arm) that opens up in the N-terminal tail (the three short arms) to form a cross-shaped laminin of 400-900 kD in size. To date, 5 α (α1-5), 3 β (β1-3) and 3 γ (γ1-3) chains, as well as additional splice variants, have been identified (for review see Colognato and Yurchenco, 2000; Miner and Yurchenco, 2004). The C-terminal end of the α-chain consists of five globular domains (LG1-5). These possess binding sites for several plasma membrane-associated molecules such as integrins, α-dystroglycan,
sulfated carbohydrates (S-CHO), fibulin-1 and sulfatides, but also for the ECM-associated molecule perlecan (for a review see Miner and Yurchenco, 2004). The coiled-coil domain has only been shown to interact with the secreted proteoglycan agrin, via the LM-β-chain. The N-terminal parts of all chains, except α3A and α4, harbor globular domains that bind similar domains on other LM molecules, in a calcium-dependent manner.

Figure 7. The major LM isoforms of the blood vascular endothelial BM. The trimers consist of one α, one β and one γ-chain. Binding partners and sites for interactions are outlined.
The multiple interactions mentioned above facilitate assembly of LMs on the cell surface, a property that plays a significant role in the formation of the complete BM. For example, in the absence of LMs, collagen IV is essentially excluded from the BM (Li et al., 2002 and study III). This may be due to that LMs normally bridge between the cell surface and the collagen network. However there is no direct connection between the LMs and collagens. Instead, these molecules bind independent sites on nidogens-1 and -2, which thereby may function as linkers (Smyth et al., 1999; Timpl and Brown, 1996). Mice with double gene deletions of nidogen-1 and -2 survive until birth. The BM formation is not severely affected in these mice, displaying only slight reduction in LM, collagen IV and perlecan in capillary BMs (Bader et al., 2005). These results indicate that nidogens are not strictly needed for general embryonic BM formation, but may alter certain properties of its function. This phenotype resembles mice with targeted deletion for the nidogen-binding site in LM-γ1 (Willem et al., 2002). The HSPG perlecan, is also important for the integrity of the BM and has been shown to bind both LMs, nidogen-1 and -2, fibronectin and collagen IV in vitro (Costell et al., 1999; Hopf et al., 1999). It is also clear from studies of collagen IV-deficient mice that this particular collagen is dispensable for early embryonic BM assembly (Poschl et al., 2004). So far, LMs are the only BM molecules whose deletion severely affects the embryonic composition and assembly of the remaining BM constituents (Li et al., 2002; Smyth et al., 1998; Smyth et al., 1999). However, in study III in this thesis we show that deposition of fibronectin as well as of HSPGs occur in the vascular BM also in the absence of LMs. This is in contrast to previous studies of an embryonic epithelial BM (Li et al., 2002). This discrepancy could be explained by differences in integrin expression on ECs and epithelial cells. Furthermore, addition of LM-111 resulted in a close to complete rescue of the epithelial BM assembly, whereas only a partial rescue was observed for the vascular BM (paper III). Li et al also demonstrated that LM-dystroglycan interactions are dispensable for LM assembly, but that interactions with HSPGs via LG4 are required (Li et al., 2002). Interestingly, differentiating embryonic stem cells with a dominant negative mutation for fgfr are unable to deposit LMs, which results in failure of BM assembly (Li et al., 2001).

This exemplifies the interplay between cell signaling and BM protein secretion and assembly. Taken together these data suggest a role for LMs in cell differentiation, whereas collagen IV is more of a mechanical support.

In 2006 it was agreed upon a change of the LM nomenclature from the previous LN-1 through -15 to LMxyz, where x is the number of the α-chain, y the number of the β-chain and z the number of the γ-chain (Aumailley et al., 2005). For example proteins previously known as laminin-1, laminin-8 and laminin-10 is now denoted laminin-111, laminin-411 and laminin-511, respectively.
There are today many different antibodies directed against the specific LM chains, but none that specifically recognizes a complete LM-trimer. The present information on LM tissue localization is therefore based on combined immunostainings or in situ hybridizations for detection of the separate chains. It is therefore more accurate to refer to the LM-chains rather than to the complete LM trimer, if not further examined.

*Laminins in vascular biology*

LM-111 is the first laminin to be detected in embryonic development with its β1- and γ1-chains expressed at the two cell stage, and the α1-chain at the 8-16 cell stage (Aumailley et al., 2000). In the adult, the LM-α-chain is restricted to BMs of epithelia, sinusoids in the liver and blood vessels within the central nervous system (Sasaki et al., 2002; Virtanen et al., 2000). Targeted deletion of the lama1 (encoding LM-α1) in mice leads to lethality at E7 due to failure in formation of Reichert’s membrane (Miner et al., 2004). The possible role of LM-111 in pathological angiogenesis is largely unknown, although several studies indicate that LM-111, or fragments thereof, affect EC function (study I, and reviewed by Hallmann et al., 2005)

LM-α4 (which combines with β1 and γ1) is relatively restricted to vascular BMs throughout development (Talts et al., 2000). Genetic deletion of this chain in mice leads to vascular bleedings and hemorrhages during the prenatal stage. One week after birth, the abnormalities are completely abolished and the mice are viable and fertile (Thyboll et al., 2002). It is believed that this phenotype is reversed by the deposition of LM-α5, a component of the other major vascular LM; LM-511. LM-α4 is also produced by various hematopoietic cells with consequence for their function. For example, production of LM-α4 by leucocytes has been reported to enhance extravasation of neutrophils into the tissue, as demonstrated by reduced migration in the LM-α4-deficient mice (Wondimu et al., 2004). LM-411-release is furthermore stimulated by the inflammatory cytokines interleukin-1 and tissue necrosis factor-α (Sixt et al., 2001). Knockdown of lama1 or lama4 (encoding LM-α1 and α4 respectively) in zebra fish embryos, did not affect intersomitic vessel growth. However, when both genes were simultaneously suppressed, the intersomitic sprouting was absent or severely delayed (Pollard et al., 2006). The authors suggest that the defects are due to absence of LM-containing substratum for EC migration in the intersomitic clefts. In accordance, LM-411 is known to enhance monocyte migration, which might apply also to ECs (Wondimu et al., 2004). LM-α4 is truncated in the N-terminus and lacks the short arm. Its ability to polymerize is therefore reduced, as compared to LM-111 and LM-511. Despite this fact, it assembles within the BM. Binding-partners are shown in Figure 7 (p23). LM-α4 has also been suggested to mediate EC survival in vitro, by activation of the β1 integrins (DeHahn et al., 2004).
LM-α5 (component of LM-511) has been observed in murine BMs of large vessels from E13, and in capillaries, 3-4 weeks after birth (Frieser et al., 1997; Sorokin et al., 1997). Mice lacking LM-α5 die at E13-17.5 with severe brain and limb defects, enlarged and disorganized placental vessels, and failed renal glomeruli vascularization (Miner et al., 1998; Miner and Li, 2000). This chain is the only intact LM-chain known to bind integrin αvβ3, an interaction dependent on the globular domain L4b in the short arm (Sasaki and Timpl, 2001). In addition, a fragment of the γ1-chain stimulates angiogenesis by binding integrins α5β1 and αvβ3 (Ponce et al., 2001).

The study on LMs in EC biology has been challenging due to the vast number of molecular interactions, the number of isoforms with overlapping functions, the potential functional contribution of proteolytic fragments and the variation between LM-preparations used (Wondimu et al., 2006). Despite such complications, the understanding of these molecules is now substantial. This has been facilitated in part by gene targeting in mouse (table 1, p19) and zebra fish and by the development of specific antibodies and high quality recombinant proteins.

The integrins
Integrins form a large family of dimeric transmembrane glycoproteins. At the time of writing, 18α- and 8β subunits have been recognized, composing 24 different integrins. As the extracellular domains bind components of the ECM such as collagens, LMs, and fibronectin, the integrin undergoes a conformational change allowing recruitment and activation of intracellular binding partners. ECs have been demonstrated to express at least integrins α1β1, α2β1, α3β1, α5β1, α6β1, α8β1, α9β1, αvβ1, αvβ3, αvβ4 and αvβ5 depending on EC type and state (for a review see Ruegg and Mariotti, 2003). Signals via integrins are dependent on integrin clustering which occurs in cell-cell, cell-matrix contacts (Clark and Brugge, 1995). All 24 integrins differ from each other in binding properties. On top of this complexity, the signals are influenced by the combination of the surrounding matrix and the cell type. For example the activation of FAK via integrins requires both ligand-binding and an intact cytoskeleton (Ruegg and Mariotti, 2003).

Glycobiology
Sugars (saccharides) are essential for life by providing energy, structural support and by modulating cell signaling and protein structure. Paper II focuses on a certain group of sugars that are members of the glycosaminoglycan (GAG) family, and their role in cellular communication. These sugars are unbranched polysaccharides composed of repeated disaccharide units, present at high copy number on all cells. They are all (except for hyaluronan, see below) synthesized in the Golgi, attached to a core protein. The GAG chains and the protein, together make up a proteoglycan of either intracellu-
lar, trans-membrane, glycosyl phosphatidylinositol (GPI)-anchored or secreted type. Frequent sulfate groups within the sugar backbone mediate binding to a number of proteins, including growth factors and morphogens. Dependent on the sugar composition, the GAGs are divided into four groups as follows: Heparin/heparan sulfate (HS), hyaluronan and keratan sulfate, chondroitin sulfate (CS) and dermatan sulfate. Since Paper II is focused on protein interactions with HS/heparin, the following sections will be dedicated to these molecules.

**Heparan sulfate and heparin**

HS and heparin are unbranched polysaccharides of the GAG type, composed of repeats of up to 100 N- and O-sulfated disaccharide units (Fig. 8). HS/heparin are synthesized in the Golgi, covalently linked to a serine of a core protein. The number of sugar chains per protein can vary from one to

![Figure 8](image-url)

*Figure 8. The synthesis and structure of heparan sulfate. Enzymes responsible for each step are indicated in the shaded boxes. EXT, exostosin family; NDST, N-deacetylase/N-sulphotransferase; OST, sulfotransferase.*
The synthesis is initiated by the attachment of a xylose-galactose-galactose-glu\c{c}uronic acid sequence to a serine of the protein. Subsequently, the HS polymer is generated by the action of HS polymerases (glycosyl transferases of the exostosin family (EXT)) that catalyze alternating additions of N-acetylglucosamin and glucuronic acid to the linkage region (for reviews see Esko and Lindahl, 2001; Hacker et al., 2005). This chain will then undergo a number of modifications: 1. In certain regions, N-acetyl groups are removed and replaced by sulfate groups. This is carried out by either of the four enzymes N-deacetylase/N-sulphotransferases (NDSTs)-1-4. 2. Transformation of glucuronic acid to iduronic acid (epimerization) preferably in N-sulfated regions. 3. 2O-, 3O- and 6O-sulfotransferases (OST) attach additional sulfate groups in the positions indicated by the names of the enzymes. As the sulfate groups are negatively charged these modifications will result in a highly negatively charged, hydrophilic molecule. The degree and pattern of sulfation, that depend on the activity of the different enzymes described, are highly variable, in a tissue-, cell-, and age-dependent manner (Dennissen et al., 2002; Feyzi et al., 1998). Heparin undergoes heavy modifications resulting in a higher degree of sulfation than is the case for HS.

Proteins with attached HS-chains are denoted HS proteoglycans (HSPGs). HSPGs can be divided into four main types depending on the core protein (Fig. 9.): 1. The intracellular, stored in granules (serglycin, carrying heparin in connective tissue-type mast cells (Kolset and Gallagher, 1990)). 2. The glypicans (six members, glypican 1-6), which are linked to the cell surface by a GPI anchor.

3. Transmembrane. syndecans, right (the dashed lines indicate chondroitin sulfate chains), 4. Secreted forms, upper.
by a glycosyl phosphatidylinositol (GPI)-anchor. 3. The transmembrane syndecans (four members, syndecan 1-4), and 4. Soluble HSPGs (three major members, perlecan, agrin and collagen XVIII) which are released from cells and deposited in the extracellular matrix. The ectodomains of both syndecans (Fitzgerald et al., 2000) and glypicans can be shed from the cell surface by various enzymes. Several studies indicate that shedding might be of great importance for cell communication (Kreuger et al., 2004). In accordance, Kato et al. present indications for diverse signaling properties of soluble contra immobilized HS. The immobilised ectodomain of syndecan-1, prepared from wound exudates, stimulated heparin-mediated FGF-induced cell proliferation whereas the soluble ectodomain opposed this action (Kato et al., 1998). Such differences are further demonstrated in paper II in which only cell surface HS was able to rescue VEGFR-2-signaling in HS-deficient ECs.

**HS/protein interactions and lessons from knockout studies**

Almost all cells in the body express HSPGs. HS or heparin have been shown to interact with over 100 proteins, including several growth factors and morphogens, such as VEGF, FGF, Wnt and Hedgehog (Burri and Djonov, 2002; Chen et al., 2004). The impact of these molecular interactions on cell biology is diverse. Formation of morphogen gradients is essential for proper embryogenesis. Here, HSPGs of both the glypican and syndecan families are known to play an important role in restricting diffusion of these proteins (Belenkaya et al., 2004; Han et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004; The et al., 1999). HSPGs also take part in formation of signaling complexes, modulating their stability (Yayon et al., 1991) and membrane trafficking (paper II: Jakobsson et al., 2006). Furthermore, HSPGs might function to protect proteins from proteolytic degradation (Rosengart et al., 1988). Whether this HS-protein binding is dependent on specific patterns of sulfation of the saccharide backbone, or if it simply relies on charge density, has been heavily debated (reviewed by Kreuger et al., 2006). In vivo data in favor of the net charge theory were presented by Kaminura and colleagues (Kamimura et al., 2006). By genetic alterations in *Drosophila* they illustrate that FGF-function depends on the HS net charge, regardless of whether it carries 6O- or 2O-sulfation. Also, Kreuger et al. demonstrated equal binding of FGF-2 to oktasaccharides irrespective of the positioning of sulfate groups, given that the number of sulfate groups was constant (Kreuger et al., 2005). There was on the other hand a strict correlation to the degree of sulfation. Although several studies favor the critical role of charge-density dependence, we cannot exclude the possibility of sequence-dependence in situations not yet investigated.

It is unclear however to what extent the core proteins themselves contribute to signaling. The importance of the core protein was illustrated by a mu-
tation of syndecan-1 to eliminate certain HS-attachment sites (Langford et al., 1998). The syndecan-1-dependent cell-cell interactions and cell adhesion were affected even when only one single site was mutated. In contrast, no clear phenotype was observed in mice with deleted syndecan-1 under normal conditions (Alexander et al., 2000). In paper II, we show that HS-mediated signaling via the core protein (syndecans) in ECs, is dispensable for vasculo- or angiogenesis. Taken together, these data suggest that these particular HSPGs primarily function as co-receptors. However, targeting syndecan-2 by morpholinos or overexpression of a syndecan-2 with a truncated intracellular domain, result in defective angiogenic sprouting in zebra fish development (Chen et al., 2004). This suggests a role for the core protein itself in signaling.

The requirement for HS in development is exemplified by a number of genetically altered mice. Gene targeting of the glycosyl transferase EXT1 results in death at E8.5 with total absence of HS but increased chondroitin sulfate synthesis (Lin et al., 2000). Inactivation of the Ndst1 gene leads to a reduction in the degree of N-sulfation of HS. Most embryos lacking this enzyme survive birth but die shortly thereafter in a condition resembling respiratory distress syndrome (Ringvall et al., 2000). The Ndst2+/- mice survive and are fertile (Forsberg et al., 1999). Elimination of Ndst2-/- results in reduction of heparin sulfation with abnormal mast cell function as a consequence. Combined deletion of Ndst-1 and -2 is accompanied by lethality at E5.5 (unpublished data, Kjellén and Holmborn). Embryonic stem cells generated from blastocysts derived from these mice, synthesized HS without N-sulfation but with low amounts of 6O-sulfation (Holmborn et al., 2004). The early lethality of EXT1 and Ndst1/2 knockout mice prohibited analysis of the general HS contribution to vascular development. In paper II, we addressed this issue employing embryonic stem cells deficient in both NDST-1 and -2 expression. These embryonic stem cells cannot, unlike wild type cells, respond to VEGF by the formation of vascular structures. However, presentation of HS to the HS-deficient ECs by adjacent cells, rescues vascular development and VEGF-induction. This will be further discussed below (see present investigation).

It is clear that invaluable information can be retrieved from gene deletions. It is however well known that defects due to gene deletions can be compensated by upregulation of other genes. It should therefore be emphasized that care should be taken in the analysis and interpretations of knockout models. Also, most genes are involved in multiple actions that might affect a variety of cells. Given this, one should be aware of possible secondary effects.
Embryonic stem cells, embryoid bodies and vascular biology

In 1981, Bradley et al. and Martin et al. presented protocols for the culture of ESCs in vitro, without loss of their pluripotency (Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981). A few years later, targeted deletion of genes by homologous recombination in ESCs was described (Thomas and Capecchi, 1987). These cells were soon thereafter shown to give germ line transmission, allowing generation of transgenic mice (Thompson et al., 1989). These techniques have been invaluable to all fields of cell biology, and since then, numerous knockout animals have been demonstrated. Gene targeted ESCs can be isolated from blastocysts of such mice, and cultured in vitro. If these cells are let to differentiate in suspension, they will form spheroids, denoted embryoid bodies. Within the embryoid bodies cells will differentiate to a variety of cell lineages, including ECs. Development in embryoid bodies has been shown to constitute a useful model for the study of vasculo- and angiogenesis (Bloch et al., 1997; Byrd et al., 2002; Doetschman et al., 1985; Feraud et al., 2001; Ferrara, 2002; Magnusson et al., 2004; Vittet et al., 1996). Vascular development in the embryoid bodies share many features with development in vivo. For example, the order of appearance of a number of vascular markers (VEGFR-2, CD31, VE-cadherin, Tie-1 and Tie-2) resembles that observed in vivo (Vittet et al., 1996). Furthermore, EC structures are surrounded by supportive smooth muscle cells and/or pericytes (paper II-IV; Lindskog et al., 2006). At later stages of embryoid body differentiation (day 10-12 after initiation), the ECs form lumensized vessels enclosed in a BM composed of LMs, fibronectin, collagen IV and HSPGs (paper III). In paper I-III we have cultured embryoid bodies either in a rather two-dimensional state on a tissue culture dish (vasculogenesis), or in a three-dimensional collagen-I matrix (invasive angiogenesis). When cultured in the two-dimensional setting vasculo-/angiogenesis are enhanced by addition of PDGF-BB, FGF-2, VEGF-A165 or VEGF-A121, each giving raise to a characteristic vessel morphology. In collagen however, only VEGF-A165 is able to induce angiogenic sprouting with the setup used in paper II and III, although induction by treatment with FGF-2 has been observed in a similar system (Feraud et al., 2001).

Mice lacking either VEGFR-2, NDST-1 and -2 or LM-γ1 are embryonically lethal before or during early vasculogenesis. We have however been able to study vascular development employing ESCs lacking these genes, in the embryoid body model. This demonstrates the potential of this in vitro differentiation model (paper II-IV).
Present investigations and future perspectives

Paper I
Laminin-1 promotes angiogenesis in synergy with fibroblast growth factor by distinct regulation of the gene and protein expression profile in endothelial cells

Aim
Remodeling of the BM of ECs is a prerequisite for angiogenesis. A major constituent of BMs are the LMs, which have been shown to affect EC biology in various ways (Hallmann et al., 2005). Previous observations that LM-111 (denoted LN-1 in paper I) affected EC morphology in vitro had not been explained; we therefore wanted to investigate possible molecular mechanisms for this action. Also, overexpression of the LM-α1-chain in a human colon carcinoma results in abundant angiogenesis and enhanced tumor growth of xenotransplants in mice, indicative of LM involvement in pathological angiogenesis (De Arcangelis et al., 2001). We wished to further investigate the role of LMs in EC signaling and differentiation.

Results
We tested the effect of LM-111 on the chorio-allantois membrane of the chicken. We applied filter discs soaked in LM-111 with or without FGF-2 and noted that LM-111 and FGF-2 induced angiogenesis to the same extent. Furthermore, differentiating embryonic stem cells were exposed to the same combination of proteins at various concentrations. At low concentrations, neither LM-111 nor FGF-2 induced an increase in endothelial structures. A combination of the two at the same concentration, however, promoted the relative EC population.

To exclude possible indirect effects by other cells in the cultures, we investigated differentiation of an EC-line. Immortalized mouse brain endothelial (IBE) cells were seeded between two layers of collagen to induce formation of tube-like structures. Inclusion of FGF-2 and LM-111 or both in the medium for seven hours, induced formation of tubules that were absent in the untreated control. After 24 hours, IBE cells treated with LM-111 alone
had undergone apoptosis, whereas treatment with FGF alone and together with LM-111 still allowed tube formation. The tubes co-treated with FGF and LM-111 were longer and less branched than with FGF alone, and seemed to have reached a higher degree of differentiation as cell-cell borders were hardly distinguishable. This implicated a role for LM-111 in promotion of differentiation, but not survival of ECs in this system. Only LM-β1 and -α5 were slightly enhanced after FGF-stimulation, which may exclude any major contribution of endogenous LMs to the initiation of EC differentiation. However, several LM-chains were detected, indicating that they still might be required for this process. Many studies have suggested that Notch4 and its ligand Jagged-1 play a central role in regulation of EC differentiation. We observed a transient upregulation of mRNA and protein-levels of Jagged-1 during tube formation of the IBE cells after treatment with LM-111 alone, FGF alone and combined. LM-111 had no effect on phosphorylation of either ERK or Akt/protein kinase B. On the other hand, LM-111 induced an up-regulation of FGF-2 and FGFR-1 transcripts. Co-treatment with LM-111 and FGF-2 induced a transient increase in the number of \textit{fgf2}, \textit{vegfa}, \textit{fgfr1} and \textit{vegfr-2} transcripts at 3 h with a drop at 7 h. This indicates an intriguing interplay between cytokines and components of the BM during differentiation of ECs.

Discussion

From data presented in paper I, it is not possible to make a statement on whether Jagged-1 triggers differentiation, or if the up-regulation is rather a consequence of differentiation. The regulation of the Notch ligands Jagged-1 and Delta-like 4 is still largely unexplored although their involvement in EC biology is apparent (Xue et al., 1999). Interestingly, in a recent publication by Hellström et al., activation of Notch by Jagged-1 results in decreased number of tip cells in the developing retina (Hellström et al., 2007). Similar observations were made concerning intersomitic EC-sprouting in the developing zebra fish (Siekmann and Lawson, 2007). It seems likely that Notch signaling contributes to vascular differentiation and maturation rather than to active growth, similar to ECs in collagen treated with LM, in paper I (for a review on the family of Notch receptors and ligands in EC biology see Iso et al., 2003).

Although LM-111/FGF-induced tube formation involves regulation of Jagged-1, signaling via integrins are likely to be of great importance, functioning either as upstream or down stream mediators. Here, at least α3- and α6-integrins seem not to be central to the differentiation, since blocking antibodies had little or no effect in the collagen assay.

It is known from previous studies that exogenous addition of LM-111 at least in part can rescue BM formation in cells lacking LM (Li et al., 2002; Li et al., 2001 and paper II). However, we do not know if exogenous addition
of LM to ECs in collagen contributes to BM formation or if it rather competes with endogenous polymerized proteins for the binding to integrins. FGF-2 clearly induced EC morphogenesis in all models used in paper I. Surprisingly however; embryoid bodies with deleted FGFR-1 display an increased EC population, despite the fact that FGF-2 induced vascular development in the wild type (Magnusson et al., 2004). It might be, that the basement membrane composition is altered in the FGFR-1-deficient cells, since FGFRs are needed for LM secretion (Li et al., 2001).

Future perspectives
It is still not clear precisely at what stage during EC differentiation that Jagged-1/Notch signaling is critical. Such knowledge would increase our understanding of EC differentiation.

Paper II
Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis

Aim
HSPGs bind numerous proteins, thereby modulating the signaling properties for some of them. Since certain VEGF-A isoforms are known to bind heparin we wished to investigate if, and possibly how, HSPGs modulate VEGF-signaling in vasculo- and angiogenesis. To address these issues we employed embryonic stem cells deficient in the enzymes NDST-1 and 2 with severely reduced sulfation of HS and heparin as a consequence.

Results
Embryonic stem cells lacking NDST-1 and 2 produce HS with deficient sulfation. We show that this HS is unable to bind VEGF-A165, in contrast to wild type-derived HS. Furthermore, these ESCs failed to differentiate to form vessels; a phenotype which could not be rescued by addition of heparin or HS. VEGFR-2-deficient ESCs are, like the Ndst1/2−/− cells, unable to form vascular structures during differentiation. However, when these cell-types were mixed at the stem cell stage to generate chimeric embryoid bodies, the formation of vessel structures was at least as prominent as for the wild type cells. In addition, the chimeric cultures responded to VEGF-A165 with the characteristic formation of a ring-like structure of ECs in the embryoid body.
periphery, when cultured on a flat surface, and by sprouting when cultured in three-dimensional (3D) collagen gels. The endothelial sprouts in the collagen matrix expressed VEGFR-2 and were hence derived from the Ndst1/2-/- cells. This indicates that EC-derived HS is dispensable for vascular development in this setting. In the wild type cultures, HS was expressed by all cells as indicated by immuno-fluorescent staining. In the chimeric embryoid bodies, the ECs lacked sulfated HS, whereas adjacent pericytes expressed high levels. To further investigate the HS contribution to invasive angiogenesis, we introduced VEGF-A121 that was shown not to bind heparin or HS. VEGF-A121 failed to induce vascular sprouting in the collagen assay. Furthermore, treatment of embryoid bodies in collagen with heparinase I-III to cleave HS into small inactive fragments reduced VEGF-A165-induced sprouting of chimeric embryoid bodies. These data further indicate that ligand/HS binding is required for proper signaling. We then investigated possible differences in VEGFR-2 signaling dependent on whether HS was positioned either in \textit{trans} or in \textit{cis} to the receptor. Stimulation of wild type embryoid bodies with VEGF-A165 resulted in a transient phosphorylation of the receptor and a decrease in the total receptor levels after 1 h. In the chimeric embryoid bodies, with HS presented in \textit{trans}, there was instead a potent phosphorylation that persisted for at least 6 h. This was accompanied by an increase in the total receptor level over a 2 h time period. Despite considerably lower amounts of total receptors in chimeric cultures compared with wild type, the phosphorylated protein levels were in the same range. This indicates that a higher percentage of the receptors is activated and/or that each receptor is tyrosine phosphorylated at increased levels (or stochiometry) when HS is presented in \textit{trans}.

These data suggest that the composition of HS, as well as its location in relation to VEGFR-2, modulate both signaling potency and kinetics. Thus, interaction of HS with the VEGF receptor and the co-receptor neuropilin-1 seems to be of greater interest than previously considered.

Discussion

In vivo, adjacent cells may display differences regarding their respective HS epitopes (Dennissen et al., 2002). In paper II, we have investigated the situation in which one cell (the pericyte) expresses high amounts of “normal” HS, whereas the neighboring cell (the EC) expresses HS with deficient sulfation. This setting will maximize the interactions in \textit{trans} between HS and the ligand/receptor-complex, and prevent interactions in \textit{cis}. In normal tissue, it is likely that there is a competition between HS on the adjacent cell, HS in matrix and HS on the same cell, for interaction with the signaling components. It should be emphasized that HS interacts not only with the ligand but also with the receptor itself and the neuropilins.
The importance of cell-cell or cell-matrix interactions in RTK signal transduction has received relatively little attention. This is not true for the cadherins, molecules that interact in a trans-cellular fashion via homophilic binding, which have been carefully studied in this aspect. Suyama et al. demonstrated reduced internalization of FGFR with potentiated signaling, in the presence of N-cadherin (Suyama et al., 2002). No mechanism responsible for this phenomenon was described. However, it is tempting to speculate that the reciprocal binding of N-cadherins, which also interacts with FGFRs, is responsible for the decrease in internalization observed in the presence of N-cadherin. Also, VE-cadherin is known to regulate internalization of VEGFR-2 as demonstrated by (Lampugnani et al., 2006). When VE-cadherin is localized at junctions, VEGFR-2 is internalized at a lower rate. In the absence of VE-cadherin, VEGF-stimulation results in rapid internalization of the receptor into endosomes where it remains active (as measured by PLC-γ activity). This indicates that a prolonged localization at the plasma membrane not necessarily leads to enhanced signaling and that the outcome of receptor activation is highly dependent on the cellular context.

The duration of a ligand-bound receptor at the plasma membrane may be crucial for the outcome of the cellular response. When VEGFR-2 binds its ligand, the intracellular kinase domains will be brought in close proximity to their substrates and trans-phosphorylation of tyrosine residues will occur. The phosphorylated tyrosines will subsequently bind different molecules that each may initiate down-stream signaling that result in specific cellular responses (migration, proliferation, survival etc.). The cellular response is known to be dependent on the cellular context. Since the phosphotyrosine-binding partners exist at different concentrations and with varying affinities for their binding-sites, it is likely that this binding is time-dependent. This would cause certain pathways to be initiated faster than others, meaning that activation of the slower pathways requires persistent activation of the receptor. If such time-dependency is of biological significance to RTK signaling remains to be investigated.

In conclusion, this implies that cell-cell contacts are central to signal transduction and that care should be taken regarding cell confluency, and model setup. Furthermore, differences in quality as well as potency of signals may be missed when only one time point is analyzed.

In addition, matrix components and coreceptors are known to affect ligand/receptor affinities and possibly conformations which can modulate RTK signaling. HS/FGF/FGFR interaction is perhaps the most studied in this aspect. Heparin has been demonstrated to increase the ligand/receptor affinity and thereby decreasing the off-rate of the signaling complex (Ibrahim et al., 2004; Yayon et al., 1991). In accordance, certain tyrosines in the receptor intracellular domain were phosphorylated only when the ligand was introduced together with heparin (Lundin et al., 2003).
The data presented in paper II suggest that interactions with HS potentiate VEGFR-signaling and hence minimizes the amount of VEGF needed to mediate survival. As discussed previously, few antiangiogenic substances have proven valuable in the treatment of cancer. Notably, it has not been elucidated if the treatments fully inhibit VEGFR-2-mediated signaling. For even more efficient inhibition it might therefore be beneficial to include soluble molecules that could compete with cell- and matrix-bound HS for the interaction with the ligand/receptor. However, it is likely that HS or heparin fragments that interact with VEGF and/or its receptor also would intervene with other signaling cascades, with possible side-effects as a consequence.

Future perspectives
We do not know if the suggested mechanism for regulation of VEGFR-signaling by HS concerns also other heparin/HS-binding ligand/receptor systems, although this is tempting to suggest. Here, the PDGFRs and ligands are clearly of interest. The possible time-dependency for initiation of certain signaling cascades is very interesting and may add in yet another dimension in cell signaling. In the long run, concepts should be proven in vivo, and we will therefore try to generate chimeric embryos from vegfr2\(^{-/-}\) and Ndst1/2\(^{-/-}\) ESCs.

Paper III
Laminins regulate vascular lumen diameter

Aim
It is known that LMs may affect EC differentiation, attachment, migration and survival in an isoform-specific manner. We aimed to study vascular development, including stability, survival, lumen formation and pericyte recruitment, in a LM-deficient model. We also intended to analyze potential LM-isoform-specific effects on EC development by addition of purified LMs.

Results
Characterization of vascular basement membranes in wild type embryoid bodies revealed the presence of LMs (including LM-\(\alpha4\)- and LM-\(\alpha5\)-chain), collagen IV, HSPGs and fibronectin. We generated embryoid bodies, derived from ESCs null for the LM-\(\gamma1\)-chain, to study vascular development. It has been previously shown that no LM deposition occurs in the absence of
LM-γ1 and as a consequence, BMs fail to assemble (Li et al., 2002). Vascular development proceeded with mild defects in EC differentiation, which could be rescued by exogenous addition of LM-111. Immunostaining for LMs revealed only intracellular deposits in the absence of LM-γ1. There was however an increase in fibronectin deposition. In contrast, collagen IV was nearly completely lost from the vascular BM. VEGFA-165-induced vascular sprouting in a 3D collagen matrix was similar to wild type regarding length and number of vascular sprouts, as well as pericyte coating. Moreover, it was evident that LMs were produced and deposited from the stalk to the filopodia of tip cells at the front of invading sprouts. Furthermore, the kinetics of vessel deterioration was similar in wild type and LM-γ1 null embryoid bodies following VEGF withdrawal. The number of vessels with large (>36 µm) lumen diameter was increased 4-fold in the absence of LM-deposition, suggesting a regulatory function independent on pressure. We conclude that LM is deposited at all stages of angiogenesis and that this deposition is dispensable for vasculogenesis but required for fine-tuning of the 3D vessel; a mechanism independent of flow.

Discussion
Deletion of sleepy (analogous to lamc1 (encoding LM-γ1) in mouse) in zebra fish results in defective intersomitic sprouting, suggesting a defect either in differentiation or migration capacity (Parsons et al., 2002). From data presented in paper III it is evident that ECs can migrate and organize in the absence of LMs. It might be that LMs that normally cover the edges of the somites are required for guidance, and function as a substrate for EC migration. This type of strict patterning does not exist in the embryoid bodies. From paper III, it is apparent that LMs are not required for EC migration per se. Furthermore, pericytes are equally well attracted to ECs and disperse over the sprout length regardless of LM presence or absence. It is still possible that there is a redundancy between some of the LMs and fibronectin, suggesting that the increase in fibronectin levels in the absence of LM-γ1 could compensate for the lack of LMs. In this paper we show that vessels grow wider in the absence of LM deposition although no flow exists. It is likely that the introduction of flow and pressure would add on to this effect.

Future perspectives
We would like to investigate the mechanism responsible for the increase in vessel diameter using different matrices and integrin-blocking antibodies. Another interesting pursuit would be to generate animals from the lamc1−/− ES cells by tetraploid aggregation, to investigate the impact of pressure and flow on vessels lacking a BM.
Paper IV
Building blood vessels: Stem cell models in vascular biology

Aim
We wished to summarize the current state of the use of embryonic stem cell differentiation as a model for vasculo- and angiogenesis, and to discuss advantages and shortcomings in relation to other systems.

Results
We conclude that the embryoid bodies constitute a suitable model for the study of vascular development. Furthermore it allows examination of signal transduction with close resemblance to the in vivo context.

Future perspectives
We will continue to refine the techniques for live imaging in the embryoid bodies. To facilitate this, we will use lentiviral-mediated introduction of various fluorescent reporters for cell lineage tracking. Moreover, we plan to generate stable cell lines from embryoid body cultures to allow expansion of certain cell types.
Concluding remarks

The intention of this introduction has been to present facts and data that are closely related to papers I-IV. The number of processes involved in angiogenesis is enormous, only a fraction could therefore be represented in this thesis.

The studies presented in this thesis have provided some answers, but also generated many questions concerning the meaning of the interplay between the molecules involved in angiogenesis. It will be very exciting to see what the scientific “evolution” will bring in the future.
Sammanfattning på svenska


Blodkärl är uppbygga av endotelceller samt glatta muskelceller som är förankrade i ett basalmembran bestående av ett nätverk av bl a lamininer, kollagen IV, fibronectin och heparansulfatproteoglykaner (HSPG). Ett antal vanter (isoformer) av laminin har påvisats i kroppen, men olika isoformers eventuella roll i utvecklingen av det vaskulära systemet har i stort varit okänd. I arbete I visar vi att laminin-111, ensamt eller i samspel med den angiogena faktorn fibroblast growth factor-2, stimulerar mognadsprocessen av både isolerade endotelceller och endotelceller i embryonala stamcellskultur. I arbete III kan vi studera angiogenes i frånvaro av lamininer i basalmembranet och kan konstatera att lamininer inte är nödvändiga för angiogenes, men att de påverkar kärlens tjocklek.

HSPG är proteiner kopplade till långa oförgrenade sockerkedjor (heparansulfat) som finns i tusental på i stort sett alla celler i kroppen. Det är känt sedan tidigare att HSPG kan påverka cellsignalering genom att förändra molekylers affinitet till varandra. I detta arbete visar vi för första gången att sockrets position i förhållande till cellsignalingskomponenterna har en
avgörande betydelse för styrkan och varaktigheten av signalen via ett tyrosinkinasreceptorsystem. Detta är en ny mekanism för reglering av cellsignalering och poängterar vikten av cell-cellkontakter i cellsignalering.

Vi vet ytterst lite om basalmembranproteiners betydelse för endotelcells-differentiering, polarisering samt stabilitet och regeneration. Vi visar i arbete III att endotelceller kan differentiera, polarisera och bilda lumen i frånvaro av samtliga lamininisofomer. Vidare ser vi att lamininer produceras i alla stadijer av den angiogena processen och att de reglerar kärldiametern oberoende av tryck.

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References


Harvey, W. 1628. Exercitatio Anatomica De Motu Cordis et Sanguinis in Animalibus.


Vittet, D., M.H. Prandini, R. Berthier, A. Schweitzer, H. Martin-Sisteron, G. Uzan, and E. Dejana. 1996. Embryonic stem cells differentiate in vi-


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine").