NRP1 Presented in \textit{trans} to the Endothelium Arrests VEGFR2 Endocytosis, Preventing Angiogenic Signaling and Tumor Initiation

Sina Koch,\textsuperscript{1,4,5} Laurens A. van Meeteren,\textsuperscript{1,4,6} Eric Morin,\textsuperscript{1,4} Chiara Testini,\textsuperscript{1} Simone Weström,\textsuperscript{1} Hanna Björkelund,\textsuperscript{2} Sébastien Le Jan,\textsuperscript{1,7} Jeremy Adler,\textsuperscript{1} Philipp Berger,\textsuperscript{3} and Lena Claesson-Welsh\textsuperscript{1,*}

\textsuperscript{1}Department of Immunology, Genetics and Pathology, Rudbeck Laboratory and Science for Life Laboratory, Uppsala University, Dag Hammarskjöldsväg 20, 75185 Uppsala, Sweden
\textsuperscript{2}Ridgeview Instruments, Skillsta 4, 740 20 Vänge, Sweden
\textsuperscript{3}Paul Scherrer Institute, Laboratory of Biomolecular Research, Molecular Cell Biology, 5232 Villigen PSI, Switzerland
\textsuperscript{4}These authors contributed equally to this work
\textsuperscript{5}Present address: Department II, Systemic Cell Biology, Max-Planck Institute of Molecular Physiology, Otto-Hahn-Straße 11, 44227 Dortmund, Germany
\textsuperscript{6}Present address: Institute for Life Sciences and Chemistry, University of Applied Science Utrecht, FC Donderstraat 65, 3572JE Utrecht, the Netherlands
\textsuperscript{7}Present address: Laboratoire de Dermatologie, Medical Faculty of Reims, 51100 Reims, France

\textsuperscript{*}Correspondence: lena.welsh@igp.uu.se
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\textbf{SUMMARY}

Neuropilin 1 (NRP1) modulates angiogenesis by binding vascular endothelial growth factor (VEGF) and its receptor, VEGFR2. We examined the consequences when VEGFR2 and NRP1 were expressed on the same cell (\textit{cis}) or on different cells (\textit{trans}). In \textit{cis}, VEGF induced rapid VEGFR2/NRP1 complex formation and internalization. In \textit{trans}, complex formation was delayed and phosphorylation of phospholipase \(C_\gamma\) (PLC\(\gamma\)) and extracellular regulated kinase 2 (ERK2) was prolonged, whereas ERK1 phosphorylation was reduced. \textit{Trans} complex formation suppressed initiation and vascularization of NRP1-expressing mouse fibrosarcoma and melanoma. Suppression in \textit{trans} required high-affinity, steady-state binding of VEGF to NRP1, which was dependent on the NRP1 C-terminal domain. Compatible with a \textit{trans} effect of NRP1, quiescent vasculature in the developing retina showed continuous high NRP1 expression, whereas angiogenic sprouting occurred where NRP1 levels fluctuated between adjacent endothelial cells. Therefore, through communication in \textit{trans}, NRP1 can modulate VEGFR2 signaling and suppress angiogenesis.

\textbf{INTRODUCTION}

Neuropilin1 (NRP1) and NRP2 are transmembrane proteins that bind vascular endothelial growth factors (VEGFs). NRP1 has critical roles in vascular development (Koch et al., 2011) and in pathological angiogenesis (Zachary, 2014). NRP1 is expressed in arterial endothelial cells, whereas NRP2 is expressed in veins and lymphatic vessels (Bielenberg et al., 2006). The NRP1s were originally identified as coreceptors for the soluble class 3 semaphorins in axon guidance (Goshima et al., 1999; Takagi et al., 1995). In addition to VEGF, growth factors such as fibroblast growth factor and hepatocyte growth factor bind NRPs (Zachary, 2014). Both NRPs are composed of an extracellular domain with binding motifs for VEGF and semaphorins and a cytoplasmic domain of 35 amino acid residues equipped with a PDZ-domain binding motif (SEA). The SEA motif binds the PDZ-domain of Galpha interacting protein, C terminus (GIPC)/synectin (Cai and Reed, 1999; Wang et al., 2003); this interaction has been shown to be critical in internalization of the NRPs (Lanahan et al., 2010; Prahst et al., 2008; Salikova et al., 2008). GIPC/synectin couples to myosin-VI (Naccache et al., 2006), and both synectin and myosin-VI have been implicated in trafficking of VEGFR2 to early endosomes (Horowitz and Seerapu, 2012; Lanahan et al., 2010). Mice expressing a C-terminally truncated NRP1 survive to adulthood without angiogenic defects (Fattin et al., 2011), indicating that NRP1 might not signal directly via its C-terminal domain in response to VEGF.

VEGF induces formation of a heterocomplex by binding to both NRP1 and VEGFR2 (Soker et al., 1998, 2002), the main VEGF receptor on vascular endothelial cells (Koch et al., 2011). Inclusion of NRP1 in the ligand-receptor complex modulates the VEGF signal output and the biological response, but the exact mechanism for NRP1’s effects on signaling downstream of VEGFR2 has not been resolved (Zachary, 2014). NRP1 has potential as a therapeutic target, and neutralizing NRP1 antibodies have been shown to block tumor growth in mice in an additive manner to VEGF neutralization (Pan et al., 2007). As NRP1 is expressed on a range of nonendothelial cells, such as tumor cells (Bielenberg et al., 2006), a better understanding of NRP1 function in tumor biology is needed to predict the clinical outcome of NRP1 blocking agents.

We hypothesized that tumor-expressed NRP1 might regulate VEGF responsiveness by presenting VEGF to VEGFR2 on endothelial cells in the tumor. This \textit{trans} (Latin prefix; across) interaction has been demonstrated in vitro (Soker et al., 2002).
However, it has remained unclear how the trans complex contributes to VEGF biology and if the signaling output differs from that of the cis (Latin prefix; on the same side) signaling complex containing VEGFR2 and NRP1 expressed on the same cell. Here, we have investigated the role of the VEGF-bridged VEGFR2/NRP1 trans complex in vitro and in vivo. Our data support a model where NRP1 modulates VEGFR2 biology differently when the molecules are expressed on the same cell or on adjacent cells, through regulation of VEGFR2 internalization and intracellular trafficking. Therefore, depending on the expression pattern and relative expression level, NRP1 may either promote or suppress angiogenesis.

RESULTS

VEGFR2 and NRP1 Engage in trans and cis Complexes with Different Kinetics

To investigate if trans presentation of VEGF to VEGFR2 by NRP1 occurs with different kinetics and signaling output compared to presentation in cis, mixed cultures of porcine aortic endothelial (PAE) cells individually expressing either VEGFR2 or NRP1 (trans configuration), were examined by immunoprecipitation/immunoblotting. In parallel, we examined cultures of PAE cells coexpressing VEGFR2 and NRP1 (cis configuration). PAE cells were chosen since they are well characterized and lack endogenous expression of both VEGFR2 and NRP1 (Soker et al., 2002).

VEGFR2/NRP1 complexes in the cis configuration formed quickly and transiently, with a maximum at 10 min of VEGF stimulation (Figures 1A and 1B). VEGFR2 and NRP1 engaged in complexes also in the trans configuration (Figures 1A and 1B), but with delayed kinetics compared to cis. Trans complex formation appeared with a maximum at 120–240 min of VEGF treatment, when cis complexes had returned to unstimulated levels.

VEGFR2/NRP1 trans Complexes Form at the Cell-Cell Interface

To examine potential spatial differences between cis and trans complex formation, we employed the proximity ligation assay (PLA), which allows visualization of complexes in situ through oligonucleotide-tagged antibody-mediated rolling circle amplification on fixed cells.

In agreement with the coimmunoprecipitation results (Figures 1A and 1B), cells coexpressing VEGFR2 and NRP1 (cis) displayed PLA signals representing VEGFR2/NRP1 complexes quickly after stimulation with VEGF, at approximately 3–10 min (Figures 1C and 1D). The cis complexes gradually decreased and returned to basal levels after 60 min.

In the mixed culture, trans VEGFR2/NRP1 complexes appeared with slower kinetics than in cis, from 30 min of stimulation and onward (Figures 1C and 1D). Whereas cis complexes were distributed evenly over the surface of coexpressing cells (Figure 1C, upper panels; Figure S1A available online), trans-complexes were confined to and persisted at the interface between adjacent NRP1- and VEGFR2-expressing cells (Figure 1C, lower panels; Figure S1).

VEGFR2/NRP1 in cis Internalize VEGF in Rab4/Rab5 Vesicles

To monitor internalization kinetics of cis and trans complexes, we used AlexaFluor 555-labeled VEGF (555-VEGF) in the different cell models. In the cis-configuration, 555-VEGF was internalized into cytoplasmic vesicle structures (Figures 2A, 2B, and S2A). In the trans-configuration, 555-VEGF did not appear intracellularly. Instead, patches of fluorescence were detected on and between cells individually expressing NRP1 and VEGFR2 (Figures 2A, 2B, and S2A). There was a significant difference in the extent of 555-VEGF internalization when NRP1 was expressed in cis or trans (Figure 2B). PAE/VEGFR2 cells internalized 555-VEGF to some extent also in the absence of NRP1. PAE/NRP1 cells bound 555-VEGF, the majority of which was retained on the cell surface (arrows in Figure S2B).

That trans complexes indeed caused an arrest in 555-VEGF internalization was further supported by the effect of expression of dominant-negative (dn) Dynamin on 555-VEGF internalization (Cao et al., 2000). As shown in Figure 2C, 555-VEGF was retained at the cell surface of PAE/VEGFR2/NRP1 cells (cis) expressing dnDynamin (visualized through coexpression of GFP), rather than becoming internalized.

In agreement with previous reports (Ballmer-Hofer et al., 2011), internalized 555-VEGF in PAE/VEGFR2/NRP1 cells colocalized with Rab5- and Rab4-positive early endosomes (Figure 2D). The 555-VEGF-positive Rab4/Rab5 vesicles also contained VEGFR2 (Figure 2D) as well as NRP1 (Figure S2C).

Distinct VEGF-Dependent Signaling in cis and trans

VEGFR2 downstream signal transduction is influenced by receptor internalization (for a review, see Koch et al., 2011). Therefore, we examined the VEGF-induced activation of major signal transduction mediators downstream of cis and trans VEGFR2/NRP1 complexes. As shown previously (Kawamura et al., 2008) in cells with VEGFR2/NRP1 cis complexes, VEGFR2-mediated phosphorylation of phospholipase Cγ (PLCγ) and extracellular regulated kinase 1 and 2 (ERK1/2) occurred transiently with a maximum at 3–5 min and 10 min, respectively, of VEGF stimulation (Figures 3A–3D, S3A, and S3C). VEGFR2/NRP1 trans complexes also allowed a rapid induction of PLCγ phosphorylation, which remained elevated for more than 1 hr (Figures 3A, 3B, S3A, and S3C). Moreover, ERK2 phosphorylation downstream of VEGFR2/NRP1 trans complexes increased steadily over a 30- to 60-min time period of VEGF treatment and remained increased compared to cis (Figures 3C and 3D). Importantly, phosphorylation of ERK1 was markedly decreased in the trans configuration (Figures 3C, 3D, and S3A).

Thus, while the trans-dependent arrest in VEGFR2 internalization allowed VEGF-induced ERK2 phosphorylation with slower kinetics but with the same magnitude as in the cis-setting, ERK1 phosphorylation was markedly suppressed in trans. Phosphorylation of PLCγ was induced by VEGF to the same extent in cis and trans, but termination was delayed in the trans-setting, most likely due to the prolonged cell surface residency of the VEGFR2/NRP1 complexes. These results indicate fundamental qualitative differences in signal transduction dependent on whether NRP1 was presented in cis and trans, summarized schematically in Figure 3E.

Despite the marked differences in downstream signaling between the cis and trans configurations, tyrosine phosphorylation of VEGFR2 occurred with similar kinetics independent of whether NRP1 was expressed on the same cell as VEGFR2 or on an adjacent cell (Figures S3A and S3B). This is in agreement
Figure 1. VEGFR2 and NRP1 Engage in cis and trans Complexes with Different Kinetics

(A) Immunoprecipitation (IP) and immunoblotting (IB) to visualize VEGFR2/NRP1 complexes.

(B) Densitometric scanning, showing fold increase compared to the 3 min time point (set to 1). n = 2 (cis), n = 3 (trans); plotted is mean ± SD.

(C and D) PLA (C) and quantification (D) of VEGFR2/NRP1 complexes (in red) in cis (C, upper panel) and trans (C, lower panel). Panels in (C) show high magnification of representative areas. n = 3–4, up to 14 images per time period in each experiment quantified. Nuclei were visualized using Hoechst 33342 (blue); cell membranes were visualized using fluorescent WGA (green) in the cis condition and VEGFR2 immunostaining (green) in the trans condition. Statistical analyses in (D) were between graph columns (presented as single data points to show non-Gaussian distribution). Scale bar, 50 μm.

See also Figure S1.

Developmental Cell
NRP1 cis and trans Regulation of VEGF Signaling


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with VEGF initiating VEGFR2 activation at the cell surface, and therefore independent of whether VEGF bound to NRP1 in cis or trans. We did not record any effect of VEGF on signal transduction in PAE/NRP1 cells (Figure S3D), indicating that VEGF does not induce signaling via NRP1 alone in this model.

The C-Terminal NRP1 Domain Regulates Efficient VEGF Binding

Previous reports have implicated the NRP1 C-terminal domain in efficient VEGF-induced cis complex formation (Prahst et al., 2008) and VEGFR2 internalization (Ballmer-Hofer et al., 2011). We hypothesized that the C-terminal domain might be essential for the stability and biology of the VEGFR2/NRP1 complex also in trans.

We first compared the capacity of wild-type NRP1 versus truncated NRP1 (deleted C terminus) (NRP1\_\text{DC}; Figure 4A) to bind VEGF in real time. For this purpose, T241 fibrosarcoma cells, which lack endogenous NRP1 expression (Figures S4A and S4B), were transduced with phosphoglycerate kinase (PGK)-human NRP1 (hNRP1)-simian virus 40 (SV40)-Neo lentivirus expressing full-length NRP1 (T241/NRP1), PGK-hNRP1\_\text{DC}-SV40-Neo (T241/NRP1\_\text{DC}) expressing a truncated NRP1 lacking most of the C-terminal tail (Figure 4A), or an empty virus (T241/EV) lacking a gene of interest. For a more thorough characterization of the T241/NRP1 cells, see below. The LigandTracer Grey high-resolution cellular uptake and retention assay (Björk and Andersson, 2006) was used to estimate the equilibrium dissociation constant $K_D$ and kinetic parameters ($k_{on}, k_{off}$) of $^{125}$I-VEGF-interaction with wild-type NRP1 and NRP1\_\text{DC}. The data were analyzed using a nonlinear regression model describing a monovalent ligand interacting with either one (the one-to-one model) or two targets (the one-to-two model) in TraceDrawer 1.5 (Björkelund et al., 2011). The one-to-one model fitted the curve data poorly, whereas the one-to-two model analysis identified two populations of VEGF binding to NRP1. One was a high-affinity interaction with a $K_D$ of $\sim$1.5 nM, in agreement with previous reports (Prahst et al., 2008), and the other a low-affinity interaction with a $K_D$ of $\sim$50 nM, not previously recognized (Figure 4B). The low-affinity interaction is a so-called “fast-on-fast-off” interaction, in which
the half-life of VEGF binding was 10 min, in contrast to the high-affinity binding which had a half-life of 15 hr. The low-affinity interaction would not be detected in conventional radioligand binding assays, as the detached ligand would be discarded. Using LigandTracer, both T241/NRP1 and T241/NRP1 ΔC showed the two populations of VEGF binding, but with different ratios. VEGF-binding to T241/NRP1 was equally distributed in high- and low-affinity populations (52% high versus 48% low), whereas T241/NRP1 ΔC cells displayed a preferential low-affinity interaction (27% high versus 73% low). These data support a model in which the C-terminal domain of NRP1 is required for the stable high-affinity binding of VEGF to NRP1, which in turn could affect the stability and signaling downstream of the trans VEGFR2/NRP1/VEGF complex.

To investigate the consequence of deletion of the NRP1 C-terminal domain in complex formation, we used PAE cells expressing VEGFR2 and NRP1 ΔC, individually or combined (Figure S4C). Remarkably, the kinetics of VEGFR2 and NRP1 ΔC complex formation (Figures 4C and 4D) and downstream signal transduction in the ERK pathway (Figures 4E and 4F) were similar between cis and trans complex formation.

In accordance, 555-VEGF was efficiently internalized both in the cis and trans configuration when cells expressed NRP1 ΔC rather than wild-type NRP1 (Figures 4G and S4D). Although the uptake was more pronounced in cis than in trans when cells expressed NRP1 ΔC, there was a significant difference between the abilities of wild-type NRP1 and NRP1 ΔC to suppress internalization in trans (Figure 4G; see also Figures 2A and 2B). When cells expressed NRP1 ΔC, 555-VEGF appeared in Rab4/Rab5-positive vesicles colocalized with VEGFR2 and NRP1 (Figures S4E and S4F). VEGFR2 and NRP1 ΔC complex formation showed a biphasic pattern with an initial peak at 10 min and a second peak at 240 min (Figures 4C and 4D). This might be due to new protein synthesis and not to recycling, as we detected VEGFR2 in Rab11-positive compartment in PAE cells expressing VEGFR2 and wild-type NRP1, but not in cells expressing VEGFR2 and NRP1 ΔC (Figure 4H). We conclude that the NRP1 C-terminal domain, necessary for stable high-affinity binding of VEGF to NRP1, was required for NRP1 to delay VEGF-induced complex formation with VEGFR2 and modulate downstream signaling in trans.

**NRP1 on Tumor Cells Suppresses Tumor Initiation and Growth**

Mutation of the VEGF binding site on NRP1 (Y297A) in mice is compatible with embryonic development but disturbs pathological angiogenesis (Fantin et al., 2014), demonstrating an
important role for VEGF binding to NRP1, e.g., in tumor angiogenesis. Therefore, to investigate if the altered VEGFR2/NRP1 trans-complex-mediated signaling could influence tumor angiogenesis, two different tumor models, B16F10 melanoma and T241 fibrosarcoma, were used for subcutaneous injection in mice with tamoxifen-regulated NRP1 expression in endothelial cells (Acevedo et al., 2008; Figure 5 A).

B16F10 melanoma cells express NRP1 endogenously. Therefore, we stably knocked down (KD) NRP1 expression using lentiviral small hairpin RNA (shRNA) to create B16F10/KD-NRP1 cells. A nontargeting shRNA was used to create the control, B16F10/KD-Ctrl. NRP1 expression in B16F10/KD-Ctrl and B16F10/KD-NRP1 cells was assessed by flow cytometry (Figure 5 B). Importantly, NRP1 knockdown was not complete in B16F10/KD-NRP1 cells, resulting in residual NRP1 expression. There was no significant difference in in vitro growth properties of B16F10 tumor cells expressing NRP1 or not (Figure S5A).

In contrast, T241 fibrosarcoma cells do not express NRP1 endogenously and were therefore transduced with PGK-hNRP1-SV40-Neo lentivirus (T241/NRP1) or with an empty virus (T241/EV), as described above (Figures S4 A and S4B). Expression levels of NRP1 were confirmed and compared between the cell lines by flow cytometry analysis (Figure 5 B). Proliferation in vitro of the different T241 cell lines with and without NRP1 expression was unaffected by the presence of VEGF (Figure S5A). Proliferation of T241/NRP1 and T241/EV cells was also similar with and without Sema3A in the culture medium (Figure S5A). VEGF was expressed to similar extents by the different cell lines and tumors. In contrast, there was little or no expression of Sema3A in vitro and, importantly, no expression in vivo in tumors (Figure S5B). Furthermore, there was no expression of vegfr2 transcript (Figure S5C) or protein (data not shown) in the B16F10 and T241 tumor cells.

Mice with conditional deletion of nrp1 in endothelial cells were generated by crossing nrp1 floxed (nrp1^fl/fl) mice (Gu et al., 2003) with endothelial-specific stem cell leukemia (EC-SCL)-Cre-ER<sup>T</sup> mice (Göthert et al., 2004). Cre recombinase excision of nrp1 was thereby controlled by the 5′ endothelial enhancer of the...
stem cell leukemia (EC-SCL) locus in a tamoxifen-dependent manner. Four different cohorts of mice were compared: Cre-negative nrp1 flox/flox mice injected with tumor cells lacking NRP1 expression (cis) or with tumor cells expressing NRP1 (cis+trans), and Cre-positive nrp1 flox/flox mice injected with tumor cells expressing NRP1 (trans) or with tumor cells lacking NRP1 expression (no NRP1; Figure 5A). NRP1 staining on tumor sections showed endothelial cell-specific knockout of NRP1 in tamoxifen-treated EC-Cre-positive nrp1 flox/flox mice, but not in tamoxifen-treated Cre-negative nrp1 flox/flox mice (Figure 5C). Moreover, PLA on tumor sections confirmed the presence of VEGFR2/NRP1 complexes in situ. The complexes were differently located, on endothelial cells (cis), in the interface between tumors and vessels (trans), or both (cis+trans), depending on where NRP1 was expressed (Figure 5D). We did not detect expression of NRP1 on cell types other than endothelial cells and tumor cells.

Tumor growth was measured over time in Cre-positive and -negative nrp1 flox/flox mice injected with the B16F10 melanoma cells. The end-point tumor weights in the cis and cis+trans groups were significantly higher than tumor weights in the trans and no NRP1 groups of mice (Figure 5E); a similar pattern was recorded for end-point tumor volumes (Figure S5D). This difference was at least in part because initiation of tumor growth was delayed in the trans and no NRP1 groups (Figure S5E). Vessel density (Figure S5F) and pericyte coverage (data not shown) were similar among cis, cis+trans, and trans groups, likely due to rescue by NRP1-independent angiogenic stimulation. Not all mice developed a palpable B16F10 tumor (Figure 5F).

Interestingly, the percentage of mice without tumors in the trans and no NRP1 groups of mice (35% and 32%, respectively) was significantly higher than in the cis and cis+trans groups (13% and 16%, respectively). This suggested a decisive role of endothelial (cis) NRP1 in tumor initiation.

In the subcutaneous T241 fibrosarcoma model, the difference in NRP1 expression between no NRP1 and overexpressed NRP1
Figure 6. Inhibition of Angiogenesis by NRP1 in trans Is Dependent on the NRP1 C-Terminal Tail

(A) Schematic overview of the experimental setups for the T241 tumor and tumor-matrigel studies.

(B–D) Quantification (B), representative overview (C), and high-magnification images (D) of CD31-positive vessels in matrigel tumors containing T241 cells.

(B) CD31-positive area normalized to total section area with data pooled from two independent experiments. Four to eight animals (with two matrigel tumors per animal) were used per experimental group in each experiment.

(C) Nuclei visualized by Hoechst 33342 staining show cell contents in the matrigel tumors (arrowheads). Scale bar, 500 μm.

(D) High magnification of images in (C). Blue, Hoechst 33342; green, CD31. Scale bar, 50 μm.
was substantial (Figure 5B), which might explain why the T241 tumor weights in cis+trans and trans groups were significantly decreased compared to the cis and no NRP1 groups (Figures 5E and 5D). The difference was at least in part due to delayed initiation of tumor growth in the cis+trans and trans groups (Figure 5E). These results confirm that tumor cell NRP1 in trans does not compensate for the loss of endothelial cis NRP1. Vessel density (Figure 5F) and pericyte coverage (data not shown) in the T241 tumors were not significantly different between cis and trans groups. We verified significant Cre-mediated excision of nrp1 from endothelial cells in the T241 tumors (Figure S5G). These observations suggested that once tumor growth was initiated, any potential difference in vessel parameters due to loss of NRP1 in endothelial cells was compensated by NRP1-independent angiogenic stimulation.

As shown in Figure 5F, the number of T241-injected mice without tumors in the cis+trans and trans groups (38% and 48%, respectively) was significantly higher than in the cis and no NRP1 groups (18% and 20%, respectively). These data indicate that the trans-expressed NRP1 (in the cis+trans and trans cohorts) efficiently suppressed tumor formation, irrespective of whether NRP1 was expressed in endothelial cells or not.

NRP1 on Tumor Cells in trans Suppresses Early Tumor Vascularization

As shown in Figure 5F, NRP1 expressed in trans on tumor cells suppressed tumor initiation. However, in the subcutaneous B16F10 and T241 models, this suppressive trans effect of NRP1 was rescued by NRP1-independent angiogenesis in a large proportion of the cohorts, resulting in tumors that eventually showed no difference in vascular density regardless of whether VEGF was presented in cis or in trans. To study the early stage of tumor development under cis and trans conditions, we injected tumor cells suspended in matrigel and harvested the matrigel tumors after 7 days. Thereby, we could monitor early tumor vascularization before tumor takeoff and non-NRP1-dependent growth. See Figure 6A for design of long-term tumor studies shown in Figure 5 versus the short-term matrigel tumor studies shown in Figure 6.

T241 cells expressing wild-type NRP1, NRP1ΔC, or transduced with empty lentivirus (EV) were suspended in matrigel and injected subcutaneously in Cre-positive and -negative nrp1fl/fl mice, treated with tamoxifen. After 7 days, the matrigel tumors were removed. Macroscopically, T241/EV matrigel tumors (cis) often appeared red, while T241/NRP1 matrigel tumors (trans) were paler (Figures S6A and S6B). Quantification of vessel density in the matrigel tumors showed that wild-type trans NRP1 inhibited CD31-positive vascularization in Cre-negative nrp1fl/fl mice (cis+trans). Vascularization in matrigel tumors where tumor cells expressed the truncated NRP1ΔC was significantly higher than when tumor cells expressed wild-type NRP1 (compare cis+trans with cis+trans ΔC versus cis; Figure 6B). These data confirm an important role for the NRP1 C-terminal tail in establishment of the trans effect (see Figure 4). Matrigel tumors from Cre-positive nrp1fl/fl mice (no NRP1 in endothelial cells) showed similar, limited vascularization, irrespective of whether tumor cells expressed no NRP1, wild-type NRP1, or NRP1ΔC. All conditions showed tumor growth in the matrigel (Figure 6C); still the CD31-positive area within the tumor-dense regions was significantly lower when NRP1 was presented in trans (Figures 6B and 6D).

The reduced vascularization of the cis+trans and trans matrigel tumors was not due to differences in VEGF accessibility. A soluble Flt1 extracellular domain (sFlt1), known to bind VEGF with high affinity (Koch et al., 2011), was used to detect VEGF in the matrigel tumors. Binding of sFlt1 showed an equal and broad expression of VEGF regardless of the expression pattern of NRP1 (Figure 6E). Taken together, these data indicate that the mechanism of tumor inhibition through trans NRP1 might be mediated by inhibition of vessel recruitment into the tumor. We asked whether this inhibition might involve the nonpermissive signal transduction regulated by VEGF binding in trans to NRP1 as was observed in vitro (see Figure 3). Analysis by immunoprecipitation/immunoblotting showed that complex formation between VEGFR2 and NRP1 was low, probably reflecting turnover, and similar in the different conditions (Figure 6F). On the other hand, immunoblotting of the tumor lysates revealed that accumulation of phospho ERK1 (pERK1) was significantly suppressed in vivo in the trans matrigel tumors (Figures 6G and 6H). All other conditions showed a higher pERK1/pERK2 ratio compared to trans, in agreement with the in vitro data on trans-modulated VEGFR2 signaling (see Figure 3). It is conceivable that this trans-effect on the pERK1/pERK2 ratio was exerted on low levels of remaining cis-expressed NRP1 activity in the tamoxifen-treated mice, explaining the higher pERK1/pERK2 ratio in the no NRP1 than in the trans condition.

NRP1 in Regulation of Endothelial Sprouting

Developmental angiogenesis in the retina follows a strictly patterned radial outgrowth of vessels headed by sprouting tip cells followed by nonsprouting stalk cells (Phng and Gerhardt, 2009). We hypothesized that NRP1 might suppress sprouting in the stalk region by trans-modulation of VEGFR2 signaling between adjacent endothelial cells. We therefore studied retinas of Cre-positive nrp1fl/fl p5 pups after suboptimal treatment with tamoxifen to create a condition of mosaic expression of NRP1. Tamoxifen-treated Cre-negative pups were examined in parallel (wild-type). The extent of angiogenic sprouting in the stalk region of the retina was determined in areas expressing NRP1 through the use of antibody and immunohistochemistry. See also Figure S6.
Figure 7. NRP1 in Regulation of Endothelial Sprouting

(A) Retinas of Cre-positive nrp1^{fl/fl} p5 pups after suboptimal treatment with tamoxifen showing mosaic areas (left) with vessel fragments expressing NRP1 (red) or not. Arrowheads point to cells with low NRP1 expression. Retinas from Cre-negative nrp1^{fl/fl} p5 pups (wild-type [WT]; right) were examined in parallel. Isolectin B4 (IB4; green) shows endothelial cells. *, filopodia extending from endothelial cells expressing NRP1 located adjacent to endothelial cells lacking NRP1.

(B) Quantification of sprouting endothelial cells in the mosaic and wild-type areas in (A). n = 25–26 (wild-type and mosaic areas), n = 12 (no NRP1). “Undefined” indicates IB4-positive structures that projected from endothelial cells but which did not extend filopodia.

(C) Immunostaining and microscopy to show NRP1 expression (red) in human umbilical vein endothelial cell cultures subjected to scratch wounding (dashed line). Panels to the right show high magnifications of the wound and the quiescent areas of the cell monolayer, respectively. Nuclei were stained with Hoechst 33342.

(D) Replicates WGA, replicates NRP1, mean WGA, mean NRP1. (E) Mean fluorescence intensity of WGA and NRP1 at the tips and stalks of the wounds.
(wild-type), in the borders between areas expressing NRP1 or not (mosaic regions), and within the NRP1-deficient area (no NRP1). Sprouting was induced in the mosaic areas of the stalk region and occurred in endothelial cells expressing NRP1 in close proximity to non-NRP1-expressing cells (Figure 7A; quantification in Figure 7B). In contrast, sprouting was efficiently suppressed in the wild-type retina as well as in areas lacking NPR1 expression.

Differences in relative expression of NPR1 may occur physiologically. NPR1 was efficiently downregulated in cells bordering the wound in a monolayer of VEGF-treated primary endothelial cell cultures (Figures 7C–7E). These data are compatible with a model where NPR1 expression suppresses induction of angiogenic sprouting between adjacent endothelial cells in a cis+trans configuration, dependent on the relative expression levels of NPR1. Moreover, endothelial quiescence can be overcome through downregulation of NPR1 on adjacent endothelial cells.

DISCUSSION

The data presented here support a model where expression of NPR1 on nonendothelial cells can suppress angiogenesis and reduce initiation of tumor growth via altered VEGFR2 internalization and signaling. This could be a critical mechanism in tumor dormancy where healthy individuals can harbor microscopic tumors and dysplastic foci for many years, which do not progress without angiogenesis (Albini et al., 2012). Moreover, our data indicate that downregulation of NPR1 expression might overcome vascular quiescence. The relative expression levels of NPR1 between neighboring endothelial cells will determine the ability of NPR1 to promote or arrest productive VEGFR2 signaling. Combined, our data identify an important trans-regulating effect of nonendothelial- and endothelial-cell-expressed NPR1 (Figure 8).

The need for VEGFR2 internalization for proper signaling has been recognized in several reports (Lanahan et al., 2010; for references, see Koch et al., 2011). Indeed, signaling in the AKT and ERK pathways can be initiated from the endosomal compartment (Sorkin and von Zastrow, 2009). We show that arrest of VEGFR2 at the endothelial cell surface by VEGF/NRP1 in trans resulted in loss of ERK1 activation, whereas ERK2 activity was prolonged. While ERK1 and ERK2 proteins are coexpressed in most tissues and have been regarded as redundant, recent data indicate that ERK2, but not ERK1, contributes to Ras-induced oncogenic signaling (Shin et al., 2010) and that ERK1 may have an inhibitory effect on ERK2 (Lloyd, 2006). Our in vitro and in vivo data indicate that balanced ERK1/ERK2 signaling might be critical in initiation of angiogenesis. In agreement, signaling in the Ras pathway is critical for angiogenic sprouting (Westenskow et al., 2013).

Less is known about sustained PLCγ activation, which was present in trans but not in cis, probably due to internalization and degradation of cis signaling complexes. PLCγ competes with phosphatidylinositol 3-kinase (PI3K) for the same substrate, phosphatidylinositol 4,5 bisphosphate (PIP2). Interestingly, PI3K is essential for tube formation in vitro, whereas PLCγ promotes regression of tubes by competing for PIP2 (Im and Kazlauskas, 2006). Therefore, trans presentation of VEGF and sustained PLCγ activation might have contributed to the reduced vascular density in matrigel tumors.

NRP1 expression has been linked to tumor aggressiveness and progression; however, information is often lacking with regard to which cell type in the tumor microenvironment expresses NRP1. A recent report (Jubb et al., 2012) confirms the expression of NPR1 in tumor cells on a subset of human cancers, such as non-small-cell lung cancer. Previous studies have addressed the consequence of NPR1 expression in tumor cells with conflicting results. NPR1 overexpression promotes tumor vascularization and growth, for example in rat prostate carcinoma (Miao et al., 2000) and U87MG glioblastoma (Hu et al., 2007). In contrast, growth of PANC-1 pancreatic cancer is reduced with NPR1 overexpression and enhanced with NPR1 suppression (Gray et al., 2005). One confounding factor may be the potential expression of class 3 semaphorins, which bind to NPR1 and induce antiangiogenic effects (for a review, see Muratori and Tamagnone, 2012). Sema3a might influence vascular properties in a NPR1-dependent manner resulting, e.g., in excess vascular permeability in diabetic retinopathy (Cerani et al., 2013). Importantly, there was no effect of Sema3a on NPR1-expressing tumor cell lines in vitro and Sema3a was not expressed in vivo in the tumors. Moreover, cells expressing NPR1 but not VEGFR2 lacked signs of VEGF-induced NPR1 signaling or biological responsiveness. Thus, in the vasculature, NPR1 modulates VEGFR2 signaling in response to VEGF but does not directly transduces signals upon binding of VEGF.

Trans expression of NPR1 in subcutaneous tumors reduced the incidence of tumor establishment (tumor initiation). Hanahan and Folkman (1996) coined the expression “angiogenic switch” to indicate the critical role for angiogenesis during tumor initiation. The ability of cells undergoing epithelial-mesenchymal transition to produce VEGF and promote angiogenesis is critical for tumor initiation (Fantozzi et al., 2014). Trans-presentation of VEGF by NPR1 expressed on tumor cells offers a mechanism whereby the switch might be delayed, e.g., to maintain tumor dormancy. It is unlikely that antitumor immunity and inflammation contributed to the NPR1-dependent tumor suppression, as there was no NPR1 expression on cells other than the endogenous expression on endothelial cells or on B16F10 tumor cells or transduced expression on T241 tumor cells. Furthermore, differences in tumor initiation were observed early (7 days after inoculation) in the subcutaneous tumor models.

Once tumors started to grow, there was no difference in the growth rate or in vascular density between the different groups (cis, cis+trans, trans, or no NPR1 expression), most likely due to compensation by other NPR1- and VEGF-independent, angiogenic stimuli. In order to facilitate the early phase of tumor growth rate or in vascular density between the different groups (cis, cis+trans, trans, or no NPR1 expression), most likely due to compensation by other NPR1- and VEGF-independent, angiogenic stimuli. In order to facilitate the early phase of tumor growth rate or in vascular density between the different groups (cis, cis+trans, trans, or no NPR1 expression), most likely due to compensation by other NPR1- and VEGF-independent, angiogenic stimuli. In order to facilitate the early phase of tumor growth rate or in vascular density between the different groups (cis, cis+trans, trans, or no NPR1 expression), most likely due to compensation by other NPR1- and VEGF-independent, angiogenic stimuli. In order to facilitate the early phase of tumor growth rate or in vascular density between the different groups (cis, cis+trans, trans, or no NPR1 expression), most likely due to compensation by other NPR1- and VEGF-independent, angiogenic stimuli.
establishment, we injected tumor cells with or without NRP1 expression suspended in matrigel as a size-standardized matrix, which allowed growth of tumor cells during the early, decisive phase. Thereby, we could analyze also the noncompensated trans and cis+trans conditions, which were characterized by reduced vascular density. Our data firmly support the conclusion that NRP1 might suppress VEGF-dependent processes through a trans-dependent mechanism. Interestingly, tumors expressing a mutant NRP1 missing most of its C-terminal domain did not show trans-suppressed angiogenesis, probably due to the short half-life of VEGF retention. VEGF was released within a few minutes from the truncated NRP1, whereas most of VEGF bound to wild-type NRP1 was very stably retained for many hours. These data raise the question why there is only a very mild angiogenic phenotype in the NRP1ΔC mouse (Fantin et al., 2011). The expression level of NRP1ΔC is elevated compared to that of full-length NRP1 in wild-type mice (data not shown); the elevated expression as well as other molecular changes in the NRP1ΔC mouse might allow a compensatory escape that conceals the consequence of the reduced VEGF retention by truncated NRP1. Moreover, although development proceeds essentially undisturbed in the NRP1ΔC mouse, tumor angiogenesis remains to be analyzed. We cannot exclude other effects of deleting the NRP1 C-terminal domain that might have influenced results presented here, such as loss of other molecular interactions and changes in the cell surface distribution. Such potential changes might underlie the rapid kinetics of VEGFR2/NRP1ΔC complex formation both in cis and trans. In another study from Fantin and colleagues, NRP1 expressed on macrophages or neuronal cells was shown not to influence vascular branching in E11 hindbrains (Fantin et al., 2013), implying that trans-effect of NRP1 described here would not play a role during development. A more recent study (Fantin et al., 2014) on the phenotype of a mouse expressing mutant NRP1 (Y297A) unable to bind VEGF shows that postnatal and pathological angiogenesis, but not embryonic development, depends on VEGF binding to NRP1. This is in agreement with the model presented here (Figure 8). Notch pathway signaling is a major determinant in the switch between angiogenic sprouting and vascular quiescence (Phng and Gerhardt, 2009). It is an interesting possibility that dynamic regulation of NRP1 expression is influenced by Notch.

Figure 8. Summary of Consequences of Presenting NRP1 in cis and in trans
Schematic outline concluding the data presented in the study, listing the parameters assessed and the different outcomes dependent on whether NRP1 was expressed in cis or in trans.
Overall, our data indicate that cis presentation of VEGF by NRP1 to VEGFR2 is favored over trans due to the rapid kinetics of complex formation in cis, at least as represented in vitro by feeding of starved cells with saturating concentrations of VEGF. However, internalization of cis complexes consisting of VEGFR2/NRP1/VEGF might reduce cell surface expression of NRP1 on the endothelial cell. VEGF produced by an adjacent cell, such as a tumor cell that also expresses NRP1, might be retained by NRP1 on the producer cell. In the NRP1-transduced T241 tumors, trans presentation of VEGF suppressed the cis response, indicating a higher trans than cis presentation. In contrast, in the B16F10 tumors where NRP1 had been silenced but not completely suppressed, cis dominated over trans. Moreover, in the developing retinal vasculature, the relative expression levels of NRP1 on adjacent endothelial cells determined the extent of angiogenic sprouting. Therefore, dependent on the local, potentially dynamically regulated ratio of NRP1 expressed in trans relative to cis, trans presentation might dominate and suppress VEGF-R2 function in a manner relevant for both physiological and pathological angiogenesis.

EXPERIMENTAL PROCEDURES

Animal Studies
Animal work was approved by the Uppsala University board of animal experimentation. Endothelial-cell-specific, tamoxifen-inducible NRP1 knockout mice were obtained by crossing nргс-fl/fl mice (Gu et al., 2003) with transgenic mice expressing tamoxifen-inducible Cre controlled by the EC-SCL locus (Gothert et al., 2004). B16F10/KO-Ctl (control), B16F10/KO-NRP1, T241/EV (empty vector), or T241/NRP1 cells were injected subcutaneously. Tumor size and weight were compared between the experimental groups using the Kruskal-Wallis test. When the p value from this overall test was < 0.05, pair-wise comparisons between the groups were performed using the Mann-Whitney test. All tests were two-tailed and p < 0.05 was considered a statistically significant result. The statistical package SAS for Windows version 9.2 was used for the calculations.

See Supplemental Experimental Procedures for additional procedures.

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.02.010.

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