Soluble Notch ligand and receptor peptides act antagonistically during angiogenesis

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Aims
Notch signalling is essential for blood vessel formation. During angiogenesis, the Notch ligand DLL4 on the leading tip cell activates Notch receptors on the adjacent stalk cells. DLL4-Notch signalling is impaired by the Notch ligand JAG1 in endothelial cells. The Delta/Serrate/Lag2 (DSL) domain of the Notch ligands binds to the EGF-like repeats 11–13 of the Notch receptor. This study aimed to elucidate how soluble proteins containing these short domains interfere with Notch signalling during angiogenesis.

Methods and results
Adenoviral vectors were generated to express the DSL domains of DLL1, DLL4, JAG1, and the Notch1 EGF-like repeats 11–13 fused to immunoglobulin-G heavy chain. These soluble ligand peptides inhibited Notch signalling in endothelial cells and this caused hyperbranching in cellular angiogenesis assays and in the neonatal mouse retina. The soluble Notch receptor peptides bound stronger to JAG1 than DLL4 ligands, resulting in increased signalling activity. This led to impaired tip cell formation and less vessel sprouting in the retina.

Conclusion
The minimal binding domains of Notch ligands are sufficient to interfere with Notch signalling. The corresponding soluble Notch1 EGF11-13 peptide binds stronger to inhibitory Notch ligands and thereby promotes Notch signalling in endothelial cells.

Keywords
Notch signalling • DSL domain • EGF-like repeats • Angiogenesis

1. Introduction
Notch signalling determines numerous cell fate decisions during embryonic development and gene mutations are causative for several syndromes.1,2 Notch signals in the vasculature are essential for the control of endothelial cell migration, tube formation, branching, regression, and anastomosis of new capillary sprouts.3,4 Vascular endothelial growth factor (VEGF) activates endothelial cells during angiogenesis. This enhances expression of the Notch ligand Delta-like 4 (DLL4) on the leading tip cells. The interaction of DLL4 with Notch1 receptors on adjacent endothelial cells induces cleavage of the receptor to release the intracellular Notch domain, which enters the nucleus. There it changes the activity of several transcriptional regulators to alter gene transcription, causing an inhibition of VEGF receptor expression.1,3,4

The Delta-Notch cascade has emerged as a potential target for tumour therapy.5 Enforced DLL4-Notch signalling (e.g. by endothelial expression of DLL4 or the intracellular domain of Notch, NICD) leads to impaired tumour angiogenesis, while blockade of Notch signalling (e.g. with the DLL4 extracellular domain fused to the Fc domain of immunoglobulin-G) results in a hyperdense but poorly perfused vascular network.6–9

There is overwhelming evidence that not all Notch ligands act redundantly, but play tissue-specific synergistic, distinct, or even opposite roles. One of the best-described examples in vascular biology is that strong endothelial expression of the JAG1 ligand antagonizes DLL4-Notch1 signalling during sprouting angiogenesis.10 The soluble Notch1 receptor comprising the complete extracellular domain (Notch1-Fc, Notch decoy) inhibits angiogenesis.11 This implies that
Notch1-Fc blocks inhibitory ligands more powerful than stimulatory ligands.

There is strong evidence that different domains of the Notch receptor selectively mediate Delta or Jagged/Serrate-signalling. The canonical Notch ligands contain a Delta/Serrate/lag-2 (DSL) domain that directly interacts with EGF-like repeats 11–13 of the Notch1 receptor. These are only the minimum-length protein structures required for protein–protein interaction; however, full signalling strength arises from additional parts of the proteins. As such, the MNNL domain and EGF-like repeats 1–3 of Dll1 and Dll4 and the EGF-like repeats 6–15 of Notch1 are essential for signalling activity. Additionally, Notch receptors get glycosylated in manifold ways. For instance, the addition of an N-acetylglucosamine by the enzyme fringe enhances binding to Delta ligands and signalling output and inhibits the interaction with Jagged/Serrate.

This study was aimed at analysing how very small soluble ligand or receptor domains, namely the DSL domains of Dll1, Dll4, or Jag1 and the EGF-like repeats 11–13 of Notch1 affect signalling outcome and vascular differentiation.

2. Methods

2.1 Plasmids

To generate vectors for IgG1-Fc fusion proteins pINFUSE-hlgG1-Fc2 (InvivoGen) was used in the first step for cloning the different Notch ligand and receptor cDNA sequences. DLL1-DSL was PCR-amplified from human umbilical vein endothelial cells (HUVEC) cDNA using primers DLL1-DSL-5Nco and DLL1-DSL-3Bgl, digested with NcoI and BglII and inserted into pINFUSE-hlgG1-Fc2 linearized with the same enzymes. Fragments for the DSL domains of DLL4 and Jag1 and for the EGF-like repeats 11–13 of NOTCH1 were generated with specific primer pairs and the same cloning strategy. Secondly, the sequences encoding the IL-2 signal peptide in frame with the Notch ligand or receptor fragments and the IgG1-Fc protein was released by Kasl-Nhel, blunted with Klenow polymerase and shunted into pENTR2B (EcORI–EcoRV, blunted) for Gateway cloning into the adenoavector pAd/CMV/VS-DEST (Invitrogen). The primer sequences are listed in Supplementary material online, Table S1.

2.2 Cell culture and virus production

HUVEC were freshly isolated from umbilical veins as described according to the Declaration of Helsinki and with approval of the Heidelberg University ethics review board. Informed consent was given for the use of the umbilical cords for the isolation and culture of cells. HUVEC were grown in ECGM2 with SupplementMix (PromoCell) and 10% heat inactivated FCS (Biochrom). HEK293 and C2C12 cells (ATCC) were cultured in high glucose DMEM supplemented with 10% FCS and GlutaMax. Adenoviruses were generated in HEK293 cells according to the ViraPower Adenoviral Expression System (Invitrogen) protocol. Semiconfluent HUVEC were transduced with multiplicity of infection (MOI) of 50, 100, or 150 and C2C12 with MOI of 200.

C2C12 differentiation was assessed by staining adenovirus infected cells on gelatin-coated glass cover slips against fast-twitch skeletal myosin heavy chain using an anti-MHC (MY-32) antibody (1:500; Sigma) and Alexa Fluor 488-coupled secondary antibodies (1:200, Invivogen). As a positive control cells were cultured with DMEM and 2.5% horse serum, which induces differentiation. Nuclei were stained with DAPI (1:5000). Images were taken with a fluorescence microscope (Zeiss Axio Imager.Z1; AxiosCam HRc camera).

2.3 Animal protocols

C57BL/6 mice were i.p. injected with 3 × 10⁷ adenoviral particles at postnatal Days 1 and 2 and sacrificed at postnatal Day 6 by decapitation. Eyes were fixed overnight in methanol at −20 °C. Retinas were prepared and permeabilized in 1% BSA and 0.3% Triton X-100 for 2 h and washed in PBLEC buffer (1% Triton X-100, 0.1 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM MnCl₂ in PBS pH 6.8), then incubated overnight in PBLEC plus isoelectric-B4 (1 mg/mL Sigma-Aldrich). After washing, retinas were mounted on slides with Fluorescent Mounting Medium (Dako). Images were taken with an LSM 700 confocal microscope (Leica) and processed using Fiji software. All animal experiments were approved by the local government committee and performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.4 Sprouting angiogenesis

Spheroid-based sprouting was performed as described. HUVEC were suspended in growth medium with 20% methocoll 48 h after viral transduction. Cells were incubated as hanging drops for 24 h to form spheroids containing 400 cells each. Spheroids were harvested, suspended in 2 mL methocoll supplemented with 20% FCS and 2 mL rat collagen, and embedded in a 24-well plate. Basal culture medium (0.1 mL) was added to assess basal sprouting. VEGF was added for stimulation (25 ng/mL final concentration). Cells were fixed after 24 h with 10% formaldehyde. The lengths of all sprots of 10 spheroids per condition were counted using an inverted microscope (Olympus IX50 with cell^P software).

2.5 Gene expression analyses

Total RNA for mRNA expression analysis by real-time PCR was isolated using the RNeasy Kit (Qiagen) and transcribed into cDNA with SuperScript II Reverse Transcriptase and random hexamer primers (Invitrogen). CDNA was diluted 1:5 and 1 μL was used for qPCR using the POWER SYBR Green Master Mix in a 25 μL reaction on an ABI StepOnePlus cycler (Applied Biosystems). Ct-values were determined using StepOnePlus software and normalization was done with the housekeeping genes OAZ1. Primers for real-time analysis are listed in Supplementary material online, Table S1. For western blotting, cells were lysed in protein lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Roche) and 1 mM DTT. After SDS–PAGE, proteins were transferred to PVDF nitrocellulose filters (Whatman). Membranes were blocked with 5% skim milk in 0.05% Tween 20 in TBST and stained with primary antibodies at 4 °C overnight. The membrane was washed and incubated with peroxidase-conjugated secondary antibody (Dako) for 2 h at room temperature. Chemiluminescence was detected with the AceGlow substrate (Peqlab). The following primary antibodies were used: anti-GAPDH (1:5000, Abbcam); anti-Fc (1:1000, Pierce); anti-DLL4 (1:1000, Cell Signaling); anti-Jag1 (1:1000, Santa Cruz); anti-myosin (1:5000, Sigma).

2.6 Microscope thermophoresis

DLL4 (Life Technologies, cat. no. 10171-H02H) and Jag1 (R&D Systems, cat. no. 1277-G7) recombinant proteins were labelled using N-Hydroxysuccinimide (NHS)–ester chemistry according to the manufacturer’s protocol (MO-L001 Monolight™ Protein Labeling Kit RED-NHS, NanoTemper Technologies, Munich, Germany). Binding interactions between recombinant Notch1-ECD (R&D Systems, cat. no. 3647-TK) or protein-A-purified N1-EGF11-13 peptide and Alexa Fluor 647-labelled recombinant DLL4 (Sigma) and Jag1-ECD (R&D Systems) were measured using microscale thermophoresis. This technique determines differences in the thermophoretic properties of ligand-receptor complexes (Monolight NT.015; NanoTemper Technologies, Munich, Germany). A titration series with Notch1-ECD or N1-EGF11-13 peptides and varying DLL4 (Sigma) and Jag1-ECD (R&D Systems) concentrations was pre-incubated for 5 min in the dark to achieve ligand-receptor binding equilibrium. All measurements were performed in PIST binding buffer.
(50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM MgCl₂, 2 mM CaCl₂, 0.05% Tween 20) using standard capillaries at room temperature. Plotting normalized fluorescence $F_{\text{norm}}$ at a given time against the Alexa Fluor-647 concentration resulted in a binding curve, and $K_D$ values were obtained using a computer-based sigmoidal fitting function.

2.7 Cell proliferation and migration assay

The proliferation rate of cells was investigated using the Cell Proliferation ELISA, BrdU (colorimetric) Kit (MTT, Roche) according to the manufacturer’s protocol. Cell migration was assessed in a modified Boyden chamber. Twenty-four hours after viral transduction, HUVEC were starved in basal medium and 2.5% FCS overnight and seeded on the upper side of the collagen-coated membrane (8 μm pore size). As a chemotactic stimulus VEGF (25 ng/mL) was added to the lower chamber.

2.8 Statistical analysis

Results are expressed as means ± standard deviations. Statistics were analysed by a two-tailed paired or unpaired Student’s t-test, repeated-measures ANOVA or one-way ANOVA followed by Dunnett post-hoc test as indicated in the figure legends. Probability values smaller 0.05 were considered significant.

3. Results

3.1 The Notch ligand DSL domains are highly conserved

The Delta ligands contain one cysteine-rich Delta/Serrate/lag-2 domain (DSL) and several EGF-like repeats in their extracellular part, while the Jagged/Serrate ligands possess an additional cysteine-rich domain (Figure 1A). The corresponding Notch receptors harbour several EGF-like domains and a negative regulatory region (NRR) close to the transmembrane domain (Figure 1B). All ligands are supposed to bind their receptors via the DSL domains. Crystallography studies have revealed that the DSL domain of JAG1 is in direct contact with the EGF-like repeats 11–13 of the Notch receptor. These binding domains (DSL and EGF-like 11–13) are highly conserved during evolution from worm and fly to man (Supplementary material online, Figure S1A).

We fused the ligand DSL or the receptor EGF-like 11–13 domains with a secretory IL-2 signal peptide and with the Fc domain of the anti-body heavy chain IgG1 (Figure 1C and D) to study the effects of soluble ligand and receptor peptides on angiogenesis. To enable assessment of the effects caused by the ligand DSL domain only, we also generated ligand and receptor peptides on angiogenesis. To enable assessment of the effects caused by the ligand DSL domain only, we also generated soluble DLL4-ECD-Fc and JAG1-ECD-Fc, which is similar to previously described soluble Notch ligands. All peptides were abundantly expressed in endothelial cells after adenoviral transduction and secreted into the cell-culture medium (Figure 1E and F).

3.2 Soluble ligands and receptor proteins inhibit Notch signalling in 2D cell monolayers

There is basal Notch activity in primary endothelial cell cultures (HUVEC) leading to induction of the target genes. Expression of Delta-Fc, Jagged-Fc, and Notch1-Fc fusion proteins decreased steady-state mRNA expression of the classical Notch target genes Hey1, Hey2 in a dose-dependent manner (Figure 2A) and treatment with conditioned medium led to similar results (data not shown). This indicated that the proteins were secreted and enabled the inhibition of Notch signalling. In contrast to previous studies, the soluble ligands and receptor proteins had no or only a minor effect on basal endothelial cell proliferation 48 h after viral transduction (Supplementary material online, Figure S2A). These studies described an increase of cell proliferation after a prolonged time (4–8 days) of Notch inhibition.

A widely used assay to determine functional Notch activity in a non-vascular cellular system is the differentiation of C2C12 myoblasts into myotubes. This differentiation step is blocked by Notch signalling. We stained C2C12 cultures with antibodies against fast-twitch skeletal myosin heavy chain. Forced differentiation stimuli with 2.5% horse serum induced myosin expression (Figure 2B). While infection with Fc-expressing adenovirus had no effects (Figure 2B), adenoviral transduction with Delta-Fc, Jagged-Fc, and Notch1-Fc vectors led to an early onset of myosin expression after 48 h followed by myogenic differentiation after 96 h (Figure 2B and Supplementary material online, Figure S2B and C). This indicated successful inhibition of Notch activity in C2C12 cells. Again, there was no obvious difference between the individual constructs (Figure 2C). In summary, soluble Notch ligands and receptors acted in the same way to inhibit Notch signalling in 2D cell culture conditions of HUVEC and C2C12 cells.

Consistently, the expression of ligand fusion proteins led to increased migration of HUVEC through an 8 μm pore-size filter even in the absence of VEGF as stimulus. Addition of VEGF could not further elevate migration rates after Notch inhibition (Supplementary material online, Figure S2D).

In contrast, no alteration in the number of migrated cells was observed after treatment with Notch1-EGF11-13-Fc compared with Fc control. Thus, in a more complex scenario in which cells need to break down cellular contacts and migrate through an extracellular matrix, soluble ligands exert different functions compared with their receptors.

3.3 Opposite roles of soluble ligands and receptors on angiogenesis in 3D cell cultures

The genetic or pharmacological inhibition of Notch signalling in endothelial cells is known to cause a hypersprouting phenotype, whereas DLL1 or Notch1 gain-of-function impairs angiogenesis. However, it has also been reported that JAG1 acts in endothelial cells—in contrast to many other cell types—as an inhibitory ligand and counteracts DLL4-Notch signalling. As such, soluble ligands would always block the receptors and promote the tip cell phenotype, whereas soluble receptors would either block DLL4 to promote tip cell differentiation or JAG1 to promote the stalk cell phenotype.

We tested this hypothesis with the newly generated constructs. Indeed, all Delta-Fc and all JAG1-Fc constructs strongly increased capillary-like tube formation under basal and VEGF-stimulating conditions, resembling previous observations after Notch inhibition. The Notch blockade induced the formation of vascular networks in such complexity as usually observed only after VEGF treatment (Figure 3A and B). The same was observed when HUVEC spheroids embedded in a collagen gel were treated with conditioned medium of endothelial cells transduced with these vectors (Supplementary material online, Figure S3A and B). Consistently, the blockade of Notch using Delta-Fc and Jag1-Fc peptides led to a decrease of the Notch target genes HEY1 and HEY2 (Figure 3C).

In contrast to the soluble ligands, the Notch1-Fc construct that contained only the EGF-like repeats 11–13 inhibited capillary formation under basal conditions and after VEGF stimulation. This was consistently seen irrespective of whether HUVEC were directly transduced with adenovirus or treated with conditioned medium containing Notch1-EGF11-13-Fc (Figure 3D, E and Supplementary material online,
Figure 1 Schematic illustration of Notch ligands and Notch receptors, and soluble Fc-constructs. (A) Jagged/Serrate and Delta-like Notch ligands share several common motifs in vertebrate species: a module at the N-terminus of Notch ligands (MNNL), the Delta/Serrate/lag-2 domain (DSL), EGF-like repeats (light blue, normal EGF-like repeat; green, Ca\(^{2+}\) binding; dark green, atypical EGF-like repeat), a transmembrane domain (TM), and a short cytoplasmic stretch. Jagged ligands harbour an additional cysteine-rich domain (CRD). Some ligands contain a C-terminal PDZ binding domain (PDZ-BD). (B) Notch receptors share a common domain organization in vertebrates. The large extracellular part contains several EGF-like repeats (green, Ca\(^{2+}\) binding; dark, atypical EGF-repeat) and the negative regulatory region (NRR), which consists of three LNR repeats and the heterodimerization domain (HD). The second fragment contains a short extracellular stretch, the transmembrane domain (TM), a RAM domain, several ankyrin repeats (ANK), a transactivation domain (TAD), and a C-terminal PEST domain. (C) Architecture of soluble Jag1- and DLL1/4-ligand fragments fused to human IgG1-Fc consisting of CH2, CH3, and hinge region. (D) Scheme of the soluble Notch1 receptor fragment fused to human IgG1-Fc. (E and F) Representative immunoblots for soluble human Fc-peptides. HUVECs were transduced with adenoviral vectors expressing the indicated Notch1-Fc ligand or receptor. Cells and supernatant were harvested after 48 h and Fc peptides purified using protein A-coupled sepharose beads.
Figure 2 Soluble Notch1 ligand and receptor peptides inhibit Notch signalling in different cell types. (A) Quantification of HEY1 and HEY2 expression in HUVECs transduced with different concentrations of adenovirus expressing the indicated peptide. Endothelial cells expressing the Fc-peptide served as control (n = 3 independent experiments). (B) Representative fluorescence microscope images of C2C12 cells double-stained for fast-twitch skeletal myosin heavy chain and DAPI after adenoviral transduction with soluble Notch ligands or Notch 1 EGF-like repeat 11–13 receptor. (C) Quantification of (B) represented by the ratio of myosin-positive C2C12 cells to total DAPI-positive cells (n = 5). DM, differentiation medium. Bar graphs show mean values, error bars indicate SD. Data were analysed using repeated measures ANOVA (A) or 1-way ANOVA (C) followed by Dunnett’s post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar equals 100 µm.
Figure 3 Soluble Notch ligands and receptor show different effects on sprouting angiogenesis. (A) Representative images of HUVEC spheroids overexpressing the indicated Fc-peptide under basal conditions or after stimulation with VEGF. (B) Quantification of the cumulative sprout length as shown in (A). Thirty spheroids per condition were measured in three independent experiments. (C) Quantification of HEY1 and HEY2 mRNA levels in HUVEC spheroids after transduction with adenovirus expressing the indicated Notch ligand peptide ($n = 3$). Fc-transduced cells served as control. (D) Microscopical images of unstimulated and VEGF-stimulated HUVEC spheroids after adenoviral Notch1-EGF11-13 overexpression. (E) Quantification of the cumulative sprout length of HUVEC spheroids after adenoviral transduction with the Notch1-EGF11-13 fragment or Fc control. Spheroids were cultured for 24 h under basal conditions or after VEGF stimulation as shown in (D). Notch-signalling was inhibited by administration of the γ-secretase inhibitor DAPT ($n = 30$ for three independent experiments). (F) Real-time PCR analysis of HEY1 and HEY2 transcript levels in Notch1-EGF11-13 transduced HUVEC spheroids ($n = 3$). Bar graphs show mean values, error bars indicate SD. Data were analysed using repeated measures ANOVA followed by Dunnett’s post-hoc test (B, C and E) and two-tailed, paired Student t-test (F). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bar equals 100 μm.
Furthermore, overexpression of Notch1-EGF11-13 led to an increase of the Notch target gene expression in HUVEC spheroids (Figure 3F). Co-administration of the γ-secretase inhibitor DAPT reversed this effect of the Notch1-EGF11-13-Fc peptide, indicating increased Notch-activity (Figure 3E). Taken together, DLL1-Fc, DLL4-Fc, and JAG1-Fc proteins inhibited Notch signalling in 2D and 3D cell cultures. In contrast, the Notch1-EGF11-13-Fc protein inhibited Notch signalling in 2D static cell cultures, but promoted Notch signalling in 3D cell cultures.

### 3.4 Opposite roles of soluble ligands and receptors on angiogenesis in vivo

To analyse if the newly generated peptides were also active in vivo, we injected adenoviral particles expressing DLL1-DSL-Fc, DLL4-DSL-Fc, Jag1-DSL, and Fc as control into newborn mice (P1 and P2) and analysed vascularization of the retina at Day P8. Retinal angiogenesis occurs within the first 2 weeks after birth in a stereotype fashion by radial outgrowth of blood vessels. The frequent selection of tip and stalk cells is under strict control of Notch signalling. Adenoviruses infect the liver and the secreted proteins are transported to the retina via the blood stream. Adenoviral expression of the Fc protein as control alone did not affect retinal angiogenesis as expected. However, DLL1-DSL-Fc, DLL4-DSL-Fc, and Jag1-DSL caused excessive vessel sprouting and branching. Consistently, more tips were observed (Figure 4A and B). Oppositely, the application of Notch1-EGF11-13-Fc inhibited tip cell formation. The resulting vascular pattern was less branched and complex (Figure 4C and D). It should be noted that changes in tip cell numbers were slightly less pronounced compared with previous studies in which Notch signalling was manipulated.
Figure 5 Notch-ligands exhibit different binding affinities to the extracellular domain of the Notch1 receptor. (A) Representative MST-signals for DLL4- and Jag1-ECD recombinant proteins after incubation with the Notch1-ECD and N1-EGF11-13 fragment. Initial fluorescence was set for 5 s (T1) before the IR-laser was turned on to measure the fluorescence decrease for 30 s (T2). Turning off the IR-laser results in back diffusion of molecules and increase in fluorescence (T3). (B and C) Determination of DLL4- and Jag1-ECD binding affinities to the Notch1-ECD and N1-EGF11-13 fragment, respectively. $K_D$-values were obtained from three independent measurements. Bar graphs show mean values and error bars indicate SD. Data were analysed by two-tailed, unpaired Student t-tests. *$P < 0.05$. 

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3.5 Soluble Notch1-EGF11-13 preferentially binds to Jagged ligands

Endothelial cells showed similar expression levels of the Notch ligands DLL4- and Jag1 in 3D cell cultures (Supplementary material online, Figure S4A and B), leading to our hypothesis that at least in endothelial cells Delta-like and Jagged Notch ligands act antagonistically. 

Since the Notch1-EGF11-13 peptide promotes Notch signalling, we hypothesized that it should inhibit JAG1-Notch stronger than DLL4-Notch signalling. To test this, we determined the binding affinity of these peptides by microscale thermophoresis. This technique allows for quantitative analysis of protein–protein interactions based on directed motion of molecules in temperature gradients.

In a first experiment, we labelled recombinant DLL4-ECD-Fc and JAG1-ECD-Fc with the dye Alexa-647 (Supplementary material online, Figure S5A) and determined binding to recombinant Notch1-ECD-Fc (Figure 5A–C). The JAG1 peptide bound 2.1-fold stronger to Notch1 than the DLL4 peptide (K$_D$ 6.04 nM $\pm$ 1.56 vs. 12.8 nM $\pm$ 2.46, $P$ = 0.0192). Secondly, we performed this experiment with the shorter Notch1-EGF11-13 peptide. Although the overall binding affinity was weaker than full-length Notch1-ECD, JAG1 again interacted stronger than DLL4 (Figure 5A–C).

In summary, this work revealed that soluble Notch ligands and receptors act antagonistically and can be employed to either promote or inhibit sprouting angiogenesis (Figure 6).

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**Figure 6** Scheme illustrating pro- and antiangiogenic properties of soluble DSL- and Notch peptides. (A) Notch signalling in endothelial cells can be interfered by soluble Fc-fusion constructs of the DSL domains of a DLL1, DLL4, or JAG1 ligand, referred to as DSL-peptide, that is supposed to bind to the EGF-like repeats 11–13 of the Notch1-ECD. (B) A soluble Fc-fusion construct consisting of the EGF-like repeats 11–13 of the Notch1 receptor domain preferentially binds to the extracellular domain of the Notch-ligand Jag1, which in turns leads to a stronger binding of DLL4 to the Notch1-receptor.
4. Discussion
Notch signalling plays a fundamental role in different aspects of blood vessel development, in particular tip/stalk cell differentiation and vascular remodelling. During sprouting angiogenesis some stalk cells can acquire tip cell characteristics and take over the lead of the new sprout. The change of tip/stalk cell identity may be guided by oscillatory gene expression loops of Notch ligands, Notch target genes, and components of the BMP signalling pathways. Many attempts have been made to manipulate Notch signalling in the tumour vasculature. On the one hand, enforced DLL4-Notch signalling strongly suppresses vascular branching and outgrowth, and is therefore seen as an option for anti-angiogenic therapy. On the other hand, a panel of neutralizing antibodies, soluble DLL4 ligands, or gamma-secretase inhibitors can mediate the blockade of Notch signalling. All these compounds lead to the formation of a hyperdense, heavily branched vascular network with strong calibre alterations. Such a chaotic network cannot sufficiently distribute blood leading to severe tissue hypoxia and even tumour necrosis at least in laboratory animals. We tested in this study how treatment of endothelial cells with very small soluble Notch ligand or receptor domains changes signalling outcome and vascular differentiation. Our results demonstrate that DLL1-Fc, DLL4-Fc, and JAG1-Fc proteins inhibit Notch signalling in 2D and 3D cell cultures, regardless of whether these proteins contained the complete extracellular domain or only the DSL domain. Several studies demonstrate for different cell types an anti-angiogenic effect of a synthetic 17 amino acid Jag1-DSL peptide. Our newly generated Jag1-DSL peptide also contains this amino acid sequence, but contains additional 26 amino acids of the Jag1-DSL domain and is therefore fused to an Fc domain which enables dimer formation in vivo. Furthermore, the expression of our peptide in eukaryotic cells enables posttranslational modification, e.g. glycosylation, which leads to a different protein conformation and modifies protein–protein and protein–cellular interactions, respectively.

Oppositely to the soluble ligands, the Notch1-EGF11-13-Fc protein had more complex functions. In 2D static cell cultures Notch signalling was inhibited, while signalling activity was increased and angiogenic sprouting disturbed in 3D cell cultures. These findings arise most likely from a different cell organization. A cell multilayer, such as in 3D cell cultures, allows more physical interactions and sometimes better resembles an in vivo-like environment.

Others reported that soluble Notch1 containing the complete extracellular domain fused to Fc (Notch1 decoy) also inhibited HUVEC sprouting and even tumour angiogenesis in mice. This suggested that Delta-like and Jagged ligands may bind Notch1 with different affinities. Indeed our experiments showed stronger binding of Notch1 to the inhibitory ligand Jagged.

In vivo, the effects of soluble Notch ligands and receptors reflected the outcome from 3D cell cultures. Application of DLL1-DSL-Fc, DLL4-DSL-Fc, and Jag1-DSL caused excessive vessel branching and increased tip cell formation in the neonatal mouse retina. These are the typical signs of Notch blockade, whereas application of Notch1-EGF11-13-Fc inhibited tip cell formation and vessel branching. This was in line with observations from JAG1 overexpressing mice, and the soluble Notch1 fragment acted in the opposite way. It inhibited sprouting angiogenesis apparently by titrating out Jagged.

Supplementary material
Supplementary Material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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Authors’ contributions
R.K. and C.B. conceived and designed experiments, performed experimental work, analysed data, and contributed to the writing of the manuscript. I.M. assisted with western blot studies. M.G.A. performed experiments and analysed data. K.M. and F.S. provided technical advice for microscale thermophoresis. H.G.A. analysed data and contributed to the writing of the manuscript. A.F. conceived and designed the overall research plan, analysed data, and wrote the manuscript.

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