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VASCULAR DEVELOPMENT

FROM EMBRYONIC STEM CELLS TO ARTERIES AND VEINS

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ABSTRACT

A fundamental question in developmental biology is how a single fertilized egg cell and its progeny are programmed or instructed to form thousands of different cell types and how these are organized into a complex and functional organism. In this thesis I have explored how a specific subset of cells is formed - the vascular endothelial cells lining our blood vessels. As the embryo grows in size, passive diffusion is not sufficient to support the developing tissues with oxygen and nutrients. The cardiovascular system is therefore the first functional organ to be established during embryonic development. Understanding the mechanism driving vascular development could have great implications to our understanding and treatment of multiple pathologies and diseases such as cancer, diabetes and ischemic heart disease. My studies have been focused on the initial *de novo* generation of endothelial lineage and further specification into arterial and venous endothelial subtypes. These processes have been explored using *in vitro* differentiation of embryonic stem cells (ESC) to mimic the sequence of events in the developing embryo.

In the first paper we addressed the function of vascular endothelial growth factor recptor-2 (VEGFR2) in the generation of endothelial cells and their integration into vascular structures. We showed that ESC lacking VEGFR2 fail to form and incorporate into vascular structures. By reintroducing VEGFR2 using lentiviral transduction we could rescue these defects. Interestingly, neither loss of or over expression of VEGFR2 seemed to influence the *de novo* generation of, at least immature, endothelial cells.

In paper II I present data showing that it is possible to generate endothelial cells with transcriptional and functional arterial and venous characteristics through *in vitro* differentiation of ESC. VEGF played a critical role where high concentration promoted arterial fate in contrast to intermediate or low VEGF concentration which supported venous differentiation. I could further show that the VEGF signal driving arterial fate was dependent on functional Notch signaling.

Having established the model system detailed above, I explored whether the hypoxic state of the embryo prior to functional circulation may influence the arterial venous lineage. Hypoxia did indeed strongly potentiate arterial over venous transcription in a Notch dependent manner. The hypoxia inducible factor (HIF) 1α and more potently 2α activated Adrenomedullin gene transcription. I showed that Adrenomedullin drives Notch signaling through transcriptional induction of the arterially expressed Notch ligand Dll4.

In the final paper I aimed to dissect the individual role of two VEGF co-receptors, Neuropillin1 and heparan sulfate (HS) proteoglycans in arterial venous specification. Surprisingly we found NDST1/2$^{-/-}$ ESC which produce HS lacking a critical modification, N-sulfation, to be resistant to *in vitro* differentiation. These cells maintained transcriptional, morphological and functional characteristics of undifferentiated ESC following four days of embryoid body differentiation in the absence of leukemia inhibiting factor (LIF). We show that N-sulfation is critical specifically for endogenous FGF4 which is responsible for down-regulation of Nanog and thereby initiation of ESC differentiation. Finally, N-sulfation of HS has a

functional importance as the efficiency of *de novo* ESC line derivation from mouse blastocysts greatly increased by the addition of a general sulfation inhibitor.

These studies suggest that *in vitro* differentiation of ESC can be an excellent model system to compliment studies performed in the actual embryo, especially when addressing lineage specifications.

LIST OF PUBLICATIONS

I. Xiujuan Li, Dan Edholm, **Fredrik Lanner**, Georg Breier, Filip Farnebo, Anna Dimberg, Lena Claesson-Welsh.
Lentiviral rescue of vascular endothelial growth factor receptor-2 expression in Flk1-/- embryonic stem cells show early priming of endothelial precursors.
Stem Cells (2007) 25, 2987-2995.

II. **Fredrik Lanner**, Marcus Sohl, Filip Farnebo.
Functional arterial and venous fate is determined by graded VEGF signaling and Notch status during embryonic stem cell differentiation.
Arterioscler Thromb Vasc Biol. (2007) 27, 487-493.

III. **Fredrik Lanner**, Marcus Sohl, Emil Hansson, Peter Carmeliet, Lorenz Poellinger, Urban Lendahl, Filip Farnebo.
Hypoxic induction of Adrenomedullin and Notch signaling promotes arterial differentiation of embryonic stem cells.
Manuscript (2008).

IV. **Fredrik Lanner**, Marcus Sohl, Katarina Holmborn, Johannes Wilbertz, Filip Farnebo.
N-sulfation of heparan sulfate is critical for embryonic stem cell differentiation.
Manuscript (2008).

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
Crlr	calcitonin receptor-like receptor
Dll	deltalike ligand
EPI	epiblast
ESC	embryonic stem cell
EXT	exostosin
FIH	factor inhibiting hif-1α
HEY	hairy enhancer of split
HIF	hypoxia inducible factor
HSPG	heparan sulfate proteoglycan
ICM	inner cell mass
LIF	leukemia inhibiting factor
NDST	N-deacetylase/N-sulfotransferase
NECD	Notch extracellular domain
NICD	Notch intracellular domain
Nrp	neuropillin
PE	Primitive endoderm
PHD	prolyl hydroxylase
PrE	primitive ectoderm
Shh	sonic hedgehog
TE	trophectoderm
TS	trophoblast stem cell
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau
XEN	extra embryonic endoderm

Introduction

All animals are dependent on oxygen for their survival. Passive diffusion of oxygen can only supply tissues of a few cell layers in thickness which has put evolutionary constrains on organism size. Animals had to either remain small and generally single layered or evolve some type of oxygen transportation system. Most large invertebrates have solved this through a so-called open circulation where the heart pumps blood/intercellular fluid relatively freely through the tissues. Vertebrates have evolved a more specialized closed circulatory system where the blood essentially never leaves the vessels. Oxygenated blood from the lung is pumped out through into the aorta and subsequent smaller arteries and arterioles. The oxygen exchange takes place in the capillary bed before the blood returns to the heart through venules and veins. This allows for a more efficient transport and can therefore support larger body size (Ruppert and Carle 1983).

As all cells need oxygen, the development of a functional vascular system is one of the earliest events in mammalian embryonic organogenesis. Understanding the processes driving embryonic vascular development has great implication for future tissue engineering where proper tissue vascularisation will be critical. Furthermore, treatment or prevention of a wide range of diseases such as diabetes, rheumatoid arthritis, ischemic heart disease and cancer would greatly benefit from increased knowledge of vascular development. Most progress to our knowledge of vascular development has been made through *in vivo* studies of mice embryos. However, these experiments have the inherent problem of secondary effects. As the function of the vascular system is to support the growing embryo with oxygen and nutrients, vascular phenotypes will severely affect the whole embryo. In addition, the early events of vascular development in mice do not easily allow for direct manipulation, visualization, and the vascular precursors are in relatively small numbers. It would therefore be useful if one could also study vascular lineage specification in isolation.

This thesis explores the potential of embryonic stem cells (ESC) to generate specified vascular lineages such as arteries and veins *in vitro* and how such a system can be used to study embryonic vascular development. ESCs are much more amendable to direct manipulation by intrinsic and extrinsic factors. Specific and transient cell populations can easily be identified or even isolated by fluorescent-activated cell sorting for further subculture or additional analysis. Mimicking vascular development by the use of ESC would therefore be an attractive complement to work in the developing embryo.

I have focused my attention to the differentiation of vascular progenitor into endothelial cells with specialized arterial and venous characteristics. These studies have examined the instructive roles of growth factors, signaling transduction pathways, transcriptional regulation as well as physical cues such as oxygen tension. It is however critical to have an understanding of the events prior to vascular development both in embryogenesis and *in vitro* differentiations of ESC. This point was well illustrated by Paper IV where we aimed to investigate the role of co-receptors for endothelial maturation but

uncovered a much earlier function for such co-receptors in the very initiation of ESC differentiation. I have therefore chosen to first give a background on the earliest events of embryogenesis leading up to gastrulation and describe the nature of various stem cells derived from the blastocyst. This will then be followed by an in depth background to arterial venous specification and vascular development before I discuss the specific papers that are presented in this thesis.

PRE-GASRTULATION DEVELOPMENT AND EMBRYONIC STEM CELLS

Formation of the first three cell lineages

During the initial cleavages of the fertilized egg cell, all daughter cells or blastomeres appear identical in morphology (reviewed by Johnson & McConnell 2004). At the 8-cell stage, the events of compaction and polarization begin. As cells divide from the 8-16 and 16-32 cell stage, the outer cells acquire a polarized phenotype, while enclosed inner core cells become apolar. An inside-out model of trophectoderm specification has emerged where the outside polarized epithelium will go on to form the trophectoderm (TE; future placenta), while the enclosed group of apolar cells goes on to form the inner cell mass (ICM) of the developing blastocyst (Rossant & Tam 2004).

Next the ICM will segregate into the eipiblast (EPI: future embryo) and primitive endoderm (PE: future yolk sac). PE forms along the blastocoels surface of the ICM. Initially it appeared that an inside-out mechanism could also be driving this segregation. Support for such a model came from the observation that during *in vitro* differentiation of embryonic stem cells (ESC) as embryoid bodies, the outer cell-layer formed PE-like cells (Martin & Evans 1975, Becker *et al.* 1992, Murray & Edgar 2001). However, recent data showed heterogeneity within the cells of the ICM before sorting of PE cells to the blastocoels surface. Non-overlapping PE and EPI gene expression, transcriptional single cell analysis, and cell tracing experiments suggest that the ICM truly is a heterogeneous cell population (Chazaud *et al.* 2006, Kurimoto *et al.* 2006). The current view is that EPI/PE lineage precedes the inside-out segregation.

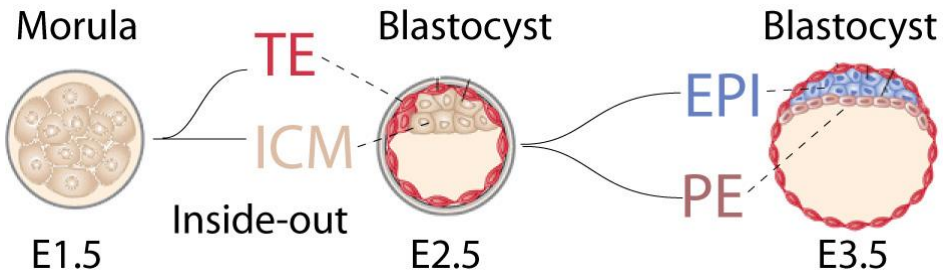

Figure 1. Development of the first three cell lineages in the mammalian embryo, the trophectoderm (TE), the primitive endoderm (PE) and the epiblast (EPI) which will form the embryo proper. ESC are derived from the inner cell mass (ICM). TE and ICM segregation involves an inside-out mechanism.

Three genes can be used to segregate the cell types of the developing blastocyst: Oct-4, Nanog, and Gata4. Oct-4 and Nanog are expressed in a subset of cells in the morula

and the ICM of the early blastocyst but not in the trophectodermal cells. At the blastocyst stage Gata-4 positive cells emerge in a salt and pepper pattern in the ICM. These cells, which express Oct-4 but not Nanog, appear to be fated for PE and are subsequently sorted to the ICM surface facing the blastocoel cavity (Chazaud *et al.* 2006). Nanog and Oct-4 expression is maintained in the remaining ICM cells (Chambers *et al.* 2003, Niwa 2005, Rosner *et al.* 1990, Schöler *et al.* 1990).

Implantation and gastrulation

In the mouse, the blastocyst hatches from the zona pellucida and attaches to the uterine wall at embryonic day (E) 4.5. This initiates a massive proliferation of the trophectoderm cells which invade the uterine wall and contribute to the placenta. The proliferation of TE stem cells is dependent upon FGF4 produced by the underlying ICM/EPI (Guzman-Ayala *et al.* 2004). In the absence of this FGF4 signal the TE stem cells differentiate prematurely (Adelman *et al.* 2000, Hemberger *et al.* 2004). A subset of the PE migrates away from the ICM to form the parietal endoderm underlying the trophoblast giant cells. The remaining PE forms the visceral endoderm which will form the endodermal tissues in the yolk sac (Thomas & Beddington 1996). By E5.5 the embryo is fully implanted and embedded in maternal decidual tissue.

The TE and PE are not only important tissues for maternal-fetal interactions, but have also important roles in establishment of the axial body plan at gastrulation. The TE derivative drives the proximo-distal patterning of the epiblast or primitive ectoderm (PrE) (Beck *et al.* 2002, Ang & Constam 2004). The anterior visceral endoderm (AVE) promotes anterior over posterior development by secretion of antagonists of nodal and Wnt signaling (Kimura *et al.* 2000, Yamamoto *et al.* 2004). PE is further suggested to have important signaling functions also at later time points during gastrulation and formation of vascular precursors.

Segregation between the three germlayers: ectoderm, mesoderm, and endoderm are generated during gastrulation which starts at E6.5. The first morphological sign of gastrulation is the formation of the primitive streak, a thickening by cells, in the region of the embryo that ultimately will form the posterior end of the embryo. Uncommitted epiblast cells migrate through the primitive streak and emerge as either mesodermal or endodermal progenitors (Tam and Behringer, 1997). Molecularly, several genes like Brachyury (Kispert and Herrmann, 1994) and Mixl1 (Hart *et al.* 2002) are expressed throughout the primitive streak. Mapping studies have shown that the first cells to egress through the posterior primitive streak form the hematopoietic, endothelial, and smooth muscle cells of the yolk sac (Kinder *et al.* 1999). Subsequently, cells contributing to cranial and cardiac mesoderm, followed by paraxial and axial mesoderm, emerge. The definitive endodermal cells transit through a more anterior region of the primitive streak while the ectodermal tissues develop from the anterior region of the epiblast, which does not migrate through the primitive streak.

Stem cells derived from the early embryo

The first stem cells to be derived from blastocysts are the embryonic stem cells (ESC) originating from the ICM (Evans and Kaufman 1981; Martin 1981). ESCs have three critical properties: self-renewal, pluripotency, and potential to generate chimeric animals. Initially ESC were derived and cultured on feeder cells. The essential function of the feeders was to produce the leukemia inhibitory factor (LIF) (Stewart *et al.* 1992). The need for feeders could be replaced by addition of recombinant LIF (Smith *et al.* 1988, Williams *et al.* 1988). LIF exert its function through activation of STAT3 (Matsuda *et al.* 1999, Niwa 1998), and one candidate STAT3 downstream gene is c-Myc (Cartwright *et al.* 2005). Interestingly, LIF also activates the Ras-erk pathway which has been suggested to promote differentiation (Burdon *et al.* 1999). The need for serum can also be replaced by addition of bone morphogenetic proteins (BMPs) (Ying *et al.* 2003).

FGF4 appears to play multiple a critical functions in the earliest embryo. FGF-Erk1/2 signalling was shown by Stavridis *et al.* (2007) and Kunath *et al.* (2007) to induce differentiation of ESC in an autocrine manner but the authors came to different conclusions concerning the precise timing of the critical FGF-signalling. Stravidis *et al..*, argues that FGF triggers the transition from an FGF5-positive post implantation primitive ectodermal (PrE) state to neuronal progenitors. Kunath *et al.* identified FGF4 as the critical ligand, being the predominant FGF species in ESC. In the absence of functional FGF4 signalling they also reported an induction of FGF5 indicative of transition to PrE. However, they could not detect any accompanying reduction in Rex1 or Nanog arguing that FGF4 may indeed be critical for the earlier transition of ESC from self-renewal to linage commitment.

The exact origin of ESC is still a subject to debate. It is widely accepted that they are derived from the ICM. But in contrast to the cells of the ICM ESC do not give rise to PE following blastocyst injections, only the embryo proper (Beddington & Robertson 1989). This would argue that ESCs are more of EPI origin. On the other hand, *in vitro* differentiations as embryoid bodies give rise to PE-like cells which would indicate an earlier more ICM like origin (Hamazaki *et al.* 2004). Human ESC will not be discussed in greater detail in this thesis but an interesting observation is that their origin appears to be more like PrE. Human ESC are derived and cultured without LIF but with added FGF2. Stem cells from mouse post-implantation embryos can indeed be derived (so called EpiSC) using these culture conditions and they do acquire transcriptional and morphological characteristics which are more like human than mouse ESC (Rossant 2008).

In addition, stem cells have also been derived from the TE, termed trophoblast stem (TS) cells, and from the PE, termed eXtra-embryonic Endoderm (XEN) cells (Tanaka *et al.* 1998 and Kunath *et al.* 2005). TS cells were initially derived from blastocysts using conditioned media and FGF4. Conditioned media has subsequently been replaced by Activin and transforming growth factor (TGF)-β (Erlebacher *et al.* 2004). If FGF4 is

removed the TS cells differentiate into syncytiotrophoblast and spongiotrophoblast derivatives (Adelman *et al.*. 2000, Hemberger *et al.* 2004), mimicking the inductive properties of the underlying ICM in the blastocyst. To efficiently generate XEN cells, the ICM was isolated using immunosurgery and cultured on embryonic fibroblasts with FGF4 (Kunath *et al.* 2005).

Transcriptional regulation of pluripotency

Three transcription factors have been suggested to maintain the pluripotent state: Oct4, Sox2, and Nanog (Chambers and Smith 2004, Niwa 2007).

Oct-4 is a POU domain transcription factor expressed in the morula and the ICM but switched off in the gastrulating embryo although expression is maintained in germ cells. Oct-$4^{-/-}$ embryos develop to blastocysts, but the inside cells fail to form an ICM and differentiate instead into trophoblast (Nichols *et al.*., 1998). Similarly, deletion of Oct-4 in ES cells results in formation of trophectoderm. Over-expression of Oct-4 by ~1.5 times resulted in differentiation into mesendoderm-like cells (Niwa *et al.*., 2000). Oct-4 has been reported to directly prevent trophectoderm differentiation through interaction with Cdx2 (Cdx2 by itself drives trophectoderm differentiation) and forming a repressor complex (Niwa *et al.* 2000). There appears to be autoregulation of these two factors through reciprocal inhibition. Oct-4 expression levels must therefore be finely balanced to allow maintenance of pluripotency.

Sox2 is an HMG box transcription factor and expressed in the ICM and trophectoderm. It is required for development of the embryonic lineage (Avilion *et al.*., 2002) but is also required for proliferation of the extra-embryonic ectoderm. Sox2 has been shown to maintain Oct-4 expression and interact with Oct-4 to influence expression of target genes (Masui *et al.* 2007).

Nanog is a homeobox transcription factor whose expression is tightly associated with the pluripotent population (Chambers *et al.* 2003). Nanog expression is undetectable in the oocyte, and appears in the inner cells of the morula. In the absence of Nanog, embryos form only trophectoderm and primitive endoderm. Deletion of Nanog in ESC promoted primitive PE-like differentiation even in the presence of LIF. Over-expression of Nanog confers LIF independence (Mitsui *et al.* 2003). Being regulated by Oct4 and Sox2, Nanog has been suggested to block PE differentiation through inhibition of Gata6 (a transcription factor promoting PE) (Hamazaki *et al.* 2004). Increasing data suggest a gate-keeper function of Nanog. Not only does Nanog repress PE lineage but it has independently been show to repress neuronal differentiation and expression of the mesodermal-specific transcription factor Barchyury (Ying *et al.* 2003, Suzuki *et al.* 2006).

ESC – a heterogenic cell population

In 2006, Susuki *et al.* reported that a fraction of ESC in normal culture media containing LIF and serum were Brachyury positive and appeared primed for mesodermal lineage. They further showed that these cells could be fully reverted to pluripotent ESC in a Nanog dependent manner. In addition, two independent groups have reported Nanog to be heterogeneously expressed and fluctuating in ESC cultures (Chambers *et al.* 2007, Singh *et al.* 2007). Chambers *et al.* showed that although genetic deletion of Nanog predisposes ESCs towards differentiation, it does not mark commitment. Indeed, $Nanog^{-/-}$ ESCs have not lost the ability to generate chimeric animals following blastocyst injection. Singh *et al.* observed that ESC cultures are heterogenic in Nanog expression and examined whether this heterogeneity reflected the observed heterogeneity in the early ICM where cells are either are Nanog or Gata6 positive (Yamanaka *et al.* 2006). They could indeed detect increased levels of Gata6 in Nanog negative ESC and further showed that Nanog directly repress Gata6 through binding to the proximal promoter region of *Gata6*.

A similar study explored heterogenic Rex1 expression in ESC (Toyooka *et al.* 2008). Toyooka *et al.*. convincingly showed that both $Rex1^{+}$ and $Rex1^{-}$ cells could give rise to negative and positive cells respectively when cultured in normal ESC conditions. Upon *in vitro* differentiation, Rex1 was quickly down regulated and transcriptional micro array analysis showed that the $Rex1^{-}$ ESCs resembled late epiblast or early primitive ectodermal cells of a post implanted embryo. The negative population expressed increased levels of genes such as *FGF5, Eomes* and *Brachyury,* whereas the positive cells were enriched in Nanog, Klf4, Sox2 and Tcl1. Oct4 and Gata6 were expressed in equal levels in both populations. Once cells differentiated to PrE they have lost their ability to make chimeric contribution through blastocyst injection. Although the $Rex1^{-}$ cells were shown to possess the potential to "revert" into positive cells, they behaved like PrE cells and failed to properly integrate following blastocyst injections. Taken together, these data strongly suggest that ESC cultures contain subpopulations of cells corresponding to ICM, EPI, PrE and possibly PE, and that Nanog plays an important gatekeeper role in the transitions between these states.

In Paper IV I will present a critical and novel function of HSPG for FGF4 driven reduction in Nanog transcription and thereby initiation of ESC differentiation.

VASCULAR DEVELOPMENT

When Leonard da Vinci studied the human vascular system he compared it to the roots of a plant which grow out from the seed (1508). We now know that although vessels do sprout in a manner like roots, they do not originate from the heart. Instead, the blood vessels form *de novo* through migration and aggregation of endothelial precursors (angioblasts) into a primitive plexus, a process termed vasculogenesis (Risau & Flamme 1995). This primary plexus subsequently undergoes an extensive remodeling process where growth, migration, branching, sprouting and pruning forms the functional circulatory system. This process is termed angiogenesis (Carmeliet 2003).

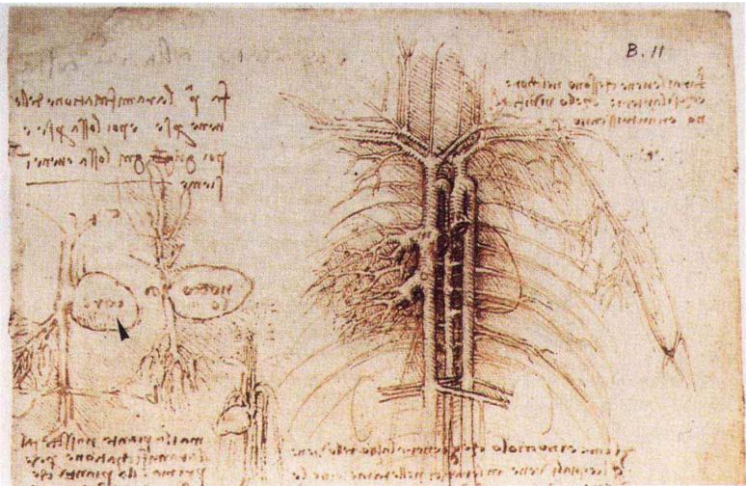

Figure 2. Leonardo da Vinci's sketch of the human cardiovascular system, (taken from "the anatomy of man: the cardiovascular system" (ca. 1508)). Da Vinci compared the heart to a seed from which the roots or vessels sprouts.

The first historical reference to a difference between arteries and veins can be traced 2000 years back, to the Greek anatomists Paraxagora and Herophilus (Wiltse and Pait 1998). However it was not until 1628 that the English physician William Harvey postulated and experimentally showed that arteries carry blood from the heart and veins back to the heart. Structurally, arteries have thicker smooth muscle walls and tend to have narrower lumens. The smooth muscle cells are crucial in controlling blood flow by constriction and dilation of the vessels. The thicker walls must also withstand the high pressure driving the flow in the arteries. The venous vessels on the other hand have thinner walls and wider lumens. In contrast to arteries, veins also have valves that helps maintain the directional flow. Interestingly, arterial walls are more susceptible to atherosclerosis whereas veins are more prone to thrombosis. Arteries and veins tend to run in parallel with close proximity to each other, but maintain their distinct characteristics. Loss of this distinction through improper arterial-venous lineage specification results in congenital arteriovenous malformations and devastating diseases such as hereditary hemorrhagic telangiectasia.

Hemangioblast

The question of where and when the endothelial and blood cells first arise in the developing embryo has been, and still is, a classical debate in developmental biology. The first structural sign of vasculogenesis is the formation of blood islands in the extraembryonic mesoderm of the yolk sack. The outer cells of the blood island are endothelial cells surrounding a loose inner core of hematopoetic precursors (His 1900). These groups of cells coalesce and form the primary plexus. The plexus also forms without involvement of blood islands in the yolk sack (Drake & Fleming 2000). The close spatial connection between the hematopoetic and the endothelial lineages together with common marker expression led to a hypothesis, which assumes that they have a common precursor, the hemangioblast (His 1900). Furthermore, blood and endothelial cells formed in the yolk sack were believed to then populate the embryo proper. However, careful histological analysis has shown that vascular structures could be independently observed in the embryo proper, arguing that endothelial cells of the embryo developed from an intraembryonic source rather then through colonization (Reagan 1915, McClure 1921, Wilms *et al.* 1991).

It is now generally accepted that mesoderm is the exclusive source of endothelial cells. The earliest marker of angioblasts precursors is Flk1 (VEGFR2), the primary receptor of vascular endothelial growth factor A (VEGF-A) (Millauer *et al.* 1993, Yamaguchi *et al.* 1993). Flk1 is expressed in a subset of Brachyury positive cells as they emerge through the primitive streak (Huber *et al.* 2004). These cells migrate and populate both the extraembryonic and intraembryonic tissues. The inductive signals driving emergence of this population is not entirely characterized. Early experiments suggest that endoderm derived signals such as FGFs, BMPs and Indian hedgehog are important for the early vascular development (Ferguson *et al.* 2005).

The first secreted molecule with endothelial specificity is VEGF. Both the receptor Flk1 and VEGF are well documented for their crucial importance for vascular development. Embryos lacking Flk1 die around embryonic day (E) 9 and show no formation of blood vessels or hematopoetic cells (Shalaby *et al.* 1995). Mutations of VEGF have similar early embryonic lethality. Interestingly, the VEGF dosage appears critical as even the heterozygote mutant is lethal with severely reduced size and underdeveloped blood vessels (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). The loss of both vascular and hematopoetic cells following genetic targeting of Flk1 further indicates that there could be a common progenitor, the hemangioblast. Flk1+ cells generated through *in vitro* differentiation of embryonic stem cells can give rise to single-cell-derived blast colonies (BL-CFCs) that can generate both endothelial and hematopoetic cells lending more support to the hemagioblast model (Choi *et al.* 1998). A similar Brachyury^{+}/Flk1^{+} cell population has been isolated from the primitive streak of the developing embryo and showed similar potential (Huber *et al.* 2004). However, this does not formally prove that single cells do indeed give rise to both blood and vasculature in vivo, although they appear to have that potential. Lineage tracing in zebrafish supports the model of bi-potential hemangioblast (Vogeli *et al.* 2007). A

similar clonal analysis in mice showed the opposite (Ueno & Weissman 2006). The clonal analysis in mice showed that the individual blood islands in the yolk sack are not derived from a single cell but had a multi-lineage origin. This study also indicated that in contrast to endothelial cells, all blood cells may not derive from $Flk1^+$ progenitors. Finally, $Flk1^+$ cells are not to restricted to endothelial and blood lineages but can also contribute to smooth muscle and cardiac lineages, indicating a more mesangioblasts characteristic (Huber *et al.* 2000, Yamashita *et al.* 2003, Motoike *et al.* 2003).

Arterial and venous lineage – physiological cues vs. genetic prespecification

During the progression from vascular progenitors to a complex and functional vascular system the cells must acquire their proper arterial or venous vessel identity. Much of our understanding of arterial-venous specification has evolved through syntheses of different experimental approaches and model systems. Chicken, zebrafish and mice have been the three primary model organisms. The chicken model pioneered vascular biology and has been instrumental to study the role of flow. Quail-chick transplantations allowed for lineage tracing and investigation of plasticity. However, most progress has been made through genetic studies in zebrafish and mice. The fast development and visibility of the zebrafish vasculature have made it possible to employ high-throughput chemical mutant screens. Much of the progress made in fish has been validated in mice through genetic targeting studies. Importantly there are differences in data produced in fish and mammals. One underlying reason for the differences may be that mice are more dependent on proper vasculature function and placental oxygen supply for early development.

The consensus view was, until not so long ago, that arterial and venous identity would be specified and maintained by hemodynamic mechanisms such as blood flow, pressure and oxygen tension. Indeed, flow is shown to drive arterial or venous identity in the developing chick embryos. Re-routing flow by ligation of the vitelline artery results in perfusion of the arterial tree with blood of venous origin, which effectively changed both morphology and gene-expression into a venous phenotype (le Noble *et al.* 2003). Conversely, perfusing veins with flow of arterial origin drives arterial characteristics.

However, before angioblasts have even formed vessel structures that could be under such hemodynamic control they appear to be pre-specified with arterial or venous cells identity. This is perhaps best illustrated in the developing zebrafish. 12 hours after fertilization zebrafish angioblasts become apparent in the lateral plate mesoderm which subsequently migrate and coalesce to form the dorsal aorta and the cardinal vein within 6 hours. Although this is several hours before circulation is initiated the vessels have differential gene expression with EphrinB2, Notch3, and Gridlock in the aorta, whereas the cardinal vein expresses EphB4 and Flt4 (Zhong *et al.* 2001). Lineage tracing experiments even indicates that the angioblasts are pre-specified to arterial or venous fate, even before they migrate to the midline.

In the chick yolk sack, individual blood islands have been shown to differentially express the arterial and venous markers Neuropillin1 and 2 (Nrp1 and 2) respectively (Herzog *et al.* 2001). Initially at the six-somite stage, blood islands express either one or both markers. In 13-somite embryos, the primitive plexus has formed and Nrp1 and 2 are restricted to arterial or venous parts of the plexus, despite the absence of flow. However, the arterially expressed EphrinB2 is not evident until the 26-somite stage when a functional vascular system has been established.

Compared to flow, oxygen tension has attracted less attention in endothelial lineage specification. Since oxygen tension is higher in arteries compared to veins, oxygen may be a crucial factor in arterial venous lineage specification and maintenance. One paper show supporting data of this model (Claxton & Fruttiger 2005). Claxton and Fruttinger report that after exposing newborn mice to an atmosphere of 10% oxygen they could detect lower levels of arterial markers Dll4 and EphrinB2 in the forming retinal vasculature (Claxton & Fruttiger 2005). These data should be interpreted with some care, since the development of retinal vasculature is quite unique and the authors could not distinguish between loss of arterial markers or an increase in venous endothelium. Furthermore, specification of arterial and venous lineage occurs early in development before the onset of circulation in a hypoxic environment. In Paper III I examine the possible role of such early embryonic hypoxia in arterial venous specification.

Although the pendulum has swung towards a genetic program driving this fate decision, environmental cues like flow and possibly oxygen tension definitely has a crucial role in shaping the developing vasculature.

A hierarchal model

Initially, genes involved in endothelial lineage decision were identified through their differential expression in the two vessel types. The first evidence for a genetic component in arterial-venous specification came from the finding that the ligand Ephrin-B2 is expressed in arteries and its receptor, EphB4, is restricted to veins (Wang *et al.* 1998). This differential expression is evident even before circulation is established and mutational studies in mice reveled symmetrical phenotypes of the ligand and receptor (Gerety *et al.* 1999). Despite the distinct expression of EphrinB2-EphB4 / ligand-receptor it does not appear to drive lineage specification (Wang *et al.* 1998). Since then, a hierarchical model for arterial venous lineage specification has been established where the key players are Shh, VEGF, Notch and COUP-TFII (Figure 3). In the following section I will discuss the different findings that have lead up to this model.

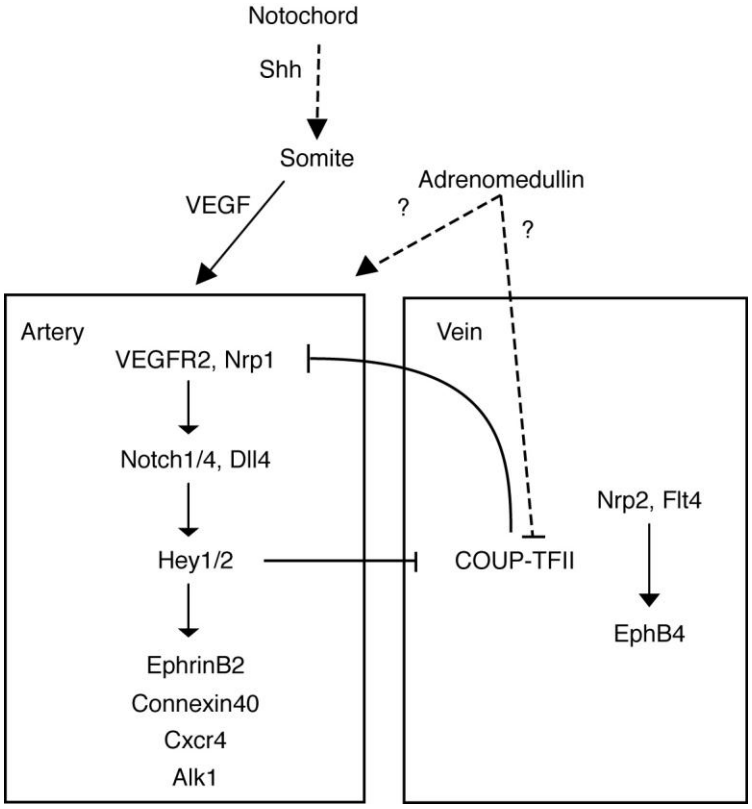

Figure 3. The hierarchal model of arterial venous specification. Shh expressed by the notochord induces the expression of VEGF in the surrounding tissues such as the somites. VEGF acts on adjacent angioblasts and activates Notch signaling which through Hey1/2 promotes an arterial fate. The transcription factor COUP-TFII is expressed in veins where it blocks arterial fate, possibly through inhibition of Nrp1, a VEGF co-receptor expressed in arteries, and thereby reduces Notch activation by VEGF. Adrenomedullin has recently been added to this model, but the mechanism through which it drives arterial fate is not clear.

Sonic hedgehog – at the top

At the top of the hierarchy driving arterial-venous specification is Sonic hedgehog (Shh), a member of the hedgehog family. This has been show by a number of elegant experiments using mRNA and morpholino antisense RNA injections, in combination with genetic mutants and transgenic zebrafish embryos. Null mutations for Shh (sonic-you, in zebrafish) or the hedgehog regulated transcription factor gli2 (you-too, in zebrafish) both show defects in trunk vasculature with loss of arterial identity and EphrinB2-expression (Brown *et al.* 2000, Lawson *et al.* 2002). Conversely, overexpression of Shh caused formation of EphrinB2 expressing vessels. Shh have been shown to induce expression of VEGF, angiopoietin-1 and angiopoietin-2 in mesenchymal cells and thereby indirectly driving vascular specification (Pola *et al.* 2001). Loss of Shh function indeed results in reduced VEGF transcription that can be rescued by ectopic overexpression of VEGF, placing Shh at the top in the hierarchy (Lawson *et al.* 2002).

In mice, hedgehog signaling also seems to be important for the formation of the aorta, as mutants of the signaling component *smoothened* or the use of signaling inhibitors results in defective aorta formation (Vokes *et al.* 2004). However, loss of Shh had no obvious vascular defect, indicating that other members of the hedgehog family could have a complementary function in mice. Indian hedgehog has been shown to have a severe phenotype in the yolk sack with very limited vascular remodeling (Byrd *et al.* 2002). Whether this is due primarily to failure in arterial-venous specification is not known.

Vascular endothelial growth factor – downstream of Shh

Vascular endothelial growth factors (VEGFs) have critical roles throughout vascular development from vasculogenesis to angiogenesis. In mammals, there are five identified ligands, which occur in several splice variants, VEGFA, B, C D and placenta growth factor (PlGF). These ligands bind in an overlapping manner to three receptor tyrosine kinases (RTKs), VEGF receptor1, -2 and -3. VEGFA, B and PlGF bind to VEGFR1, VEGFA binds to VEGFR2 (also called Flk1 in mice) and VEGFC and D binds to receptor 3. VEGFC and D can after proteolytic processing also bind to VEGFR2 (Figure 4) (Olsson *et al.* 2006).

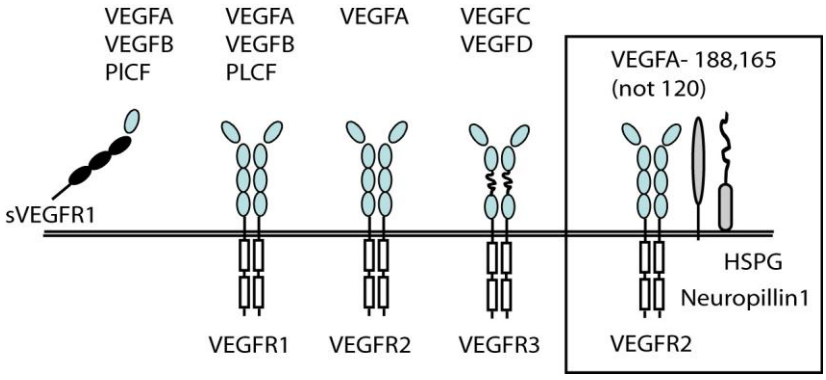

Figure 4. The VEGFs and the receptors to which they bind. Proteolytic processing of VEGFC and VEGFD can bind also to VEGFR2 although not indicated in the figure. The shorter VEGF120 isoform fail to interact with either Neuropillin1 of HSPG.

VEGFA splice variants, heparan sulfate and Neuropillin1

The single *VEGF-A* gene gives rise to multiple isoforms, through alternative splicing, primarily VEGF120, VEGF165, and VEGF188 (Ferrara *et al.* 1992). All isoforms are secreted as covalently bound homodimers. The three variant differ in the presence or absence of exon 6 and 7, which have been shown to contain the heparan sulfate (HS) and Nrp1 binding domain. This gives them distinct gradient distributions and signaling

capabilities. VEGF188 that binds the strongest to extra-cellular matrix or HS is also the least soluble. VEGF120 fails to bind to extra-cellular HS and is therefore the most soluble of the three where VEGF165 has an intermediate distribution (Park *et al.* 1993). The function of HS in VEGF signaling has been studied primarily in terms of gradient formation (Houck *et al.* 1992). However, not only the VEGF ligand but also the VEGFR2 has HS binding domain (Dougher *et al.* 1997). This has opened the possibility that HS may function as a co-receptor and more directly modulate the VEGFA-VEGFR2 interaction and downstream signal transduction.

Along these lines it has recently been suggested that HS presented in *trans* (from the adjacent cell) is critical for VEGF induced sprouting (Jakobsson *et al.* 2006). They reported that embryoid bodies from HS deficient ESC failed to sprout in response to VEGF stimulation. The phenotype could not be rescued by addition of soluble heparin. In contrast, the sprouting defect was perfectly rescued in chimeric cultures including 10% ESC expressing wild type HS. This indicates that trans presentation is indeed crucial for the VEGF-VEGFR2 function. The authors further speculated that *trans* presentation might stabilize the ligand-receptor complex and possibly reduce complex internalization, yielding a stronger VEGFR2 signaling. No function of HS has yet been described in arterial venous lineage specification.

In contrast to VEGF165, VEGF120 also fail to bind the VEGF co-receptor Neuropillin1 (Nrp1) (Soker *et al.* 1998). Nrp1 and Nrp2 are transmenbrane proteins with small cytoplasmatic domains, which probably lack catalytic function (Fujisawa *et al.* 1997). Nrp1 was originally characterized as a semaphorin receptor involved in axon guidance (Kolodkin *et al.* 1997, He & Tessier-Lavigne 1997). It has subsequently been shown that Nrp1 and Nrp2 are differentially expressed in arteries and veins, respectively (Herzog *et al.* 2001). Co-expression of VEGFR2 and Nrp1 in porcine aortic endothelial cells strongly enhances VEGF165 binding to VEGFR2 and mediates chemotactic activity (Soker *et al.* 2002). Genetic targeting of Nrp1 in mice have indeed resulted in vascular defects similar to reduced VEGF signaling, however it is not clear whether the malformations are truly due to defects arterial venous specification (Kawasaki *et al.* 1999). Our unpublished data indicate that VEGF121 does not support full arterial differentiation in our *in vitro* embryonic stem cell system. This would fit with a model in which Nrp1 facilitates strong VEGF signaling and arterial differentiation. However, no obvious arterial venous defects are reported from mice engineered to selectively express VEGF120 (Carmeliet *et al.* 1999, Stalmans *et al.* 2002). In contrast to the Nrp1 null mice the VEGF120 mice actually survive to term but do show postnatal vascular phenotypes. Examination of the coronary vessels of VEGF120 mice surprisingly revealed an increased expression of arterial markers (van den Akker *et al.* 2008). Van den Akker *et al.* suggest that VEGF120 was able to diffuse further then VEGF165 (VEGF165 is the dominant isoform in the heart) and thereby ectopically increasing the local VEGF concentration and convert vein to arteries. Interestingly, human coronary arterial endothelial cells cultured *in vitro* with VEGF165 or VEGF121 showed the opposite effect. In this system VEGF121 failed to induce arterial transcripts. Notably, a recent paper suggests that endogenous VEGF120, in contrast too commercial and widely used, VEGF120 do interact bind to Nrp1 although it does not appear to facilitate the VEGFR2-Nrp1 complex formation (Pan *et al.* 2008).

This may explain differences between results generated with recombinant VEGF121 in cell culture compared to mouse models with overexpressed VEGF120.

The VEGF receptors

VEGFR1 exists in a soluble and membrane bound form. The membrane bound receptor possesses all the motifs that are classically required for kinase activity. However, the phosphorylation following ligand activation is very low and difficult to detect unless the receptor is expressed at artificially high levels (Seetharam *et al.* 1995, Waltenberger *et al.* 1994). Indeed, genetic removal of the tyrosine kinase domain is compatible with normal vascular development (Hiratsuka *et al.* 1998). However, complete genetic removal of the full-length receptor results in vascular disorganization, angioblasts overpopulation and embryonic lethality, indicative of increased VEGF signaling (Fong *et al.* 1995). It has therefore been suggested that the receptor acts as a soluble and membrane-tethered VEGF-sink, controlling the level of free VEGF.

VEGFR3 is shown to be critical for lymphatic development (Dumont *et al.* 1998). The lymphatic cells develop from the cardinal vein at E10.5 as prox-1^{+} and $VEGFR3^{+}$ cells bud off and migrate toward a VEGFC gradient (Olivier & Srinivasan 2008). *Vegfc*$^{-/-}$ embryos lack lymphatic sacks and die of severe edema (Karkkainen *et al.* 2004). *Vegfr3*$^{-/-}$ mice die already at E10.5, prior to lymphatic formation and show severe vascular remodeling defects. This indicates that VEGFR3 also have an important role in the blood endothelial development. VEGFR3 has further been shown to form heterodimers with VEGFR2, which probably modulates the downstream signaling events of both receptors (Dixelius *et al.* 2003). In zebrafish, the two receptors have been reported to have an interesting expression pattern. The dorsal aorta expresses VEGFR2, but not VEGFR3. In contrast, endothelial cells lining the venous trunk vessels express VEGFR3, but not VEGFR2. Finally, sprouting segmental arterial tip cells express both receptors (Siekmann *et al.* 2008). This could indicate that depending on receptor combinations, a VEGF signal could be interpreted differently in separate endothelial compartments.

Among the known VEGFs, VEGFA signaling through VEGFR2 is the most important, or at least most studied, in vascular development and arterial-venous specification. Again, studies in zebrafish placed VEGFA signaling downstream of Shh but upstream of Notch promoting arterial fate. VEGF antisense morpholino treatments led to a specific defect in arterial differentiation (Lawson *et al.* 2002). This phenotype could be rescued by activation of Notch signaling. Loss of VEGF or VEGFR2 in mice gives a more severe phenotype with complete loss of blood vessels. Interestingly, VEGF haploid mice are capable of making vessels but these develop with reduced size and caliber (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). Whether Notch signaling or arterial-venous specification was affected in these haploid mice was not examined. There are however additional data in mice that points to a role for VEGF in specification of arteries. Arteries but not veins often align with developing peripheral nerves, which produce VEGF (Mukouyama *et al.* 2002). Mukouyama *et al.* also show that *in vitro* culture of endothelial progenitors with VEGF further induced EphrinB2 expression. In

an additional study VEGF was overexpressed in the heart of mice with either an arterial EphrinB2-LacZ or venous EphB4-LacZ reporter. VEGF overexpression robustly increased the ephrinB2^{+} endothelial cells from 50% to 90% and reduced ephB4^{+} cells to 10% (Visconti *et al.* 2002). In paper II we show that the dose of VEGF is important for proper arterial differentiation (Lanner et 2007). Low or intermediate levels of VEGF allow venous differentiation whereas high levels potently drove arterial differentiation.

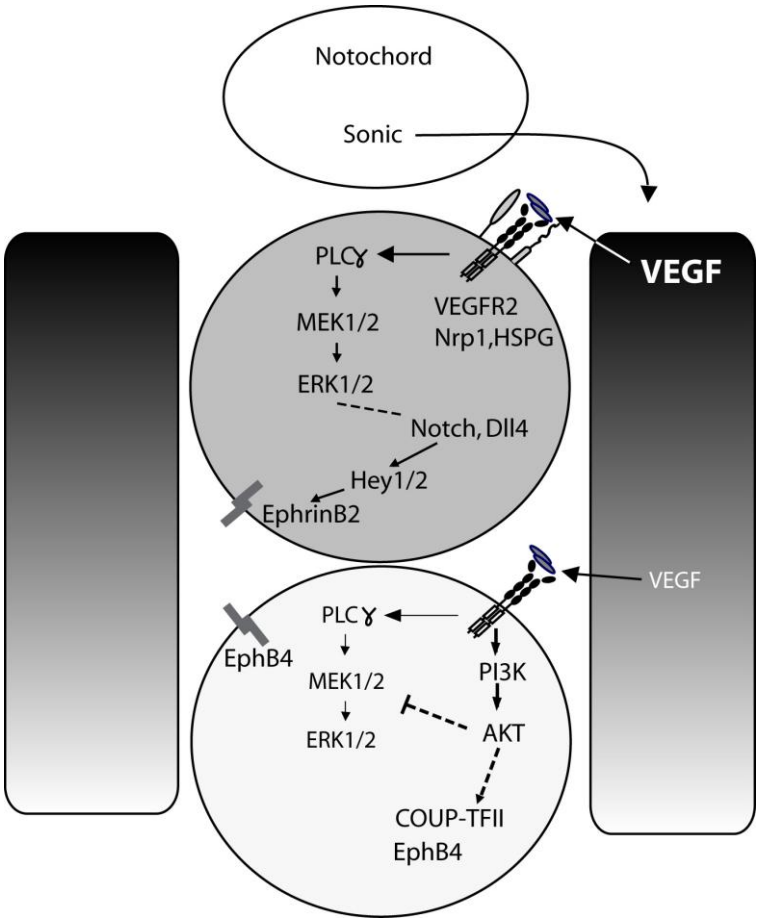

Figure 5. VEGFR2 activates several signal transduction pathways upon ligand activation. Two of these, the ERK and the PI3K pathway have been shown in zebrafish to have opposite arterial venous outcome. The PLCγ-MEK1/2-ERK pathway activates in an unknown manner Notch signaling and promotes arterial differentiation. The PI3K-AKT pathway will block the ERK signaling and promote/allow venous differentiation. How differential pathway activation is achieved is unknown but could be due to the VEGF gradient or possibly through involvement of Nrp1 and HSPG.

Dual VEGFR2 signal transduction pathway

Interesting observations have been made in the downstream VEGFR2 signaling transduction pathway with regard to arterial-venous differentiation. Upon ligand binding, the dimerized receptor is phosphorylated at several tyrosine residues. Phosphorylation of Tyr1173 is essential for VEGFR2 signaling as genetic targeting of

this residue phenocopies the full *Vegfr2*$^{-/-}$ vascular defect (Sakurai *et al.* 2005). This residue activates two signaling cascades, the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways (for review Olsson *et al.* 2006). In zebrafish, Akt activation (downstream of PI3K) has been shown to antagonize the signaling through the Erk pathway and block arterial endothelial differentiation (Figure 5). This antagonism was first discovered in a zebrafish using a small molecules screens (Peterson *et al.* 2004). Incubation of zebrafish embryos with PI3K inhibitors, LY294002 and wortmannin, resulted in activation of ERK/MAPK and expansion of arterial fate (Hong *et al.* 2006). Conversely, inhibition of MEK, an upstream activator of ERK, results in loss of arterial structures. In an alternative approach, mosaic expression of dominant negative AKT along with green fluorescent protein (GFP) resulted in preferential aorta localization. This was in contrast to cells expressing constitutively active AKT that was enriched in the posterial cardinal vein. It should be noted though that in mammalian endothelial cell-culture experiments these observation did not appear to hold true. In contrast to zebrafish, VEGF-induced notch signaling together with Dll4 and Hey2 expression was induced through PI3K, rather than ERK/MAPK (Liu *et al.* 2003).

Notch signaling – downstream of VEGF

A large body of experimental data suggests that Notch signaling is a key downstream mediator of VEGF, driving arterial specification. This signaling pathway is evolutionary conserved and involved in several critical cell-fate decisions such as lineage segregation and establishment of borders throughout embryonic development. The *Notch* gene was first discovered in *Drosophila* as a notched wing trait (Artavanis-Tsakonas *et al.* 1999, Kojika & Griffin 2001).

The receptor is a single-pass membrane protein with large extracellular domains containing epidermal growth factor (EGF)-like repeats (Artavanis-Tsakonas *et al.* 1999). In mammals, four different Notch receptors (Notch1-4) which interact with five ligands have been identified. The ligands are the homologues of the *Drosophila* Delta and Serrate, named Delta-like (Dll) 1, 3, 4, Jagged1, and Jagged2 (Nye & Kopan 1995). These ligands are also single-pass membrane proteins, containing EGF-repeats and bind to the receptor through the common DSL (Delta, Serrate, Lay) motif, located in the extracellular domain of the protein.

The Notch receptor is transcribed as a single protein that is cleaved in the *trans*-Golgi network, producing non-covalently linked Notch extracellular (NECD) and intracellular (NICD) domains. The ligand is also processed by proteolytic cleavage. Furthermore, ligand endocytosis following binding and ubiqutination of the ligand intracellular domain by Neuralized and Mindbomb is also critical for activation (Nichols *et al.* 2007).

Upon ligand binding, the NECD is trans-endocytosed into the ligand expressing cell. Recent data suggest that trans-endocytosis generate a pulling force that facilitates the following ADAM and γ-secretase cleavage, resulting in liberation of the active NICD (Nichols *et al.* 2007). The NICD tranlocates to the nucleus where it interacts with the DNA-binding protein CSL and its coactivator, Mastermind-like (MAML). This event releases the CSL of its corepressors and allows for transcription (Figure 6).

The ligands appear to be competent to signal through all receptors with two exceptions. Firstly, Dll3 has been suggested to lack the ability to interact with any receptors but may cell autonomously attenuate signaling induced by other ligands (Ladi *et al.* 2005). Secondly, Notch receptors are modulated by glycosylation within several of the EGF repeats. This modification inhibits the activation by Jagged/Serrate ligands but potentiates activation by Delta ligands (Haines and Irvine 2003, Bray 2006).

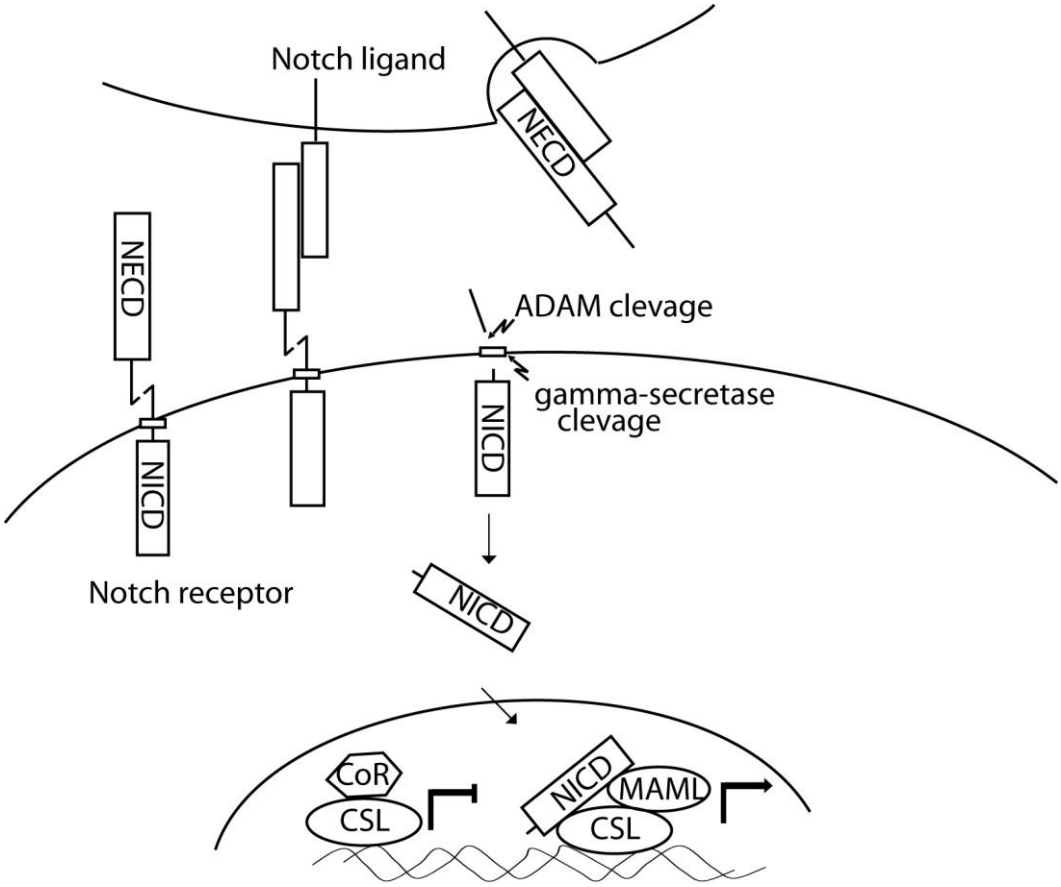

Figure 6. The Notch receptor consists of an extracellular (NECD) and an intracellular domain (NICD) that are non-covalently bound to each other. Upon ligand binding the NECD is trans-endocytosed into the ligand-expressing cell. This generates a pulling force, which has been suggested to facilitate ADAM and gamma-secretase cleavage of the NICD. These two proteolytic events liberates the NICD from the cell membrane and allows transport into the nucleus. The NICD removes co-repressors (CoR) from the DNA binding CSL and recruits the transcriptional co-activator MAML, resulting in activated transcription.

Differential expression patterns in the developing blood vessels suggest however that different ligands and receptors truly have specific functions. Notch1, 3, 4, Dll4, Jagged1 and Jagged2 are expressed in the embryonic mouse vasculature (Villa *et al.* 2001). Arterial but not venous endothelium expresses Notch1, Notch4, Dll4, Jagged1 and Jagged2. Notch3 is expressed selectively in arterial whereas Jagged1can be found in both venous and arterial smooth muscle cells (Shirayoshi *et al.* 1997, Shutter *et al.*. 2000, Uyttendaele *et al.* 1996).

Targeting Notch receptors, ligands and downstream genes

Targeted mutational studies in mice strongly support the importance of Notch signaling in the developing vasculature. Homozygous mutations of *Notch1, Notch4, Dll, Dll4* and *Jagged1* all display severe phenotypes. *Notch1* $^{-/-}$ embryos die by E9.5 with defects in somitogenesis and cardiovascular anomalies (Conlon *et al.*. 1995, Swiatek *et al.* 1994). Krebs *et al.* performed a closer inspection of the vascular system and reported pale yolk sacks where the primary plexus formed but failed to remodel properly. In the intra-embryonic vasculature, the aorta and the other major vessels were underdeveloped (Krebs *et al.*. 2000). *Notch4*$^{-/-}$ embryos posses no detectable vascular phenotype but compound homozygote loss of *Notch1* and *Notch4* has a more pronounced phenotype than embryos only deficient in *Notch1*.

Interestingly, Dll4 was shown to be heterozygote lethal, revealing an important dosage function (Duarte *et al.* 2004, Gale *et al.* 2004). Some heterozygote mice did survive and could be crossed to generate homozygote embryos that showed a stronger phenotype. Similarly to the Notch1 mutants, Dll4 mice fail to remodel the primitive vascular plexus, both in the yolk sack and in the embryo proper. Close inspection showed a general reduction in arterial vessel caliber and branching. The aortas of Dll4 homozygote mice were devoid of the arterial lineage markers EphrinB2, Connexin37 and Connexin40. They could also detect a venous expansion with EphB4 expression in the dorsal aorta. In some occasions the cardinal vein and the dorsal aorta had actually merged and formed one large vessel. This showed that the vascular defects could at least partially be attributed to loss of vessel identity. Trindade *et al.* (2008) created a Dll4 overexpressing mouse, which showed severe vascular phenotypes with ectopic expression of arterial genes. Targeting of the ligand Jagged1 show a similar but slightly later phenotype (Xue *et al.* 1999). Detailed expression analysis revealed that Dll4 and Jagged1 are expressed in a more complimentary than overlapping pattern in the developing retinal vasculature, which could explain the modest phenotypic difference. (Hoffmann & Iruela-Arispe 2007). During the last two years Dll4 has attracted enormous interest not only as a regulator of arterial venous identity but also of tip versus stalk cell identity and angiogenic sprouting (Hellstrom *et al.* 2007, Noguera-Troise *et al.* 2006, Siekmann & Lawson 2007, Suchting *et al.* 2007).

In keeping with a *Notch3* expression in smooth muscle cells, *Notch3*-deficient mice lack the specialized VSMC layers in the wall of muscular arteries (Domenga *et al.*

2004) Furthermore, mutations in the human *Notch3* gene result in CADASIL (Louvi *et al.* 2006). Jin *et al.* (2008) recently showed that *PDGFR*-β is an immediate Notch downstream gene and Notch-induced *PDGFR*-β upregulation was reduced in VSMCs from CADASIL patients, suggesting that Notch regulated PDGF expression plays a role in the pathogenesis of CADASIL.

Studies performed in the zebrafish model support the role of Notch signaling in arterial venous specification. Lawson *et al.* (2001) reported that Notch signaling deficient *mindbomb* mutant embryos showed a loss of arterial vessel identity. In the *mindbomb* embryos arterial Notch3 and EphrinB2 were lost. Instead, venous identity was expanded with increased expression of VEGFR3 and EphB4. It should be mentioned that Notch signaling was targeted indirectly as *mindbomb,* being an E3-ligase, is needed for functional ligand activation in the signal-sending cell. However, the same result was obtained through overexpression of a dominant negative CSL. Conversely, ectopic expression of the Notch pathway repressed venous expression of EphB4 and VEGFR3.

Hairy and Enhancer-of-split-related basic helix-loop-helix (bHLH) transcription factors, (Hey and Hes in mammals and *gridlock* in zebrafish) are direct Notch downstream transcriptional targets and constitute the main transducers of Notch signaling (Iso *et al.* 2003). In zebrafish, vascular progenitors have been shown to be pre-specified to the arterial or venous compartment prior to vessel formation, as discussed above. Genetic targeting of *gridlock* resulted in failed arterial contribution of these progenitors and an expansion of venous gene expression (Zhong *et al.* 2001). Overexpression of *gridlock* generated the opposite phenotype with reduced venous gene expression. Zhong *et al.* suggested that Notch-gridlock is critical to reduce default venous identity and thereby indirectly promote arterial differentiation. Mice express two *gridlock* homologues, Hey1 and Hey2. Targeting them individually failed to reproduce the *gridlock* phenotype indicating functional redundancy. Indeed, combined loss of Hey1 and Hey2 mimicked the results reported in zebrafish with loss of arterial Nrp1 and EphrinB2 expression (Fischer *et al.* 2004,). Although *gridlock* clearly has an important role in arterial venous specification and is classically regarded as a direct downstream target of Notch, this axis still remains elusive. Surprisingly, *gridlock* expression was not changed in Notch loss- or gain-of-function experiments (Lawson *et al.* 2001). Furthermore, Notch activation in zebrafish did not induce arterial markers. This indicates that Notch might cooperate with additional pathways to mediate arterial differentiation.

EphrinB2 and its cognate receptor EphB4 were the first two genes shown to be differentially expressed in arteries and veins. EphrinB2 is, as discussed above, regulated by Notch signaling, possibly through Hey1 and Hey2. Ephrin/Eph signaling is known to mediate migration and repulsion cues in neuronal tissues (Egea 2007). However, the function of the ligand/receptor expression in arteries and veins has been unresolved. Recently Kim *et al.* (2008) proposed a new and interesting model where Notch and EphrinB2/EphB4 signaling regulates the individual and reciprocal size of the arterial and venous compartment. Mice with a gain-of function Notch1 mice showed enlarged dorsal aorta and reduced cardinal veins. In contrast, loss-of-function had the

opposite phenotype. Similar results have been reported earlier but the reciprocal nature has not been appreciated (Krebs *et al.*. 2000, Duarte *et al.* 2004, Gale *et al.* 2004). Loss of EphrinB2 showed increased arterial and reduced venous compartment together with extopic localization of venous cells, expressing EphB4, in the enlarged arterial vessels. This would indicate that repulsive forward EphrinB2-EphB4 is critical to maintain the venous cells in the venous compartment. This is a very attractive mode and it will be interesting to follow how it holds true to further analysis.

COUP-TFII - a venous transcription factor

The majority of genes involved in arterial-venous specification are both expressed in arteries and drive arterial lineage. Venous fate on the other hand has been viewed as the default program (Figure 7). One exception is the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II, COUP-TFII. Biochemical analysis has suggested that both COUP-TFI and II can bind as dimers to different direct, inverted or everted repeat elements (Cooney *et al.* 1992). COUP-TFs can work as transcriptional repressors and activators (Shibata *et al.* 1997). There appears not to be much of a difference in the two transcription factors but they are differentially expressed during development. COUP-TFI is highly expressed in the central nervous system whereas COUP-TFII is expressed in mesenchymal cells (Qiu 1994, Pereira 2000).

The COUP-TFII null mutation displays defects in angiogenesis and heart development (Pereira 1999). It was not until 2005 that You *et al.* discovered the differential expression in the vascular system. A *COUP-TFII/lacZ* knock-in mouse shows expression restricted to venous endothelium from E8.5. Interestingly, smooth muscle cells of arteries but not veins also showed strong expression. Chimera analysis demonstrated that *COUP-TFII*$^{-/-}$ ESC give rise preferentially to arterial and not venous endothelium. Pereira *et al.* were also excluded from arterial smooth muscle cells. Endothelial specific *Tie2-Cre*; *COUP-TF*$^{flox/flox}$ deletion leads to an expansion of arterial markers such as Neuropillin1, Notch1, Jagged1and EphrinB2 in the venous endothelium. There was also a slight increase in Hey1 and moderate reduction in venous EphB4. This would indicate that venous identity was not completely lost. As loss of COUP-TFII allowed expansion of Neuropillin1, the authors argued that COUP-TFII may act upstream of Neuropillin1 and thereby inhibit Notch signaling. However, the mechanism leading to this repression has not been investigated in a more stringent manner.

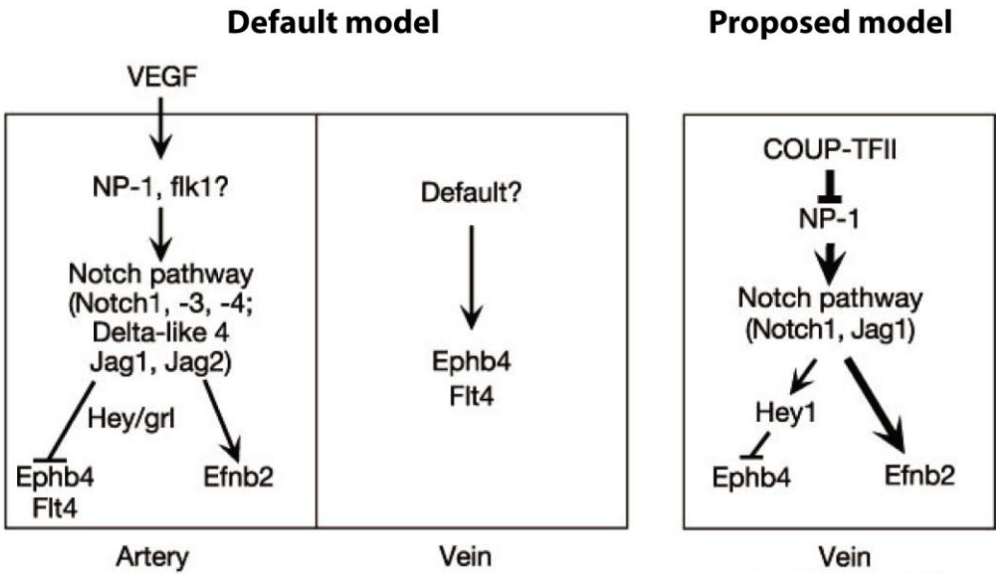

Figure 7. Venous fate has been viewed as the default lineage. You *et al.*, 2005 proposed a new model in which COUP-TFII is suggested to promote venous fate through indirect inhibition of Notch signaling. COUP-TF inhibits Neuropillin1 (NP-1) and thereby reduces downstream Notch signaling.

COUP-TFII has recently been implicated also in lymphatic development (Lee *et al.* 2008). There has been an intense debate over the origin of the lymphatic system, arguing whether they are of a single or dual, venous or hematopoetic origin. Genetic lineage tracing support a model, where lymphatic endothelial cells (LECs) are derived from a subset of venous endothelium. Endothelial cells expressing the homeodomain transcription factor Prox1 migrate out and form the primitive lymphatic vessels (Wigle JT 2002, Srinivasan RS 2007). Overexpression of Prox1 in blood endothelial cells (BECs) represses BEC-specific markers and upregulates LEC-specific genes (Shin *et al.* 2006).

Lee *et al.* (2008) showed the venous transcription factor COUP-TFII to physically and functionally interact with Prox1 in the developing lymphatic cells. siRNA experiments in LEC suggest that COUP-TFII acts as a co-regulator and positively regulating lymphatic genes like VEGFR3, LYVE-1, FGFR3 and Neuropillin1. It is interesting to note that COUP-TFII in these cells positively regulates Nueropillin1. This is in contrast to the observed Neuropillin1 expansion in veins following the loss of COUP-TFII.

Adrenomedullin – in parallel with VEGF?

The vasoactive peptide Adrenomedullin has recently emerged as an additional key regulator of arterial venous differentiation. Initially, Adrenomedullin was isolated from human pheochromocytoma cells on the basis of its ability to elevate cAMP in rat platelets (Kitamura 1993). The peptide consists of 52 amino acids and exhibits a potent

and long lasting vascular relaxant activity. In order to elucidate the functions of Adrenomedullin, Caron and Smithies (2001) replaced the coding region of the Adrenomedullin gene with GFP and found that *Adm* $^{-/-}$ embryos died at midgestation with extreme hydrops fetalis and cardiovascular abnormalities, including overdeveloped ventricular trabeculae. The arterial vessels of the *Adm* $^{-/-}$ embryos had significantly reduced investment of smooth muscle cells, suggesting a potential role in arterial maturation.

Adrenomedullin signal through a receptor complex consisting of the calcitonin receptor-like receptor (crlr) and two different receptor activity-modifying proteins 2 and 3(RAMP-2/3). Yurugi-Kobayashi *et al.* 2006, reported Adrenomedullin to be critical for arterial *in vitro* differentiation of embryonic stem cell derived Flk1^{+} vascular precursors. They further argued that coordinated signaling of VEGF, Notch, and Adrenomedullin-induced cAMP is required to induce arterial differentiation. The effect of cAMP was shown to be dependent on functional Notch-signaling and addition of a cAMP analog, 8-bromo-cAMP, induced nuclear localization of cleaved NICD. Mechanistically, they suggested that Adrenomedullin/cAMP activated Notch signaling through suppression of COUP-TFII expression. Their hypothesis builds on the previous study in which *COUP-TFII*$^{-/-}$ mice that displayed increased Neuropillin1 expression and expanded arterial expression. It should be noted that the effect of 8bromo-cAMP was much stronger than Adrenomedullin alone.

A study in zebrafish lends support to the role of Adrenomedullin signaling in arterial differentiation (Nicoli 2008). Loss of the putative Adrenomedullin receptor *crlr* resulted in severe vascular defects and remarkable loss of arterial endothelial cell identity in the dorsal aorta, indicated by the loss of EphrinB2, DeltaC and Notch5. The receptor was shown to be downstream of Shh and could be induced by Shh. Interestingly, the arterial phenotype following Crlr deficiency could be rescued by overexpression of VEGF. This would argue that Adrenomedullin acts in parallel with VEGF. However, there are even studies suggesting that Adrenomedullin signaling converge with and activatesVEGFR2 signaling pathway (Guidolin *et al.* 2008).

Hypoxic signaling - a potential role in arterial venous specification

As the embryo grow its tissues becomes increasingly hypoxic before a functional vascular system is established. Genetic targeting has shown that molecular responses to O_2 gradients play an important role for proper vascular development.

Central to the hypoxic response is hypoxia inducible factor (HIF) 1α and 2α. HIF1/2α are members of the helix-loop-helix family. Under normoxic conditions (> 5% O_2), the HIF-1/2α proteins are targeted for proteosomal degradation (Ivan *et al.* 2001, Jakkola *et al.* 2001). Under hypoxic conditions HIF-1/2α is stabilized and binds, together with HIF-1β/ARNT, to hypoxia responsive elements (HRE) which activate gene transcription (Poellinger and Johnson 2004). The true sensors of oxygen levels are believed to be a set of prolyl hydroxylases (PHDs). The PHD executes hydroxylation of

specific prolyl residues on HIF-1/2α through an oxygen dependent reaction. This modification serves as a recognition tag to recruit and bind the E3 ubiqutin ligase, von Hippel-Lindau (VHL) tumor suppressor protein that directs the HIF-1/2α for proteolytic degradation. The activity of HIF-1/2α is not only regulated via protein stability. Factor inhibiting HIF-1α (FIH-1) has been revealed to hydroxylate a conserved asparaginyl residue on HIF-1/2α. This modification disrupts the interaction with the critical transcriptional co-activator p300 and blocks activation HRE containing promoters.

Mice deficient in ARNT die by E10.5 with massive vascular defects. The initial vasculogenesis on formation of the primitive plexus appears normal but the subsequent remodeling into functional vessels is disrupted. This was particularly evident in the yolk sac, brachial arteries and the placenta (Adelman *et al.* 1999). *Hif-1α*$^{-/-}$ embryos show similar phenotypes whereas *Hif-2α*$^{-/-}$ mice show a more divergent phenotype depending on genetic background (Iyer *et al.* 1998, Ryan *et al.* 1998, Compernolle *et al.* 2002, Peng *et al.* 2000, Tian *et al.* 1998). Much of the remodeling defects have been attributed to a loss of hypoxic induction of VEGF. This makes sense since even a reduction in VEGF levels cause vascular phenotypes, illustrated by the lethality of VEGF heterozygote mice (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). Although arterial differentiation is shown to be driven by VEGF signaling, a direct alteration in vessel identity has not been described in either hypoxic signaling mutants or the VEGF heterozygote mice.

As discussed above, Notch and hypoxic signaling appear is critical for vascular development and genetic targeting show similar phenotypes. Interestingly, recent data suggest that the two pathways actually converge. Hypoxia was shown to maintain the myogenic and neuronal stem/progenitor state and promote epithelial-to-mesenchymal transition in tumor cell lines in a Notch signaling dependent manner (Gustafsson *et al.* 2005 & Sahlgren *et al.* 2008). Several points of interaction have been suggested. HIF-1α have been shown to interact with NICD and be recruited to CSL binding sites. This could increase NICD stability and/or modulate the properties of the NICD transcriptional complex. An additional mechanism, which could promote HRE dependent transcription, is NICD-titration of FIH-1. FIH-1 has been shown to bind stronger to NICD than to HIF-1α. This could result in a NICD sequestering of FIH-1 and relieve HIF-1α from FIH-1 repression (Coleman *et al.* 2007, Zheng *et al.* 2008). Diez *et al.* (2007) reported a hypoxia induced Dll4-Notch-Hey2 signaling axis in endothelial cells. Using promoter constructs the suggested that dual CSL and HRE-binding site activation was critical for Hey1 activation by hypoxia. These findings further indicate that hypoxia could have an important role in arterial venous specification and spurred us to explore this possibility in Paper III.

PRESENT INVESTIGATION

Aims

The overall aim of my PhD studies has been to establish an *in vitro* model that would allow exploration and lead to increased understanding of vascular development with focus on arterial and venous lineage specification. In the course of this pursuit I have been involved in several projects and collaborations, four of which I present in this thesis. The individual aims of these projects have been to:

- Investigate whether lentivirally expressed VEGFR2 could rescue vascular development of *Flk1*$^{-/-}$ ESC.

- Investigate whether endothelial cells derived through ESC *in vitro* differentiations acquire arterial and venous characteristics. If they do, could *in vitro* differentiation be a new and useful model system for endothelial lineage specification?

- Explore whether embryonic oxygen tension (hypoxia) modulate the endothelial lineage specification using our *in vitro* system.

- Elucidate what potential role the VEGFR2 co-receptor, heparan sulfate proteoglycan (HSPG), plays in endothelial differentiation.

PAPER I: Viral rescue of vascular development

VEGF-A and its receptor VEGFR2/Flk1 play a fundamental role in vascular development and *VEGFR2* deficient mice die between E8.5 and E9.5 of defective hematopoesis and absence of a vascular structures (Shalaby *et al.* 1995, Shalaby *et al.* 1997). *In vitro* differentiation suggests that the *Flk1*$^{-/-}$ ESC do maintain the potential to generate at least early vascular progenitors or immature endothelial cells (Schuh *et al.* 1999). In agreement with these observations, chimeric mice suggested that migration of vascular progenitors from the primitive streak to the extra and intra-embryonic sites of vasculogenesis is defective in *Flk1*$^{-/-}$ cells. VEGFR2 activates several downstream signaling transduction pathways, driving several specific responses. VEGF induced proliferation has been suggested to be mediated through tyrosine (Y) 1173 in the mouse VEGFR2 (Takahashi *et al.* 2001). In addition, the Y949 regulating Src activation and Y1212 is responsible for the MAPK pathway have been suggested to drive vascular permeability and migration respectively (Matsumoto *et al.* 2005).

In an attempt to shed more light on the function of VEGF-VEGFR2 signaling in the formation of endothelial cells and structures, we explored whether function of the *Flk1*$^{-/-}$ ESC could be restored by reintroduction of wild type Flk1 using lentiviral transduction. If this strategy proves successful, it will be possible to further dissect the functions of the individual signal transduction pathways by reintroducing of VEGFR2 with point mutations in the different tyrosine phosphorylation sites.

In this paper we showed that wild type and *Flk1*$^{-/-}$ ESC were readily transduced with lentivirus expressing Flk1 from a constitutively active ubiqutin promoter followed by an internal ribosomal entry site facilitating simultaneous expression of GFP. GFP^{+} ESC were sorted to more than 90% purity. No significant silencing was detected during routine culture or subsequent differentiation. The phosphorylation kinetics of the transduced *Flk1*$^{-/-}$ cells appeared to be normal when compared to the wild type. Interestingly, forced expression or absence of Flk1 did not appear to influence the size of the endothelial cell population during *in vitro* differentiation as embryoid bodies. This indicates that endothelial commitment is independent of VEGFR2 signaling.

We used two *in vitro* assays that resemble vasculogenesis and sprouting angiogenesis respectively. In the 2-D vasculogenesis model, embryoid bodies are grown on a glass slide. Addition of VEGF will drive endothelial cells migration to the periphery and form a primitive plexus-like ring structure. If the embryoid bodies are placed in a 3-D collagen type-I matrix, VEGF will instead drive sprouting angiogenesis (Figure 8). Lentiviral-rescued *Flk1*$^{-/-}$ cell regained VEGF responsiveness with migration of endothelial cells to the rim of plated embryoid bodies in the 2-D assay. This is in agreement with the migration defect of *Flk1*$^{-/-}$ cells in chimeric embryos. Reconstitution of Flk1 expression could also rescue sprouting angiogenesis in response to VEGF stimulation. Although sprouting was regained, the sprouting frequency was substantially reduced in the rescued cultures compared to wild type. Finally we examined if the Flk1 deficient cells could contribute to the vascular structures in chimeric cultures. We mixed *Flk1*$^{-/-}$ or reconstituted ESC with wild type ESC at a ratio of 9:1. The cultures nicely formed vascular structures but the deficient cells failed to incorporate at all in these structures. The reconstituted cells did not exhibit such

negative selection. These *in vitro* data suggest that VEGFR2 is not primarily required for the *de novo* generation of endothelial cells but is needed for the formation and integration into vascular structures.
A more detailed analysis will be required to establish if this defect is the result of a failure in migration or in endothelial maturation. However, this system is now primed to further dissect the specific functions of the different signaling transduction pathways in vasculogenesis, sprouting and possibly in arterial and venous specification.

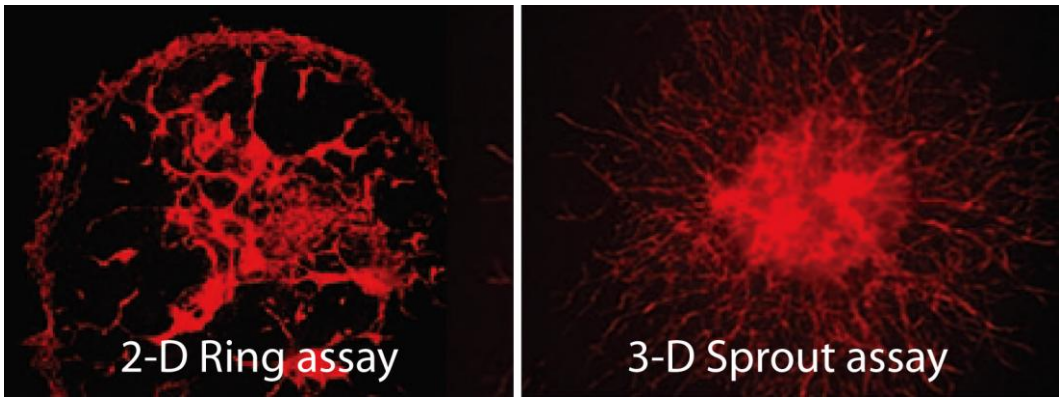

Figure 8. Pictures showing 2-D ring assay and 3-D sprout assay. Endothelial cells are stained with CD31 antibody.

PAPER II: Establishing an *in vitro* differentiation model of arterial venous specification

The primary aim of my PhD studies has been to establish an *in vitro* models system that would allow faster and more convenient exploration of arterial venous lineage specification.

In vitro differentiations of ESC have been extensively and reproducibly used as a cellular assay to study mechanisms of early embryonic development (Murry & Keller 2008). The first indication that vascular development occurred in differentiations of ESC in an *in vivo*-like fashion was first provided by Doetschman *et al.* (1985) and subsequently characterized in more detail by Wang *et al.* (1992). In 2000, Yamashita *et al.* (2000) showed that $Flk1^+$ cells derived through *in vitro* differentiations, give rise to smooth muscle/mural and endothelial cells when cultured on collagen type IV-coated plates. I decided to explore whether *in vitro* differentiation of ESC could be adopted to generate specialized endothelial cells with functional characteristics of arteries and veins.

We set up a protocol were ESC were differentiated for four days, from which $Flk1^+$ cells were isolated by flow cytometry. Sub-culture of these vascular progenitors, robustly generated $CD31^+$ and acLDL incorporating endothelial cells in a VEGF dose-dependent manner. Examining the endothelial transcriptional profiles reveled that arterial transcripts were driven by high VEGF concentration whereas venous transcripts were promoted in differentiations using mediate to low concentrations. The arterializing effect of VEGF was shown to be Notch dependent as gamma-secretase inhibition reduced these arterial transcripts. Next we examined whether morphological and functional characteristics that are associated with arteries and veins could be captured in our *in vitro* differentiation system. Explants from veins have been shown to generate longer sprouts and to recruit less pericytes than explants from arteries (Nicosia *et al.* 2005). Furthermore, leukocyte adhesion and infiltration as a response to inflammatory cues is only seen in veins (Kalogeris *et al.* 1999). We could show that all of these characteristics supported the observed transcriptional profile. With this paper we confirms the possibility of generating specialized endothelial lineages and that this system can be used to further our understanding of arterial venous differentiation.

One major advantage of this system, compared to work done *in vivo*, is the ease to purify cell populations from intermediate stages of vascular development. Comparing global transcriptional and proteomic profiles of $Flk1^+$ progenitors, smooth muscle cells and endothelial cells of arterial and venous identity would provide interesting cues to the mechanistic regulation of vascular lineages. Furthermore, it would be interesting to explore the mechanism for the observed dose-dependent VEGF response. Could the graded response be due to preferential receptor activation of the PI3K or ERK pathways? Work in zebrafish has suggested that ERK activation is critical for arterial fate, whereas PI3K drives venous fate. An attractive and testable model would be that strong VEGFR2 activation, either by high VEGF concentration or/and potentiation though co-receptors like Neuropillin1 and HSPG is critical for ERK activation and arterial differentiation.

PAPER III: Oxygen tension fine tunes lineage decision

Although hypoxia is widely associated with adult pathogenesis, it is also a physiological process that regulates cell differentiation during embryogenesis (Simon & Keith 2008). Prior to the establishment of a functional vascular circulation system, the embryonic development occurs in a highly hypoxic environment. Genetic targeting of several hypoxic response components has revealed severe vascular remodeling defects, which has often been attributed to a loss of hypoxic VEGF induction. Although these embryonic phenotypes resemble and could possibly be attributed to loss of proper arterial venous specification, this has not been specifically addressed. Recently, the hypoxic response has been shown to be partly mediated by Notch signaling (Gustafsson *et al.* 2005). The established role of Notch signaling in vascular and arterial venous specification spurred us to investigate the possible role of hypoxia using our novel differentiation system.

We show that hypoxia (1.5-2% O_2) strongly potentiates arterial differentiation. In the previous paper we identified graded VEGF signaling to be critical for arterial differentiation. Although an increase in VEGF transcripts was detected, the hypoxic effect did not appear to be dependent on hypoxic VEGF induction. Instead, it was largely driven by hypoxia potentiated Notch signaling. A 12xCSL Notch signaling reporter was strongly activated in hypoxic cultures and addition of pharmacological Notch signaling inhibitor potently attenuated the observed transcriptional arterialization. However, the regulation of the arterial transcripts Dll4 and EphrinB2 initially appeared somewhat enigmatic. Although gamma-secretase inhibitor reduced the hypoxic induction, the Dll4 activation could not be completely blocked. Furthermore, Notch signaling deficient *Csl*$^{-/-}$ ESC still revealed a potent increase of both Dll4 and EphrinB2. Activating Notch signaling in normoxic cultures failed to robustly activate EphrinB2 and showed only a moderate induction of Dll4. Transfection with stabilized HIF-1/2α activated VEGF, Flk1, Hey2, Connexin40, and EphrinB2 but not Dll4. Our interpretation of these data is that EphrinB2 is regulated through combined HIF-1/2α and Notch activation. This did not explain the observed Notch-independent Dll4 induction.

Adrenomedullin has recently been implicated in vascular development with a potential role in arterial induction. Interestingly, we notice that HIF-2α transfection strongly activated Adrenomedullin transcripts. Reexamining hypoxic cultures showed a similar induction of Adrenomedullin in a Notch independent manner. It has been suggested by Yurugi-Kobayashi *et al.* (2006) that Adrenomedullin induced cAMP is critical for arterial differentiation together with VEGF and Notch signaling. The authors suggested that Adrenomedullin indirectly potentiated Notch signaling through reduction of the venous promoting transcription factor COUP-TFII. COUP-TFII has been suggested to antagonize Notch signaling through a reduction of Neuropillin1 (You *et al.* 2005). We could indeed detect an increased 12xCSL reporter activity but no transcriptional reduction of COUP-TFII following addition of exogenous Adrenomedullin to normoxic cultures. Instead, our data suggest a more direct Notch-activation through induction of

Notch ligand Dll4. Targeting Adrenomedullin by siRNA in normoxic and hypoxic cultures supported a direct Adrenomedullin-Dll4 axis.

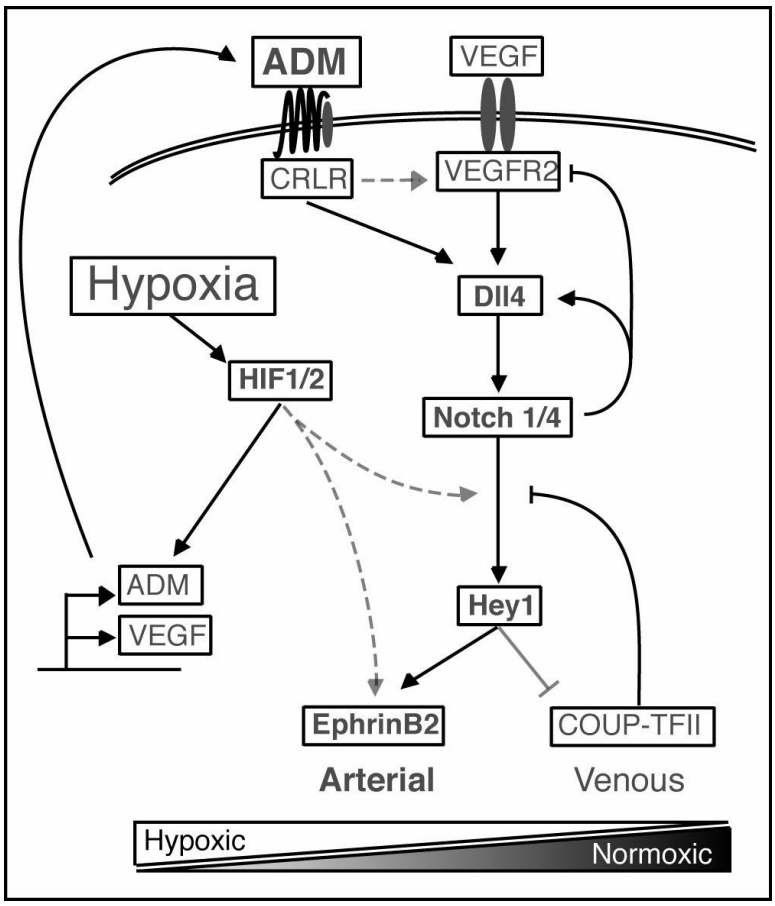

Figure 9. Interaction model of HIF-1/2α, Adrenomedullin, VEGF and Notch signaling pathways in arterial-venous specification. HIF-1/2α potentiates Notch signalling but appears also to directly regulate EphrinB2. The Notch ligand Dll4 is positively regulated by HIF-2α induced Adrenomedullin. Notch signalling further regulates its own ligand Dll4 and promotes arterial over venous differentiation.

This paper has opened several interesting potential leads for further investigation. Firstly, it would make sense to re-examine the vascular phenotype observed in *HIF-1/2α*$^{-/-}$ or *ARNT*$^{-/-}$ embryos to determine whether theses mice indeed display failure in arterial lineage specification. Secondly, the arterial venous lineage segregation appears to be well suited for further detailed dissection of the mechanisms responsible for the observed hypoxia-Notch cross-talk. The role of Adrenomedullin in vascular development has been discovered only recently and could benefit from more detailed studies. Particularly the Adrenomedullin signal transduction pathway would be interesting to dissect. Classically, Adrenomedullin is thought to signal through cAMP induction but recent studies suggest that there could be a direct involvement of the VEGF signaling pathway (Guidolin *et al.* 2008). Finally it would be interesting to look at the potential role of Adrenomedullin in tip-cell regulation since this mediator potently activated Dll4 that plays a key function in tip versus stalk-cell formation.

PAPER IV: HSPGs takes an unexpected turn

During our examination of graded VEGF induced arterial transcripts in Paper II we detected a differential effect of the various VEGF isoforms. The shorter $VEGF_{121}$ that lacks the exons containing the Nrp1 and HSPG binding domains exhibited a different transcriptional response compared to $VEGF_{165}$. We therefore set out to dissect the individual role of Nrp1 and HSPG for VEGF induced arterial transcripts.

Heparan sulfate (HS) covers the surface of almost every type of animal cell during development and in the adult. They are incredibly complex structures consisting of linear chains of negatively charged polysaccharides that range in size from ~40 up to 400 sugars (Haltiwanger & Lowe 2004). These structures are found in high copy number and have been shown to interact with more than 100 proteins. The biosynthesis starts in the Golgi compartment with the linker region consisting of a tetrasaccharide attached to the core protein via a serine-residue (Esko & Lindahl 2001). Exostosin (EXT) 1 and 2 polymerase the polysaccharide backbone (Haltiwanger & Lowe 2004). Next, the chains are subjected to several modification steps such as removal of N-acetyl groups followed by replacement of these with sulfate groups. The enzymes responsible for both these actions are the N-deacetylase/N-sulfotransferase (NDST) 1-4. Further modifications of the chains include several types of O-sulfations (Haltiwanger and Lowe 2004). These types of modifications create a multitude of protein binding motifs critical for their function (Kreuger *et al.* 2005).

A paper by Jakobsson *et al.* (2006), suggested that HSPGs are indeed critical for VEGF-induced vascular sprouting. We obtained the HSPG deficient (*Ndst1/2*$^{-/-}$) ESC used in that study to examine the role in arterial differentiation. Surprisingly, we soon realized that the *Ndst1/2*$^{-/-}$ ESC were not competent to initiate differentiation at all, thus offering an alternative interpretation to the reported vascular phenotype.

We show that *Ndst1/2*$^{-/-}$ maintained transcriptional, morphological and functional properties of ESCs in spite absence of LIF and culture-conditions promoting differentiation. Differentiation was rescued in chimeric cultures where HS was presented in *trans* or by addition of soluble heparin. Chambers *et al.* (2007) showed that Nanog is heterogeneously expressed in ESC cultures and negative cells are more posed for differentiation. Interestingly, we found *Ndst1/2*$^{-/-}$ ESCs to have greatly elevated, almost homogenous, Nanog expression indicative of a more naïve pluripotent state. Addition of sodium chlorate, a known heparan sulfation inhibitor, to wild type cultures copied the *Ndst1/2*$^{-/-}$ phenotype by increasing Nanog positive cells from 50-93% and completely blocking differentiation. As it is able to both block differentiation and induce a more naïve Nanog positive pluripotent state, sodium chlorate also improved efficiency of *de novo* ESC derivation by 100%.

Two recent papers examined the role of EXT1, the polymerase responsible for HS chain synthesis, in mouse ESC with contrasting results. Sasaki *et al.* (2008) reported that reduction in HS chains length as the result of siRNA against *Ext1* actually reduced Nanog transcription and promoted primitive endoderm differentiation. In contrast, and

more in line with our data, are results by Johnson *et al.* (2007) who reported a loss of neuronal differentiation in *Ext1-/-* ESC.

Mechanistically, FGF4 has recently been reported to promote differentiation of ESCs (Kunath *et al.* 2007) and we show that *Ndst1/2*$^{-/-}$ ESCs do not respond to FGF4 stimulation. We show that unlike FGF4, FGF2 that is only expressed later in development does not exhibit the same dependency of N-sulfation and is capable of initiating *Ndst1/2*$^{-/-}$ differentiation. These data are further strengthened by the findings that pharmacological blocking of FGFR robustly induced Nanog and blocked differentiation mimics treatment with sodium chlorate.

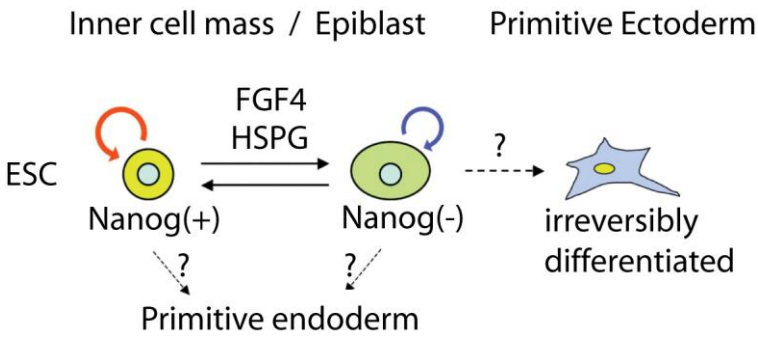

Figure 10. Embryonic stem cells (ESC) are a heterogeneous cell population with Nanog positive and negative cells. We suggest that FGF4 signalling drives the down-regulation of Nanog in a N-sulfated HSPG dependent manner. The Nanog negative cells in ESC cultures are not yet irreversibly differentiated but have been primed to make the transition to primitive ectoderm. Cells of the primitive ectoderm fail to contribute to chimeric mice following blastocyst injection, indicating that they are irreversibly differentiated. Whether the Nanog negative cells also are primed toward primitive endoderm remains to be established.

Having established the role of N-sulfated HS in ESC initiation we are now in a good position to address our initial question of the relative importance of HS and Nrp1 in arterial venous specification. FGF2 could as mentioned in the text above rescue differentiation of the NDST1/2 deficient cells. However, this rescue was only partial and failed to support the formation of Flk1^{+} cells indicating that additional signaling is dependent on proper N-sulfation. Further investigation is needed to identify this signal.

Although the first ESC lines were derived more than 25 years ago we still do not fully understand the mechanisms maintaining pluropotency or the signals that initiate differentiation. Indeed, it was only recently discovered that ESC are a highly plastic and heterogeneous population of cells. It would therefore be very interesting to further study the role of HSPG and FGF4 signaling in establishment of the first lineages in the early mouse embryo.

CONCLUDING REMARKS

In this thesis I have shown that ESC can be used to efficiently generate endothelial cells with transcriptional and functional characteristics of arteries and veins. I have also shown that this system can be used to further our understanding of the mechanisms driving these two lineages and identified a novel function of hypoxia in this segregation.

Recent papers have revealed that this *in vitro* differentiation system can be adopted for generation of lymphatic cells and it will be interesting to explore whether establishment of additional endothelial subtypes like hemogenic endothelium and endocardial cells can be addressed. A critical aspect of these types of *in vitro* differentiation studies will be to establish assays for morphological and functional validation in addition to the more easily attainable transcriptional profiles.

However, being able to direct differentiation into clinically useful cell-types holds great promise for cell transplantation therapies, especially with the recent advances in generation of patient specific induced pluripotent stem cells.

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