VEGFR-2 in Endothelial Differentiation and Vascular Organization

DAN EDHOLM
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, April 11, 2008 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

The cardiovascular system is the first functional organ to develop during embryogenesis. As the embryo reaches above a certain size, passive diffusion of gases and nutrients is no longer compatible with efficient growth. During embryogenesis, endothelial progenitor cells (angioblasts) are recruited from the primitive streak mesoderm and instructed to express vascular endothelial growth factor receptor-2 (VEGFR-2). This thesis examines the roles played by VEGFR-2 in the events through which a subpopulation of embryonic stem (ES) cells differentiate into endothelial cells and form the vasculature.

We show that ES cells gene targeted for VEGFR-2 (flk1-/-) develop immature endothelial cells (ECs), precursors, when differentiated in vitro as embryoid bodies (EBs). The flk1-/- ECs are unresponsive to VEGF-stimulation and consistently fail to form vessels. However, when co-cultured with wild type ES cells in chimeric EBs, flk1-/- endothelial precursors are guided by wild type ECs to form transient, chimeric vascular structures. Use of lentivirus in an add-back approach allowed reconstitution of VEGFR-2 expression in flk1-/- ES cells, and rescue of vasculogenesis and sprouting angiogenesis. We propose that recruitment to the endothelial lineage is not dependent on VEGFR-2, although this receptor tyrosine kinase appears indispensable for EC integrity, survival and for differentiation of endothelial precursors into mature ECs forming a stable vasculature.

Neuropilin-1 (NRPI) and heparan sulfate proteoglycans (HSPGs) function as co-receptors for VEGFs. The co-receptors influence, qualitatively and quantitatively, the intracellular signal relayed by VEGFR-2 but it is unclear how. We examined the contribution of NRPI to VEGFR-2 signaling in EB cultures, in zebrafish and in mice. Only NRPI-binding VEGFs were able to promote sprouting angiogenesis and formation of properly branched vascular tubes, supported by pericytes. Downstream of VEGFR-2/NRP1 activation, we identified recruitment of p38MAPK in signal transduction regulating sprouting angiogenesis.

Keywords: Embryonic stem, VEGFR-2, Flk1, KDR, VEGF, vasculogenesis, angiogenesis, embryoid body

Dan Edholm, Department of Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden

© Dan Edholm 2008

ISSN 1651-6206
ISBN 978-91-554-7133-0
urn:nbn:se:uu:diva-8579 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8579)
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


III. Edholm, D., Li, X., Claesson-Welsh, L. Fate and Performance of flk1-/- Endothelial Precursors. (manuscript)

Reprints were made with the permission of the publishers.
Contents

Introduction................................................................................................................................. 9
The Cardiovascular System ...................................................................................................... 10
  History of Discovery .............................................................................................................. 10
  Anatomy of the cardiovascular system .............................................................................. 12
  Structure of vessels .............................................................................................................. 13
Embryogenesis & Vascular Development .............................................................................. 14
  From zygote to primitive streak .......................................................................................... 14
  From mesoderm to blood islands ...................................................................................... 17
  Vasculogenesis .................................................................................................................... 18
  Cell-cell junctions ................................................................................................................ 19
  Hematopoiesis ..................................................................................................................... 21
  Angiogenesis ........................................................................................................................ 21
  Totipotency of stem cells ..................................................................................................... 22
VEGFs and VEGF-receptors ................................................................................................. 23
  VEGF ....................................................................................................................................... 23
  VEGF receptors .................................................................................................................... 24
    VEGFR-2 .............................................................................................................................. 24
    VEGFR-1 .............................................................................................................................. 26
    VEGFR-3 .............................................................................................................................. 26
  Co-receptors ........................................................................................................................ 26
    Heparan sulfate .................................................................................................................. 27
    Neuropilins ........................................................................................................................ 28
Present Investigations .............................................................................................................. 29
  Paper 1: Lentiviral rescue of vascular endothelial growth factor receptor-2 expression in flk1-/- embryonic stem cells shows early priming of endothelial precursors ........................................... 29
  Paper 2: Neuropilin-1 in Regulation of VEGF-Induced Activation of p38MAPK and Endothelial Cell Organization .................................................................................................................. 31
  Paper 3: Fate and Performance of flk1-/- Endothelial Precursors ........................................ 33
Concluding Remarks and Future Perspectives ...................................................................... 36
Acknowledgements .................................................................................................................. 39
References ................................................................................................................................. 41
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM</td>
<td>Aorta-gonad-mesonephros region</td>
</tr>
<tr>
<td>E#</td>
<td>Embryonic day, number of days post-coitum</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonal stem</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</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>Flk1</td>
<td>Fetal liver kinase-1 (VEGFR-2 in mouse)</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase domain receptor (VEGFR-2 in human)</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NRP</td>
<td>Neuropilin</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Octamer-4</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1 (also CD31)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B (Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase Cγ</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>SCL/Tal-1</td>
<td>Stem cell leukemia / T-cell acute lymphocytic leukemia protein</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology-2</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TSAd</td>
<td>T-cell-specific adapter (also VRAP, Lad)</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor (also Flk1 and KDR)</td>
</tr>
<tr>
<td>Wnt-3</td>
<td>Wingless/Int-3</td>
</tr>
</tbody>
</table>
This thesis examines the process through which embryonic stem cells differentiate into endothelial cells and how these cells organize themselves into blood vessels, a development labeled vasculogenesis. In particular, the role played by vascular growth factor receptor-2 (VEGFR-2) has been experimentally addressed; both during vasculogenesis and in subsequent and ongoing remodeling of existing vasculature, a process denoted angiogenesis.

The understanding of these processes will be essential for the promising field of tissue engineering. Future techniques may permit production and transplantation of complete organs or ways to direct a patient’s own cells into formation of new tissues. For any such treatment, control of the vasculature will be imperative for success. In addition, very few ailments lack a vascular component and few treatments lack a vascular perspective. Knowledge of the biological processes that control the vasculature has an unlimited potential to empower the field of medicine with new treatments.
The Cardiovascular System

Tissues constantly consume oxygen and nutrients in metabolic processes that generate waste materials, which become toxic to the cell if not removed. The cardiovascular system solves these problems by allowing transports within the animal body. Gases, nutrients, metabolites and hormones may be deposited in the blood by one organ and received and processed by another, providing means for tissue specialization and communication. Immune system components may use the cardiovascular system for deployment at sites where pathogens are encountered. In addition, by controlling the flow of blood, the cardiovascular system is important as a thermal regulator.

History of Discovery

Many attempts to understand the function of the cardiovascular system have been made throughout history. In the 2nd century AD, the Greek physician Galen proposed that venous blood originates in the liver and arterial blood in the heart. From these organs the two substances flows via vessels to tissues where they are mixed and consumed. These ideas dominated both Europe and the Middle East. The fall of the Roman Empire brought an era of scientific stagnation in Europe while Islamic scholars conserved and continued work on the Greek ideas. In 1242, the Arabian physician Ibn al-Nafis became the first to correctly describe the pulmonary circulation (Al-Dabbagh 1978). This finding challenged Galen’s ideas, but it is uncertain to what extent this critique spread among European scholars. With the Renaissance, Europe revived its interest in science through the Arabic translations of the Greek scholars. The teachings of Galen prevailed and formed the foundation for blood-letting as a remedy of illness. Then in 1552, the Spanish physician Miguel Serveto rediscovered the pulmonary circulation and this was experimentally confirmed in 1559 by the Italian surgeon Realdo Colombo.

Half a century later, William Harvey became the first to correctly describe the systemic circulation (1628). Soon after, French Jean Pecquet correctly described the flow of the lymphatic vasculature in animals (1651). An intense priority dispute then ensued between Danish Thomas Bartholin (1652) and Swedish Olof Rudbeck (1653) on who should be credited for the discovery of lymphatics in humans. Marcello Malpighi discovered capillaries in 1661.
Table 1. References to History of Discovery Chapter.

<table>
<thead>
<tr>
<th>Scholar</th>
<th>Publication</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibn al-Nafis</td>
<td>Commentary on Anatomy in Avicenna’s Canon</td>
<td>1242</td>
</tr>
<tr>
<td>Miguel Serveto</td>
<td>Christianismi Restitutio</td>
<td>1552</td>
</tr>
<tr>
<td>Realdo Colombo</td>
<td>De Re Anatomica</td>
<td>1559</td>
</tr>
<tr>
<td></td>
<td>Exercitatio anatomica de motu cordis et sanguinis in animalibus</td>
<td></td>
</tr>
<tr>
<td>William Harvey</td>
<td>Exercitatio anatomica de motu cordis et sanguinis in animalibus</td>
<td>1628</td>
</tr>
<tr>
<td>Jean Pecquet</td>
<td>Experimenta Nova Anatomica</td>
<td>1651</td>
</tr>
<tr>
<td></td>
<td>De lacteis thoracis in homine brutisque nuperrime observatis</td>
<td>1652</td>
</tr>
<tr>
<td>Thomas Bartholin</td>
<td>Nova excercitatio anatomica, Exhibens ductus hepaticos aquosos et vasa glandularum serosa</td>
<td>1653</td>
</tr>
<tr>
<td>Olof Rudbeck</td>
<td>De Pulmonibus</td>
<td>1661</td>
</tr>
<tr>
<td>Marcello Malpighi</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** *Anatomia del corpo humano* by Juan Valverde de Amusco. A flayed cadaver holds his skin in one hand and a dissecting knife in the other. The skin’s distorted face has the appearance of a ghost or a cloud, suggesting that spirit has been separated from, or peeled off of, the fleshy inner man. Copperplate engraving, Rome 1559. National Library of Medicine, Rome. Public domain.
Anatomy of the cardiovascular system

The cardiovascular system encompasses all the vessels that propel and contain blood within the animal organism. Blood is a fluid tissue consisting of suspended cells and chemical compounds in a water solvent. The cells include erythrocytes, platelets, leukocytes, lymphocytes and bone marrow-derived stem cells. The water phase of blood is denoted plasma and carries salts, gases, proteins, sugars, lipids and other compounds.

Figure 2. A) Schematic representation of the pulmonary and systemic circulation. B) Cartoon showing blood-flow through the capillary bed. Images adopted from U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program (public domain).

The heart is a muscular pump that maintains circulation of blood through the vasculature. The right side of the heart collects the deoxygenated blood returning from the tissues, and pumps this blood via the pulmonary circulation through the capillaries of the lungs. Here gaseous exchange, between the blood and the inhaled air of the lung alveoli, oxygenates the blood and clears it of dissolved carbon dioxide. The pulmonary circulation is completed as oxygenated blood is collected by the left atrium of the heart. In the systemic circulation, the left ventricle pumps oxygenated blood via arteries and arterioles into the capillaries of all tissues. The thin capillary vessels have diffusion-access to all tissues and thus grant exchange of molecules between blood and tissue. The oxygen-depleted blood is returned to the heart via venules and veins and this closes the systemic circulation (See figure 2).

The closely related lymphatic vasculature serves to drain tissues from excess fluid, extravasated proteins and cells, and recycles this fluid called lymph to the systemic vasculature (see figure 3).
Structure of vessels

A mono-layer of endothelial cells (ECs), sitting on a basement membrane, lines the lumen of all blood and lymph vessels. Cells of the endothelium are joined to each other by intercellular connectors called tight junctions and adherens junctions. This prevents unregulated leakage of blood cells and proteins to surrounding tissue.

Lymphatic endothelium is referred to as discontinuous because lymphatic endothelial cells are arranged in an overlapping manner that produces valve-like structures between the cells of the layer. These valves allow fluid to enter the vessel from the surrounding tissue while preventing backflow.

Arteries have a large diameter and serve mainly to regulate and sustain pressure and transport blood to tissues. Connective tissue, elastic fibers and layers of smooth muscle cells surround the arterial endothelium for support against the high pressure. Arteries branch out into thinner arterioles that in turn divide into yet thinner capillaries (5-15 μm in diameter). The capillary bed, also referred to as the microcirculation, contacts almost all tissues and allows molecules to be exchanged between the tissues and the blood.

The supporting pericytes are mesenchymal smooth muscle cells that wrap around small vessels and regulate blood flow. The capillary bed feed into venules that converge into larger veins that have less coating of elastic fibers and smooth muscle cells than arteries. Venous blood pressure is low therefore veins contain valves that prevent backflow. In addition blood flow is aided by the skeletal-muscle pump or the venous pump, in which muscles in the legs contract to squeeze the veins, forcing the blood onwards towards the heart.

Figure 3. Cartoon showing how the vessels connect. Blood enters the capillary bed from the arterial side (red) and leaves via the venules (blue). Excess liquid is drained from the tissue by the one-way directed lymph vessels (green). Image adopted from U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, (public domain).
The cardiovascular system is the first functional organ to develop during embryogenesis. As the embryo grows above a certain size, passive diffusion of gases, nutrients and wastes is no longer compatible with efficient growth. The mouse embryo must develop a functional cardiovascular system by E9 in order to survive (Copp 1995). Through a relatively rapid and complex procedure, and involving a plethora of morphogens, growth factors and signal receptors of which a few examples are given by this text, the cardiovascular system is developed as the principle body plan is being patterned.

For practical reasons, mouse development has received most of the attention in the efforts to elucidate the mechanics of mammalian embryogenesis. This thesis will consequently discuss mouse development unless otherwise indicated. Although the processes and the molecules involved in mammalian embryogenesis are principally similar between species, there are some features that differ. Research on the fruit fly *Drosophila melanogaster*, the amphibian *Xenopus laevis* and zebrafish *Danio rerio* has contributed greatly to the discovery of many morphogens and to the assertion of their roles during development.

### From zygote to primitive streak

The fertilized egg is totipotent; it has the potential to generate all cells composing the embryo as well as the extra-embryonic tissues that support its growth in the uterus wall. The zygote divides through successive cleavages until a fluid-filled cavity (blastocoel) forms in the center of this spheroid of cells. At this stage, referred to as blastula or blastocyst (E3-3.5), the embryo consists of an outer cell layer labeled the trophectoderm and attached to its interior is a clump of cells identified as the inner cell mass (ICM) (see figure 4). The trophectoderm expresses caudal type homeobox-2 (CDX2), a prerequisite for placental development (Strumpf 2005). The trophectoderm will give rise to the extra-embryonic ectoderm, the ectoplacental cone and the giant cells, while the embryo itself and all other extra-embryonic lineages develop from the ICM. Fibroblast growth factor-4 (FGF-4) is expressed by the ICM in order to sustain normal trophectoderm development (Goldin 2003).
Figure 4. A) Schematic representation of the blastocyst just before implantation in the uterus wall. B) Microphotograph of blastocyst. Images adopted from Open source.

The blastocoel-facing cells of the ICM differentiate into primitive endoderm that will later form the visceral and parietal endoderm of the yolk sac. The remaining ICM is referred to as the epiblast and will generate all lineages of the fetus, including the germ-line (Gardner 1978). During implantation (E4.5), the trophoblasts lyse and invade the endometrial epithelium, bringing the blastocyst into place within the endometrial interstitium. The trophoblasts will continue migration inwards and form lacunae in conjunction to the maternal blood vessels, the seed for uteroplacental circulation.

During gastrulation (E6.5), cells of the epiblast will separate into either of the three definite germ layers of the vertebrate; the ectoderm (later forming skin and nervous system), the mesoderm (muscles, cardio-vasculature, connective tissues, excretory organs and gonads) and the endoderm (epithelium of the gut). Recent studies have revealed that tissue patterning within the epiblast is observable on a molecular level before implantation (reviewed by Tam 2007). Gastrulation starts with a thickening of the epiblast, forming a vertical furrow along the side of this cup-shaped entity (see figure 5). This thickening is located along the posterior side of the embryo, prompting the embryo’s anteroposterior axis. The bulge is caused by a directed migration of epiblast cells through a structure called the primitive streak. The migrating cells settle in the space between the epiblast and the visceral endoderm and form the mesoderm and the definite endoderm in a spatial-temporal directed fashion (Kinder 1999). The descendants of the non-migratory epiblast cells will form the ectoderm. This text will follow the fate of the mesoderm; however the induction of endoderm has been reviewed elsewhere (Shivdasani 2002).
Several gene-products are implicated in the formation of the primitive streak. The transcriptional activator Brachyury was initially discovered due to spontaneous mutation (Dobrovksaia-Zavadksaia 1927). It has been noted as a mesodermal marker gene necessary for proper gastrulation (Herrmann 1990; Smith 1991). Recent studies reported that both Brachyury and Wingless/Int-3 (Wnt-3) were expressed in the posterior visceral endoderm (E5.5), prior to expression in cells of the epiblast primitive streak (Perea-Gomez 2004; Rivera-Perez 2005). The first sign of the anteroposterior axis within the embryo was expression of Wnt-3, and indeed proper expression proved necessary for this polarity (Barrow 2007). In the absence of Wnt-3, the epiblast will fail to differentiate due to continued expression of the homeodomain transcription factor Octamer-4 (Oct-4), which maintains pluripotency (Liu 1999).

Many morphogens are involved in gastrulation and induction of mesoderm. For instance, lack of the secreted Transforming growth factor-β (TGF-β)-related factor Nodal results in premature epiblast differentiation into neural cell types and an absence of primitive streak formation (Iannaccone 1992; Brennan 2001; Camus 2006). Another secreted TGF-β-related factor, Bone morphogenetic protein-4 (BMP-4) is crucial for formation of mesoderm (Winnier 1995). FGF-2 and the FGF-receptor-1 (FGFR-1) (Deng 1994; Yamaguchi 1994; Deng 1997; Faloon 2000; Ciruna 2001) and Activin A have been implicated in mesodermal induction at this stage (Johansson 1995). In
amphibian *Xenopus laevis*, Nodal and Activin A were able to induce mesoderm when ectopically expressed in ectodermal explants (Heasman 1997).

**Figure 6.** Vasculogenesis and blood islands. A) Blood island - the peripheral cells differentiate into endothelial cells and elongate (green) while central cells differentiate into primitive hematopoietic cells and detach. B) Endothelial cells encapsulate the primitive hematopoietic cells of blood islands. C) Although angioblast settle and form a primitive vascular plexus over the entire visceral endoderm of the yolk sac – the emergency of blood islands is restricted to the blood island region (boxed). D) Fluorescence microphotograph depicting angioblast settlements (green) and hematopoietic settlements (red). Images adopted from Open source.

**From mesoderm to blood islands**

In the beginning of the 20th century, Wilhelm His found that endothelial cells and clusters of primitive nucleated hematopoietic cells always appeared together during embryogenesis. Thus he proposed that common ancestors, whom he named angioblasts, gave rise to both lineages (His 1901). Through experiments with developing chicken embryos, Florence Sabin managed to show that angioblasts were derived from the mesoderm and that these progenitors migrated to sites in the visceral endoderm of the yolk sac where they proliferated into clusters. The outer cells of these clusters developed into endothelial cells, while the inner cells progressed to form blood islands or hematopoietic cell clusters (Sabin 1917, 1920). The pluripotent mesodermal precursor later became known as the hemangioblast (Murray 1932) and
the concept has been validated in more recent studies (Haar 1971; Pardanaud 1989; Choi 1998; Huber 2004).

Brachyury-positive hemangioblasts / angioblasts will appear in the posterior primitive streak and start to express vascular endothelial growth factor–receptor-2 (VEGFR-2). This tyrosine kinase receptor enables the hemangioblasts / angioblasts to migrate in a chemotactic fashion against a gradient of its cognate ligand, VEGF (Cleaver 1998; Ash 2000; Hiratsuka 2005). VEGF is produced by the tissues that attract hemangioblasts (Ramirez-Bergeron 2006). The mechanisms of VEGF-VEGFR-2 signaling will be examined in more detail in the next chapter. In addition to VEGF, other growth factors, morphogens, extracellular matrix components and cell-cell interactions may provide guiding cues for migrating hemangioblasts / angioblasts.

As VEGFR-2-positive hemangioblast /angioblasts migrate to the visceral endoderm of the yolk sac, they start expression of the basic-loop-helix transcription factor Stem cell leukemia / T-cell acute lymphocytic leukemia protein (SCL/Tal-1) by E7.5 (Elefanty 1999), which is critical for hematopoietic development (Shivdasani 1995; Porcher 1996). SCL/Tal-1 is not essential for endothelial development but loss of its expression will severely reduce the EC population (Visvader 1998).

Hemangioblasts / angioblasts settle in the visceral endoderm of the yolk sac as polyclonal proliferating clusters (Ueno 2006). At E8.0, the central cells of these clusters accumulate hemoglobin and lose their attachments, while the outer cells are flattened to form an endothelial outer layer (Haar 1971). ECs are formed as VEGFR-2-positive precursor cells down-regulate SCL/Tal-1, and the SCL/Tal-1-positive hematopoietic cells are generated through down-regulation of VEGFR-2 (Chung 2002).

Although the entire visceral endoderm of the yolk sac is colonized by angioblasts, development of hematopoietic cells from hemangioblasts is confined to the blood island region as depicted in figure 6. It has been proposed that angioblasts and the pluripotent hemogenic hemangioblasts may be two separate precursor cell-types, with a shared mesodermal origin (Kinder 1999; Ema 2003). Another possibility is that they both belong to a single precursor population that may produce variable outcomes dependent on cues from the tissues they settle in. In addition it has been proposed that hemangioblasts may contribute to vessel-supporting smooth muscle cells, when stimulated with platelet-derived growth factor-BB (Yamashita 2000) or if expression of SCL/Tal-1 is down-regulated (Ema 2003).

**Vasculogenesis**

Vasculogenesis is defined as the process through which blood vessels are formed *de novo* from precursor cells. The first step of the process is the re-
entification of endothelial cells which has been described above. Although most research has focused on vascularization of the yolk sac, a similar process occurs simultaneously within the embryo proper (reviewed by Yancopoulos 2000). Mesodermal angioblasts migrate to intra-embryonic sites and seed the major vessels.

Next, vasculogenesis also encompasses the process whereby the generated endothelial cells connect to each other and form a continuous layer that surrounds a vessel-lumen, a process also referred to as tubulogenesis. Clusters of ECs migrate in a coordinated fashion and connect to each other, thus forming a continuous network of primitive vessels or a vascular plexus. This process involves chemotactic migration and is guided by VEGF and dependent on endothelial expression of VEGFR-2 as described previously. The rough vascular plexus is thereafter pruned and remodeled into vessels of various dimensions through a process named angiogenesis, which will be described later. While large vessels such as the dorsal aorta and the cardinal vein form through vasculogenesis, the smaller intersomitic vessels develop from sprouting angiogenesis in zebrafish (Childs 2002).

ECs commit to an either arterial or venous fate before the onset of circulation. This is achieved through exclusive expression of the reciprocally repulsive ephrinB2 (arterial) or its receptor EphB4 (venous) and may indicate stronger genetic patterning component of vasculogenesis than initially expected (Wang 1998). Likewise, neuropilin-1 is mainly expressed on arterial ECs while neuropilin-2 is found only on venous ECs (Herzog 2005). Circulation initiates at E8.5 and becomes fully functional by E10 (McGrath 2003). The direction of flow and shear forces will govern arterial-venous identity by overriding previous patterns (le Noble 2004).

**Cell-cell junctions**

Cells connect to each other through intercellular connections such as tight junctions, adherens junctions, gap junctions and desmosomes (Farquhar 1963) (reviewed by Vestweber 2000; Tsukita 2001; Aijaz 2006). The organization and status of these intercellular connections between ECs affect vascular permeability - the leakage of solutes from the blood to the surrounding tissues, and EC mobility. In addition, cell-cell junctions may convey signals such as survival, apoptosis, migration, polarization (reviewed by Sakisaka 2007).

Organized tight junctions polarize the cell by blocking transmembrane proteins and membrane lipids from diffusing freely between the apical and basolateral regions of the plasma membrane (Dragsten 1981). Tight junctions are formed by transmembrane proteins that connect the plasma membrane of one cell with that of another. The proteins; occludin (Furuse 1993), claudins (Furuse 1998) and JAMs (Martin-Padura 1998), bind as trans-
connecting homodimers. The intracellular domains of these proteins interact with ZO-1 whereby the junction is connected to the actin cytoskeleton (Bazzoni 2004).

Adherens junctions constitute the adhesive apparatus between neighboring cells (Gumbiner 1996). The adhesion is produced by trans-connecting homodimers of Ca\(^{2+}\)-dependent transmembrane glycoproteins called cadherins (reviewed by Vestweber 2008) and nectins (Takai 2003). Nectins interact not only with nectins but also with other Ig-like molecules and may transfer signals (reviewed by Sakisaka 2007). Catenins mediate the connection between the cadherin and the intracellular microfilaments (Boller 1985). Endothelial adherens junctions are built mainly with EC-specific vascular endothelial cadherin (VE-cadherin) which are connected intracellularly via \(\alpha\) and \(\beta\)-catenin to actin microfilaments (Lampugnani 1995; Jou 1995). ECs also express N-cadherin but it is not localized to these junctions (Navarro 1998).

Desmosomes are made up of other cadherins; namely heterodimers of desmogleins and desmocollins. These are linked to the intermediate filament cytoskeleton via interactions with plakophilins and desmoplakin. Desmosomes are designed to support heavy mechanical stress but they have also been found to signal. Desmosomes connect to vimentin intermediate filaments in ECs although they are not as prevalent between ECs as among epithelial cells (reviewed by Green 2000).

The organization of the cell-cell junctions between ECs vary with the type of vessel the cells are forming. In ECs of normal microvasculature, the tight junctions are overlapped by adherens junctions in a manner that allows for a higher degree of permeability (Ruffer 2004) compared to that of brain microvasculature. In brain microvasculature, tight and adherens junctions are spatially separated along the apico-basolateral axis of the cleft between cells. With the addition of desmosomes this produces a high trans-endothelial electric resistance which upholds the blood-brain barrier (Ruffer 2004).

The transmembrane glycoprotein, Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is expressed by almost all cells of the vascular compartment; ECs, platelets, granulocytes and leukocytes (Favaloro 1989). This Ig-like proteoglycan forms homodimers in trans and mediates leukocyte transmigration through which leukocytes exit blood and enter tissues. Expression on both the extravasating leukocyte and on the surface of the paracellular cleft between the endothelial cells of the blood vessel aids this migration (reviewed by Woodfin 2007). In addition, extravasating leukocytes depend on CD31 for migration through the basement membrane of the vessel (Wakelin 1996).

CD31 is tyrosine-phosphorylated by Src-family kinases and serine-phosphorylated by Protein kinase C (PKC). It has been reported that CD31 may mediate F-actin assembly via \(\beta\) and \(\gamma\)-catenin. Matsumura et al. proposed that CD31 and Vascular endothelial cadherin (VE-cadherin) may con-
trol tubulogenesis of ECs (Matsumura 1997). CD31 has also been reported to mediate cell survival signals (for review see Newman 2003).

**Hematopoiesis**

During the hematopoietic activity of the blood islands, hematopoietic progenitor cells will down-regulate VEGFR-2 and form primitive nucleated erythroblasts in a process referred to as primitive hematopoiesis (Chung 2002). CD41 serves as the earliest marker of primitive erythroid progenitor cells of the yolk sac at E7 (Ferkowicz 2003). These cells express both embryonic and adult hemoglobins in a temporal pattern following maturation of the blood stream (Leder 1992). Multipotential hematopoietic cells are later found both in the yolk sac and in the aorta-gonad-mesonephros region (AGM) as early as E8.5-9.5 (Godin 1995; Medvinsky 1996).

Definitive hematopoiesis starts around E10.5 and is mostly an intra-embryonic event, although it may be difficult to ascertain the level of extra-embryonic involvement (Palis 2001). Definite hematopoietic precursor cells are found in the AGM, where after they colonize the fetal liver at E11.0 (Muller 1994). Sometime between E14-16 the bone marrow starts to serve as the site of hematopoiesis (Godin 1999). GATA-1 and -2, Lmo2 and Runx1 are selectively implicated in hematopoietic precursor cell development (Tsai 1994; Warren 1994; Fujiwara 1996; Yamada 1998; Chung 2002 and Lacaud 2002).

**Angiogenesis**

The formation of new vessels from an existing vascular network may occur by two distinct processes; sprouting angiogenesis and intussusceptive angiogenesis.

The process of sprouting angiogenesis is regulated by growth factors, such as VEGF, and is initiated by proteolytic degradation of the basement membrane surrounding an existing vessel and a loosening of adhesion to neighboring cells. Endothelial cells are then able to escape the existing vessel and form an invasive sprout, connected at its base to the original vessel. The sprout is composed of proliferating stalk cells and a path-finding tip cell, sensing chemo tactic growth factor gradients by probing its surroundings with filopodia extensions (Gerhardt 2003). As it extends, the sprout promptly recruits supporting pericytes and produces an encapsulating basement membrane. Extracellular fusion of vacuoles produces a connecting lumen within the sprout, starting at the vessel base (Lawson 2002). Capillary sprouts finally interconnect and fuse with each other as they are spatially
gathered at the crest of the gradient. Sprouting angiogenesis has an invasive capacity to vascularize a previously non-vascularized space and is implicated during embryogenesis, as well as in various pathological conditions such as cancer or rheumatoid arthritis.

Intussusceptive angiogenesis refers to the splitting of an existing vessel, a mode of angiogenesis far from being as extensively studied. Insertion of a transluminal tissue pillar through a vessel brings opposite endothelial cells of the same vessel in direct contact with each other. This contact induces the endothelial cells to connect, forming a hole through the vessel (Caduff 1986; Patan 1992). As growing tissue expands and enlarges this hole, the vessel may be split like a zipper into two. Possibly, any vessel may be subjected to a constant tug-of-war between surrounding tissues and their intrinsic oxygen and nutrient requirements. As the tension on a vessel becomes equally strong in two directions, the vessel may flatten out along the plane of tension. This may initiate or alleviate the insertion of a tissue pillar along an axis perpendicular to the tension-plane. Intussusceptive angiogenesis has been estimated to play a major part during vascularization of the lung (Burri 2004).

Cells will normally only proliferate in proximity to vessels, where there is sufficient supply of oxygen and nutrients. Through the process of intussusception, vessels will expand in tune with the tissue and vice versa. Intussusceptive angiogenesis has been shown accountable for angiogenesis in tissue expansion. There is of yet no mechanism that describes how intussusceptive angiogenesis could vascularize non-vascularized tissues. Likely, both sprouting angiogenesis and intussusception play significant roles in different contexts during embryogenesis.

**Totipotency of stem cells**

As the blastocyst-embryo is struggling for implantation, the maternal endometrium expresses a cytokine named leukemia inhibitory factor (LIF) which sustains the status of totipotency in the cells of the embryo. In mice but not in humans, LIF maintains pluripotency through activation of the transcription factor STAT3 (Schuringa 2002). In order to maintain the totipotency in the laboratory, murine embryonic stem (ES) cells are cultured on a monolayer of mouse embryonic fibroblasts in the presence of LIF. Pluripotency may be maintained in culture for many days in the absence of embryonic fibroblasts if LIF is present. Embryonic fibroblasts secrete factors such as Activin A, which may sustain pluripotency for several weeks if supplemented to serum-containing medium (Beattie 2005).
VEGFs and VEGF-receptors

VEGF

VEGF has received much attention since it was identified by independent groups (Senger 1983, Ferrara 1989 and Ferrara 1991). The members of the VEGF family (VEGF-A, -B, -C, -D and Placenta growth factor (PIGF)) and virus encoded VEGF-E stimulate cellular responses by binding to and activating cell-surface expressed VEGF-receptor tyrosine kinases (reviewed by Robinson 2001, Cross 2003, Olsson 2006). VEGF-A is the main regulator of blood vasculature and its mRNA is alternatively spliced to produce isoforms with variable ability to bind co-receptors. Gene inactivation of even a single vegfa-allele was embryonic lethal due to vascular defects at E11-12 of mouse development, and homozygotes for the knock-out allele, generated through tetraploid aggregation, died at around E10 (Ferrara 1996, Carmeliet 1996). This implies a stringent dose-dependency of VEGF-A. In addition, vegfa<sup>120/120</sup> mice - engineered to exclusively express the VEGF-A<sub>120</sub>-splice variant - were lethal shortly after birth due to severe vascular defects involving internal bleeding and vascular malformations (Carmeliet 1999).

![Figure 7](image-url). The VEGFs and their ability to bind cognate VEGFRs. Arrows specify direct binding partners and a dashed line indicates that the interaction requires proteolytic cleavage of the ligand. Neuropilin-1 and -2, and heparan sulfate proteoglycans (HSPGs) are VEGF co-receptors. Courtesy of Lars Jakobsson.
The most abundant isoform VEGF-A\textsubscript{165}, commonly referred to as VEGF, has affinity for both heparan sulfate proteoglycans (HSPGs) and neuropilin-1 (NRP1) which will be discussed in more detail below. Expression of VEGF-A is regulated by the availability of oxygen. As a cell experiences hypoxia, the transcription factor HIF-1 accumulates and binds to a hypoxia-responsive element in the \textit{vegfa}-gene, initiating its expression. VEGFs are secreted as disulfide-bridged homodimers and selectively bind VEGFRs according to the scheme depicted in figure 7.

### VEGF receptors

VEGFRs -1 and -2 have an extracellular part consisting of 7 immunoglobulin-like domains while VEGFR-3 has the 5\textsuperscript{th} immunoglobulin-like domain substituted by a disulfide bridge. All VEGFRs pass the plasma membrane with a single transmembrane region which is followed by an intracellular chain that contains a split tyrosine-kinase domain. A VEGF dimer is able to bind two cognate VEGF receptors and this causes the receptors to come in contact with each other (Muller 1997). This proximity allows kinase domain-mediated reciprocal trans-phosphorylation of intracellular tyrosines which is referred to as activation of the receptors. The phosphorylated tyrosines serve as docking sites for various intracellular signal mediators, bound directly or via adaptors. Bound signaling molecules are regulated by the kinase activity of the receptor.

#### VEGFR-2

VEGFR-2 (Flk1 - fetal liver kinase - in mouse/ KDR - kinase domain receptor - in human) is recognized as a main regulator of vascular endothelial cells as well as of their precursors. It relays a multitude of signals affecting vascular permeability, migration, proliferation and survival. Tyrosines 951, 1054, 1059, 1175, and 1214 have been shown to be phosphorylated in human VEGFR-2 (reviewed by Matsumoto 2001); these correspond to 949, 1052, 1057, 1173 and 1212 respectively, in the mouse receptor. Targeted inactivation of the \textit{vegfr}-2-gene in mice is embryonic lethal at E9.0, resulting in an absence of hematopoietic and endothelial cells (Shalaby 1995).

Phospholipase C\textsubscript{γ1} (PLC\textsubscript{γ1}) becomes phosphorylated and active through its Src-homology-2- (SH2)-domain-mediated binding to phospho-tyrosine 1175 of human VEGFR-2 (see Figure 8) (Takahashi 2001). This signal is relayed via Protein kinase C (PKC) and Mitogen-activated protein kinase (p44/42-MAPK), also known as extracellular signal-regulated kinase-1 and -2 (ERK1/2), to stimulate cell proliferation. Alternatively, phospho-tyrosine 1175 may recruit Shb which regulates cell migration (Holmqvist 2004). Phospho-tyrosine 1175 has been associated with activation of phosphoinosi-
tol 3-kinase (PI3K) (Dayanir 2001) which may activate Akt / protein kinase B (PKB) and mediate survival. Knock-in mutation of tyrosine 1173 to phenylalanine is embryonic lethal at E9 of mouse gestation with similar effects as the knock-out (Sakurai 2005).

Phospho-tyrosine 951 recruits T-cell-specific adapter (TSAd, also called VEGFR-associated protein (VRAP), or Lad) (Spurkland 1998; Wu 2000). TSAd has been found to mediate migration in T-cells (Park 2007) and in ECs (Matsumoto 2005). The latter study identified TSAd -Src-interaction and suggested effects on vascular permeability. TSAd knockout mice were viable and developed apparently normally, but were defective in T-cell activation (Rajagopal 1999).

VEGF-mediated activation of p38MAPK has been reported to depend on phosphorylation of tyrosine 1214 of VEGFR-2, and this pathway is implicated in cell migration (Lamalice 2004; Lamalice 2006). Knock-in mutation of tyrosine 1212 to phenylalanine was not associated with observable defects in mice (Sakurai 2005).

**Figure 8.** Schematic of the intracellular signaling downstream of activated VEGFR-2. The numbers represent phospho-tyrosines of VEGFR-2, ovals correspond to signal mediators and boxes indicate outcomes associated with the various signal transduction pathways. DAG, diacylglycerol; EC, endothelial cell; 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; Shh, SH2 and β-cells; TSAd, T-cell-specific adaptor. Adapted from Olsson et al., 2006, © Maximillian Publishers Ltd.
When mixed with wild-type ES cells, flk1−/− (VEGFR-2-deficient) ES cells did not contribute to the vasculature or the blood of chimeric mouse embryos (Shalaby 1997). However, when flk1−/− ES cells were differentiated in vitro, both hematopoietic and endothelial progenitor cells were detected using the blast colony-forming cell assay (Schuh 1999). In vitro cultures of differentiating vegfa−/− ES cells revealed that cells expressing certain endothelial markers (VEGFR-2, CD31, VEGFR-1 and Tie-2) were formed although vasculogenesis was halted (Bautch 2000).

**VEGFR-1**

VEGFR-1 (Flt1) is expressed on endothelial cells, hematopoietic stem cells and monocytes and has a higher affinity for VEGF-A than VEGFR-2 (de Vries 1992; Sawano 1996). In addition, VEGFR-1 is activated by PlGF and VEGF-B. Targeted deletion of VEGFR-1 is embryonic lethal at E9 due to vascular malformation and an overproduction of endothelial cells (Fong 1995). The function of VEGFR-1 for endothelial cells remains somewhat secluded as stimulation produces only weak cellular responses, if any (Waltenberger 1994; Seetharam 1995). Surprisingly, deletion of the intracellular domain of VEGFR-1 (tyrosine kinase dead) (Flt1 tk−/−) is compatible with normal vascular development (Hiratsuka 1998). It is therefore generally believed that VEGFR-1 regulates VEGFR-2-activation by sequestering its ligand (VEGF-A). In fact, an alternatively spliced soluble form of VEGFR-1 (sVEGFR-1) has been found (Kendall 1993) that may support that interpretation. Outside of the endothelial lineage, VEGFR-1 is essential for VEGF-A-guided migration of monocytes (Barleon 1996).

**VEGFR-3**

VEGFR-3 (Flt4) is activated by VEGF-C and -D and is expressed on lymphatic endothelium where it induces proliferation, migration and survival (Makinen 2001). In addition, VEGFR-3 is expressed on embryonic blood vasculature and plays a regulatory role in the development of blood vessels during embryogenesis. Gene inactivation in mice is embryonic lethal at E10, resulting in cardiac failure and obstructed and disorganized larger vessels. Defects in vessel remodeling generated a leaky vasculature which resulted in a fluid-filled pericardial cavity prior to lymphatic development (Dumont 1998).

**Co-receptors**

VEGF activates VEGFR-2 through complex-formation and the participation of co-receptors in these complexes influence and direct the signaling out-
come (see figure 9). It has been suggested that such effects may be attributed to stabilization of the signaling complex or to affect third party mediators.

Heparan sulfate

Heparan sulfates are un-branched polysaccharide chains made up of disaccharide repeats which may be secreted or are attached to serine residues of certain proteins. The glycosylation takes place in the Golgi where the sugar chains also undergo modifications, such as sulfation of N-acetyl groups (reviewed by Esko 2001). Proteins with attached heparan sulfate are denoted heparan sulfate proteoglycans (HSPGs). Many ligand-receptor interactions are dependent or affected by the HSPGs. It has been proposed that HSPGs stabilize and take part in the ligand-receptor complex (Esko 2002). In fact, HSPGs restricted to participation in \textit{trans} produced a stronger activation of VEGFR-2 upon VEGF-stimulation, possibly by tethering the complex and preventing internalization and degradation (Jakobsson 2006).

\textbf{Figure 9.} VEGFR-2 activation is dependent on ligand and co-receptor complex. A) Only ligand (red box) produces weak activation of VEGFR-2. B) Ligand and receptor in complex with HSPG (core protein – green, HS-chain – blue) and neuropilin-1 (grey) is more stable, resulting in a stronger phosphorylation of major tyrosines (yellow discs marked “P”). C) When ligand and receptor form a complex with neuropilin-1 and a HSPG located on an adjacent cell the activation of the receptor is even stronger (as proposed by Jakobsson 2006). Adopted from "VEGF signal transduction in angiogenesis" by Kawamura H, Li X, Welsh M and Claesson-Welsh L. Chapter 17 in "Angiogenesis: An Integrative Approach from Science to Medicine" (Springer Verlag, eds Douglas Figg, Judah Folkman). In press.
Neuropilins

NRP1 and -2 are transmembrane glycoproteins that may interact with both certain VEGF isoforms and class 3 semaphorins (reviewed by Neufeld 2002). NRP1 plays an essential role in axon guidance; binding to Semaphorin-3A (Sema3A) induces the collapse of neuronal growth cones (He 1997). In addition NRPs functions as co-receptors for VEGF-A isoforms containing exon 7 (Soker 1997; Soker 1998; Soker 2002, Gluzman-Poltorak 2000 and Favier 2006) and to PlGF (Migdal 1998). Since VEGFR-2 binds to exon 3-4 of VEGF-A_{165} (Keyt 1996) it has been suggested that the three molecules form a signaling complex. NRP1 modulate VEGFR-2-signaling (Whitaker 2001). NRP1 has a cytoplasmic domain that was first believed not to signal directly (Fujisawa 1997), but a recent study proposed that the NRP1 c-terminal is required for NRP1-mediated angiogenesis via G-protein signaling (Wang 2006). Targeted NRP1 inactivation produce embryos that display severe vascular and neuronal defects (Kawasaki 1999) while ectopic over-expression give rise to excessive angiogenesis and embryonic lethality at E17.5 (Kitsukawa 1995). From a study done using zebrafish, knockdown of NRP1 resulted in formation of main vessels (dorsal aorta and cardinal vein) but interfered with formation of the intersomitic vessels (Lee 2002). NRP2 knock-out mice are viable but carry a reduction in lymphatics (Yuan 2002) and double knock-outs (NRP1 and NRP2) die at E8.5 (Takashima 2002).
Present Investigations

Paper 1: Lentiviral rescue of vascular endothelial growth factor receptor-2 expression in flk1-/- embryonic stem cells shows early priming of endothelial precursors.

Aim

flk1-/- embryos failed to develop both vasculature and hematopoietic cells (Shalaby 1995; Shalaby 1997), but in vitro differentiated ES cells from the gene-targeted animals produced both (Schuh 1999). In previous analyses of flk-/- EBs, we had noticed that flk-/- ECs failed to connect into a vascular plexus (Magnusson 2004). We were interested in the detailed role played by VEGFR-2 during these differentiation events. We decided to restore its expression using a lentivirus vector in order to shed some light on these processes.

Results

flk-/- ES cells were transduced with a lentivirus that conferred ectopic and constitutive expression of VEGFR-2. We noted that expression of the transgene was stable and at a level similar to that found in ECs from wild-type EBs. Furthermore, transgenic VEGFR-2 was activated upon VEGF-stimulation and the activation displayed wild-type-like kinetics. With all evidence pointing towards a functional transgenic receptor, we decided to analyze its potential to restore capillary plexus formation in EBs, a feature which was absent in flk-/- EBs. The 2-D vasculogenesis assay revealed that reconstituted EB cultures underwent normal vasculogenesis and responded to VEGF treatment by allocating ECs to the periphery. Overall, the endothelial response was similar to that of wild-type EBs, especially when the dose of VEGF was increased to 2.5 nM. Despite the ectopic expression of VEGFR-2, from day 0 of differentiation and onwards, no visible disturbance was noted for non-endothelial lineages.

We further studied the angiogenic potential of wild-type and reconstituted ES cultures by placing EBs in a 3-D collagen matrix in the presence of
VEGF. Thereby EBs formed vascular sprouts invading the surrounding collagen. In this 3-D assay, reconstituted EBs produced fewer endothelial sprouts than wild-type EBs, but the sprouts that were formed were of equal length. Even though an increased number of sprouts were formed by reconstituted cultures when the VEGF-concentration was raised, the frequency was still higher for wild-type cultures. We observed that the endothelial sprouts of reconstituted EBs had pericytes arranged as semi-detached aggregates in comparison to the smooth interplay between endothelial sprouts and pericytes presented by wild-type EBs.

FACS-analysis showed that the size of the VE-cadherin-positive EC-pool of EBs at day 14 was unaffected by the presence of VEGFR-2. Transcript analysis on the sorted ECs and the cells of the flow-through, confirmed that endothelial transcripts were confined to the endothelial pool, apart from the deregulated VEGFR-2 transcripts of reconstituted cultures. Thus, expression of VEGFR-2 neither rescued nor promoted endothelial recruitment of differentiating ES cultures. We also transduced wild-type ES cells with the VEGFR-2-expressing lentivirus. Interestingly, these cultures appeared normal in all assays tested, apart from a deregulated expression of VEGFR-2.

We mixed \( \text{flk1}^{-/-} \) and wild-type ES cells (9:1) to make chimeric EBs and found that VEGFR-2-expressing ECs of wild-type origin were favored in the vascular plexus when analyzed on day 10 of differentiation. In chimeras composed of reconstituted \( \text{flk1}^{-/-} \) and wild-type ES cells, the reconstituted \( \text{flk1}^{-/-} \) ECs were represented to a higher degree than \( \text{flk1}^{-/-} \) ECs.

**Discussion**

Our results showed that it is possible to use lentivirus to reconstitute \( \text{flk1}^{-/-} \) ES cells and that this indeed rescues vasculogenesis and angiogenesis. The data suggests that endothelial commitment occurs independently of VEGFR-2-expression. However, VEGFR-2 is crucial for proper maturation of the endothelium and formation of an interconnected vascular plexus. It remains unclear whether VEGFR-2 has any effect at all during mesodermal commitment to the endothelial lineage.

After being recruited, hemangioblasts express the lineage marker VEGFR-2. Secreted VEGF-gradients are sensed by VEGFR-2 of hemangioblasts / angioblasts as they migrate and settle in the various tissues where they subsequently will form vessels (Ash 2000; Hiratsuka 2005). Possibly, hemangioblasts require additional cues for survival and differentiation and these signals may only be received once the cells have reached the correct position. Since \( \text{flk1}^{-/-} \) ECs are unable to respond to this migration cue, it may explain why \( \text{flk1}^{-/-} \) ECs failed to contribute in vivo (Shalaby 1995; Shalaby 1997).
In order to explain why $flk1^{-/-}$ ES cells are able to form ECs in vitro, we propose two possibilities. In contrast to the spatially organized development of the embryo, the development of the EB cultures is spatially very chaotic, much like that of teratomas. If the environment that conveys survival and differentiation of hemangioblasts is randomly distributed, there should be no requirement to migrate. Secondly, fetal bovine serum is used during in vitro culture of cells. Components of the serum may aid and protect the development of $flk^{-/-}$ ECs.

**Paper 2: Neuropilin-1 in Regulation of VEGF-Induced Activation of p38MAPK and Endothelial Cell Organization**

**Aim**
Since VEGF ligands have different affinities for the three VEGF-receptors, they produce different effects in stimulated ECs. In addition, VEGF ligands may bind to co-receptors, which also influence the signaling outcome of activated VEGF-receptors. We were interested in the signaling influenced by the VEGF co-receptors Neuropilin-1 (NRP1) and heparan sulfate proteoglycans (HSPGs) on ECs.

**Results**
Porcine aortic endothelial (PAE) cells were transduced to express human VEGFR-2 and/or Neuropilin-1 (NRP1), and all three cell lines expressed similar level and composition of HS. VEGF-A$_{165}$ (bound both HS and NRP1), VEGF-A$_{121}$ (bound neither HS nor NRP1) and the pox virus derived VEGF-E$_{NZ2}$ (bound NRP1 but not HS) were all able to induce tyrosine phosphorylation of VEGFR-2 in PAE/VEGFR-2, NRP1 cells. VEGF-E$_{NZ2}$, and to a lesser degree VEGF-A$_{165}$ but not VEGF-A$_{121}$, produced VEGFR-2-NRP1 complexes. VEGF-A$_{121}$-NZ2 is a fusion protein composed of canine VEGF-A$_{121}$ and the NRP1-binding domain of VEGF-E$_{NZ2}$ (Scheidegger 1999). Stimulation of PAE/VEGFR-2, NRP1 cells with the chimeric protein produced VEGFR-2-NRP1 complexes, suggesting that complex formation is dependent on the ligand’s ability to bind NRP1. In PAE/VEGFR-2 cells, that expressed only low amounts of porcine NRP1, VEGF-E$_{NZ2}$ proved to be a weak stimulator of VEGFR-2, although the degree of stimulation with VEGF-A$_{121}$ was undiminished.

All three ligands induced EC proliferation in EBs. VEGF-A$_{165}$ and VEGF-E$_{NZ2}$ were able to promote organization of endothelial cells into a vascular plexus which suggests that these ligands were acting as migration
cues for ECs. VEGF-A_{121} produced only central sheet-like endothelial structures, indicative of proliferation. VEGF-A_{165} and less effectively VEGF-E_{NZ2}, but not VEGF-A_{121}, induced sprouting angiogenesis in EBs placed in collagen. Subcutaneously injected matrigel plugs were soaked with either ligand and the host's vascular invasion into the plug was assayed; VEGF-A_{165} induced well-branched and pericyte-covered vessels, VEGF-E_{NZ2} gave pericyte-clad vessels that were less branched but induction with VEGF-A_{121} produced detached ECs or at best poor vascular structures with disorganized support from pericytes. In contrast, matrigels soaked with the fusion protein VEGF-A_{121-NZ2} (able to bind NRP1) contained branched pericyte-clad vessels.

Morpholino knock-down of VEGF-A in zebrafish embryos prohibited formation of the caudal plexus and the intersomitic vessels. The latter vessels are normally formed through sprouting angiogenesis from the dorsal aorta and the posterior cardinal vein. These defects were fully rescued with injections of mRNA encoding vegfa_{165} or vegfe_{NZ2}, but not with mRNA encoding vegfa_{121}.

We used signal transduction antibody arrays to identify which signaling pathways that were differently activated by VEGFs due to binding NRP1. PAE/VEGFR-2, NRP1 cells were stimulated with either VEGF-A_{165} or VEGF-A_{121} and lysates were incubated with the antibody array and this was subsequently probed using phospho-tyrosine antibody. Activation of p38MAPK was found to be dependent on stimulation with NRP1-binding VEGF and this was confirmed by western blot. We used the p38MAPK-inhibitor SB203580 on VEGF-stimulated EBs in collagen and confirmed that sprouting angiogenesis indeed was suppressed. The inhibitor did not inhibit VEGFR-2 phosphorylation nor did it affect formation of VEGFR-2 expressing ECs in differentiating EBs. We also produced matrigel plugs soaked with both SB203580 and VEGF-A_{165} and analyzed the invasion of host vasculature. The effects were very similar to the use of VEGF-A_{121}; sprouting angiogenesis was repressed and the vessels formed displayed poor pericyte coverage.

Discussion
We show that NRP1 and NRP1-binding VEGF ligands are prerequisites for proper sprouting angiogenesis. Additionally, recruitment of pericytes to newly formed vascular sprouts was dependent on stimulation with VEGFs having affinity for NRP1, although this may have been an indirect consequence of compromised vascular sprouting. VEGFR-2-NRP1 complexes were formed exclusively upon stimulation with VEGFR-2 ligands with affinity for NRP1. The same ligands were required for VEGF-mediated activation of p38MAPK which suggests a connection between the presence of NRP1 in
the VEGFR-2 signaling complex and activation of p38MAPK. Inhibition of p38MAPK-activation prohibited sprouting angiogenesis similarly to stimulations with VEGFR-2 ligands lacking affinity for NRP1.

Earlier studies have implicated HS as a prerequisite for development of endothelial cells, vasculogenesis and sprouting angiogenesis (Gitay-Goren 1992; Jakobsson 2006). HS is implicated in signaling of a multitude of other growth factors such as FGF-2 and PDGF-BB (Yayon 1991, Rolny 2002). Therefore, lack of HS may produce developmental defects at an earlier stage which indirectly influences shortcomings observed at later stages of development. This complicates the assessment of what is a direct consequence of a compromised HS production.

Genetically engineered mice that exclusively express VEGFs in the form of VEGF-A_{120} (the mouse homologue of human VEGF-A_{121}) die around or shortly after birth due to defects in vascular development. Most major vessels are formed but remain dilated and poorly branched (Carmeliet 1999). Gene targeted inactivation of NRP1 in mice is embryonic lethal at E10.5-12.5 with similar vascular defects and in addition neural malformations. However, the authors noted no defects concerning endothelial cell development and stated that most major vessels were formed as in wild type animals (Kawasaki 1999). These findings suggest that a deficiency in NRP1-expression or in the repertoire of expressed VEGFs indeed causes vascular defects, but these occur at a later developmental stage compared to the defects generated by flaws in HS production.

Paper 3: Fate and Performance of flk1/- Endothelial Precursors

Aim
In paper 1 (Li 2007), we showed that flk1/- EBs gave rise to EC precursors but that these cells were unable to form vascular structures. We noted that flk1/- EC precursors were able to integrate with wild-type vasculature and wished to study this further. In particular we were interested in the kinetics of flk1/- EC participation and if possible what mechanisms that regulated this.

Results
Chimeric EBs were produced by mixing flk1/- and wild-type ES cells at a 9:1 ratio. Wild-type ECs dominated the vascular plexus from day 8, but flk1/- ECs integrated transiently forming a chimeric vasculature. With time the
flk1-/- ECs became completely depleted, suggesting that some sort of regulation was acting against ECs lacking VEGFR-2. This negative selection was observed also for EC precursors in EBs made entirely of flk1-/- ES cells.

Upon closer inspection it became clear that the endothelial markers separated with time in the flk1-/- EC population. The markers used were β-galactosidase (a reporter expressed from the flk1-locus, indicative of flk1-/- ECs), CD31 and VE-cadherin. This disintegration may reflect a collapse of the EC population that lack VEGFR-2. While CD31 normally covers the entire plasma membrane of cultured endothelial cells evenly, many flk1-/- ECs instead displayed a spotted distribution of this adhesion protein, often along the boundary to an EC neighbor. In addition to disappearing, flk1-/- ECs detached from wild-type vessels with time.

Staining for cleaved caspase-3 on day 8 revealed that apoptosis was more prevalent in flk1-/- ECs compared to wild-type ECs. We also found an unexpected high rate of apoptosis in vascular structures compared to other tissues, even in wild-type EBs when measured on day 8. The reconstituted flk1-/- ES cells, described in paper 1, were mixed with wild-type ES cells to make in chimeras and the reconstituted flk1-/- ECs displayed a stronger durability. This finding emphasizes that VEGFR-2 is indeed regulating EC integrity.

Analysis of the 9:1 (flk1-/-: wild-type) chimeras indicated that wild-type ES cells contributed greatly to the vasculature, despite a starting contribution of only 10%. Therefore we analyzed 99:1 chimeras (flk1-/-: wild-type) and found that wild-type contribution to the vasculature was similar to that of wild-type EBs. Interestingly, there was a very potent vascularization of chimeric EBs even if only a very small proportion (1-10%) of ES cells were of wild-type origin.

**Discussion**

We showed in paper 1 that VEGFR-2 was dispensable for endothelial recruitment, but required for endothelial maturation and vessel formation. In this study we mixed wild-type and flk1-/- ES cells to form chimeric EBs and found that the flk1-/- ECs were transiently associated with the wild-type vasculature. Since flk1-/- ECs are unresponsive to VEGF-mediated migration, this association may be due to the expression of endothelial adhesion proteins in the flk1-/- ECs, such as VE-cadherin which forms adherens junctions between ECs. A preliminary time-lapse microscopy experiment suggested that wild-type vasculature was subjected to constant remodeling within the EB (data not shown). Possibly, flk1-/- ECs become integrated in the rearranging wild-type vasculature upon contact, due to basic adhesiveness mediated by the shear stickiness of the expressed adherens junction proteins.

After being initially integrated, lack of VEGFR-2 resulted in a temporal detachment of flk1-/- ECs from the chimeric vasculature of chimeric EBs.
EC-markers were simultaneously separating in the \textit{flk1/-} EC population, revealing a break-down in EC integrity. We also noted a spot-like distribution of CD31 and with time, \textit{flk1/-} ECs disappeared from cultured EBs. These data suggest that VEGFR-2 plays an important part in regulating CD31 and VE-cadherin and that the combined effect of this is crucial for endothelial maintenance.

Regardless of genotype and context, the vasculature displayed an increased sensitivity to apoptosis compared to other tissues on day 8. Although the mechanisms that regulate the vasculature are not entirely understood, it is obvious that any promoting signal is likely to be countered by a repressive signal. Such a signal may subject ECs to the high rate of apoptosis that we observed, and might be a part of vascular remodeling.

The degree of apoptosis was higher for \textit{flk1/-} ECs compared to wild-type ECs. Since VEGFR-2 is known to mediate both proliferation and survival of ECs, these findings are not contradicting previous studies on the subject. Taken together, the deficiency of VEGFR-2-signaling and the consequences this has inflicted on adherens junction proteins and cell survival may be enough to collapse the entire population of \textit{flk1/-} ECs. We can not rule out that the disappearance of \textit{flk1/-} ECs has occurred through trans-differentiation, although it seems unlikely regarding the results on apoptosis.

Although we found an increased rate of apoptosis in \textit{flk1/-} endothelium, we lack important information on proliferation. However, the results from the 99:1 (\textit{flk1/-} : wild-type) chimeras suggest that VEGFR-2 acts as a very powerful mitogen for the endothelial lineage at this stage.

An alternative explanation would perhaps suggest a recruitment process that favored VEGFR-2-expressing cells over \textit{flk1/-} cells. However, when the endothelial area was compared, both \textit{flk1/-} and wild-type ES cells produced roughly the same amount of EC precursors regardless of context - alone or in chimeric EBs. We therefore propose that our results indicate that wild-type ECs propagate and dominate the vascular niche in chimeras due to the mitogenic potential of VEGFR-2.

We therefore propose that the \textit{flk1/-} EC-population disappears or becomes limited due to a lack of survival-promoting and proliferative signals mediated by VEGFR-2, acting directly or indirectly. It is probable that VEGFR-2 serves many purposes in this context which are currently indiscernible.
Concluding Remarks and Future Perspectives

We report that VEGFR-2 was dispensable for recruitment of endothelial precursors in differentiating EBs, a finding which is corroborated (Schuh 1999; Bautch 2000). The \( flk1^-/- \) ECs expressed endothelial markers (CD31, VE-cadherin and the β-galactosidase reporter - indicative of an active \( flk1 \) promoter). Chimeric EBs composed of \( flk1^-/- \) and wild-type ES cells, revealed and the \( flk1^-/- \) ECs were able to integrate transiently with wild-type vasculature. With time the \( flk1^-/- \) ECs regressed and lost their endothelial identity. We measured a 3-fold higher rate of apoptosis among \( flk1^-/- \) ECs compared to wild-type EC. The increased sensitivity of \( flk1^-/- \) ECs to apoptosis may be directly or indirectly influenced by the lack of VEGFR-2.

Wild-type ECs displayed an astonishing mitogenic activity as judged from experiments with chimeric EBs. To certify that this proliferation is directly dependent on VEGF-VEGFR-2 signaling is difficult, since indirect effects are difficult to separate. Also, to effectively prove that recruitment of endothelial precursors or angioblast is completely independent of VEGFR-2, we need to go further. To shed some light on this issue one may study chimeras at earlier time points. If VEGF-driven proliferation and survival are the main causes of wild-type domination, then \( flk1^-/- \) ECs would be expected to be in majority just after the recruitment. It has been difficult to immunostain and study very young EBs so one may opt for an alternative approach. It is possible to tag ES cultures with fluorescence expressed from artificial endothelial-specific promoters and introduced using lentivirus. Vascular cells of either genotype may then be followed using real-time microscopy on chimeric EBs. Such a fluorescent tag could be used to study how the \( flk1^-/- \) ECs integrate and separate from the wild-type vasculature in chimeras.

Another issue to resolve is to verify that the increased rate of apoptosis, observed for the \( flk1^-/- \) ECs, really is VEGFR-2-dependent. It is possible, although unlikely, that \( flk1^-/- \) ES cells in general are more prone to apoptosis than wild-type ES cells. We plan to perform quantitative PCR to assess the level of transcripts from the neomycin resistance gene which is constitutively expressed by the \( flk1^-/- \) ES cells. A fluorescent cell line tracer would be useful also here.

We re-introduced VEGFR-2 in \( flk1^-/- \) ES cells, using lentivirus delivery and the human Ubiquitin C promoter to drive the expression. This rescued
the vascular defects in flk1-/− EBs, but assays involving exogenous stimula-
tion demanded more VEGF-A ligand to produce effects of the same quality
as for wild type EBs. Since we could not see this flaw in EBs composed of
wild type ES cells transduced with the same lentivirus construct, we can
exclude explanations that involve ectopic VEGFR-2-expression and over-
consumption of the ligand. Instead two possibilities remain; 1) the proper
vegfr-2 promoter is crucial for fine-tuning VEGFR-2-expression and is re-
quired for optimal migration or sprouting response, or 2) the flk1-/− ES cells
carry a secondary unknown defect, perhaps due to prolonged culture. To
confirm this, we aim to perform our assays using another flk1-/− ES cell line.
We also have strategies to use a cloned vegfr-2 promoter to drive expression
from the lentivirus construct, instead of the ubiquitin C promoter currently
used. This may give clues on the need for properly regulated VEGFR-2-
expression.

It is well established that VEGFR-2 plays an important role in both de-
velopment and maintenance of the vasculature. A current effort in the re-
search field focuses on how the receptor selectively feeds its signals into a
number of intracellular pathways. For this purpose both cell cultures and in
vivo manipulations have been very useful and generated important data.
However, these strategies have limitations. Immortalized cell lines do not
recapitulate differentiation. They are homogeneous cultures and thus poorly
suited for studies on how the different cell types within tissues interact. Al-
though gene-inactivation studies in mice generate very solid data, embryonic
lethal manipulations prohibit further assessments on the functions of the
gene of interest.

We managed to reconstitute flk1-/− ES cells and rescue vasculogenesis
and sprouting angiogenesis in EBs. It is therefore possible to study mutant
VEGFR-2 in the same system. We are primarily interested in the tyrosine
residues which become phosphorylated upon activation and serve as scaf-
folds for intracellular signal mediators. Targeted point-mutation of wild type
cDNA allows expression of mutant receptors that incorporate a phenyla-
lanine residue instead of a tyrosine which effectively negates the signaling
functions of that specific tyrosine. Our method of using EBs would allow us
to investigate the function of the mutated receptors in a more permissive
environment compared to in vivo manipulations, but still in a context of
differentiation and where interactions between various cell types occur.

VEGF co-receptors are influencing the signal conveyed by VEGFR-2. We
show that VEGF ligands that bind both VEGFR-2 and NRP1 allowed
activation of p38MAPK which was associated with sprouting angiogenesis.
The exact mechanism through which this signaling is achieved is yet un-
known and warrants further investigations. If we were to truncate NRP1 so
that it lacks the cytoplasmic domain, we should be able to answer whether
NRP1 signals directly. Through chimeric cell cultures we may present NRP1
exclusively in trans, situated on an adjacent cell to the VEGFR-2-expressing
cell. This may solve whether NRP1 is able to trap activated VEGFR-2 in the membrane which may result in a different signal. ES cells in which the *nrp1* gene has been gene targeted would provide a very useful reagent in order to better understand its role in VEGFR-2 signaling.

It is clear that VEGFR-2 is capable of playing many different roles in regulation of ECs; inducing and directing migration, proliferation, survival and vascular permeability. The net result of signaling through VEGFR-2 will depend on a multitude of factors such as:

a) The affinity of the ligand for the dimerized receptors,
b) The co-receptor’s availability and ability to bind the specific ligand-VEGFR-2 complex and the result of this interaction,
c) The “mind-set” of the cell - reflected in its current portfolio of expressed downstream signaling molecules, adaptors and transcription factors as a result of previous directions,
d) The rate and route of VEGFR-2 internalization,
e) Other external signals received by the cell which may interplay with the signaling downstream of activated VEGFR-2,
f) The status of the terminal signal-receivers (such as junction proteins or cytoskeletal regulators) that net signaling may be exerted on.
Acknowledgements

I would like to express my gratitude to everyone who has helped and supported me during this process. In particular I would like to thank:

Lena - my supervisor for your profound engagement, your solid support and your fearless attitude that transforms difficulties into possibilities and of course for paying me. Your 24-7 devotion to your operation is truly an amazing achievement, both on a personal level and in what you manage to accomplish.

Xiujuan – my co-supervisor and co-worker for your great and skilled efforts on the projects, your relentless support, our discussions on science, cooking and culture (both in the sense of cells and in the more widespread definition). You have an enormous heart with an equaled talent.

Co-authors and collaborators for contributions and team play.

Co-workers of the Vascular biology unit, past and present (and in alphabetical order):
Anna for discussions and joint struggles with the Amaxa
Anna-Karin for organizing seminar series
Charlotte R for the vibe
Charlotte W for early lunches, putting up with my messy bench and taking care of the lab
Chunsik for being a “lazy guy”
Eduar for being so liberating open and easy to talk to
Else for pepparkakor and talks
Emma for order in the class room
Fuad for discussions on the Middle East
Harukiyo for persistent and skilled efforts on the Neuropilin project, and your expertise on were to get acceptable sushi.
Ingrid for protocols and discussions on science and politics
Irja for early lunches, putting up with my messy desk and taking care of the lab
Irmeli for no limits
Jan for technical assistance far beyond what duty requires
Johan D for open-minded discussions
Johan K for support and inspiring enthusiasm
Kerstin for lunch talks
Lars J for extraterrestrial support, techniques and friendship – lets race for the train again some day?
Lars L for all the cookies
Laurens for good conversation and help
Lothar for upholding the social order, friendship and much more
Ludvig for your relaxed ways
Mike for hilarious UK humor
Pacho for organizing the Gräsö kayak-trip, friendship and much more
Peder for briefing me on contemporary metal music
Peetra for being an exhilarating Energizer-bunny
Peter - “Do it” and move your “pawns”
Petter - long time no see
Pär for support and scientific discussions
Sebastién for great times
Sofie for all the fun times
Stefan – former co-supervisor for pep-talks and beer-associated fun times
Svante for vibrant discussions on most things
Svetlana for always being helpful
Taro for showing me the art of Japanese perfection
Witek for crazy days and fresh Popperian thinking
Zuyue for your easy laughter and good food
Åsa for great discussions on politics
And all other colleagues at Rudbeck lab for always providing a great atmosphere, suitable for both work and party.

Friends for still being friends - I hope you know who you are despite all my neglect. No one mentioned means that I avoid forgetting anyone - yes I can be very practical.

Mom, Dad, David, Frida & Hildur, Ulf & Katri, David & Sara, Petter & Cilla and the rest of my family for unconditional support and great company always.

Last but certainly not least,
Kaijsa – for making anything worth everything. Endless love.
References.


Holmqvist, K., M. J. Cross, et al. (2004). "The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) recep-


Muller, Y. A., H. W. Christinger, et al. (1997). "The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 A resolution:


Sabin, F. R. (1917). "Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick." *Anat Rec* **13**: 199–204.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 322

Editor: The Dean of the Faculty of Medicine

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”.)