**Institution/Department**

# Hypoxia in arterial and venous specification during vascular development

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i CMB auditorium, Berzelius väg 21

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# Abstract

A developing embryo needs a constant supply of oxygen and nutrients in order to survive and grow into a functioning organism. During the earliest embryonic stages passive diffusion is enough to sustain the embryo. At later embryonic stages however, a system for delivering the necessary oxygen and nutrients to all parts of the embryo is needed. As a consequence, the vasculature is the earliest functional organ to form during embryonic development. The area of interest for my research has been vascular development, with a specific aim to uncover mechanisms of arterial and venous specification during embryogenesis. This research could be important for understanding underlying mechanisms behind several severe pathologies such as cancer, diabetes and atherosclerosis.

In the first study we developed a system for generating endothelial cells with arterial or venous characteristics from ESCs (Embryonic stem cells). We showed that VEGF play a critical role in determining both arterial and venous fate. Differentiation of ESCs to endothelial cells using high levels of VEGF promoted arterial specification, while low levels of VEGF induced venous fate. In addition we could show that the VEGF signaling was dependent on Notch signaling for driving arterial fate.

In the second paper we characterized the promoter region of ephrinB2, a gene specifically expressed in arteries but not in veins, in order to identify transcription factors involved in arterial specification. In this study we identified the minimal promoter region of ephrinB2 and proved that the transcription factors MAZ, Meis1 and NFY bind to the promoter and induce EphrinB2 expression in MAE cells. In addition, a TATA-box necessary for ephrinB2 expression was identified.

In the third paper we showed that ephrinB2 is up regulated in response to hypoxia in mouse arterial endothelial (MAE) cells, and aimed to reveal the mechanism for hypoxic regulation of ephrinB2. We proved that neither hypoxia inducible factor (Hif) 1a nor Hif-2a was responsible for inducing ephrinB2 expression in MAE cells. Instead we showed that Sp1 binds to the promoter during hypoxic conditions but not in normoxia, while the opposite is true for MAZ. Also, knocking down Sp1 proved to reduce ephrinB2 expression in hypoxic MAE cells.

In the fourth manuscript we used the *in vitro* ESC differentiation system developed in the first study to investigate how hypoxia affects arterial/venous differentiation of vascular progenitor cells. We showed that hypoxia activates an arterial transcriptional program and that this response is not driven by classic VEGF signaling, but rather by Notch and Adrenomedullin signaling.

# List of publications

1. 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.
   Arteriosclerosis, Thrombosis, and Vascular Biology. (2007) 27, 487-493

2. **Marcus Sohl**, Fredrik Lanner, Filip Farnebo.
   Characterization of the murine Ephrin-B2 promoter.
   Gene (2009), 437, 54-59.

3. **Marcus Sohl**, Fredrik Lanner, Filip Farnebo.
   Hypoxia induced expression of ephrinB2 is independent of Hif-1.
   Manuscript (2009)

4. Fredrik Lanner, **Marcus Sohl**, Emil Hansson, Peter Carmeliet, Lorentz Poellinger, Urban Lendhal, Filip Farnebo.
   Hypoxic induction of Adrenomedullin and Notch signaling promotes arterial differentiation of embryonic stem cells.
   Manuscript (2009)

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# List of Abbrevations

| | |
|---|---|
| Adm | Adrenomedullin |
| Chip assay | Chromatin immunoprecipitation assay |
| cAMP | cyclic adenosine monophosphate |
| Dll | Deltalike ligand |
| DNMT | DNA methyl transferase |
| EC | Endothelial cell |
| ESC | Embryonic stem cell |
| HDAC | Histone deacetylase |
| HIF | Hypoxia inducible factor |
| HRE | Hypoxia responsive element |
| ICM | Inner cell mass |
| LIF | Leukemia inhibiting factor |
| MAE cells | Mouse arterial endothelial cells |
| MAZ | MYC-associated zink finger protein |
| Nrp | Neuropillin |
| PE | Primitive endoderm |
| qPCR | Quantitative polymerase chain reaction |
| Shh | Sonic hedgehog |
| siRNA | Small interfering RNA |
| Sp1 | Stimulating protein 1 |
| TE | Trophectoderm |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |

# Introduction

The most fundamental question for developmental biologists is how one cell can give rise to a functioning multi cellular organism. During embryonic development a wide variety of tissues and cell types is formed and a lot of effort is being put into figuring out the mechanisms behind this process. My research has been focused on vascular development, with a specific aim to uncover mechanisms for how arteries and veins are specified and how hypoxia influences this process.

In order for correct embryonic development to take place, distribution of oxygen and nutrients to all parts of the embryo is vital. In vertebrates the cardio-vascular system is responsible for this distribution both during embryogenesis and in the adult. The vasculature is first formed during early embryonic development in a process called vasculogenesis, but during later stages of vascular development new blood vessels are mainly formed through sprouting from existing vessels in a process called angiogenesis. During embryogenesis, forming new blood vessels is a vital part in creating a functioning organism. In the adult on the other hand, angiogenesis is often connected to pathologic processes like cancer and ischemic heart disease. Thus, figuring out mechanisms for vascular development may not only be important for understanding embryonic development but also for learning more about the mechanisms behind several diseases.

In the vascular system arteries and veins differ both morphologically and at the molecular level. The differences between arteries and veins are specified early during vascular development but also adult vasculature exhibit differential gene expression between arteries and veins (Gerety et al 1999, Adams et al 1999, Mukhopadhyay et al 2008). Vasculogenesis starts when embryonic tissues become hypoxic as the embryo grows and passive diffusion of oxygen is no longer enough to sustain it, and throughout the entire process of vascular development hypoxia is a major driving force (Hänze et al 2007). Therefore, my major approach of studying vascular development has been to investigate how hypoxia influences gene expression and function in the context of arterial/venous differentiation.

Even though transcriptional regulation is important for virtually every cellular function, little is known about the transcription factors that regulate vascular development. In an attempt to find transcription factors that are important in arterial/venous differentiation and hypoxic regulation we decided to study the promoter region of the gene ephrinB2. We chose ephrinB2 because it (1) is specifically expressed in arteries but not veins (Adams et al 1999), (2) is vital for developing a functioning vasculature (Gerety et al 1999), (3) it has been shown to be up regulated in response to hypoxia (Vinhanto et al 2005). We also developed an *in vitro* differentiation system of embryonic stem cells for studying arterial and venous development. This system was subsequently used for studying the effect of hypoxia in establishing vascular lineage fate.

# Early embryonic development and embryonic stem cells

## Early lineage specification

In mammalian embryos the three first distinct cell lineages to form are the trophectoderm (TE), the primitive endoderm (PE) and the epiblast. In mouse embryos early lineage specification begins around day E3.0 by generating two subpopulations of cells, the inner cell mass (ICM) and the TE in a process mediated by the genes OCT3/4 and CDX2 (Figure 1) (Niwa et al 2005). Later in development the OCT3/4 expression is abolished in the ICM and the outermost layer forms the PE while the rest of the ICM forms the epiblast. The endoderm differentiation is driven by GATA6 (Fujikura et al 2002), while Nanog together with SALL4 is responsible for maintaining pluripotency in epiblast progenitors (Chambers et al 2003, Mitsui et al 2003, Zhang et al 2006)(Figure 1). Eventually the TE will connect the embryo to the uterus and form the placenta, while the PE will produce the yolk sac tissue, and the epiblast gives rise to amnion, extra embryonic mesoderm and the fetus.

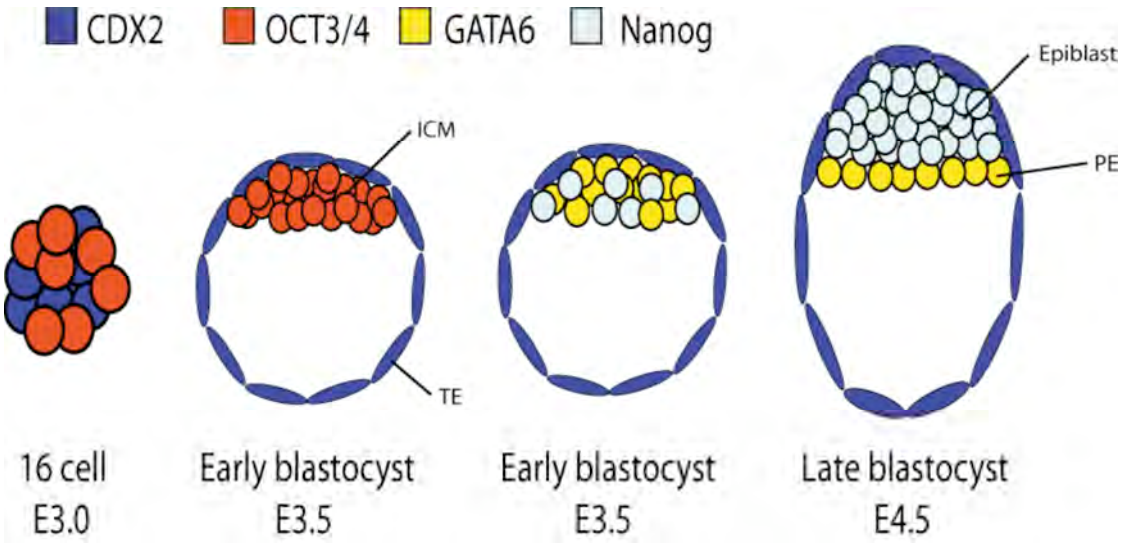


**Figure1.** During embryogenesis the first three lineages that forms are the trophectoderm, primitive endoderm and epiblast. Embryonic stem cells are derived from the inner cell mass (ICM) of the early blastocyst.

## Implantation and gastrulation

At around E4.5 during the mouse gestation the blastocyst attaches to the uterine wall in a process called implantation. This triggers proliferation of the trophectoderm cells, which invade the uterine wall and eventually form the placenta. By E5.5 the embryo is fully implanted in the uterine wall. At E6.5-E7.5 epiblast cells differentiate and form the three primary germ layers of the embryo, the endoderm, mesoderm and ectoderm, in a process known as gastrulation. The first visible sign of gastrulation is the formation of the primitive streak in the posterior region of the embryo (Lawson et

al 1991). Then epiblast cells ingress through the primitive streak and form the mesoderm and endoderm while descendants of the epiblast cells that do not ingress give rise to the ectoderm. The ectoderm form neural tissues, neural crest and skin, while the endoderm produces the digestive system and internal organs like lungs, liver and pancreas. The mesoderm gives rise to blood, vasculature, muscle, bone and connective tissues.

## Embryonic stem cells

Mouse embryonic stem cells (ESCs) were the first to be described and were isolated in 1981 (Evans and Kaufman 1981, Martin 1981). ESCs can be isolated from the ICM of an embryo at the early blastocyst stage (Figure 1). In order to be considered true ESCs the cells must have the following characteristics: (1) Pluripotency, the ability to differentiate and form all of the around 200 cell types that constitute the fetus. (2) Germline competency, the ability to become precursors of the gametes (oocytes, sperm cells). (3) Self- renewal, being able to produce identical copies of themselves. Mouse ESCs do not only show pluripotency *in vitro* but can also give rise to a complete fertile mouse (Poueymirou et al 2007). Also, the proliferation and self-renewal of mouse ESCs *in vitro* is dependent on leukemia inhibitory factor (LIF), a cytokine that is not required for normal early embryonic development (Stewart et al 1992, Ware et al 1995). Until recently mouse was the only species from which true ESCs could be isolated, but now rat ESCs have been obtained. Rat ESCs share several key features with mouse ESCs, including pluripotency, germline competency, morphology, molecular signature and LIF dependence (Li et al 2008, Buehr et al 2008). Human ESCs were first isolated in 1998 (Ware et al 1998) and they show comparable pluripotency with mouse ESCs, but the gene expression profile and growth requirements differ between the two cell populations.

## Origin of endothelial cells

Endothelial cells are derived from the extra embryonic mesoderm of the yolk sac and the mesoderm within the embryo proper (Risau and Flamme 1995, Quinn et al 1993). There are several studies that provide indirect evidence that endothelial and hematopoetic lineages share a common progenitor, referred to as hemangioblast. VEGFR2 knockout mice die at an early embryonic stage due to defects in development of both hematopoetic and endothelial cells (Shalaby et al 1995). The zebrafish gene Cloche has also been shown to be required for development of both hematopoetic and endothelial lineages (Stainier et al 1995). Also, using ESC differentiation, one progenitor derived embryoid body has been shown to contain hematopoetic, endothelial and smooth muscle cells (Choi et al 1998). More direct evidence of the existence of hemangioblasts *in vivo* has also emerged lately. Through fate mapping and lineage tracing, Kinder and colleagues observed that hematopoetic precursors emerge earlier than endothelial progenitors in the yolk sac, suggesting that hematopoetic and endothelial lineages arise independently (Kinder et al 1999). However, single cell resolution fate mapping in zebrafish has demonstrated the existence of hemangioblast cells in the developing embryo. The hemangioblasts only

give rise to a small part of the endothelial lineage though, instead specific endothelial progenitors or angioblasts give rise to most of the endothelium (Vogeli et al 2006). In vivo experiments in mouse and chicken have given similar results, supporting a model where primarily angioblasts but also hemangioblasts contribute to the endothelial lineage (Ueno et al 2006, Weng et al 2007)

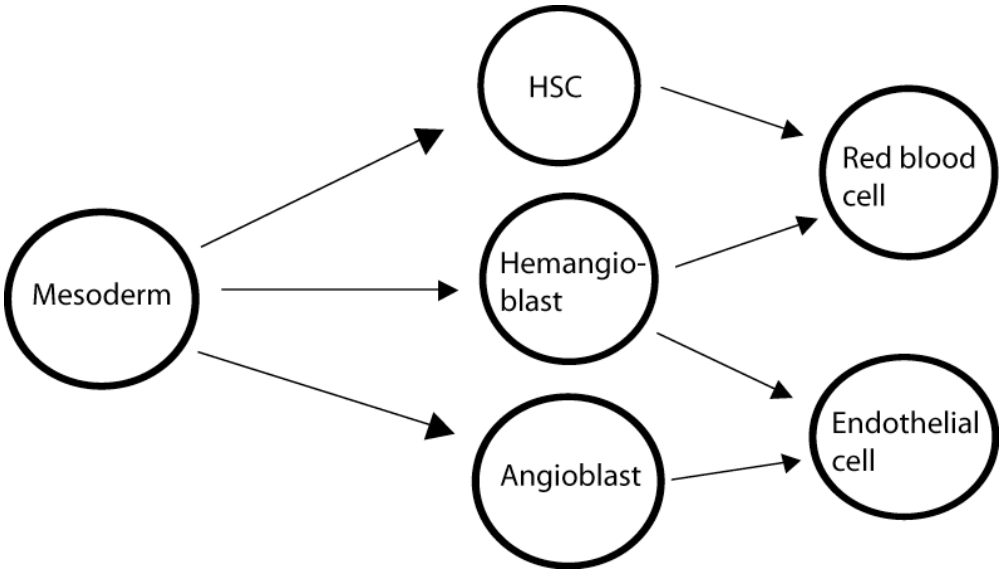


**Figure 2**. The endothelial and hematopoetic lineages emerges from mesodermal tissues and are in part derived from common progenitors.

# Vascular development

A developing embryo needs a constant supply of oxygen and nutrients in order to survive and grow into a functioning organism. During the earliest embryonic stages passive diffusion of is enough to sustain the embryo. However, early during embryogenesis a state of oxygen depravation referred to as hypoxia occurs in the embryonic tissues. Therefore the cardiovascular system is the first organ to develop during embryogenesis and formation of the vasculature in the developing embryo and the adult takes place through two distinct processes called vasculogenesis and angiogenesis. The formation of new vasculature from vascular progenitor cells is called vasculogenesis, while angiogenesis is the process by which new vasculature is formed through sprouting from, or remodeling of, already existing vessels.

## Vasculogenesis

The first sign of vasculogenesis is formation of blood islands in the extra embryonic mesoderm of the yolk sac. Blood islands consist of endothelial progenitor cells (angioblasts), surrounding a core of hematopoetic precursor cells. The angioblasts coalesce and form the primary plexus (His 1900). Because the primitive vasculature first appear in extra embryonic tissue, it was hypothesized that that the blood vessels of the embryo proper were generated through in-growth from extra embryonic vasculature. It was later proven though that the embryonic vasculature is derived from tissues within the embryo (Reagan 1915).

During embryonic vasculogenesis FGF-2 is secreted from the endoderm and promotes differentiation of FGF-receptor expressing mesodermal cells into endothelial progenitor cells (hemangioblasts, angioblasts) (Cox and Poole 2000). Sonic hedgehog (Shh) is expressed in the notochord, and Shh induces expression of VEGF in the somites (Lawson et al 2002). VEGF promotes migration of endothelial progenitor cells from the lateral plate mesoderm to the trunk midline by signaling through the VEGF receptors VEGFR-2 and Nrp1 which are expressed in the endothelial progenitors (Ash and Overbeek 2000, Hirutsaka et al 2005). Then the primary plexus is formed in a series of events, including endothelial cell (EC) maturation, EC proliferation, tube formation, cell-cell junction formation, recruitment of mural cells and arterial/venous specification (Ribatti et al 2009).

It was previously believed that after the formation of a primary plexus, all additional vascular development takes place through angiogenesis. However, it has now been proven that vasculogenesis is involved also at later stages of embryogenesis. Vascularisation of kidneys, lungs and the liver have been shown to take place through vasculogenesis (Robert et al 1998, Gebb and Shannon 2000, Matsumoto et al 2001). There is also evidence that vasculogenesis occurs in the adult and several sources of endothelial progenitors, including hematopoetic stem cells, myeloid cells and tissue residing stem cells have been suggested (Kässmeyer et al 2009).

## Angiogenesis

As tissues grow, the available supply of oxygen may not be enough and a state of hypoxia is induced. As a response those tissues release VEGF, which triggers an angiogenic response, thus restoring the oxygen availability. The cell guiding an angiogenic sprout is called a tip cell, and the other ECs in the sprout are refered to as stalk cells. The tip cells have numerous filopodia that extend into the extracellular matrix and sense attractive and repulsive cues (Gerhardt et al 2003, Lu et al 2004). Tip cells do not divide during vascular sprouting. Instead the stalk cells are responsible for proliferation and formation of the new vascular lumen (Ausprunk and Folkman 1977).

Endothelial cells transform into tip cells as a response to a VEGF gradient. When this happens the tip cell starts to express Dll4. Dll4 subsequently activates Notch-1 in the adjacent stalk cells and Notch-1 suppresses tip cell differentiation, thus preventing formation of excessive amounts of tip cells (Hellström et al 2007).

During vascularisation of the mouse retina VEGF expression is induced in astrocytes through hypoxia dependent regulation (Stone et al 1995). VEGF creates a gradient that acts as an attractive cue and signals through VEGFR-2 and Nrp1, which are expressed in the tip cells (Gerhardt et al 2003, Gerhardt et al 2004). Thus the angiogenic sprout is guided to the site of VEGF expression. The receptor UNC5B is also expressed in tip cells and can control repulsive guidance events. Activation of UNC5B by its ligand netrin-1 leads to filopodia retraction and reduces the number of tip cells (Lu et al 2004).

# Genetic specification and environmental cues in vascular lineage decision

## Genetic specification

Previously it was assumed that the morphological distinctions between arteries and veins were due to the different hemodynamic environments in the two types of blood vessels. This view changed when the genes EphB4 and EphrinB2 was proven to be differentially expressed in arteries and veins. EphB4 is specifically expressed in veins and EphrinB2 in arteries. Also, the differential expression of eprinB2 and ephB4 in arteries and veins was shown to be induced before the onset of circulation and before morphological differences can be detected (Wang et al 1998, Adams et al 1999, Wang H et al 1998). EphB4 and EphrinB2 were the first molecular distinctions between arteries and veins to be discovered, but recently several other genes that are differentially expressed in arteries and veins have been identified. These findings indicate that arterial and venous fate is induced through genetic specification already during vasculogenesis at an early embryonic stage.

## The hemodynamic environment

Although arteries and veins are molecularly distinct before onset of circulation there is evidence that also the hemodynamic environments in arteries and veins influence the genetic expression. A study has shown that when arterial and venous ECs are cultured *in vitro* they lose their asymmetrical EphrinB2 or EphB4 expression (Korff et al 2006). Recently it has also been shown that when endothelial progenitor cells are subjected to shear stress *in vitro*, Sp1 binds to the EphrinB2 promoter and induces expression of EphrinB2 (Obi et al 2009). This study suggests a role for Sp1 in arterial specific expression of EphrinB2 in response to the hemodynamic environment.

## Hypoxia

A condition called hypoxia occurs when the oxygen demand becomes greater than the oxygen supply in tissues or whole organisms. This condition is induced early during embryogenesis when passive diffusion of oxygen no longer can sustain the embryo. Hypoxia plays a major role in vascular development as the expression of some of the most important genes that control vasculogenesis and angiogenesis are induced by hypoxia. The most studied hypoxia regulated proteins is a transcription factor called hypoxia inducible factors (HIF). HIF have been shown to regulate the activity of major vasculogenic and angiogenic factors such as vascular endothelial growth factor (VEGF) and Notch. The HIF pathway represents a major mechanism for hypoxic regulation in vascular development but other mechanisms has also been identified.

*Hypoxia-inducible factor*

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that consists of one a and one ß subunit (Wood et al 1996, Wang et al 1995). HIF binds to DNA sequences called hypoxia regulated elements (HREs) in the promoters or enhancers of target genes. Three different isoforms of the a-subunit has been identified, and during normal oxygen conditions these subunits are rapidly degraded in the cells. The degradation process begins by oxygen dependent hydroxylation by prolyl hydroxylases of proline residues in the HIF-a subunit (Epstein et al 2001, Fukada et al 2007). Then the von Hippel-Lindau tumor suppressor protein (VHL) recognizes the hydroxylated proline residues. VHL is the recognition component of a multiprotein ubiquitin ligase complex, which targets HIF-a for ubiquitin mediated proteolysis in the proteosome (Salceda & Caro 1997, Huang et al 1998, Ivan et al 2001, Jaakkola et al 2001). The hydroxyl groups used for the HIF-a modification is derived from molecular oxygen, thus the degradation process is completely oxygen dependent (Bruick & McKnight 2001, Epstein et al 2001, Ivan et al 2001). During low oxygen conditions (hypoxia), HIF-a is not hydroxylated and therefore not degraded. When HIF-a proteins accumulates in the cell they dimerize with HIF- ß subunits, the HIF-a/HIF-ß complex binds to the HRE of target genes and activates transcription (Bruick & McKnight 2002, Jiang et al1997). Hypoxia does not only affect HIF-a stability but also the transcriptional activity of the HIF-complex, as co-activators like p300/CBP can only be recruited under hypoxic conditions. This mechanism is induced by hydroxylation of an asparagine residue in the HIF-a subunit by an asparaginyl hydroxylase. Hydroxylation of the asparagine residue prevents HIF-a from interacting with the p300/CPB co-activator and the transcriptional activity is reduced.

Knocking out Hif-1ß or HIF-1a in mice results in death by E10.5 due to defects in vascular remodeling although the initial development of vascular beds is intact (Maltepe et al 1997, Ryan et al 1998). These studies prove that HIF proteins are necessary for correct vascular development. Several angiogenic factors, including VEGF have been shown to be direct targets of the HIF transcription factors (Fong 2008). VEGF and its downstream target Notch have been implicated in regulating arterial and venous fate. However, in spite of that, there is no direct evidence that hypoxia is involved in influencing vascular lineage decision through the HIF proteins or any other mechanism.

*HIF independent mechanisms of hypoxic regulation*

Even though VEGF is a direct target gene of HIF-1, knocking down HIF-1 in different angiogenesis models does not significantly affect vessel density. It has also been shown that VEGF is still highly expressed in HIF-1 deficient cells during hypoxia. Another angiogenic factor, interleukin 8 (IL8), is specifically induced in hypoxic HIF-1 deficient tumors (Mizukami et al 2005, Hopfl et al 2002). In human ovarian cancer cells hypoxic induction of IL-8 is mediated by NF-kB. NF-kB is a transcription factor controlled by the RAS-effector pathway P13/Akt (Xu et al 2004). NF-kB can also be induced through generation of reactive oxygen species (ROS)

(H2O2) (Chandel et al 2000) or through decreased prolyl hydroxylation and subsequent degradation of IkB kinase-ß (Cummings et al 2006). It has also been shown that NF-kB can regulate VEGF transcription (Huang et al 2000). In addition, HIF-1 independent activation of VEGF in hypoxia can be mediated through the Rho/Rho kinase pathway (Mizukami et al 2006).

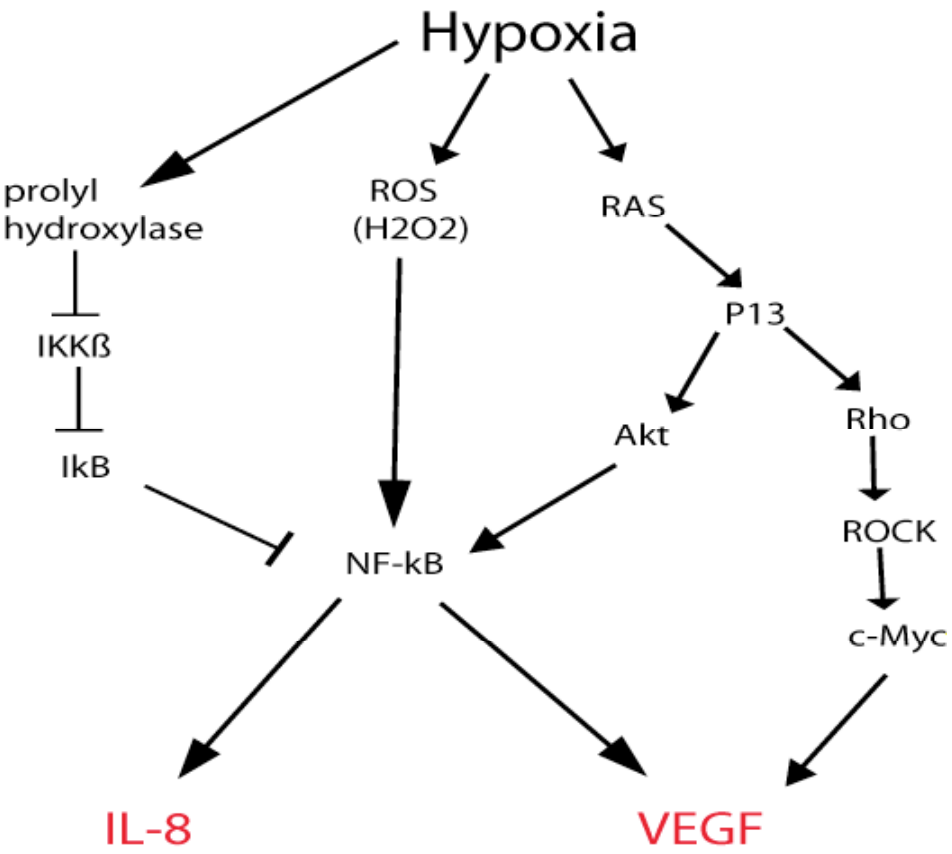


**Figure 3**. VEGF and IL-8 can be induced in hypoxia through HIF- independent mechanisms in tumors.

# Factors involved in arterial and venous fate determination

Several proteins have been shown to be involved in determining vascular lineage fate. The most important factors that have been implicated so far are Sonic hedgehog, VEGF, Notch and the transcription factor COUP-TFII. During the last few years, significant progress has been made in understanding the process of arterial and venous specification and a hierarchal model have emerged. In this section the most important molecules that are involved in specifying vascular lineage fate will be discussed.

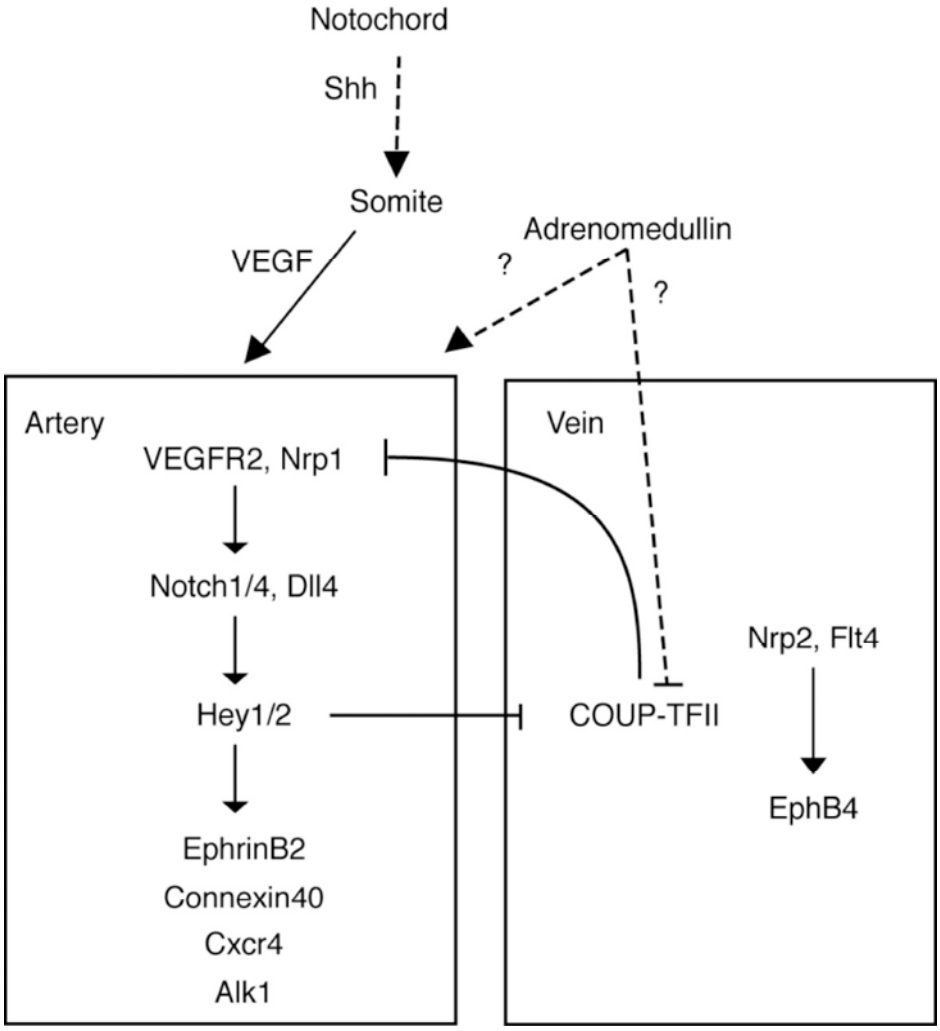


**Figure 4**. A hierarchal model of vascular lineage specification. Shh is expressed in the notochord and induces VEGF expression in the somites. VEGF signals to vascular progenitor cells through VEGFR2 and Nrp1. This leads to arterialization promoted by the transcription factors Hey1 and Hey2. COUP-TFII is expressed in venous endothelial cells have been suggested to block arterial fate by inhibiting Nrp1 and thus preventing Notch signaling.

## Sonic hedgehog

Sonic hedgehog (Shh) is a member of the gene family Hedgehog (Hh), which consists of three ligands (sonic, Indian, and desert hedgehog). All Hh ligands signal through the same receptors, patched-1 and patched-2 (Lavine et al 2007). Sonic hedgehog (Shh) is the first factor driving vascular development to be expressed during embryogenesis. Downstream genes of Shh signaling include VEGF and Notch. During gastrulation Shh is secreted from the notochord and this signal promotes VEGF-mediated migration of vascular endothelial progenitor cells (Hiratsuka et al 2005). Shh deficient zebrafish embryos lack arterial ephrinB2 expression and fail to undergo arterial differentiation. Instead they retain one big blood vessel that expresses venous markers, indicating a role for Shh in arterial differentiation (Lawson et al 2002). Also, Hh signaling has been shown to be essential for endothelial tube formation during vasculogenesis in mouse embryos (Vokes et al 2004).

## VEGF and its receptors

### *VEGF*

Vascular endothelial growth factor (VEGF) is a tyrosine kinase family that is a major regulator of vascular development. It consists of the members, VEGF-A, B, C, D and E, and placental growth factor (PIGF). The VEGFs convey signaling through five tyrosine kinase receptors, VEGF receptor (VEGFR) 1, 2 and 3 plus neuropilin-1 and 2 (Nrp1/2). Also, heparan sulfate (HS) has been shown to modulate VEGF signaling. Like other tyrosine kinases, VEGFRs needs to dimerize in order to be active. Nrp1 and Nrp2 act as co-receptors for VEGFs by forming heteromultimeric complexes with VEGFR-2. VEGF-A is the most studied of the VEGFs and several splice forms has been identified. The most abundantly expressed and most studied of these isoforms are VEGF 121, 165 and 189; the numbers represent the amount of amino acids present in the proteins. VEGF 121 is a soluble, secreted molecule. VEGF-A expression is induced in hypoxia by HIF, but HIF-independent mechanisms can also induce VEGF expression in response to hypoxia. VEGF-A signaling is vital for several processes during vascular development. It has been shown that deletion of VEGF-A, VEGFR1 and VEGFR2 in mutant mice leads to an almost complete lack of blood vessels in the embryo (Carmeliet et al 1996, Ferrara et al 1996, Fong et al 1995, Shalaby et al 1995). Also, studies have shown that migration of vascular progenitor cells is regulated by VEGF-A signaling during vasculogenesis. Sonic hedgehog is secreted from the notochord, and in response VEGF-A is secreted from the somites. Then migrating VEGFR1/2 positive vascular progenitors are guided by the VEGF gradient to the midline of the embryo, where they form the primitive plexus (Cleaver et al 1998, Ash et al 2000, Hiratsuka et al 2005). VEGF signaling also drives tip cell formation and angiogenic sprouting, and has been shown to function upstream of Notch in a signaling pathway regulating early arterial differentiation (Hellström et al 2007, Gerhardt et al 2003, Lawson et al 2002).

*VEGF receptor 1*

VEGFR-1 is mainly expressed on endothelial cells and is up regulated in hypoxia through activation of HIF (Gerber et al 1997). It binds to VEGF-A/B and PIGF. VEGFR-1 is only weakly phosphorylated upon interaction with VEGF-A or PIGF (Seethram et al 1995). Also, deletion of the intracellular tyrosine kinase domain allows normal vascular development (Hiratsuka et al 1998), indicating that VEGFR-1 functions through a different mechanism than phosphorylation. Also, a soluble form of VEGFR-1 has been identified (sVEGFR-1) which inhibits VEGF-A activity (Kendall et all 1996).

VEGF receptor 2

VEGFR-2 is expressed on endothelial cells, hematopoetic stem cells, megakaryocytes and osteoblasts. It binds to VEGF-A, C, D and E, and is strongly phosphorylated upon interaction with its ligands. This phosphorylation is responsible for activating several important processes including: (1) Activation of the mitogen- activated protein kinase/extracellular signal- regulated pathway (MAPK/ERK1/2 pathway), which results in subsequent proliferation of endothelial cells (Takahashi et al 2001). (2) Inducing the P13K and AKT pathway that is essential for endothelial survival (Gerber et al 1998). Several studies have proved the importance of this pathway in vascular morphogenesis, integrity and maturation (Somonath et al 2006, Ackah et al 2005, Chen et al 2005). Also there are P13K isoforms that are important for regulation of endothelial cell migration (Granpera et al 2008). (3) Binding of VEGFR associated protein (VRAP), also important for regulating endothelial cell migration (Matsumoto et al 2005). (4) Sequential activation of CDC42 and p38MAPK which has been implicated in actin remodeling (Lamalice et al 2004), inhibition of p38MAPK increases VEGF-induced angiogenesis (Issbrucker et al 2003, Matsumoto et al 2002). (5) VEGFR-2 also induces some kinases of the Src family, including Fyn, Yes and Src. These proteins play an important role in VEGF-A induced angiogenesis and permeability (Eliceiri et al 1999, Gavard et al 2006).

*VEGF receptor 3*

VEGFR-3 binds to VEGF-C and D and is up regulated in hypoxia. It has mainly been studied in the context of lymphatic development although it is expressed in all endothelia during embryogenesis and only becomes restricted to the lymphatic compartment in the adult. Some studies have demonstrated the importance of VEGFR-3 in blood vessel development. For example, VEGFR-3 knockout mice die due to vascular defects before the onset of lymphatic development (Dumont et al 1998). Also, another study has proven VEGFR-3 to be important for angiogenic sprouting and vascular network formation (Petrova et al 2002).

*Neuropilin 1 and 2*

During vascular development Nrp1 functions as a co-receptor by forming hetero-multimeric complexes with VEGFR-2. Nrp1 expression is induced by hypoxia in endothelial cells and it binds to VEGF-165. Even though it lacks catalytic activity, interaction between Nrp1 and VEGFR-2 increases VEGF induced chemotaxis, signaling and migration (Whitaker et al 2001). Notch is a downstream target of Nrp1/VEGFR-2 signaling. Nrp2 functions as a co-receptor for VEGF-A165, VEGFA145, VEGF-C and D, and forms complexes with VEGFR-2 and 3. Even though Nrp2 mainly functions in lymph-angiogenesis, it is expressed in veins while Nrp1 is expressed in arteries (Yuan et al 2002).

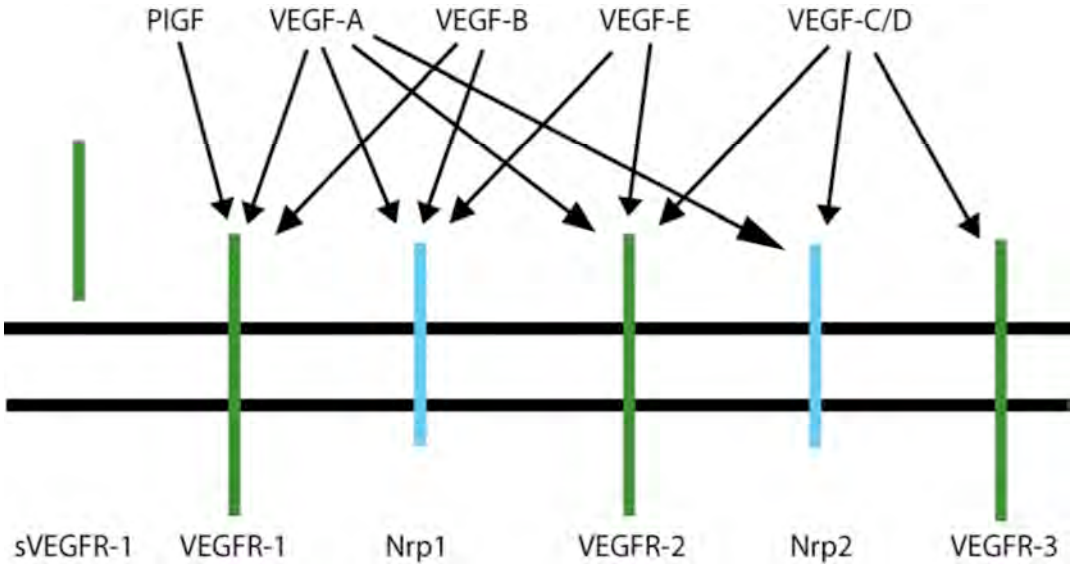


**Figure 5.** Binding specificities between VEGFs and their receptors. sVEGFR-1 has the same binding specificity as its membrane bound form.

*Heparan sulfate proteoglycans*

Heparan sulfate proteoglycans (HSPGs) is a family of cell surface and matrix molecules consisting of protein and carbohydrate components. HSPGs modulate the signaling of many growth factors and during vascular development they can promote VEGF signaling in trans through cell-cell interactions. VEGF-A188 and 165 binds heparan sulfate (HS) with high affinity (Robinson et al 2006) and VEGFR-2 also binds to HS (Ashikari-Hada et al 2005), indicating that HSPG may play a role in forming the signaling complex. HSPG play an important role in capillary formation and patterning (Kjellen 2003, Jakobsson et al 2006).

## Notch and its ligands

Notch receptors are transmembrane proteins that direct cell fate decisions during embryogenesis (Artavanis-Tsakonas et al 1999). There are 4 Notch receptors, Notch1 to 4, and they interact with 5 ligands, Jag1 and 2 and Dll1, 3 and 4. All receptors interact with all 5 ligands but not all receptor/ligand interactions result in signaling (Ladi et al 2005). Notch signaling is usually activated in trans through cell-cell contact but binding to ligands in cis also occurs (Li et al 2004). Upon ligand binding Notch is activated through a series of proteolytic events where ADAM and g–secretase are involved. These events result in release of the Notch intracellular domain (NICD) into the cell, and trans-endocytosis of the extracellular domain (NECD) into the ligand-binding cell. After the NICD is released it is translocated into the nucleus where it binds to the transcription factor CSL. In this process NCID displaces corepressors of CSL and recruits coactivators to form a transcriptionally active complex, which activates promoters containing a CSL-binding sequence.

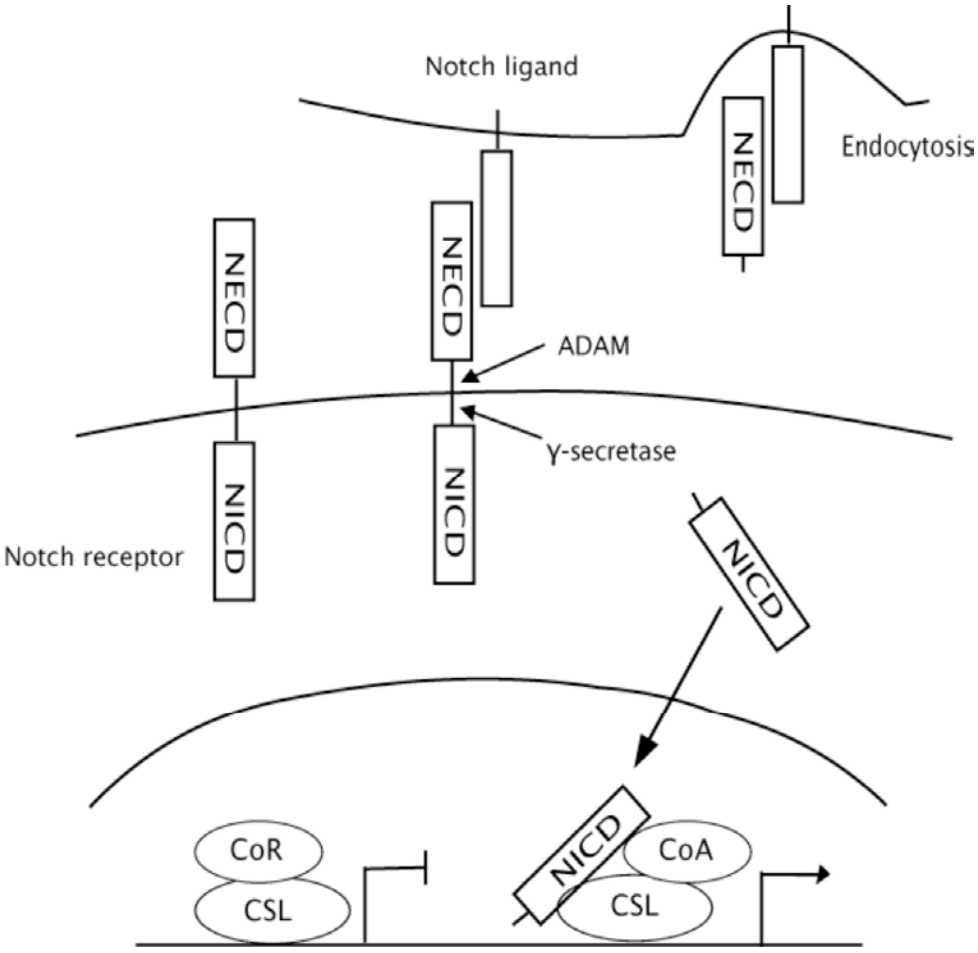


**Figure 6.** Upon ligand binding, the NICD and NECD are proteolytically released by g-secretase and ADAM. The NECD is trans-endocytosed by the ligand-binding cell while the NICD is translocated into the nucleus where it dislocates co-repressors of CSL and induces transcription by interacting with CSL and recruiting co-activators.

In mice Notch1, Notch4, Jag1, Jag2 and Dll4 are all specifically expressed in arterial but not venous ECs (Villa et al 2001). Notch4 deficient mice show no major vascular defects while Notch1 knockout mice die by E9.5 due to severe defects in vascular remodeling. Also, Notch1/4 double mutants exhibit more severe vascular defects than Notch1 deficient mice (Krebs et al 2000). Dll4 deficient mice also die due to severe vascular defects and in at least some genetic backgrounds even heterozygous mice are not viable. These heterozygous mice show increased EphB4 expression and decreased EphrinB2 expression (Duarte et al 2004). In addition, double mutants of the notch downstream targets Hey1 and Hey2 show similar phenotypes to the Notch1 and Dll4 knockouts (Fischer et al 2004). In zebrafish embryos lacking Notch activity, ephrinB2 expression is abolished and venous marker EphB4 is ectopically expressed (Lawson et al 2001). These studies indicate a role for Notch1/4-Dll4 signaling in promoting arterial fate. In Notch deficient zebrafish embryos some artery specific markers are still expressed though, suggesting that additional upstream factors are involved in arterial specification (Lawson et al 2001). During early vascular development Shh and VEGF have been shown to function upstream of Notch signaling. Nrp1 is a co-receptor for VEGFR-2 that is necessary for activating the Notch pathway. The transcription factor COUP-TFII is specifically expressed in veins and has been suggested to suppresses arterial fate by inhibiting Nrp1 expression and the downstream Notch pathway (You et al 2005).

**Adrenomedullin**

Adrenomedullin (Adm) belongs to a family of regulatory peptides that include amylin, calcitonin and calcitonin gene-related peptide. Adm acts via selective receptors derived from the calcitonin receptor-like receptor (CRLR). CRLR functions as a receptor for either CGRP or Adm, depending on its interaction with a family of transmembrane proteins called receptor-activity-modifying proteins (RAMPs). RAMP1 interact with CRLR to form CGRP receptors, while RAMP2 and RAMP3 generate Adm receptors (Ribatti et al 2005). Adm deficient mice die around E13.5 due to hemorrhage under the skin and in visceral organs. The embryos also exhibited poorly developed vasculature in the yolk sac, umbilical cord and placenta, indicating an important role for Adm in vascular development (Chindo et al 2001). Adm has also been proposed to promote arterial differentiation by indirectly enhance Notch signaling by reducing the expression of venous transcription factor COUP-TFII (Yurugi-Kobayashi et al 2006).

**Ephs and Ephrins**

The Eph receptors are a family of 15 tyrosine kinases that have been identified to date. They are divided into two subclasses, the EphAs (EphA1-A9) and EphBs (EphB1-B6), based on their ligand-binding affinity and structure. The Eph ligands are called Ephrins and they are also divided into two subclasses EphrinA (1-6) and EphrinB (1-3). The Ephrins are capable of binding multiple Ephs but EphrinBs binds primarily to EphBs and EphrinAs bind to EphAs. EphA4 is an exception as it binds ligands of both classes. Ephs and Ephrins generate bidirectional signaling upon

interaction with each other, this means that the signaling affects both the receptor and ligand expressing cells. Eph/Ephrin interaction leads to clustering of the molecules, which in turn induces autophosphorilation of several tyrosine residues in the intracellular domain of Eph receptors through its tyrosine kinase domain. The tyrosine kinase domain is also capable of phosphorylating other proteins, and Eph receprors can also mediate signaling through an intracellular PDZ-binding motif. The Eph intracellular domain activates a complex network of downstream targets, including Abelson kinase, Src family kinases (SFKs), phosphotyrosine-binding adaptors, PDZ domain proteins and modulators of RAS and Rho family small GTPases. The intracellular domains of EphrinB ligands are also phosphorylated upon binding to Ephs, but the mechanism for this phosphorylation is unknown. EphrinBs also mediates signals through a PDZ-binding motif. Little is known about the signaling mechanisms of EphrinA ligands but they may require other transmembrane proteins for signal transduction. Signal transduction of both EphrinA and B subclasses involves SFKs (Kullander and Klein 2002, Pasquale 2005, Poliakov et al 2004). Ephs and ephrins control many processes during embryonic development by regulating cell repulsion, adhesion and motility. Several Ephs and Ephrins (EphB2, B3, B4, A2 and EphrinB1, B2, B3 A1) are important for proper vascular development, but EphB4/EphrinB2 signaling is the most extensively studied example of Eph/Ephrin signaling in the context of vascular development (Zhang and Hughes 2006).

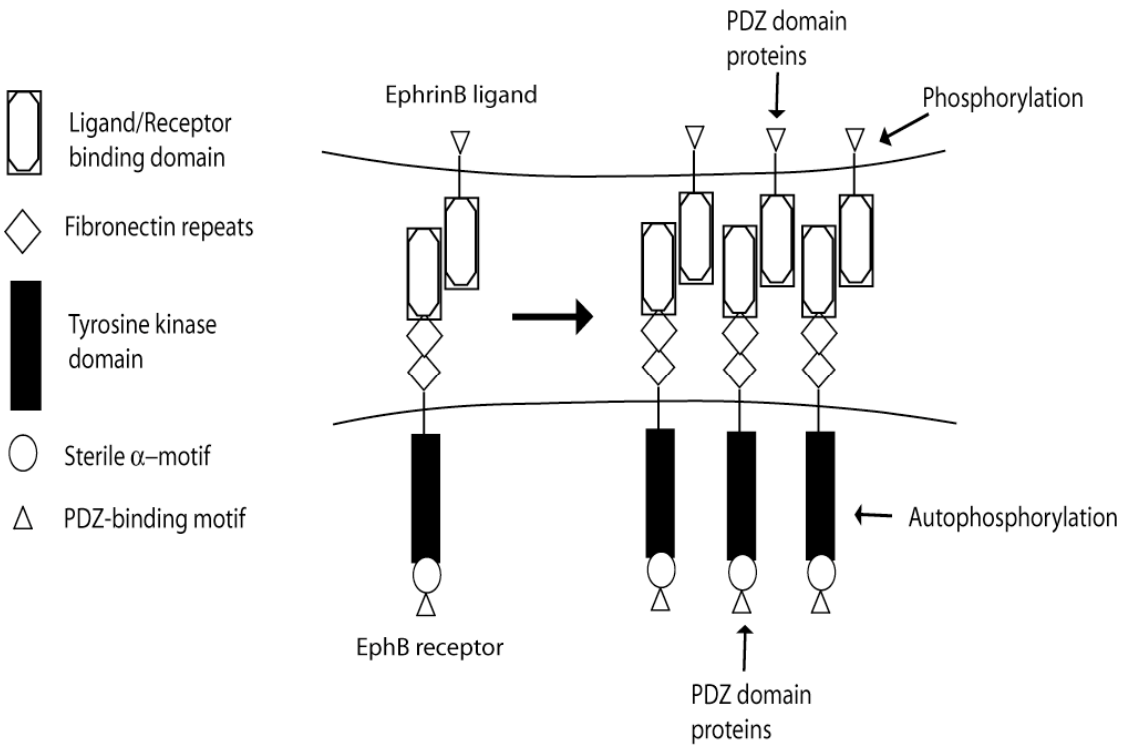


**Figure 7.** EphB/EphrinB bidirectional signaling. Receptor/ligand interaction leads to clustering of the molecules. This in turn leads to autophosphorylation of the receptor by its tyrosine kinase domain, which also is capable of phosphorylating other proteins. The ligand is also phosphorylated upon interaction with the receptor, but the mechanism is unknown. Both the receptor and ligand contain PDZ-binding motifs that can promote signaling through proteins containing PDZ domains.

*EphB4/EphrinB2*

EphB4 and its ligand EphrinB2 are vital for vascular development. Knocking out either EphrinB2 or EphB4 in mouse leads to death at an early embryonic stage due to defective remodeling of the vasculature (Gerety et al 1999). Other studies have proven EphB4/EphrinB2 signaling to be involved in EC migration, adhesion and sprouting *in vitro* and postnatal angiogenesis *in vivo* (Adams et al 1999, Oike et al 2001, Foo et al 2006). Little is known about the signaling pathways through which EphB4/EphrinB2 signaling mediates their function but it has been shown that EphB4 signaling promotes EC migration and proliferation at least in part through P13-kinase and Akt/PKB kinase (Maekawa et al 2003, Steinle et al 2002).

EphB4 and EphrinB2 were the first molecular distinction between arteries and veins to be discovered. EphB4 is expressed on veins while EphrinB2 is expressed on arteries (Wang et al 1998). The arterial-venous distinction at the molecular level by EphB4/EphrinB2 precedes the onset of blood flow and morphological distinction between arteries and veins (Adams et al 1999, Wang H et al 1998). This indicates that during vasculogenesis, arterial/venous fate is determined through genetic specification. Upon *in vitro* culture arterial and venous cells lose their asymmetrical EphB4/EphrinB2 expression, whereas contact with smooth muscle cells and stimulation with VEGF increases EphrinB2 expression (Korff et al 2006). Subjecting endothelial progenitor cells to shear stress *in vitro* up regulates EphrinB2 expression (Obi et al 2009), and EphrinB2 can also be up regulated in response to hypoxia (Vihanto et al 2005). These studies indicate that in addition to genetic specification in early development, microenvironmental determinants influences arterial/venous fate by controlling the expression of EphB4 and EphrinB2. In addition, Notch signaling simultaneously down regulates EphB4 expression and induces EphrinB2 expression (Heroult et al 2006). The transcription factor COUP-TFII is specifically expressed in veins and is believed to induce venous fate by inhibiting expression of the arterial marker nrp-1 and the downstream Notch pathway (You et al 2005).

# Transcription factors in vascular development

Even though a lot of progress has been made in the area of vascular development lately, little is still known about the transcription factors that are involved in regulating vasculogenesis and angiogenesis. In this section the transcription factors that have been shown to influence vascular development will be discussed. The hypoxia inducible factors (HIFs) are also transcription factors but they will not be included here since they have been described previously in this thesis.

## Transcription factors in endothelial development

Tal1 is a transcription factor that is important for both blood and endothelial cell development (Bloor et al 2002). Tal1 is expressed early in the developing embryo and disruption of Tal1 in mouse results in severe vascular defects. However, blood vessels do form in the absence of Tal1, indicating that it is not required for the initial specification of endothelial cells (Kallianpur et al 1994, Visvander et al 1998). Tal1 activates gene enhancers through E-box binding elements, a transcriptional element present in enhancers of several genes involved in endothelial development including VEGFR2, Fli1, Ve-cadherin and Gata2 (Kappel et al 2000, Primada et al 2007, Deluze et al 2007, Khandekar et al 2007). Gata2 is also an important regulator of hematopoetic and endothelial genes. Experiments with embryonic stem cells has shown that GATA2 is important for the development of hemangioblast –like cells and for the induction of endothelial-specific genes (Lugus et al 2007).

Several members of the forkhead (Fox) family of transcription factors are expressed in endothelial cells or their precursors (Papanicolaou et al 2008). Inactivation of FoxO1 or FoxF1 in mice results in embryonic lethality due to vascular defects (Furyama et al 2004, Mahlapuu et al 2001). The mechanism through which FoxO1 controls endothelial gene expression is unknown, but it has been shown that it can function as both a positive and negative regulator of transcription (Daly et al 2004, Paik et al 2007). FoxF1 is expressed before endothelial specification and has been suggested to be involved in controlling Hedgehog signaling (Astorga and Carlsson 2007). FoxH1 has been shown to be a negative regulator of VEGFR2 (Choi et al 2007).

Ets transcription factors are important regulators of endothelial gene expression. At least 19 different Ets proteins are expressed in human endothelial cells (Hollenhorst et al 2004). Deletion or mutation of most Ets proteins in mouse or zebrafish does not result in a severe vascular phenotype. This is probably due to redundancy of Ets factors in endothelial development because they all bind to the same GGA(A/T) sequence in promoters (Graves and Petersen 1998). An exception to this redundancy is Etv2 since deletion of this gene in mice results in severe impairment of vasculogenesis (Lee et al 2008). Etv2 has also been shown to induce expression of several early endothelial genes including VEGFR2 and Tie2 (De Val et al 2008).

## Transcription factors in arterial specification

The transcription factors Hey1 and Hey2 are Notch target genes that are important for arterial differentiation. Knockdown of the zebrafish ortholog of the Hey proteins, Gridlock (grl), causes loss of the dorsal aorta while the axial vein increases in size. Also, arterial markers are down regulated in grl mutants but venous markers are up regulated. Over expression of grl has the opposite effect, as the size of the axial vein is reduced (Zhong et al 2001). Also, Hey1/Hey2 double knockouts in mice lack expression of arterial markers and die due to severe vascular defects (Fischer et al 2004).

Forkhead transcription factors FoxC1 and FoxC2 are also essential for arteriovenous specification. FoxC1/FoxC2 double mutant mice show vascular fusions between arteries and veins, and the mutant embryos also lack expression of several arterial markers (Seo et al 2006). In addition, expression of FoxC1 or FoxC2 in endothelial cell lines up regulates expression of arterial marker genes (Seo et al 2006).

Members of the Sox family of transcription factors may also be involved in arteriovenous specification. Knocking down the orthologs of Sox7 and Sox18 simultainiously in zebrafish results in severe vascular defects. The double knockdown fish fail to acquire correct arteriovenous identity and there are several fusions between the major axial vessels (Cermenati et al 2008).

## COUP-TFII regulates venous fate

The transcription factor COUP-TFII is a transcription factor that is activated by retinoic acid, and has been shown to promote venous identity (Kruse et al 2008, You et al 2005). In vascular endothelium COUP-TFII is expressed in veins and lymphatics but not in arteries. Deletion of COUP-TFII in mice causes lethality due to vascular defects, and these mice also express arterial markers including Nrp1 and Notch1 in veins, indicating a role for COUP-TFII in establishing venous identity (Pereira et al 1999, You et al 2005). Also, over expression of COUP-TFII in endothelial cells in mice caused defects in angiogenesis, large fused vessels and lack of arterial/venous distinction (You et al 2005). In this paper it was proposed that COUP-TFII promotes venous fate by suppressing Nrp1 expression and thus inhibiting Notch signaling, which results in suppression of arterial genes and promotes expression of venous genes.

## Sp1 and MAZ

Sp1 is a ubiquitously expressed gene that previously was believed to function mainly as a constitutive activator of housekeeping genes. However, there is now evidence that several posttranslational modifications including phosphorylation, acetylation sumoylation, ubiquitylation and glycosylation affect the stability, transcriptional activity and DNA binding affinity of Sp1 (Tan and Khachigian 2009). Sp1 has also been shown to suppress transcriptional activity through interactions with DNA methyl

transferase (DNMT) and histone deacetylases (HDACs) (Song et al 2003, Xu et al 2008). Phosphorylation of Sp1 has been shown to induce transcription of VEGF (Milanini-Mongiat et al 2002), and recently Sp1 was proven to bind the promoter of ephrinB2 and induce transcription in endothelial progenitor cells in response to shear stress (Obi et al 2009). MAZ is a transcription factor that is known to regulate transcriptional activity together with Sp1 (Parks and Shenk 1996). HDACs can repress transcriptional activity also through MAZ and MAZ has been shown to bind to the same sites as Sp1 within promoters (Song et al 2003)

# Present investigation

## Aims

The aim of my Phd studies has been to unravel mechanisms involved in lineage decision of endothelial cells in the vascular system. My main focus has been to investigate the role of hypoxia in establishing arterial and venous fate. In the individual studies of my thesis the aims has been to:

- Establish an *in vitro* system for differentiating ESC to endothelial cells with arterial and venous characteristics, and to investigate weather this *in vitro* system could be used as a model system for studying arteriovenous differentiation.

- Find out which transcription factors are involved in regulating the expression of EphrinB2, an arterial specific gene.

- Uncover the mechanism for hypoxic regulation of EphrinB2

- Investigate the role of hypoxia in vascular lineage decisions.

**Paper I: Establishing an *in vitro* differentiation system for studying arteriovenous specification**

ESCs have the potential to give rise to any cell type of the body. This charasteristic has made *in vitro* differentiation assays of ESCs useful tools for studying embryonic development. By subjecting ESCs to different growth conditions it is now possible to generate and study a wide variety of differentiated cell types *in vitro* (Murry and Keller 2008). It has previously been shown that *in vitro* differentiation of ESCs can give rise to smooth muscle and endothelial cells (Yamashita et al 2000). In this study we aimed to develop an *in vitro* assay for differentiating ESC to endothelial cells with arterial and venous characteristics.

We developed a system where ESCs were cultured for 4 days on collagen type IV coated dishes, and VEGFR2+ cells could be isolated through flow cytometry. This population of endothelial progenitors was cultured for another 4 days in medium containing varying concentrations of VEGF. This protocol robustly yielded CD31+ cells, and we could show that high VEGF levels induced an arterial transcript profile while cells subjected to low or intermediate VEGF levels produced a venous transcript profile. Inhibition of Notch signaling using a ɣ-secretase inhibitor showed that the arterializing effect of VEGF signaling was Notch dependent, since the inhibition reduced arterial transcripts.

We also investigated weather our differentiated cells showed morphological and functional characteristics associated with arterial and venous cells. It has been shown previously that explants from veins that are cultured in collagen type I gel generate longer sprouts and recruit less pericytes than explants from arteries (Nicosia et al 2005). Our differentiated cells showed arterial and venous characteristics in this assay. It has also been shown that leukocyte adhesion and infiltration as a response to inflammatory cues are seen in venous but not arterial endothelium (Kalogeris et al 1999). By using a monocyte adhesion assay we could further confirm that our differentiated cells show functional arterial and venous characteristics. This study shows that it is possible to differentiate ESCs to endothelial cells with arterial and venous transcript profiles and functional arterial and venous characteristics.

Most of the work in the area of embryonic vascular development is done in zebrafish and mouse. One important advantage of our *in vitro* differentiation system compared to the more widely used *in vivo* model systems is the ease to purify cells from intermediate stages of development. By comparing progenitor cells at different stages and mature arterial and venous endothelial cells, mechanisms of vascular development may be revealed. Since ESCs also are easy to manipulate genetically, molecular pathways can be readily dissected through *in vitro* differentiation systems. In addition, because it is easy to obtain a lot of cells through *in vitro* differentiation, different cell populations can easily be compared at a global transcriptional and proteomic level using microarrays.

**Paper II: Transcriptional control of EphrinB2 expression**

Even though a lot of progress has been made in understanding the mechanisms involved in vascular development during the last decade, little is still known about the transcription factors that regulate arterial and venous specification. The aim of this study was to identify transcription factors that are involved in controlling arterial/venous endothelial fate. The transcription factors that have been implicated in arterial specification so far are Hey1, Hey2 and members of the Fox and Sox families of transcription factors (Fischer et al 2004, Seo et al 2006, Cermenati et al 2008). However, the mechanisms these transcription factors promote arterial fate through are still poorly understood. The only transcription factor that has been implicated in establishing venous fate is COUP-TFII, which has been suggested to promote venous fate by suppressing Nrp1 expression and thus inhibit Notch signaling (Kruse et al 2008, You et al 2005).

In an attempt to find out more about the transcriptional regulation of genes involved arterial/venous fate decision, we decided to study the regulatory elements of EphrinB2. We chose EphrinB2 for this study because it is specifically expressed in arteries but not veins (Wang et al 1998), it is up regulated in response to hypoxia (Vihanto et al 2005), and the hemodynamic environment influences the expression of EphrinB2 (le Noble et al 2004). The aims for this study were to: 1) Identify the transcription start site of EphrinB2. 2) Isolate the minimal promoter. 3) Identify specific transcription factors involved in regulating the transcriptional activity of the promoter.

The transcription start site for EphrinB2 was identified through 5`race experiments. Deletion constructs made by fusing upstream sequences of EphrinB2 to the luciferase gene of pGL3 vectors, revealed that all transcriptional information necessary for EphrinB2 expression was contained within the first 180 bp of upstream sequence. Bioinformatics methods revealed several putative transcription factor-binding sites within the promoter region. Mutation analysis of these sites indicated that a TATA-box was necessary for transcriptional activity. Also, binding sites for the transcription factors NFY, MAZ, Meis1 and Sp1 were important for transcriptional activity. Chromatin immunoprecipitation (Chip) assays proved that NFY, MAZ and Meis1 interacted physically with the promoter. The chip assays did not reveal any binding of Sp1 to the promoter even though the mutational analysis indicated that 2 putative Sp1 sites were transcriptionally active. Sp1 and MAZ can bind to the same regulatory elements in promoters (Song et al 2003), so MAZ may be responsible for transcriptional activation through the Sp1 binding elements. This hypothesis was strengthened when we knocked down Sp1 and MAZ using siRNA. siRNA against MAZ reduced EphrinB2 expression, while siRNA against Sp1 did not affect EphrinB2 expression.

Both Sp1 and MAZ can regulate gene expression negatively, DNMT and HDACs can suppress gene expression through Sp1 while HDAC 1, 2 and 3 are associated with MAZ repression (Xu et al 2008, Song et al 2003). Thus, this study reveals possible mechanisms for blocking EphrinB2 expression in venous cells or other cells that do not express EphrinB2. Also, several genes upstream of EphrinB2 in vascular

development have been identified. These include sonic hedgehog, notch, VEGF and neuropilins, and they can now be studied in the context of EphrinB2 gene regulation. In addition to genetic specification, the environmental cues oxygen tension and hemodynamic flow are involved in vascular fate decision. Our findings in this study can be used to investigate how environmental cues influences vascular lineage decision at the transcriptional level, using the EphrinB2 promoter as a model system.

**Paper III: Uncovering the mechanism for hypoxic regulation of EphrinB2**

Hypoxia is associated with pathologies like ischemia, inflammation and solid tumors in the adult, but hypoxia is also an important factor during embryogenesis. The HIF transcription factors are the most studied and probably the most important proteins that are regulated by oxygen tension. During vascular development hypoxia play a major role in establishing a functional vasculature and the Hif transcription factors has been shown to be necessary for correct vascular development. Important factors involved in vasculogenesis and angiogenesis like VEGF and angiopoetin 1 and 2, are direct targets of Hif. However, there are other mechanisms for oxygen dependent transcriptional regulation (Simon and Keith 2008).

EphrinB2 expression can be regulated at the transcriptional level in an oxygen dependant manner (Vihanto et al 2005). In this study we aimed to reveal the mechanism for hypoxic induction of EphrinB2. Recently it was shown that Sp1 binds to and activates the promoter of EphrinB2 in endothelial progenitor cells upon exposure to fluid shear stress, indicating that Sp1 can regulate EphrinB2 expression in response to changes in the hemodynamic environment (Obi et al 2009). It has also been shown recently that Sp1 can induce transcription of other genes in response to hypoxia (Eltzschig et al. 2009, Kato et al. 2008). These studies in combination with our results in paper II of this thesis prompted us to hypothesize that Sp1 binds to the EphrinB2 promoter and induces expression in response to hypoxia.

We showed that EphrinB2 expression is up regulated in response to hypoxia in mouse arterial endothelial (MAE) cells. We identified a putative hypoxia responsive element (HRE) less than 1 kb upstream of the transcription start site of ephrinB2, but mutational analysis of this site showed that it was not involved in the hypoxic response. Also, transfecting the cells with plasmids expressing stabilized versions of Hif-1a or Hif-2a did not induce EphrinB2 expression, further indicating that the Hif proteins are not involved in the observed up regulation of EphrinB2 in hypoxia. Chromatin immunoprecipitation (Chip) assays proved that Sp1 but not MAZ bound to the EphrinB2 promoter after the cells were incubated in hypoxic conditions. In paper II we showed that MAZ but not Sp1 bound to the promoter when the cells were incubated in normoxia. In addition, silencing of Sp1 expression using siRNA in hypoxia treated cells resulted in down regulation of EphrinB2. This effect was not seen in the normoxic cells used in paper II.

In this paper we reveal a Hif-independent mechanism for hypoxic regulation of EphrinB2. This mechanism may prove to influence vascular development by regulating the expression of EphrinB2 and perhaps also other genes during embryogenesis. Because Sp1 can induce EphrinB2 expression in response to both hypoxia and shear stress (Vihanto et al 2005, Obi et al 2009), this mechanism might also explain how EphrinB2 expression is maintained when blood flow is switched on in the embryo and the arterial cells go from a hypoxic to an oxygen rich environment.

**Paper IV: Investigating the role of hypoxia in vascular lineage decision**

Even though a lot of effort has been put into studying the role of hypoxia in vascular development, little is known about how hypoxia influences vascular lineage decision. It has been suggested that hypoxia promotes arterial differentiation by inducing Dll4-Notch-Hey2 signaling in endothelial progenitor cells (Diez et al 2006). In this study we used our ESC differentiation system described in paper I to investigate how hypoxia influences arterial/venous differentiation.

In paper I we showed that graded VEGF signaling influenced arterial and venous differentiation. When VEGFR2+ cells were cultured under hypoxic conditions and subjected to high VEGF levels, the arterial transcript levels were strongly enhanced. *Vegf-/-* ESC showed a similar hypoxic response, indicating that hypoxic induction of VEGF is not responsible for the arterial differentiation. By using a 12xCSL reporter vector we showed that Notch signaling is strongly induced in hypoxic cells. Also, inhibition of Notch using a γ-secretase inhibitor strongly reduced the arterial transcriptional response, indicating that hypoxic induction of notch is responsilble for at least part of the observed arterialization. However, strong hypoxic induction of Dll4 and EphrinB2 could be detected in Notch signaling deficient *Csl-/-* cells. Induction of Notch in normoxic cultures failed to enhance EphrinB2 expression and only weakly increased Dll4 levels. Transfection of vectors expressing stabilized versions of Hif-1α or Hif-2α resulted in activation of VEGF, VEGFR2 (Flk1), Hey2, Cx40 and EphrinB2 but not Dll4. These data indicates that EphrinB2 expression can be regulated by a combination of HIF proteins and Notch. The Notch independent induction of Dll4 could however not be explained by these experiments.

A role for Adrenomedullin (Adm) in arterial differentiation has been proposed previously (Yurugi-Kobayashi et al 2006). We could detect strong activation of Adm in response to Hif-2α transfection and to hypoxia. However, γ-secretase inhibitor did not suppress the hypoxic response, indicating a Notch independent mechanism. Yurugi-Kobayashi et al proposed that Adm indirectly enhanced Notch signaling by reducing the expression of venous transcription factor COUP-TFII, a factor that has been suggested to block Notch signaling by down regulating Nrp1 (You et al 2005). Adding exogenous Adm to normoxic cultures did increase 12xCSL reporter activity, but no reduction of COUP-TFII transcripts could be detected. Instead we could see a strong induction of Notch ligand Dll4 in both wt and *Csl-/-* cells, and targeting of Adm using siRNA reduced Dll4 expression. Thus our results suggests a more direct activation of Notch through its ligand Dll4 rather than through down regulation of COUP-TFII.

A role for Adm in vascular development was discovered only recently and Adm has not been extensively studied in this context. In this paper we suggest a possible mechanism for involvement of Adm in hypoxia-mediated arterialization. Adm is known to signal through the receptor ARNT and can activate several pathways including cAMP, NO-cGMP and P13K/Akt (Kato et al 2005). It has also recently been suggested that the VEGF signaling pathway is involved in Adm function (Guidolin et al 2008). Dissecting the Adm signaling pathway in the context of vascular development may reveal important mechanisms for vascular lineage

decision. Also, it would be interesting to investigate a potential role for Adm in tip cell formation since Dll4 plays an important role in tip cell specification (Hellström et al 2007).

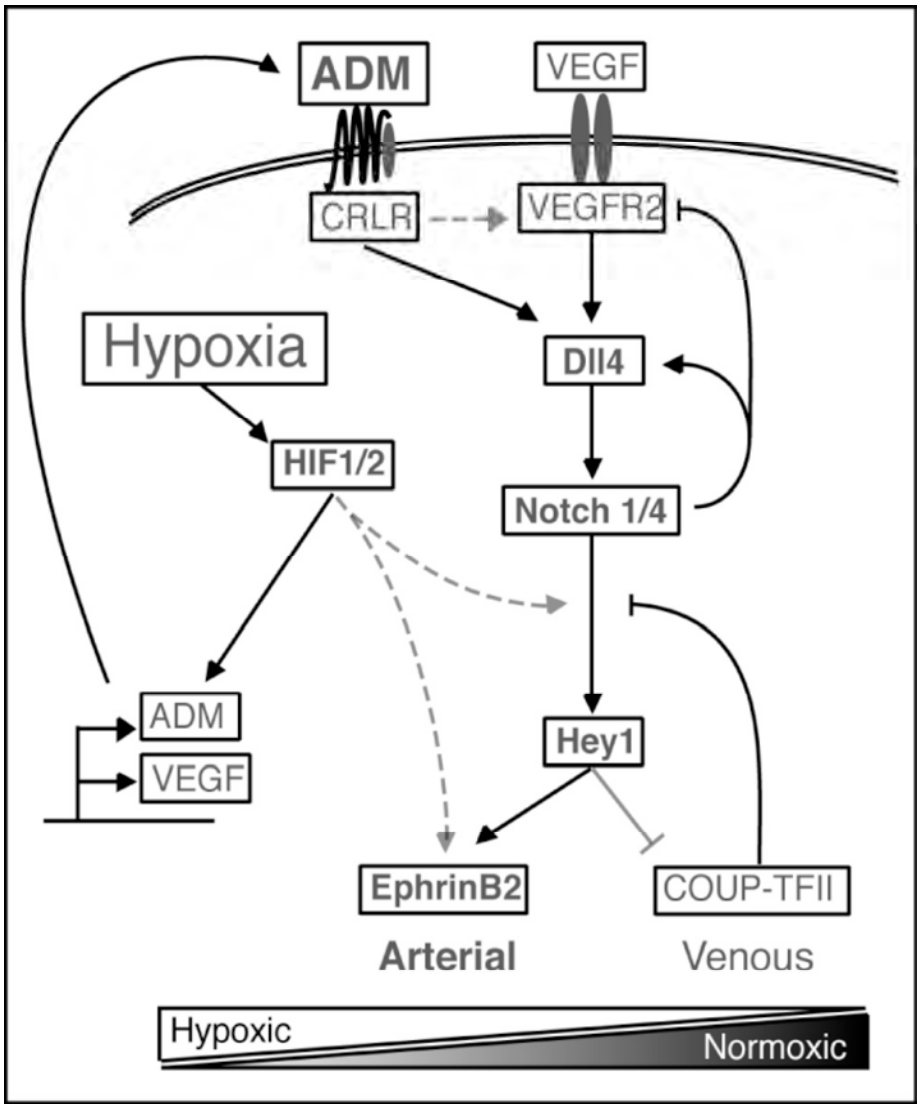


**Figure 8.** A model for interaction between HIF-1/2a, Adm, VEGF and notch signaling in arterial lineage decision. HIF-1/2a induces Notch signaling and up regulates EphrinB2 expression. HIF-2a induced Adm enhances Dll4 transcription. Notch further promotes its ligand Dll4 and promotes arterial fate.

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