VEGF and NOTCH in blood vessels, an intricate and fascinating interplay

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VEGF and NOTCH in

BLOOD VESSELS,

an INTRICATE and

FASCINATING INTERPLAY

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DISSERTATION

to obtain the degree of Doctor at Maastricht University,
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in accordance with the decision of the Board of Deans,
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Chapter 1

General Introduction

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The NOTCH Signaling Pathway

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VEGF-signaling

The role of VEGF and NOTCH in Artery/Venous Differentiation of Endothelial Cells

VEGF and NOTCH in Endothelial Cell Sprouting

NOTCH in Vascular Smooth Muscle Cells (vSMC) Differentiation

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Chapter Outline

NOTCH Signaling Pathway in Vascular Development

The formation of a functional vascular network is essential for the growth and maintenance of tissues. During embryogenesis the earliest stages of vascular development occur when endothelial cell (ECs) precursors (angioblasts) migrate and differentiate into blood islands, which then fuse to form the primitive capillary plexus in a process termed vasculogenesis^{1,2}. The newly formed plexuses grow as a result of angiogenesis, i.e. vascular sprouting and tube formation by single ECs or by longitudinal division of existing capillaries, called intussusception. This process involves reorganization of the inter-endothelial cell junctions and central perforation of the bilayer, followed by interstitial pillar core formation and subsequent pericytes and myofibroblast invasion^{3,4}. During the process of arteriogenesis, the formed channel of endothelial cells is covered by multiple layers of pericytes or smooth muscle cells (SMCs) to respectively generate small or large vessels of the vascular system. A large number of intercellular signaling pathways are implicated in these processes. These pathways include the families of vascular endothelial growth factors (VEGF), transforming growth factor- β (TGF- β), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), angiopoietins and the ephrins. During the last decade several studies have made clear that the NOTCH pathway also plays an essential part in developmental neovascularization².

The NOTCH pathway comprises a highly conserved intercellular signaling mechanism that is expressed in all metazoans and controls cell fate by cell-cell interaction⁵. In vertebrates, components of the NOTCH signaling pathway are expressed in various organs including blood vessels. The importance of NOTCH signaling in vascular development has been underlined by the observation that mutations of NOTCH members can cause serious cardiovascular defects. Knockout mice of several components of the NOTCH pathway, such as *Notch1*, *Notch1* plus *Notch4* and *Jagged1* all resulted in embryonic lethality related to vascular defects^{6,7}. Mice homozygous for a single point mutation that prevents the intracellular processing of Notch1 resembles the null Notch1 phenotype, indicating that Notch1 signaling is essential for embryonic viability and proper vascular morphogenesis⁸. Also both *Delta-like 1 (Dll1)* deficient and *Notch2*-hypomorphic mice embryos presented with hemorrhage resulting from poor development of vascular vessels^{9,10}. *Dll4* mutant mice even exhibited embryonic lethal haploinsufficiency because of vascular remodeling defects¹¹⁻¹³.

In human, the Alagille syndrome, an autosomal dominant disorder characterized by developmental abnormalities, is caused by mutations of the NOTCH ligand JAGGED1¹⁴. The cardiac defects associated with Alagille syndrome include pulmonary outflow tract anomalies and tetralogy of Fallot. This congenital cardiac malformation predisposes to aortic valve calcification, which can impair blood flow

through the valve. A second human disease, CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is related to a mutation of the NOTCH-3 receptor¹⁵ and affects vascular smooth muscle cell (VSMC) development. CADASIL is the most common genetic disorder found in patients with stroke and vascular dementia¹⁶.

The NOTCH Signaling Pathway

The NOTCH pathway is an evolutionary highly conserved signaling system. Four different NOTCH receptors, NOTCH-1 to -4, and five ligands, DELTA-LIKE (DLL) -1, -3, -4 and JAGGED (JAG) -1, -2, have been identified in vertebrates. The NOTCH members are single-pass transmembrane proteins. NOTCH receptors are synthesized as single-chain precursors that, after glycosylation by protein o-fucosyl transferase (POFUT1) in the endoplasmic reticulum, are processed into non-covalently linked extracellular (NECD) and intracellular (NICD) domains in the trans-Golgi¹⁷⁻¹⁹. The NECD is characterized by 29–36 epidermal growth factor (EGF)-like repeats which mediate the interaction with the ligand, and three cysteine-rich NOTCH/LIN12 repeats that prevent ligand independent signaling¹⁷⁻¹⁹. The NICD is characterized by a RAM (RBP-Jk associated module), a motif containing a NLS (nuclear localization sequence) that facilitates translocation and constitutes the primary binding domain to RBP-Jk. The RAM motif is followed by six tandem ankyrin repeats (ANK), which are highly conserved among NOTCH proteins of various species and mediate protein-protein interaction. Downstream of the ANK repeats lies a glutamine-rich domain (OPA), followed by a C-terminal PEST (proline/glutamic acid/serine/threonine-rich) sequence which facilitates rapid turnover of the protein²⁰. The ligands, also referred to as DSL (Delta/Serrate/Lag-2) are characterized by an extracellular motif, which mediates the binding with the receptor, EGF-like repeats, and a cytoplasmatic domain containing a conserved C-terminal PDZ (postsynaptic density; discs large; zonula occludens-1) -binding motif¹⁸. The receptor-ligand interaction induces two proteolytic cleavages of the receptor. The first is mediated by extracellular proteases, known as A Disintegrin And Metalloprotease (ADAM), TNF- α Converting Enzyme (TACE) or kuzbanian. Subsequently, the γ -secretase complex mediates a second proteolytic cleavage that releases the NOTCH Intracellular Domain (NICD) from the membrane. Next, the NICD translocates to the nucleus, where it associates with the DNA binding protein RBP-Jk (also named CSL after mammal CBF1, *Drosophila* Su(H), and *Caenorhabditis elegans* LAG-1) and its co-activator Mastermind (MAML1) to initiate the transcription of its downstream targets, such as the basic Helix-Loop-Helix proteins Hairy/Enhancer of Split (HES) and Hairy Related Transcription factors (HRT, HERP, HEY)^{18,21-24}, which in turn regulate the transcription of downstream genes.

Figure 1 Schematic Representation of NOTCH signaling pathway

Mammals possess 4 NOTCH receptors (NOTCH1–4) and five ligands (Jagged1 and 2, and Delta-like1, 3, 4). NOTCH signaling is triggered upon receptor-ligand interaction, which induces two sequential proteolytic cleavages. The first cleavage, in the extracellular domain, is mediated by metalloproteinases of the ADAM family, and the second, within the membrane domain, is mediated by the γ -secretase complex. The second cleavage allows the release and translocation of the NOTCH intracellular domain (NICD) to the nucleus where it associates with the RBP-Jk co-transcription factor. The binding of NICD to RBP-Jk leads to transcriptional activation of the HES and HERP/HEY NOTCH target genes by displacement of a co-Repressor (CoR) and recruitment of the co-Activator, Mastermind Like protein (MAML1). NOTCH ligand and receptor domain compositions are also illustrated in the figure.

NOTCH Signaling in ECs, a Direct Mediator of VEGF Signaling

Activation of NOTCH signaling in the endothelium controls important processes such as arterial/venous differentiation and sprouting of vessels. Within this context the role of NOTCH signaling is intertwined with another essential regulator of vascular development; Vascular Endothelial Growth Factor (VEGF)^{25, 26}.

VEGF-signaling

The VEGF family members are secreted as dimeric glycoproteins of approximately 40 kDa. There are five characterized VEGF subgroups in mammals (VEGF-A through

VEGF-D, and placenta growth factor, (PlGF)). Homologues of VEGF have also been identified in the genome of the parapoxvirus Orf, VEGF-E, and in snake venom, VEGF-F. Alternative mRNA splicing of VEGF-A in human generates six different VEGF-A isoforms: VEGF-A₁₂₁, VEGF-A₁₆₅ and VEGF-A₁₈₉ being the most important, which are represented by VEGF-A₁₂₀, VEGF-A₁₆₄ and VEGF-A₁₈₈, respectively, in the mouse. The VEGF ligands display different interactions with the three VEGF receptor tyrosine kinases (RTK): (VEGFR-1/Flt1, VEGFR-2/KDR/Flk1 and VEGFR-3/Flt4)²⁷⁻²⁹. The activities of VEGF-A isoforms are dictated by their different ability to interact with the VEGF co-receptors, such as NPs (neuropilins) and HSPGs (heparin sulphate proteoglycans)³⁰. Heparan-sulfate proteoglycans and the neuropilins bind VEGFs but do not induce biological responses on their own in the absence of the tyrosine-kinase receptors²⁷. VEGF-A₁₂₁ lacks the heparin binding domain and is freely diffusible; whereas VEGF-A₁₈₉ is strongly bound to the extracellular matrix; VEGF-A₁₆₅ has intermediate properties. VEGF-A₁₆₅ is secreted, however a significant fraction remains bound to heparan sulphates of matrix proteins situated on the cell surface. The VEGF importance in regulating vascular development has been well established³¹⁻³³. VEGF-A haploinsufficiency is lethal in mouse embryos, presenting with a prominent vascular phenotype including a reduced number of blood vessels^{32, 33}. On the other hand, increased VEGF-A levels also lead to severe abnormalities in the heart and subsequently embryonic lethality in mice³⁴.

Among the VEGF receptors, VEGFR-1 and VEGFR-2 are expressed in vascular endothelium of both arterial and venous origin, whereas VEGFR-3 expression is restricted to lymphatic endothelium. VEGFR-1 is also abundant in macrophages/monocytes. VEGFR-1 and -2 are structurally similar, consisting of an extracellular domain with seven immunoglobulin Ig-like motifs, a single transmembrane domain, a juxtamembrane domain, a kinase domain split by a kinase insert, and a carboxyl terminus. The VEGF ligands display different interactions with the VEGF Receptors (Figure 2). VEGF-A and VEGF-F (a snake isoform) interact with both VEGFR-1 and VEGFR-2; VEGF-B and PlGF interact exclusively with VEGFR-1, VEGF-E (a viral isoform) binds only to VEGFR-2, whereas VEGF-C and VEGF-D interact with VEGFR-2 and R-3^{28,35}.

It is generally accepted that VEGFR-2 is the major mediator of the VEGF induced effects in endothelial cells, showing a stronger activation than VEGFR-1, and stimulating a variety of signaling pathways and biological responses in endothelial cells³⁶. VEGFR-2 deficient mice fail to form blood-island and show impaired vasculogenesis³⁷. VEGFR-1 is considered to be a kinase-impaired RTK, since autophosphorylation of its tyrosines is rather poorly induced. This poor kinase activity of VEGFR-1 is probably the result of an aspartic acid to asparagine mutation in the activation loop of the tyrosine kinase domain³⁸. The redundancy of the VEGFR-1 is illustrated by minor effect of the deletion of the tyrosine kinase domain on vascular development in mice³⁹. The observation that targeted mutation of PlGF,

as a specific VEGFR-1 ligand, does not affect embryonic vascular development further supports the redundancy of VEGFR-1 signaling in this context³¹. Moreover, VEGFR-1 deficient embryos show normal endothelial differentiation but an increased number of endothelial cells and disorganized primary blood vessels^{39,40}, likely resulting from excessive VEGF/VEGFR-2 signaling. These studies indicate VEGFR-1 as a negative regulator of endothelial proliferation. These observations together with the biochemical characteristic of its tyrosine kinase domain suggest that during embryogenesis, VEGFR-1 in ECs may serve as a decoy receptor, effectively reducing the availability of VEGF for the VEGFR-2 receptor, rather than as a signaling receptor.

It is worth to note that despite its secondary role in ECs, active VEGFR-1 is the sole VEGF receptor expressed on the surface of circulating monocytes⁴¹, where it mediates chemotaxis in response to VEGF or PlGF-1⁴².

Figure 2 Growth Factors and Receptors of the VEGF family

The three signaling tyrosine-kinase receptors of the VEGF family (VEGFR-1, VEGFR-2, and VEGFR-3), the accessory isoform specific receptors neuropilin-1 and neuropilin-2, and VEGF binding heparan-sulfate proteoglycans are displayed together with their major structural features. The different isoforms of the VEGF family members that bind to each of these cell membrane proteins are also shown (modified after Hoeben Pharmacological Reviews 2004).

The role of VEGF and NOTCH in artery/venous differentiation of endothelial cell

Although blood flow has for long been considered the major factor to regulate the differentiation of arteries and veins, recent studies have shown that genetic pre patterning, mainly mediated by the NOTCH pathway, play a primary role in inducing arterial-venous differentiation.

The importance of the NOTCH signaling pathway in regulating arterial differentiation was initially shown in Zebrafish^{43,44}. NOTCH signaling deficient embryos showed a poorly formed dorsal aorta and posterior cardinal vein. These embryos also showed loss of arterial marker expression, such as EphrinB2, and ectopic expression of venous markers in the arterial compartment. Reduction of VEGF-A signaling also resulted in loss of arterial marker in the dorsal aorta and ectopic expression of venous markers, whereas injection of VEGF-A mRNA induced the expression of the arterial marker EphrinB2 in the posterior cardinal vein⁴³. Moreover, induced expression of NOTCH-1 in VEGF-A-deficient embryos rescued the expression of EphrinB2, whereas injection of VEGF-A mRNA into NOTCH signaling deficient embryos was unable to restore arterial differentiation⁴³. These studies provide evidence that both NOTCH and VEGF are involved in a signaling cascade that is essential for arterial-venous differentiation in which NOTCH signaling acts downstream of the VEGF pathway.

The VEGF-A/NOTCH axis in regulating arterial endothelial differentiation has also been confirmed in mice. Vegf164 overexpression in cardiomyocytes and primary embryonic endothelial cells⁴⁵ leads to arterial differentiation with an increased EphrinB2/EphB4 ratio. Likewise in murine vasa nervorum, Schwann cell derived Vegfa also stimulates arterial markers^{46,47}. NOTCH activation induced arterial differentiation in mice as shown by loss and gain of function studies. Surviving *Dll4* heterozygous mice displayed a lack of arterial markers in their vasculature^{11-13,48}, as well as NOTCH signaling deficient mice such as the RBP-Jk and Hey1 and Hey2 double-mutants^{11-13,49,50}. Studies in cultured endothelial cells confirmed that DLL4-induced NOTCH signaling up-regulates EphrinB2 expression⁵¹. Furthermore, the overexpression of activated NOTCH-4 (NICD-4) in adult mice also induced the expression of EphrinB2 in the venous compartment resulting in arteriovenous malformation and this phenotype was reversible after repression of transgene expression⁵².

The close interaction of VEGF and NOTCH signaling in arterial differentiation has been defined in mammal cells. Liu et al.⁵³ showed that NOTCH-1 receptor and DLL4 ligand are induced by VEGFA in human arterial but not in venous endothelial cells. VEGF-A induced DLL4, NOTCH-4, EphrinB2 and downregulation of venous markers COUP-TFII and EphB4 expression was also described for mouse embryonic⁵⁴ and human bone-marrow derived mesenchymal stem cells⁵⁵. Also ablation of the venous marker and repressor of NOTCH signaling, COUP-TFII, in endothelial cells enabled veins to express NOTCH signaling components, acquiring an arterial phenotype, whereas COUP-TFII ectopic expression resulted in fusion of arteries and veins (AV-shunts) in transgenic mouse embryos⁵⁶.

As anticipated above, besides genetic factors, environmental factors such as hemodynamics and oxygen tension also intervene in defining artery and venous identity. Blood vessels preserve a certain degree of plasticity which allows them to

respond to external stimuli as fluid mechanic forces and lack of oxygen. Masumura et al.⁵⁷ have reported that shear stress regulates the arterial-venous specification of murine embryonic stem (ES) cells-derived ECs. They showed that EphrinB2 expression increased when these cells were exposed to shear stress, whereas EphB4 expression decreased. However, the prevention of the effects in the presence of VEGFR-2 and NOTCH signaling inhibitors showed that shear stress induced arterial-differentiation requires the VEGF-NOTCH signaling pathway. Similarly, low oxygen levels can trigger VEGF expression through the hypoxia inducible factor (HIF) signaling cascade⁵⁸⁻⁶¹. Moreover, promoter analysis revealed that *Hey1*, *Hey2* and *Dll4* are induced by HIF-1 alpha and NOTCH signaling⁶² and hypoxia-mediated Dll4 and Hey2 upregulation repressed COUP-TFII expression in endothelial progenitor cells (EPCs).

VEGF and NOTCH in Endothelial Cell Sprouting

During angiogenesis, new capillaries sprout from pre-existing blood vessels. In this process, specialized endothelial cells, called tip cells, react to a VEGF gradient^{63, 64} by migrating and extending filopodia. This response is highly localized as the stalk cells -that follow the tip cell- already respond differently to VEGF²⁶. Several recent studies have shown that NOTCH signaling plays an important role in regulating the formation and function of these tip cells. Endothelial tip cells present with high levels of VEGFR-2⁶⁵. In response to VEGF stimulation Dll4 expression is upregulated in these cells^{66, 67}. The tip cell expressing Dll4 interact with the neighbouring cells, thereby activating NOTCH signaling, which in turn prevents migration and filopodia extension that will functionally define these neighbouring cells as stalk cells. Thus, in the absence of NOTCH signaling, endothelial cells continue to form sprouts in response to VEGF resulting in more sprouts and subsequently, branches per blood vessel. This indeed has been observed in different model systems such as mouse retina and hindbrain^{66, 68-70}, zebrafish embryos^{71, 72}, and xenograft tumor models^{70, 73, 74}. Consistently, *Dll4*^{+/−} mice^{66, 68, 69} present serious vascular defects in the retinal vasculature with excessive capillary density, diameter and filopodia extension of endothelial cells, while similar defects have been observed with NOTCH inhibition either in an inducible NOTCH-1 knockout⁶⁸, pharmacological blockade by anti-Dll4 antibodies^{69, 70} or γ-secretase inhibitors^{66, 68}. Stalk cells that are unchecked by DLL4 also remain highly proliferative as shown in Dll4 or RBP-Jk morphant zebrafish embryos⁷².

NOTCH in Vascular Smooth Muscle Cells Differentiation

Besides affecting arterial ECs differentiation, NOTCH signaling has been reported to specify arterial-venous identity of vascular SMCs as well⁷⁵. Although several reports

have been published during the last years with respect to NOTCH and SMC differentiation, the results have been contradictory.

Among the receptors, Notch3 is expressed in vascular SMCs from arteries but not in those from veins⁷⁵. Notch3^{-/-} mice show serious arterial defects, such as enlarged arteries with a thinner layer of SMCs⁷⁵ that are poorly differentiated⁷⁵⁻⁷⁷ compared to wild-type animals. This is consistent with in vitro data showing that ligand induced NOTCH signaling up-regulated the SMA (Smooth Muscle Actin) and SM-MHC (Smooth Muscle myosin heavy chain) expression, promoting VSMC differentiation⁷⁸⁻⁸⁰ via RBP-Jk. Among the ligands, endothelial Jag1 appears to be essential in regulating vascular morphogenesis by inducing NOTCH signaling in the neighbour SMC. Endothelial-specific deletion of Jag1 caused serious cardiovascular abnormalities and embryonic lethality in mice⁸¹. Surprisingly, these embryos differ from embryos in which general NOTCH signaling has been abrogated as they still present with normal NOTCH activation and arterial differentiation of the endothelium. This suggest that endothelial Jag1 mainly acts by inducing the differentiation of the adjacent SMCs⁸¹. NOTCH3 activation by endothelial Jag1 is further supported by enhanced expression of NOTCH3 in SMC co-cultured with Jag1 expressing endothelial cells, suggesting a positive feedback loop. Indeed, Liu et al⁷⁹, demonstrated that activated NOTCH-3 signaling initiates a positive feedback loop that promotes its own expression and propagated NOTCH signaling to other SMCs, meanwhile inducing SMC differentiation markers, such as α -SMA, CALPONIN and SM-MHC. These data are in contrast with other studies in which NOTCH target genes have been reported to antagonize SRF (Serum Response Factor) and myocardin, the main transcription regulators of VSMC gene expression and SMC differentiation^{82 83}. The controversy between these reports could relate to the balance between NOTCH ICD that induces the expression of VSMC markers and, the contemporary expression of NOTCH target genes such as Hey1 and Hey2 that would turn off these factors as part of a negative feedback loop. The validity of this model has been experimentally addressed by using ECs and SMC cocultures^{83,84}. Hey1 and Hey2 were indeed able to repress SMA expression and to antagonize the NOTCH-induced SMA expression in vitro by decreasing NOTCH ICD/RBP-Jk binding and transactivation of the SMA promoter.

Vascular Remodeling and Notch Signaling

In the adult vascular remodeling can occur upon vascular damage (inward remodeling) or altered blood flow (outward remodeling). During inward remodeling excessive migration and proliferation of SMCs in response to vascular injury induces the formation of a thickened layer of arterial intima from the media referred to as neointima. Increased blood flow is the main trigger of an endothelial-monocyte driven expansion of the vessel wall referred to as outward remodeling. This process

in which the increase in both ECs and SMCs number facilitate the expansion of the vessel is also defined as arteriogenesis.

As regulator of SMC proliferation and migration, the NOTCH signaling pathway has been associated with both arteriogenesis and neointima formation. With respect to collateral growth, Limbourg et al.⁸⁵ have underlined the importance of NOTCH signaling in postnatal arteriogenesis in response to hindlimb ischemia. Mice heterozygous for Dll1 showed absence of endothelial NOTCH activation and EphrinB2 induction after hindlimb ischemia, with subsequent lack of arterial collateral growth and impairment of blood flow recovery of the hindlimb. Similarly, postnatal angiogenesis and blood flow recovery was impaired in haplo-insufficient general or endothelial specific Notch1+/- mice⁸⁶. In the arterial response to injury, the effects of NOTCH signaling and of its downstream targets on VSMC differentiation, migration and proliferation make it a suitable candidate in regulating neointima formation. The expression of different NOTCH members is regulated in a temporal manner after vascular injury⁸⁷⁻⁸⁹. It appears that NOTCH1, 2 and 3 together with Herp-1 and Herp-2 are downregulated in the first 7 days after balloon injury, after which at least NOTCH 1 and 4 together with Jagged-1 and -2 are upregulated at the leading edges of the regenerating endothelium⁸⁸. Loss of function studies with general as well as smooth muscle cell specific NOTCH1 heterozygous mice support the notion that NOTCH activation is required for neointima formation⁹⁰. Accordingly, in Hey2-/- mice⁹¹ VSMC proliferation was reduced resulting in diminished neointima formation. Consistently, gain of function studies with overexpression of HRT1 (also named Hey1/HERP2/Hesr-1/CHF2)⁹² or constitutive expression of Notch 1 and 3⁹³ induced SMCs proliferation and migration capacity with enhanced neointima formation as a result.

An intact and functional endothelial layer appears to maintain VSMC in a quiescent state^{94,95}. Although several mechanisms have been reported, including NO (Nitric Oxide) and heparin-like substances, definitive proof for a particular mechanism has not been established yet. The NOTCH pathway might be a suitable intercellular signaling to mediate this mechanism. Under certain conditions, Delta and Jagged can be cleaved from the cell membrane generating a soluble antagonist of NOTCH activity⁹⁶. Duarte et al⁴⁸ have shown that thrombin mediates the proteolytic release of Jagged1, which subsequently inhibits NOTCH signaling. SMC migration and proliferation are positively regulated by NOTCH signaling and are induced following vascular injury. Lindner et al⁸⁸ have shown that soluble Jagged decreased cell-matrix interaction, focal adhesion formation and migration in NIH3T3 cells. Altogether these observations suggest a possible therapeutic application for soluble ligands to limit the response to vascular injury, by interfering with NOTCH signaling and SMCs proliferation and migration.

Chapter Outline

The aim of this thesis was to study the NOTCH signaling pathway in vascular biology. First we investigated the molecular mechanisms of VEGF-NOTCH signaling interaction in ECs and the role of ADAMs in this process. Furthermore, we aimed to study the effect of VEGF-NOTCH signaling on coronary arteries development and arterial specification. Finally, we studied the effect of a soluble NOTCH ligand, sJag1, on SMCs behaviour and neointima formation following vascular injury.

Chapter 1 provides background information on the multiple roles of NOTCH signaling in blood vessels. The interaction between NOTCH and VEGF signaling in regulating artery/vein identity and sprouting of endothelial cells is introduced. The role of NOTCH in regulating VSMC differentiation in normal or injured vessels is outlined.

Chapter 2 provides a detailed molecular analysis of the VEGF and NOTCH signaling interaction. We demonstrated that VEGFR-2-induced DLL4 expression is NOTCH mediated. Accordingly, VEGF also induced NOTCH signaling activation. Moreover, DLL4 induced its own expression in a NOTCH-dependent manner unrelated to VEGF-signaling, indicating a feed-forward mechanism downstream of NOTCH in ECs.

Chapter 3 shows the functional interaction between the VEGFR-2 pathway and ADAM-10, a metalloproteinase involved in NOTCH processing and activation. VEGF induces ADAM-10 expression and activation in ECs, increasing vascular permeability and facilitate ECs migration. Furthermore, ADAM-10 expression in atherosclerosis lesions is associated with plaque progression and neovascularization.

Chapter 4 reports on the effect of altered VEGF-signaling in *Vegf*120/120 mouse embryos on coronary development. Mutant coronary artery endothelium showed decrease expression of arterial marker such as NOTCH-1, DLL4 and EphrinB2 and an increase of venous marker expression, whereas the venous endothelium showed opposite effects. Coronary arteriogenesis was also anomalous in mutant embryos with decreased arterial and increased venous medial development.

Chapter 5 describes the role of the soluble NOTCH ligand Jag1 in inhibiting neointima formation in a rat injured carotid artery model. Soluble-Jagged reduced SMC proliferation and migration following vascular injury, by interfering with NOTCH signaling activation and NOTCH target gene *Herp2* expression.

Chapter 6 discusses the regulation of the NOTCH pathway and ADAM-10 activity by VEGF signaling together with the existence of a NOTCH feed-forward mechanism in ECs; the role of VEGF and NOTCH signaling in coronary endothelial differentiation and maturation; the negative effect of sJag1 on neointima formation.

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Chapter 2

Feed-forward signaling by a membrane bound ligand receptor circuit: The case of NOTCH-DELTA LIKE 4 in endothelial cells

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Abstract

The Delta like-4 (DLL4) ligand belongs to the highly conserved NOTCH-family and is specifically expressed in the endothelium. DLL4 regulates crucial processes in vascular growth, including endothelial cell (EC) sprouting and arterial specification. Its expression is increased by VEGF-A. In the present study we show that VEGF-induced DLL4 expression depends on NOTCH activation. VEGF-induced DLL4 expression was prevented by the blockage of NOTCH-signaling with γ -secretase or ADAMs inhibitors in Human Cardiac MicroVascular ECs. Similar to VEGF-A, recombinant DLL4 itself stimulated NOTCH-signaling and resulted in up-regulation of DLL4, suggesting a positive feed-forward mechanism. These effects were abrogated by NOTCH inhibitors, but not by inhibition of VEGF-signaling. NOTCH activation alone suffices to induce DLL4 expression as illustrated by the positive effect of Notch Intracellular Domain (NICD) -1 or -4 overexpression. To discriminate between NICD/RBP-Jk and FOXC2 regulated DLL4 expression, DLL4 promoter-activity was assessed in promoter deletion experiments. NICD induced promoter activity was dependent on RBP-Jk site but independent of the FOXC2 binding site. Accordingly, constitutively active FOXC2 did not affect *DLL4* expression. The notion that the positive feed-forward mechanism might propagate NOTCH-activation to neighboring ECs was supported by our observation that DLL4-eGFP transfected ECs induced DLL4 expression in non-transfected cells in their vicinity. In summary, our data provide evidence for a mechanism by which VEGF or ligand-induced NOTCH-signaling up-regulates DLL4 through a positive feed-forward mechanism. By this mechanism DLL4 could propagate its own expression and enable synchronization of NOTCH expression and signaling between ECs.

Introduction

The NOTCH family encompasses a fundamental signaling pathway involving four receptors (NOTCH-1, -2, -3, -4) and, in vertebrates, five cognate ligands (DLL-1, -3, -4 and JAGGED-1, -2). Cell-cell contact is a prerequisite for NOTCH signaling as all members are membrane bound. The first activation step upon receptor-ligand interaction involves cleavage of the extracellular domain of the NOTCH receptor by A Disintegrin And Metalloprotease (ADAM). Subsequently, the γ -secretase complex instigates a second proteolytic cleavage resulting in the release of the NOTCH intracellular domain (NICD) into the cytoplasm. Next, the NICD translocates to the nucleus, where it associates with the Recombinant Signal binding protein for Immunoglobulin kappa J region (RBP-Jk or CSL, CBF1/RBPJk,Su(H), Lag1) transcription factor to initiate the transcription of its downstream targets basic Helix-Loop-Helix proteins Hairy/Enhancer of Split (HES) and Hairy Related Transcription factors (HRT, HEY, HERP)¹.

Among the ligands, DLL4 is specifically expressed in the arterial endothelium and plays a major role during embryonic vascular development. *Dll4* haploinsufficiency is embryonically lethal due to severe vascular abnormalities related to a loss of arterial and venous specification²⁻⁷. DLL4 overexpression on the other hand induces arterIALIZATION even in the venous compartment that typically lacks NOTCH expression⁸.

Notch signaling is important for the development of endothelial cell sprouting. In this process VEGF induces the formation of new sprouts, whereas DLL4 signaling appears to reduce their formation^{9, 10}. Since DLL4 is upregulated by VEGF¹¹ this signaling pathway might serve as a negative feedback loop with respect to endothelial sprouting. This feedback could be bidirectional as NOTCH reduces VEGF responsiveness through downregulation of VEGFR-2¹² and subsequently, suppresses the sprouting phenotype in the adjacent stalk cell, thus limiting the number of sprouts. Accordingly, loss of DLL4 function promotes excessive endothelial cell sprouting and endothelial cell migration¹³. VEGF-A has also been reported to induce the activation of ADAM-10 and -17¹⁴, which are the protease required for the first step in NOTCH activation. This interrelation between VEGF-A and Notch signaling suggests that VEGF not only induces the expression of DLL4 but also stimulates NOTCH signaling. However, alternative pathways have been suggested for VEGF-induced DLL4 expression. Upregulation of DLL4 by VEGF has been considered the result of activated FOXC (Forkhead box C) transcription factors by a PI3K and ERK/MAPK dependent pathway¹⁵. FOXC2 has been shown to activate the DLL4 and HEY2 promoters, which both contain a forkhead binding element (FBE)^{15, 16}. In particular, FOXC2 functionally interacts with RBP-Jk and NICD to activate the HEY2 promoter¹⁵. Similarly to HEY2, the DLL4 promoter presents with multiple RBP-Jk binding sites¹⁷, suggesting that NOTCH might regulate DLL4 expression as well. Altogether these observations prompted us to speculate that NOTCH-signaling

could play a role in up-regulating the expression of its own ligand DLL4, which in turn would activate NOTCH signal transduction, thus creating a positive feed-forward signaling that propagates the activation to adjacent cells.

In the present study we show that in endothelial cells, VEGF-induced DLL4 up-regulation is NOTCH activation dependent. VEGF positively affects DLL4 expression and NOTCH signaling mainly by activating VEGFR-2, whereas inhibition of NOTCH signaling abrogated VEGF-induced DLL4 expression. Consistently, immobilized recombinant DLL4 also induced endogenous DLL4 expression in a NOTCH-dependent manner unrelated to VEGF-signaling, indicating a feed-forward mechanism downstream of NOTCH. The increase in DLL4, along with transactivation of the DLL4 promoter by NICD-1 and NICD-4 confirmed the role of NOTCH signaling in this process, further suggesting that NOTCH activation is sufficient for DLL4 induction. In this endothelial system, we found no evidence for FOXC2 dependent DLL4 expression by VEGF. Finally, we show that DLL4 transfected endothelial cells were able to increase DLL4 expression of surrounding cells providing a mechanism by which NOTCH signaling is distributed between communicating endothelial cells.

Experimental Procedures

Reagents

Human Cardiac Microvascular Endothelial Cells (HCMvECs) (Lonza, Vervies, Belgium) were cultured in EGM-2MV, with 5% FBS (FBS; Lonza). Recombinant VEGF-A₁₆₅ was purchased from RELIATech GmbH (Braunschweig, Germany); DLL4 (rDLL4) extracellular domain was from R&D System (Abingdon, Oxfordshire, UK); L685,458 (γ -secretase inhibitor) from Calbiochem (Nottingham, UK); IMC-1121b (human VEGFR-2 inhibitor) and IMC-18F1 (human VEGFR-1 inhibitor)^{18, 19} were both kindly provided by ImClone Systems Corporation (New York, NY, USA). ADAM inhibitors GI250423X (ADAM-10 inhibitors) and GW28026X (ADAM-10/ADAM-17 inhibitor)²⁰ were both kindly provided by GlaxoSmithKline.

Cell Culture and Cell Treatment

HCMvECs were cultured according to manufacturer's protocol (Lonza). Cells were cultured in EGM-2MV medium without basic Fibroblast Growth Factor (bFGF) and VEGF-A₁₆₅. Cells were stimulated for 24 hours with 100 ng/mL of human recombinant VEGF-A₁₆₅. For rDll4 stimulation experiments HCMvECs were grown for 48 hours on 0.2% gelatin coated plates containing 1 μ g/mL rDll4 or BSA as control²¹. For inhibitor treatment, HCMvECs were incubated for 16 hours with 5 μ M L685,458, GI250423X, GW28026X or 20 μ g/mL IMC-1121b or IMC-18F1 antibodies prior to stimulation with VEGF-A₁₆₅. PAECs (Porcine Aortic Endothelial Cell), VEGFR-1/PAECs and VEGFR-2/PAECs²² were cultured in EGM-2MV medium without bFGF and VEGF-A₁₆₅ (overnight) and, subsequently stimulated with 100 ng/mL VEGFA₁₆₅ for 24

hours. Cells were subcultured according to the manufacturer's protocol (Lonza) and viable cells count was determined using trypan blue staining and the Countess Automated cell Counter (Invitrogen).

Plasmids and transfection

Mouse *Notch-ICD-1* and human *NOTCH-ICD-4* were both cloned in pcDNA expression vectors. The pIRES2-EGFP (Clontech) containing the full-length human *DLL4* coding region, *DLL4*-promoter constructs pGL3-h*DLL4* 6kb, the pGL3-h*DLL4* (-2616), the pGL3-h*DLL4* *SacI* (-1517) and the pGL3-h*DLL4* *MscI* (-931) were kindly provided by Prof. Dr Manfred Gessler and have been previously described in ¹⁷. To obtain a minimal *DLL4* promoter fragment lacking any putative RBP-Jk binding site the pGL3-h*DLL4* *MscI* (-931) was digested with *NruI* and *PstI* to remove the RBP-Jk binding site, blunted and religated. Constitutively active FOXC2-VP16 (caFOXC2) pCB6 expressing vector was kindly provided by Prof. Ormond A. MacDougald and has been previously described ²³. All transfection experiments were carried out in triplicate. HCMvECs were grown on 0.2% gelatin coated plates. Transfections were performed with lipofectamine LTX and PLUS reagent (Invitrogen). After 24 or 48 hours of culture RNA or proteins were isolated and analyzed by RT-qPCR or Western Blotting, respectively.

Luciferase Reporter Assay

HCMvECs seeded on 0.2% gelatin in PBS coated plates were transiently transfected with 0.6 µg of either inducible RBP-Jk responsive firefly luciferase construct (SuperArray Bioscience Corporation, Frederick, MD, USA). After 24 hours stimulation with VEGF-A₁₆₅ or inhibitors, the cells were harvested using Dual Glo luciferase assay system (Promega, Leiden, the Netherlands) and reporter activity was measured on a PE Victor3 plate reader (Perkin Elmer, Groningen, the Netherlands). PAECs were transiently transfected with pcDNA NICD-1, NICD-4 or vector alone along with the 6kb *DLL4*- promoter firefly luciferase construct or with the deletional constructs of this promoter (-2616, -1587, -931 or -931-Deletion (D), respectively). The renilla luciferase reporter plasmid was cotransfected as internal control for defining transfection efficiency. After 24 hours of transfection, cells were harvested and luciferase activity was assessed as reported above.

RNA isolation and RT-qPCR and Protein Extraction.

After each experiment RNA was isolated by using RNeasy micro-kit Qiagen (Qiagen, GmbH, Hilden, Germany). A total of 100 ng RNA per sample was subjected to reverse transcription (RT). qPCR was performed using SuperscriptTMIII Platinum Two-step qRT-PCR kit with SYBR green (Invitrogen) and primer concentration of 10 µM according to Van den Akker et al ²⁴. PCR Primers used were. human β -*ACTIN*-sense: 5'-ATCCTCACCCTGAAGTACCC-3', β -*ACTIN*-antisense: 5'-CACGCAGCTC

ATTGTAGAAG-3'; GADPH-sense: 5'-GCCTCAAGATCATCAGCAAT-3' GADPH-antisense: 5'-GGACTGTGGTCATGAGTCCT-3'; DLL4-sense: 5'-ACAACCTGTCGGACTTCCAG-3', DLL4-antisense: 5'-CAGCTCCTTCTTCTGGTTTG-3'; HES-1-sense: 5'-CCAAAGACAGCATCTGAGCA-3', HES-1-antisense: 5'-GCCGCGAGCTATCTTTCTT-3'; porcine β -ACTIN -sense: 5'-gcacccctgacccctcaagtac-3, β -ACTIN -antisense: 5'-caccgcagctcgttgtagaag-3'; GADPH-sense: 5'-gtgtcggttgtggatctga-3', GADPH-antisense 5'-cctgcttcaccaccttctt-3'; DLL-4-sense: 5'-tgtcgcaatggaggtagctgcaag-3', DLL-4-antisense 5'-aagggtgctatgttcgagtcgagg-3'. Primers were designed with oligoperfect™ Designer (Invitrogen), Primer3 and Mfold (<http://www.idtdna.com/scitools/Application/mfold/>) and were synthesized by Eurogentec (Seraing, Belgium). qPCR reactions were run on a BioRad MiQ Real Time PCR detection System (BioRad, Veenendaal, the Netherlands). Proteins were isolated using RIPA lysis buffer supplemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz, Santa Cruz, USA). The protein concentration was measured using Micro BCA (Pierce, Rockford, USA). For Western Blot analysis, XT reducing agent and sample buffer (Biorad) were added to the total cell lysates, samples and were heated at 95°C for 5 minutes, loaded on a 4–12% gradient Criterion XT Bis-Tris gel and were, subsequently, subjected to electrophoresis in MOPS running buffer (BioRad) at 200 V for 55 minutes. Proteins were transferred to a PVDF membrane (BioRad) at 30 V overnight using Tris-glycin transfer buffer. Non-specific binding of the antibodies was blocked by incubating with 3% milk powder in TBS supplemented with Tween-20 (TBS-T) for 1 hour. The membranes were exposed to anti-NOTCH1 (Santa Cruz sc-6014) or anti-NOTCH-4 (Santa Cruz sc-5594), or anti-DLL4 (R&D, MAB1389) antibodies and were, subsequently, incubated with biotinylated Goat-anti-Rabbit IgG (Vector Labs, Burlingame, USA BA-1000) or biotinylated Rabbit anti-Rat (Vector BA-9001) secondary antibody. The detection was performed by using SuperSignal West Femto or Pico substrate (Pierce) and was visualized with the ChemiDoc XRS System (BioRad).

Immunofluorescence Staining for DLL4 and eGFP.

HCMVECs (Lonza, Switzerland) were cultured on 4 wells slides (BD, Franklin Lakes, USA) until confluence and transfected with pIRES2-EGFP or pDLL4IRES2-EGFP. After 48 hours cell were incubated for 1 hour with the primary rabbit-anti human DLL4 antibody (Ab; R&D; MAB1389). Slides were washed and incubated with 2% rabbit serum and secondary Ab Alexa-Fluo594 Rabbit anti-Rat (Invitrogen). Photos were taken using a Leica DFC350 FX digital camera.

Statistical Analysis.

Statistical significance was determined by applying one-way ANOVA/Tuckey (SigmaStat Software, San Jose, USA) testing. Values are expressed as mean $\pm$ SEM of 3 experiments. Probability values of < 0.05 were considered significant.

Results

VEGFR-2 mediated DLL4 expression involves NOTCH signaling in endothelial cells.

To investigate the effect of VEGFR-1 and VEGFR-2 signaling on DLL4 expression, HCMvECs were exposed to receptor specific ligands, i.e. Placenta Growth Factor-2 (PlGF-2) for VEGFR-1, VEGF-E for VEGFR-2, and VEGF-A165 for both receptors²⁵⁻²⁷. Both VEGF-A165 and VEGF-E increased *DLL4* expression, whereas PlGF-2 had no significant effect, suggesting a predominant involvement of VEGFR-2 (Fig. 1A). In accordance, specific inhibition of VEGFR-2 by the IMC-1121b antibody (Fig. 1B) prevented VEGF-A165 induced *DLL4* expression, whereas VEGFR-1 inhibition with IMC 18F1 had no effect (Fig 1B). Final confirmation for the dominance of VEGFR-2 signaling, came from PAEC cell lines that overexpress either the human VEGFR-1 or VEGFR-2²². In line with the HCMvEC results, VEGF-A165 induced *DLL4* only in VEGFR-2 transfected but not in VEGFR-1 transfected PAECs (Fig.1C).

VEGFA induced *DLL4* expression in HCMvECs was abrogated by blockage of NOTCH signaling with γ -secretase- and ADAMs inhibitors (Fig. 1D), suggesting a role for NOTCH signaling. To further substantiate that VEGF-A165 activated NOTCH-signaling, RBP-Jk luciferase activity and NOTCH target gene *HES-1* expression were assessed. VEGF-A165 induced RBP-Jk promoter activity and *HES-1* expression (Figs. 1E & 1F), an effect that was prevented by NOTCH inhibition (Figs.1E & 1F).

Activated NOTCH-signaling induces DLL4 expression by activating DLL4 promoter.

NOTCH-1 and NOTCH-4 are the main mediators of NOTCH-signaling in the endothelium^{28, 29}. In order to define a direct effect of NOTCH signaling on *DLL4* expression, HCMvECs were transfected with the intracellular domain of NOTCH-1 and -4 (NICD-1 and NICD-4, respectively) or empty vector. Both NICD-1 and -4 induced *DLL4* mRNA and protein expression (Figs. 2A & C lower panel). Endogenous NOTCH signaling was not required for this stimulation as NICD-1 and -4 overexpression had the same effect in the presence of γ -secretase inhibition (Fig. 2B).

The *DLL4* promoter has previously been described to contain a forkhead binding element (FBE) and *DLL4* expression was reported to be induced by VEGFA in a FOXC mediated manner^{15, 16}. Furthermore, the *DLL4* promoter sequence also contains several RBP-Jk binding sites¹⁷, suggesting that NOTCH signaling might regulate *DLL4* expression as well.

Figure 1 VEGF-A165-induced DLL4 expression is VEGFR-2 and NOTCH-signaling mediated.

A. DLL4 mRNA expression in HCMvECs treated with VEGF-A165, VEGF-E or PIGF-2 compared to the expression of non treated cells as control. B. DLL4 mRNA expression in HCMvECs treated with VEGF-A165 or VEGF-A165 along with IMC-1121b (hVEGFR-2 inhibitor), or IMC-18F1 (hVEGFR-1 inhibitor) or IMC-1121b and IMC-18F1. C. DLL4 mRNA expression in VEGFR-2/PAECs (right) and VEGFR-1/PAECs (left) upon stimulation with or without VEGF-A165. D. DLL4 mRNA expression in HCMvECs stimulated with or without VEGF-A165 and VEGF-A165 along with L685,458 (γ -secretase inhibitor), GI254023X (ADAM-10 inhibitor) or GW280264X (ADAM-10/ADAM-17 inhibitor). E. Luciferase activity of HCMvECs cell lysates transiently transfected with RBP-Jk-luciferase reporter construct and cultured with or without VEGF-A165 or VEGF-A165 along with L685,458 (γ -secretase inhibitor). F. HES-1 mRNA expression in HCMvECs stimulated with or without VEGF-A165 and VEGF-A165 along with L685,458 (γ -secretase inhibitor), GI254023X (ADAM-10 inhibitor) or GW280264X (ADAM-10/ADAM-17 inhibitor). Data represent mean $\pm$ SE (n=3). One way Anova test: * = significantly different from control $p < 0.05$; # = significantly different from VEGF-A165 $p < 0.05$.

To discriminate between FOXC and NICD/RBP-Jk regulated DLL4 expression several deletional DLL4 promoter fragments were analyzed for their transactivation activity upon NICD-1 and -4 activation in PAECs. These promoter fragments lacked the FBE site and contained either three (-2616), two (-1587), one (-931) or no (-931-D) RBP-Jk binding site, respectively¹⁷. The 6 kb DLL4 promoter fragment was significantly stimulated when co-transfected with NICD-1 or NICD-4 (Fig. 3A). FBE deletion in the presence of all three RBP-Jk sites resulted in NICD-1 transactivation that was comparable to the full length promoter. Significant transactivation was also apparent after NICD-4 overexpression, although less effective than NICD-1 (Fig. 3A).

The shorter -1587 and -931 constructs, containing two and one RBP-Jk binding sites, respectively, showed reduced but still significant induction by NICD-1. Although NICD-4 transactivated the -1587 fragment that contained two RBP-Jk binding sites, NICD-4 was unable to induce promoter activity in the -931 DLL4 promoter fragment that presented with only one RBP-Jk binding site. As expected, no NICD transactivation effect was observed for the -931-D fragment that lacked both the FBE and all RBP-Jk binding sites (Fig 3A).

Like in HCMvECs, NICD-1 and -4 overexpression (Fig. 3C) upregulated *DLL4* mRNA expression (Fig. 3B) in PAECs. Transfection of both HCMvECs and PAECs with a constitutively active FOXC2 (caFOXC2) expressing vector did not alter *DLL4* expression (Fig.3D), underlining that in endothelial cells the FOXC2 pathway seems redundant for NOTCH stimulated *DLL4* expression.

Figure 2 NICD-1 and NICD-4 induced *DLL4* expression in HCMvECs. A and B *DLL4* mRNA expression in HCMvECs transiently transfected with NICD-1, NICD-4 or pcDNA empty vector as control with or without L685,458. Data represent mean $\pm$ SE (n=3). One way Anova test: * = significantly different from control (mock) $p < 0.05$. C. Western Blot analysis of protein extracts from HCMvECs transfected with NICD-1 and NICD-4. Membranes were probed with anti-NOTCH-1 (upper left panel), anti-NICD-4 (upper right panel) or anti-DLL4 (lower panel).

Figure 3 Induction of DLL4 promoter activity by NICD-1 and NICD-4.

A. Luciferase assay activity from PAE cells cotransfected with a 6kb DLL4 promoter luciferase reporter or shortened fragments of the 6 kb promoter containing three (-2616), two (-1587), one (-931) or none (-931-D) RPB-Jk binding site along with NICD-1, NICD-4 or empty pcDNA (mock) vector. Schematic drawing of the 6 kb DLL4 promoter and of the shortened fragments -2616, -1587, -931 and -931-D (relative to the transcriptional initiation site) with potential RPB-Jk (R) sites indicated by red bars, FBE (Forkhead binding site) indicated by a green bar and deletion of (R) indicated by the black cross. B. *DLL4* mRNA expression in PAECs transiently transfected with NICD-1 and NICD-4 and pcDNA as control. Data represent mean $\pm$ SE (n=3). One way Anova test: * = significantly different from control (mock), $p < 0.05$. C. Western Blot analysis of protein extracts from PAECs transfected with NICD-1 (upper panel) and NICD-4. (lower panel). Results are from one representative experiment. D. *DLL4* mRNA expression in HCMvECs (left panel) and PAECs (right panel) transiently transfected with constitutively active FOXC2 (caFOXC2) and empty vector as control.

DLL4-induced NOTCH-signaling upregulates DLL4 expression

To address a potential DLL4-driven positive feed-forward loop, rDLL4 protein was used to stimulate the NOTCH pathway in HCMvECs. Similar to VEGFA, rDLL4 was able to increase the expression of DLL4. NOTCH signaling blockage by γ -secretase and ADAMs inhibitors prevented rDLL4-induced *DLL4* expression (Fig. 4A). The activation of NOTCH signaling by rDLL4 was further assessed by RBP-Jk luciferase promoter analysis. rDLL4 stimulation induced transactivation of RBP-Jk promoter, that was prevented by NOTCH inhibitors (Fig. 4B). To investigate whether rDLL4-

induced DLL4 expression depends on VEGFR-2 signaling we cultured HMCvECs on rDLL4 along with specific VEGFR-2 or/and VEGFR-1 inhibitors and analyzed DLL4 expression. The inhibition of VEGFR-2, of VEGFR-1 or of both receptors did not affect rDLL4-induced DLL4 expression suggesting that VEGF signaling is not required for the positive feed-forward circuit (Fig. 4C).

Figure 4 rDLL4 activated NOTCH-signaling induced DLL4 expression.

A. DLL4 mRNA expression in HCMvECs stimulated with BSA as control, rDLL4 or rDLL4 along with L685,458 (γ -secretase inhibitor), or GI254023X (ADAM-10 inhibitors) or GW280264X (ADAM-10 and ADAM-17). B. Luciferase activity of HCMvECs cell lysates transiently transfected with RBP-Jk-luciferase reporter construct and stimulated with BSA as control, rDLL4 protein or with rDLL4 and L685,458. C. DLL4 mRNA expression in HCMvECs stimulated with BSA as control, rDLL4 or rDLL4 along with IMC-1121b (hVEGFR-2 inhibitor), or IMC-18F1 (hVEGFR-1 inhibitor) or IMC-1121b and IMC-18F1. Data represent mean $\pm$ SE (n=3). One way Anova test: * = significantly different from control (BSA) $p < 0.05$; # = significantly different from rDLL4.

DLL4 induces propagation of endogenous DLL4 expression in adjacent cells

As NOTCH is an inter-cellular signaling pathway, we hypothesized that NOTCH-induced DLL4 expression taking place in one specific cell would eventually induce NOTCH signaling and, subsequently DLL4 expression in its neighboring cell. To test this hypothesis, HCMvECs were transfected with pIRES2-eGFP or pDLL4IRES2-eGFP with or without NOTCH signaling inhibitor (L685,458) and DLL4 expression was analyzed by immunofluorescence staining. eGFP (green) signal allows to

discriminate between transfected and non-transfected cells (Figs. 5A-C GFP). pIRES2-eGFP transfected control cells presented relatively weak DLL4 expression (red staining) in their surrounding cells (Fig. 5A, DLL4). In contrast, the pDLL4IRES2-eGFP transfected cells revealed high DLL4 level and prominent DLL4 staining was also detected in the non-transfected surrounding cells (Fig. 5B, DLL4). The pDLL4IRES2-eGFP expressing cells treated with L685,458 had high DLL4 expression, however no DLL4 was detected in the surrounding non-transfected cells (Fig. 5C, DLL4) indicating that inhibition of NOTCH signaling was sufficient to prevent the DLL4-induced positive feed-forward loop.

Figure 5 DLL4 overexpressing cells induce endogenous DLL4 expression in the neighboring and adjacent cell. DLL4 immuno-fluorescent labeling pIRES-eGFP transfected HCMVECs versus pIRES-DLL4-eGFP transfected HCMVECs with or without L685,458. eGFP (green) signal allows to discriminate between transfected and non-transfected cells (Fig. 5 A, B and C; GFP). pIRES-DLL4-eGFP transfected ECs revealed higher DLL4 staining (red) (B and C; DLL4) compared to pIRES-eGFP transfected cells (A; DLL4). Cells surrounding pIRES-eGFP-DLL4 transfected cells also showed increased DLL4 expression (B; DLL4) compare to cells surrounding pIRES- eGFP transfected cells (A; DLL4). This effect was prevented by adding the NOTCH-signaling inhibitor L685,458 (C, DLL4). Merged images between eGFP and DLL4 staining (Fig. 5, A,B,C; Merge) and PCI (Phase Contrast microscopy) images (Fig. 5, A,B,C; PCI) are also reported. Scale bars: 50 μ m.

Discussion

The VEGF and NOTCH signaling pathways and their intricate interactions are indispensable for regulating blood vessel formation. VEGF-A has been reported to modulate the expression of NOTCH members in arterial endothelial cells. VEGFR-2 acts as the main receptor in mediating VEGF signaling in vascular endothelial cells^{22, 30, 31} and likewise has been suggested to positively regulate NOTCH/DELTA expression^{10, 11, 21, 24, 32}. Also a reciprocal effect of NOTCH signaling on the expression of VEGFR-2 has been reported, i.e. a reduction in VEGFR-2 and an increase in VEGFR-1 expression with subsequent drop in VEGFA responsiveness³².

Accordingly, we show that in primary adult HCMvECs, the expression of *DLL4* is stimulated by the VEGFR-2 acting ligands VEGF-A165 and VEGF-E, and not by PlGF-2. Intriguingly, no other NOTCH ligands beside *DLL4* were affected by VEGF-A-related endothelial signaling (data not shown) suggesting a selectivity of VEGF-A-related signaling in primary adult microvascular ECs. The essential role of VEGFR-2 was confirmed in porcine aortic endothelial cells expressing only one of the receptors and with a specific VEGFR-2 inhibitor. The presented data clearly show that upregulation of *DLL4* by VEGF-A is NOTCH signaling dependent. VEGF-A failed to induce *DLL4* expression when NOTCH signaling was inhibited, indicating the requirement of activated NOTCH for this process. In addition, NOTCH signaling alone was sufficient to induce *DLL4* expression as shown by NICD-1 and NICD-4 overexpression. A comparable effect of VEGF-A induced *DLL4* expression was described for embryonic³³ and human bone-marrow derived mesenchymal stem cells⁴.

We show that both NICD-1 and NICD-4 were able to transactivate the *DLL4* promoter. Previous reports^{15, 16} indicated that the FOXC2 transcription factor can serve as a mediator for VEGF-A induced *DLL4* expression by interacting with a FBE located in the *DLL4* promoter. Besides the FBE, multiple RBP-Jk binding sites are present in the *DLL4* promoter sequence, providing the potential molecular cues for NOTCH induced expression. Indeed, NICD-1 effectively transactivated both the full length *DLL4* promoter and FBE deleted *DLL4* promoter sequences providing evidence that the FOX transcription factor pathway does not mediate VEGF-induced NOTCH activation in the endothelium. Accordingly, overexpression of a constitutively active FOXC2 transcript did not increase *DLL4* expression. The FBE lacking -2616, -1587 and -931 promoter constructs, showed a transactivation profile that suggests dependency on the number of RBP-Jk binding sites. As expected, both NICD-1 and -4 were unable to stimulate the -931- *DLL4* promoter fragment that lacked any RBP-Jk binding site. NICD-4 had a similar profile of transactivation as NICD-1 with respect to -2616 and -1587 fragments but, in accordance with previous studies, at lower levels³⁴. This difference likely relates to the presence of a C-terminal autonomous transactivation domain (TAD) in the NICD-1, which acts independently of RBP-Jk. Such TAD sequence is absent in the NICD-4 and

consequently, it requires RBP-Jk to initiate transcription³⁵. Therefore, the reduced induction capacity of NICD-4 is likely related to the number of RBP-Jk that are present in the shortened -2616 and -1587 DLL4 promoter fragments. Accordingly, no effect was shown by NICD-4 for the -931 DLL4 promoter fragment, that contained only one RBP-Jk binding site, whereas NICD-1 could still effectively transactivate this promoter.

NOTCH is typically activated by one its ligands, including membrane bound DLL4. Similar to VEGF-A therefore, NOTCH activation by surface bound recombinant DLL4 induced *DLL4* expression in HCMvECs. Inhibition of NOTCH signaling, but not of VEGF signaling, was sufficient to prevent this induction. These data indicate that activated NOTCH can induce DLL4 expression and transactivate the DLL4 promoter in absence of FOXC transcription factors and support the observation that NOTCH can induce DLL4 expression independently of FOXC2-mediated VEGF signaling. To our knowledge, these results show for the first time that DLL4 induces its own expression in endothelial cells, most likely through direct NICD mediated transcriptional regulation. VEGF-signaling also induced the upregulation of HES-1 and RBP-Jk transactivation, indicating that VEGF-A activates NOTCH signaling. This activation is likely not secondary to ligand overexpression, as NOTCH inhibition prevented the VEGF-A-induced expression of DLL4.

The link between VEGF-A and NOTCH signaling most likely relates to VEGFR-2 increased ADAM expression and activity. In a previous study we showed that ADAM-expression and protease activity (i.e. sheddase) in HUVECs and VEGFR-2/PAEC was increased upon VEGF-A₁₆₅ stimulation³⁶. VEGF-A has also been reported to induce the expression of *ADAM-10* and to enhance shedding of VEGFR-2 and its co-receptor NRP1 by activating ADAM-10 and ADAM-17, respectively¹⁴. Although this mechanism has been postulated to modulate VEGF-A signaling¹⁴, VEGF-A increased ADAM sheddase activity might potentiate NOTCH signaling as well. In line with our observations Hainaud et al³⁷ showed an accumulation of cleaved NOTCH-4 in VEGF-A-treated HUVECs with concomitant upregulation of the ADAM and Presenilin (part of the γ -secretase complex) expression.

The involvement of ADAM-10 and ADAM-17 in NOTCH processing and signaling has been unequivocally established³⁸⁻⁴². ADAM-10 mutant mice phenotypically resemble *Notch-1* null mutants and die at embryonic day 9.5, whereas *ADAM-17* mutants are viable⁴³. Among others Bozkulak and Weinmaster⁴¹ showed that NOTCH1 is a substrate for both ADAM-10 and ADAM-17 and, although ADAM-10 was essentially required for ligand induced NOTCH-1 signaling, ADAM-17 mediated ligand independent signaling. The importance of ADAM in NOTCH signaling was also reflected by our experiments showing that inhibition of ADAM activity abrogated the VEGF-A induced expression of *DLL4* and the NOTCH transcription factor *HES-1*. In addition, rDLL4 induced *DLL4* expression was depressed by blockage of ADAM

activity as well. Altogether these data indicate that VEGF-A activates ADAM, which is required for NOTCH signaling activation.

The ability of the activated NOTCH receptor to specifically induce the expression of its ligand would provide endothelial cells a mechanism by which NOTCH signaling is distributed and propagated between communicating cells with initially a limited amount of ligand. A comparable positive feed-forward mechanism has recently been described to regulate the interaction between endothelial and mural cells. JAGGED-1 ligand expressing endothelial cells induced NOTCH-3 signaling activation in mural cells⁴⁴, that in turn promote JAGGED-1's own expression. In this way mural cells can interact more efficiently with endothelial cells and can activate NOTCH signaling of neighboring cells, maintaining and distributing the signal to other mural cells. In the setting of membrane bound ligands the positive feed-forward mechanism is especially attractive to expand the signal to distant cells. The regulation of ligand expression by NOTCH receptors has already been described in *Drosophila*⁴⁵. NOTCH signaling was found to increase the expression of Delta and Serrate through a positive feedback loop during the regulation of the dorsoventral boundary of the developing wing⁴⁵. Likewise, in vertebrates, Delta-like ligands are required during somitogenesis for the maintenance of somite borders and accumulate in a Notch-dependent manner⁴⁶⁻⁴⁸.

The expression pattern of DLL4 ligand in arteries and sprouting vessels affects spatial distribution of NOTCH signaling in endothelial cells. During sprouting, tip endothelial cells will express high DLL4 levels and low NOTCH activity, whereas their neighbor stalk cells will have high NOTCH activity but low DLL4 expression. However, a static situation in which a subpopulation of cells presents high level of DLL4 and low NOTCH signaling is unlikely. NOTCH signaling is in fact required for arterial specification and lack or reduction of NOTCH signaling would cause loss of arterial identity. Here we show, that in contrast to eGFP-transfected cells, DLL4-eGFP ECs were able to induce DLL4 expression of non-transfected surrounding ECs, through a feed-forward mechanism, that was NOTCH signaling dependent. Coordinated activation of NOTCH signaling would produce a wave of DLL4 expression that provides periodic NOTCH signaling activation in each cell⁹. Such mechanism could be important to maintain an arterial phenotype along a blood vessel and for regulating spatial patterning of branching as well^{9, 49}.

In summary, stimulation of VEGFR-2 by VEGF-A165 up-regulates *DLL4* expression by activating NOTCH signaling in HCMvECs, independently of FOXC2. The NOTCH involvement in regulating DLL4 expression indicates the existence of a feed-forward mechanism by which NOTCH signaling can be propagated between adjacent endothelial cells.

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Chapter 3

ADAM-10 is a novel mediator of VEGF-induced endothelial cell function in angiogenesis and associated with atherosclerosis

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Abstract

Neovascularization has been associated with atherosclerotic plaque growth, plaque instability, and elevated risk of clinical complications. VEGF is a key angiogenesis factor associated with plaque angiogenesis. Using a Yeast-two-hybrid assay we identified A Disintegrin And Metalloprotease 10 (ADAM-10) as novel binding partner of VEGFR-2. ADAM-10 is a metalloprotease with sheddase activity involved in cell migration, however, its exact function in endothelial cells (EC), angiogenesis and atherosclerosis is largely unknown. We show for the first time ADAM-10 expression in human atherosclerotic lesions, associated with plaque progression and neovascularization. We demonstrate ADAM-10 expression and activity in EC to be induced by VEGF and ADAM-10 to mediate ectodomain shedding of VEGFR-2. Furthermore, VEGF induces ADAM-10-mediated cleavage of VE-cadherin, which could increase vascular permeability and facilitate EC migration. Indeed, VEGF increases vascular permeability in an ADAM-10- and ADAM-17-dependent way and inhibition of ADAM-10 reduces EC migration and chemotaxis. These data provide the first evidence of ADAM-10 expression in atherosclerosis and neovascularization. ADAM-10 plays a functional role in VEGF-induced EC function. These data open perspectives for novel therapeutic interventions in vascular diseases.

Introduction

Angiogenesis is associated with tumor growth, metastasis, and with the growth and instability of atherosclerotic plaques. The latter contributes to plaque rupture and its clinical complications¹. Expression of the major angiogenic factor Vascular Endothelial Growth Factor-A (VEGF) increases during atherogenesis. In EC, VEGF exerts its stimulatory effect mostly via VEGFR-2 (KDR/Flk-1) and is a potent angiogenic mediator of EC migration, proliferation and survival². VEGF binding induces VEGFR-2 dimerization followed by (auto)phosphorylation on tyrosine residues. Activation of VEGFR-2 induces signaling via PLC γ , activation of the MAPK/ERK1/2-pathway and EC proliferation. Furthermore, activation of p38-MAPK or PI3K leads to EC migration, survival and increased permeability^{2,3}.

Besides angiogenesis, VEGF and its receptors are associated with development of atherosclerosis, plaque angiogenesis and plaque instability. Recently, it was suggested that VEGF may induce a more vulnerable plaque phenotype by promoting leukocyte recruitment⁴. DNA vaccination against VEGFR-2 in LDLr^{-/-} mice inhibited atherosclerotic plaque growth by reducing plaque neovascularization⁵. Treatment with a neutralizing anti-VEGFR-1 antibody reduced atherosclerosis in ApoE^{-/-} mice via inhibition of leukocyte infiltration, whereas anti-VEGFR-2 was ineffective⁶. The neutralizing VEGF-antibody bevacizumab (AvastinTM) is an effective anti-cancer therapy that inhibits tumor angiogenesis, but is associated with an increase in cardiovascular side effects based on thrombosis and plaque instability⁷. Unraveling downstream VEGFR signaling mechanisms involved in angiogenesis and atherosclerosis will identify novel therapeutic targets and will therefore open perspectives for more specific and more effective therapeutic interventions.

In this study we aimed to identify novel binding partners of VEGFR-2 involved in downstream VEGFR signaling by using a yeast-two-hybrid screening. We identified ADAM-10, a metalloprotease with alpha-secretase activity, as a novel candidate interacting with VEGFR-2. ADAM-10 is required for cleavage of membrane proteins involved in adhesion, migration and chemotaxis and has been implicated in cancer progression⁸. ADAM-10-deficient mice are embryonically lethal due to defects in cardiovascular and neural development⁹.

Our data show for the first time a functional interaction between VEGFR-2 and ADAM-10, and a role of ADAM-10 in (VEGF-induced) endothelial cell function. In addition, we provide initial evidence for the expression of ADAM-10 in human atherosclerotic plaques, associated with neovascularization.

Methods

Co-immunoprecipitation and in vitro kinase assay

These assays, combined with SDS-PAGE, were performed as previously described² with minor modifications. For details, see supplement.

Fluorogenic peptide cleavage assay

EC were cultured in a 96-well plate and serum starved overnight once 70–90% confluency was reached. Cells were pre-treated for 30 min with 10 μ mol/L LY294002, PD98059 or SB203580. Medium was replaced by Hanks' Balanced salt solution (Sigma) containing 10 μ mol/L Mca-PLAQAV-Dpa-RSSSR-NH₂ fluorogenic peptide substrate (R&D systems, ES003). EC were stimulated with 5 or 50 ng/mL VEGF, 20 ng/ml PMA or 5 μ mol/L ionomycin in presence of 5 μ mol/L GI54023X, 5 μ mol/L GW280264X or dimethyl sulfoxide (DMSO). Increase of fluorescence was measured at 405 nm (excitation at 320 nm) for 6 hrs using a fluorescence microplate reader.

Statistical Analysis

Values are expressed as mean $\pm$ SEM of at least n=3 experiments and a two-tailed unpaired Student's t-test was used to compare groups. Probability values <0.05 were considered significant.

For an extensive description of methods see supplements page 64.

Results

Identification of ADAM-10 as novel VEGFR-2 binding partner

Using a yeast-two-hybrid screening with the intracellular domain of human VEGFR-2 as bait, we aimed to identify novel binding partners involved in downstream VEGFR-2 mechanisms. Five screens were performed using a cDNA library of human placenta as angiogenic tissue. As depicted in table I (Supplement), a total of 93 out of 990 clones were found positive and were re-transfected for confirmation of the interaction, which yielded 20 candidates for interaction with VEGFR-2. Sequence analysis of these clones revealed A Disintegrin And Metalloprotease 10 (ADAM-10) as novel binding partner of VEGFR-2, with sequence coverage of 94% and identity of 100%. As positive controls for our screening, NCK adaptor protein and PI3K were found, both proteins known to be involved in VEGFR-2 signaling^{2, 10}.

ADAM-10 is expressed in human atherosclerotic lesions and associated with angiogenesis

Since a role of ADAM-10 in vascular function and disease was largely unknown, we first investigated ADAM-10 expression in human vascular wall and atherosclerotic plaques using immunohistochemical staining. ADAM-10 expression is low in normal vessel wall (mammary artery, supplementary figure 1A) and early human atherosclerotic lesions (Figure 1A, n=5) but significantly increased during plaque progression from early and advanced to ruptured atherosclerotic plaques (Figure 1B-C, n=7-8), as was determined by semi-quantitative analysis (table II, supplement) and confirmed by quantitative PCR analysis of human atherosclerotic plaque specimen ($p < 0.01$, $n = 6-7$, Figure 1D). ADAM-10 was expressed in most cell types, i.e. smooth muscle cells (SMC) and macrophages, especially in foam cells (Figure 1E) and most interestingly highly expressed in EC of plaque microvessels (Figure 1F), as confirmed by double staining for ADAM-10 and CD31⁺/CD34⁺ EC (Suppl. Figure 1B). Additionally, we demonstrated high expression of ADAM-10 in human colon cancer cells as well as in the microvessels within, but not surrounding the colon tumor (Figure 1 G-H, $n = 5$ and supplementary table III). These data further strengthen our finding of a functional association between ADAM-10 and angiogenesis.

Figure 1. Immunohistochemical staining of ADAM-10 in human early (A), advanced stable (B) and ruptured (C) atherosclerotic plaques, quantified by PCR (D, normalized to GAPDH). High expression was found in foam cells (E) and microvessels (F). L=lumen, IEL=internal elastic lamina. ADAM-10 expression in normal human colon (G) and carcinoma with microvessels (H).

ADAM-10 interacts with VEGFR-2 and mediates VEGFR-2 ectodomain shedding

To validate the interaction of VEGFR-2 and ADAM-10 as identified in our yeast-two-hybrid screening, we performed co-immunoprecipitation experiments. VEGFR-2 or ADAM-10 were precipitated from Porcine Aortic Endothelial Cells overexpressing human VEGFR-2 (PAEC-KDR) or untransfected PAEC stimulated with 0–50 ng/mL

VEGF. Tyrosine-phosphorylated proteins were detected in precipitated immune complexes using an *in vitro* tyrosine kinase assay and separation of protein complexes by SDS-PAGE. In VEGFR-2 precipitates of PAEC-KDR, a strongly phosphorylated protein corresponding to the size of human VEGFR-2 (~250 kD) was detected, which was absent from untransfected PAEC that do not express detectable levels of endogenous VEGFR-2 (Figure 2A). As expected for VEGFR-2, phosphorylation of this protein significantly increased after VEGF stimulation, strongly indicating this is indeed VEGFR-2. In ADAM-10 precipitates, several phosphorylated proteins could be detected. In unstimulated PAEC-KDR, phosphorylated VEGFR-2 could barely be detected, whereas after VEGF stimulation phosphorylated VEGFR-2 was clearly detectable as a co-precipitate of ADAM-10, supporting our Yeast-2-hybrid results of VEGFR-2 and ADAM-10 interaction. Since VEGFR-2 expression in primary human EC is much lower than in PAEC-KDR, VEGFR-2 co-immunoprecipitating with ADAM-10 in these cells was below detection limit (data not shown).

ADAM-10 is involved in the juxtamembrane cleavage of several cell surface molecules and receptors, thereby releasing their ectodomains (shedding)¹¹. Since the related metalloprotease ADAM-17 has been implicated in VEGFR-2 shedding, we examined whether the interaction between ADAM-10 and VEGFR-2 provides a basis for ADAM-10-mediated ectodomain shedding of VEGFR-2. PAEC-KDR cells were incubated for 5 hrs with two hydroxamate-based inhibitors of ADAM-10. The inhibitor GI254023X has been shown to block ADAM-10 with a more than 100-fold higher potency compared to ADAM-17, whereas compound GW280264X has been shown to block both ADAM-10 and ADAM-17¹². Inhibition of ADAM-10 by 5 μ mol/L GI254023X, a concentration that selectively blocks ADAM-10 activity, as well as inhibition of both ADAM-10 and ADAM-17 by GW280264X significantly reduced constitutive release of VEGFR-2 ectodomain in PAEC-KDR culture medium compared to DMSO-treated controls (Figure 2B-C, $p < 0.001$). Release of sVEGFR-2 slightly increased after VEGF stimulation, which was again significantly decreased after inhibition of ADAM-10 only or both ADAM-10 and ADAM-17. Since PAEC-KDR cells overexpress the human VEGFR-2 and the proportion shed by the (limited) amount of ADAM-10 available in these cells will be limited, no differences in amount of full length VEGFR-2 in PAEC-KDR lysate were found (Figure 2B, lower panel).

Figure 2. (A) Co-immunoprecipitation and in vitro kinase detection of VEGFR-2 of PAEC and PAEC-KDR. (B) Representative Western blot of soluble and full length VEGFR-2 in PAEC-KDR. ADAM-10 inhibition (GI254023X, 5 μ mol/L) or ADAM-10 and ADAM-17 inhibition (GW280264X, 5 μ mol/L) inhibits both constitutive and VEGF-induced VEGFR-2 shedding (B). ** $p < 0.001$

VEGF induces ADAM-10 expression in endothelial cells

Besides a physical interaction we examined the functional interaction between ADAM-10 and the VEGFR-2 pathway to determine whether ADAM-10 is involved in downstream VEGFR-2 functions. VEGF increased ADAM-10 mRNA expression up to 34% in PAEC-KDR (data not shown) and HUVEC (Suppl. figure II, $p \leq 0.01$). Besides mRNA, we analyzed VEGF-induced ADAM-10 protein expression in ECs. In HUVECs ADAM-10 is expressed as a ~135 kDa pro-enzyme which is processed into a ~90 kDa mature and active enzyme (Figure 3A and B). VEGF stimulation of HUVECs for 24 hrs

significantly induced total ADAM-10 protein expression up to 1.95 fold (figure 3C, $p<0.01$). Most interestingly, short-term stimulation with VEGF resulted in a 2.1 fold increase of processed, mature i.e. active ADAM-10 in HUVECs (Figure 3D, $p<0.01$). Similar results were found in PAEC-KDR (data not shown). These data indicate that VEGF stimulation of EC not only induces total ADAM-10 expression at both the mRNA and protein level, but could also induce the processing of the latent enzyme into the activate ADAM-10 protein.

ADAM-10 activity in endothelial cells is induced by VEGF

To confirm ADAM-10 activation by VEGF stimulation in EC, we used a fluorogenic peptide as a substrate, which emits a fluorescent signal when degraded. As expected, PMA and ionomycin induced peptide degradation in HUVEC. Stimulation with 50 ng/ml VEGF significantly induced peptide degradation in 6 hrs by 1.4 fold in HUVECs (Figure 3E, $p<0.05$). Since this peptide can also be degraded by ADAM-17 and ADAM9, the inhibitors GI254023X and GW280264X were used to determine the contribution of ADAM-10 to VEGF-induced peptide degradation. Inhibition of ADAM-10 alone by GI254023X reduced basal peptide degradation by 10% ($p=0.08$). Interestingly, VEGF-induced peptide degradation was not significantly stimulated anymore when ADAM-10 was inhibited by GI235024X (Figure 3E). Combined ADAM-10 and ADAM-17 inhibition by GW280264X reduced basal and VEGF-induced peptide degradation (18%, $p<0.01$ and 41%, $p<0.001$ respectively). Similar results were found in PAEC-KDR (data not shown).

VEGF stimulates several signaling pathways by VEGFR-2 activation^{2, 3}. Therefore we used specific inhibitors to identify the signaling pathways required for VEGF-induced ADAM-10-activity. In non-stimulated HUVEC, inhibition of PI3K, p38 and ERK1/2 activation slightly reduced basal peptide degradation but not significantly. Interestingly, VEGF-dependent increase of peptide degradation was significantly inhibited by ERK1/2 inhibition ($p<0.05$). Similar results were found by PI3K inhibition, while no significant effects were seen with p38 inhibition (Supplementary figure III). These data indicate that VEGF induces ADAM-10 (and ADAM-17) activity in ECs via ERK1/2 and potentially PI3K signaling.

Figure 3. VEGF increases HUVEC total protein (A-C) and mature ADAM-10 expression (B-D, * $p < 0.05$). (E) VEGF-induced fluorogenic peptide degradation (6 hrs; # $p < 0.05$ vs DMSO without stimulus) is inhibited by GI254023X (5 $\mu\text{mol/L}$). GW280264X (5 $\mu\text{mol/L}$) reduces basic and VEGF-induced peptide degradation (* $p < 0.05$).

VEGF induces ADAM-10-mediated VE-cadherin cleavage, thereby increasing permeability

ADAM-10 can cleave several adhesion molecules, e.g. N-cadherin, E-cadherin and the endothelial specific VE-cadherin¹³. Cleavage of VE-cadherin was shown to increase endothelial permeability and T-cell transmigration, two important processes in angiogenesis and atherosclerosis. To investigate whether VEGF could induce this functional activity of ADAM-10, we stimulated HUVEC for 24 hrs with VEGF in presence or absence of GI254023X or GW280264X. The γ -secretase inhibitor DAPT (10 $\mu\text{mol/L}$) was used to prevent further intracellular processing of VE-cadherin after initial cleavage by ADAM-10, allowing quantification of the cleaved intracellular fragment (Figure 4A). Both GI254023X and GW280264X

completely blocked cleavage of VE-cadherin, confirming indeed the role of ADAM-10 in cleavage of this EC adhesion molecule (Figure 4B). Interestingly, VEGF stimulation increased VE-cadherin cleavage by 41% ($p=0.02$), which was prevented by ADAM-10 inhibition ($p<0.01$). Results of pharmacological inhibition of ADAM-10 and ADAM-17 on VE-cadherin cleavage were confirmed using an RNAi approach. HUVEC transfection with specific ADAM-10 siRNAs resulted in specific knockdown of ADAM-10 protein (70–80%) without affecting ADAM-17 expression (Supplementary figure IV). Similarly, specific ADAM-17 knockdown (50–60%) was found without a significant effect on ADAM-10 expression. Interestingly, specific knockdown of ADAM-10 significantly inhibited VE-cadherin cleavage in HUVEC (60% reduction, $p<0.0001$ for unstimulated HUVEC, Figure 4C-D). ADAM-17 knockdown in HUVEC did not block VE-cadherin. This indicates the involvement of ADAM-10, but not ADAM-17 in VE-cadherin cleavage in HUVEC.

Since ADAM-10 mediated VE-cadherin cleavage was shown to increase vascular permeability and leukocyte transmigration, we investigated whether VEGF could mediate its effect on vascular permeability by ADAM-10. HUVEC were grown to confluency and electrical resistance was measured using the Electric Cell-substrate Impedance Sensing (ECIS) system. Electrical resistance of HUVEC without VEGF-treatment remained constant throughout the experiment (data not shown). As expected, after a transient increase in resistance, VEGF induced a gradual decline in cellular resistance corresponding to an increased permeability (Figure 4E). A similar VEGF-induced decline in resistance was found when only ADAM-10 was inhibited by GI254023X. However, combined inhibition of ADAM-10 and ADAM-17 by GW280264X attenuated the VEGF-induced decline in resistance (Figure 4E). This indicates that, whereas ADAM-10 inhibition alone is sufficient to block VE-cadherin cleavage, VEGF-induced EC permeability is regulated by both ADAM-10 and ADAM-17, probably involving cleavage of other cell-cell adhesion molecules besides VE-cadherin.

Figure 4. VEGF induces VE-cadherin cleavage in HUVEC, which is completely blocked by GI254023X or GW280264X (5 μ mol/L, A-B) or ADAM-10 siRNA, but not ADAM-17 siRNA (C-D). (E) ECIS permeability measurement of HUVEC pre-treated with 5 μ mol/L GI254023X, GW280264X or DMSO followed by 100 ng/ml VEGF stimulation. * p <0.05, ** p <0.01, n =3.

ADAM-10 is required for endothelial cell and monocyte migration and chemotaxis

Since cleavage of adhesion molecules is essential for cell migration, we examined the requirement of ADAM-10 for EC migration and chemotaxis using a Boyden chamber. Baseline EC migration (chemokinesis) was significantly reduced (39%) when ADAM-10 was inhibited by GI254023X, whereas VEGF-induced chemotaxis was reduced by 41% (Figure 5A, p =0.005 and p =0.02, respectively). Besides EC, monocytes are critical players in cardiovascular disease, not only in atherosclerosis,

but also by participating in neovascularization¹⁴. ADAM-10 inhibition also significantly reduced both monocyte chemokinesis (50%, Figure 5B) and chemotaxis (52% for VEGF and 36% for fMLP), indicating that ADAM-10 is required for cell migration, most likely by facilitating cell detachment by cleavage of cell adhesion molecules.

Using Electric Cell-substrate Impedance Sensing (ECIS) we measured the migratory healing response of HUVEC after wounding by a high electrical field. In control cells, resistance returned to steady state conditions within 8 hrs after wounding, indicating that the response is rather a result of EC migration than proliferation (Figure 5C). Inhibition of ADAM-10 by GI254023X delayed this wound healing response which was even more pronounced when both ADAM-10 and ADAM-17 were inhibited by GW280264X.

Figure 5. (A) GI254023X (5 μ mol/L) reduces PAEC-KDR migration and chemotaxis towards 10 ng/ml VEGF. (B) GI254023X reduces monocyte migration and chemotaxis towards 10 ng/ml VEGF and 10^{-8} mol/L fMLP. * $p < 0.05$, $n = 3$. (C) GI254023X and GW280264X (5 μ mol/L) reduces HUVEC migration response after applying a high electrical field.

Discussion

In this study we identified ADAM-10 as a novel mediator of VEGF-induced EC function and provide first evidence of ADAM-10 interaction with VEGFR-2. VEGF induces both ADAM-10 expression and activity. We showed ADAM-10 to be required for VEGF-induced VE-cadherin cleavage, vascular permeability and EC migration. This is the first report to document ADAM-10 expression in human atherosclerotic lesions, associated with atherosclerotic plaque progression and neovascularization.

Using a yeast-two-hybrid screening we identified ADAM-10 as novel binding candidate for VEGFR-2, which was supported by co-immunoprecipitation of VEGFR-2 with ADAM-10 in PAEC-KDR. This interaction could be of functional significance in endothelial biology. Recently, Swendemann *et al.* showed the highly related ADAM-17 to mediate VEGFR-2 shedding, which could be induced by VEGF¹⁵. For ADAM-10 they reported a role in shedding of neuropilin-1, the co-receptor for VEGFR-2. In this study, we showed not only ADAM-17, but also ADAM-10 to be involved in VEGFR-2 ectodomain shedding. Shedding of VEGFR-2 and neuropilin-1 could provide a mechanism to modulate VEGFR-2-signaling, e.g. as a negative feedback loop to dampen the VEGF stimulatory signal. Such an interaction would be transient, which might also explain why we could only find a small amount of VEGFR-2 co-precipitating with ADAM-10.

Besides a physical interaction, we demonstrated a functional interaction between ADAM-10 and the VEGFR-2-pathway. We found VEGF to induce not only ADAM-10 expression, but more importantly, to stimulate its proteolytic activity. The exact mechanism of activation of ADAM-10 by VEGF remains to be elucidated. In this study, we found evidence of ERK1/2 involvement in VEGF-induced peptide degrading activity of ADAM-10 (although the involvement of ADAM9 and ADAM-17 can not be excluded in this assay). VEGF is known to increase intracellular calcium levels by activating PLC¹⁶. Increased intracellular calcium has been shown to induce ADAM-10 processing (i.e. activation) by furin-like proteases¹⁷. Whether induction of ADAM-10 activity by VEGF is indeed dependent on calcium influxes and activity of furin or other proteases remains to be determined.

In this study, we showed VEGF to stimulate cleavage of the endothelial specific adhesion molecule VE-cadherin by ADAM-10, but not ADAM-17, using both pharmacological inhibitors and specific siRNAs for ADAM-10 and ADAM-17. Recently, VE-cadherin cleavage has been shown to increase EC permeability and T cell transmigration¹⁸. VEGF is well known to induce endothelial permeability by promoting phosphorylation, endocytosis of VE-cadherin and subsequent disruption of adherens junctions¹⁹. Our data clearly demonstrate a role for ADAM-10, but also ADAM-17, in VEGF-induced EC permeability. Whereas VE-cadherin cleavage was nearly completely abrogated when only ADAM-10 was inhibited, VEGF-mediated HUVEC permeability was only attenuated by inhibition of both ADAM-10 and

ADAM-17. This might be explained by ADAM-10/17-mediated cleavage of other EC junctional adhesion molecules that are involved in regulating endothelial permeability. Recently, Koenen *et al.* showed that cleavage of Junctional Adhesion Molecule-1 (JAM-1) in EC to be predominantly mediated by ADAM-17 and to a lesser extent by ADAM-10²⁰. Since JAM-1 is also involved in regulating endothelial permeability²¹, this might explain why it is necessary to inhibit both ADAM-10 and ADAM-17 to effectively block VEGF-mediated increase in permeability, even though ADAM-10 inhibition alone is sufficient to block cleavage of individual adhesion molecules, i.e. VE-cadherin. Increased EC permeability has also been implicated in progression and rupture of atherosclerotic lesions, where the microvasculature has been proposed to be immature and 'leaky'²². Consequently, leukocytes can transmigrate through the endothelium more easily and enter the atherosclerotic plaque, where they can contribute to plaque progression and destabilization.

Besides adhesion molecules, ADAM-10 is known to be involved in shedding of numerous chemotactic and inflammatory molecules such as CXCL16, fractalkine, IL-6 receptor, TNF α , RAGE and CD40L²³, all molecules known to contribute to atherogenesis and plaque progression. It is therefore not surprising that we found increased ADAM-10 expression in advanced human atherosclerotic plaques with a high number of infiltrating cells and a high degree of inflammation. Furthermore, we found high ADAM-10 expression associated with plaque neovascularization as well as tumor neovascularization. ADAM-10 has been implicated in the proteolytic cleavage of several proteins regulating arteriogenic and angiogenic processes, e.g. members of the Notch pathway²⁴ and Ephrin (ligands)²⁵. Recently, VEGF was shown to induce shedding of the angiopoietin receptor Tie-2, which likely is mediated by ADAM-10²⁶. Furthermore, VEGF-induced Notch activation in EC was ADAM-10 dependent (own, unpublished data). Activation of Notch has been shown to be involved in the regulation of vessel sprouting²⁷, which, besides inhibiting EC migration, provides another mechanism by which ADAM-10 inhibition could modulate neovascularization responses. These functional data of a role of ADAM-10 in (VEGF-induced) endothelial cell functions, together with our finding of a direct interaction between ADAM-10 and VEGFR pathway, which by shedding of the VEGFR-2 could modulate VEGFR-2-signaling and downstream endothelial cell functions, strengthen the hypothesis that ADAM-10 plays a crucial role in various mechanisms underlying angiogenic and atherosclerotic processes. Furthermore, it may also have important implications for other diseases in which neovascularization contributes to disease progression such as cancer.

Besides ADAM-10, other ADAM proteins may be involved in the angiogenic and atherosclerotic processes. As mentioned above, ADAM-17 is related to ADAM-10 and shares several substrates. Nevertheless, differences in regulation between ADAM-10 and ADAM-17 functions have been reported, i.e. in pathways leading to activation of the proteases, in constitutive versus regulated proteolytic activity and

substrate specificity^{13, 15, 28}. Interestingly, ADAM-17 expression has been associated with both atherosclerotic lesion progression and resistance to atherosclerosis in mice^{29, 30}. Recently, ADAM-17 was also shown to be involved in endothelial tube formation *in vitro* and pathological neovascularization *in vivo*³¹. Pathological retinal neovascularization and growth of heterotopically injected tumor cells was reduced in mice that lack ADAM-17 specifically in the endothelial cells. Furthermore, ADAM15 has been shown to mediate inflammation and was found to be associated with atherosclerosis³². Therefore, it will be important to further elucidate the exact mechanisms of ADAM functions in cardiovascular diseases, and most challenging to determine specificity of ADAM-10 functions compared to related ADAMs. In this study, we used two approaches to inhibit ADAM-10 and ADAM-17 activities, i.e the hydroxamate-based compounds GI254023X and GW280264X, which were designed to discriminate between ADAM-10 and ADAM-17¹², and specific knockdown of either ADAM-10 or ADAM-17 using siRNA. Both approaches showed similar results. However, to identify the involvement of other distinct ADAM proteases in (VEGF-induced) endothelial cell functions additional selective inhibitors are needed. Furthermore, it will be an important task to determine the role of ADAM-10 in neovascularization and cardiovascular diseases *in vivo*. Since ADAM-10^{-/-} mice are embryonically lethal⁹, this will require the use of conditional knockout models.

In conclusion, this study shows - for the first time - expression of ADAM-10 in atherosclerotic lesions, associated with neovascularization. This provides the first evidence of an interaction between the VEGFR-2 pathway and ADAM-10, and a functional role of ADAM-10 in (VEGF-induced) endothelial cell functions. Therefore these data open perspectives for novel therapeutic interventions in neovascularization and vascular diseases.

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Supplement

Methods

Compounds

Recombinant human VEGFA₁₆₅ (VEGF) was purchased from Reliatech. The metalloprotease inhibitors GI254023X and GW280264X were a kind gift from GlaxoSmithKline. Inhibitors LY294002, PD98059 and SB203580 were purchased from Sigma and γ -secretase inhibitor IX (DAPT) was purchased from Calbiochem.

Yeast-two-hybrid screening

The nucleotide sequence corresponding to amino acids 790–1356 of the intracellular domain of human VEGFR-2 (VEGFR-2cyt) was amplified by PCR performed on the full length VEGFR-2 cDNA sequence. The following primers were used for PCR: sense primer: 5'-GGGTTTCATATGAAGCGGGCCAATGGAGGG-3' (NdeI restriction site) antisense primer: 5'-TGCGGTCGACTTAAACAGGAGGAGAGCTCAG-3' (Sall restriction site). As bait, this VEGFR-2cyt sequence was cloned between NdeI and Sall restriction sites into the pGTBKT7 plasmid and transformed into the AH109 yeast strain. A human placenta cDNA library was cloned into the pACT2 plasmid and transformed into the AH109 yeast strain according to the Matchmaker Gal4 Two-Hybrid system 3 (Clontech). From β -galactosidase-positive clones, cDNA was isolated and retransformed together with VEGFR-2cyt-pGBKT7 into the AH109 yeast strain to confirm the interaction. Confirmed positive clones were selected, sequenced and aligned with the human genome using BLASTn (NCBI).

Immunohistochemistry

Formalin-fixed paraffin sections or RNA samples of human atherosclerotic plaques were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage and use of tissue and patient data were performed in agreement with the 'Code for Proper Secondary Use of Human Tissue in the Netherlands'. Sections were (double) stained after antigen retrieval by boiling in 1x citrate pH 6 antigen retrieval buffer (Dako) with anti-ADAM-10 antibody (Chemicon 19026, 1:20), followed by incubation with biotin-labeled swine anti-rabbit antibody (1:1000, Dako). ABC-alkaline phosphatase and vector red/blue were used for visualization. For double stainings, sections were subsequently stained with anti-CD31 (1:50) combined with anti-CD34 (1:200) for EC and 1:200 biotin-labeled rabbit anti- mouse as secondary antibody. ABC^{hrp} and diaminobenzidine (DAB) were used for visualization.

Cell culture

Porcine Aortic Endothelial Cells overexpressing human VEGFR-2 (PAEC-KDR)¹ were grown in Ham's F-12 medium containing 10% FCS and gentamycin G418 until ~70–

90% confluency. Before stimulations, cells were serum starved overnight in Ham's F-12 medium containing 0.01% BSA and G418.

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained by trypsin perfusion of umbilical cords from the department of Obstetrics and Gynecology at the University Hospital Maastricht, as described previously². Cells were grown on gelatin in RPMI medium containing 10% FCS, 10% human serum and streptomycin/penicillin. Cells were serum starved in RPMI medium containing 1% FCS, 1% human serum and streptomycin/penicillin and used when subconfluent.

RNAi

HUVEC (70% confluency) were transfected with 10 nM of either ADAM-10 or ADAM-17 ON-TARGET plus siRNA SMARTpools or non-targeting siRNA pool (Dharmacon) using Interferin (polyplus, Illkirch, France) following manufacturer's protocol. After 48 hrs of transfection, cells were used for experiments. From all transfections, a fraction was lysed and analysed on Western blot for protein knockdown.

Co-immunoprecipitation and in vitro kinase assay

These assays were performed as previously described¹ with minor modifications. After overnight starvation, PAEC-KDR and PAEC were pre-incubated with 100 μ M sodium orthovanadate for 10 min and stimulated with 0–50 ng/ml VEGF for 3 min. Cells were lysed at 4°C in 0.05% NP40 lysisbuffer (containing 150 mmol/L NaCl, 20 mmol/L Tris pH 7.4, 10 mmol/L EDTA, 0.05% NP40 and protease cocktail (Roche)). After pre-clearance, KDR or ADAM-10 were precipitated with 2.5 μ g rabbit-anti-human VEGFR-2 antibody (Santa Cruz C1158) or affinity-purified rabbit-anti-human ADAM-10 antibodies (A3226, Sigma), respectively. As negative control, precipitation without primary antibody was performed using equal amounts of protein. Immune complexes were precipitated for 45 min at 4°C with protein A Sepharose beads (Amersham Biosciences) and incubated for 7 min at RT with 5 μ Ci/ μ l ³²P- γ -ATP in kinase buffer (50 mmol/L HEPES, 10 mmol/L MnCl₂, 1 mmol/L DTT, 0.05% Triton X100). Kinase reactions were stopped by boiling in loading buffer with β -mercaptoethanol. Samples were run on 8% SDS-PAGE gels, gels were fixed in 2.5% glutaraldehyde, and incubated for 1 hr. at 55 °C with 1 mol/L KOH to hydrolyze serine/threonine phosphorylations (to visualize only tyrosine phosphorylations), after which gels were dried and exposed to an X-ray film at -80 °C.

RNA isolation and quantitative real-time PCR

After overnight starvation, cells were stimulated for 8 or 20 hrs with 0, 0.5, 5, or 50 ng/ml VEGF. Cells were lysed and total RNA was isolated using the RNeasy mini kit (Qiagen) and DNase treatment according to manufacturer's instructions. cDNA was

synthesized from 10 ng RNA using random hexamer primers and oligoDT primers (Fermentas) and the Omniscript RT polymerase kit (Qiagen). Quantitative real time-PCR was performed on a MyiQ system (Bio-Rad) using Platinum SYBR Green qPCR Supermix UDG (Invitrogen) containing 1:50 Fluorescein (Molecular Probes) and the following primers with annealing temperatures of 60 °C: human ADAM-10: Fw 5'-TTGCCTCCTCTAAACCACTTCCA-3' and Rev 5'-AGGCAGTAGGAAGAACCAAGGCAA-3'; human ADAM-17: Fw primer 5'-GGGAAGTGAAGTACAGATG-3' and Rev. 5'-CTAGATTCACCTTCACCTTACC-3'; human β -actin: Fw primer 5'-ATCCTCACCTGAAGTACCC-3' and Rev 5'-CACGCAGCTCATTGTAGAAG-3'; human GAPDH: Fw 5'-GCCTCAAGATCATCAGCAAT-3' and Rev 5'-GGA CTGTGGTCATGAGTCCT-3'; porcine β -actin: Fw 5'-GCATCTGACCCTCAAGTAC-3' and Rev 5'-CACGCAGCTCGTTGTAGAAG-3'; porcine GAPDH: Fw 5'-GTGTCGGTT GTGGATCTGA-3' and Rev 5'-CCTGCTTCACCACCTTCTT-3' (Eurogentec). Data were analyzed with the iQ5 software (Bio-Rad) and expressed relative to the housekeeping gene expression using the ddCt- method.

VEGFR-2 ectodomain shedding

PAEC-KDR were cultured in T25 cell culture flasks and serum-starved overnight once 70–90% confluency was reached. The next day, cells were given 2 ml fresh starvation medium containing either 5 μ mol/L GI54023X, 5 μ mol/L GW280264X or DMSO as control and stimulated for 5 hrs with 0 or 50 ng/ml VEGF. Cell culture medium was collected and concentrated 40x by 10 min centrifugation at 3000 g using Vivaspins columns with 10000 MWCO PES filter (Sartorius). Complete concentrated media were loaded on 8% SDS-PAGE gels and release of soluble VEGFR-2 ectodomain was detected by Western blotting.

Western Blotting

ECs were lysed by cell scraping and rotating for 30 min at 4°C in a 1% NP40 lysisbuffer containing 1x protease inhibitor cocktail (Roche) and 100 μ mol/L NaVO₃. Equal amounts of proteins were separated on 8% SDS-PAGE gels and transferred to a nitrocellulose membrane (Amersham). Blots were blocked with 5% milk in PBS-0.1% Tween, and incubated with 1:5000 rabbit anti-human ADAM-10 antibody (Chemicon), 1:500 mouse anti-human VEGFR-2 antibody (Acris) or 1:5000 mouse monoclonal anti-human VE-cadherin antibody (Santa Cruz) in blocking solution, followed by addition of the appropriate horseradish peroxidase labelled secondary antibody and West Dura ECL detection (Pierce). As loading control, β -actin expression was detected using 1:15000 mouse anti- β -actin monoclonal antibody (Sigma). Chemiluminescence was detected using LAS 1000 imaging system (Fuji), analyzed using Aida software analysis and results were expressed relative to β -actin expression.

Electric Cell-substrate Impedance Sensing (ECIS)

HUVEC were grown to confluency on fibronectin-coated electrode arrays. When basal electrical resistance of the monolayer reached a plateau, cells were pre-incubated with 5 $\mu\text{mol/L}$ GI54023X, 5 $\mu\text{mol/L}$ GW280264X or DMSO as control for 30 min. After steady state was reached, cells were treated with 0 or 100 ng/ml VEGF and electrical resistance was monitored on-line with the ECIS-Model-100 Controller (BioPhysics, Troy, USA) and changes in resistance were analyzed as previously described³.

At steady state conditions, an elevated field (3 V at 40,000 Hz for 10 s) was applied to a confluent layer of HUVEC to wound the cells on the electrode and wound healing was monitored for 10–20 h by ECIS measuring transendothelial electrical resistance.

Cell migration

Monocytes were isolated from human peripheral blood by negative selection using the MACS monocyte isolation kit II (Miltenyi Biotech) as described previously⁴. ECs were serum starved overnight and migration was assayed using a modified Boyden chamber⁵. A total of 25000 cells treated with 0–5 $\mu\text{mol/L}$ GI54023X (selective ADAM-10 inhibitor) were loaded in quadruplicate onto a 5–8 μm pore membrane (Whatmann) uncoated for monocytes or coated with 5 $\mu\text{g/ml}$ fibronectin (Sigma) for EC, respectively. Migration was stimulated with 10 ng/ml VEGF or 10^{-8} mol/L fMLP (N-formyl-Met-Leu-Phe, Sigma). After 1.5 hrs (monocytes) or 4 hrs (EC) migration, membranes were fixed with 100% ethanol and stained with Giemsa (Merck). Non-migrated cells on the upper membrane surface were removed before embedding the membrane in Neomount (Merck). Migrated cells were counted in five random high power fields per well.

Figures

Supplemental figure I .Immunohistochemical staining of ADAM-10 in human mammary artery (A) and double-staining of ADAM-10 (blue) and CD31 (brown) in advanced atherosclerotic plaque (B). L=lumen, IEL=internal elastic lamina.

Supplemental figure II. VEGF induces ADAM-10 mRNA expression after 8 hrs stimulation in HUVECs as assessed by quantitative real-time PCR and expressed relative to β -actin and GAPDH housekeeping gene expression. * $p < 0.05$, $n = 3$.

Supplemental figure III. Using 10 $\mu\text{mol/L}$ of the specific inhibitors LY294002 (PI3K), PD98059 (ERK1/2) and SB203580 (p38) we showed VEGF-induced fluorogenic peptide degradation was significantly reduced by inhibition of ERK1/2 (* $p=0.02$, $n=3$) and showed similar results for PI3K inhibition ($p=0.06$, $n=3$).

Supplemental figure IV. ADAM-10 Western blot (left) and ADAM-17 Western blot (B) detection shows reduction of ADAM-10 expression by ADAM-10 siRNA transfection, but not ADAM-17 or control siRNA, while ADAM-17 expression is specifically reduced by ADAM-17 siRNA transfection in HUVEC ($n=3$).

Table I. Results of Yeast-two-hybrid screening using the intracellular domain of human VEGFR-2 as bait and screening against a human placenta cDNA library.

Screen nr.	Number of transfections	Number of colonies	Number of β -gal positive clones
1	1.64×10^4	0	0
2	120	2	2
3	3361	24	4
4	3.4×10^4	401	40
5	2.36×10^4	563	47
total		990	93

Table II. Semi-quantitative analysis of ADAM-10 expression in human atherosclerotic plaques. Staining scores: 0 = negative, 1= faint, 2= moderate, 3= strong. * $p < 0.05$ vs early and advanced stable.

Plaque number	Normal vessel wall (n=2)	Early (n=5)	Advanced (n=8)	stable	Ruptured (n=5)
1	0	1	2		2
2	0	2	2		3
3		2	3		3
4		1	2		3
5		2	3		3
6			2		
7			1		
8			2		
Mean $\pm$ SEM	0 ± 0	1.6 ± 0.2	2.1 ± 0.2		$2.8 \pm 0.2^*$

Table III. Semi-quantitative analysis of ADAM-10 expression in human colon tissue. Staining scores: 0 = negative, 1= faint, 2= moderate, 3= strong. * $p < 0.05$ vs normal.

Tissue number	Colon tissue		Microvasculature		Tumor Stroma (n=5)
	Normal (n=5)	Carcinoma (n=5)	Normal (n=5)	Carcinoma (n=5)	
1	1	2	1	2	1
2	2	2	1	2	2
3	1	3	2	3	1
4	1	3	1	2	1
5	1	3	2	3	2
Mean $\pm$ SEM	1.2 ± 0.2	$2.6 \pm 0.2^*$	1.4 ± 0.2	$2.4 \pm 0.2^*$	1.4 ± 0.2

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Chapter 4

Developmental Coronary Maturation is Disturbed by Aberrant Cardiac VEGF-expression and Notch-Signaling

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Abstract

Currently, many potential cardiac revascularization therapies are targeting the Vascular Endothelial Growth Factor (VEGF)-pathway, with variable success. Knowledge regarding the role of the VEGF/Notch/ephrinB2-cascade in (ab)normal coronary development will provide information on the subtle balance of VEGF-signaling in coronary maturation and might enhance our therapeutic possibilities. The effect of VEGF-isoforms on coronary development was explored *in vivo* using immunohistochemistry and RT-qPCR on *Vegf120/120* mouse embryos, solely expressing VEGF120. *In vitro*, human arterial coronary endothelial cells were treated with VEGF121 or VEGF165 upon which RT-qPCR was performed. *In vivo*, mutant coronary arterial endothelium showed a decrease in protein expression of arterial markers such as cleaved Notch1, Delta-like4 and ephrinB2 concomitant with an increase of venous markers such as chicken ovalbumin upstream promoter transcription factor II. The venous endothelium showed the opposite effect, which was confirmed on mRNA-level. *In vitro*, mRNA-expression of arterial markers highly depended on the VEGF-isoform used, with VEGF165 having the strongest effect. Also, coronary arteriogenesis was anomalous in the mouse embryos with decreased arterial and increased venous medial development as shown by staining for smooth muscle α -actin, Delta-like1 and Notch3. We demonstrate that VEGF-isoform related spatiotemporal cardiac alterations in the VEGF/Notch/ephrinB2-cascade lead to disturbed coronary development. This knowledge can contribute to optimizing therapies targeting VEGF-signaling by enabling balancing between angiogenesis and vascular maturation.

Introduction

Morphological diversity between different populations of endothelial cells (ECs) has long been recognized to relate to functional variation and as such proper physiology. More recently, differential expression profiles between arterial, microcirculatory, venous and lymphatic endothelial populations have been described¹, further underscoring endothelial diversity. Both intrinsic and microenvironmental differences are instructive for these endothelial characteristics². Abnormalities can lead to endothelial dysfunction and subsequent pathogenesis, as found for maternal diabetes-related fetal endothelial dysfunction³, increased nuchal translucency⁴, tumor angiogenesis⁵ but also for coronary atherosclerosis⁶ and adult venous bypass graft disease⁷.

Numerous factors associate with endothelial functioning in processes such as vasculogenesis, angiogenesis and arteriogenesis, with one of the key factors being Vascular Endothelial Growth Factor (VEGF)⁸. Therefore, it is not surprising that many potential therapies for ischemic cardiovascular disease target VEGF, although clinical success is less than expected⁸. VEGF exerts its main effects through binding to the VEGF receptor VEGFR-2⁸. Its coreceptor neuropilin-1 (NP-1) can enhance this effect⁹. VEGF-signaling can upregulate members of the Delta-like/Jagged/Notch-family, proteins highly important in cardiovascular development^{10, 11}. Several members of this family are specifically expressed by arterial ECs, including Notch1, Notch4 and Delta-like4 (Dll4)¹². Upon stimulation of the Notch-receptor, its cytoplasmic domain becomes cleaved and translocated to the nucleus. There, it forms a complex with other nuclear proteins, which upregulates transcription of Notch-signaling specific transcription factors (i.e. Hes, Hey). These can enhance arterial EC-specific ephrinB2-expression¹⁰, suggesting a role for the VEGF/Notch/ephrinB2-cascade in arterial differentiation¹¹.

The development of the coronary vasculature is of eminent importance for the final phases of cardiogenesis. Disbalance in these processes might lead to a dysfunctional vascular bed which will predispose for coronary diseases. Coronary development starts with the occurrence of a subepicardially located primitive endothelial network. This becomes connected to the systemic circulation downstream to the right atrium and upstream to the ascending aorta through coronary orifices at the level of the left and right sinus of the semilunar valves¹³. After connection to the circulation is established, arterial differentiation of the most proximal ECs becomes obvious and, subsequently, arteriogenesis is stimulated by inducing vascular smooth muscle cell (vSMC) migration and differentiation^{13, 14}. In the coronary system, vSMCs are mainly recruited from the population of epicardium-derived cells (EPDCs), which arises through epithelial-to-mesenchymal transformation of cells of the epicardium¹⁵. In addition, microcirculatory and venous endothelial differentiation and remodeling of the primitive endothelial network into a fully functional coronary system will take place.

We recently described the *Vegf120/120* mouse model that solely expresses the non-heparin binding isoform VEGF120. Structural cardiac abnormalities such as Tetralogy of Fallot (TOF) were found concomitant with alterations in VEGF and Notch-signaling^{16, 17}. This mouse model presents postnatally with impaired myocardial angiogenesis¹⁸ and altered retinal arterio-venous differentiation¹⁹. In this paper, we show that changes in spatiotemporal distribution of VEGF and Notch-signaling -due to altered VEGF-isoform expression in *Vegf120/120* embryos- lead to anomalous coronary EC differentiation and disturbed arteriogenesis.

Methods

Mouse Experiments

All animal experiments were approved by the Animal Ethics Committee of the Leiden University and executed according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996). For an extensive description of mouse experiments see¹⁷.

Immunohistochemistry, in situ hybridisation and microscopy

Immunohistochemistry was performed as described¹⁷. In short, immunohistochemistry was carried out on sections of 5 µm of 4%PFA/0.01 Mol/L phosphate buffered fixed, paraffin embedded tissue. Antigen retrieval was achieved either by heating the sections (12 minutes to 98° C) in citric acid buffer (0.01 Mol/L, pH6.0) or by incubating them for 6 min in 0.1 mg/mL Pronase E (1.07433.0001, Merck, Darmstadt, Germany) in PBS. Visualization was performed with the DAB-procedure and Mayer's hematoxylin was used as a counterstaining. Differences between genotypes were scored per immunostaining in at least 3 different embryos per genotype per age-group (i.e. E10.5-E14.5 or E15.5-E19.5) that were processed within one and the same experiment. The primary antibodies were checked for false positive signals. An extensive description of the primary antibodies used is listed in Table S1. The α-Notch4-antibodies listed in Table S1 were tested to bind aspecific to the material used and therefore no characteristics were mentioned. For in situ hybridisation, sense and anti-sense ³⁵S-radiolabelled *Vegf*-A RNA probes were transcribed using a 451-bp clone encoding for the mouse *Vegf*-120 isoform (pVEGF2; kindly provided by Dr. G. Breier, University of Technology, Dresden, Germany). Micrographs were made using an Olympus AX70-microscope fitted with Olympus UPlanApo-objectives (Olympus, Tokyo, Japan). The camera used was an Olympus DP12 and micrographs were fitted into a panel using Adobe Photoshop CS2 (Adobe, San Jose, USA).

3D-reconstructions

3D-reconstructions were performed as described⁴. In short, micrographs were made of E18.5 wild type and *Vegf120/120* hearts and converted to 3D using ResolverRT™-software (Template Graphics Software Inc., San Diego, USA).

Morphometry

For morphometry, VEGFR-2 stained sections of 8 embryos (4 *Vegf*^{+/+} and 4 *Vegf120/120*; E17.5-E19.5) were used. To calculate the total heart volume and the ratio myocardial to vascular volume, we performed volume measurements for total heart volume, myocardium and coronary veins/microvasculature using the Cavalieri method²⁰. Statistical analysis was performed using a Mann-Whitney test with SPSS 11.0 (SPSS Inc., Chicago, USA) software.

Cell culture

Human coronary artery endothelial cells (HCAECs) were obtained from Cambrex (Verviers, Belgium) and cultured according to the protocol of the manufacturer. Cells at passage 9 were seeded at a density of 3500 cells/cm² and cultured to a 70% subconfluent monolayer in EGM-2-MV BulletKit without bFGF and VEGF165 supplemented with 5% Fetal Bovine Serum (Cambrex). Subsequently, HCAECs were stimulated for 24 hours with 100 ng/mL recombinant human VEGF165 or VEGF121 (RELIATech GmbH, Braunschweig, Germany). Cells were harvested according to the manufacturer's protocol using a trypsin/EDTA and a Trypsin neutralization solution (Cambrex). Cell number was determined using trypan blue dye and the Neubauer improved chamber method. mRNA was isolated as described below. Per sample, 100,000–140,000 cells were harvested. Samples stimulated with either VEGF165 or VEGF121 provided higher cell numbers than unstimulated samples (cultured without VEGF).

RT-qPCR

Total RNA from triplicate HCAEC cultures or from cardiac tissue of E18.5 wild type and *Vegf120/120* embryos (n=3 per group) was isolated by using the RNeasy micro-kit Qiagen with DNase treatment (Qiagen, GmbH, Hilden, Germany). Sample with sufficient RNA quality (OD260/280>1.9, RIN>8.0) and content were approved for analysis. A total of 100 ng total RNA per sample was subjected to reverse transcription (RT). qPCR was performed by using Superscript™III Platinum Two-step qRT-PCR kit with SYBR green (Invitrogen, Paisley, UK) and primer concentration of 10 µM. qPCR reactions were run on a MyiQ Single-Color Real time PCR detection System (Bio-Rad, Veenendaal, the Netherlands). Primers were designed with oligoperfect™ Designer (Invitrogen), Primer3 and Mfold (<http://www.idtdna.com/scitools/Application/mfold/>) and were synthesized by Eurogentec (Seraing, Belgium). All primers used met the requested qPCR efficiency

(between 80–105%) and were found to be gene-specific. Primer sequences are found in Table S2. Data analysis was performed using a Microsoft Excel spreadsheet based on the qBase program²¹. Statistic significance was determined by applying one-way ANOVA/Tukey (SigmaStat v2.03, Systat Software, San Jose, USA) testing. Sample with a probability value (p) <0.05 were regarded to be significant different between groups. HCAEC samples were normalized for input based on both β -actin and GAPDH-values. For the E18.5 embryos normalization was based on the pan-endothelial marker *Tie2*. No significant differences were found for *Tie2* between wild type and *Vegf120/120* littermates as determined after normalization for β -*Actin* and *Gapdh* expression (correction for cDNA input). Using immunohistochemistry, no altered myocardial expression of the genes investigated was found. Myocardial Notch4-expression was not determined due to aspecific binding of the antibodies tested (see table S1), however, Notch4 has been reported to be highly selective for ECs during development²².

Results

Anomalous coronary morphology in Vegf120/120 mouse embryos

In line with literature on murine coronary development²³, the first appearance of coronary ECs and vessels was seen at E11.5 when using an α -VEGFR-2-staining for visualization of the ECs. We observed that at this stage, primitive coronaries already connected (in)directly to the right atrium in all embryos, suggesting normal coronary development in mutant embryos (Figures 1A-B,E,H). However, abnormally large coronary veins were discerned in 22/33 early-stage (E11.5-E13.5) mutant cases (Figure 1A-B,F-G).

In wild type embryos, two definitive coronary orifices, originating from the left and right aortic sinus respectively, were observed in 24/24 cases of E15.5 and older. In contrast, only 15/28 *Vegf120/120* embryos of these stages presented with 2 coronary orifices, whereas others showed only one (2/28), three (7/28) or in one case four orifices (1/28 and Table 1). After formation of the coronary orifices, maturation of coronary arteries was obvious in normal embryos. In 22/28 mutant embryos between E15.5 and E19.5, arteriogenesis was severely impaired (Table 1; Figure 1C-D and movies online). Half of the mutant embryos showed abnormalities in anatomy of the primary coronary branches (Table 1; Figure 1C-D and movies online), with the main artery supplying the interventricular septum (IVS) either having its own coronary orifice and/or originating from the left instead of the right sinus, which is normal²⁴. All embryos in which the IVS-branch originated (in)directly from the left sinus were previously diagnosed with TOF (5/28)¹⁷. For a more detailed description of the abnormalities in coronary branching patterns we refer to the online supplement.

In 50% of E15.5-E19.5 *Vegf120/120* embryos (14/28) enlarged coronary veins (Table 1; Figure 1J-K), already evident at earlier stages, and enlarged cardiac lymphatics (16/28; Table 1; Figure 1L-M), visualized using staining for LYVE-1 of which expression is specific for lymphatic ECs, were encountered. Also, in late stage embryos, ventriculo-coronary arterial communications (VCACs) and/or coronary arterio-venous shunts were evident (Table 1; Figure 1N,O). Arterio-venous shunts between the aortic arch and vena cava superior were found in 3 cases (all E19.5; Figure 1P).

Table 1. Coronary abnormalities in *Vegf120/120* embryos of E15.5-E19.5.

Coronary Anomaly	No/Total	%
Abnormal number of coronary orifices	10/28	36
< 2	2/28	7
> 2	8/28	29
Small coronary arteries	22/28	79
Abnormal origin IVS-branch	14/28	50
Large coronary veins	14/28	50
Large cardiac lymphatics	16/28	57
Increased microvascular density	17/28	61
VCAC	10/28	36

IVS = indicates interventricular septum; VCAC = indicates ventriculo-coronary artery communication

Spatiotemporal distribution of Vegf in normal and mutant embryonic hearts

During early cardiac development (E10.5-E14.5), the distribution of *Vegf*-expression has been described before¹⁷ and is likely mainly related to myocardial and to a lesser extent to coronary development. In later stages (E16.5 and older) the predominant area of *Vegf*-expression was the borderline of compact to trabecular myocardium, overlapping with the region where prominent coronary angiogenesis and arteriogenesis takes place at this stage²⁵. *Vegf* levels in this area were increased in *Vegf120/120* embryos (Figure 1Q-R).

Loss of coronary venous endothelial differentiation in Vegf120/120 embryos

Besides morphological differences in mutant veins, abnormal differentiation of coronary venous ECs was clearly distinguishable from E15.5 onwards (Table S3). High expression per EC of VEGFR-2 was still seen at later stages of development (Figure 1J-K), together with anomalous expression of arterial markers including cleaved (nuclear/activated) Notch1, Notch2, Dll4 and ephrinB2 (Figure 2A-B,E-F,I-J,M-N), suggesting increased VEGF and Notch-signaling per EC. Expression of the venous EC-markers chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and EphB4 (Figure 2Q-R,U-V) was clearly reduced in mutant coronary venous ECs.

RT-qPCR, normalized for endothelial-specific Tie2-expression, was performed on whole hearts of either wild type mouse embryos or *Vegf120/120* littermates of E18.5. A significant increase of *Notch1*, *Notch4* and *Hes1* mRNA-levels could be found (Figure 3A). Furthermore, a significant decrease in *Ephb4* mRNA was obvious, while a positive trend was noticed for expression of *Dll4* and *Hey1* (Figure 3A).

← **Figure 1. Abnormal coronary and aortic arch morphology and *Vegf*-distribution in *Vegf120/120* embryos.** Wild type (+/+) and mutants (120) are compared (upper left corner, age in embryonic days (E)) with the staining indicated upper right. At E11.5, a direct (arrow) and an indirect via the left cardinal vein (LCV; arrowhead) connection between the coronary system and the right atrium (RA) are seen in +/+ and 120 embryos (schematic drawings in A and B, respectively). The indirect connection is also shown in E (+/+) and H (120) and arrows in Ei and Hi. The stainings indicated in E and H do not correspond, as differentially stained sections had to be chosen due to the fact that the connection can only be seen in a very small area of the embryos. Mutant coronary arteries are smaller and reveal abnormal morphology of the main branches (arrows in C-D), especially in embryos with TOF (D). Both the aortic arch (AoA) and coronary arteries are shown in red, the pulmonary trunk (PT) in green. At E11.5, an increase in size of the coronary veins (CV) is seen in mutant embryos (compare F and G). This is maintained throughout development (J-K). An increase in size of cardiac lymphatics (CL), as visualized using a staining for the lymphatic EC-specific marker LYVE-1, is obvious (L-M). In mutant embryos, ventriculo-coronary artery connections (VCAC), visualized using an ephrinB2-staining (N-Ni; with Nii being a consecutive section of Ni stained for α -SMA and Niii a section 35 μ m caudal from Nii) and arterio-venous shunts in both the coronary system visualized using a Notch3-staining (O and arrow in Oi) and the AoA (P and dotted lines in Pi) were seen. Locally increased levels of *Vegf*-mRNA are observed in mutant embryos (arrows in Q-R). Abbreviations: AAo = ascending aorta; CA = coronary artery; CM = compact myocardium; LA = left atrium; LV = left ventricle; PC = pericardial cavity; RCV = right cardinal vein; RV = right ventricle. Scale bar = 60 μ m (E,H.Ni-P); 20 μ m (Ei,Hi,Oi); 30 μ m (F-G,J-K,L-M); 200 μ m (N,Pi,Q-R).

Figure 2. Abnormal EC-differentiation and microvascular morphology in *Vegf120/120* embryos. Stainings performed are indicated in the upper right corner and the genotype (+/+ wild type and 120 mutant) and age in embryonic days (E) upper left. ECs of the mutant coronary veins (CV) showed an increase in staining per cell for arterial markers cleaved Notch1 (A-B), Notch2 (E-F), Dll4 (I-J) and ephrinB2 (M-N) and a decrease for the venous markers COUP-TFII (Q-R) and EphB4 (U-V). Increased capillary size is seen (Y-Z). ECs of the coronary arteries (CA) show decreased expression of cleaved Notch1 (C-D), Jagged1 (G-H), Dll4 (K-L) and ephrinB2 (O-P) concomitant with an increase in COUP-TFII (S-T), EphB4 (W-X) and VEGFR-2-expression (AA-AB). Abbreviations: Cl. Notch1: cleaved Notch1; PC: pericardial cavity. Scale bars 20µm.

Abnormalities in mutant coronary microvasculature

Not only the coronary veins, but also the capillaries were enlarged and dilated in *Vegf120/120* embryos (Figure 2Y-Z). To quantify these differences morphometry was performed on late-stage embryos. Total heart volumes did not differ significantly between groups (data not shown). The ratio myocardial to coronary vascular volume was significantly decreased in mutant mouse embryos with a p-value of 0.021 (Figure 3B). The differentiation of microvascular ECs in *Vegf120/120* mouse embryos, however, was indistinguishable from normal.

Figure 3. Quantification of alterations in mRNA expression in vivo and in vitro with regard to VEGF-isoforms and abnormalities in microvascular density in *Vegf120/120* embryos. A significant (*) increase in *Notch1*, *Notch4* and *Hes1*, a significant decrease in *Ephb4* and a trend in increase of *Dll4* and *Hey1* mRNA expression is seen when performing RT-qPCR normalized for *Tie2* on mRNA isolated from wild type (+/+) and mutant (120) hearts of E18.5 (A). Using morphometry, a significant difference ($p = 0.021$) in myocardial/vascular volume is found (B). When human coronary arterial ECs (HCAECs) are cultured with VEGF165 or VEGF121, it is obvious that VEGF165 can while VEGF121 cannot significantly increase expression of arterial markers (*NP-1*, *NOTCH1*, *NOTCH4*, *DLL4*, *HES1* and *EPHRINB2*) and decrease expression of the venous marker *COUP-TFII* when compared to HCAECs cultured without VEGF (of which expression is set to 1) (C).

Coronary arterial endothelial differentiation is impaired in Vegf120/120 embryos

In mutant embryos of E15.5 and older, obvious differences in differentiation of coronary arterial ECs were observed (Table S3). Protein expression levels indicative for arterial maturation, including cleaved Notch1, Jagged1, Dll4, and ephrinB2 (Figure 2C-D,G-H,K-L,O-P) were lower per EC, suggesting decreased Notch-signaling. Increased expression per EC of the venous markers COUP-TFII and EphB4 (Figure 2S-T,W-X) was observed. Due to lack of Notch4-specific antibodies its expression profiles could not be determined (see online information). Although arterial endothelial VEGFR-2-expression normally decreased upon induction of Notch-signaling²⁶, this decrease was much less prominent in mutant embryos (Figure 2AA-AB).

To determine whether differences in arterial marker expression were VEGF-isoform related, expression of differentiation-specific markers (i.e. arterial; *NP-1*, *NOTCH1*, *NOTCH4*, *DLL4*, *HES1*, *EPHRINB2* and venous; *COUP-TFII*) was analyzed in HCAECs in vitro. RT-qPCR on HCAECs cultured with or without VEGF165 or VEGF121 showed that VEGF121 was much less able to affect expression than VEGF165. VEGF165 significantly induced expression levels of arterial markers and decreased expression of *COUP-TFII* (Figure 3C).

Abnormal arteriogenesis is evident in Vegf120/120 mouse embryos

Anomalous coronary endothelial differentiation observed in mutant mouse embryos was concomitant with abnormal arteriogenesis. Coronary arteries of mutant embryos of E15.5 and older showed a decrease in medial development with a reduction in number of medial cells and abnormal morphologic and differentiation characteristics as evident by staining for the SMC-marker α SMA (Figure 4A-B). The spatiotemporal protein expression of NP-1, Dll1, Jagged2 and Notch3 was analyzed and a decrease in number of cells positive for these markers was seen in the medial layer of the coronary arteries of mutant embryos (Figures 4E-F,I-J,M-N,Q-R). In contrast, the coronary veins of mutant embryos were surrounded by a larger number of α SMA positive cells when compared to wild type littermates (Figure 4C-D). These cells were also positive for the SMC-related markers NP-1, Dll1 and Jagged2 (Figures 4G-H,K-L,O-P). Additionally, the pericyte-coverage of the coronary microvasculature as determined by Notch3-staining showed a marked decrease in number of positive cells in mutant mouse embryos (Figure 4S-T).

Figure 4. Abnormal coronary arteriogenesis in *Vegf120/120* embryos. The expression of several different markers (upper right) in vSMCs/pericytes was compared between wild type (+/+) and mutants (120; upper left together with embryonic age in days (E)). A decrease in medial cells expressing α SMA (A-B), NP-1 (E-F), Dll1 (I-J), Jagged2 (M,N) and Notch3 (I-J) is obvious in the coronary arteries media (CA) of mutants while an increase of α SMA (C-D), NP-1 (G-H), Dll1 (K-L) and Jagged2-positive cells (O-P) surrounds the coronary veins (CV). A decrease in number of Notch3-positive pericytes is seen in mutant coronary microvasculature (K-L). Abbreviation: PC = pericardial cavity. Scale bars = 20 μ m.

Discussion

The loss of the *Vegf164* and *Vegf188* isoforms in *Vegf120/120* mutants results in spatiotemporal alterations in VEGF and Notch-signaling in the heart. These alterations coincide with abnormalities in coronary endothelial differentiation and subsequent arteriogenesis. The data provide us with further insight on the role of both VEGF-isoform and spatiotemporal VEGF-distribution on coronary differentiation and maturation.

Anomalous endothelial differentiation of the Vegf120/120 coronary system

VEGF-signaling influences vasculogenesis and angiogenesis, but also arterial endothelial differentiation^{11, 27}. VEGF-signaling in ECs can upregulate expression of members of the Notch-signaling family, which instructs ephrinB2-expression and defines arterial differentiation^{11, 27}. In venous ECs, expression of these proteins is inhibited by the vein-specific transcription factor COUP-TFII, thereby favoring EphB4-expression and venous differentiation²⁸. We show in vitro that addition of VEGF165, but not of VEGF121, leads to significant decrease in COUP-TFII-expression in human coronary arterial ECs, indicating a possible mechanism through which VEGF-signaling might favor arterial endothelial differentiation. The exact pathway by which VEGF-signaling regulates COUP-TFII-expression remains to be elucidated. The main isoform expressed during normal murine cardiac development is *Vegf164*¹⁷. VEGF164 has a proximate bioactivity to its place of production due to high heparin-binding capacities²⁹ (Figure 1Q and 5A,C,E). We show that cardiac *Vegf*-production in vivo overlaps in later stages ($\geq$ E16.5) with the region where coronary arteries develop (i.e. deep in the myocardium of the ventricular free walls in the mouse). We consider that this spatial distribution will result in relatively high levels of VEGF and subsequent Notch-signaling in ECs localized in the region of the developing coronary arteries due to 1) high local levels of VEGF, combined with 2) amplification of signaling levels by artery-specific NP-1²⁸ (Figure 5C). This is reflected in vivo by inhibition of COUP-TFII and induction of Dll4, cleaved Notch1 and ephrinB2 staining in coronary arterial ECs in late embryonic stages, suggesting increased Notch-signaling, and (potentially Notch-induced²⁶) reduced VEGFR-2 expression. In contrast, veins develop subepicardially and therefore, venous ECs are mainly subjected to the less expressed and more diffuse VEGF120-isoform, with resulting low levels of Notch-signaling as indicated by low cleaved Notch1 and high COUP-TFII and EphB4-staining in wild type embryos. In combination with the fact that venous ECs do not express NP-1²⁸, this leads to the assumption that coronary venous ECs experience lower levels of VEGF and Notch-signaling (Figure 5E). This spatiotemporal determination of arterial and venous specification also corresponds with the situation in zebrafish. Here, VEGF-expression is induced in the somites next to the notochord, which is located directly caudal from the dorsal aorta. The

posterior cardinal vein is positioned more ventrally, so further away from the place of VEGF-production²⁷.

In *Vegf120/120* mouse embryos, only the highly soluble non-heparin-binding VEGF120 isoform is produced²⁹. This will affect the VEGF-gradient in the myocardium giving relative low levels of VEGF in the area of the coronary arteries and higher levels around the coronary veins (Figure 5B,D,F). Because of this, combined with the fact that VEGF120 cannot induce formation of the signaling potent NP-1/VEGFR-2 complexes³⁰, lower VEGF and Notch-signaling levels are expected in the ECs in the area of the coronary arteries (Figure 5D), and higher levels in the ECs of the coronary veins (Figure 5F). This could lead to the alterations in EC-differentiation observed in these embryos. Besides altered differentiation, a severe decrease in coronary arterial size coincides with an increase in venous size. As VEGF is involved in endothelial proliferation and vascular fusion³¹, locally increased *Vegf*-expression in the venous region could lead to enlarged veins as observed in the mutant mouse embryos. Therefore, we speculate that the RT-qPCR data normalized for endothelial input on embryonic hearts indicate mainly venous expression. This underscores that VEGF-signaling in venous ECs might induce expression of arterial markers such as *Notch1* and *Notch4*. Unfortunately, we could not confirm alterations in Notch4 protein-expression as the anti-Notch4-antibodies tested bind aspecific (Table S1).

Our in vitro data show that the expression of arterial markers in HCAECs varies for the VEGF-isoform applied, with VEGF165 having a more prominent effect than VEGF121. This probably relates to higher levels of VEGF-signaling through VEGFR-2 with VEGF165 due to NP-1-mediated amplification^{9, 28, 30}. Unfortunately, we could not directly test the effect of VEGF-signaling on coronary venous ECs in vitro, as these cells cannot be obtained for culturing. However, earlier published data on HUVEC show that treatment with VEGF induces arterial phenotype of venous ECs^{11, 32} as does ectopic VEGF-expression in zebrafish³³.

Total heart volumes are unchanged in mutant embryonic hearts, while a decrease in myocardial/vascular volume-ratio is seen, together with abnormal (dilated) microvascular morphology. This pleads for an increase in vascular volume rather than a decrease in myocardial volume as the underlying cause for the altered myocardial/vascular volume-ratio. This is likely caused by a disturbed VEGF-gradient, as it has been described that loss of a VEGF-gradient leads to alterations in microvascular branching morphogenesis³⁴ and increased availability of VEGF induces hyperfusion of vessels³¹. Furthermore, VEGF is able to induce proliferation of lymphatic ECs³⁵, located subepicardially in the heart. Another function of VEGF as Vascular Permeability Factor³⁶ might additionally lead to the appearance of the increased size of the cardiac lymphatic system in mutant embryos.

Figure 5. VEGF-expression and signaling during embryonic coronary development in normal and *Vegf120/120* hearts. A-B: Schematic drawings of transverse sections of the left ventricle (LV) of wild type (+/+) and mutant (120/120) embryos of E16.5 are shown. In +/+ embryos, *Vegf*-production mainly takes place in the borderzone between compact and trabecular myocardium (A; see also Figure 1Q), where coronary arteries (red) develop. As this is mainly *Vegf164*, protein distribution (yellow) is expected to give high protein levels around arteries and low around veins (blue). As mutants (B) only produce *Vegf120*, protein levels are expected to be more diffuse due to lack of heparin-binding domains. This will lead to relative low protein levels around arteries and high levels around veins. C-F: Schemes indicating the expected VEGF-signaling levels in +/+ and 120 coronary arterial endothelial cells (AECs) and venous endothelial cells (VECs) are shown. In a +/+ AEC high VEGF protein-levels combined with neuropilin-1 (NP-1)-mediated amplification leads to high levels of VEGF-signaling (C), while low VEGF-concentrations and lack of NP-1-expression in VECs leads to low levels of VEGF-signaling (E). In mutant embryos, relative low VEGF-levels around the AECs in combination with lack of NP-1-mediated amplification leads to relative low levels of VEGF-signaling in a 120/120 AEC (D), while relative high VEGF-levels around the VECs lead to high signaling levels (F). NOTE: Due to resolution, in A different VEGF-isoform are indicated with yellow dots of the same size.

Alterations in Vegf120/120 coronary arteriogenesis

We show that incorrect coronary arterial EC-differentiation coincides with impaired development of the media, together with a gain of arterial phenotype in coronary venous ECs and an increased number of venous pericytes. The differentional state of ECs influences recruitment or proliferation and maturation^{37, 38}, suggesting a role for VEGF-signaling on arteriogenesis via manipulation of endothelial performance. A direct role for VEGF-signaling in arteriogenesis is supported by the described instructive role for NP-1³⁹ and Notch-signaling^{38, 40} in differentiation of vSMCs. Concomitantly, we show that the amount of cells expressing α SMA, NP-1, Dll1, Jagged2 and Notch3 in the coronary arterial media and of Notch3-positive microvascular pericytes was impaired in mutant embryos, while an increase in number of cells surrounding the coronary veins positive for these markers was seen. Besides a positive role for Notch-signaling in arteriogenesis, inhibiting roles have been described as well⁴¹. Our data plead for an instructive rather than an inhibiting role. We suggest that lack of larger VEGF-isoforms leads to a deficient development of the vSMC-layer of the coronary arteries. This can occur either indirectly via altered endothelial differentiation, or directly through a decrease in VEGF-related Notch-signaling in the mutant coronary vSMCs.

Morphological abnormalities in the coronary system of mutant mouse embryos

Congenital coronary anomalies are often correlated with primary cardiac defects. In all *Vegf120/120* mutants with TOF¹⁷ the IVS was supplied by an artery coming from the left sinus, in contrast to normal where the IVS is supplied by a branch of the right coronary artery²⁴. The anomalies specific for TOF (overriding and/or dextroposition of the ascending aorta) might favor the coronary IVS-branch to connect to the left sinus⁴². As in humans with TOF, a single orifice was occasionally encountered in mutant mouse embryos. More often an increased number (3 or 4) of coronary orifices was found, which is regarded to be a rare variation in both humans and mice^{24, 43}. Nevertheless, we never saw more than 2 orifices in the 24 wild type embryos of E15.5 and older.

The presence of VCACs in cardiac anomalies related to pulmonary atresia are not uncommon, even before complete atresia has developed⁴⁴. A substantial part of coronary vSMCs originates from the EPDC-population¹⁵ and EPDC-performance has been suggested to play part in the appearance of VCACs⁴⁴. Abnormal development and/or differentiation of EPDCs due to altered VEGF and/or Notch-signaling might explain the occurrence of VCACs in *Vegf120/120* embryos. Besides VCACs, coronary arterio-venous shunts were observed. Alterations in VEGF and Notch-signaling in *Vegf120/120* embryos may cause arterio-venous shunts, as the presence of such shunts has been described in Notch-mutants⁴⁵. Extracardiac arterio-venous shunts were observed in the aortic arches of perinatal *Vegf120/120* mouse embryos, suggesting that alterations in Notch-signaling are not heart-restricted.

We already observed at E11.5, both in wild type and mutants, two connections of the coronary venous system (in)directly to the right atrium. This is the first report on such early coronary venous drainage in mice. We describe that the connection of the coronary system to the right atrium arises before the coronary connection to the aorta. This is feasible as it enables circulation throughout the coronary system at the onset of systemic flow and pressure, which occurs several days later, without obstruction¹⁴.

Conclusions

We show that loss of *Vegf164* and *Vegf188* in *Vegf120/120* embryos leads to spatiotemporal alterations in VEGF and Notch-signaling in the heart reflected by altered expression of several markers involved in and regulated by these pathways. These alterations coincide with anomalous coronary EC-differentiation and ensuing arteriogenesis. The effect of different VEGF-isoforms on Notch-signaling and coronary EC-differentiation could be confirmed in vitro.

From our study it can be concluded that the spatiotemporal VEGF-distribution as well as specificity of the isoforms are important for proper coronary vascular development. These new insights on instructive roles of VEGF(-isoform) distribution on angiogenesis, endothelial functioning, and arteriogenesis can be of use to improve the efficacy of current therapies targeting VEGF-signaling^{8, 46} in tissue revascularization.

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Supplement

Results

Anomalous coronary morphology in Vegf120/120 mouse embryos

A remarkable observation was that half of the mutant embryos showed abnormalities in the anatomy of the main coronary branches. In all wild type embryos the main artery supplying the interventricular septum (IVS) originated from the first branch of the right coronary artery (RCA), with sometimes an additional smaller branch coming from the left coronary artery (LCA), which corresponds with earlier observations in normal mice¹. In 8/28 mutant embryos the IVS-supplying branch had its own coronary orifice, which originated in 6 cases from the right and in 2 cases from the left facing sinus. In addition, in 3/28 cases the IVS was solely supplied by a branch that arose from the LCA. All embryos in which the IVS-branch originated (in)directly from the left facing sinus were diagnosed with TOF (5/28)². In contrast to mice, the IVS in humans is normally supplied by the left anterior descending artery and it has been described that patients with TOF often (up to 19%³) present with coronary abnormalities such as bilateral or right-originating anterior descending arteries^{3, 4}. The differences between mouse and man, both in normal and pathological coronary morphology might lie in the fact that the angle of the aorta relative to the IVS-axis differ between the two species⁵. Furthermore, in 2/28 *Vegf120/120* cases no major coronary artery could be observed supplying the IVS and in 1/28 cases both a branch splitting from the RCA and from the LCA with the left branch being most prominent was observed.

Table S1. Characteristics of primary antibodies used for immunohistochemistry.

Antigen	Cat. no. (company)	Antigen re-retrieval	ABC-incubation	CSA-II kit (DAKO)	Reference
VEGFR-2	AF644 (R&D Systems, Minneapolis, USA)	HR	Yes	No	www.rndsystems.com /ihc
LYVE-1	103-PAB150 (ReliaTech, Braunschweig, Germany)	P	Yes	No	Mäkinen <i>et al</i> ⁶
cleaved Notch1	2421 (Cell Signaling, Beverly, MA, USA)	HR	No	Yes	Tanaka <i>et al</i> ⁷
Notch2	sc-5545 (Santa Cruz, Santa Cruz, USA)	-	Yes	No	Okuyama <i>et al</i> ⁸
Notch3	sc-7424 (Santa Cruz, Santa Cruz, USA)	HR	Yes	No	Karlstöm <i>et al</i> ⁹
Jagged1	sc-6011 (Santa Cruz, Santa Cruz, USA)	HR	Yes	No	Sestan <i>et al</i> ¹⁰
Jagged2	sc-8158 (Santa Cruz, Santa Cruz, USA)	-	Yes	No	Rivolta <i>et al</i> ¹¹
Dll1	sc-9102 (Santa Cruz, Santa Cruz, USA)	HR	Yes	No	Conboy and Rando ¹²
Dll4	AF1389 (R&D Systems, Minneapolis, USA)	HR	Yes	No	www.rndsystems.com
ephrinB2	AF496 (R&D Systems, Minneapolis, USA)	HR	Yes	No	www.rndsystems.com /ihc
EphB4	AF446 (R&D Systems, Minneapolis, USA)	HR	Yes	No	www.rndsystems.com
COUP-TFII	PP-H7147-00,2ZH7147H (PPMX, Tokyo, Japan)	HR	Yes	No	You <i>et al</i> ¹³
NP-1	sc-7239 (Santa Cruz, Santa Cruz, USA)	HR	Yes	No	Serini <i>et al</i> ¹⁴
α -SMA (1A4)	A2547 (Sigma-Adrich, Missouri, USA)	HR	No	No	Skalli <i>et al</i> ¹⁵
Notch4	sc-8645 (Santa Cruz, Santa Cruz, USA)	N.A.	N.A.	N.A.	N.A.
Notch4	sc-5594 (Santa Cruz, Santa Cruz, USA)	N.A.	N.A.	N.A.	N.A.

Abbreviations; HR = heat retrieval; N.A. = not applicable; P = pronase treatment

Table S2. Primer sequences used for RT-qPCR.

Species	Gene	Forward	Reverse
Homo Sapiens	<i>β-ACTIN</i> (BC002409)	5'- ATCCTCACCTGAAGTACCC- 3'	5'-GGACTGTGGTCATGAGTCCT- 3'
	<i>GAPDH</i> (NM_002046)	5'- GCCTCAAGATCATCAGCAAT - 3'	5'-GGACTGTGGTCATGAGTCCT- 3'
	<i>NP-1</i> (NM_003873)	5'-TGAGCCCTGTGGTTTATTC -3'	5'-CGTACTCCTCTGGCTTCTGG- 3'
	<i>NOTCH1</i> (NM_017617)	5'- CTGGAGGACCTCATCAACTC - 3'	5'- TTCTTCAGGAGCACAACCTGC -3'
	<i>NOTCH4</i> (NM_004557)	5- GTGGGTATCTCTGCCAGTGT- 3'	5'-CTCAGGTTTGGGAGTACAG- 3'
	<i>DLL4</i> (NM_019074)	5'- ACAACTTGTCGGACTTCCAG- 3'	5'-CAGCTCCTTCTTCTGGTTTG- 3'
	<i>HES1</i> (NM_005524)	5'- CCAAAGACAGCATCTGAGCA- 3'	5'-GCCGCGAGCTATCTTCTT-3'
	<i>EPHRINB2</i> (NM_004093)	5'- GTTCCGAAGTGGCCTTATTT- 3'	5'-CTCCGGTACTTCAGCAAGAG- 3'
	<i>COUP-TFII</i> (BC014664)	5'- AGCAAGTGGAGAAGCTCAAG -3'	5'- CACATGGGCTACATCAGAGA -3'
Mus Musculus	<i>β-actin</i> (NM_007393)	5'- actcctatgtgggtgacgag -3'	5'- ccagatcttctccatgtcgt -3'
	<i>gapdh</i> (BC083149)	5'- aagtggagattgttccatc -3'	5'- cgtgagtgagtcatactgg -3'
	<i>Tie2</i> (X71426)	5'- gccatcaagaggatgaaaga -3'	5'- catgctccaagagattgat -3'
	<i>Notch1</i> (NM_008714)	5'- agcctctcccaatacctt -3'	5'- ggctggagctgtaagtctg -3'
	<i>Notch4</i> (NM_010929)	5'- agaggacgggactacacctt -3'	5'- cctttatccctggctccta -3'
	<i>Hes1</i> (NM_008235)	5'- cctctgagcacagaaagta -3'	5'- gccgggagctatctttctta -3'
	<i>Ephb4</i> (NM_010144)	5'- gccatcaagatgggaagata -3'	5'- cacactggccaagatttct -3'
	<i>Dll4</i> (NM_019454)	5'- actctgtctgcaactgtcct -3'	5'- cagcaccagcagtaaccata -3'
	<i>Hey1</i> (AF232241)	5'- gtacccagtgccctttgagaa -3'	5'- tttcaggtgatccagta -3'

Table S3. Overview of alterations in coronary EC-expression patterns in *Vegf120/120* mouse embryos.

Marker	Venous ECs	Arterial ECs
VEGFR-2	↑	↑
cleaved Notch1	↑	↓
Notch2	↑	=*
Dll4	↑	↓
Jagged1	=*	↓
ephrinB2	↑	↓
EphB4	↓	↑
COUP-TFII	↓	↑

The protein expression patterns as determined using immunohistochemistry of ECs in *Vegf120/120* mouse embryos are indicated, when compared to wild type littermates, as shown in Figure 1, 2 and data not shown (*). To indicate a difference, this difference was seen in at least 3 mutant vs 3 wild type embryos per age-group (E15.5-E19.5).

Chapter 5

Soluble JAGGED-1 Inhibits Neointima Formation by Attenuating NOTCH-HERP2 Signaling

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Abstract

Notch has been implicated in neointima formation as reflected by increased Notch/Jagged expression upon vascular injury and the pro-migratory effect of Notch signaling on smooth muscle cells (SMC). Soluble Jagged-1 (sJag1) has been shown to inhibit Notch-signaling *in vitro*, however its capacity to suppress neointima formation remains unknown. Balloon injury of rat carotid arteries induced Notch1, Notch3 and Jagged-1 expression at day 3 and 14 post-injury. Notch signaling was activated as shown by increased expression of the Notch target gene Herp2. Adenoviral-sJag1 (Ad-sJag1) transfection reduced neointima formation in carotid artery and enhanced re-endothelialization, whereas adenoviral-full length Jagged-1 (Ad-Fl-Jag1) or -LacZ had no effect. Injury induced Herp2 expression was absent in vessels treated with Ad-sJag1. Consistently, Herp2 expression was reduced in Ad-sJag1 infected or recombinant sJag1 treated Coronary Artery Smooth Muscle Cells (CASMCs). Ad-sJag1 had no effect on Human Umbilical Endothelial Cells (HUVECs) behavior, but significantly reduced proliferation and migration of CASMCs. Overexpression of Herp2 in sJag1 treated CASMCs rescued the migratory and proliferative capacity *in vitro*. Our results demonstrate that sJag1 can inhibit neointima formation after balloon injury by decreasing SMC proliferation and migration through interference with Notch-Herp2 signaling.

Introduction

Occlusive arteriopathies, such as coronary artery and peripheral artery disease are a consequence of atherosclerosis related neointima formation involving maladapted migration and proliferation of smooth muscle cells (SMCs). As vascular SMCs are not terminally differentiated, they exhibit substantial plasticity in phenotypic modulation that can be activated upon a vascular insult^{1, 2}. Members of the Notch family can regulate SMC plasticity and have been associated with neointima formation³⁻⁷.

The Notch family encompasses an evolutionary highly conserved intercellular signaling pathway known to regulate cell fate decision (i.e. apoptosis, proliferation, differentiation and migration)⁸ in the cardiovascular system^{9, 10}. This family consists of four Notch receptors (Notch1-4) and two ligand groups, Jagged (Jagged1, -2) and Delta Like (Dll1, -3, -4). Both receptors and ligands are transmembrane proteins containing an extracellular domain with which they facilitate communication between cells. After binding of the ligand to the receptor -present on adjacent cells- a proteolytic cascade is activated involving the γ -secretase/ADAM (A Disintegrin And Metalloprotease) complex. Cleavage of the intracellular part of the receptor results in the release of the Notch intracellular domain (NICD) that translocates to the nucleus and together with its co-transcription factor CSL (CBF1/RBPJk, Su(H), Lag1) induces the transcription of the basic helix-loop-helix transcription factors HES (Hairy/Enhancer of Split) and HERP (Hairy Enhancer of Split-related Repressor Protein)¹¹⁻¹³. Also the ligand is cleaved upon receptor interaction by the same proteases, giving rise to a soluble extracellular ligand domain that can influence NOTCH signaling as well¹⁴. For example, soluble Jagged1 (sJag1) inhibits migration and proliferation of the fibroblast cell line NIH3T3^{15, 16}.

Notch1, -3 and Jagged1 along with the downstream HES/HERP-proteins are expressed in adult SMCs¹⁷. Jagged1 and Notch1 play important non-redundant roles during vascular development as their respective knockouts die early *in utero* due to vascular remodeling defects of the yolk sac^{18, 19}. Notch3 mutants are viable and fertile but present with structural vascular defects showing reduced arterial size and deficient SMC differentiation post natally (i.e. low SM22 α and smoothelin expression)^{20, 21}, suggesting a function of Notch3 in vascular maturation. A role for Notch3 in arterial maturation is also supported by the phenotype of *NOTCH3* mutations in men (i.e. CADASIL, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy)²². *In vitro* experiments by Sweeney and colleagues underline this notion further, showing that constitutive expression of Notch1/3ICD (IntraCellular Domain) in SMCs increased proliferation and inhibited apoptosis⁶. Consistently, Jagged1 increased the expression of smooth muscle-myosin heavy chain (SM-MHC)²³, while the Notch down-stream transcription factors

HERP1 and HERP2 were able to stimulate SMC migration, proliferation⁹ and differentiation^{24–26}.

As migration of SMCs is a prerequisite for neointima formation after arterial injury, we hypothesized that blockage of Notch signaling with sJag1 could reduce neointima formation. Here, we show that neointima formation in a balloon injury carotid rat artery model was inhibited by sJag1. Accordingly, sJag1 was able to reduce the SMC proliferation and migration capacity, by interfering with Notch signaling involving depression of Herp2 expression.

Methods

A detailed description of methods can be found in the supplemental materials.

Statistical analysis

For inter-group comparison, one-way ANOVA with Tukey posthoc test using SPSS software was applied (version 12.0). Values are expressed as mean±SEM. Probability values of < 0.05 were considered significant.

Results

Notch1, Notch3, Jagged1 and Herp2 were Highly Expressed in Injured Arteries

The expression of Notch1, Notch3, Jagged1 and Herp2 in intact and injured carotid arteries was determined at day 3 (early stage injury response) and day 14 (late stage injury response) post injury by immunohistochemistry and was compared to shams (Figure 1). Upon injury neointima formation progressed rapidly over time, beginning at day 3 and showing a profound and cell rich neointima at day 14 post injury (intima to media ratio >1.7). This injury response was associated with clear spatial-temporal changes in the expression of Notch members.

In non-injured carotid arteries, the Notch receptors (Figure 1A) were differentially expressed with Notch1 being present throughout the vessel wall, but more pronounced in the endothelium. Notch3 was expressed at a much lower level with moderate presence in the media and relative high signal in adventitial myofibroblasts. Jagged-1 was heterogeneously expressed with relative low levels in the intima and with individual cells containing a higher protein content in the media and adventitia. For Herp2 a relative low and scattered expression was apparent for the vessel wall with a higher level for the adventitia.

Three days after injury (Figure 1B), Notch1 was reduced throughout the wall with exception of the peri-adventitial layer of SMCs. In contrast, Notch3 was increased in the injured media as well as the adventitia with a typical high expression in the peri-endothelial SMC layer. Jagged-1 and Herp2 expression was also increased with a

Figure 1. Notch1, Notch3, Jagged1 and Herp2 expression in non-injured (0d, A) and injured carotid arteries at three (3d, B) and fourteen days (14d, C) following injury. High magnification images of cross sections with similar orientation, i.e. lumen to the right and adventitia (A) to the left. A, At 0d, Notch1 (N1) is expressed throughout the vessel wall, but especially in the endothelium (arrowheads). Notch3 (N3) is barely detectable in the media. Only adventitial myofibroblasts expressed N3 at high level (arrows). Jagged-1 (Jag1) and Herp2-expression was found in the vessel wall. B, At 3d, N1 expression was reduced and N3 was increased, showing a high content in adventitia and the more luminal SMC layers (arrowheads). Also Jag-1 and Herp2 were present in the wall, Jag-1 was expressed by all SMCs and the Herp2-pattern highly resembled N3. C, 14d after injury the neointima layer (asterisk) abundantly expressed all Notch members.

prominent signal in the peri-endothelial SMCs and adventitia, overlapping the Notch3 expression pattern. At day 14, the cell rich neointima expressed all Notch members stronger than the media and adventitia (Figure 1C). At this stage the expression of Herp2 remained high, reflecting a considerable degree of Notch signaling in the injured vessel wall. Interestingly, the vasa vasorum surrounding the vessel were highly Notch positive (not shown).

sJag1 negatively affected the Proliferation and Migration of SMCs

To define the potential effects of sJag1 on EC and SMC behavior, both primary cell types were transduced with adenoviral vectors for either sJag1 (Ad-sJag1), Full-length Jag1 (Ad-Fl-Jag1) or LacZ (Ad-LacZ, control), cultured and subsequently analyzed for their migratory and proliferative capacity (Figure 2). Standard microscopy on cell morphology and viability revealed no confounding cytotoxicity. Transduction efficiency was established by Western Blotting for c-myc and Jagged-1 on protein extracts from CSMCs and HUVECs that were infected with Ad-sJag1 and Ad-FlJag1, respectively or Ad-LacZ as control. All infected cells revealed clear increased expression of the protein of interest as compared to the LacZ control (Figure 2E and 2F and Suppl. Figure IIB).

Figure 2. Effect of Jagged-1 on CSMCs and HUVECs function. A, Ad-sJag transduced CSMCs migrated at significant lower rate (asterisk, $p < 0.01$) compared with Ad-LacZ or Ad-Fl-Jag1 transduced CSMCs. B, HUVECs migration was unaffected by the treatments. C, CSMCs proliferation was significantly reduced by Ad-sJag1 (asterisk, $p < 0.01$). D, Proliferation rate of HUVECs between treatments was comparable. E and F, Western blots of Ad-sJag1 and Ad-Fl-Jag1 transduced CSMCs and HUVECs showing clear protein over-expression. RFU (Relative Fluorescence Units). Data presented are mean values $\pm$ SD of three independent experiments.

Ad-sJag1, but not Ad-Fl-Jag, considerably reduced migration of CASMCs (Figure 2A sJag1: $6,701 \pm 227$ RFU; LacZ: $11,986 \pm 557$ RFU; FlJag: $13,240 \pm 1,053$ RFU, $p < 0.01$). Ad-sJag1 also significantly depressed the proliferation of CASMCs as compared with Ad-LacZ and Ad-Fl-Jag1 infected cultures at both 48 (Figure 2C ratio to 24 hours: 1.34 ± 0.024 versus 1.67 ± 0.051 and 1.63 ± 0.077 , respectively, $p < 0.01$) and 72 hours post transduction (1.60 ± 0.059 versus 2.08 ± 0.024 and 2.02 ± 0.10 , respectively, $p < 0.01$). In contrast, migration and proliferation of endothelial cells (HUVECs) were unaffected by Ad-sJag1 or Ad-Fl-Jag (Figure 2B and 2D). A comparable lack of effect was also obtained with human coronary artery endothelial cells (data not shown). Interestingly, Lindner et al¹⁶ reported that NIH3T3 cells stably transfected with soluble Jag1 migrated at a slower rate than cells transfected with a control vector. However, when cells were cultured on a fibronectin substrate this phenotype was partially rescued. To rule out matrix dependency of sJag induced effects we therefore tested the migration capacity of Ad-LacZ and Ad-sJag1 infected HUVECs that were cultured on fibronectin or plastic, but we were unable to detect a difference (Suppl. Figure 1A and 1B). Likewise, no difference in proliferation rate between sJag1 and LacZ infected HUVECs cultured on fibronectin was apparent (Suppl. Figure 1C).

sJag1 Reduced Neointima Formation

To determine the effect of sJag1 on the development of neointima formation, injured carotid arteries were instilled with Ad-sJag1, Ad-Fl-Jag1 or Ad-LacZ control. In vivo efficiency defined by X-Gal staining of Ad-LacZ infected day 3 post injury rat carotid arteries was sufficient (Suppl. Figure 1IA). The efficiency of infection was confirmed in cultured CASMCs infected with Ad-Fl-Jag1 by immunofluorescent staining performed against the extracellular domain of Jagged1 protein (Suppl. Figure 1IB).

Morphometrical analysis was performed on carotid artery cross sections 14 days after injury that were infected with Ad-sJag1, Ad-Fl-Jag1 or Ad-LacZ. Neointima formation in control Ad-LacZ treated carotid arteries was comparable to injured non-transduced carotid arteries, excluding cytotoxic effects of the viral intervention in vivo (data not shown). In accordance with the observed in vitro reduction in SMC proliferation and migration, Ad-sJag treatment resulted in more than 40% (1.02 ± 0.19) reduction in intima/media ratio compared with the control and Ad-Fl-Jag1 (1.74 ± 0.18 and 1.73 ± 0.14 , respectively; $p < 0.01$) (Figure 3A and B). Immunohistochemical staining against proliferating cell nuclear antigen (PCNA) was performed to examine the effect of sJag1 on SMC proliferation in vivo. Smooth muscle cells proliferative index at 3 days (calculated as the percentage of positive cells among total nucleated cells) was significantly lower in the media of injured carotid arteries from Ad-sJag1 group as compared with Ad-LacZ group ($40 \pm 8\%$

Figure 3. Effect of sJag1 and Fl-Jag on neointima formation. A, Eosin stained cross sections of non-injured (native; control) and denudated carotid arteries at 14 days post-injury. Smaller neointima was apparent for the Ad-sJag1 (lowest-panel) as compared with control (Ad-LacZ; upper-panel) and Ad-Fl-Jag1 (middle-panel) B, Morphometric analysis showed significant lower average intima/media ratios (asterisk, $p < 0.05$) for the Ad-sJag1 as compared with both Ad-LacZ and Ad-Fl-Jag1 transduced arteries. C, Effect of in vivo sJag1 on proliferation as assessed by proliferating cell nuclear antigen staining (PCNA) staining. Proliferative index at 3 days after balloon injury and gene delivery was significantly lower for carotid artery section of Ad-sJag1 as compared with Ad-LacZ group. D, Representative en face AgNO₃ stained carotid arteries showing the EC-borders in untreated control (upper left panel, black arrow points to EC mitosis); control immediately after balloon injury presenting complete removal of the endothelial layer (upper-right panel); 2 weeks post arterial injury with Ad-LacZ (lower-left panel) revealing disorganized and incomplete endothelial coverage (asterisks) and Ad-sJag1 treatment with good endothelial recovery (lower right panel, asterisks indicating areas lacking endothelial recovery). E, Small though significant (asterisks, $p < 0.05$) higher degree of re-endothelialization was apparent for the Ad-sJag1 treated group at 14 days post injury.

versus $28.7 \pm 4.2\%$, $p < 0.05$; Figure 3C). No difference in SMC proliferation rate between treatments was observed at 14 days after injury. A significantly higher degree of reendothelialization was apparent for the Ad-sJag1 treated group after 14 days, whereas the Ad-Fl-Jag1 treated group did not differ from the control ($92.4 \pm 1.0\%$ versus $81 \pm 3.3\%$ and $83 \pm 4.4\%$; Figure 3E and en face staining Figure 3D). Expression analysis of Notch members in the adenoviral treated groups revealed no clear differences in expression of Notch1 at day 3 and 14 day post-

injury. As expected, Jag1 was higher expressed in the Ad-FlJag1 treated group at day 3 (Suppl. Figure III). For all other conditions and time points no differences in Jag1 expression were apparent. The majority of the 3 day Ad-sJag1 treated group (3 out of 4) revealed a lower Notch3 expression for the media (Suppl. Figure IV, B). This difference in expression was not found at day 14 post-injury. The degree of SMC differentiation of the injured carotid arteries was analyzed by examining the α -smooth muscle actin (α SMA) expression in the vessel wall. Three days after injury α SMA was observed in all SMC layers of the vessel wall. In contrast, the 3 day Ad-sJag1 treated vessels revealed a reduction in α SMA expression as reflected by patches of low actin signal in the vessel wall (Suppl Fig V, C and D). At day 14 post-injury, strong α SMA expression was observed in the media and intima of injured vessels, with less clear and inconsistent differences between Ad-sJag1 and Ad-LacZ groups (Suppl. Figure V, E and F).

Notch Related Herp2 Signaling in Neointima Formation and SMC function

Treatment of injured carotid arteries with Ad-sJag1 resulted in a sharp decrease in medial expression of the Notch target gene Herp2 three days after injury (Figure 4A, right panel) compared to Ad-LacZ group (Fig.4A, left panel). In contrast, Ad-Fl-Jag1 was unable to prevent the up-regulation of Herp2 expression induced by injury (Suppl. Figure VI A).

The inhibitory effect of sJag1 on Herp2 expression was further assessed *in vitro*. CASMCs treated with 5 μ g/mL of sJag1 recombinant protein or infected with Ad-sJag1 decreased Herp2 expression (right and left panels, Figure 4B). Conversely, CASMCs infected with Ad-Fl-Jag1 showed increased Herp2 expression (Suppl. Figure VI B).

The functional relevance of sJag1 inhibited Herp2 for the migration and proliferation of SMC was studied by a rescue experiment in which Herp2 was over expressed. As expected, sJag1 gave a strong reduction in SMC migration in comparison with mock treated control for all time points analyzed (Figure 4C). At 24 hours a more than 10-fold difference between control and sJag1 treated control was apparent (8.8% versus 100%). This depressed SMC migratory capacity could partially be rescued by inducing Herp2 expression (52.5% after 24 hrs), indicating that Notch-Herp2 signaling is involved in SMC migration. Similarly, the negative effect of sJag1 on CASMC proliferation was abrogated by Herp2 overexpression (Figure 4D). Soluble Jag1 treatment, at 72 hours post transfection, resulted in a significant reduction of SMC proliferation as compared to non treated mock condition (Ctrl) (Figure 4D ratio to 24 hours sJag1, 1.78 ± 0.031 versus Ctrl, 2.37 ± 0.002 ; $p < 0.001$). Herp2 overexpression was able to rescue SMC proliferation of the sJag1 treated samples (Herp2+sJag1, 2.65 ± 0.018 versus sJag1, 1.78 ± 0.031 ; $p < 0.001$), and increased the proliferation in untreated cells (Herp2, 3.22 ± 0.005 versus Ctrl, 2.37 ± 0.002 ; $p < 0.001$).

Figure 4. Cross section balloon injured and Ad-LacZ (A, left panel) and Ad-sJag1 (A, right panel) transduced carotid arteries showing Herp2 expression at 3 days post-injury. Ad-sJag1 treated arteries (A, right panel) showed a decreased medial Herp2 expression as compared with Ad-LacZ treated arteries (A, left panel). Scale bar: 200 μ m. B. *Herp2* mRNA expression in CASMCs receiving mock or recombinant sJag1 and CASMCs infected with Ad-sJag1 or Ad-LacZ control. *Herp2* mRNA expression was significantly reduced by recombinant sJag1 (left panel). Similarly, Ad-sJag1 infected cells showed a significant decrease of *Herp2* mRNA expression (right panel). Data presented as mean $\pm$ SD of three independent experiments. C, Migration of Herp2- or Mock-transduced (control, ctrl) CASMCs treated with or without recombinant sJag1. Migration was depressed in CASMCs treated with sJag1 and Herp2 over-expression partly rescued this reduced migration capacity. Data presented as mean $\pm$ SD of three independent experiment, values are normalized for mock group at 24 hours of migration and was set at 100%. D. Proliferation of Herp2- or Mock-transduced (control, ctrl) CASMCs treated with or without recombinant sJag1. Proliferation was decreased (#) in CASMCs the sJag1 group, whereas Herp2 overexpression rescued (*) this effect. Data presented as mean $\pm$ SD of three independent experiments.

Discussion

In this study we present evidence that soluble Jag1 effectively inhibits neointima formation upon vascular injury through attenuation of Notch signaling and subsequent reduction of SMC proliferation and migration.

The development of intimal hyperplasia is associated with a temporal spatial change in Notch1, Notch3, Jagged1 and Herp2. Already three days post-injury a clear up-regulation of Notch3, Jagged1 and Herp2 was obvious for the vessel wall, indicating a quick induction of Notch expression and activation upon insult at the locus of neointima formation. The up-regulation of Notch members is in agreement with previously published data^{4, 16}. However, two studies^{27,25} have reported a down regulation of Notch members at mRNA and protein level²⁷ or low levels of Herp2 expression²⁵ during the early phases of vessel remodeling. This discrepancy most likely relates to the region of the injured vessel examined as we focused on the border zone of the regenerating endothelium and mid-sections, whereas others analyzed the mRNA and protein content of the entire vessel²⁷ or studied the aorta²⁵. Neointima formation is considered the result of excessive SMC proliferation and migration that can be regulated and contained by regenerating endothelium^{28, 29}. The marked up-regulation of Jagged and Notch expression in the regenerating endothelium and underlying media of balloon injured rat carotid arteries point towards a localized Notch signaling circuitry that is activated upon vascular injury. The positive role of Notch signaling in SMCs proliferation and migration has been shown in models of constitutive overexpression of intracellular *Notch1* and *Notch3*⁶. Consistently, abrogated Notch signaling in hemizygous *Notch1* and *Herp1* knockouts reduced SMC proliferation and migration as well as neointima formation^{4, 30}. Moreover, sJag1 is known to interfere with Notch signaling^{31,14} and to reduce cell migration and proliferation of NIH3T3 fibroblastic cells¹⁶. We showed that sJag1 is able to diminish SMC migration and proliferation *in vitro*. Accordingly proliferation of SMCs was reduced in the media of carotid artery from Ad-sJag1 group at day 3 as compared to Ad-LacZ group.

In vivo re-endothelialization was slightly increased at 14 days after vascular injury, whereas sJag1 treated ECs *in vitro* revealed no obvious changes in proliferation or migration. In view of the small *in vivo* difference found and the lack of a clear effect *in vitro*, it is unlikely that sJag1 plays a major role in endothelial regeneration. It is worth to note that Jag1 appears to be the main ligand for vascular SMCs related Notch signaling²³, whereas DLL4 has been reported the major inducer of NOTCH activation in EC^{32, 33}. Accordingly, we show that stimulation of ECs with immobilized recombinant DLL4 increased the expression of the Notch target genes *HES1*, *HERP1*, *-2* and *-3* (Suppl. Figure VII and ³⁴). Conversely, stimulation with immobilized recombinant Jag1 had no effect on these Notch target genes in ECs except for *HERP2*. Nonetheless, this increase in *HERP2* expression following recombinant Jag1 stimulation was far less profound than after recombinant DLL4 stimulation (Suppl.

Figure VII). Thus, other Notch ligands, beyond Jagged1, are likely involved in regulating endothelial regeneration following vascular injury, with DLL4 being a possible candidate. Lindner et al¹⁶ showed matrix protein dependency of sJag1 effects on NIH3T3. Specifically, NIH3T3 migrated at much slower rate than cells transfected with vector control when seeded on plastic, but on fibronectin this effect was much reduced. Therefore, we tested the effect of fibronectin coating on the migration and proliferation of EC in presence of sJag1. We found that HUVECs cultured on fibronectin failed to respond to sJag1 as HUVECs cultured on plastic, indicating that the lack of effect of sJag1 on ECs function is not related to the coating used.

From *in vitro* and *in vivo* studies it has become evident that both Herp1 and Herp2 act downstream of the Notch/ICD (IntraCellular Domain) signaling^{35, 36} and regulate SMC differentiation, migration and stimulate vascular remodeling in response to trauma³⁻⁷. In a previous study the Notch1-Herp1 axis was reported to stimulate vascular SMC proliferation and migration *in vitro* and neointimal formation in rodents after vascular injury^{3, 4, 25}. In accordance, we observed up-regulation of Herp2 expression in the media and neointima of injured vessels, which was reduced in injured vessels treated with sJag1. *In vitro*, Ad-sJag1 infection or recombinant sJag1 stimulation reduced *Herp2* mRNA expression in SMCs, suggesting an inhibitory effect on Notch signaling. The observation that the hampered migration and proliferation of the sJag treated SMCs could be partially rescued by Herp2 overexpression, suggests that Notch-Herp2 signaling is required for SMC function and that sJag is inhibitory. Considering the overlap in expression and function between Herp1 and Herp2 in cardiovascular development and SMC function, together with our data, it can be concluded that Herp2 plays a role in neointima formation as well.

Notch signaling has been reported to promote SMC differentiation^{23, 26}. Recently, Liu et al⁵ demonstrated that Jag1-induced Notch3 signaling initiates an autoregulatory loop that results in Notch3 overexpression and simultaneously enhanced the expression of SMC markers such as α SMA, CALPONIN and SM-MHC. We also showed that Notch3 expression in carotid artery sections in the Ad-sJag1 group was reduced concomitant with the SMC marker α SMA expression at day 3, confirming the positive role of Notch in regulating SMC differentiation. In contrast, other studies have shown that NOTCH can negatively affect SMC differentiation by interfering with SRF (Serum response factor) and myocardin^{7, 25}, that are both essential for the expression of SMC markers. The apparent controversy in data likely relates to the balance between NICD/RBPk⁷ and Notch target genes (Herp1 and 2)³⁷ that can induce and inhibit SMC differentiation, respectively.

In conclusion, we report that sJag1 inhibits neointima formation in a rat carotid artery injury model by interfering with Notch-Herp2 signaling. The sJag1 inhibitory effect on Notch activation is likely related to its interference with membrane bound

ligand induced signaling. Notch ligands induce signaling only when expressed on the cell surface or immobilized to a substrate^{31, 37}, whereas they show none or antagonistic activity when expressed as soluble forms^{31, 37, 38}. Here, we describe an interesting approach to reduce neointima formation by blocking Notch signaling with sJag1, providing a potentially applicable clinical tool for lowering the risk of restenosis in patients with peripheral artery disease.

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Supplement Material

Methods

Balloon angioplasty and adenovirus infection of rat carotid arteries

All animal studies were approved by the Animal Welfare Committee of the University of Maastricht. Animals were handled in accordance with the American Physiological Society guidelines for animal welfare and the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health. Sprague Dawley rats (n=48) were anesthetized with 80 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 8 mg/kg of xylazine (Phoenix Pharmaceutical, St. Joseph, MO, US) and for pain medication Temgesic (Shering-Plough, Houten, the Netherlands) was applied (0.01 mg/kg 30 minutes pre-operative and 3 times within 24 hours post-operative) all i.p. Vascular injury was performed as described by Clowes¹. Next the common carotid artery was instilled with 100 μ L 5.0×10^{11} VP/mL Adenoviral LacZ (Ad-LacZ), adenoviral full length Jagged1 (Ad-Fl-Jag1) or adenoviral soluble Jagged1 (Ad-sol-Jag1) for 10 minutes.

Immunohistochemistry

Carotid artery tissue of non-treated and day 3 and 14 post denudation (in total n=25) was paraffin imbedded and cross sectioned at 5 μ m, deparaffinated, rehydrated and subjected to antigen retrieval by heating the sections (12 minutes to 98° C) in citric acid buffer (0.01 Mol/L, pH 6.0). Endogenous peroxidase activity was blocked by incubating the sections for 20 minutes in PBS, 0.3% H₂O₂ before adding the primary antibody for Jagged-1 (sc-6011), Notch-1 (sc-6014R), Notch-3 (sc-7424) (all Santa Cruz Biotechnology, US) or Herp2, which was kindly provided by Dr Larry Kedes. For α SMA staining sections were incubated with A2547 Ab (Sigma-Aldrich, Missouri, US) as previously reported by Skalli et al² and for PCNA staining sections were incubated with the PCNA PC10 Ab (Roche Diagnostics, Indianapolis, US). Subsequently each section was incubated for 1 hour with peroxidase-labelled antibody (rabbit- α -mouse; P0260, DAKO, Glostrup, Denmark) or biotin-labelled secondary antibody (goat- α -rabbit (BA-1000), horse- α -mouse (BA-2000) and horse- α -goat (BA-9500), all Vector Labs, Burlingame, US). When using biotin labelled antibody, additional incubation with the Vectastain ABC staining kit (PK-6100, Vector Labs, Burlingame, US) was performed for 45 minutes. The sections were mounted with Entellan (Merck, Darmstadt, Germany). Visualization was performed with the DAB procedure and Mayer's hematoxylin was used as a counterstaining.

Adenoviral transduction and treatment with recombinant Jag1 or DLL4 proteins.

Human umbilical vein endothelial cells (HUVECs), human coronary arterial endothelial cells (HCAEC) and human coronary artery smooth muscle cells (CASMCS) (all from Lonza, Verviers, Belgium) were transduced with 5×10^9 VP/mL Ad-LacZ, Ad-sJag1 (c-myc tagged)³ or Ad-Fl-Jag1. For stimulation experiments with attached ligand rDll4 or rJag1, ECs were grown for 48 hours on 0.2 % gelatin coated plates containing 1 μ g/mL recombinant rDLL4 or Jag1 (R&D System; Abingdon, Oxfordshire, UK) as previously described by Williams et al⁴. Stimulation of CASMCs with soluble Jag1 was performed by adding 5 μ g/mL of recombinant sJag1 (R&D System) to the medium. ECs and CASMCs, infected or treated with recombinant protein, were cultured in EGM-2 and SmGM-2 medium (Lonza), respectively at 37 °C with 5 % CO₂. Forty eight hours after infection cells transduced with Ad-sJag1 were treated with Brefeldin A (10 ng/mL, Sigma, St. Louis, MO, US) for 4 hours. Cells were lysed with RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 20 mM NaPyrophosphate, 1 % Triton X-100, 1 mM PMSF, 1 mM Na₃VO₄). Immunoprecipitates were separated on a 10 % SDS-polyacrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA, US). Membranes were stained with anti-c-Myc or anti-Jagged1 antibody as described previously⁵.

Infection efficiency

The infection efficiency in vivo was determined by performing a β -galactosidase enzymatic staining at 3 day post injury in carotid artery sections that have been instilled with 100 μ L 5.0×10^{11} VP/mL. The infection efficiency was also assessed by immunofluorescent staining of cultured cells upon Ad-Fl-Jag1 infection. CASMCs were cultured on 4 wells slides (BD, Franklin Lakes, USA) until confluence and infected with Ad-Fl-Jag1 or Ad-LacZ. After 24 hours cells were incubated for 1 hour with the primary goat polyclonal anti-Jagged1 (Santa Cruz sc-6011). Slides were washed and incubated with 2 % rabbit serum and secondary Ab Alexa-Fluo594 Rabbit anti-Goat A-11080 (Invitrogen). Photos were taken using a Leica microscope with DFC350 FX digital camera.

Proliferation and migration assays in HUVECs or CASMCs

To assess HUVECs or CASMCs proliferation CellTiter 96® AQueous One solution Cell Proliferation Assay (Promega, San Luis Obispo, CA, US) was used. Per well 2×10^3 cells were plated in a 96 well tissue culture plate for 48 hours after adenoviral transduction and were incubated overnight in appropriate growth medium. Cell Proliferation Assay was added directly to culture wells, incubating for 2 hours and then recording absorbance at 490 nm on a Victor-3 fluorescent microplate reader (Perkin Elmer). The same protocol was used to determine migration of CASMCs previously transfected with pcDNA3.2-Hey1 (Herp2) or mock and incubated with or

without 5 µg/mL recombinant Jagged1 (R&D technologies). Proliferation of HUVECs seeded on fibronectin coating 96 well tissue culture plate after Ad-sJag1 transduction was also assessed. Proliferation was defined as the average of four independent wells.

For the migration wounding assay, HUVECs were grown to confluence in 6-well plates and the monolayer was scratched with a pipette tip. Gap width was measured at marked locations from images taken by inverted microscope (TMS-F, Nikon), immediately after the scratching and 10 hours later. Relative migratory distances were calculated. Independent experiments were performed 3 times. Migration of HUVECs cultured on fibronectin was assessed by performing an Oris™ Cell Migration Assay (Platypus Technologies, LLC, USA). Per well 1.3×10^4 cells were seeded on 96 wells plate coated or not with fibronectin. The next day cells were infected with Ad-LacZ or Ad-sJag1. After 24 hours cells were stained according to protocol instructions and migration was assessed by measuring fluorescence on a Victor-3 fluorescent microplate reader (Perkin Elmer, Groningen, the Netherlands) in bottom read mode. For transmigration assays, CASMC were labeled with calcein-AM (5 µL/mL, Molecular Probes, Eugene, OR) and 5×10^4 cells were placed in the FALCON HTS FluoroBlock transwell system inserts (pore size 8 µm; Becton Dickinson, Bedford, MA) in DMEM supplemented with 0.5 % fetal bovine serum (Invitrogen, Carlsbad, California) and incubated for 8 hours at 37 °C. The lower chamber of the FluoroBlock contained DMEM supplemented with PDGF-BB (10 ng/mL) and 0.5 % fetal bovine serum. The extent of cell migration was estimated by measuring fluorescence on a Victor-3 fluorescent microplate reader (Perkin Elmer, Groningen, the Netherlands) in bottom read mode. The same protocol was employed to determine migration of CASMCs that were transfected with pcDNA3.2-Hey1 (Herp2) or mock and were incubated with or without 5 µg/mL recombinant Jagged1 (R&D technologies). Three independent experiments were performed. SMC proliferation in vivo was assed by counting the number of PCNA positive nuclei in a given area of the vessel of day 3 and 14 Ad-LacZ and Ad-sJag1 treated balloon injured carotid arteries (n=6 per group).

RNA expression analysis

Twenty four hours after infection or treatment with recombinant sJag1 protein, RNA was isolated by using RNeasy micro-kit Qiagen (Qiagen, GmbH, Hilden, Germany). A total of 100 ng RNA per sample was subjected to reverse transcription (RT). qPCR was performed using Superscript™ III Platinum Two-step qRT-PCR kit with SYBR green (Invitrogen) and primer concentration of 10 µM according to van den Akker et al⁶. PCR Primers used were: human β -ACTIN-sense: 5'-ATCCTCACCCTGAAGTACCC-3', β -ACTIN-antisense: 5'-CACGCAGCTCATTGTAGAAG-3'; GADPH-sense: 5'-GCCTCAAGATCATCAGCAAT-3'; GADPH-antisense: 5'-GGACTGTGGTCATGAGTCCT-3'; HES1-sense: 5'-CCAAAGACAGCATCTGAGCA-3', HES1-antisense: 5'-

GCCGCGAGCTATCTTTCTT-3'; *HERP1*-sense: 5'-GAAGATGCTTCAGGCAACAG-3', *HERP1*-antisense: 5'-GCAACTTCTGTTAGGCACTCTC-3'; *HERP2*-sense: 5'-CGCCCTTGCTATGGACTATC-3', *HERP2*-antisense: 5'-TGCGAAACCAGTCGAACTC-3'; *HERP3*-sense: 5'-CGCATCAACAGTAGCCTTTCT-3', *HERP3*-antisense: 5'-GGCATGGAGCATTTTCAAGT-3'. Primers were designed with oligopercetTM Designer (Invitrogen), Primer3 and Mfold (<http://www.idtdna.com/scitools/Application/mfold/>) and were synthesized by Eurogentec (Seraing, Belgium). qPCR reactions were run on a BioRad MiQ Real Time PCR detection System (BioRad, Veenendaal, the Netherlands).

Carotid artery perfusions and silver staining

Two weeks after carotid artery injury upper body perfusion was performed through cannulation of the ascending aorta. In brief, after rinsing with heparinized saline, the upper circulation was perfused with fixative (1% paraformaldehyde (PFA) and 0.5 % glutaraldehyde in 75 mmol/L cacodylate, pH 7.4), followed by saline and 5 % glucose and 0.1 % silver nitrate under 90 mm Hg pressure. Thereafter carotid arteries were removed and post fixed, opened longitudinally, exposed to light, and dehydrated and mounted in Permaslip mounting medium (Alban Scientific Inc, St Louis, MO, US). Endothelial regeneration was assessed using an Olympus BH-2 microscope with a Sony SSC C374 camera and NIH Image-J[®] software. Endothelial covering was calculated by two independent investigators blinded to the experimental groups (Inter-observer variability was <2 %) as the percentage regenerating endothelial cells divided by the total area.

Morphometric analysis

Carotid artery segments of 5 mm were fixed in 4 % PFA for 30 minutes and imbedded in Tissue-Tek OCT media (Sakura Finetek USA, Inc. Torrance, CA, US). Sections of 5 µm were stained with Elastin van Gieson, hematoxylin and eosin (H&E). From each section, luminal area, inside the internal elastic lamina, and the area encircled by the external elastic lamina were traced manually and measured with standard computer assisted planimetry in a double blinded manner. Medial area was calculated as area encircled by external elastic lamina minus area inside the internal elastic lamina. Intimal area was calculated as area inside the internal elastic lamina minus luminal area. From these calculations the ratio of intimal to medial area was defined.

Supplemental Figures

Figure I Migration and proliferation assay on HUVECs infected with Ad-sJag1 (sJag1) or Ad-LacZ (LacZ) and cultured on plastic or fibronectin. A) No difference in migration rate was observed in HUVECs infected with Ad-LacZ or Ad-sJag1 when cultured on plastic. B) Likewise, Ad-sJag1 had no effect on HUVECs cultured on a fibronectin coated surface. C) Proliferation rate of HUVECs transduced with Ad-LacZ or Ad-sJag1 and cultured on fibronectin was comparable. Data represent mean $\pm$ SD of three independent experiments.

Figure II β -galactosidase and Jagged1 staining showing the infection efficiency *in vivo* and *in vitro*, respectively. A) β -Galactosidase expressed as blue cells at day 3 post-injury in the rat carotid artery that had been instilled with 5×10^{11} VP/mL Ad-LacZ for 10 minutes. X-gal staining showed blue stained cells in the transfected artery. B) Immunofluorescence analysis of CSMCs infected with Ad-LacZ or Ad-FI-Jag1 5×10^9 VP/mL for 24 hours for Jagged-1. CSMCs infected with Ad-FI-Jag1 showed higher Jagged1 staining (red) (panels III and IV, 10X and 20X magnification, respectively) as compared with Ad-LacZ infected cells (panel I and II, 10X and 20X magnification, respectively) showing only the expression of endogenous Jagged1.

Figure III Cross sections of balloon injured and Ad-LacZ (A), Ad-Fl-Jag1 (B) transduced carotid arteries showing Jag1 expression at 3 days post injury. A higher Jag1 signal was apparent for the Ad-Fl-Jag1 section as compared with the Ad-LacZ control. Asterisk represents the media. Scale bar: 200 μ m.

Figure IV Cross sections of balloon injured and Ad-LacZ (A), Ad-sJag1 (B) and Ad-Fl-Jag1 (C) transduced carotid arteries showing Notch3 expression at 3 days post injury. Notch3 expression was lower in Ad-sJag1 (B) than in Ad-LacZ control (A) or Ad-Fl-Jag1 (C) groups. Asterisk represents the media. Scale bar: 200 μ m.

Figure V Cross sections of balloon injured and Ad-LacZ (A, B and E) and Ad-sJag1 (C, D, F) transduced carotid arteries showing alpha-smooth muscle actin (α SMA) expression at 3 and 14 days post injury. α SMA was expressed at a lower level in the media of Ad-sJag1 (C) infected arteries as compared to Ad-LacZ controls (A) at 3 days post-injury. (B) and (D) are higher magnifications of the boxed area in (A) and (C) respectively. Scale bar: 200 μ m.

(C), respectively. (E) At 14 days following vascular injury, the SMCs within the neointima strongly express α SMA at a level comparable to the SMCs of the media. (F) Ad-sJag1 transduced carotid arteries showing comparable α SMA level in the media and neointima as the control Ad-LacZ (E). Asterisk represents the neointima. Scale bar: A and C 800 μ m; B and D 150 μ m; and E-F 350 μ m.

Figure VI Cross sections of balloon injured and Ad-LacZ or Ad-Fl-Jag1 transduced carotid arteries showing Herp2 expression at 3 days post-injury (A) and *HERP2* mRNA expression in CASMCs infected with Ad-LacZ or Ad-Fl-Jag1 (B). Ad-Fl-Jag treated arteries (A, right panel) showed a comparable medial Herp2 expression to Ad-LacZ treated arteries (A, left panel). *HERP2* mRNA expression was significantly increased in Ad-Fl-Jag1 transfected CASMCs as compared to Ad-LacZ infected cells (B). Data are presented as mean $\pm$ SD of three independent experiments. Scale bare is 200 μ m.

Figure VII mRNA expression of Notch target genes of ECs stimulated with immobilized rDLL4 or rJag1. rDLL4 stimulated ECs showed a significant (*) increase in *HES1*, *HERP1*, 2 and 3 mRNA expression (rDLL4, black bars) compared to non-stimulated cells (Ctrl, open bars). Cells cultured on rJag1 showed only an increase in *HERP2* expression (rJag1, grey bars) that was significantly less (#) than the increase induced by rDLL4. Data presented as mean $\pm$ SE of three independent experiments.

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Chapter 6

General Discussion

New insights in the NOTCH Signaling Pathway

VEGF induced DLL4 is mediated by VEGFR-2 and NOTCH signaling

A positive feed-forward loop is responsible for the VEGF-A induced DLL4 expression

NOTCH-induced DLL4 expression: feed-forward or feed-back signaling?

VEGF induced NOTCH signaling and DLL4 overexpression by activating ADAM-10

Consequence of altered NOTCH signaling at Tissue/Organ Level

DLL4 blockade: potential therapeutic implication for inhibition of tumour growth

VEGF and NOTCH in coronary artery development: effects on endothelial differentiation and development of the media

Inhibition of NOTCH-signaling by soluble Jagged1: a new tool to inhibit neointima formation

Conclusion

New insights in the NOTCH Signaling Pathway

The role of NOTCH in cardiovascular development and its implication in regulating physiological and pathophysiological vascular biology has received particular attention over the last years¹⁻¹⁰. In this thesis the role of the NOTCH signaling pathways in vascular biology has been investigated with special focus on the effects of VEGF on DLL4 expression, NOTCH signaling and ADAM activity in endothelial cells (ECs), and the consequence of altered NOTCH signaling for vessel function.

VEGF induced DLL4 is mediated by VEGFR-2 and NOTCH signaling

DLL4-induced NOTCH signaling is required for endothelial sprouting and branching of blood vessels. Among endothelial cell (EC), the ones that are highly VEGFR-2 positive will localize at the sprouting edge and will develop into endothelial tip cells. In response to VEGF-A, these cells will start sprouting by elongating filopodia, and will upregulate DLL4 expression. Subsequently, interaction of the DLL4 positive tip cells with adjacent ECs triggers NOTCH signaling in the latter and, eventually, differentiation into a stalker cell¹¹. The stalker cell exhibits low migration and filopodia capacity^{1, 2, 5, 11-14} and will become the endothelium lining the lumen of the newly branched vessel structure. The VEGF signaling appears self-limiting as DLL4-induced NOTCH signaling in turn triggers the downregulation of VEGFR-2^{6, 8}. VEGF-signaling is further silenced by enhancing the expression of VEGFR-1 and soluble VEGFR-1 (sVEGFR-1) that both are considered negative regulators of VEGF signaling in ECs by sequestration of VEGF^{15, 16}. VEGFR-2 shows a stronger tyrosine kinase activity than VEGFR-1 and is regarded the main receptor to mediate VEGF signaling in vascular ECs¹⁷⁻¹⁹. VEGFR-2 has therefore been considered the major player in facilitating the VEGF-A induced DLL4 expression^{6, 8}, although no direct evidence was provided that fully justified this assumption. We established that the expression of *DLL4* in ECs is specifically stimulated by VEGFR-2 and not by VEGFR-1 mediated signaling (Chapter 2). Intriguingly, VEGF-A165-induced DLL4 expression was prevented by NOTCH signaling inhibitors, indicating that NOTCH signaling itself is required for increased DLL4 expression downstream of VEGF/VEGFR-2. These observations strongly suggest that VEGF induces DLL4 expression in a manner that is dependent on VEGF-induced NOTCH signaling.

A positive feed-forward loop is responsible for the VEGF-A induced DLL4 expression

In addition to the VEGF-A induced NOTCH activation, the FOXC forkhead transcription factors have also been implicated to mediate DLL4 expression downstream of VEGF/VEGFR-2 and independent of NOTCH activation. Although Seo et al²⁰ have shown that FOXC1 and more efficiently FOXC2 transactivated the DLL4 promoter in a dose dependent manner, mutation of the Forkhead Binding Element (FBE) did not attenuate the induction of DLL4. The authors concluded that a

combinatorial regulation by different classes of transcription factors in addition to FOXC could be responsible for the regulation of DLL4 transcription. We found that inhibition of NOTCH signaling perturbed VEGF-induced DLL4 expression, suggesting a direct role for NOTCH in this process. Furthermore, we showed that increased NOTCH signaling by overexpression of NICD-4 and NICD-1 effectively transactivated deletional fragments of the DLL4 promoter that contained several RBP-Jk binding sites, but lacked the FBE²¹ (Chapter 2). Moreover, overexpressing a constitutively active FOXC2 construct in endothelial cells was without clear effect on DLL4 expression (Chapter 2). Hayashi and Kume²² showed that FOXC2 and more effectively NICD were individually able to transactivate the promoter of the NOTCH target gene Hey2. However in combination they act synergistically inducing a more potent transactivation of the promoter. We can not exclude that a similar mechanism could affect DLL4 expression as well.

NOTCH-induced DLL4 expression: feed-forward or feed-back signaling?

Once ascertained that NOTCH induces DLL4 expression we wondered whether DLL4 overexpression could serve as a positive or a negative regulation mechanism of NOTCH signaling. NOTCH ligands can be shed from the plasma membrane and serve as inhibitor of NOTCH signaling. Thus, the potential negative effect of soluble DLL4 (sDLL4) on NOTCH signaling was tested in ECs (unpublished data Caolo et al 2010). Theoretically, sDLL4 would inhibit the DLL4-induced expression in cells cultured on immobilized DLL4 by competing with immobilized DLL4 for the same membrane bound NOTCH receptor without activating signaling. We found that the inhibition displayed by sDLL4 was not as effective as that observed with γ -secretases blockers. Furthermore, we were unable to detect any sDLL4 in immuno-precipitated medium from cells that were previously treated with immobilized DLL4 or NICD, which presented with higher DLL4 expression. These data, together with those from chapter 2, are in favor of a mechanism by which induced DLL4 is produced as an activating membrane bound ligand rather than a soluble inhibitor.

Stimulation of ECs with immobilized recombinant DLL4 triggered a positive feed-forward loop that resulted in the activation of NOTCH signaling and subsequent upregulation of DLL4. Such mechanism was VEGF-signaling independent as the inhibition of VEGFRs did not prevent this effect (Chapter 2). The ability of an activated NOTCH receptor to induce the expression of its ligand DLL4 provides ECs with a mechanism by which NOTCH signaling might be distributed between communicating ECs. NOTCH signaling activation, initially potentiated by external stimuli such as VEGF, would induce DLL4 up-regulation and in turn NOTCH signaling and DLL4 expression in the surrounding cells²³ (Figure 1). As DLL4-induced NOTCH signaling is essential for arterial specification, propagation of NOTCH signaling through the positive feed-forward loop, could be a suitable mechanism to induce and maintain arterial marker expression along a blood vessel. Besides inducing

arterial marker expression DLL4-induced NOTCH signaling is required for regulating sprouting of newly forming vessels. Loss of DLL4-Notch signaling *in vitro*²⁴ and in zebrafish and mouse models of angiogenesis led to ectopic sprouting and increased tip cell number^{7, 11, 25-27}. A mechanism, by which DLL4 expression and NOTCH signaling are propagated, would prevent filopodia extension in a subpopulation of cells rather than exclusively in the stalk cells immediately adjacent to the tip cells. A balance between tip cells and stalk cells will eventually contribute to a proper branching patterning and to the formation of a functional blood vessel network.

VEGF induced NOTCH signaling and DLL4 overexpression by activating ADAM-10

VEGF-induced RBP-J κ transactivation and NOTCH target gene HES-1 expression indicated that NOTCH signaling was activated directly downstream of VEGF. This activation was not secondary to DLL4 upregulation, suggesting that VEGF might stimulate NOTCH signaling in a ligand independent way (Chapter 2). Swendeman et al recently showed that VEGF-A enhanced shedding of its own receptor VEGFR-2 and co-receptor NP1 by activating ADAM-17 and ADAM-10, respectively²⁸. As ADAMs are essential metalloproteinases involved in the activation of NOTCH signaling - mediating the first proteolytic cleavage of the receptor - we speculated that VEGF-A induced NOTCH signaling by activating ADAM. Accordingly, VEGF increased both the expression of ADAM-10 at mRNA and protein level as well as ADAM related protease activity in endothelial cells (Chapter 3). Furthermore, we showed that the link between VEGFR-2 signaling and ADAM activity is likely a direct result of VEGFR-2 phosphorylation and related kinase activity. In particular, the phosphorylation of tyrosine 1175 within the C-terminus recruits PLC γ , which mediates the activation of the mitogen-activated protein kinase (MAPK)/extracellular-signaling-regulated kinase-1/2 (Erk1/2) cascade and the phosphorylated Tyr1175 adaptor molecule Shb²⁹ that activates Phosphatidylinositol 3' kinase (PI3K). Both ERK1/2 and PI3K are essential kinases involved in cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. Another important phosphorylation site in VEGFR-2 is Tyr1214 which initiates the p38-MAPK pathway³⁰. Blockage of these main signal transduction pathways showed that inhibition of the PI3K or ERK1/2 pathway prevented VEGF-A165 induced ADAM-10 activity, whereas p38 inhibition was without consequences. These results indicate that VEGF/VEGFR-2 signaling, through PI3K and ERK1/2 kinase pathways, specifically induced ADAM expression and activity, which subsequently mediates VEGFR-2 shedding, thus limiting the duration of signaling. As ADAMs mediate NOTCH signaling activation even in the absence of typical NOTCH ligands³¹, VEGFA induced ADAM activity will likely result in enhanced NOTCH signaling, with subsequent increase of DLL4 expression, and shutdown of VEGF signaling by VEGFR-2 shedding. In support of this model, we showed that VEGF induced NOTCH signaling and DLL4 expression was prevented by ADAM blockers addition (Chapter 2).

This interrelation between VEGF and ADAM activity could explain the spatial and/or temporal regulation that causes the “salt and pepper” pattern of DLL4 expression and associated tip and stalk cell distribution described for the mouse retina model^{11, 32}. The VEGFRs expression appears to determine whether a cell will become a tip or a stalk cell. Computational models have been used to allow various combinations of ECs heterozygous for VEGFR-1 or VEGFR-2 to compete with wild-type cells for the tip position^{12, 33, 34}. Cells heterozygous for *Vegfr-2* showed poor contribution to the tip cell population and predominantly became stalk cells in competition with wild-type cells, whereas cells heterozygous for *Vegfr-1* dominated the tip cell population. The balance of VEGFR-2 and VEGFR-1 expression in individual endothelial cells appears to affect their potential to become tips cell during sprouting angiogenesis. In response to VEGF, cells which are supposed to have high VEGFR-2 signal will upregulate DLL4 and adopt tip cells to initiate sprouting¹². Thus it can be speculated that in these cells, VEGF/VEGFR-2 induced ADAM activates NOTCH signaling and secondarily DLL4 expression, initially independent of a NOTCH ligand as was proven by Delwig et al³¹. This assumption is in agreement with the observation that the surrounding stalk cells will –initially- present with low DLL4 levels. Subsequently, expressing DLL4 cells would induce NOTCH signaling and DLL4 expression in the neighbor cells. Eventually, activated NOTCH would turn down VEGFR-2 signaling⁸ and thus reduces sprouting.

Altogether, these data provide new insights into the molecular mechanism that characterizes VEGF and NOTCH signaling pathway interaction, indicating that VEGF-A activates ADAMs, that will initiate NOTCH signaling. Eventually, cleaved NICD induces DLL4 upregulation initiating a feed-forward loop that results in the propagation of NOTCH signaling and ligand expression between communicating endothelial cells (Figure1).

Figure 1 Proposed model of propagated NOTCH-signaling and DLL4 expression downstream of VEGF in Endothelial Cells. The stimulation of VEGFR-2 by VEGFA induces ADAM activity. Such process is mediated by PI3K and ERK1/2 intracellular kinase pathways. Activated ADAM will attenuate VEGF-signaling by shedding VEGFR-2, and will initiate NOTCH signaling, by shedding the extracellular region of the NOTCH receptor. Following ADAM, the γ -secretase complex mediates the second proteolytic cleavage of the NOTCH receptor leading to NICD release. Activated NICD translocates to the nucleus where it induces DLL4 and represses VEGFR-2 expression. Subsequently, newly expressed DLL4 activates the NOTCH receptor on the neighbor cell, which in turn up-regulates DLL4 expression.

Consequence of altered NOTCH signaling at the Tissue/Organ Level

DLL4 blockade: potential therapeutic implication for inhibition of tumour growth

Over the last years, research efforts have focused on the identification of new therapeutic agents that disrupt functional angiogenesis in tumours. The crucial role of DLL4 in regulating vascular development and its high expression level in tumour vasculature^{9, 35–39} make it a suitable therapeutic target. Indraccolo et al⁴⁰ reported that the escape of human T-cell acute lymphoblastic leukaemia (T-ALL) cells or colorectal cancer cells from dormancy is associated with DLL4 expression in the tumour microenvironment and increased Notch3 signaling in tumour cells. Conversely, neutralization of DLL4 greatly reduced EC-induced Notch3 activation in T-ALL and blocked tumour growth⁴⁰. Moreover, DLL4 seems to play a role in the differentiation of Bone Marrow (BM)-derive pericytes/vSMCs during vasculogenesis in Ewing's Sarcoma^{41, 42}. The BM cells migrate into the tumour and differentiate into endothelial cells and pericytes supporting the growth of the Ewing's sarcoma. Inhibition of DLL4 correlated with decreased numbers of BM-derived cells in tumour vessels and increased tumor hypoxia^{41, 42}. From these studies it emerges that DLL4 plays a role in tumour vasculature formation, suggesting that interference with DLL4/NOTCH signaling might provide an approach to prevent formation of functional vessels in tissue surrounding tumours^{43, 44}. Consistently, several recent studies revealed that inhibition of DLL4/NOTCH signaling reduced the growth of various solid tumours in mice^{45–52}. As observed in zebrafish and in the retina mouse model, despite the increase in tumour blood vessels density following anti-DLL4 therapy, these vessels were not functional^{45–47}.

Although most of the current therapies aim to block the VEGF pathway, not all tumours are responsive to VEGF blockers³. Remarkably, anti-DLL4 therapies were effective against tumours that were resistant to anti-VEGF treatment³. Differently, DLL4 inhibition increase vessels density by inducing branching^{45, 48}. Despite the increase in vessel number, these vessels are disorganized and poorly perfused resulting in tumour size degradation (Figure 2). Therefore DLL4 inhibition could provide synergistic effects when combined with anti-VEGF therapy against certain tumours^{45, 46, 53}, providing a more exciting approach to inhibit tumour growth⁵⁴. Anti-DLL4 antibodies have recently entered clinical trials. However, the toxic side effects of DLL4 are still poorly understood⁵⁵. Chronic DLL4 blockade has recently been reported to abnormally activate endothelial cells, disrupt normal organ homeostasis and produce significant pathology in multiple organs, inducing vascular neoplasms⁵⁶. From this study, it emerges that a diligent strategy is required to use the DLL4-NOTCH signaling pathway as a powerful tool against tumours, avoiding undesired side effects. Moreover, our data provide evidence for a role of VEGF-induced ADAM activity in the NOTCH signaling activation. We showed that VEGF-induced DLL4 expression was prevented by ADAM blockers (Chapter 2). Interfering at the level of NOTCH receptor activation - i.e. by using ADAM inhibitors - might

provide a more potent tool to prevent blood vessels formation in tumours, than sole DLL4 inhibition.

Figure 2 Comparison of VEGF versus DLL4-NOTCH inhibition in tumor angiogenesis. The tumor vasculature consists of actively growing and abnormal blood vessels. Addition of VEGF inhibitors results in decreased tumor perfusion and decreased tumor growth, although the tumor vasculature is normalized and the perfusion of vessels remains efficient. Conversely, DLL4 inhibitors addition results in increased tumour vessel density. However, these vessels are not functional, thus resulting in decreased tumour perfusion and growth (modified after Thurston Nature Review Cancer 2007).

VEGF and NOTCH in coronary artery development: effects on endothelial differentiation and development of the media

Arterial specification, i.e. differentiation into venous or arterial endothelium is characterized by specific gene expression patterns. Arteries tend to express EphrinB2 and NP1 whereas venous expression includes EphB4 and COUP-TFII. VEGF-induced expression of DLL4 and increased NOTCH signaling results in overexpression of EphrinB2 and repression of EphB4 and COUP-TFII in endothelial cell and thus contributes to an arterial phenotype^{6, 8, 10, 25, 57}. Ablation of COUP-TFII in endothelial cells likewise enables veins to acquire an arterial phenotype, including the expression of arterial markers NP1 and NOTCH signaling. Moreover, ectopic expression of COUP-TFII in ECs results in the fusion of veins and arteries in transgenic mouse embryos⁵⁸.

In addition to the two VEGF receptors, several coreceptors (NP1, NP2 and heparin sulphate) are involved in the regulation of VEGF signaling in ECs⁵⁹⁻⁶². NP1 and NP2 are membrane bound receptors containing a small cytoplasmatic domain lacking intrinsic catalytic functions⁶³⁻⁶⁶. NP1 is preferentially expressed in arterial endothelial cells⁶⁷⁻⁶⁹ and its mutation causes vascular defects in mouse embryos⁷⁰. Although NP1 has initially been shown to guide the development of the nervous system, its importance in the development of the vascular system has been firmly established^{63, 65, 66}. Coexpression of neuropilins with VEGFR-2 in transfected cells enhances VEGF-A165 binding to VEGFR-2⁶⁸. Heparin sulphate is another crucial VEGFR-2 coreceptor^{59-61, 71}. Heparan sulfate proteoglycans (HSPGs) are transmembrane proteins and modulate the activity of a large number of secreted signaling molecules by stabilizing the complex between ligand and receptor⁷². HPSG can physically interact with VEGF modulating its interaction with VEGFR-2, and determining the duration of downstream signaling⁷¹. The VEGF ligands and isoforms display different interactions with the VEGF receptors and coreceptors, which results in diverse downstream effects. The three main human VEGF-A splice variants VEGF-A121, VEGF-A165 and VEGF-A189 are represented by Vegf120, Vegf164 and Vegf188, respectively in the mouse. We analyzed the effect of altered VEGF-signaling on NOTCH signaling and endothelial differentiation and on artery development by using a *Vegf120/120* knock-in mouse model that lacks the other splice variants (Chapter 4). The coronary arterial differentiation was clearly altered in this mouse model. Arterial endothelial cells of *Vegf120/120* transgenics showed decreased expression of several NOTCH members such as *Notch1*, *Dll4* and *Jag1* accompanied by a reduction of the arterial marker *EphrinB2* and concomitant increase of the venous markers *Coup-TFII* and *EphB4*. Conversely, the coronary venous ECs showed the reverse effect with increased expression of arterial markers and decreased venous marker expression. In vivo, in the *Vegf120/120* mouse, a severe decrease in coronary arterial size with an increase in venous size was observed. We concluded that the altered expression of these endothelial markers

was due to lack of the Vegf164 isoform. Stimulation of cultured human cardiac endothelial cells with either VEGF-A121 or VEGF-A165 confirmed the different effects of Vegf120 and Vegf164 on arterial specification observed in vivo. VEGF-A165-stimulated ECs showed higher arterial marker EphrinB2 and lower venous marker COUP-TFII expression compared to VEGF-A121 stimulated cells. In *Vegf120/120* mouse embryos, where Vegf120 is the sole VEGF isoform produced⁷³, the VEGFR-2 and NOTCH signaling is likely negatively affected in arterial ECs. Moreover, due to its limited heparin-binding capacity, Vegf120 is more soluble than Vegf164 and will cause an ectopically higher VEGF concentration around the venous vessels than normally present. We assume that this increase in Vegf120 concentration will increase VEGF signaling as well and, subsequently will result in NOTCH signaling. Future research is required to unravel the link between EC signaling and the expression of NOTCH receptors in which VEGF will likely participate as a co-activator. From our study in *Vegf120/120* mice it emerges that alteration of VEGF and NOTCH signaling during development affects maturation, morphology and function of the coronary system. Consistently, polymorphisms in the VEGF and VEGFR-2 genes in humans are associated with Kawasaki Disease⁷⁴, in which children can develop coronary aneurysms upon inflammation⁷⁵, and with coronary heart disease⁷⁶. Our findings indicate an instructive role for VEGF isoforms distribution in coronary development which could be used for optimizing current revascularization therapies by targeting VEGF-signaling in the heart⁷⁷.

Besides inducing arterial ECs specification, NOTCH signaling has been implicated in vSMC-development^{78,79}. Diminished arteriogenesis was also obvious in *Vegf120/120* embryos, whereas coronary veins showed a gain of pericytes/vSMCs. The media of coronary arteries showed decreased number of cells expressing α SMA, NP1, Dll1, Jagged2 and Notch3, whereas coronary veins showed an increase in cells positive for these markers (Chapter 4). On the basis of this data and of the reported link between NOTCH-signaling and SMC differentiation⁸⁰, we proposed that alterations in the NOTCH-signaling pathway of ECs in *Vegf120/120* mice, caused by defective VEGF-signaling, contributed to decreased differentiation of vSMC⁸¹ and therefore to aberrant arteriogenesis in these animals. A comparable link between SMCs differentiation induced by neighboring ECs through NOTCH signaling has been shown by Liu et al. They have shown that Jagged1 expressing endothelial cells can induce the expression of NOTCH-3 and Jagged1 in adjacent SMCs and this was essential for SMC differentiation⁸⁰.

Altogether data from literature and our results underlie the crucial role of VEGF-signaling in inducing NOTCH expression and signaling, contributing to arterial differentiation of the endothelium. Conversely, the expression of COUP-TFII appears to be sufficient to repress VEGF and NOTCH signaling promoting a venous phenotype (Figure 3).

Adapted from You et al, Nature 2005

Figure 3 Role of VEGF-signaling in arterio-venous differentiation (a) High levels of VEGF-signaling, amplified by VEGF co-receptor NP1, leads to high NOTCH-signaling, which subsequently, induces the expression of the arterial EC-specific marker EphrinB2 and represses the expression of the vein-specific marker EphB4, resulting in an arterial phenotype of the EC. Notch activation also initiates a feed-forward loop that results in the upregulation of its own signaling independent of VEGF-signaling. (b) The presence of COUP-TFII, a vein specific transcription factor, decreases NP-1 expression and negatively affects NOTCH expression and thus signaling. This gives rise to lower EphrinB2 and higher EphB4 levels, concomitant with a venous phenotype.

Inhibition of NOTCH-signaling by soluble Jagged1: a new tool to inhibit neointima formation

The behaviour of SMC in a blood vessel is strongly affected by their neighboring ECs. After vascular injury, SMCs in response to signals delivered from the injured endothelial and accumulating inflammatory cells start to migrate from the media to the intima and proliferate, forming an intima lesion in a process referred to as neointima formation. Lindner et al showed that the NOTCH circuit is activated following vascular injury⁸², and others reported a positive role for NOTCH in the neointima formation process^{83–85}. Specifically, Li et al⁸³ demonstrated that Notch1 mediates SMC proliferation and neointima formation after vascular injury through a NOTCH specific CHF1/Hey2 signal pathway. The relation between NOTCH and neointima formation was further supported by analyses of Notch1^{+/-} and SMC-specific Notch1 heterozygous deficient (smN1^{+/-}) mice that showed decreased neointima formation after carotid artery ligation. Also Sakata et al⁸⁴ reported that neointima formation was dramatically reduced in the Hey2^{-/-} mice and that

CHF1/Hey2-deficient VSMC showed decreased proliferation and ability to migrate in response to growth factors such as PDGF and heparin-binding epidermal growth factor (HB-EGF), which have been reported to accumulate at the site of vascular injury⁸⁶⁻⁸⁸. Lamellopodia formation and membrane ruffling induced by these growth factors were reduced in *Hey2* mutant VSMC compared to wild type SMC⁸⁴. Moreover, *Sos1* expression and *Rac1* activation, essential proteins for cell proliferation and cell motility^{89, 90}, were reduced in *Hey2* knockout VSMCs⁸⁴. Other possible mechanisms that include regulation of cell cycle genes and apoptosis have been reported to play a role in vascular remodelling after injury. Wang et al (72) showed that the overexpression of another NOTCH target gene *HRT1* (also named *Hey1/HERP2/Hesr-1/CHF2*) induces VSMC growth by inhibiting the expression of a crucial cell cycle inhibitor, *p21*^{WAF1/CIP1}, and by up-regulating *Akt* expression/activity, a key modulator of apoptosis. Taken together, these data suggest that inhibition of NOTCH signaling can prevent neointima formation.

Soluble forms of NOTCH ligands, Delta and Jagged, have been isolated and can serve as natural inhibitors of this pathway^{91, 92}. Lindner et al⁸² have shown that soluble Jagged1 (sJag1) ligand negatively affected migration of NIH3T3 cells by interfering with cell-matrix interaction and focal adhesion formation. A similar mechanism could affect SMC as well. We showed that sJag1 reduced neointima formation after vascular damage in a rat carotid artery injury model (Chapter 5). In this process sJag prevents the activation of NOTCH signaling by competing with the membrane bound ligand, thereby inhibiting NOTCH-signaling, SMC proliferation and migration as schematized in figure 4. We observed that among the NOTCH target genes, *Herp2* was up-regulated in the media and neointima of injured vessel and was reduced in injured vessels that were treated with sJag1. The reduced neointima formation and *Herp2* expression in animals treated with sJag strongly suggested a link between these two processes. Consistently, we showed that sJag was able to inhibit SMC migration and proliferation by interfering with *Herp2* mediated NOTCH signaling. Decreased migration of the sJag treated SMC could be rescued by *Herp2* overexpression, suggesting that Notch-*Herp2* signaling is sufficient for SMC migration. Although further studies are needed to fully elucidate the molecular mechanism underlying the inhibition of NOTCH signaling by sJag1, it might provide a potential therapeutic tool to reduce restenosis in patients who have been subjected to percutaneous coronary intervention (PCI). The application of drug-eluting stent (DES) technology to improve clinical outcomes following PCI procedures represents one of the greatest successes in cardiology^{93, 94}. The use of stents capable to release agents such as antibodies and soluble ligands that interfere with NOTCH signaling, combined with anti-inflammatory drugs delivery, would probably benefit to limit restenosis after stent implantation. However, as previously discussed for DLL4, potentially toxicity of NOTCH ligand inhibition should be carefully assessed.

Figure 4 Model of NOTCH-signaling inhibition by sJag1 ligand. a) NOTCH receptor expressed by SMC interacts with Full length membrane bound Jag1 (FlJag1) located on the plasmatic membrane of the neighbor cell inducing NOTCH signaling. Next, NICD translocates to the nucleus where it interacts with its co-transcription factor RBP-Jk to initiate the transcription of HES/HERP target genes. Such activation positively affects SMC migration and proliferation. b) sJag1 competes with FlJag1, subsequently preventing NOTCH signaling activation and SMC migration and proliferation.

Conclusion

Our results provide new insights in the molecular mechanisms that characterize the interaction between VEGF and NOTCH signaling in endothelial cells. We found that VEGF-induced DLL4 expression is NOTCH mediated. Furthermore, DLL4 initiated a feed-forward mechanism downstream of NOTCH, which resulted in the up-regulation of its own expression. These data indicate that VEGF-induced NOTCH signaling is not subsequent to DLL4 ligand overexpression per se (Chapter 2). Consistently, VEGF could induce the expression and the activity of ADAM-10, a crucial metalloproteinase involved in NOTCH receptor processing and activation (Chapter 3), providing an alternative mechanism by which VEGF can potentiate NOTCH receptor activation prior to and possibly independent of ligand overexpression. The interaction between VEGF and NOTCH signaling with respect to

arterial specification has also been investigated (Chapter 4). We found that altered VEGF-signaling within the developing heart of the *Vegf120/120* mouse embryo affected NOTCH signaling, endothelial differentiation and arteriogenesis, and as a consequence coronary artery development. Finally, we reported that sJag1 inhibited neo-intima formation in a rat carotid artery injury model (Chapter 5) following vascular injury by interfering with NOTCH-HERP2 signaling and, based on the *in vitro* data, we showed that this effect related to reduced SMC proliferation and migration. These results might provide a potential approach to reduce restenosis after PCI.

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Summary

In this thesis, the role of VEGF and NOTCH Signaling Pathways in cardiovascular development and function has been investigated. The effects of VEGF on DLL4 expression, NOTCH signaling and ADAM activity in endothelial cells (ECs) have been explored. Also, the consequences of altered NOTCH signaling for vessel function have been studied at cellular as well as the organ level.

In **Chapter 1**, a general introduction of this thesis is provided. The interaction between VEGF and NOTCH signaling pathway is described together with their effects on arterial endothelial cell differentiation and sprouting. The NOTCH signaling role in SMCs behavior is introduced along with potential implications of NOTCH signaling modulation for vascular remodeling.

In **Chapter 2** we provide new insights into the molecular interaction between the NOTCH and VEGF signaling pathways in ECs. We demonstrated that VEGF-induced DLL4 expression depends on NOTCH activation in HCMvECs. Accordingly, NOTCH signaling was activated by VEGF, as was shown by overexpression of the NOTCH target gene HES-1 and transactivation of a promoter containing multiple RBP-Jk binding sites. Next, activation of NOTCH signaling, by NICD overexpression or recombinant DLL4 stimulation, induced the expression of DLL4 ligand. Also, we reported that NICD-induced DLL4 promoter activity was dependent on RBP-Jk sites but independent of the FOXC2 binding site, previously reported to be required in regulating DLL4 expression downstream VEGF. Moreover, constitutively active FOXC2 did not affect *DLL4* expression.

We speculated that the induction of DLL4 by activated NOTCH might provide a positive feed-forward signaling mechanism that propagates NOTCH-signaling between neighboring ECs. Indeed, DLL4-eGFP transfected ECs induced DLL4 expression in non-transfected cells in close vicinity in a NOTCH-dependent way. As DLL4 regulates crucial processes in vascular growth, such as EC sprouting and arterial specification, a deeper understanding of the molecular mechanisms underlying its regulation will undoubtedly help to develop new strategies for the treatment of blood vessel-related pathologies.

In **Chapter 3** the VEGF effect on ADAM-10 activation, an essential metalloproteinase involved in NOTCH processing and activation, has been studied. Using a Yeast-two-hybrid assay, ADAM-10 was identified as a novel binding partner of VEGFR-2. Such interaction was further confirmed by immuno-precipitation experiments. Remarkably, VEGF stimulation induced ADAM-10 expression and its activity in ECs. VEGF-activated ADAM-10 mediated ectodomain shedding of VEGFR-2, providing a negative feed-back mechanism that shuts down VEGF signaling in ECs. VEGF induced-ADAM-10 also resulted in cleavage of VE-cadherin, increasing

vascular permeability and, facilitated EC migration. Therefore, VEGF increased vascular permeability in an ADAM-10 dependent manner and inhibition of ADAM-10 reduced EC migration and chemotaxis. For the first time, we reported that ADAM-10 is expressed in human atherosclerotic lesions and associated with plaque progression and neovascularization. Altogether, these data provide evidence of a role for ADAM10 in atherosclerosis and neovascularization. As ADAM-10 is involved in NOTCH signaling proteolysis and activation, and VEGF has been reported to activate NOTCH signaling, these results provide an important link between VEGF and NOTCH signaling activation as well.

In **Chapter 4** the role of VEGF and NOTCH signaling in arterial endothelial differentiation is addressed. In mutant *Vegf120/120* mouse embryos, the endothelial cells of coronary arteries failed to gain a proper arterial phenotype (i.e. high level of activated Notch1, Jagged1, Dll4 and ephrinB2 and low levels of VEGFR-2, EphB4 and COUP-TFII), while the endothelial cells of coronary veins showed ectopic expression of the arterial markers together with low expression of the venous markers. Moreover, the media of coronary arteries was underdeveloped with decreased number of pericytes expressing α SMA, NP1, Dll1, Jagged2 and Notch3, whereas coronary veins showed an increase in cells positive for these markers.

Vegf is mainly expressed in the area where coronary arteries develop in normal mouse embryos. This part of the coronary system is expected to be subjected to high levels of VEGF-signaling due to retention of the larger VEGF splicing variants in the heparin-containing extracellular matrix and to NP-1-modulated amplification. In the subepicardially located coronary veins therefore, low VEGF-signaling levels are expected. Due to lack of retention of VEGF120 in the extracellular matrix and lack of NP-1-mediated amplification in *Vegf120/120* mouse embryo, the inverse is likely the case, leading to the observed coronary anomalies. These data imply that VEGF and the downstream NOTCH signaling are essential for arterial endothelial differentiation and arteriogenesis with consequences for coronary artery development and performance.

In **Chapter 5** the negative effect of a soluble NOTCH ligand, sJag1, on NOTCH signaling and neointima formation is studied. Rat carotid arteries subjected to balloon injury showed increase of Notch1, Notch3 and Jagged-1 expression at day 3 and 14 post-injury. In these injured vessels, Notch signaling was activated as was shown by increased expression of the NOTCH target-gene Herp2. We found that adenoviral-sJag1 transfection reduced neo-intima formation in the carotid artery and enhanced re-endothelialization, whereas adenoviral-full length Jagged-1 (Fl-Jag1) or -LacZ had no effect. As neointima is the result of uncontrolled proliferation and migration of SMCs, we analyzed the effect of sJag1 on SMCs behavior by transducing CSMCs with sJag1. In line with our expectations, sJag1 reduced both migration and proliferation of CSMCs compared to Fl-Jag1 or LacZ used as control,

whereas no effect on ECs was observed. Moreover, injury-induced Herp2 expression was dramatically reduced in injured vessels treated with adenoviral-sJag1. Accordingly, CSMCs infected with Ad-sJag1 or treated with sJag recombinant protein showed decreased Herp2 expression compared to their respective controls. These observations prompted us to speculate that sJag1 prevents SMC migration and proliferation by interfering with Herp2 mediated NOTCH signaling. In line with these speculations, overexpression of Herp2 rescued the reduced migratory capacity of sJag1-treated CSMCs. Thus, sJag1 can inhibit neo-intima formation after balloon injury by decreasing SMC proliferation and migration through interference with NOTCH-Herp2 signaling.

Chapter 6 provides a general discussion of the data presented in this thesis. First we discussed the new insights in the NOTCH and VEGF signaling molecular interaction provided by our studies. In particular, the major role of VEGFR-2 and the existence of a positive feed-forward loop - that involves activated NOTCH signaling - in VEGF-induced DLL4 expression was examined. The activation of ADAM-10 by VEGF and its implication in NOTCH signaling activation downstream of VEGF has been discussed. Second we focused on the consequence of altered NOTCH signaling at the tissue/organ level. The DLL4 blockade as potential therapeutic tool in tumor growth was introduced. In addition, the implication of VEGF and NOTCH signaling pathway in arterial differentiation and development of coronary artery was discussed. Finally we considered therapeutic use of sJag to inhibit neointima formation following PCI.

Samenvatting

In dit proefschrift zijn de vascular endothelial growth factor (VEGF) en NOTCH signaal wegen bestudeerd wegens hun rol in de ontwikkeling en functie van het bloedvat stelsel. Meer specifiek hebben we het effect van VEGF op de expressie van Delta-like 4 (DLL4) en de activiteit van NOTCH en “a desintegrin associated metalloprotease” (ADAM) in endotheel cellen onderzocht. Tenslotte hebben we enkele functionele consequenties van veranderde NOTCH activatie bekeken in vasculaire cellen als ook in bloedvaten.

Hoofdstuk 1 leidt het onderwerp van het promotieonderzoek in. Hierin worden de VEGF en NOTCH signaalwegen beschreven en wordt tevens het verband geschetst met differentiatie en het spruiten – bijvoorbeeld bij nieuwvorming van bloedvaten- van slagaderlijk endotheel. Vervolgens wordt de rol van NOTCH activatie op het gedrag van gladde spiercellen besproken en de mogelijk gevolgen daarvan voor vaatwand remodelering.

In **Hoofdstuk 2** verschaffen we nieuw inzicht in de moleculaire interactie tussen de NOTCH and VEGF signaalwegen in endotheelcellen. In humane coronaire microvasculaire endotheel cellen (HCMvECs) hebben we laten zien dat NOTCH activatie nodig was voor de expressie van DLL4 door VEGF. Deze NOTCH activatie bleek uit de inductie van het NOTCH afhankelijke “hairy enhancer of split” (HES-1) door VEGF, evenals uit reporter assays met een promotor rijk aan RBP-Jk transactivatie domeinen. Het intracellulaire domein van NOTCH (NICD) dat verantwoordelijk is voor de transcriptie-activatie, leidde ook tot overexpressie van DLL4, evenals recombinant DLL4 (rDLL4) zelf dat als ligand van NOTCH zorgt voor NOTCH activatie. In tegenstelling tot studies van anderen hebben we geen aanwijzingen gevonden dat FOXC2 betrokken is bij DLL4 expressie. Promotor constructen zonder het FOX bindende domein lieten toch activatie zien, terwijl overexpressie van FOXC2 niet leidde tot DLL4 expressie.

De observatie dat rDLL4 aanzet tot DLL4 expressie suggereert een positieve terugkoppelfunctie waarin initiëring van NOTCH activatie snel leidt tot het overdragen van de activatie op naburige cellen. Het bleek inderdaad dat DLL4 overexprimerende cellen leidde tot NOTCH activatie van naburige cellen met DLL4 expressie tot gevolg.

Omdat DLL4 betrokken is bij spruiten van nieuwe bloedvaten en bij differentiatie in slagaderlijke bloedvaten zal beter inzicht in dit proces kunnen leiden tot nieuwe therapieën.

In **Hoofdstuk 3** is het effect van VEGF op activatie van ADAM10 bestudeerd. ADAM-10 is een essentieel metalloprotease voor NOTCH activatie. In een ‘Yeast-two-hybrid’ systeem werd ADAM10 als bindingspartner van VEGFR-2 geïdentificeerd en

dit werd bevestigd door immuunprecipitatie. VEGF stimuleerde ADAM-10 expressie en activiteit in endotheelcellen. Aangezien ADAM10 op zijn beurt leidt tot afbraak van het extracellulaire deel van VEGFR-2 en daarmee de receptor inactieveert, lijkt ADAM-10 onderdeel te zijn van een negatief terugkoppelingsmechanisme. VEGF geïnduceerde ADAM-10 resulteerde ook in de afbraak van VE-cadherin en dus in verhoogde permeabiliteit en endotheelcel migratie. Deze laatste effecten bleken ook door ADAM-17 te kunnen worden bewerkstelligd. ADAM-10 blijkt in human atherosclerotische plaques voor te komen en is geassocieerd met plaque-groei en vaatnieuwvorming in de plaque. Aangezien ADAM-10 activatie noodzakelijk is voor NOTCH activatie en VEGF ook leidt tot activatie van NOTCH, is het waarschijnlijk dat ADAM-10 een noodzakelijk onderdeel is van de VEGF-NOTCH as.

In **Hoofdstuk 4** wordt de rol van VEGF en NOTCH in slagaderlijke differentiatie gedurende de embryonale ontwikkeling besproken. In *Vegf120/120* muize-embryo's vormen ondergaan endotheelcellen onvoldoende slagaderlijke differentiatie. Hun expressie van slagaderlijke kenmerken als Notch1, Jagged1, Dll4 en EphrinB2 bleef laag, terwijl aderlijke kenmerken als EphB4 en COUP-TFII hoog bleven. Aan de andere kant, werden plaatselijk slagaderlijke kenmerken gevonden in typische aderlijke posities als de kransadren. De media van de slagaders ontwikkelde zich ook onvoldoende met verlaagde aantallen pericyten die α SMA, NP1, Dll1, Jagged2 en Notch3 exprimeren, terwijl de aderen juist meer van deze cellen recruteerden. In normale embryo's wordt vegf vooral gevonden in de gebieden waar kransslagaders worden gevormd. Deze hoge expressie leidt tot retentie van heparine bindende varianten van vegf (zoals vegf164, 188) en deze hoge expressie wordt mogelijk nog versterkt door neuropilin-1 (NP-1) gemedieerde activatie. De aderen ontwikkelen zich in de subepicardiale laag waar lage vegf spiegels heersen. Echter, Vegf120 bindt niet aan de heparine en wordt ook niet versterkt door NP-1, zodat slagaderlijke differentiatie verminderd is. Deze observaties impliceren dat VEGF en NOTCH activatie essentieel zijn voor slagaderlijke differentiatie en daarmee voor de functie van het bloedvat stelsel als geheel.

Hoofdstuk 5 beschrijft de inhibitie van NOTCH door een niet membraan gebonden ligand, het zogenaamde 'soluble Jagged-1' (sJag1), die in een model van vaatwandschade leidt tot verminderde vorming van een neo-intima. Vaatwandschade leidt tot expressie van NOTCH1, NOTCH3 en Jagged-1 in 3–14 dagen na de schade. Bovendien was NOTCH geactiveerd aangezien we Herp-2 in deze vaten konden aantonen. Behandeling met sJag-1 verminderde de vorming van neointima en versnelde de regeneratie van endotheel in deze halsslagaders terwijl membraan gebonden Jagged-1 of LacZ geen effect hadden. Neointima is het gevolg van gladde spiercel migratie, proliferatie en matrixeiwit synthese. In gladde spiercellen van kransslagaders (CASCs) leidde sJag1 inderdaad tot verminderde proliferatie en migratie alsook tot verminderde Herp-2 expressie. Gelijktijdige overexpressie van Herp-2 kon de sJag-1 verminderde migratie en proliferatie

tegengaan, wat suggereert dat NOTCH activatie inderdaad door sJag1 werd geïnhibeerd. Er werd dus geconcludeerd dat sJag1 neointima vorming remt door de migratie en proliferatie van gladde spiercellen te verminderen doordat NOTCH-Herp-2 activatie wordt verhinderd.

Hoofdstuk 6 is een algemene bespreking van alle data die in dit proefschrift worden gepresenteerd. Eerst worden de nieuwe inzichten in de interactie tussen VEGF en NOTCH signaalwegen besproken. In het bijzonder wordt het positieve terugkoppelingsmechanisme van VEGF, NOTCH activatie en DLL4 expressie bediscussieerd. ADAM-10 activatie lijkt noodzakelijk te zijn voor dit proces, terwijl afbraak van de VEGF receptor zou kunnen leiden tot een natuurlijke rem op dit systeem. Vervolgens hebben we de rol van veranderde NOTCH activatie in cel- en orgaan-systemen besproken, waarbij DLL4 blokkade therapeutisch wordt ingezet bij kanker. Bovendien blijkt tijdens de embryonale ontwikkeling NOTCH activatie nodig te zijn voor normale ontwikkeling van met name het slagaderlijke deel van het vaatstelsel. Tenslotte hebben we de mogelijkheden besproken om remming van NOTCH activatie therapeutisch in te zetten tegen excessieve neointima vorming na PCI.

List of Abbreviations

ADAM	A Disintegrin and Metalloprotease
AEC	Arterial Endothelial Cell
ANK	Ankyrin Repeats
AoA	Aortic Arch
AV	Arterial Venous
bFGF	basic Fibroblast Growth Factor
BM	Bone Marrow
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CASMC	Coronary Artery Smooth Muscle Cell
CL	Cardiac Lymphatic
Cl Notch1	Cleaved Notch1
CM	Compact Myocardium
Co-R	Co-Repressor
COUP-TFII	Chicken Ovalbumin Upstream Promoter Transcription Factor II
CSL	C _{BF} 1/RBPJk, S _u (H), L _{ag} 1
CV	Coronary Vein
DAB	3,3'- Diaminobenzidine
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester
Dll or DLL	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSL	Delta/Serrate/Lag-2
E	Embryonic Day
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
eGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene Glycol Tetraacetic Acid
EPC	Endothelial Progenitors Cell
EPDC	Epicardium-derived Cell
Eph	Ephrin Receptor
ERK/MAPK	Extracellular signal-regulated kinase / mitogen-activated protein kinase
FBE	Forkhead Binding Element
Fl-Jag 1	Full Length Jagged1

FLK1	Fetal Liver Kinase 1
FLT1	FMS-like Tyrosine Kinase
FOXC	Forkhead Box C
GADPH	Glyceraldehyde 3-Phosphate Dehydrogenase
HB-EGF	Heparin-Binding Epidermal Growth Factor
HCAEC	Human Coronary Arterial Endothelial Cell
HCMvEC	Human Cardiac Microvascular Endothelial Cell
HEPES	N'-2-Hydroxyethylpiperazine-N'-2 Ethanesulphonic Acid
HERP	Hairy Enhancer of Split-related Repressor Protein
HES	Hairy/Enhancer of Split
HIF	Hypoxia Inducible Factor
HR	Heat Retrieval
HRT (HEY)	Hairy Related Transcription Factor
HSPG	Heparine Sulphate Proteoglycans
HUVEC	Human Umbilical Vein Endothelial Cell
Il-6	Interleukin 6
IVS	Interventricular Septum
Jag or JAG	Jagged
KDR	Kinase insert Domain Receptor
LCV	Left Cardinal Vein
LDL	Low-Density Lipoprotein
LYVE	Lymphatic Vessel Endothelial Hyaluronan Receptor
MAML1	Mastermind like protein 1
MOPS	3-(N-morpholino)propanesulfonic acid
NCK	Non-catalytic Region of Tyrosine Kinase Adaptor Protein
NECD	NOTCH Extracellular Domain
NICD	NOTCH Intracellular Domain
NLS	Nuclear Localization Sequence
NO	Nitric Oxide
NP	Neuropilin
OPA	Glutamine-rich domain
p21 ^{WAF1/CIP1}	cyclin-dependent kinase inhibitor
PAEC	Porcine Aortic Endothelial Cell
PBS	Phosphate-Buffered Saline
PC	Pericardial Cavity
PCI	Percutaneous Coronary Intervention
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-Derived Growth Factor
PDZ	Postsynaptic density; Disc large; Zonula Occludens-1
PEST	Proline/ Glutaminic Acid/ Serine/ Threonine-rich
PFA	Paraformaldehyde

PI3K	Phosphatidylinositol 3' Kinase
PIGF	Placenta Growth Factor
PLC γ	Phospholipase C gamma
PMA	Phorbol 12-Myristate 13-Acetate
P38-MAPK	p38/ Mitogen-Activated Protein Kinase
PMSF	Phenylmethanesulfonylfluoride
POUFUT1	Protein O-Fucosyl Transferase
PT	Pulmonary Trunk
PVDF	Polyvinylidene Difluoride
qPCR	quantitative Polymerase Chain Reaction
RA	Right Atrium
RAM	RBP-Jk Associated Module
RCA	Right Coronary Artery
rDLL4	Recombinant Delta like 4
RBPJk	Recombining Binding Protein suppressor of hairless
RCV	Right Cardinal Vein
RIPA	Radioimmunoprecipitation Assay
RNAi	RNA interference
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
RUNX2	Runt related transcription factor 2
RV	Right Ventricle
SDS-PAGE	Sodium Dodecyl Sulfate - Poly Acrylamide Gel Electroforese
siRNA	Small interfering RNA
sJag1	soluble Jagged1
SM22 α	Smooth Muscle 22 alpha
SMA	Smooth Muscle Actin
SM-MHC	Smooth Muscle Myosin Heavy Chain
SRF	Serum Response Factor
TACE	TNF- α Converting Enzyme
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline Tween
TGF β	Transforming Growth factor β
TNF α	Tumor Necrosis Factor alpha
TOF	Tetralogy of Fallot Growth Factor
VCACs	Ventriculo-coronary Arterial Communication
VEC	Veins Endothelial Cell
VE-cadherin	Vascular Endothelial - cadherin
VEGF	Vascular Endothelial Growth Factor
VEGF	Vascular Endothelial Growth Factor Receptor
vSMCs	vascular Smooth Muscle Cells

List of Publications

1. van den Akker N.M., Caolo V., Wisse L.J., Peters P.P., Poelmann R.E., Carmeliet P., Gittenberger-de Groot A.C., Molin D.G. Developmental coronary maturation is disturbed by aberrant cardiac VEGF-expression and Notch-Signaling. *Cardiovasc. Res.* 2008;78:366–75.
2. Donners M.M., Wolfs I.M., Olieslagers S., Mohammadi-Motahhari Z., Tchaikovsky V., Heeneman S., van Buul J.D., Caolo V., Molin D.G., Post M.J., Waltenberger J. ADAM-10 is a novel mediator of VEGF-induced endothelial cell function in angiogenesis and associated with atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 2010;30:2188–95.
3. Caolo V., van den Akker N.M.S., Verbruggen S., Donners M.M.P.C., Swennen G., Schulten H., Waltenberger J., Post M.J., Molin D.G.M. Feed-forward signaling by membrane bound ligand receptor circuit: The case of NOTCH-DELTA LIKE 4 in endothelial cells. *Journal of Biological Chemistry*, 2010;285:40681–89.
4. Caolo V., Schulten H.M., Zhuang Z.W., Murakami M., Wagenaar A., Verbruggen S., Molin D.G.M., Post M.J. Soluble Jagged-1 inhibits Neointima Formation by attenuating Notch-Her2 signaling. *Arterioscler. Thromb. Vasc. Biol.* 2011, In press.

Published Abstracts

Vincenza Caolo, Henny Schulten, Sanne Verbruggen, Geertje Swennen, Johannes Waltenberger, Daniël G. Molin, Mark J. Post. Abstract 3941: VEGF-A Induces Arterial Endothelial Differentiation By Increasing Both NOTCH-expression And NOTCH-related Signaling. *Circulation*. 2008;118:S_506.

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Curriculum Vitae

Vincenza Caolo was born on March 13th 1982, in Sapri, Italy. After finishing the high school at the Liceo Scientifico “Galileo Galilei” in Sapri in 2000, she studied Molecular Biology at the Bologna University. In March 2006 she obtained her Master Degree *cum laude*. Since July 2006 she was appointed as a Marie Curie PhD student at the Physiology Department of the Cardiovascular Research Institute Maastricht (CARIM) of Maastricht University under the supervision of Prof. Mark J. Post. Since January 2011 she is working as Postdoctoral Research Fellow at the Physiology Department of Maastricht University.