The role of Notch signaling in postnatal neovascularization

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ABSTRACT

Notch signaling pathway is an evolutionarily conserved signaling system that is required for normal embryonic development, the regulation of tissue homeostasis, and the maintenance of stem cells in adults. Growth of functional arteries is essential for the restoration of blood flow to ischemic organs. Previously it has been demonstrated that Notch signaling regulates arterial differentiation upstream of ephrin-B2 during embryonic development, however its role during postnatal arteriogenesis is unknown.

In this study it was identified that the Notch ligand *Delta-like 1* (*Dll1*) is an essential regulator of postnatal arteriogenesis. *Dll1* expression was specifically detected in arterial endothelial cells (ECs), but not in venous ECs or capillaries. During ischemia-induced arteriogenesis endothelial *Dll1* expression was strongly induced, Notch signaling activated and ephrin-B2 upregulated, while perivascular cells expressed pro-angiogenic vascular endothelial growth factor (VEGF), and the ephrin-B2 activator EphB4. In heterozygous *Dll1* mutant mice endothelial Notch activation and ephrin-B2 induction after hindlimb ischemia were absent, arterial collateral growth was abrogated and recovery of blood flow was severely impaired, but perivascular VEGF and EphB4 expression was unaltered.

In vitro, angiogenic growth factors synergistically activated Notch signaling by induction of *Dll1*, which was necessary and sufficient to regulate ephrin-B2 expression and to induce ephrin-B2 and EphB4-dependent branching morphogenesis in human arterial EC. Thus, *Dll1*-mediated Notch activation regulates ephrin-B2 expression and postnatal neovascularization.

Based on the finding that Notch signaling is critical for postnatal neovascularization and on literature that bone marrow derived endothelial progenitor cells (EPC) contribute to neovascularization and vascular repair, next the molecular function of Notch signaling in the endothelial progenitor cells was addressed.

Human EPC expressed *Notch1* and *Notch2*, the Notch ligands *Delta-like 1*, *Jagged1* and the Notch target genes *Hey1* and *Hey2*, indicative of active Notch signaling in cultured EPC. In contrast, when Notch signaling was blocked, by using the gamma secretase inhibitor, DAPT, EPC numbers were reduced. Furthermore, Dll1 was identified as a critical activator of Notch signaling in human EPC. Knockdown of Dll1 by siRNA recapitulated the effects of Notch inhibition. This confirms that Notch signaling in EPC is initiated by Dll1-Notch interaction. Using genetic analysis, it was shown that Notch signaling is mediated by the canonical, *RBP-Jκ* dependent pathway, since ablation of *Rbp-jκ* severely impaired EPC differentiation under culture conditions.

In addition, CXCR4 was identified as an important downstream target of canonical Notch signaling in EPC. Notch signaling was required and sufficient to regulate CXCR4 levels, which determined responsiveness of EPC to SDF-1 stimulation in EPC differentiation, migration and adhesion to ECs.

The *in vivo* relevance of Notch signaling in EPC vascular repair was evaluated in the mouse carotid injury model. Injection of cultured EPC enhanced reendothelialization compared to PBS and EPC where Notch activation or CXCR4 signaling, respectively was inhibited. These results demonstrate a requirement for Notch signaling for EPC differentiation, function and vascular repair through regulating the expression of CXCR4, a receptor of SDF-1.

ZUSAMMENFASSUNG

Der Notch Signalweg ist ein evolutionär konserviertes Signalsystem, welches für die Embryonalentwicklung, die Regulation der Gewebehomöostase und die Erhaltung von Stammzellen im Erwachsenen notwendig ist. Das Wachstum von funktionellen Arterien ist essentiell für die Wiederherstellung des Blutflusses in ischämischen Organen. Es ist bereits gezeigt worden, dass der Notch Signalweg während der Embryonalentwicklung die arterielle Differenzierung oberhalb von EphrinB2 in der Signalkaskade reguliert. Seine Rolle in der postnatalen Arteriogenese ist allerdings noch unbekannt.

In dieser Studie konnte gezeigt werden, dass der Notch Ligand Delta-like 1 (*Dll1*) ein essentieller Regulator der postnatalen Arteriogenese ist. Die Expression von *Dll1* wurde speziell in arteriellen Endothelzellen (ECs), nicht aber in venösen Endothelzellen oder Kapillaren nachgewiesen. Während der durch Ischämie induzierten Arteriogenese wurde die endotheliale *Dll1* Expression stark induziert, die Notch Signalübertragung aktiviert und die EphrinB2 Signalübertragung verstärkt, während in perivaskulären Zellen proangiogenetische Faktoren wie Vascular Endothelial Growth Factor (VEGF) und der EphrinB2 Aktivator EphB4 exprimiert wurden. In heterozygoten *Dll1* mutanten Mäusen fand keine Aktivierung von Notch und keine Induktion der EphrinB2 Expression im Endothel nach Hinterlaufischämie statt. Das Wachstum von arteriellen Kollateralen und die Wiederherstellung des Blutflusses waren stark vermindert, wobei die perivaskuläre VEGF und EphB4 Expression unverändert waren.

In vitro aktivieren angiogenetische Wachstumsfaktoren synergistisch über die Induktion von *Dll1* die Notch Signalübertragung, was notwendig und ausreichend ist, um die EphrinB2 Expression zu regulieren und EphrinB2 und EphB4 abhängige Morphogenese von Verzweigungen in humanen arterialen Endothelzellen zu induzieren. Folglich reguliert die *Dll1* vermittelte Notch Aktivierung die EphrinB2 Expression und die postnatale Gefäßneubildung.

Basierend auf den Ergebnissen, dass der Notch Signalweg für die postnatale Gefäßneubildung wichtig ist und auf der Literatur, in der beschrieben worden ist, dass im Knochenmark entstandene endotheliale Vorläuferzellen (EPC, endothelial progenitor cells) zur Gefäßneubildung und Reparatur beitragen, wurde als nächstes die molekulare Funktion des Notch Signalweges in endothelialen Vorläuferzellen untersucht.

Humane EPC exprimieren *Notch1*, *Notch2*, *Delta-like 1* (*Dll1*), *Jagged1* und die Notch Zielgene *Hey1* und *Hey2*. Dies weist darauf hin, dass der Notch Signalweg in kultivierten endothelialen Vorläuferzellen aktiv ist. Wenn allerdings die Aktivierung des Notch

Signalweges z.B. mit dem γ-Sekretase Inhibitor DAPT blockiert wurde, war die Anzahl an endothelialen Vorläuferzellen reduziert. Zusätzlich wurde Dll1 als ein kritischer Aktivator des Notch Signalweges in humanen EPC identifiziert. Der Knockdown von Dll1 mit siRNA zeigte den gleichen Effekt wie eine Notch Inhibierung. Dies bestätigt, dass der Notch Signalweg in EPC durch eine Dll1-Notch Interaktion initiiert wird. Mit genetischen Analysen konnte gezeigt werden, dass die Notch Signalübertragung über den kanonischen, *RBPJκ*-abhängigen Signalweg vermittelt wird, da die Entfernung von RBPJκ die Differenzierung von EPC unter Kulturbedingungen stark vermindert.

Zusätzlich wurde CXCR4 als wichtiges Zielgen des kanonischen Notch Signalweges in EPC identifiziert. Die Notch Signalübertragung war notwendig und ausreichend, um das CXCR4 Niveau zu regulieren, welches die Ansprechbarkeit von EPC auf SDF-1 Stimulation bei der EPC Differenzierung, Migration und Adhäsion von Endothelzellen bestimmt.

Die *in vivo* Relevanz der Notch Signalübertragung in endothelialen Vorläuferzellen bei der Gefäßreperatur wurde in einem Modell untersucht, in dem die Halsschlagader verletzt wird (carotid injury model). Injektion von kultivierten EPC verstärkte die Reendothelialisierung verglichen zur Injektion von PBS oder EPC, in denen Notch Aktivierung oder CXCR4 Signalübertragung unterdrückt worden war. Diese Ergebnisse zeigen deutlich, dass die Notch Signalübertragung eine Bedingung für die Differenzierung von endothelialen Vorläuferzellen, die vaskuläre Funktion und Reparatur von Gefäßen ist, indem sie die Expression des SDF-1 Rezeptors CXCR4 reguliert.

Keywords

Dll1, arteriogenesis, EPC

Schlagworte

Dll1, Arteriogenese, EPC

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ABBREVIATIONS

%	percent
AMD3100	1,1′-[1,4-Phenylenebis (methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride
BF	bright field
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary DNA
DAPT	N-[N-(3,5- difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester
ddH_20	double deionised H2O
DNA	desoxynucleotidtriphosphate
Dnase	desoxiribonuclease
dNTP	desoxynucleotidtriphosphate
DMSO	dimethyl sulfoxide
dpc	days post coitum
EC	endothelial cell
EDTA	ethylendiamintetraacetate
EGTA	ethylene glycol tetraacetic acid
EPC	endothelial progenitor cell
EtOH	ethanol
FCS	fetal calf serum
Fig.	Figure
FGF-2	basic fibroblast growth factor
g	gram oder gravity
GFP	green fluorescent protein
GSI	gamma secretase inhibitor
h	hour
HLI	hind limb ischemia
hPBMNC	human peripheral blood mononuclear cell
HPF	high power field
IF	immunofluorescence
kb	kilo base

l	liter
LacZ	beta-galactoside
LDBF	laser Doppler blood flow
mM	milli molar
mA	milli Ampere
ml	milli liter
µM	micro molar
min	minute
mRNA	messenger ribonucleic acid
n	number of samples
nM	nano molar
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	power of the hydrogen
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT	Reverse Transkription
SDF-1	stromal cell-derived factor 1
SDS	sodiumdodecylsulfate
sec	seconds
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
V	volt
VEGF	vascular endothelial growth factor
VF	combination of VEGF and FGF-2
WB	Western blot
wt	wildtype
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-galaktopyranosid

CHAPTER -1

Functional analysis of Notch ligand, Delta-like 1 in adult arterial growth in response to ischemia

1 INTRODUCTION

1.1 Formation of blood vessels

Formation of the vascular system is one of the earliest and most important events during embryogenesis in mammals. Blood vessel formation supports tissue growth and organ function in development, physiology, and disease. Blood vessels in the embryo form through vasculogenesis; that is, through *in situ* differentiation of undifferentiated endothelial precursor cells, the angioblasts, into endothelial cells that assemble into a vascular labyrinth (Figure 1; Risau, 1997; Bautch, 2002). After formation of the primary capillary plexus, endothelial cells extend filopodia and sprout in response to the vascular endothelial growth factor (VEGF) from the plexus, leading to a mature vascular plexus. Endothelial cell proliferation is high during embryonic and postnatal development but is very low in the adult organisms under normal physiological conditions (Risau and Flamme; 1995).

Once the primary vascular plexus is established it is remodeled into arteries, veins and capillaries via the process called angiogenesis (Figure 1). Angiogenesis involves the breakdown of the extracellular matrix, sprouting of cells from pre-existing vasculature, survival and proliferation of these cells, migration of cells away from the existing vessels, morphogenesis to form tubes, and recruitment of accessory cells. Disruption of any of these steps of angiogenesis will disrupt the remodeling of the primitive vasculature (Shawber and Kitajewski, 2004). Angiogenesis occurs mainly during embryonic development and in postnatal tissue growth. The driving force for angiogenesis is ischemia (Risau, 1997, Gale and Yancopolous, 1999; Krebs et al., 2000). An insufficient supply of nutrients and oxygen prompts the formation of new vessels from the walls of existing vessels in a process called angiogenic sprouting. Hypoxic tissues secrete growth factors and chemokines that stimulate the endothelial cells to break out of their stable position in the vessel wall and jointly coordinate sprouting, branching, and new lumenized network formation, until supply meets demand and quiescence can be re-established. Once it is initiated by environmental growth factor signals, the sprouting process is spearheaded by leading endothelial tip cells. The endothelial cells that follow the tip cells are called stalk cells. Stalk cells form the vascular lumen, they also establish adherens and tight junctions to maintain the integrity of the new sprout and proliferate when stimulated with VEGF-A (Gerhardt and Betsholtz, 2003). Endothelial tip and stalk cells differ in their gene expression profile and both cells have specialized functions during sprouting angiogenesis (Gerhardt and Betsholtz, 2003).

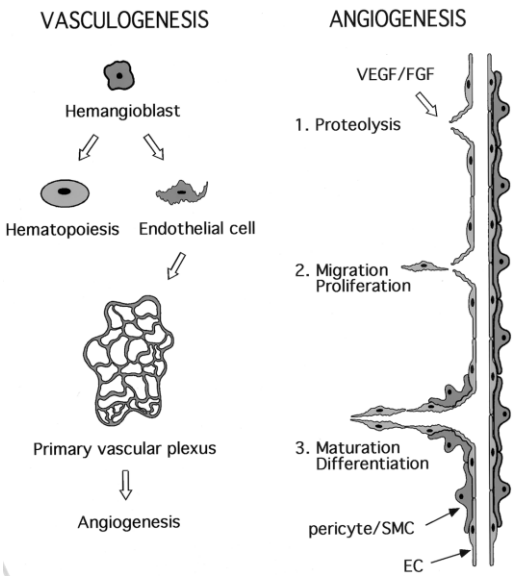


Figure 1: Schematic outline of vasculogenesis and angiogenesis (Adopted from Gerwins et al., 2000). In vasculogenesis, hemangioblasts develop into endothelial cells and hematopoietic cells. The endothelial cells form the primitive vascular plexus, which is further developed and processed to form the mature vascular system. Angiogenesis can be divided into a series of temporally regulated responses, including protease induction, migration, proliferation and differentiation.

1.2 Arteriogenesis

In contrast to embryonic development the formation of blood vessels in adult organisms proceeds in addition to vasculogenesis (mobilization of bone marrow-derived endothelial stem cells) and angiogenesis via arteriogenesis (Risau, 1997; Gale and Yancopolous 1999; Krebs et al., 2000). Postnatal neovascularization is an important mechanism, especially in pathological processes in ischemic cardiovascular disease, such as myocardial and limb ischemia, and wound repair, as well as in physiological processes in the female reproductive cycle and in pregnancy (Hayashi et al., 2005).

Arteriogenesis describes the growth of functional collateral arteries from pre-existing arterio-arteriolar anastomoses to completely developed and functional arteries. Preexistent arterioles are essential (Schaper and Scholz, 2003). Growth of collateral blood vessel is potentially able to preserve structure and function of limbs and organs after occlusion of a major artery (Figure 2; Heil et al., 2006; Cai and Schaper, 2008). Growth is controlled by a complex of subsequent mechanisms. Initial triggers are physical forces such as altered shear forces which appear within the collateral arteriole after a blood flow increase. The blood flow increase is caused by the large pressure difference in the pre-existing arterioles connecting upstream with downstream branches as the result of an arterial occlusion. It comprises the induction of vascular wall cell proliferation and migration and includes wall remodeling processes (Heil et al., 2006). Fluid shear stress (FSS) is the major candidate for the early trigger of arteriogenesis, which initiates a complex cascade of molecular and cellular events leading to

increased vessel lumen and wall thickness. Sensors for FSS may be integrins on the cell surface by which the endothelium is anchored to the extracellular compartment of the vascular wall, and in addition tyrosine kinase receptors and ion channels. It is known that endothelial cells in the collateral wall are activated in response to FSS. This activation is indicated by a number of processes conditioning for attractions of circulating blood cells (Heil et al., 2006). Nitrogen oxide (NO) is the best known transmitter that is released from the stressed endothelium. NO may have a role in permeabilizing the vascular wall during the initial stages of arteriogenesis (Schaper and Scholz, 2003). Activated endothelium also shows increased monocyte chemoattractant protein (MCP-1), TGF-β, and the adhesion molecules ICAM-1 and VCAM (Scholz, 2000, Cai and Schaper, 2008). Increases in vascular radius and wall thickness restore fluid shear stress and circumferential wall stress to normal levels and growth stops. Although increases in collateral vessel size are very substantial their maximal conductance amounts to only 40% of normal. Forced increases in FSS can reach almost 100% (Heil and Schaper, 2004).

Arteriogenesis shares some features with angiogenesis, but the pathways leading to it are different, as are the final results: arteriogenesis is potentially able to fully replace an occluded artery whereas angiogenesis cannot. Arteriogenesis is not organ- or species-specific. In contrast to angiogenesis, arteriogenesis is not dependent on the presence of hypoxia/ischemia, it takes place in a normoxic environment (Ito et al., 1997; Scholz et al., 2002; Schaper and Scholz, 2003). A collateral vessel, resulting from the arteriogenic process, always conducts arterial blood flow and cannot, by definition, become hypoxic. Collateral vessels in the vascular periphery are surrounded by normoxic tissue even after femoral artery occlusion (Helisch et al., 2006). Also the locales of arteriogenesis and angiogenesis are far apart. After the occlusion of the femoral artery, collateral vessels develop in the upper leg between proximal and distal side branches, relatively close to the site of occlusion. In contrast, ischemia and angiogenesis occur in the lower leg and in the foot, at a long distance from the arterial growth (Ito et al., 1997; Scholz et al., 2000; Scholz et al., 2002; Schaper and Scholz, 2003). While it is evident that fluid shear stress is a strong stimulator of arteriogenesis, the genetic program that regulates postnatal arteriogenesis remains largely unknown.

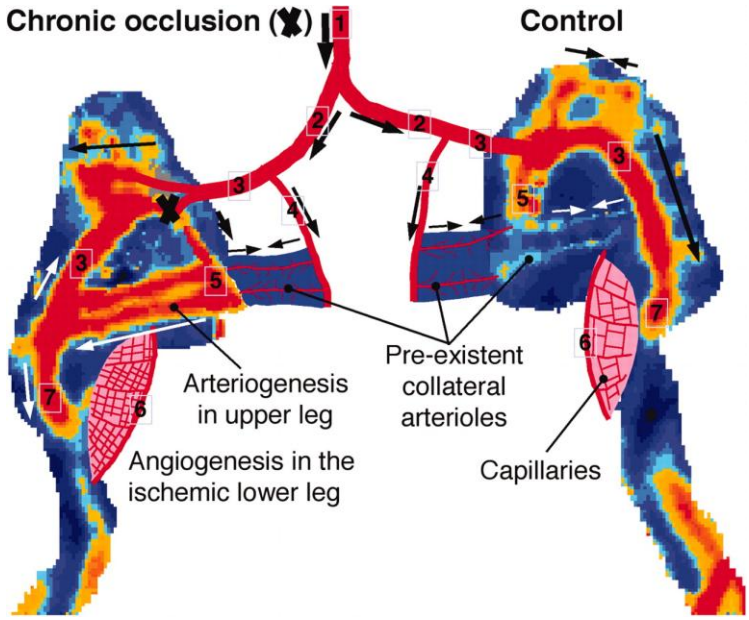


Figure 2: Collateral growth occurs in preexistent arterioles (Adopted from Schaper and Scholz, 2003). Laser Doppler image of collateral blood flow in an anesthetized mouse with exposed upper thigh skeletal muscles with chronic occlusion (7 days) of the left femoral artery. Shown are 2 preexistent arterioles that exhibit a faintly visible flow signal but a very strong signal on the occluded side, ie, the effect of growth after 7 days of occlusion. 1 indicates aorta; 2, A. iliaca; 3, A. femoralis; 4, A. pudenda externa; 5, A. profunda femoris; 6, A. tibialis posterior; and 7, A. saphena.

1.3 Blood vessel morphology

Blood vessels form an extensive hierarchical network of arteries, capillaries, and veins, that provides a conduit system for the transport of liquids, solutes, gases, macromolecules, immunity factors, and cells within the vertebrate body (Carmeliet, 2003; Jain, 2003; Roca and Adams, 2007). Arteries transport oxygenated blood from the lungs through arterioles and finally through capillaries into cells within different tissues. Veins lead oxygen-depleted blood back to the lungs to become oxygenated.

Blood vessels are comprised of two cellular layers: an inner layer of endothelial cells attached to a basement membrane and an outer layer of supportive muscle cells (Risau and Flamme, 1995; Wang et al., 1998). Endothelial cells are centrally involved in each process: they migrate and proliferate and then assemble into tubes with tight cell-cell connections to contain the blood (Hanahan, 1997). Arteries and veins are surrounded by one or more layers of vascular smooth muscle cells (vSMC), which attach in one or multiple layers to the external side of the endothelial basal lamina. vSMCs produce extracellular matrix and elastic fibers that provide vessels with the necessary structural stability and elasticity (Foo et al., 2006). The supporting cells, pericytes, are confined to newly formed vascular structures, capillaries and postcapillary venules (Gerhardt and Betsholtz, 2003). Pericytes extend numerous cellular

processes that are in direct contact with ECs. This interaction leads to a quiescent phenotype of the vasculature and prevents leakage through the vessel wall by reducing the permeability of the endothelial monolayer and promotes endothelial cell survival (Uyttendaele et al., 2001; Shawber and Kitajewski, 2004). Pericytes are thought to stabilize capillaries, whereas vascular smooth muscle cells are critical for arterial function (Figure 3; Dufraine et al., 2008).

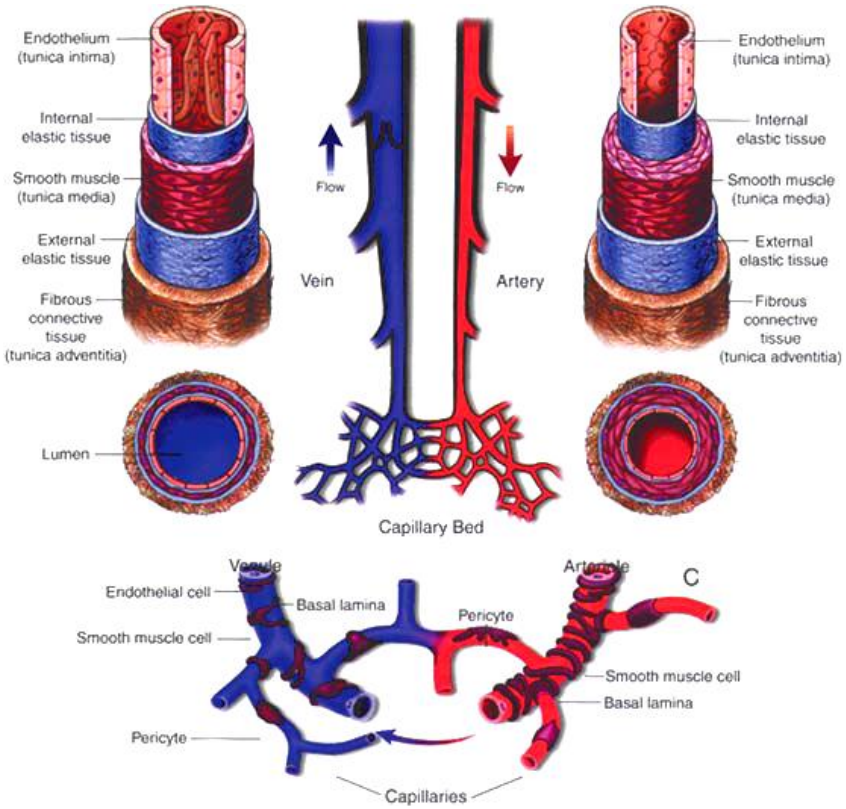


Figure 3: Arteries and veins (Adopted from Torres-Vazquez et al., 2003). Arteries and veins are both composed of an inner endothelium (tunica intima) surrounded by internal elastic tissue, smooth muscle cell layer (tunica media), external elastic tissue, and fibrous connective tissue (tunica adventitia). Larger caliber arteries have thicker smooth muscle cell layers. The two networks of tubes are completely separate at the level of the larger vessels but are linked together distally, in a system of fine capillaries found throughout all tissues.

1.4 Molecular regulation of blood vessels identity

Arteries and veins are morphologically, functionally and molecularly unique. Specification of endothelial cells into arterial and venous cells is a crucial process of vascular development. Physiological parameters such as hemodynamic flow and oxygen tension were, until recently, considered to be the main factors in establishing arterial and venous identity. This thinking changed when the membrane-bound ligand ephrinB2 was found to be specifically expressed in arteries and its receptor EphB4 more restricted to venous endothelium (Wang et al., 1998), alluding to the possibility of genetic programming of arterial and venous identity before the onset of circulation. Embryonic vascular development into mature arteries and veins is

regulated by arterial specific expression of ephrin-B2 with intrinsic signaling capabilities, whereas veins preferentially express the cognate receptor EphB4, which is known to interact only with ephrin-B2 (Wang et al., 1998). Symmetrical mutant phenotypes after targeted null mutations in both genes have demonstrated that reciprocal interactions between ephrin-B2 and EphB4 mediate angiogenic remodeling of arteries and veins through bidirectional signaling (Adams et al., 1999; Gerety et al., 1999). Vascular defects are phenocopied by endothelial specific deletion of ephrin-B2, and by deletion of the cytoplasmic domain of ephrin-B2, which functionally restricts the ligand to forward signaling via Eph receptors, but impaires reverse signaling by its cytoplasmic domain (Adams et al., 2001). In vitro, activation of ephrin-B2 reverse signaling by EphB4 promotes endothelial cell (EC) sprouting and migration (Adams et al., 2001; Hamada et al., 2003). Thus, reverse ephrin-B2 signaling in endothelial cells mediates arterial remodeling and angiogenesis. The inactivation of either gene results in early embryonic lethality with similar vascular abnormalities to Notch (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999). The link between EphB4/ephrinB2 and Notch was provided by Lawson and colleagues (Lawson et al., 2002) in experiments performed in zebrafish. This work demonstrated that Notch acts upstream of EphB4/ephrinB2 and is necessary for the expression of artery specific genes and the subsequent repression of venous specific genes in arteries.

Taken together, these data establish Notch signaling pathway as a key regulator of embryonic vascular development. Given the extent of aspects that are regulated by Notch pathway in vascular development and function it seems plausible to speculate that similar molecular mechanism may be in action in the postnatal arteriogenesis.

1.5 Overview of the Notch signaling pathway

1.5.1 The Notch family and its ligands

Notch signaling pathway is an evolutionarily conserved signaling system that is required for normal embryonic development, the regulation of tissue homeostasis, and the maintenance of stem cells in adults. Notch signaling has been implicated in cell fate decisions by providing inhibitory, inductive, or proliferative signals. It regulates cell-fate decisions by altering patterns of gene expression (Bray, 2006; Louvi and Artavanis-Tsakonas, 2006; Radtke et al., 2006; Karsan, 2005). Notch signaling impacts cell fate either by initiating differentiation processes or by maintaining the undifferentiated state of progenitor cells (Artavanis-Tsakonas et al., 1999).

The precise numbers of Notch paralogues differ between species but the basic paradigm is common throughout (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Radtke and Raj, 2003). In mammals, there are four distinct Notch receptors (Notch1 to Notch4) and five ligands. Ligands are classified in two distinct families: homologues of the *Drosophila* Serrate protein (Jagged1, Jagged2) and homologues of the *Drosophila* Delta protein (Delta-like1, Delta-like3, Delta-like4) (Gallahan and Callahan, 1997; Shutter et al., 2000; Lardelli et al., 1994; Lindsell et al., 1995; Dunwoodie et al., 1997). The ligands are collectively referred to as DSL (for Delta/Serrate/Lag-2) and they have an N-terminal, extracellular DSL motif mediating receptor binding, followed by a variable number of EGF-like repeats in their extracellular domains (eight in mammalian Delta-like protein and 15-16 in Jagged ligands) (Bray, 2006).

1.5.2 Post-translational modifications of the Notch receptors

Before presentation on the cell surface the Notch receptors are modified by several intercellular enzymes. The post-translational modifications take place in the endoplasmatic reticulum (ER) and in the Golgi. Proper modification pattern is prerequisite for both, the correct receptor presentation on cell surface as well as for the pathway activation.

Notch proteins have a large extracellular domain that consists of multiple EGF-like repeats, which are sites for glycosylation. The enzyme O-fucosyl transferase (O-Fut) adds the first fucose and is essential for the generation of a functional receptor (Shi and Stanley, 2003; Sasamura et al., 2003; Okajima and Irvine, 2002). O-Fut also functions as a chaperone to promote the folding and transport of Notch from the endoplasmic reticulum to the cell membrane (Okajima et al., 2005). The activity of O-Fut is critical for the subsequent glycosylation of the Notch extracellular EGF repeats by the glycosyltransferase Fringe in the Golgi. Fringe inhibits the ability of Notch to be activated by Jagged/Serrate ligands, whereas they potentiate the activation by Delta-like proteins (Haines and Irvine, 2003; Bray, 2006).

1.5.3 Notch signaling pathway activation

Depending on the cellular context, Notch signaling has been found to inhibit as well as induce differentiation, proliferation and promote cell survival (Weinmaster, 1997; Artavanis-Tsakonas et al., 1999; Lewis, 1998). Unlike most other paracrine cell signaling pathways, Notch signaling represents juxtacrine signaling with receptors and their ligands both being transmembrane proteins with large extracellular domains mediating communication of adjacent cells (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Radtke and Raj, 2003).

The cell surface localization of both ligand and receptor is consistent with their regulation of cell-fate decisions via direct cell-cell interactions. In general, association between Notch ligands and receptors occurs between cells (homotypic or heterotypic) resulting in trans-signaling events. However, binding to receptors in cis (i.e., within the context of the plasma membrane of the same cell) can also occur (Glittenberg et al., 2006; Li and Baker, 2004). Specificity between the ligands and receptors has not been reported. Thus, all 4 vertebrate Notch receptors are thought to be able to interact with all 5 ligands (Ladi et al., 2005).

The activation of Notch requires a series of proteolytic events that are trigged by binding to cell surface receptors. The mature Notch receptor is a heterodimer composed of amino-terminal N^{EC} and carboxy-terminal N^{TM} fragments (Figure 4; Shawber et al., 1996; Blaumueller et al., 1997; Rand et al., 2000). N^{EC} and N^{TM} fragments are generated via proteolytic cleavage of the primary translational product of the *Notch* gene (Shawber et al., 1996; Blaumueller et al., 1997; Rand et al., 2000). This proteolysis occurs in the secretory pathway by a furin-like convertase in the trans-Golgi network that cleaves the full-length Notch (N^{FL}) at a site aminoterminal of the TM domain. Furin cleavage of Notch1 does not require ligand binding (Weinmaster, 2000). Ligand binding promotes two proteolytic cleavage events in the Notch receptor. The first cleavage is catalysed by the metalloprotease TACE in mammals. Subsequently, the final intramembrane cleavage is mediated by γ-secretase, an enzyme complex that contains presenilins (PS1 and PS2), nicastrin, presenilin enhancer protein 2 (PEN2) and anterior pharynx-defective 1(APH1) (Fortini, 2002; Selkoe and Kopan, 2003; Mumm and Kopan, 2000; Baron, 2003). The second cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus due to the presence of nuclear localization signals located in the NICD (Figure 5).

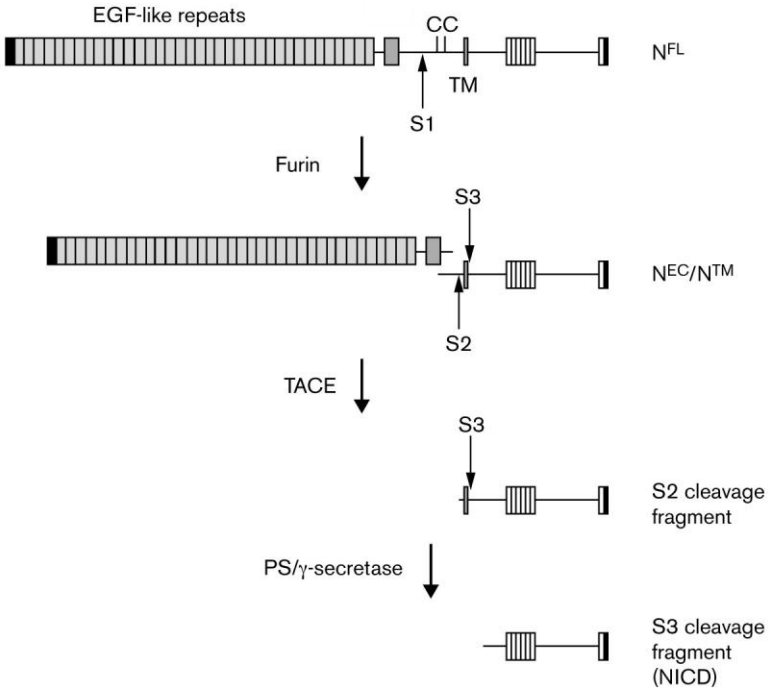


Figure 4: Schematic view of proteolytic cleavage sites identified in Notch1 (Adopted from Weinmaster, 2000). S1 represents the proteolytic cleavage site recognized by furin upstream of the single transmembrane domain (TM). Cleavage at S1 generates an aminoterminal fragment that contains most of the extracellular sequences (N^{EC}) and a carboxyterminal fragment (N^{TM}) that contains a pair of conserved cysteines (CC), the TM and cytoplasmic sequences. S2 in N^{TM} represents the putative TACE cleavage site upstream of the TM domain that, in response to ligand binding, would produce a membrane-associated S2 cleavage fragment. Cleavage at S2 facilitates S3 cleavage within the TM domain that is dependent on PS/γ-secretase activity. Cleavage at S3 releases the Notch intracellular domain (NICD) from the membrane.

Once in the nucleus, binding of NICD to DNA-binding protein CSL (named after CBF1, Su(H), and LAG-1) (also known as Rpbsuh or RBP-Jκ) displaces the repressor complex and recruits nuclear coactivator mastermind-like (MAML) and histone acetyltransferases (Alva and Iruela-Arispe, 2004) and converting CSL to a transcriptional activator. The above series of events describe the classic or CSL-dependent signals are induced via the conserved canonical Notch signaling pathway (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Radtke and Raj, 2003). However, although poorly understood, there is evidence for alternative actions, for instance CSL-independent signaling or Notch-independent CSL auto-activation (Matsuno et al., 1997).

Notch- mediated transcription results in the expression of various target genes, including the *Hes* (*Hairy/Enchancer of Split*) and *Hey* (*Hairy/Enchancer of Split* related with YRPW, also known as *HesR, HRT,* and *HERP*) families of transcription factors (Iso et al., 2003; Curry et al., 2006). *Hes* (*-1, -3, -5*) and *Hey (-1, -2, -L*) family members have been identified as immediate downstream targets of Notch activation. These proteins are transcriptional repressors that act by negatively regulating expression of target genes such as tissue-specific

transcriptional activators and can form both homo- and heterodimers, which is believed to extend their individual repression activity (Iso et al., 2003).

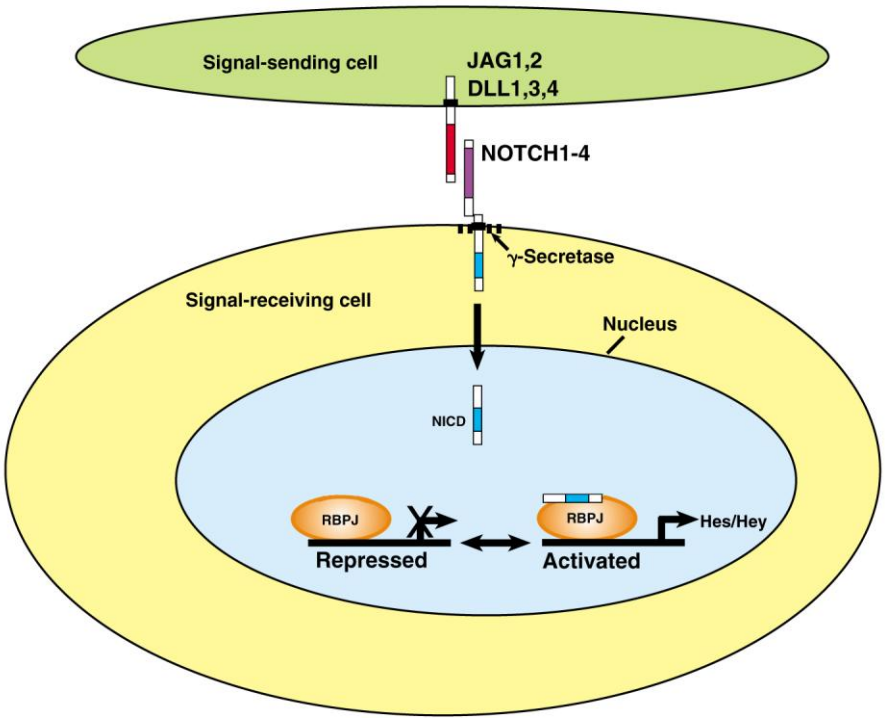


Figure 5: Schematic view of Notch signaling (Adopted from Gridley, 2007). Upon binding of the ligand on the signaling cell to the receptor Notch on the responding cell the Notch receptor is cleaved by a γ-secretase on the intracellular side which releases the intracellular domain of Notch (NICD). NICD translocates into the nucleus where it associates with the RBPj and activates target genes.

1.6 Notch signaling in vascular remodeling and stabilization

A number of observations indicate that Notch signaling pathway plays a key role during vascular development, homeostasis and tumor angiogenesis. The importance of Notch signaling in vascular morphogenesis has been highlighted by the severity of the phenotypes resulting from genetic ablation targeting receptors, ligands, and downstream targets of this pathway (Karsan, 2005; Shawber and Kitajewski, 2004; Alva and Iruela-Arispe, 2004). These results show that proper regulation of the Notch pathway is indispensable for vascular development during embryogenesis in mammals. The common theme is that inactivation of the Notch signaling pathway prevents the transition from the primitive vascular plexus to the hierarchical progression of a defined highly branched network of arteries, capillaries, and veins (Hoffmann and Iruela-Arispe, 2007).

Embryos homozygous for a null allele of ***Notch1*** die by embryonic day 9.5 (E9.5) with defects in somitogenesis and severe cardiovascular anomalies (Conlon et al., 1995; Swiatek et

al., 1994; Krebs et al., 2000). The target tissue of global *Notch1* knockout mice with respect to embryonic lethality is the endothelium. Both increased and decreased activation of Notch signaling result in impaired vascular development (Limbourg et al., 2005). Endothelial-specific *Notch1*$^{-/-}$ mice exhibit a similar phenotype to global *Notch1*$^{-/-}$ mice, suggesting that endothelial Notch1 is critical for embryonic vascular development and viability (Limbourg et al., 2005; Swiatek et al., 1994).

The expression of another Notch receptor ***Notch4*** has been also reported to be endothelium specific. Loss-of-function experiments for *Notch4* showed that *Notch4*-deficient mice were viable and fertile and exhibited no obvious mutant phenotype (Krebs et al., 2000). However, *Notch1*$^{-/-}$*/Notch4*$^{-/-}$ double mutant mice exhibit a more severe vascular phenotype than *Notch1*$^{-/-}$ mice, suggesting that Notch1 and Notch4 may have overlapping roles in vascular remodeling and morphogenesis during development (Krebs et al., 2000). Also endothelium-specific ectopic expression of activated *Notch4* in mice results in embryonic death with vascular defects in mice (Uyttendaele et al., 2001). The role of Notch1 and Notch4 signaling in postnatal vascular angiogenesis and remodeling remains to be determined.

Expression of ***Notch2*** is critical for the vascular morphogenesis of a more selective group of vascular beds. *Notch2* function is required for glomerulogenesis in the kidney, as well as for development of heart and eye vasculature (McCright et al., 2001; Hamada et al., 1999).

The ***Notch3*** null mouse is viable and fertile and this gene is necessary for the differentiation and acquisition of arterial identity of vascular smooth muscle cells and is absent from endothelial cells (Domenga et al., 2004).

Characterization of the expression patterns of the genes encoding Notch ligands revealed that only the ***Delta-like4* (*Dll4*)** gene (Shutter et al., 2000) is expressed in the early embryonic vasculature, the phenotype has several similarities with *Notch1* null mice (Krebs et al., 2000). *Dll4*$^{+/-}$ heterozygous embryos exhibit lethal haploinsufficiency due to vascular defects at E9.5 (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). It is known that Dll4/Notch signaling controls endothelial cell specification toward the arterial or venous phenotype in zebrafish and mouse (Duarte et al., 2004). Recent studies in the mouse retina and in tumor angiogenesis demonstrate that the specification of endothelial cells into tip and stalk cells is regulated by Dll4/Notch signaling (Hellström et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006). The *Dll4* is expressed in tip cells, whereas the Notch pathway activation is observed in the stalk cells (Hellström et al., 2007; Hoffmann and Iruela-Arispe, 2007). Suppression of Notch signaling or genetic deletion of one *Dll4* allele in the mouse dramatically augments sprouting and branching of the capillary network as a result of

excessive tip cell formation. Notch is cell autonomously required for stalk cell specification by actively suppressing the tip cell phenotype (Hellström et al., 2007). *Dll4* encodes a pivotal Notch ligand during arterial development and tumor angiogenesis. However, *Dll4* is not expressed in major adult arteries but only in microvessels (Gale et al., 2004), suggesting that other as yet unknown ligands regulate Notch signaling in adult arteries.

A second Notch ligand expressed in the developing vasculature is ***Delta-like1*** (***Dll1***). *Dll1* is essential for early embryonic development, targeted disruption of *Dll1* leads to early lethality at E12 with generalized hemorrhagic events (Hrabe de Angelis et al., 1997). Although, its expression has been reported in the developing vascular endothelium starting at midgestation (Beckers et al., 1999), *Dll1* postnatal vascular expression pattern is unknown.

The third Notch ligand to display embryonic lethality with significant vascular defects is ***Jagged1*** (*Jag1*). Jag1 is a ligand thought to be involved in early cardiovascular development by regulation of endothelial and vascular smooth muscle cells (Dufraine et al., 2008). Global as well as endothelium-specific knockouts of *Jag1* induce embryonic death with vascular defects at E10.5 (Xue et al., 1999; High et al., 2008).

Mice lacking **CSL** displayed defects similar to *Notch1/Notch4* double knockout mice with severe growth retardation (Oka et al., 1995) and a primitive vascular plexus lacking vessel remodeling. Additionally, an endothelial-specific knockout of CSL also resembled the *Notch1*, *Notch1*/*Notch4*, and *Dll4* knockout phenotypes with arteriovenous malformations, pericardial effusion, and the absence of vascular remodeling, demonstrating that regulation of specific levels of Notch activity is necessary for proper vascular development (Krebs et al., 2004).

Similarly, the combined deletion of the downstream Notch effectors ***Hey1*** and ***Hey2*** leads to disruption of typical vascular defects in Notch signaling, such as hemorrhage, defects in arterial/venous specification, enlarged pericardial sacs, heart abnormalities, lack of vessel remodeling, and enlarged vessels in the embryo and yolk sac (Fischer et al., 2004; Kokubo et al., 2005). *Hey1* and *Hey2* have been reported to promote arterial fate, possibly by upregulating *ephrinB2* and inhibiting *EphB4* expression (Fischer et al., 2004; Liu et al., 2003).

1.7 The role of Notch signaling in arterial/venous specification

The evolutionary conserved Notch signaling pathway regulates arterial development downstream of VEGF and upstream of ephrin-B2 (Lawson and Vogel, 2002; Shawber and Kitajewski, 2004). The role of the Notch pathway in regulating early embryonic vascular development is intertwined with another major regulator of vascular development and

physiology, the vascular endothelial growth A (VEGFA) pathway. VEGFA is a secreted glycoprotein that is a potent inducer of angiogenesis that also regulates multiple other aspects of blood vessel homeostasis (Byrne et al., 2005; Coultas et al., 2005; Shibuya and Claesson-Welsh, 2006). Gain-of-function transgenic experiments have demonstrated a role of *Vegfa* in regulating arterial endothelial cell differentiation in mice.

In mammals, a role for Notch in arterial/venous specification has been inferred from expression studies. Notch family members and Notch ligands are expressed throughout the vasculature early in development, but later become restricted to the arteries. This transition in expression suggests a role for Notch in the regulation of arterial/venous endothelial cell specification or in the maintenance of the arterial phenotype. Arterial fate is acquired by the combined effect of the forkhead transcription factors Foxc1 and Foxc2 and VEGF signaling (Lawson and Vogel, 2002; Seo and Kume, 2006; Lawson et al., 2001). Simultaneous inactivation of both *Foxc1* and *Foxc2* results in arterial/venous shunts and a lack of arterial markers, whereas upregulation of either transcription factor results in increased expression of *Dll4*, *Notch*, and *ephrinB2* (Seo and Kume, 2006). Genetic experiments in mice have also shown that the orphan nuclear receptor COUP-TFII (encoded by the gene *Nr2f2*) promotes venous EC differentiation by suppressing Notch signaling. Veins acquire arterial features in *Nr2f2* knockout mice, whereas arterial markers are suppressed by EC-specific overexpression of the orphan receptor (You et al., 2005).

1.8 Ephrin-B2 and EphB4 role in postnatal neovascularization

Recently has been also demonstrated that ephrin-B2 is expressed on arterial ECs and some smooth muscle cells in vascular organs of adult mammals (Shin et al., 2001; Gale et al., 2001). Hayashi and colleagues (Hayashi et al., 2005) demonstrated that ephrin-B2 is a functional molecule that plays a role in the angiogenic cascade in postnatal neovascularization. They suggested that ephrin-B2 signaling system may be required for the subsequent response of triggered angiogenic ECs before blood vessel stabilization in the process of neovascularization, especially in governing the proper process of communication between arterial and venous neovascularization for structural maturation of blood vessels (Hayashi et al., 2005).

1.9 Notch signaling pathway defects and cardiovascular pathologies

In addition to its contribution to early vascular morphogenesis and arterial-fate specification, the Notch signaling pathway also impacts vascular homeostasis. The connection between Notch and vasculature was first recognized when mutations in members of the pathway were found to be responsible for two congenital diseases that affect the vasculature in humans.

CADASIL (OMIM #125310) is a hereditary vascular degenerative disorder caused by mutations in the human *NOTCH3* gene. This syndrome is characterized by autosomal-dominant arteriopathy that affects mainly the small cerebral arteries and leads to stroke, migraines and progressive dementia in middle-age adults (Domenga et al., 2004, Ruchoux and Maurage, 1997; Joutel et al., 1997; Joutel et al., 2004). Consistent with the expression patterns of *Notch3* within blood vessels, degeneration and loss of the smooth muscle layer surrounding cerebral and skin arterioles, are main features of CADASIL (Joutel and Tournier-Lasserve, 1998). These processes cause the thickening of the vessel wall and narrow the lumen of the affected arteries (Roca and Adams, 2007). The genetic defects in CADASIL are typically missense mutations, most of which translate into amino acid exchanges within the first five EGF-like repeats of the NOTCH3 ectodomain (Joutel et al., 1997). It is thought that CADASIL mutations alter the intacellular trafficking and maturation but not the signaling by NOTCH3 (Karlstrom et al., 2002).

Alagille syndrome (AGS, OMIM #225750) is a congenital disorder caused by mutations in the human gene for *Jagged1*. Symptoms include abnormalities affecting the liver, heart, eye, and skeleton (Oda et al., 1997). Patients with AGS exhibit also abnormally formed blood vessels, and arterial stenosis (Piccoli and Spinner, 2001; Kamath et al., 2004; McElhinney et al., 2002). In humans, mutations in the Jagged1 gene have been identified in 60-70% cases of AGS. AGS is caused by JAG1 haploinsufficiency, but the exact role of *Jagged1* in the affected tissues is unclear (Spinner et al., 2001). While heterozygous *Jagged1* knockout mice do not recapitulate AGS, the additional introduction of a single *Notch2* hypomorphic allele leads to Alagille-like developmental abnormalities (Xue et al., 1999; McCright et al., 2001).

2 RESULTS

2.1 Specific expression of *Dll1* in postnatal arterial endothelium

To analyze expression of *Dll1* in adult vasculature we initially analyzed *lacZ* expression in *Dll1*$^{lacZ/+}$ heterozygous mice, in which one *Dll1* allele has been replaced by the *lacZ* gene and whose expression mimicks endogenous *Dll1* expression (Beckers et al., 1999; Hrabe De Angelis et al., 1997). These mice show a normal phenotype on gross pathologic examination and normal survival rates. Staining of whole hearts and sections revealed distinct *Dll1* expression in coronary arteries and inner aortic lining, while no staining was observed in *wildtype* (*wt*) mice lacking the *lacZ* gene (Figure 1A and G). *Dll1* expression was examined in all examined arteries, such as the aorta and the proximal carotid arteries, in large muscular conductance arteries, such as the coronary and renal arteries, and in small muscular arteries (Figure 1A, C, E, F-G). Interestingly, *Dll1* expression was specific for arterial endothelial cells (ECs), and not detected in smooth muscle cells of the arterial wall, nor in venous endothelium or capillaries (Figure 1C-H). The specificity of lacZ staining was confirmed by immunofluorescence using anti DLL1 antibody (Figure 1F).

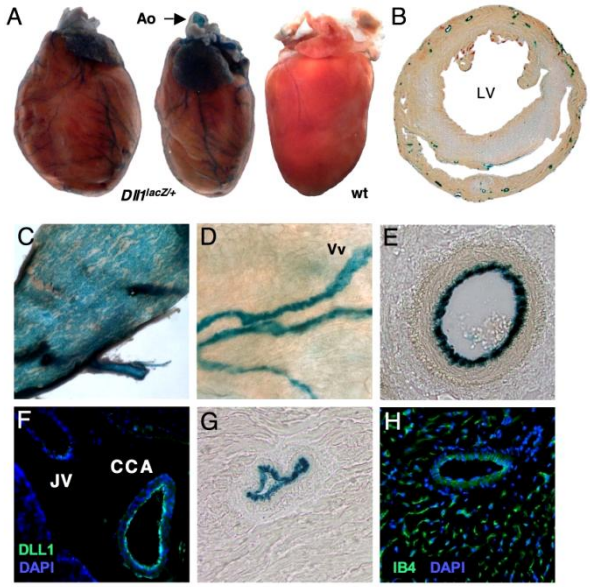


Figure 1. Specific vascular expression of *Dll1* in postnatal arterial endothelium.
(A-E, G) *Dll1* expression shown by lacZ staining in *Dll1*$^{lacZ/+}$ mice. (A, B) *Dll1* expression in coronary arteries and aorta (Ao) in whole hearts (A) and heart sections (B). No staining in wt animals. (C-H) Expression of *Dll1* in endothelium of arteries, but not veins or capillaries. (C) En face staining of aorta. (D) En face staining of vena cava with arterial vasa vasorum (Vv). (E) Renal artery. (F) Anti-DLL1 immunofluorescence, common carotid artery (CCA) and jugular vein (JV). (G) *Dll1* expression within a muscular artery and (H) whole endothelial staining with isolectin B4 (IB4) in a parallel tissue sections. Magnification: (C) 25x, (D) 100x, (F) 200x, (E, G, H) 400x.

2.2 Impaired postnatal arteriogenesis, but not microvascular angiogenesis, in heterozygous *Dll1* mice

To determine the role of *Dll1* in vascular regeneration we subjected mice to hindlimb ischemia (HLI) by ligation of the superficial branch of the femoral artery, which triggers growth and remodeling of preexisting collaterals from the deep femoral artery (Scholz et al., 2002). The hindlimb ischemia model is the most extensively used animal model for studying adult neovascular formation and strategies aimed to improve revascularization (therapeutic angiogenesis) *in vivo*. In this model, revascularization of the ischemic hindlimb occurs spontaneously through angiogenesis and arteriogenesis. The functional significance of the new formed vessels on the perfusion and tissue viability of the ischemic muscles is a major endpoint of studies performed with the model.

Quantification of blood flow by Laser Doppler measurements revealed comparable levels of postprocedure ischemia in heterozygous $Dll1^{lacZ/+}$ mice and nontransgenic *wt* littermates. Serial laser Doppler measurements in *wt* mice revealed a rapid increase in ischemic blood flow within three days of HLI, followed by more gradual recovery over 28 days. In contrast, initial blood flow recovery in $Dll1^{lacZ/+}$ mice was severely blunted and ischemic flow remained severely depressed, in fact never returning to preischemic perfusion levels, which suggested defective arteriogenesis (Figure 2A).

To investigate the role of *Dll1* in postnatal arteriogenesis we quantified collateral arterial growth. Histomorphometry of contralateral collaterals revealed no difference in vessel lumen or wall area in both genotypes. In response to HLI there was a significant increase in collateral lumen and wall area in *wt* mice, demonstrating active arterial remodeling and outward growth during arteriogenesis. In contrast, there was no significant lumen increase in collaterals of $Dll1^{lacZ/+}$ mice after HLI, and collateral wall area was significantly smaller compared with *wt* mice (Figure 2B). These data demonstrate *Dll1* haploinsufficiency in postnatal arteriogenesis.

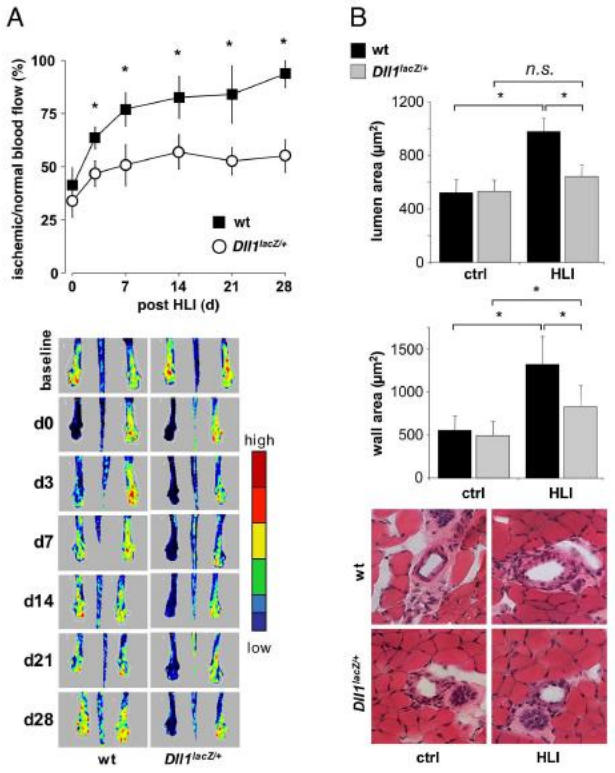


Figure 2. Impaired postnatal arteriogenesis in heterozygous *Dll1* mice.
(A) Impaired blood flow recovery of ischemic hindlimbs of heterozygous *Dll1*$^{lacZ/+}$ mice shown by laser Doppler blood flow (LDBF) measurements, expressed as ischemic to normal (contralateral) LDBF ratios (upper panel), and representative color-coded LDBF images (lower panel, dark blue represents low flow). n=11-12, *=p<0.01.
(B) Impaired collateral artery growth in *Dll1*$^{lacZ/+}$ mice demonstrated in histomorphometric analysis of collateral lumen (upper panel) and wall area (middle panel) and representative hematoxylin-eosine stained sections (lower panel) three days after HLI. n=11-12, *=p<0.01.

The pathophysiologic consequences of *Dll1* haploinsufficiency were evaluated after aggravated HLI by complete proximal femoral artery occlusion, which induces more severe ischemia. Again, the initial level of ischemia was comparable between groups (ischemic/normal perfusion [%], wt: 28±5; *Dll1*$^{lacZ/+:}$ 22±7, p=n.s., n=10 to 11). However, only *Dll1*$^{lacZ/+}$ mice sustained severe necrosis or autoamputation in the majority of cases, whereas *wt* mice recovered from ischemic insult without major damage (Figure 3A; *wt* versus *Dll1*$^{lacZ/+,}$ amputation: 0/10 versus 6/11, necrosis: 2/10 versus 2/11, complete recovery: 8/10 versus 3/11; p<0.05).

In contrast, capillary density in ischemic muscles was significantly increased in *Dll1*$^{lacZ/+}$ mice (Figure 3B), which probably reflects increased and persistent ischemia in the affected limb. Although increased capillary sprouting lead to perfused capillary beds, as shown by FITC-labeled dextran perfusion studies (Figure 3B), this did not compensate for the perfusion defect caused by impaired arteriogenesis in *Dll1* haploinsuffient mice. Taken together, these results demonstrate a crucial role of *Dll1* in postnatal arteriogenesis and ischemic limb salvage.

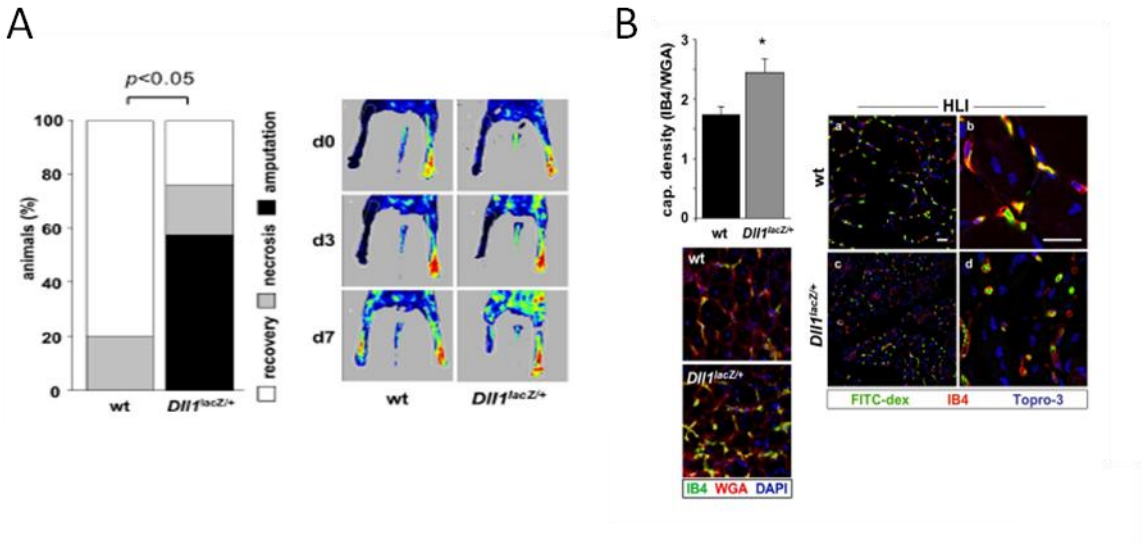


Figure 3: Inadequate response to ischemic damage in *Dll1*$^{lacZ/+}$ mice.
(A) Severe ischemic tissue damage in *Dll1*$^{lacZ/+}$ mice scored seven days after complete femoral artery occlusion (upper panel) and LDBF images showing autoamputation of the ischemic limb in a *Dll1*$^{lacZ/+}$ mouse (lower panel). n=10-11.
(B) Increased capillary density in ischemic gastrocnemic muscle of *Dll1*$^{lacZ/+}$ mice ten days after HLI shown by quantification of capillaries (IB4^{+}) per myofiber (wheat germ agglutinine+, WGA) and representative immunofluorescence stainings. n=3, *=p<0.01 (left panels). FITC-labled dextran perfusion demonstrating perfused capillary beds in Dll1-heterozygous mice (right panel).
Scale bar: 10 µm. Magnification: (A and B) 400x.

2.3 Induction of *Dll1*-dependent Notch signaling regulates Ephrin-B2 expression during postnatal arteriogenesis

It has been known previously that endogenous VEGF is required for postnatal arteriogenesis (Jacobi et al., 2004). Analysis of growing collaterals revealed strong VEGF expression in perivascular stromal cells and within vessels without apparent differences in expression levels between *wt* and *Dll1*$^{lacZ/+}$ groups (Figure 4A).

Analysis by confocal microscopy showed that endothelial DLL1 expression was low in steady-state arteries, but strongly upregulated in response to HLI in *wt* mice, whereas DLL1 levels in heterozygous *Dll1* mice remained below the detection limit of immunostaining (Figure 4B, a, b, d, e). DLL1 expression within vessels was limited to the inner endothelial lining, as shown by nonoverlapping staining with smooth muscle α-actin (SMA) (Figure 4c and 4g). *Dll1* expression in ischemic collaterals of *Dll1*$^{lacZ/+}$ mice was confirmed by endothelial restricted β-galactosidase staining (Figure 4f). In addition, DLL1 expression was also found in perivascular cells surrounding growing arteries (Figure 4b, 4g, 4h).

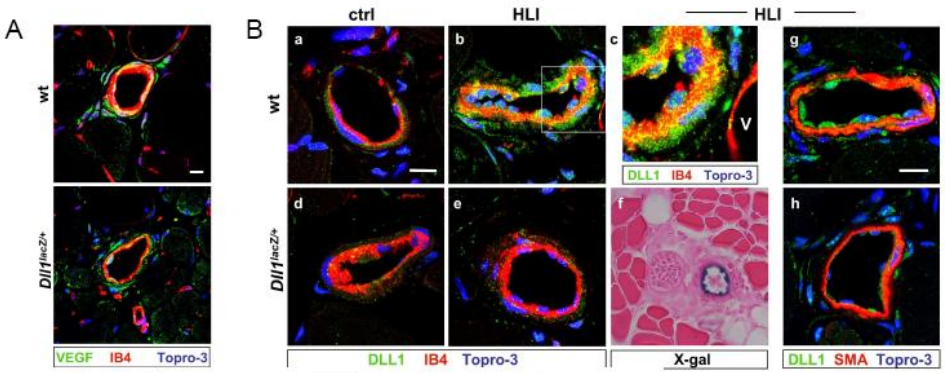


Figure 4. Induction of *Dll1*-dependent signaling during postnatal arteriogenesis.
Confocal microscopy of collateral arteries. (A) Comparable endothelial and perivascular VEGF expression in wt and $Dll1^{+/-}$ collateral arteries after HLI. (B) Marked endothelial and perivascular DLL1 upregulation in *wt* collaterals after HLI (compare a with b, c and g), but not in $Dll1^{+/-}$ collaterals (compare d with e and h). Endothelial expression of Dll1 within vessels was demonstrated by overlapping staining with endothelial IB4 (b, c), and complementary staining with smooth muscle α-actin (SMA) (g), and confirmed by endothelial restricted β-galactosidase staining in $Dll1^{lacZ/+}$ mice (f). (c) Magnification from (b), V: vein. Scale bar: 10 μm.

In contrast, the Notch ligand DLL4 was expressed in capillaries and small vessels, but was not detected in larger collateral arteries. Furthermore, there was no evidence for specific *Dll4* regulation in collaterals after HLI, or compensatory upregulation in Dll1 heterozygotes (Figure 5).

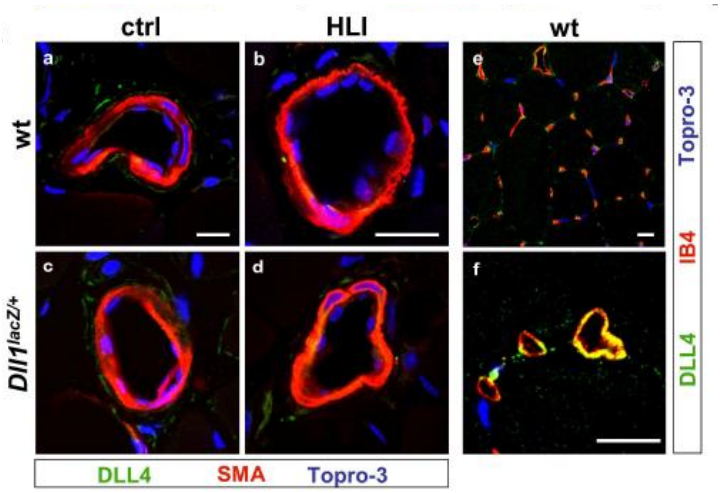


Figure 5: DLL4 expression in blood vessels.
Confocal microscopy of collateral arteries. DLL4 is not expressed in large, collateral arteries (a-d), but is present in capillaries and small vessels (e, f). Scale bar: 10 μm.

Upregulation of DLL1 coincided with activation of endothelial Notch1 in *wt* mice, but not $Dll1^{lacZ/+}$ mice, as shown by specific immunostaining for γ-secretase-cleaved Notch1 (Val1744) (Figure 6A, b–c and e–f). Consistent with *Dll1*-dependent Notch activation there was strong endothelial, but also significant perivascular, expression of the Notch target gene *Hes1* in arteries of *wt* mice, but not in heterozygous mice (Figure 6B). The Notch target gene

Hey1, which is involved in embryonic arterial development (Fischer et al., 2004), was also induced in growing collaterals in a *Dll1*-dependent manner (Figure 6C).

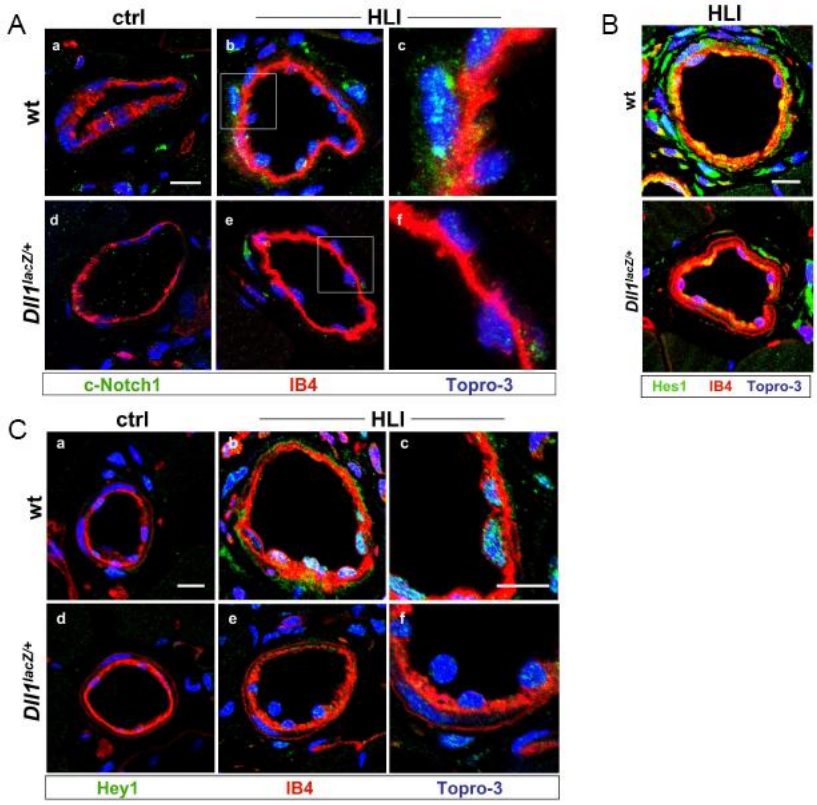


Figure 6. *Dll1*-dependent Notch signaling during postnatal arteriogenesis.
Confocal microscopy of collateral arteries. (A) Notch1 activation in growing wt collaterals (compare a with b, c), but not $Dll1^{lacZ/+}$ collaterals (compare d with e, f), shown by staining with an antibody specific for γ-secretase cleaved Notch1 (c-Notch). Predominantly endothelial and sporadic perivascular staining with marked perinuclear accumulation (b). (c and f) Magnifications from (b) and (e). (B) Marked endothelial and peri-vascular upregulation of Hes1 after HLI in *wt* collaterals. (C) Marked endothelial and peri-vascular upregulation of Hey1 after HLI in wt collaterals, right panels magnification of left panels. Scale bar: 10 μm.

Ephrin-B2 on ECs interacts with EphB4 in bidirectional signaling during vascular remodeling (Adams et al., 2001; Gerety and Anderson, 2002). No ephrin-B2 expression was detected in steady-state arteries, but there was strong induction in endothelium and vessel wall of growing *wt* collaterals, but not in $Dll1^{lacZ/+}$ collaterals (Figure 7A). Expression of EphB4, which serves as activator for ephrin-B2 reverse signaling, was not detected in steady-state arteries (Figure 7B, a and d), whereas constitutive venous endothelial EphB4 was readily detected (Figure 7B, a). After HLI, there was strong and comparable perivascular induction of EphB4 in both genotypes (Figure 7B, b, c, e, f). Thus, induction of EphB4 is regulated in a complementary fashion to ephrin-B2, but independent of *Dll1*.

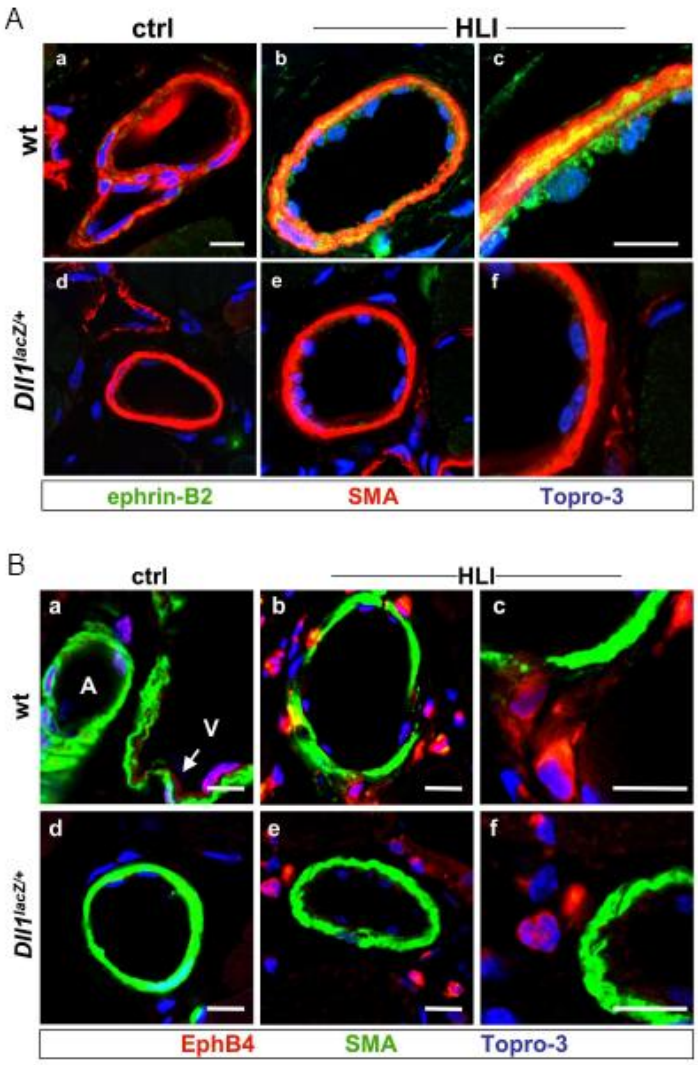


Figure 7. *Dll1*-dependent induction of ephrin-B2 during postnatal arteriogenesis.
Confocal microscopy of collateral arteries.
(A) Ephrin-B2 upregulation in growing *wt* collaterals (upper panels), but not in *Dll1*$^{lacZ/+}$ collaterals (lower panels). Partially overlapping staining with SMA indicates endothelial and mural expression after HLI. (c and f) Magnifications from (b) and (e). (B) Peri-arterial EphB4 induction after HLI in *wt* and *Dll1*$^{lacZ/+}$ mice (b, c, e and f). (a) Endothelial EphB4 staining in veins (V), but not arteries. (c and f) Magnifications from (b) and (e).
Scale bar: 10 µm.

Together, these results demonstrate the spatially coordinated induction of a pro-angiogenic signaling cascade, consisting of VEGF, DLL1-dependent Notch signaling, and the downstream effectors ephrin-B2 and EphB4. These results also suggest that ephrin-B2 mediates arteriogenesis downstream of *Dll1*-dependent Notch activation, whereas VEGF and EphB4 are regulated independent of *Dll1*.

2.4 Angiogenic growth factors regulate Ephrin-B2 expression in arterial EC via *Dll1*-dependent Notch activation

Coexpression of VEGF and DLL1 in growing arteries suggested regulation of *Dll1* by angiogenic growth factors. In cultured human aortic EC (HAEC), VEGF treatment transiently increased DLL1 protein levels in a time- and dose-dependent manner (Figure 8). Furthermore, FGF-2, which is expressed around growing collaterals and acts synergistically with VEGF in postnatal arteriogenesis (Kondoh et al., 2004; Ziegelhoeffer et al., 2004) also induced DLL1 transiently in a time- and dose-dependent manner. However, the combination of VEGF and FGF-2 (VF) increased and prolonged DLL1 upregulation synergistically (Figure 8).

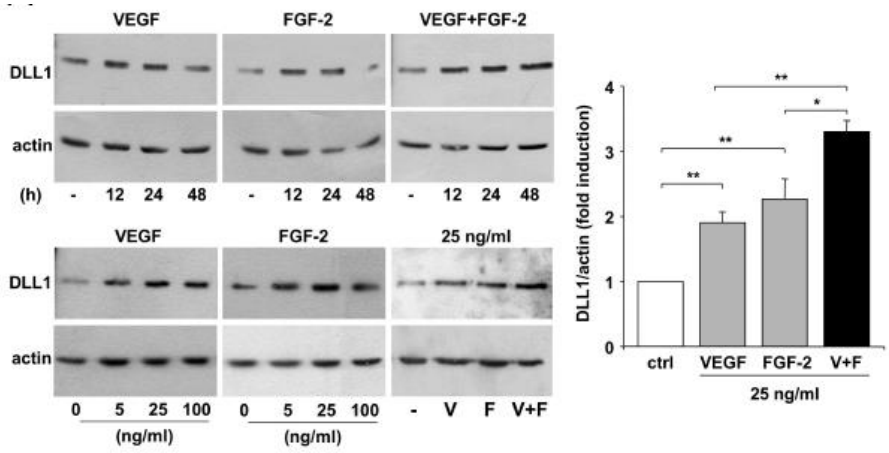


Figure 8. DLL1 protein levels are increased in a time-and dose-dependent manner in human.
Immunoblots showing time course and dose response of DLL1 induction in HAEC by VEGF (V) and FGF-2 (F) and synergistic induction by combination treatment (VF) (left panels). Membranes were stripped and reprobed for actin to ensure equal loading. Densitometric analysis of DLL1 levels normalized to actin (graph), n=3, *=p<0.05, **=p<0.01.

Next was addressed the role of *Dll1*-dependent Notch signaling in ephrin-B2 regulation. Combined growth factor treatment also synergistically upregulated ephrin-B2 in a dose-dependent manner (Figure 9A).

DLL1 upregulation induced γ-secretase-dependent cleavage and activation of Notch1, and upregulated ephrin-B2 in endothelial cells (Figure 9B). Notch1 signaling and ephrin-B2 expression, but not DLL1 upregulation, was blocked by a specific γ-secretase inhibitor (GSI), N-[N-(3,5- difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), a potent inhibitor of Notch signaling (Bettenhausen et al., 1995). In accordance with an essential role of *Dll1*-dependent signaling in ephrin-B2 regulation, *Dll1* knockdown in HAEC with small interfering (si)RNA also abrogated the induction of ephrin-B2 by VF (Figure 9B). Ephrin-B2 expression was further blocked by adenoviral overexpression of a dominant-negative (dn) inhibitor of Notch signaling, RBPJ (R218H) (Raya et al., 2004) whereas overexpression of

constitutive-active Notch1 (NICD) strongly upregulated ephrin-B2 expression independent of VF (Figure 9C). Thus, *Dll1*-dependent Notch signaling is necessary and sufficient for ephrin-B2 induction in arterial EC.

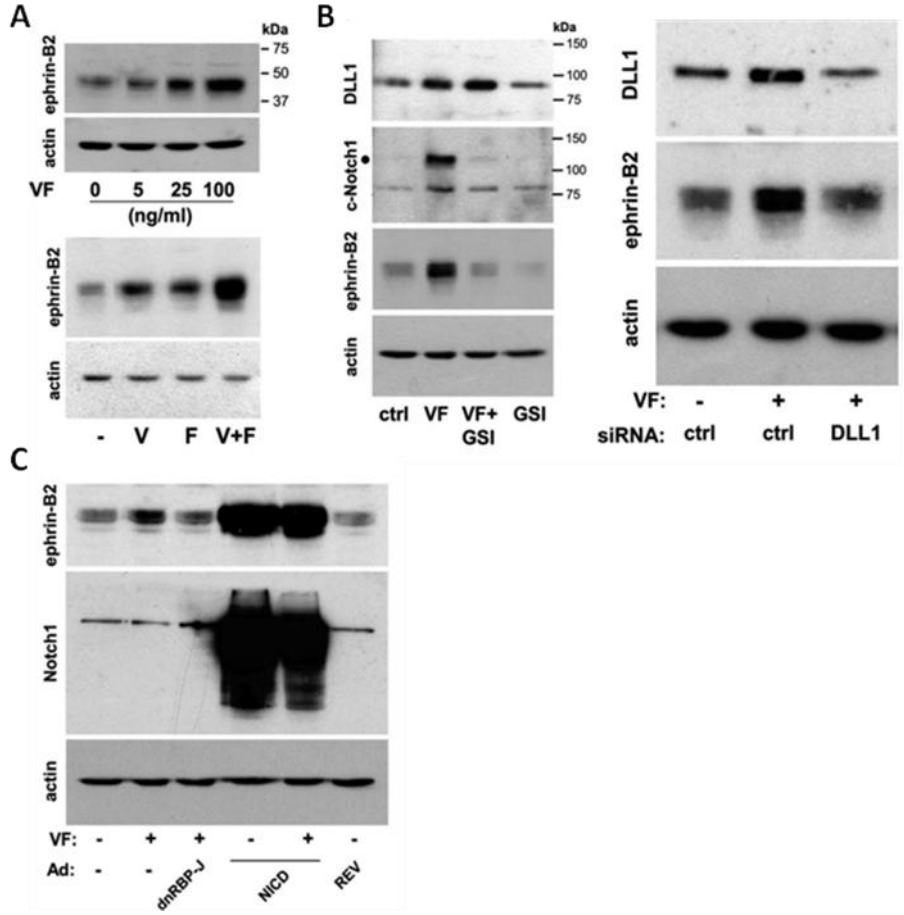


Figure 9. *Dll1*-dependent regulation of ephrin-B2 by pro-angiogenic growth factors in human arterial EC. (A) Immunoblots showing dose-dependent and synergistic ephrin-B2 upregulation bycombination treatment (VF). (B) Left panel: immunoblots showing Notch-dependent induction of ephrin-B2 by growth factors. VF-induced Notch1 cleavage (c-Notch1, ●) and upregulation of ephrin-B2, but not DLL1 induction, was abrogated by a γ-secretase inhibitor (GSI), DAPT (1 μM). Right panel: immunoblots showing impaired DLL1 and ephrin-B2 upregulation after *Dll1* siRNA treatment. (C) Immunoblots showing inhibition of ephrin-B2 induction by dominant-negative (dn) RBPJ (R218H) and VF-independent upregulation of ephrin-B2 by Notch1 intracellular domain (NICD). Ephrin-B2 and Notch1 levels after adenoviral transduction (Ad) of indicated constructs or control Ad (REV).

2.5 Dll1-dependent Notch signaling regulates branching and vascular network formation via Ephrin-B2

To study the influence of *Dll1* and external matrix on endothelial cell function *in vitro* we performed vascular network formation assays. HAEC were seeded in a matrigel matrix containing embedded EphB4-Fc fusion protein, which exclusively interacts with ephrin-B2 ligands (Brambilla et al., 1996), or control IgG-Fc fragments. In a dose-dependent manner, EphB4-Fc enhanced EC branching and total network length, while decreasing mean tube length, demonstrating increased network complexity in an EphB4-rich milieu (Figure 10A).

EphB4-Fc matrix significantly increased branching and total network extension and decreased mean tube length after growth factor stimulation compared with control matrix (Figure 10A). These effects were abrogated by inhibition of Notch signaling with GSI (Figure 10A). Futhermore, knockdown of *Dll1* by siRNA also strongly reduced branching morphogenesis and network formation, which was recapitulated by ephrin-B2 siRNA, but not by control, scrambled siRNA (Figure 10B).

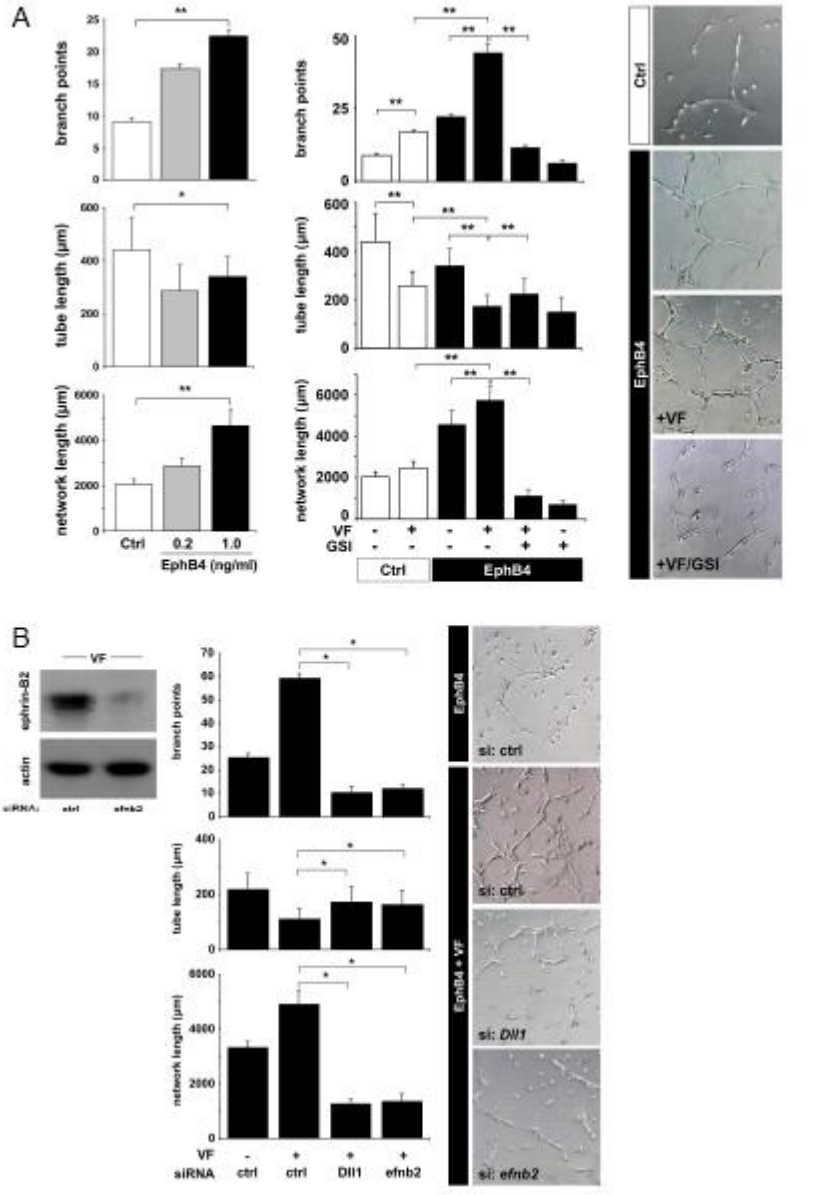


Figure 10. *Dll1* regulates branching morphogenesis and network formation via Notch dependent induction of ephrin-B2.
Quantitative analysis and representative microscopic images of branching and network formation by HAEC.
(A) Increased branching and total network length, and decreased tube length, indicative of increased network complexity, in matrix enriched with increasing concentrations of EphB4- Fc compared to control IgG-Fc (left panel). Efficient network formation following VF treatment for 24 h requires EphB4-enriched matrix (right

panel, compare white and black bars), and is abrogated by treatment with GSI (DAPT, 1 μM). n=4, *=p<0.05, **=p<0.01.
(B) Left panel: immunoblots showing downregulation of ephrin-B2 by specific siRNA (efnb2), but not by scrambled (ctrl) siRNA. Right panels: increased network formation by VF treatment in an EphB4-enriched matrix is blocked by *Dll1* and ephrin-B2 (efnb2) siRNA transfection, but not control transfection. n=4, *=p<0.01. Magnification: 50x.

3 DISCUSSION

The genetic regulation of the postnatal neovascularization is currently largely unknown. Especially poor is our understanding of the mechanisms that govern the formation of new arteries (termed arteriogenesis) from existing collateral vessels. In this study, we have examined the role of the Notch ligand *Dll1* in adult arteriogenesis. We demonstrate here that Dll1 is not simply a molecular landmark of arterial blood vessels in embryonic and postnatal life but is also a functional molecule that plays a role in the angiogenic cascade in postnatal neovascularization. Specifically, we show that heterozygous *Dll1* mice fail to properly answer to the occlusion of the arterial flow due to inability to activate Notch signaling pathway. Furthermore, we determine that angiogenic growth factors regulate *ephrin-B2* expression in arterial endothelial cells via *Dll1*-dependent Notch activation.

3.1 Dll1 is essential for adult arteriogenesis

Delta-Notch signaling is central for regulating angiogenesis during embryonic development, however, its role in postnatal neovascularization is not known. Here we have shown that *Dll1*, a ligand of Notch signaling, is expressed at low levels also in adult steady-state arteries. We addressed the functional requirement of Dll1 in adult arteries by performing hind-limb ligation that similarly to normal arteriogenesis induces a perivascular pro-angiogenic milieu up-regulating the expression of VEGF, FGF-2 and EphB4 among others. We observed a strong endothelial up-regulation of *Dll1* expression that led to Notch signaling activation and ephrin-B2 induction in wild type mice. In contrast, ligation of the hind-limb arteries of the Dll1 heterozygous mice exhibited severe haploinsufficiency characterized by the failure to significantly upregulate Dll1, consequently impairing Notch activation and ephrin-B2 expression and abrogating arterial growth, while perivascular VEGF and EphB4 expression remained unaltered. Although, ephrin-B2 expression is known to be regulated by Dll4 activated Notch signaling in the embryonic vasculature (Gale et al., 2004) we found that Dll1 plays the same role in adult arteries.

As arterial endothelium is activated by locally released cytokines and growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) resulting in arterial remodeling and growth (Jacobi et al., 2004), we were interested whether these factors are also involved in regulating postnatal arteriogenesis. Our cell culture experiments by using HAE cells demonstrated that VEGF and FGF-2 synergistically induce *Dll1*-dependent Notch signaling, which is necessary and sufficient to induce ephrin-B2.

Furthermore, the ability to induce branching morphogenesis depends on *Dll1*-dependent Notch signaling and is mediated by ephrin-B2. Endothelial ephrin-B2 reverse signaling has been shown to mediate EC migration, adhesion and sprouting *in vitro* and embryonic and postnatal angiogenesis *in vivo* (Adams et al., 2001; Hamada et al., 2003; Hayashi et al., 2005).

Based on our results we propose a model of postnatal arteriogenesis in which induction of *Dll1* by angiogenic growth factors leads to Notch-dependent expression of ephrin-B2, which mediates remodeling and outward growth towards an EphB4 enriched milieu. On the other hand, the normal baseline vascular phenotype can be explained by low *Dll1*-dependent signaling under steady-state conditions. The observed transient induction of *Dll1* by growth factors is consistent with reciprocal *Dll1* inhibition by activated Notch receptors (Artavanis-Tsakonas et al., 1999), which, we speculate, could provide a possible mechanism for termination of arteriogenesis through *Dll1* down-regulation following prolonged Notch activation. On the other hand, the lack of Dll1 induction in the heterozygous state may suggest an autostimulatory action of *Dll1* in the initiation of Notch signaling. Alternatively, it is possible that Dll1 heterozygous vessels lack the ability to respond to arteriogenic stimuli. Clearly, these interesting questions require further studies.

3.2 Dll1 and Dll4 are region specific regulators of blood vessels

Our findings also suggest that the mechanisms of postnatal arteriogenesis are reminiscent of arterial development, where Notch signaling regulates arterial differentiation downstream of VEGF and upstream of ephrin-B2 (Lawson et al., 2002). However, while *Dll4* is a crucial Notch ligand in arterial development in the embryo, our analysis of adult mice indicates expression in small vessels and capillaries, which is consistent with previous reports (Patel et al., 2005). Furthermore, we found no evidence for specific regulation of Dll4 during postnatal arteriogenesis, where *Dll1* is a key regulator. Our finding of impaired arteriogenesis but increased microvascular angiogenesis in *Dll1* heterozygous mice can be explained by the restricted expression of *Dll1* in the arterial domain and demonstrates *Dll1*-independent regulation of microvascular angiogenesis, possibly through *Dll4*, which is expressed in microvessels and tumor vasculature and regulates ephrin-B2 expression and angiogenesis (Hayashi et al., 2005; Gale et al., 2004; Patel et al., 2005; Iso et al., 2006). However, this also emphasizes the importance of arteriogenesis in blood flow restoration and ischemic organ rescue.

Finally, given the crucial role of *Dll* in postnatal arteriogenesis, interventions or drugs that upregulate vascular *Dll1* during ischemic stress might greatly enhance arteriogenesis and contribute to organ rescue and regeneration. Our findings establish *Dll1* as an essential regulator of postnatal arteriogenesis.

CHAPTER-2

Analysis of Notch signaling in endothelial progenitor cells

4 INTRODUCTION

4.1 EPC phenotypes

Growth of new blood vessels in the adult occurs through arteriogenesis, angiogenesis, or vasculogenesis. Angiogenesis refers to the growth process of mature endothelial cells sprouting from pre-existing postcapillary venules through migration and proliferation, arteriogenesis is used to describe the maturation of collateral vessels via recruitment of mural cells (Carmeliet, 2000). Until recently, the term vasculogenesis described the process of blood vessel formation in the embryo. This occurs via differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation, which then assemble into a primitive vascular network. This dogma was overturned in 1997, when Asahara and his group (Asahara, 1997) published that purified CD34+ hematopoietic progenitor cells from adults peripheral blood can differentiate *ex vivo* to an endothelial phenotype. These cells represented a heterogeneous population and were named endothelial progenitor cells (EPC) (Urbich and Dimmeler, 2004). This unique cell fraction among peripheral blood mononuclear cells (MNCs) derived from bone marrow has a similar profile to that of an embryonic angioblast, which proliferates and/or migrates in response to angiogenic growth factors and differentiates into mature endothelial cells in situ for blood vessel formation (Asahara and Kawamoto, 2004). EPC are a heterogeneous group of cells that can be characterized by the expression of surface markers, such as CD34, CD133 (AC133), von Willebrand factor (vWF), VE-cadherin, eNOS, by the uptake of acetylated LDL (DiI-Ac-LDL) and by binding lectins (Aicher et al., 2005). CD133 is a novel hematopoietic stem cell marker, which is expressed on EPC sets, but not on mature EC (Yin et al., 1997). Its expression is rapidly downregulated as hematopoietic progenitors and EPC differentiate (Yin et al., 1997; Miraglia et al., 1997). Recently, it has been shown that the expression of VEGFR2 (KDR) on pheripheral blood monocytes is essential for their endothelial-like functional capacity and support the notion of a common precursor for monocytic and endothelial cell lineage (Elsheikh et al., 2005). Rehman and colleagues (Rehman et al., 2003), reported that the peripheral-blood endothelial-like cells express monocyte/macrophage markers and secrete angiogenic growth factors. It is known that Notch receptor signaling regulates differentiation of primitive hemangioblasts during vascular development (Kumano et al., 2003) but its role in EPC related postnatal vasculogenesis is largely unknown.

Based on ex vivo culture conditions two different EPC subpopulations have been described, denoted as early EPC and late EPC (also called endothelial outgrowth cells (EOCs), with

distinct cell growth patterns, ability to secrete angiogenic factors and differential proliferation potential (Gulati et al., 2003; Hur et al., 2004). Early EPC are spindle-shaped cells derived from monocytic cells, which have a peak growth in culture at 2-3 weeks and which die after approximately 4 weeks *in vitro* and secrete an array of angiogenic, antiangiogenic and neuroregulatory cytokines (Hur et al., 2004). Early EPC have low proliferative capacity and adopt characteristics of ECs such as expression of eNOS. Importantly, although these cells may incorporate into the endothelial monolayer, they fail to form perfused vessels *in vivo*. . Usually cultured peripheral blood mononuclear cells form EPC colonies that are called colony-forming unit endothelial cells (CFU-ECs or CFU-EPC). Recently was shown the presence of a subset of T-cells at the centre of the EPC colony. These angiogenic T cells play a role in colony formation and differentiation of early EPC and have function of both early EPC and endothelial cells by secreting angiogenic cytokines. Interestingly, the number of angiogenic T cells in blood was correlated with the number of EPC colonies cultured from human peripheral blood and was influenced by donor's age and cardiovascular risk factors (Hur et al., 2007).

Late EPC are cobblestone shaped and usually appear after 2-3 weeks of culture, show exponential growth at 4 to 8 weeks and can be maintained for up to 12 weeks in culture (Gulati et al., 2003; Hur et al., 2004). Although early EPC and EOCs share common features such as expression of CD31, CD34, lectin binding and LDL uptake and comparable *in vivo* vasculogenic capacity, however they have distinct characteristics with respect to morphology, proliferative potential, and *in vitro* functional characteristics such as capillary tube formation (Lin et al., 2000; Bompais et al., 2004; Yoon et al., 2005; Ingram et al., 2004). Molecularly it is known that CD14+ cells give rise to early EPC, whereas late EPC develop exclusively from the CD14- subpopulation (Gulati et al., 2003).

4.2 Non-bone marrow-derived EPC

In addition to bone marrow, EPC can be generated from different sources in an organism. For example, the spleen is an organ particularly rich in EPC. Spleen-derived mononuclear cells can transdifferentiate into cells with an endothelial phenotype after stimulation with endothelial growth factors *in vitro* and can form tubular-like structures. Intravenous transfusion of spleen-derived MNCs or in vitro cultured EPC could sufficiently enhance re-endothelialization and diminish neointima formation after carotid artery injury (Wassmann et al., 2006; Werner et al., 2003).

Recently high levels of mobilized tissue-residing progenitor cells (TPC) have been discovered in the intestine and the liver using a rat model of intestine and liver transplantation. Systemic infusion of progenitor cells derived from the perivascular niche in the liver had the capacity to form CFU-EC, incorporated into vascular structures and enhanced neovascularization with improved blood flow recovery in ischemic hindlimbs. Incorporation of TPC was not restricted to capillaries as they were also found incorporating into the endothelium of larger vessels. By contrast, TPC derived from the transplanted organ were not found in the nonischemic limbs (Aicher et al., 2007).

Moreover, in the human adipose tissue, the presence of stem cells that can differentiate *in vitro* to endothelial cells under endothelial cell culture conditions has been reported. These cells changed their morphology and organization and expressed endothelial cell-specific markers such as CD31 and vWF (Sengenes et al., 2007; Miranville et al., 2004). *In vivo*, their injection led to an increase in blood flow and capillary density within the ischemic leg and improve postnatal neovascularization (DiMuzio and Tulenko, 2007).

The presence of progenitor cells in the adult vascular wall was also demonstrated in humans. In an area between the smooth muscle and adventitial layer, high numbers of EPC were identified, which were capable of forming capillary sprouts and may contribute to the endothelial repair (Zengin et al., 2006).

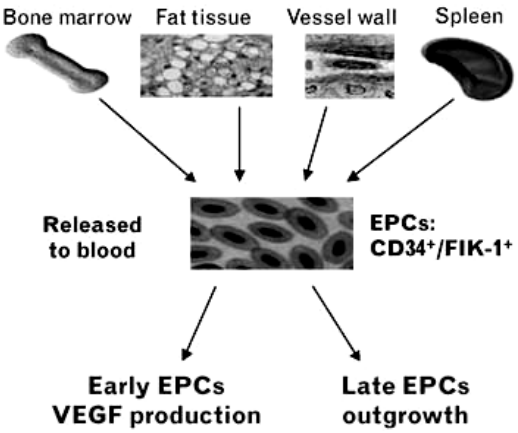


Figure 1: Endothelial progenitor cell origins (Adopted from Xu, 2007). Endothelial progenitor cells (EPC) could be released from bone marrow, fat tissue, vessel wall, and spleen, into blood, where they express CD133 at the early stage, and then CD34/Flk-1. Circulating EPC can form two types of cell in vitro: early and late EPC, characterized according to their abilities to produce vascular endothelial growth factor (VEGF) and to proliferate.

4.3 Mobilization of EPC

After birth, adult stem cells reside in a special microenvironment termed the stem cell niche, where they can either remain in an undifferentiated and quiescent state or are instructed to differentiate and mobilize to circulation following specific signals (Li and Xie, 2005; Yin and

Li, 2006; Heissig et al., 2002). Bone marrow is a major reservoir for adult organ-specific stem cells, including HSCs (Reya et al., 2001) and endothelial progenitors (Lyden et al., 2001). The stem cell niche is composed of a specialized population of stromal cells like fibroblasts, osteoblasts, and endothelial cells, that maintain the stem cell niche and play an essential role in regulating adult stem cell self-renewal and differentiation. In adults, osteoblasts (responsible for bone growth) and HSCs are closely associated in bone marrow, suggesting a reciprocal relationship between the two. The equilibrium between these two compartments is dictated by the bioavailability of stem cell-active cytokines, which are bound to the extracellular matrix or tethered to the membrane of stromal cells. In response to vascular injury or stress, such as BM ablation by cytotoxic agents, stem cells have to be rapidly mobilized from their niches to the circulation, where they home to respective organs and either contribute to restore organ function or regenerate the stem and progenitor cell pool (Yin and Li, 2006; Heissig et al., 2002).

EPC mobilized from the bone marrow into the blood stream may be recruited and incorporated into sites of active neovascularization during tissue ischemia, vascular trauma, or tumor growth. Moreover, expansion, and mobilization of EPC may augment the resident population of EC competent to respond to exogenous angiogenic cytokines (Isner and Asahara, 1999).

Mechanistically, cytokines inducing mobilization interfere with the interactions between stem cells and bone marrow stromal cells, which allow stem cell disengage the bone marrow, and to pass through the sinusoidal endothelium to enter the blood stream (Aicher et al., 2005).

VEGF is an effective mobilize of EPC from bone marrow and a potent inducer of adult angiogenesis. After tissue injury, when formation of new blood vessels is urgently required, VEGF mediates proliferation, differentiation, and chemotaxis of endothelial cells (Asahara et al., 1999; Hanahan and Folkman, 1996). A rapid elevation of circulating VEGF leads to recruitment of EPC to the site of injury and promotes neovascularization. VEGF induced progenitor cell mobilization is dependent on local secretion of MMP-9 by the hematopoietic and stromal compartments of the bone marrow which results in release of soluble Kit ligand (sKitL; also known as stem cell factor). The release of sKitL permitting the transfer of endothelial progenitors and hematopoietic stem cells from the quiescent osteoblastic niche to the vascular-enriched proliferative niche and favouring differentiation and mobilization to the peripheral circulation (Heissig et al., 2002).

Active MMP-9 is a major target for NO. Basal expression and activity of MMP-9 is dramatically reduced in endothelial nitric oxide synthase knockout (*NOS3* knockout) mice.

These mice have impaired ischemia-induced neovascularization and exhibit impaired EPC mobilization and incorporation to the ischemic areas. eNOS contributes to blood vessel relaxation in the periphery and is essential in the bone marrow microenvironment (Aicher et al., 2003). Treatment with exogenous sKitL can augment EPC numbers, suggesting that release of sKitL by the activity of MMP-9 accounts for the defective hematopoietic recovery and progenitor cell mobilization (Aicher et al., 2003).

Increased NO and activation of MMP-9 mediates the angiogenic response that is initiated by other chemokines or reagents. The chemokine SDF-1 and its receptor CXCR4 play a major role for bone marrow retention of stem cells and recruitment of EPC to sites of ischemic areas (Kollet et al., 2003; Askari et al., 2003; Lapidot et al., 2005). SDF-1 is constitutively expressed, but its levels are rapidly upregulated by a range of stimuli such as inflammatory mediators, changes in the extracellular matrix, altered mechanical forces, and hypoxia (Ceradini et al., 2004).

Platelets are crucial for homeostasis and limit blood loss after vascular injury because the early vascular response is platelet adhesion to the exposed subendothelium. It was recently reported that that CD34+ and cKit+Sca-1+Lin- EPC directly adhere to platelets after vascular injury in a process that involves P-selectin and GPIIb integrin. Once activated, platelets secrete high levels of SDF-1, which supports primary adhesion of EPC on the surface of arterial thrombi *in vivo* (Massberg et al., 2006). However, increased levels of SDF-1 cannot induce neovascularization in the absence of injury. Intramuscular gene transfer of SDF-1 into ischemic limbs enhanced ischemia-induced neovascularization in mice associated with mobilization and partial incorporation of EPC into neovessels, but in the absence of ischemia these effects were ablated, indicating that other signals from the ischemic limb are required. VEGF may be this additional signal as inhibition of VEGF signaling abrogated all SDF-1-induced effects. Similar results were obtained when *NOS3* knockout mice were used, suggesting that it involves VEGF/eNOS-related pathway (Hiasa et al., 2004)).

A cytokine clinically used for the mobilization of CD34+ cells in patients is G-CSF, which releases the proteinases elastase and cathepsin G from neutrophils. These proteinases induce cleavage of adhesive bonds on stromal cells, which interact with integrins on hematopoietic stem cells (Aicher et al., 2005).

In addition to the use of cytokines to mobilize EPC from the bone marrow, similar results were observed for a number of other bioactive proteins. Estrogen accelerates reendothelialization and attenuates medial thickening after carotid artery injury in part by augmenting mobilization and proliferation of progenitor cells and their incorporation into the

recovering endothelium at the site of injury (Iwakura et al., 2003). These events are diminished in *NOS3* knockout mice suggesting that they are NO mediated.

Erythropoietin and physical exercise can also increase mobilization of EPC numbers, promoting tissue neovascularization, and at the same time inhibit neointimal formation via an NO-dependent mechanism. The observed protective effects were abolished in eNOS-deficient mice (Urao et al., 2006; Laufs et al., 2004).

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins have been developed as lipid-lowering drugs and are well established to reduce morbidity and mortality from coronary artery disease, but besides that statins are also capable induce EPC mobilization form bone marrow (Dimmeler et al., 2001). The increase of circulating EPC numbers by statin treatment requires the eNOS (Aicher et al., 2005).

Adverse effects of EPC mobilization have been described as contribution of EPC to tumor neovascularization in some tumor models (Lyden et al., 2001).

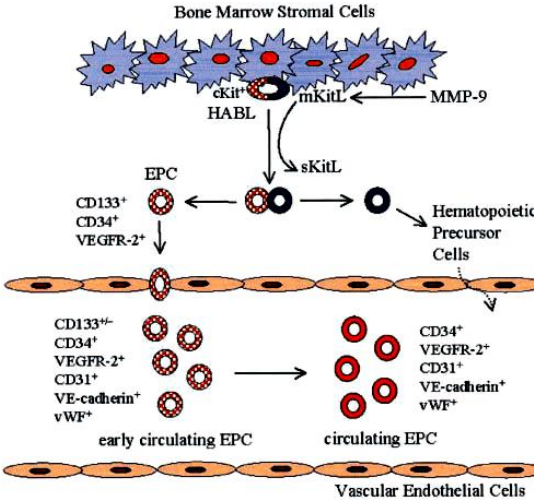


Figure 2: EPC mobilization (Adopted from Hristov et al., 2003). The mobilization of EPC from the bone marrow is a complex process, regulated by a variety of factors. The activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane-bound Kit ligand (mKitL) to a soluble Kit ligand (sKitL), is an early step in this process. Early EPC in the bone marrow are positive for CD133/CD34/VEGFR-2. Circulating EPC obviously lose CD133 and are positive for CD34/VEGFR-2/CD31/VE-cadherin/von Willebrand factor (vWF).

4.4 EPC homing

Cell therapy is a promising therapeutic option to improve neovascularization. The efficiency of cell therapy to augment recovery after ischemia depends on the sufficient recruitment of applied cells to the target tissue (Chavakis et al., 2008). Activation by chemokines is an important step during recruitment of a reasonable number of progenitor cells to the ischemic

tissue. SDF-1 has been shown to stimulate the recruitment of progenitor cell from the bloodstream to the ischemic tissues (Lapidot, 2001). The expression of SDF-1 is upregulated during ischemia (Ceradini et al., 2004; De Falco et al., 2004). Inhibition of the SDF-1/CXCR4 axis partially blocks the homing of progenitor/stem cells to the ischemic myocardium (Abbott et al., 2004). Moreover, overexpression of SDF-1 enhanced stem cell homing and incorporation into ischemic tissues (Askari et al., 2003; Yamaguchi et al., 2003), supporting that SDF-1 plays a crucial role for recruitment of circulating or intravenously infused cells.

Recent studies determined the involvement of additional other chemokines. CXC-chemokine IL-8 and its cellular receptors CXCR2 and CXCR1 contribute to homing of intravenous infused CD34+ progenitor cells to the ischemic myocardium (Kocher et al., 2006). IL-8 is an inflammatory chemokine, which is able to stimulate angiogenesis. Neutralizing anti-IL-8 antibodies or antibodies against the IL-8 receptors, CXCR1 or CXCR2, reduced CD34+ cell-mediated improvement of neovascularization, establishing a role for IL-8 for homing and neovascularisation improvement by CD34+ cells (Chavakis et al., 2008).

Futhermore, ischemia-induced VEGF acts as a chemoattractant to EPC (Kalka et al., 2000). Interestingly, VEGF is sufficient to induce the organ recruitment of bone-marrow-derived circulating myeloid cells and their perivascular localization via induction of SDF-1 expression by perivascular myofibroblasts, suggesting that different cytokines may cooperate during homing of bone marrow cells (Grunewald et al., 2006).

The next step of homing consists of the adhesion molecules activation by chemokines and the arrest/firm adhesion of the progenitor cells on the endothelium. P-selectin and E-selectin seem to mediate the initial steps of this process. It has been shown that also β2-integrins expressed on the cell surface of EPC mediate the firm adhesion and transmigration of EPC to the damaged endothelial monolayer and improve the neovascularisation in ischemic tissues (Chavakis et al., 2005).

The mobilization, recruitment and invasion of stem- and progenitor cells during vasculogenesis involve proteolytic activity. To deliver a beneficial effect on neovascularization, EPC need to transmigrate to the injured tissue and thus their invasive capacity is crucial for tissue repair and restoration of organ function. *In vitro* studies using knockout mice revealed an important role of the lysosomal cycteine protease cathepsin L for vasculogenesis. This protease is highly expressed in EPC and it seems to be essential for matrix degradation and invasion. The improvement of neovascularization after hind limb ischemia was significantly impaired in *cathepsin* $L^{-/-}$ mice and infused *cathepsin* $L^{-/-}$ bone

marrow mononuclear cells failed to home to sites of ischemia and to augment neovascularization (Urbich et al., 2005).

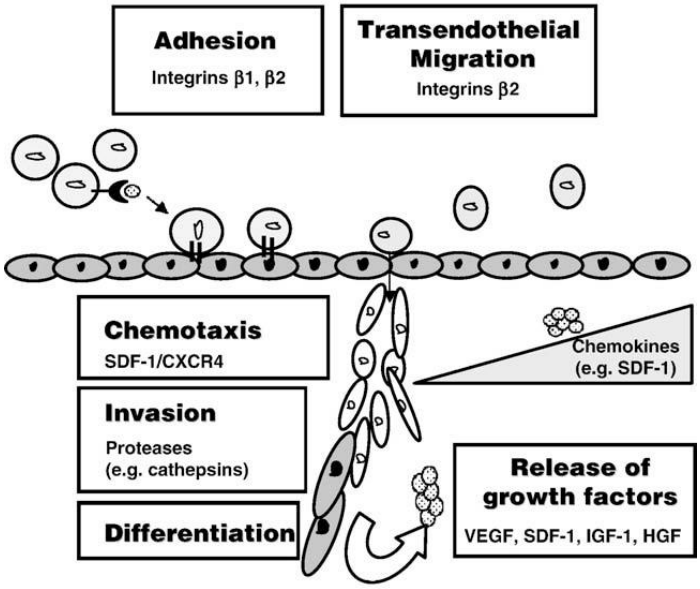


Figure 3: EPC homing (Adopted from Chavakis et al., 2008). After vascular injury, the endothelial monolayer is activated. Recruitment and incorporation of progenitor cells into ischemic or injured tissue requires a coordinated multistep process including adhesion to the endothelium, transendothelial migration, chemotaxis, matrix degradation and invasion and in situ differentiation.The factors which are proposed to regulate the distinct steps are indicated.

4.5 Role of EPC in neovascularization

Postnatal neovascularization is an important process to rescue tissue from critical ischemia or after myocardial infarction (Isner and Asahara, 1999; Urbich and Dimmeler, 2004). The finding that bone marrow-derived cells can home to sites of ischemia and express endothelial markers has challenged the use of isolated hematopoietic stem cells or EPC for therapeutic vasculogenesis.

Both in mice and rabbits with hind limb ischemia, mobilization of EPC can remarkably promote new blood vessels formation in the injured areas, enhance perfusion, and lead to recovery of ischemic tissue (Kalka et al., 2000; Takahashi et al., 1999).

Ex vivo expanded EPC, isolated from peripheral blood mononuclear cells, can also incorporate into the foci of myocardial neovascularization and augment vascularity in ischemic hearts (Kawamoto et al., 2001). Whereas intracoronary infusion of progenitor cells (either peripheral blood or bone marrow-derived progenitors) in patients with acute myocardial infarction was shown to improve blood flow and cardiac function and to associate with significant beneficial effects in post-infarction left ventricule remodeling via the release of various growth factors attracting both circulating and tissue resident cardiac progenitor

cells, thereby enhancing endogenous repair mechanisms of the myocardium (Britten et al., 2003; Schächinger et al., 2004).

EPC numbers can also be used as a predictive biomarker for cardiovascular risk and vascular function. In a large clinical study, Hill and colleagues (Hill et al., 2003) reported that high-risk individuals have fewer EPC and become senescent more rapidly compared with their low-risk counterparts. Presumably, risk factors, by modulating the levels of oxidative stress, nitric oxide activity, or other physiologic processes, could directly influence the mobilization or half-life of EPC. Another group identified a significant association between increasing numbers of EPC and decreased risk of a major cardiovascular event and hospitalization in patients with coronary artery disease (Werner et al., 2005). EPC numbers can also predict severe endothelial dysfunction in patients with coronary heart disease (Werner and Nickenig, 2007). Additionally, the low EPC levels were reported in diabetic patients and in individuals who suffered an acute stroke (Tepper et al., 2002; Ghani et al., 2005). Finally, in a case study of approximately 550 patients, results indicated that EPC number is inversely correlated with the serum cytokine levels of SDF-1a, MMP-9 and the extent of carotid artery atherosclerosis (Xiao et al., 2007).

Conditions associated with cardiovascular diseases have an effect on the functional activity of progenitors (Fadini et al., 2007). EPC from type II diabetes patients exhibit alterations in functions important for blood vessel growth like impaired proliferation, adhesion, and tubulization (Tepper et al., 2002). Similar functional features like impaired proliferation, migration and survival have been reported in EPC isolated from aged individuals, although no quantitative differences in EPC were observed (Heiss et al., 2005). The patients with postinfarction heart failure have a selective functional exhaustion of their hematopoietic progenitor cells in the bone marrow niche characterized by a profoundly impaired colony-forming capacity, but a preserved progenitor cell number (Vasa et al., 2001; Kissel et al., 2007).

The efficiency of EPC-induced neovascularization may not solely be due to the incorporation of EPC into newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner Thus, EPC may act similar to monocytes/macrophages, which can increase arteriogenesis by providing cytokines and growth factors (De Palma et al., 2003; Urbich et al., 2005). The release of growth factors in turn may influence the classical processes of angiogenesis, like the proliferation and migration as well as survival of mature endothelial cells (Urbich and Dimmeler, 2004).

4.6 EPC repair damaged endothelial cells

Endothelial cells cover the luminal surface of blood vessels and maintain multiple vascular functions (Urao et al., 2006). Activation and damage of the endothelial monolayer seem to trigger the development of the vascular diseases, including atherosclerosis.

Atherosclerosis is an inflammatory disease characterized by accumulation of mononuclear cells, smooth muscle cell migration and proliferation, and neointima formation. The endothelial dysfunction is the first step in atherosclerosis. Endothelial damage can be induced by oxidized lipids, free radicals, cytokines, hemodynamic stress and high concentrations of blood cholesterol. All these events lead to acute stress injury which results in apoptosis/necrosis of endothelial cellular layer (Ross, 1999). Initially, it was thought that the damaged endothelial cells were replaced by the adjacent intact endothelium but recent studies demonstrated the recruitment and incorporation of vascular progenitor cells into atherosclerotic lesions and thus provided evidence in support of the role of vascular cells in the development of the disease. Direct incorporation of circulating EPC into the vessel wall was detected in mice. In a model of transplant atherosclerosis, regenerated endothelial cells from arterial allografts were found to originate from recipient circulating blood but not the remaining endothelial cells of the donor vessels (Hu et al., 2003).

Importantly, there is an atheroprotective property of bone marrow that is “exhausted” with aging and prolonged exposure to risk factors. This exhaustion involves EPC mediated vascular repair (Rauscher et al., 2003).

In addition to bone marrow, spleen derived EPC also have the capacity to repair damaged endothelium. For example, intravenous transfusion of spleen-derived MNCs improves endothelium-dependent vasodilatation in atherosclerotic mice, indicating that progenitor cells play an important role in repairing the ongoing vascular injury (Wassmann et al., 2006). EPC derived from spleen homogenates also enhance re-endothelialization and reduce neointima formation after induction of endothelial cell damage using the carotid artery model (Werner et al., 2003).

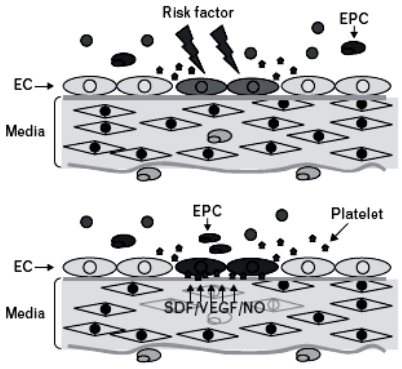


Figure 4: Schematic representation for endothelial progenitor cells replacing dead endothelial cells (Adopted form Xu, 2007). Dead endothelial cells are replaced by endothelial progenitor cells (EPC). In this process, platelets and monocytes can simultaneously attach to subendothelial matrix membrane. SDF, stromal cell-derived factor; VEGF, vascular endothelial growth factor.

4.7 EPC role in tumor angiogenesis

Tumour angiogenesis/vasculogenesis can be initiated and maintained also by bone marrow derived endothelial progenitor cells. Studies have found that the level and function of EPC in multiple myeloma correlate with disease activity. Data from patients showed that levels of EPC in peripheral blood are higher in patients that in healthy controls (Zhang et al., 2005). An increased number of CD133+ cells that contribute to the formation of capillaries were identified in lung cancer and in breast cancer (Hilbe et al., 2004).

Thereby, certain inhibitors of tumor neovascularization may act by inhibiting mobilization and homing of EPC to the developing vascular network of tumors. The in vitro proliferation and colony-forming ability of human EPC are markedly decreased in the presence of angiostatin, a proteolytic cleavage product of plasminogen with antiangiogenic properties (Ribatti, 2007).

Based on our previous work (Limbourg et al., 2007) demonstrating the importance of the Notch signaling pathway in postnatal neovascularization, we investigated the role of Notch signaling in EPC.

5 RESULTS

5.1 Expression of Notch ligands and receptors in human EPC

To characterize the repertoire of Notch receptors and ligands expressed in human EPC we generated EPC by differentiation from peripheral blood mononuclear cells of healthy donors. EPC were characterized as adherent cells double-positive for DiI-acLDL uptake and lectin binding (Fig. 1A). The endothelial phenotype of the EPC was confirmed by documenting the expression of well established cell surface markers like VEGFR2 (KDR), vWF, CD31 and VE-Cadherin by fluorescence-activated cell sorting (FACS, Fig. 1B). In addition, the expression of the endothelial marker endothelial nitric oxide synthase (eNOS) in EPC was demonstrated by RT-PCR analysis, endothelial cells (ECs) were used as a positive control (Fig.1C).

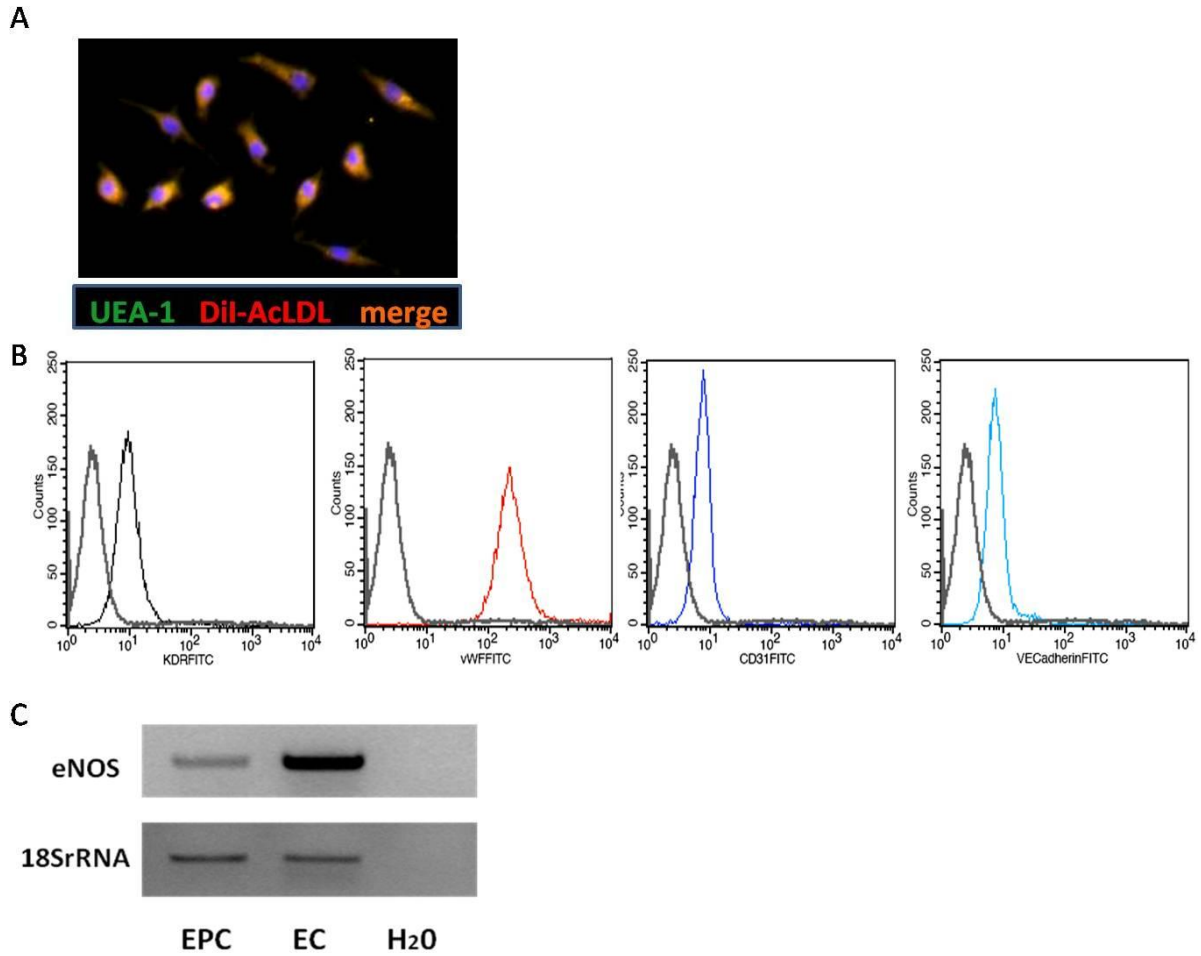


Figure 1. Characterization of human EPC. A, Fluorescence microscopy (40x objective) illustrates that adherent cells were postive for uptake of DiI-labeled acetylated LDL (red) and binding of FITC-ulex-lectin (green). All acLDL(+) cells were also postive for ulex-lectin binding, as can be seen in overlay (orange). B, Expression of, monocyte and endothelial marker CD31, endothelium-specific markers VEGFR2 (KDR), VE-Cadherin and vWF were assessed on cultured EPC (day 7) by FACS analysis. Corresponding negative isotype controls are shown in grey. C, eNOS mRNA expression was detected by semiquantitative RT-PCR. As internal control was used 18SrRNA.

Next, we determined the expression of Notch signaling component in EPC by RT-PCR analysis. We found that EPC expressed *Notch1* and *Notch2* whereas human endothelial cells (ECs) expressed *Notch1*, *Notch2* and *Notch4*, and smooth muscle cells (SMC) expressed *Notch2* and *Notch3* (Fig. 2A). Analysis of Notch ligands revealed expression of the ligand *Dll1*, *Jagged1* (*Jag1*) but not *Dll4* or *Jagged2 (Jag2)*, in EPC, while ECs expressed *Dll1*, *Dll4*, *Jag1* and *Jag2* (Fig. 2B). Together, these results demonstrate expression of *Notch1*, *Notch2* and the ligands *Dll1* and *Jag1* in human EPC.

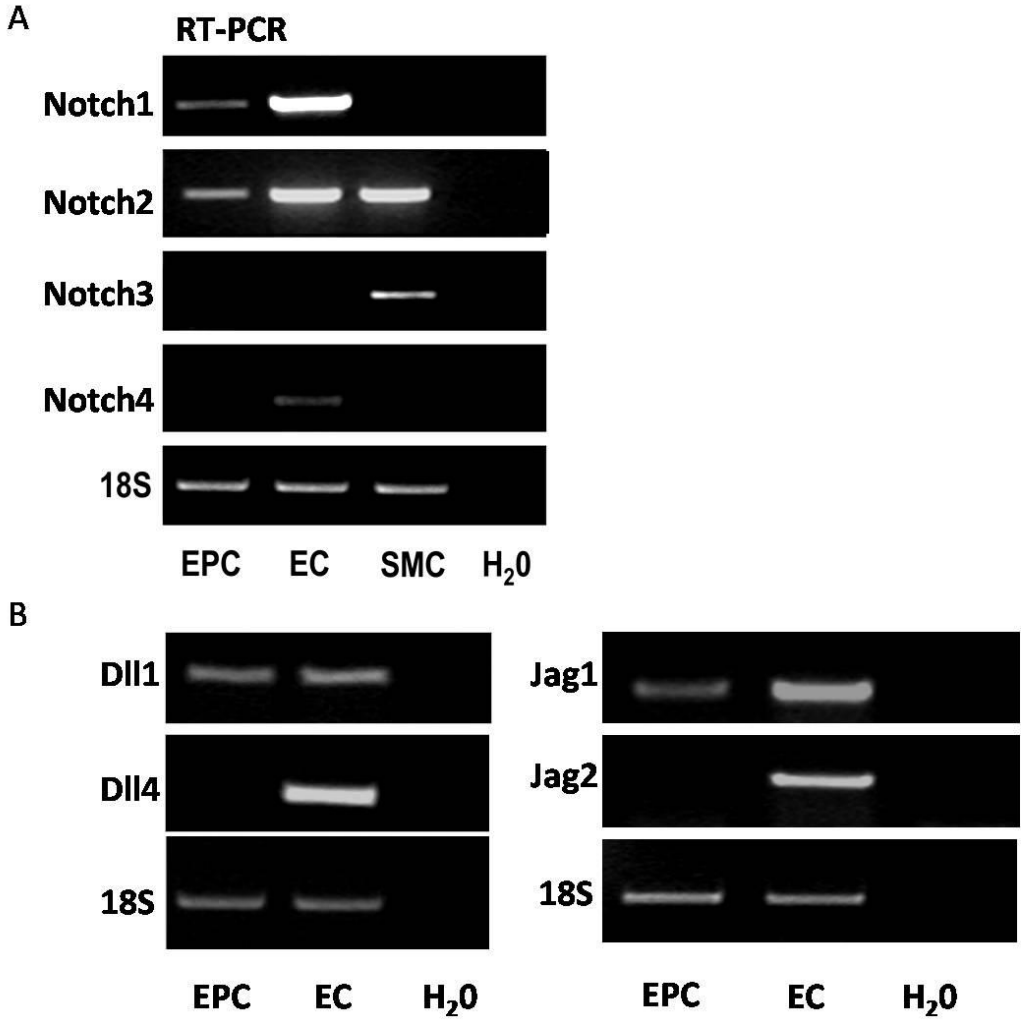


Figure 2. Expression of Notch receptors and ligands. Expression of Notch receptors (A) and Notch ligands (B) was detected by RT-PCR in cultured EPC (day 7). Cultured hEPC expressed *Notch1*, *Notch2*, *Dll1* and *Jag1*. Gene expressions were controlled by 18SrRNA.

5.2 Notch inhibition reduces EPC number

To investigate the role of Notch signaling in human EPC differentiation we inhibited ligand-induced Notch receptor activation by γ-secretase with a soluble γ-secretase inhibitor (GSI), N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), which prevents

the generation of the active Notch intracellular domain (NICD) receptor fragment from the full-length receptor, thereby acting as a potent inhibitor of Notch signaling (Raya, 2004). Counting double fluorescent staining positive cells revealed that DAPT treatment strongly reduced EPC numbers generated in culture compared to control culture conditions (Fig. 3A, EPC/hpf, ctrl: 95±9 vs. DAPT: 53±7, n=3, $p<0.05$). To confirm that Notch activation is inhibited by DAPT treatment, we performed RT-PCR of the well-known Notch target genes *Hey1* and *Hey2*. RT-PCR results indicated that *Hey1* and *Hey2* were abrogated by DAPT treatment in cultured EPC (Fig. 3B).

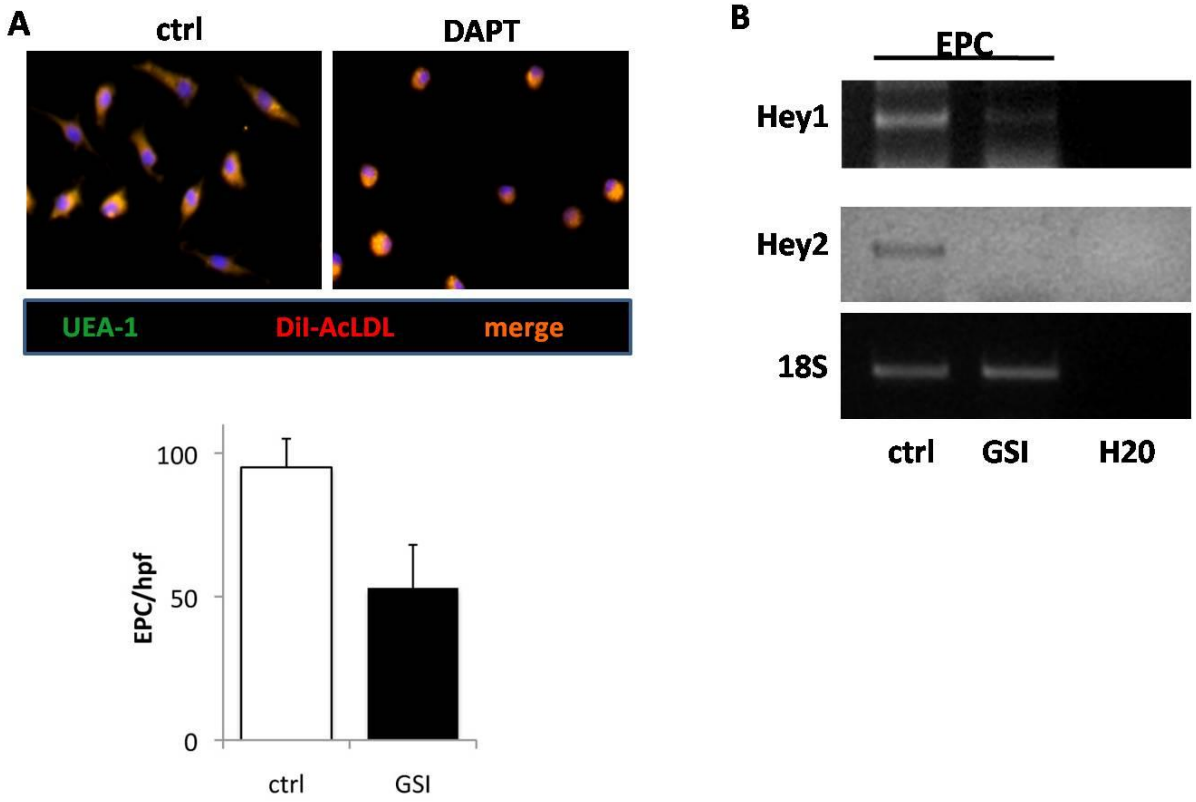


Figure 3. EPC number is reduced after Notch inhibition. A, Counting double fluorescent staining postive cells indicated that DAPT treatment reduced cultured EPC numbers compared to control. B, Expression of human-specific *Hey1* and *Hey2* was downregulated by Notch inhibitor DAPT as detected by RT-PCR. As internal control was used 18SrRNA. Magnifiation (A) 400x.

Flow cytometric analysis of light-scattering properties demonstrated that Notch inhibition by GSI in the majority of treated EPC had dramatically increased cell size and granularity compared to controls (Fig. 4A).

Interestingly, the monocytic marker CD14 was expressed at lower levels by Notch inhibition, while macrophage markers CD64 and CD86 were expressed at higher level after Notch inhibition compared to control. These results suggesting that Notch acts as a specific modulator of EPC differentiation (Fig. 4B).

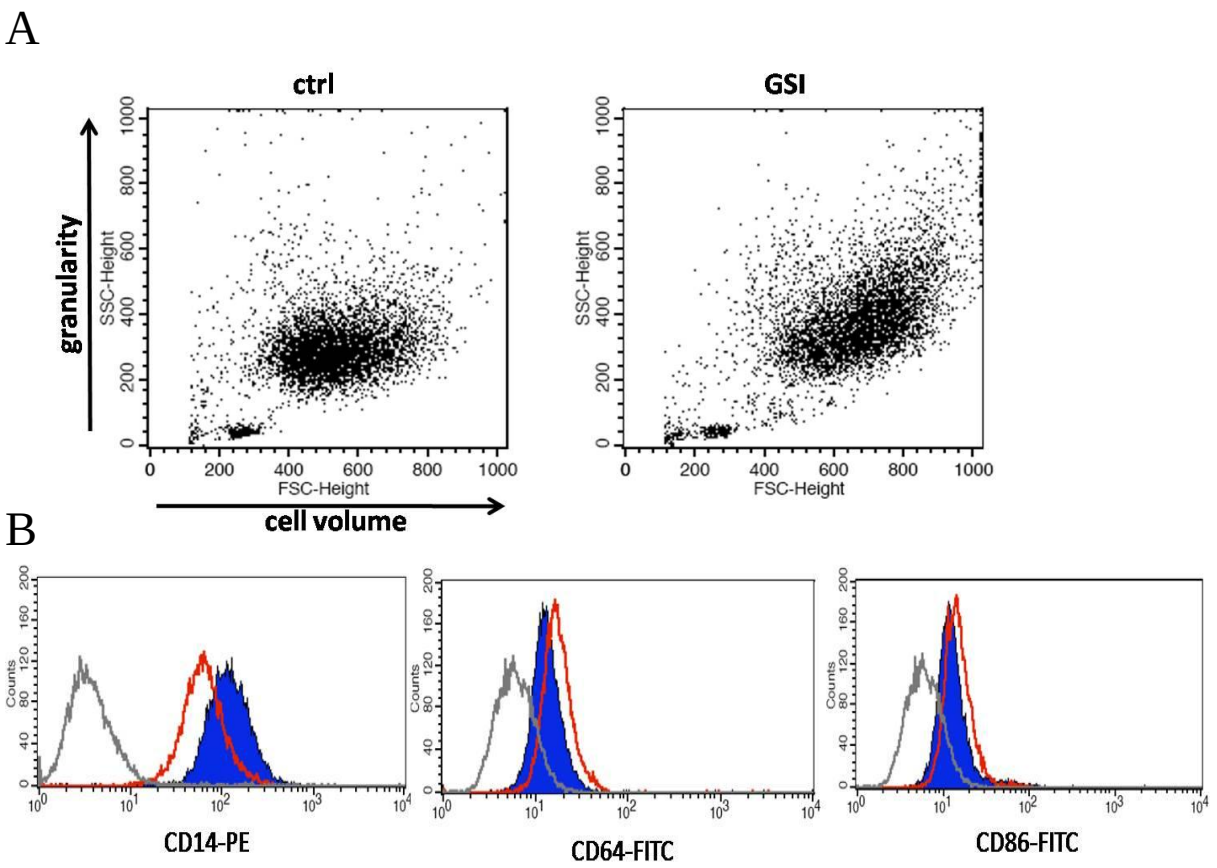


Figure 4. Notch inhibition changes EPC morphology and fate. A, Representative forward and side scatterplot of cultured EPC reveals that after inhibition of Notch with GSI the cells had bigger size and were more granular compared to controls. B, After Notch inhibition monocytic marker CD14 was expressed at lower level and macrophage markers CD64 and CD86 were expressed at higher level compared to control. Control is shown in blue, GSI in red and corresponding negative isotype controls are shown in grey.

5.3 Canonical Notch signaling regulates EPC differentiation

To prove the significance of Notch ligand *Dll1* for EPC biology, we analyzed EPC using Dll1siRNA. Counting double fluorescent staining positive cells revealed that knockdown of endogenous Notch ligand Dll1 in EPC by siRNA also markedly reduced EPC numbers (Fig. 5A). This was also confirmed by Western blot analysis. DLL1 protein expression was downregulated dose-dependently (Fig. 5B).

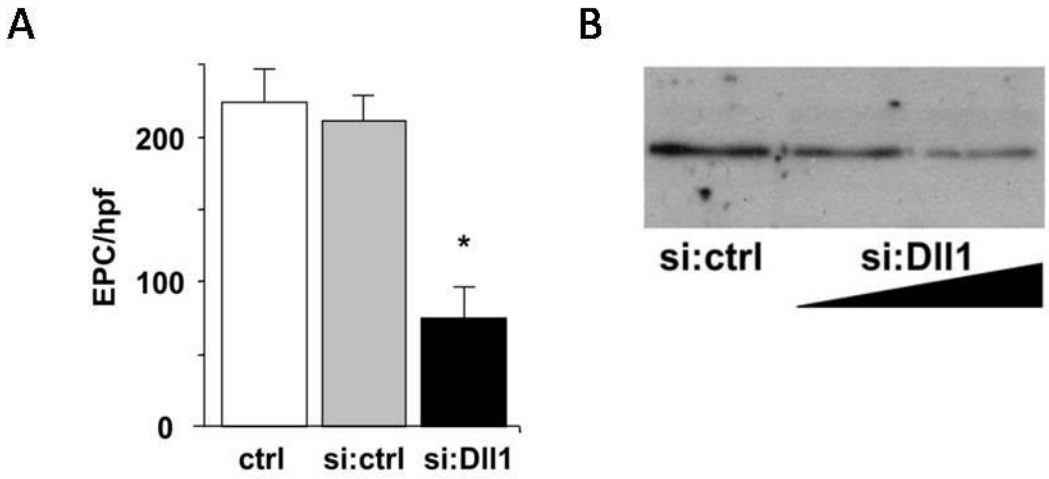


Figure 5. Notch pathway activation is required for EPC differentiation. Counting double fluorescent staining postive cells revealed that Dll1siRNA (A) strongly decreased EPC numbers compared to controls. DLL1 expression by Dll1siRNA was downregulated dose-dependently as shown by Western blot (B)

Canonical Notch signaling is mediated by the Notch nuclear effector RBP-Jκ. To investigate whether EPC differentiation is mediated by canonical Notch signaling we employed genetic ablation of *Rbp-jκ* by adenoviral transduction of Cre recombinase in EPC from *Rbp-jκ*$^{flox/flox}$ mice. Transduction of AdCre, but not AdGFP, resulted in loss of RBP-Jκ protein in EPC and impaired EPC generation (Fig. 6A), confirming an essential role of canonical, RBP-Jκ mediated, Notch signaling in EPC differentiation. Together, these data substantiate a critical role of canonical Notch signaling in EPC differentiation, which is dependent on Notch ligand Dll1 and mediated by RBP-Jκ.

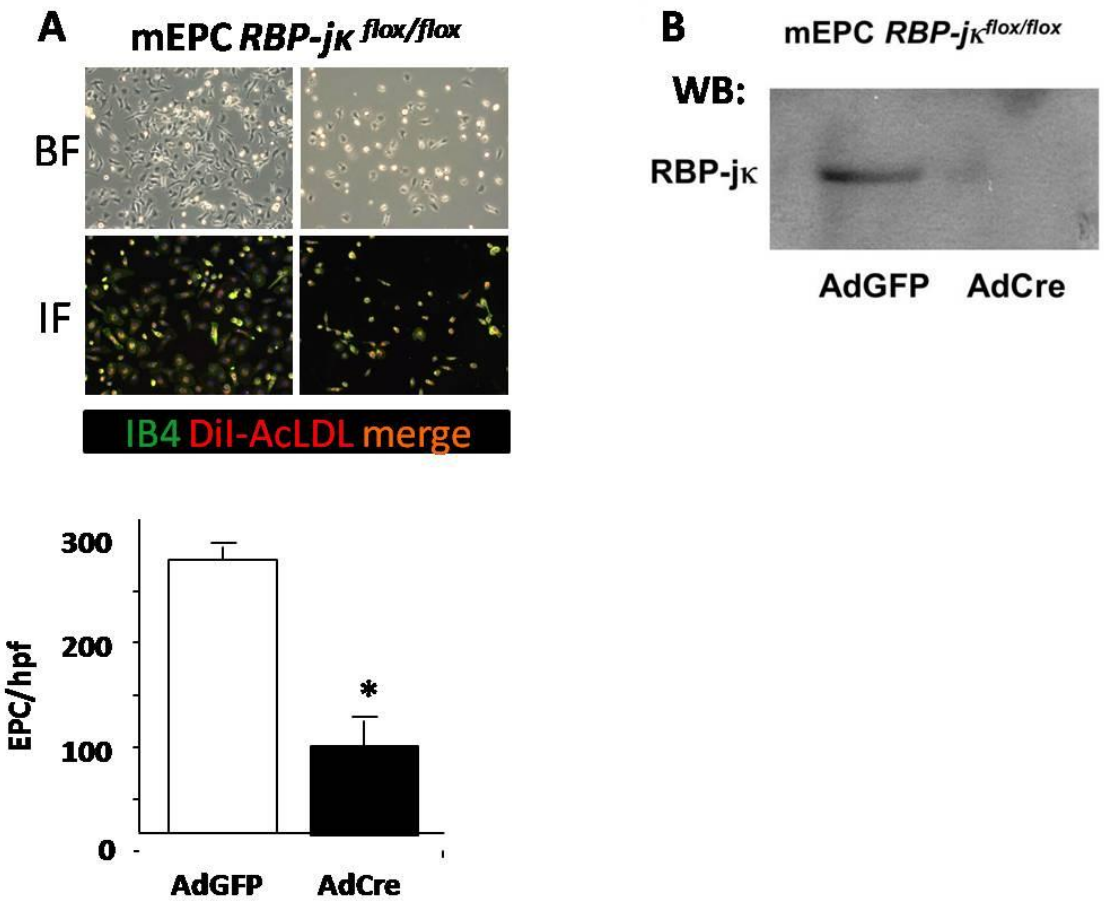


Figure 6. Canonical Notch signaling regulates EPC numbers. A, Genetic ablation of *Rbp-jκ* by adenoviral transduction of Cre recombinase (AdCre) in EPC from *RBP-jκ*$^{flox/flox}$ mice impaired EPC generation, but not AdGFP. B, Western blot analysis confirmed the loss of RBP-Jκ protein by transduction with AdCre. BF: Bright field, IF:Immunofluorescence. Magnification (A, upper panel) 200x.

5.4 Notch regulates CXCR4 expression

Given the importance of CXCR4 signaling for EPC interaction with EC, we tested the potential regulation of CXCR4 by Notch. FACS analysis and Western blot data indicated that Notch inhibition by GSI strongly reduced surface expression, and total cellular protein levels, of CXCR4 in human EPC (Fig. 7A). In addition, genetic ablation of the Notch effector Rbp-jκ in *RBP-Jκ*$^{flox/flox}$ mouse EPC also markedly reduced Cxcr4 surface expression (Fig. 7B). In contrast, constitutive Notch activation by NICD overexpression upregulated CXCR4 protein in human EPC (Fig. 7C). These results demonstrate that Notch signaling is required and sufficient to regulate CXCR4 levels in EPC.

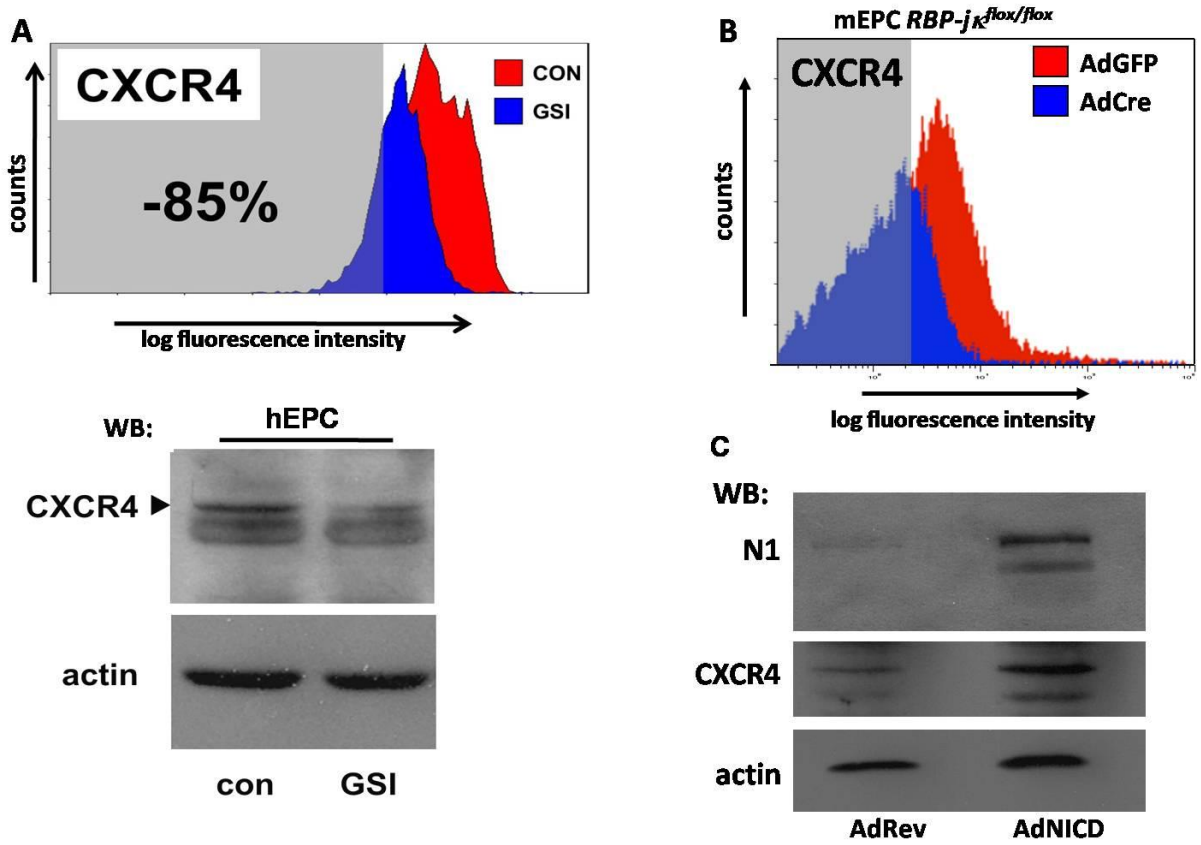


Figure 7. CXCR4 expression is regulated by Notch signaling. A, FACS (upper panel) and Western blot (lower panel) analysis indicated that Notch inhibition by GSI strongly downregulated CXCR4 expression in hEPC. B, Genetic ablation of the Notch effector Rbp-jκ with AdCre virus in *RBP-Jκ*$^{flox/flox}$ mouse cultured EPC also markedly reduced Cxcr4 surface expression as shown by FACS analysis. C, Constitutive Notch activation by NICD overexpression upregulated Notch1 and also CXCR4 protein expression in hEPC. Actin staining was used as a loading control.

To verify that Notch inhibition impaires CXCR4 signaling we used serum starvation overnight and then stimulated EPC with the CXCR4 ligand SDF-1 and analyzed activation of protein kinase Akt and JAK-2. Akt and JAK-2 are known as important downstream targets of CXCR4 signaling (Dimmeler et al., 2001; Vila-Coro et al., 1999). We measured the phosphorylation of Akt and JAK-2 by Western blot using specific antibodies. Akt phosphorylation was detected as early as 15 minutes after exposure to SDF-1 and JAK-2 after 2 minutes. As revealed in Fig. 8, SDF-1 induced robust Akt and JAK-2 activation in human EPC, which was blocked by coincubation with the CXCR4 antagonist AMD3100 and by pretreatment with GSI (Fig. 8). Together, these data demonstrate that Notch regulates CXCR4 levels and signaling in EPC.

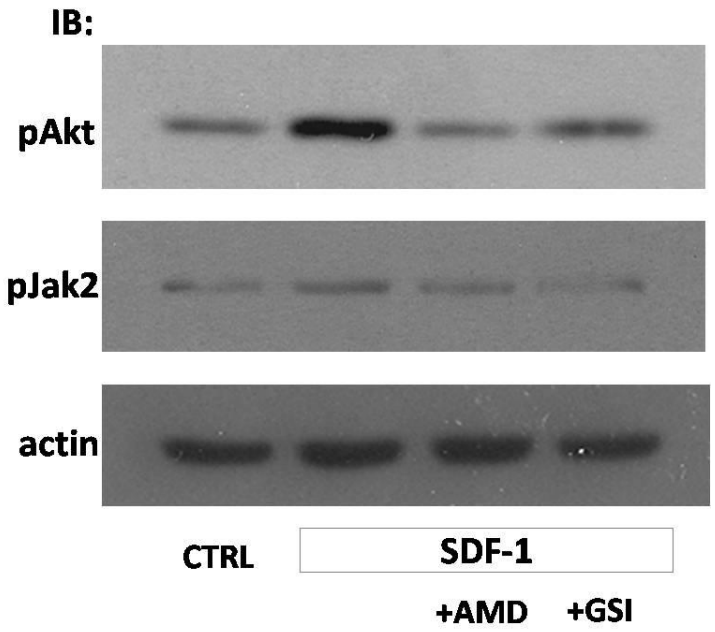


Figure 8. Inhibition of SDF-1 reduces phosphorylation of Akt and Jak2. SDF-1 induces the phosphorylation of Akt and JAK-2, which is blocked by coincubation with CXCR4 antagonist AMD and by pretreatment with GSI. Actin staining was used as a loading control.

5.5 Notch regulates CXCR4-dependent signaling

It is known that the CXCR4 ligand SDF-1 supports angiogenesis by regulating migration, adhesion and survival of EPC. To investigate the functional importance of Notch-dependent CXCR4 regulation we stimulated EPC with SDF-1 and blocked Notch activation by culturing EPC with GSI or inhibited CXCR4 signaling with a specific small molecule CXCR4 antagonist, AMD3100 (De Clercq, 2003).

Stimulation of human EPC with SDF-1 increased EPC numbers and morphology significantly, which was blocked by AMD3100 and by pretreatment of EPC with GSI, demonstrating that CXCR4 signaling is downstream of Notch activation (Fig. 9).

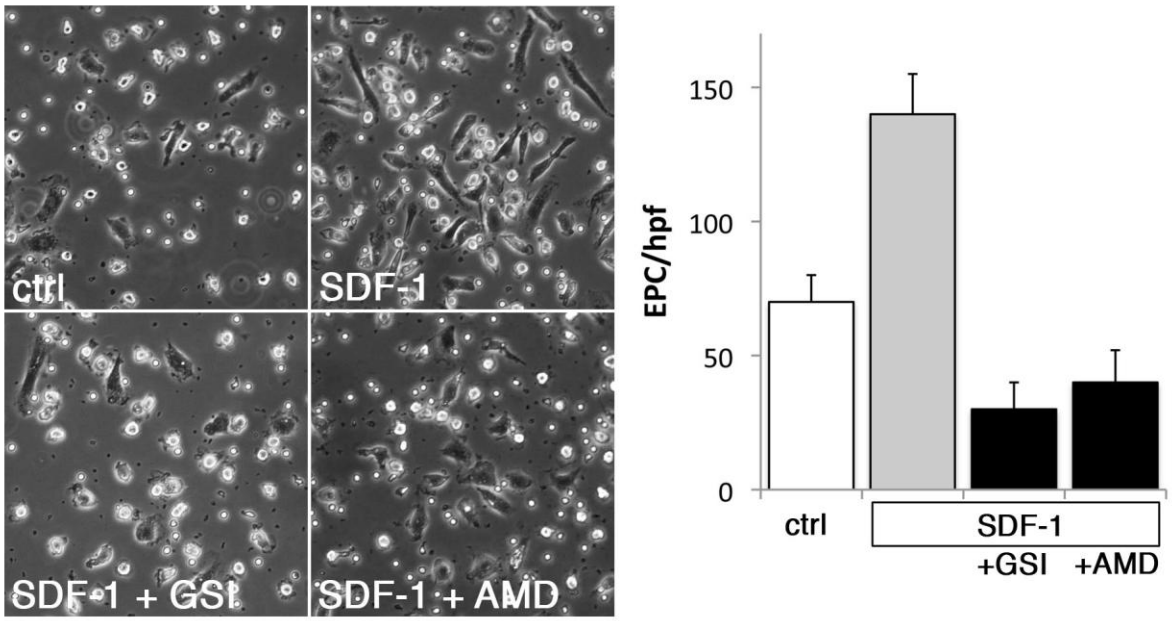


Figure 9. CXCR4 mediates Notch signaling in EPC. Stimulation of hEPC with SDF-1 increases EPC numbers and changes their morphology. This effect of SDF-1 is blocked by adding AMD and by pretreatment of EPC with GSI. Bright field image, magnification 200x.

As VEGF was also an established factor for EPC, we investigated the effect of AMD3100 and GSI on the VEGF- and SDF-1-mediated EPC adhesion to ECs. Data revealed that EPC adhesion to EC was significantly enhanced by SDF-1 and completely abrogated by either coincubation with AMD3100 or by pretreatment of EPC with GSI (Fig. 10A). Interestingly, VEGF-induced adhesion of EPC was not only abrogated by GSI pretreatment, but also AMD treatment (Fig. 10B), which suggests that the effects of VEGF on EPC adhesion are mediated by SDF-1-CXCR4 axis.

Furthermore, stimulation of EPC with SDF-1 in a modified Boyden chamber markedly enhanced migration when compared to control and VEGF treated cells, which was blocked by coincubation with AMD3100 or preatreatment of EPC with GSI. Again, VEGF-induced migration was also inhibited by CXCR4 or Notch inhibition, suggesting a contribution of CXCR4 signaling to VEGF-induced migration (Fig. 10C). Together, these data demonstrate that SDF-1 responsiveness and pro-angiogenic features of EPC are dependent on Notch signaling.

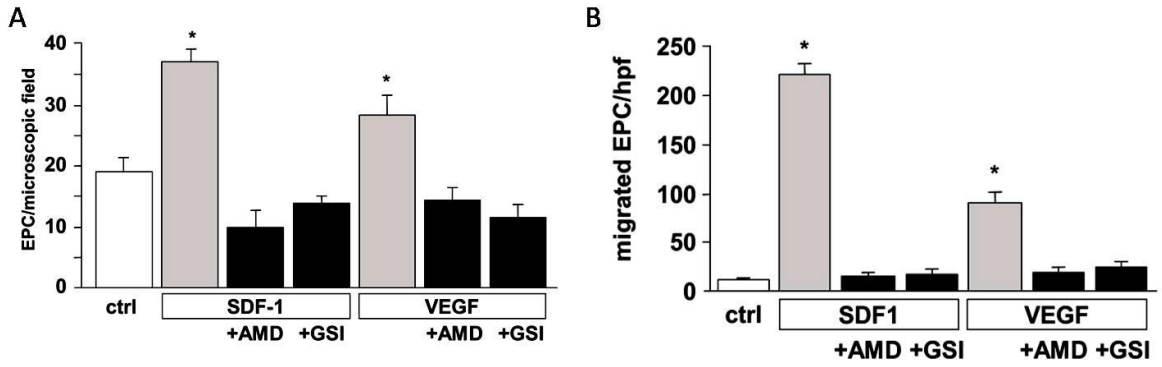


Figure 10. Stimulation with SDF-1 and VEGF increases EPC adhesion and migration. A, SDF-1 and VEGF enchanced EPC adhesion to ECs, which was blocked by AMD and GSI. B, SDF-1 and VEGF enchanced EPC migration. EPC migratory activity was decreased by AMD and GSI.

5.6 Notch signaling is required for EPC vascular repair

To evaluate the role of Notch signaling in EPC homing and vascular repair *in vivo* we employed a mouse model of carotid artery re-endothelialization, in which EPC have been shown to home to the regenerating endothelium and perivascular space and to enhance re-endothelialization (Sorrentino et al., 2007; Brouchet et al., 2001). The common carotid artery was de-endothelialized by external electric injury (Fig. 11A), and nude mice were injected with PBS, human EPC, or EPC pretreated with GSI. To evaluate the role of CXCR4 signaling, some mice received EPC pretreated with AMD3100 and daily intraperitoneal injections of AMD3100.

The injured carotid treatment by EPC markedly enhanced carotid re-endothelialization compared to control treatment three days after vascular injury, which was completely abolished by inhibition of Notch or CXCR4 signaling in EPC (Fig. 11B). These findings demonstrate an essential role of Notch signaling in EPC dependent endothelial regeneration.

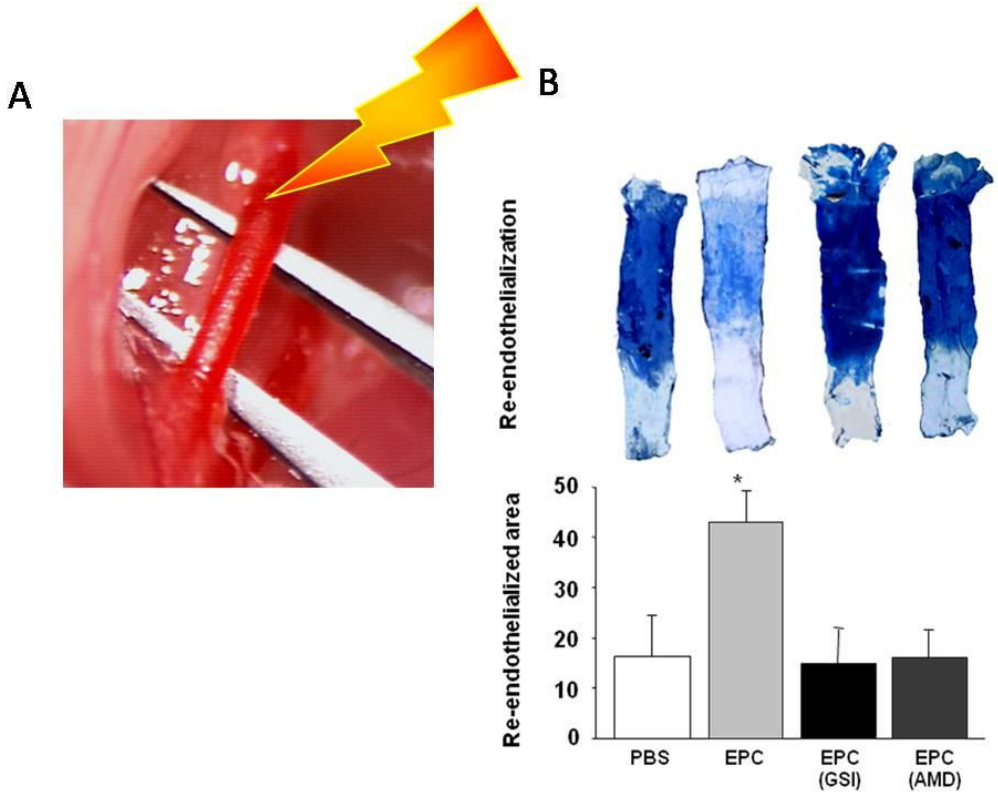


Figure 11. Notch signaling is important for EPC vascular repair. A, The common carotid artery was de-endothelialized by external electric injury. B, Re-endothelialized arteries at day 3 after carotid injury in nude mice. The injured carotid treatment by EPC markedly enhanced carotid re-endothelialization compared to control treatment three days after vascular injury, which was completely abolished by inhibition of Notch or CXCR4 signaling in EPC.

6 DISCUSSION

The role and mechanisms of Notch signaling in postnatal vascular regeneration are largely unknown. Our findings establish a critical role of Notch signaling in EPC differentiation and vascular repair by regulating CXCR4, the receptor for SDF-1.

6.1 EPC are regulated by Notch signaling

Human EPC expressed the Notch ligands *Dll1*, *Jag1*, the *Notch1* and *Notch2* receptor and the Notch target genes *Hey-1* and *Hey-2*. Blocking Notch signaling at different molecular levels, such as the γ-secretase-dependent receptor activation, or in the nucleus, abrogated Notch activation, Hey-1 and Hey-2 expression and decreased EPC numbers during EPC differentiation in culture. Importantly, Notch inhibition impaired angiogenic functions of EPC in vitro and re-endothelialization capacity in vivo.

Interestingly, Notch inhibition in EPC resulted in cells with dramatically changed morphology and indicated a likely change in their fate, most likely towards macrophage lineage.

Our data further demonstrate a critical role for the Notch ligand Dll1 in human EPC. We found that knockdown of Dll1 by siRNA recapitulates the effects of Notch inhibition. This suggests that Notch signaling in EPC is initiated by Dll1-Notch interaction. Furthermore, we show that Notch activity in EPC is mediated by the canonical RBP-Jκ dependent pathway, since genetic ablation of *Rbp-jκ* severely impairs EPC differentiation in culture.

6.2 CXCR4 is a downstream effector of Notch signaling in EPC

Despite the well-established role of Notch signaling in cell differentiation and vascular development, few target genes are known that mediate these effects. We identify CXCR4 as an important downstream target of canonical Notch signaling in EPC. Notch signaling was required and sufficient to regulate CXCR4 levels, which determined responsiveness of EPC to SDF-1 stimulation in EPC differentiation, migration and adhesion to ECs. Blocking CXCR4 recapitulated the effects of Notch inhibition on pro-angiogenic functions both *in vitro* and *in vivo*. These results demonstrate that CXCR4 is functionally downstream of Notch. Our findings thus confirm and significantly extend a previous report showing a requirement for Notch signaling in CXCR4 regulation in an embryonic stem (ES) cell differentiation system recapitulating early vascular development in vitro (Yurugi-Kobayashi et al., 2006).

The significance of CXCR4 regulation in EPC was further underscored by our findings that CXCR4 inhibition also blocked the pro-angiogenic effects of VEGF, which is consistent with

previous findings and suggests that CXCR4 signaling is an important mediator downstream of VEGF (Walter et al., 2005; Grunewald et al., 2006). This would also explain our finding of impaired VEGF responsiveness of EPC after Notch inhibition.

Interestingly, SDF-1, which is constitutively secreted by EPC and acts as an autocrine growth factor (Urbich et al., 2005; Yamaguchi et al., 2003) activated protein kinase Akt, which was blocked by Notch and CXCR4 inhibition. The Akt pathway has critical functions in cell migration, cell cycle regulation and survival in many cell types including ECs and EPC. Recently, regulation of Akt activity by Notch was demonstrated in various cell types (Liu et al., 2006; Ciofani and Zúñiga-Pflücker, 2005). Regulation of CXCR4 might thus provide a link between Notch and Akt signaling.

CXCR4 stimulation by SDF-1 is classically activated by the JAK/STAT signaling pathway (Vila-Coro et al., 1999) and is intimately involved in angiogenic and migratory processes (Zhang et al., 2001). Thus, we investigated whether the CXCR4-linked JAK-2 signaling pathway is dysregulated in EPC after Notch signaling inhibition. Indeed, we found that blocking Notch signaling with DAPT treatment reduced JAK-2 phosphorylation levels as well as the responsiveness to SDF-1. Recently, one group has also provided the evidence for crosstalk between two signal transduction pathways - Notch-Hes and JAK-STAT in the developing central nervous system (Kamakura et al., 2004).

CXCR4, which is expressed on diverse cell types, such as hematopoietic and vascular precursors, lymphoid and endothelial cells, has key functions in vascular development and neo-angiogenesis as a principal mediator of cellular trafficking and engraftment. During vascular development CXCR4 is expressed in developing arteries, but not veins, in an organ specific manner where it is thought to contribute to vascular branching and stabilize endothelial-mural interactions, while loss of endothelial CXCR4 causes defects in arterial remodeling (Yurugi-Kobayashi et al., 2006). This is reminiscent of the expression and role of Notch in arterial development and suggests that, in certain organs, Notch regulates arterial development by regulating endothelial CXCR4. During adult neovascularization CXCR4 is a key regulator of EPC mobilization and cellular retention at sites of ischemia or vascular injury, thus regulating the spatial distribution of cells (Petit et al., 2007). The effects of Notch signaling, on the other hand, have so far been ascribed to predominantly local regulation of cell fate decisions and cellular differentiation in situ (Artavanis-Tsakonas et al., 1999). Our findings suggest that Notch signaling not only guides cell fate locally but, by regulating CXCR4, also influences cell recruitment to specific sites, which might serve as an additional mode by which Notch regulates progenitor cell numbers.

Together, our findings suggest a novel role for Notch signaling in coordinating EPC differentiation and trafficking.

7 MATERIALS AND METHODS

7.1 Materials

7.1.1 Primers

Primers were synthesized by MWG Biotech AG and Sigma.

Primers used for semiquantitative RT-PCR:

Name	Product size	Sequence
DLL1 left	495 bp	TTG CTG TGT CAG GTC TGG AG
DLL1 right		ACA CAC GAA GCG GTA GGA GT
DLL4 left	458 bp	GAC CAC TTC GGC CAC TAT GT
DLL4 right		CCA TCC TCC TGG TCC TTA CA
ENOS left	385 bp	TGC TGG CAT ACA GGA CTC AG
ENOS right		TAG GTC TTG GGG TTG TCA GG
HEY1 left	423 bp	CGA GGT GGA GAA GGA GAG TG
HEY1 right		TTG TTG AGA TGC GAA ACC AG
HEY2 left	413 bp	GAA CAA TTA CTC GGG GCA AA
HEY2 right		CGC AAG TGC TGA GAT GAG AC
JAG1 left	428 bp	GGG GTA TTC AGG ACC CAA CT
JAG1 right		TAA CCA AAT CCC GAC AGG AG
JAG2 left	481 bp	GCT CCT TTA CCC TCA TCG TG
JAG2 right		CCC CAG TTG GTC TCA CAG TT
NOTCH1 left	666 bp	GAC ATC ACG GAT CAT ATG GA
NOTCH1 right		CTC GCA TTG ACC ATT CAA AC
NOTCH2 left	512 bp	ATG ACT GCC CTA ACC ACA GG
NOTCH2 right		TTT TCC TGC ATG CTC ACA AG
NOTCH3 left	491 bp	CAG GTG AGA GCT GCA GTC AG
NOTCH3 right		AAT GTC CAC CTC GCA ATA GG
NOTCH4 left	500 bp	AGC CGA TAA AGA TGC CCA
NOTCH4 right		ACC ACA GTC AAG TTG AGG
18SrRNA left	510 bp	CCT GCG GCT TAA TTT GAC TC
18SrRNA right		GGC CTC ACT AAA CCA TCC AA

***Primers used for genotyping the mice*:**

Name	Sequence 5' to 3'
Melta38	ATC CCT GGG TCT TTG AAG AAG
LacZ1/Dll1 KO	CAA ATT CAG ACG GCA AAC
RBPJFloxPrim1	GAA GGT CGG TTG ACA CCA GAT AGC
RBPJFloxPrim2	GCA ATC CAT CTT GTT CAA TGG CC
RBPJFloxPrim3	GTT CTT AAC CTG TTG GTC GGA ACC
RBPJFloxPrim4	GCT TGA GGC TTG ATG TTC TGT ATT GC

Buffers and solutions

Name	pH	Composition
TE	8	10 mM Tris-HCl, 1 mM EDTA
Tail buffer	8	0.1 M Tris-HCl, 5 mM EDTA, 0.5% SDS, 200 µg/ml proteinase K
10x PCR reaction buffer	8.8	500 mM KCl, 100 mM Tris-HCl, 0,1% Triton
PBS	7.4	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4
TAE	8	40 M Tris-Ac, 1 mM EDTA
Blocking solution		5% goat serum, 0.2% Triton X-100 in PBS
10X Tris buffered saline (TBS)	7.6	1 liter: 24.2 g Tris base, 80 g NaCl
TBST		1X TBS, 0.1% Tween-20
Tris-HCl	6.8	121.1g Tris base in 800ml H2O, adjust pH to 6.8 with HCl (32%)
Tris-HCl	8.8	121.1g Tris base in 800ml H2O, adjust pH to 8.8 with HCl (32%)
Lysis buffer		PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1´ complete inhibitor cocktail
Laemmli sample buffer		50% glycerol, 0.16 M Tris-HCl pH 6.8, 5% 2-Mercaptoethanol, 2% SDS, 0.01% Bromphenol blue
Electrode buffer	8.3	25 mM Tris-HCl, 192 mM glycine, 0,1% SDS
Transfer buffer		25 mM Tris base, 0.2 M glycine, 20% methanol
Stripping buffer	6.8	62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol
Fixing solution for X-gal staining		10 mL 1M MgCl2, 25 mL 0.2M EGTA, 400 µl 50% Glutaraldehyde fill in until 100 ml with PBS
washing buffer for X-gal staining		PBS supplemented with 2 mM $MgCl_2$
X-gal staining solution		1,25 ml X-gal (20 mg/ml), 0,5 ml 5 mM potassium ferrocyanide (K4Fe(CN)6-3H20, 0,5 ml 5 mM potassium ferricyanide (K3Fe(CN)6, 48 ml washing buffer

7.1.2 Primary cells

Human aortic endothelial cells (HAEC) and human umbilical venous cells (HUVEC) were purchased from Cambrex. Endothelial progenitor cells (EPC) were isolated from healthy donors.

7.1.3 Cell culture media

Endothelial growth media (EGM): EBM + EGM-BulletKit (bovine brain extract with heparin, hEGF, hydrocortisone, GA-1000 (gentamycin, amphotericin B)).

HAE and HUVE cells (ECs) were grown in EGM+10% FCS and EPC in EGM+20% FCS. EBM and EGM-BulletKit were obtained from Cambrex. Prior to use, fetal bovine serum (FCS, Biochrom) was heat inactivated by incubation at 56°C in a water bath for 30 min to destroy complement proteins. Freezing media was prepared by adding 10% DMSO (Sigma) and 10% FCS to the normal medium.

7.1.4 Antibodies

Primary and secondary antibodies used for Western blot analysis (WB), immunofluorescence (IF), Flow cytometry are listed below.

Primary antibodies:

Antibody	Host species	Source	Application, dilution
anti-**actin** (A2066)	rabbit, polyclonal	Sigma	WB, 1:5000
anti-**pAkt** (Ser473)	rabbit, monoclonal	Cell signaling	WB, 1:1000
anti-human **CD14**-Phycoerythrin	mouse IgG1	Immunotools	Flow cytometry
anti-human **CD31**-FITC	mouse IgG1	Immunotools	Flow cytometry
anti-human **CD64**-FITC	mouse IgG1	BD Pharmingen	Flow cytometry
anti-human **CD86**-FITC	mouse IgG1	BD Pharmingen	Flow cytometry
anti-**cleaved Notch1** (Val1744)	rabbit, polyclonal	Cell signaling	WB, 1:1000, IF 1:150
anti-**CXCR4** (ab2074)	rabbit, polyclonal	Abcam	WB, 1:1000
Anti-human **CXCR4**-Phycoerythrin	mouse IgG2A	R&D systems	Flow cytometry
DiI-Ac-LDL		Tebu-bio	IF, 1:100
anti-**Dll1** (H-265)	rabbit, polyclonal	Santa Cruz	WB, 1:1000, IF, 1:100

anti-**Dll4** (H-70)	rabbit, polyclonal	Santa Cruz	IF, 1:100
Biotinylated Anti-mouse **Ephrin-B2**	goat IgG	R&D systems	IF, 1:100
Biotinylated Anti-mouse **EphB4**	goat IgG	R&D systems	IF, 1:100
anti-**Hes-1**	rabbit, polyclonal	Tetsuo Sudo, TORAY Corporation, Yokohama, Japan	IF, 1:100
Anti-**Hey 1/HRT1**	rabbit, polyclonal	Chemicon	IF, 1:100
biotinylated **Isolectin B4** (IB4)		Vector Laboratories	IF, 1:100
Isotype control-FITC	mouse IgG1	Miltenyi Biotec	Flow cytometry
Isotype control-Phycoerythrin	mouse IgG2A	Miltenyi Biotec	Flow cytometry
Isotype control-Phycoerythrin	mouse IgG1	Miltenyi Biotec	Flow cytometry
Isotype control-Phycoerythrin	mouse IgG2B	Miltenyi Biotec	Flow cytometry
Isotype control-Phycoerythrin	rabbit IgG1	Santa Cruz	Flow cytometry
anti-**pJak2** (Tyr1007/1008)	rabbit, polyclonal	Cell signaling	WB, 1:1000
Notch1 (bTan)	rat, monoclonal	Developmental Studies Hybridoma Bank, Iowa	WB, 1:300 , IF, 1:10
anti-mouse **RBPjκ**	rat, monoclonal	Institute of Immunology Co., LTD, Japan	WB, 1:500
Anti-Actin, **α-Smooth Muscle**-Cy3	mouse, monoclonal	Sigma	IF, 1:300
Anti-Actin, **α-Smooth Muscle**–FITC	mouse, monoclonal	Sigma	IF, 1:300
Ulex europaeus agglutinin, **UEA-I**, FITC conjugate		Sigma	IF, 1:50
Anti-human **VE-Cadherin**-Phycoerythrin	mouse IgG2B	R&D systems	Flow cytometry
anti-**VEGF** (147)	rabbit, polyclonal	Santa Cruz	IF, 1:100

Anti-human **VEGF R2/KDR**-Phycoerythrin	mouse IgG1	R&D systems	Flow cytometry
anti-**Von Willebrand Factor (vWF)**	rabbit, polyclonal	Dako	Flow cytometry
Wheat Germ Agglutinin (WGA)- Rhodamine		Vector Laboratories	IF, 1:750

Secondary antibodies:

Antibody	**Host species**	**Source**	**Application, dilution**
Avidin D: FITC		Linaris	IF, 1:150
Avidin D: Texas Red		Linaris	IF, 1:150
anti-biotin HRP		R&D sytems	WB, 1:500
anti-rabbit HRP	donkey	Santa Cruz	WB, 1:4000
anti-rat HRP	donkey	Santa Cruz	WB, 1:4000
anti-rabbit Alexa Fluor® 488	goat	Molecular Probes	IF, 1:150

7.1.5 Data bases

In the course of these two projects I used the following data bases:

Ensembl genome browser (http://www.ensembl.org)

Primer3 Input 0.4.0 (http://frodo.wi.mit.edu)

National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov)

7.1.6 Computer programs

In the course of these two projects I used the following computer programs:

Text processing Word 2003 (Microsoft)

Image processing Photoshop CS3 (Adobe)

Online search Internet Explorer (Microsoft)

7.2 Methods

7.2.1 Mice

Dll1$^{lacZ/+}$ (Hrabe de Angelis et al., 1997) heterozygous mice were bread on an isogenic 129s1/SvImJ backround. *NMRI*$^{nu/nu}$ and *RBPjκ*$^{flox/flox}$ (Tanigaki et al., 2002) mice were kept on homozygous genetic background. Non-transgenic littermates served as controls where appropriate.

7.2.2 Methods of nucleic acid biochemistry

DNA isolation from tail biopsies

To isolate DNA from mice tail biopsies the tail clips were taken at two weeks of age and were incubated overnight in 300 µl tail buffer at 56°C. Next day the equal volume of phenol/chloroform (Applichem) was added and then briefly suspended with vortex. The samples were centrifuged for 15 min at full speed. The supernatant was taken off slowly and transferred to a fresh tube. Genomic DNA in the supernatant was precipitated by addition of 450 µl absolute ethanol (JT Baker). After removing the supernatant the DNA pellet at the bottom of the tube was washed with 1000 µl of 70% EtOH. It was then centrifuged at 7500g for 5 minutes. The supernatant was removed and the pellet was reconstituted in 100 µl TE buffer. Incubation of the DNA for 1-2 h at 56°C inactivated DNases and completely dissolved the DNA. Genomic DNA solution was stored at -20°C.

Genotyping by PCR

All reactions were performed in thin-walled PCR tubes and were run in PCR cyclers (Biometra).

Dll1lacZ-*PCR*

PCR program

1x	94°C	5 min
	94°C	30 sec
40x	53°C	30 sec
	72°C	30 sec

PCR reaction set up:

32.5 µl ddH_20
5 µl 10 x PCR reaction buffer
5 µl 15 mM $MgCl_2$
2.5 µl DMSO
1 µl Melta38 (see 2.1.1)
1 µl LacZ/Dll1Ko (see 2.1.1)
1 µl dNTPs (10 mM)
1 µl Taq polymerase

1 µl genomic DNA

After amplification a 580 bp PCR product indicated the *Dll1*^{lacZ} allele.

RBPjκ$^{flox/flox}$-*PCR*

PCR program			PCR reaction set up
1x	94°C	3 min	9.5 µl ddH_20
	94°C	30 sec	2.5 µl 10 x PCR buffer (Qiagen)
35x	60°C	30 sec	0.5 µl 10 mM dNTPs (Invitrogen)
	72°C	90 sec	1.5 µl 25 mM $MgCl_2$
			1 µl RBPJFloxPrim1 (see 2.1.1)
			1 µl RBPJFloxPrim2 (see 2.1.1)
			1 µl RBPJFloxPrim3 (see 2.1.1)
			1 µl RBPJFloxPrim4 (see 2.1.1)
			5 µl Q-solution (Qiagen)
			1 µl Taq polymerase
			1 µl genomic DNA

A band for the wildtype allele is at 500 bp, and for mutant allele at 600 bp.

Isolation of RNA

1000 µl Trizol (PeqLab) was added on to the cells and then briefly suspended with a pipette. The suspension was left to stand for 5 minutes at room temperature. 200 µl chloroform was added to the mixture and vortexed for 15 seconds, then left again to stand at room temperature for 3 minutes. The samples were centrifuged at 11 900g for 15 minutes and the aqueous phase was transferred to a fresh tube and 250 µl of isopropyl alcohol (JT Baker) was added and left to incubate for 10 minutes at room temperature. The mixture was centrifuged at 11 900g for 15 minutes. After removing the supernatant the RNA pellet at the bottom of the tube was washed with 1000 µl of 70% ethanol. It was then centrifuged at 7500g for 5 minutes. The supernatant was removed and the pellet was reconstituted in 12 µl of ddH_20. RNA concentration and quality was measured by spectrophotometry at 260 nm. Integrity of the RNA was checked on a gel. RNA samples were stored at -80°C until use.

Reverse transcription-PCR and PCR

For the RT-PCR a standard protocol was used.

Reverse transcription:

Master Mix:

5µl M-MLV RT 5x reaction buffer (Promega)

1.25µl 10mM dNTPs (Invitrogen)

0.25µl RNase OUT (Invitrogen)

1µl M-MLV reverse transcriptase (Promega)

4 µg total RNA is transferred into a 0.2 ml PCR tube and the volume is adjusted to 11 µl with ddH_20. 1µl of $oligo(dT)_{18}$ primer (MWG) was added and the mixture was incubated at 70°C for 5 min, followed by immediate cooling on ice. Next 14 µl of the Master Mix is added to the sample and incubated at 42°C for 60 min. Then the reaction is stopped by heating at 70°C for 15 min to inactivate M-MLV reverse transcriptase.

PCR program

1x 95°C 5 min
95°C 60 sec
60°C 60 sec
72°C 90 sec

PCR reaction set up

2.5µl 10 x PCR buffer (Qiagen)
1.5µl 25mM $MgCl_2$
0.5µl 10mM dNTPs
1.0µl Primer left
1.0µl Primer right
5.0µl Q-Solution (Qiagen)
11.3µl ddH_20
0.2µl Taq polymerase (Qiagen)
2.0µl cDNA

The amplification products were detected by electrophoresing 2 µl of product through a 1.0% agarose gel in TAE buffer.

7.2.3 Methods of protein biochemistry

Isolation of protein

Cells were washed once with PBS and all excess fluid was removed carefully. Per 6well dish was added 100 µl 1x lysis buffer. Cells were scraped off and transferred to a new tube. The mix was incubated for 30 minutes on ice and then centrifuged for 10 minutes at full speed at 4°C. The supernatant was transferred to a fresh tube. Protein samples were stored at -80°C until use. Protein concentration was determined using the Bradford method.

SDS-PAGE

Preparation of 10% resolving SDS-polyacrylamide gel:

4.0 ml H_20

2.5 ml 1.5M Tris (pH 8.8)

3.3 ml Acrylamid/Bisacrylamid (30:1)

0.1 ml 10% SDS

0.1 ml 10% ammonium persulfate

0.004 ml TEMED

Use approximately 8 ml resolving gel, pipette 1 ml H_2O on it until the gel has polymerized.

Preparation of 5% stacking gel:

3.4 ml H_20

0.63 ml 1.0 M Tris (pH 6.8)

0.83 ml Acrylamid/Bisacrylamid (30:1)

0.05 ml 10% SDS

0.05 ml 10% ammonium persulfate

0.005 ml TEMED

Remove the water from the resolving gel, add 4 ml of stacking gel and insert a comb.

Samples were boiled in Laemmli sample buffer for 5 min, cooled on ice and loaded on the gel together with protein ladder (Biorad). Proteins were resolved in an electrophoresis chamber of the "SE 260 Mini-Vertical Unit" (Amersham) at 115V ~1.5 hrs in electrode buffer. After that the gels were transferred to a membrane.

Western blot

The SDS-polyacrylamide gel was transferred to a nitrocellulose membrane (Roth) by wet tank blotting using the "TE 22 Mini Tank Transfer Unit" (Amersham). For this purpose the gel, membrane, filter paper and fiber pads were soaked in transfer buffer for 5 min. Next the fiber pad, then the filter paper were placed on the anode. The nitrocellulose membrane (Roth) was placed on the filter paper. Then the gel was placed onto the nitrocellulose membrane and covered with filter paper and fiber pad soaked with transfer buffer. Air bubbles were squeezed out using a 15 ml falcon. A transfer to nitrocellulose membrane was performed at 360 mA for 1h 50 min.

After transfer the membrane was washed with 10 ml TBST for 5 minutes at RT and incubated shaking for 60 min in 5% milk powder (Bio-Rad) in TBST to block unspecific binding sites

for antibodies. Subsequently, the membrane was incubated with the primary antibody solution diluted in 5% milk in TBST overnight at 4°C in. Next day the blots were washed three times with TBST for 5 min and incubated at room temperature with the secondary antibody diluted in 5% milk in TBST for 1 hr at RT. Afterwards the blots were washed again three times with TBST for 5 min.

For the immunodetection Western lightning-ECL (Perkin Elmer) was used. Detection solutions A and B were mixed in a ratio of 1:1, for each blot 2000 µl solution was used. Excess washing solution was removed from the blots by putting the edges of the blot on a filter paper. The blot was placed protein side up into a dish and the detection solution was pipetted onto it and incubated for 1 min at room temperature. Then excess liquid was removed as described above and the blot was placed in an x-ray film cassette. In the dark room a sheet of film (Kodak) was placed on top of the membrane for 15 sec to 20 min, depending on the intensity of the expected signal. After development of the first film it was estimated if a second film needs to be exposed and for how long this would be necessary.

Stripping of Western Blots

In order to reuse the Western blots they were stripped, removing the primary and secondary antibodies. Thus they were submerged in stripping buffer shaking for 60 min at 60°C. Then the membrane was washed three times for 10 min in TBST at room temperature with large volumes of solution. After that the membrane was blocked for 60 min in 5% milk powder in TBST and incubated with the primary antibody as described above.

7.2.4 Methods of mouse surgery

Hindlimb Ischemia Model

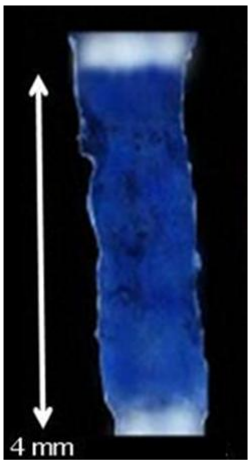
Mutant *Dll1*$^{lacZ/+}$ mice (Hrabe de Angelis et al., 1997) were bread on an isogenic 129S1/SvImJ background, non-transgenic littermates served as controls. Briefly, mice were anesthetized by intraperitoneal injection of a mixture of ketamine (2 mg/kg body weight, Pfizer) and xylazine (13 mg/kg body weight, Bayer) and the femoral artery was ligated distal to the origin of the deep femoral artery and proximal to the popliteal artery. Blood flow measurements in mouse feet were performed on 37°C heated pads before and immediately after surgery, and on post-operative days 3, 7, 14, 21 and 28 using a laser Doppler perfusion imager (PIM II, Perimed, Sweden). Perfusion was expressed as the ratio of ligated-to-non-ligated side. For induction of severe ischemia the right femoral artery was ligated proximal to the origin of the deep femoral artery (Ziegelhoeffer et al., 2004).

Perfusion fixation and tissue sampling

To analyze collateral arteries from the deep femoral artery, which follow a constant course on the surface of the adductor muscles, mice were perfused with 0.1% adenosine (Sigma), in situ fixed with 4% PFA (Sigma), and semimembranous and gastrocnemic muscles were excised, cryoprotected in sucrose, and embedded in OCT compound (Tissue-Tek; Ziegelhoeffer et al., 2004). For perfusion studies mice were also perfused with a 5 mg/ml FITC-dextran solution ($2x10^6$ avg. weight, Sigma).

In vivo reendothelialization assay

Male *NMRI*$^{nu/nu}$ athymic nude mice, aged 7-10 weeks old were used to allow injection of human EPC. EPC induced endothelial regeneration was analyzed in a mouse model of electric carotid artery injury that allows accurate quantification of reendothelialization (Brouchet et al., 2001). Animals were anesthetized with ketamine (2 mg/kg) and xylazine (13 mg/kg). The left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimeter of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery.



EPC ($5x10^5$ cells) were resuspended in 100 µl of prewarmed PBS (37°C) and transplanted 3 hours after carotid injury via tail vein injection with a 27-gauge needle. The same volume of PBS was injected into placebo mice. Three days after carotid injury, endothelial regeneration was evaluated by staining denuded areas with 50 µl of solution containing 5% Evans blue dye via tail vein injection 10 minutes before euthanization, followed by fixation with a perfusion of 4% paraformaldehyde for 5 minutes (Lindner et al., 1993). Blood, saline, and fixative were removed through an incision in the right atrium. The left common carotid artery was dissected

with an adjacent portion of the aortic arch and carotid bifurcation. The artery was then opened longitudinally and the reendothelialized area was calculated as difference between the blue-stained area and the total injured area by using the Axiovision Rel 4.4 software and a Zeiss Axiovert 200 microscope (Zeiss). Of note, this model has been shown to allow accurate quantification of reendothelialization (Brouchet et al., 2001). The total area between the distal bifurcation and the proximal aortic arch and blue stained areas of deendothelialization were measured and the ratios calculated.

7.2.5 Methods of tissue histochemistry

Tissue analysis

Histomorphometry of collateral arteries was performed on H&E stained semimembranous sections using the Axiovision Rel 4.4 software and a Zeiss Axiovert 200 microscope (Zeiss). The arterial wall area was calculated by subtracting the lumen area from the outer circumference of the tunica media.

Laser scanning confocal microscopy after immunostaining was carried out with a Leica TCS SP2 AOBS (Leica Microsystems).

Capillary density

7 μm-thick cryosections of the midportion of the semimembranosus muscle from the ischemic hindlimb were obtained capillary density, gastrocnemic muscle sections were stained with IB4 and rhodamine-coupled wheat germ agglutinin (WGA), FITC-conjugated Avidin D and nuclei counter-stained with 4`,6-diamino-2-phenylindole dilactate (DAPI, Sigma). At least 12 sections per muscle were analyzed by fluorescence microscopy and results expressed as the ratio of IB4 endothelial cells to WGA muscle fibers. Capillary density was quantified on gastrocnemius muscle frozen sections by fluorescence microscopy.

Immunofluorescence staining

For immunofluorescence analysis, cryosections were fixed in 4% PFA for 10 min at room temperature. After three washes with PBS, the sections were blocked with blocking solution for 1h at RT. Then the sections were incubated with primary antibodies diluted with blocking solution overnight at 4°C. Next day the slides were washed three times (each washing was for 10 min) with PBS, incubated with fluorescent secondary antibodies for 1h at room temperature. Sections were again rinsed with PBS three times and mounted with fluorescent

mounting medium (Dako) for fluorescence microscopic analysis. Pictures were processed and assembled using Photoshop CS3.

LacZ staining

For LacZ staining, muscles were fixed in fixing solution at 4°C for 2 hours and washed with PBS, followed by overnight incubation in 30% sucrose (Roth) and then 50% sucrose in PBS. The muscles were mounted in OCT compound at –80°C and cryosectioned. The slides were refixed in PBS/0.2% glutaraldehyde for 10 min at room temperature and washed with LacZ washing buffer. Staining was done at 37°C for 4-8 hours in the LacZ staining solution, washed 3 times in PBS and then dehydrated dehydrated in ascending alcohol solutions (70% 1 minute, 80%, 90%, 95%, 100% 2 minute) and cleared with xylene. Next the slides were either mounted in vitro-clud (R. Langenbrinck) or immunohistochemically stained.

Hematoxylin and eosin staining

For eosin counterstain the LacZ-stained slides were after washing with PBS, dipped in 70% ethanol and continued with step 10.

1. Immerse cryosections in 70% alcohol for 1 minute
2. Rinse with ddH_20
3. Mayer's Hematoxylin solution (Sigma) for 3 minute
4. Rinse with tap water
5. HCl 0,5% in Ethanol
6. Rinse with tap water for 5 minute
7. Rinse with tap water for 5 minute
8. 80% Ethanol for 15 seconds
9. 96% Ethanol for 15 seconds
10. Immerse in Eosin Y solution (Sigma) for 3 seconds
11. 90% Ethanol 2 min
12. 96% Ethanol 2 min
13. 100% Ethanol 2 min
14. 100% Ethanol 2 min
15. Clear with Xylol for 10 minute

Coverslips were mounted onto a labeled glass slide with vitro-clud.

7.2.6 Methods of cell culture assays

Cell culture

Cells were grown 37°C and 5% CO_2 in cell culture dishes.

Endothelial cells were always grown on gelatin-coated and EPC on fibronectin-coated cell culture dishes. Endothelial cells were passaged with standard trypsinization. For long term storage cells were frozen as cell suspension with freezing medium in a cryovial. Rapid thawing of the cell cryostocks was achieved with prewarmed cell culture medium. Endothelial cells and EPC were treated with following human growth factors: 100ng/ml of VEGF165, 100 ng/ml FGF-2 and 500 ng/ml SDF-1. All growth factors were purchased from Tebu-bio.

EPC culture

Total peripheral blood mononuclear cells (hPBMNCs) were isolated from blood of healthy humans by density-gradient centrifugation with lymphocyte separation medium (LSM 1077, PAA Laboratories, Germany). $10^6/cm^2$ hPBMNCs were plated on culture dishes coated with human fibronectin (Roche) and maintained in endothelial basal medium EGM-20%FCS. After 4 days in culture, nonadherent cells were removed by washing twice with PBS and new media was applied, and the culture was maintained until day 7 before cells were trypsinized for *in vitro* and *in vivo* experiments.

EPC characterization

For immunocytochemistry cells were grown on fibronectin-coated coverslips. Fluorescent chemical detection of human EPC was performed on attached hPBMNCs after 7 days in culture. Direct fluorescent staining was used to detect dual binding of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled acetylated low density lipoprotein (acLDL) and FITC-labeled *Ulex europaeus* agglutinin (UEA)-1. Cells were first incubated with acLDL at 37°C for 1,5 hours and later fixed with 4% paraformaldehyde for 10 min. After three washes with PBS, blocking with PBS/3%BSA for 45 minute at RT. After blocking the cells were reacted with UEA-1 for 1h (Asahara et al., 1997). Then the samples were analyzed using a Zeiss Axiovert 200 microscope and the Axiovision Rel 4.4 software. Cells demonstrating double-positive fluorescence were identified as EPC.

Isolation of mouse bone marrow cells

Bone marrow cells were obtained by flushing the mice tibias and femurs. Low-density mononuclear cells were isolated by density-gradient centrifugation with lymphocyte

separation medium (LSM 1077, PAA Laboratories, Germany). Immediately after isolation, $5x10^6$ bone marrow–derived mononuclear cells were also plated on 35-mm cell culture dishes coated with fibronectin (Sigma) and maintained in EGM-20%FCS. After 4 days in culture, nonadherent cells were removed and adherent cells were cultured until day 7 before cells were trypsinized for in vitro experiments.

siRNA transfection

EC:

One day before transfection 120 000 HAEC or HUVEC cells were seeded in 6-well dishes. Transfection was performed using solutions A and B.

Solution A: Predesigned annealed siRNA (10nM, Ambion) was mixed with 100 µl OptiMEM.

Solution B: 5 µl of Oligofectamine was mixed with 100 µl OptiMEM.

EPC:

Mononuclear cells were seeded in 6well dishes and on day 5 the transfection to attached cells was performed using solutions A and B.

Solution A: Predesigned annealed siRNA (600nM) was mixed with 250 µl OptiMEM.

Solution B: 7 µl of Oligofectamine was mixed with 250 µl OptiMEM.

Solutions A nad B were incubated for 10 min at room temperature, mixed and the mixture further incubated for 30 minutes at room temperature. At the same time the cells were washed twice with OptiMEM and 800 µl (EC) and 500 µl (EPC) OptiMEM were left on to the cells. The siRNA-mix was added dropwise to the cells and incubated for 4 hours at 37°C. Then 500 µl (EC) and 1000 µl (EPC) of EGM-30% FCS was added and incubated overnight. Next day the medium was changed to EGM containing 10% (EC) or 20% (EPC) FCS. At least two different siRNA sequences were evaluated for suppression of target gene expression by immunoblots, and transfection efficiency was determined to be >95%. The following siRNAs were used: Dll1 (#133773, #133774); ephrin-B2 (#14087, #14268). Scrambled siRNA (Ambion) was used as a control.

In Vitro Branching Morphogenesis and Network Formation

HAEC were serum starved in 1% FCS overnight, seeded on matrigel (BD Biosciences) at a density of $1x10^4$ cells/well and stimulated with or without VEGF and FGF-2 (100 ng/ml) for 24 h. In some experiments, matrigel was supplemented with 0.2 or 1.0 ug/ml of EphB4-Fc

(R&D Systems), in which case 1.0 ug/ml IgG-Fc (Dianova) served as control. Three random microscopic fields per well were analyzed using a Zeiss Axiovert 200 microscope and the Axiovision Rel 4.4 software (Zeiss). Branch points were defined as EC intersections of at least three tubes, total network length was defined as all tubes connected by branch points.

Immunocytochemistry for mouse EPC

The cells were rinsed twice with PBS and then incubated with acLDL at 37°C for 1,5 hrs. Again the cells were rinsed twice with PBS and then fixed with 4% PFA for 10 min at RT. After three washes with PBS, cells were blocked with PBS/3%BSA for 45 min at RT. Thereafter washed once with PBS and incubated with primary antibody against IB4 for 1h at RT and, after three washes with PBS, the cells were incubated with the fluorochrome-conjugated secondary antibody FITC-Avidin D for 1h at RT. After washing, the coverslips were mounted with fluorescent mounting medium (Dako) for fluorescence microscopic analysis. Pictures were processed and assembled using Photoshop CS3.

Notch signaling modulation in EC and EPC

Notch signaling was activated or inhibited in cultured cells.

Notch signaling was blocked using 3 different approaches:

- Pharmacologically using the specific and potent γ-secretase inhibitor (GSI), N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) (Calbiochem), which was added daily to the culture starting at day 0 to a final concentration of 1 μM in EC and 3 μM in EPC . γ-secretase inhibitor was dissolved in DMSO resulting in a 1 mM or 3 mM stock solution. The stock solution was aliquoted and kept at -20°C until usage. DAPT was added every day.
- An adenoviral dominant negative mutant dom. neg. AdRBP-j (R218H) was used to inhibit the nuclear transcriptional effector of Notch signaling RBP-j.
- An adenoviral Cre was used to conditionally ablate *RBPjk* expression in $RBPjk^{flox/flox}$ mice.

For Notch activation the cells were transduced with an adenoviral construct encoding the activated form of Notch, Notch intracellular domain (NICD).

CXCR4 modulation in EPC

CXCR4 signaling was blocked by preincubating cells with 5 μg/ml of CXCR4 antagonist AMD3100 for 24 hours.

EPC migration

EPC migration was evaluated using a modified Boyden chamber assay (BD Biosciences). Cultured EPC ($5x10^4$) were starved overnight in EBM and plated to fibronectin-coated Transwells (8-μm pore). Cell migration was assayed in the absence or presence of 100ng/ml VEGF and SDF-1 in the lower chamber in EBM-0%FCS. Cells were allowed to migrate for 16 hours. The filter was removed and the upper side of the filter containing the non-migrating cells was scraped with a rubber policeman. Migrated cells on the membrane surface were fixed with 4% PFA, nuclei stained with DAPI and counted in 5 HPF. Each experiment was performed in triplicate wells and was repeated 3 times.

EPC adhesion

EPC adhesion was analysed by EPC attachment to cultured human umbilical vein endothelial cells (HUVEC). A monolayer of HUVEC was prepared 48 h before the assay by plating 2 x 10^4 cells in each well of 24-well glass slides. 5 x 10^4 EPC that had been preincubated with DiI-AcLDL were seeded into chamber glas slides with confluent HUVEC and incubated overnight at 37°C. Cell migration was assayed in the absence or presence of 100ng/ml VEGF and 500ng/ml SDF-1. Non-attached cells were gently removed with PBS. Adherent EPC were fixed with 4% PFA and nuclei stained with DAPI in order to stain for HUVEC. Both EPC (red) and HUVEC (blue) were then analysed and counted in five random fields by fluorescence microscopy and percentage of EPC among total cells per random microscopic field was calculated.

Flow cytometry of EPC

Fluorescence-activated cell sorting (FACS) detection of EPC was performed after 7 days in culture. Adherent cells were washed twice with PBS, detached with accutase (PAA) followed by repeated gentle flushing through a pipette tip. 1-$2x10^6$ cells were resuspended with 100 µl of PBS/1%BSA and then incubated for 30 min at 4°C with primary antibody Von Willebrand Factor (vWF) or to phycoerythrin-conjugated monoclonal antibodies against CXCR4, KDR, VE-Cadherin, CD14 and FITC-conjugated antibody against CD31, CD64, CD86. Isotype-identical antibodies were served as negative controls. After staining the cells were washed and spun down for 15 min at 1200 rpm. Where appropriate the cells were then incubated with FITC-conjugated anti-rabbit secondary IgG, washed and fixed in 1% paraformaldehyde. All groups were studied in triplicates. Single- and 2-color flow cytometric analyses were performed by using a BD FACSCalibur flow cytometer (BD Biosciences). Data was evaluated

by Cellquest software (BD Biosciences). Histograms of cell number vs logarithmic fluorescence intensity were recorded for 15 000 cells per sample.

Viral transduction

All recombinant adenoviruses were generated by my colleague Diana Elligsen.

EC: After 2 days in culture, EC in 6well plate were transduced with an adenovirus for 4 hours in EBM-0% FCS. After transduction, EGM-2% FCS was added. Next day the cells were washed with PBS and got fresh EBM-1% FCS. 48 hours after viral transduction the cells were harvested for subsequent experiments.

EPC: After 5 days in culture, $2x10^6$ EPC were transduced with an adenovirus for 4 hours in OptiMEM (Invitrogen). After transduction, cells were washed with PBS and incubated with EPC media (EGM-20% FCS) for 48 hours before harvesting the cells for subsequent experiments.

Viruses used:

Name	**Particles per cell**	
	EC	**EPC**
AdNICDRev	3000	10 000
constitutive-active NICD	3000	15 000
dominant-negative RBP-J	3000	25 000
AdCre		20 000
AdGFP		10 000

AdNICDRev and AdGFP served as controls.

Statistics

All values are expressed as mean ± SD. Two treatment groups were compared by the unpaired Student's t test; one-way ANOVA was performed for serial analysis. The comparative incidence of limb amputation was evaluated by x^2 test. P values less than 0.05 were considered statistically significant.

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PUBLICATIONS

Papers

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ERKLÄRUNG ZUR DISSERTATION

Hierdurch erkläre ich, dass die Dissertation **The role of Notch signaling in postnatal neovascularization** selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

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