Esm1 Modulates Endothelial Tip Cell Behavior and Vascular Permeability by Enhancing VEGF Bioavailability

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ABSTRACT

**Rationale:** Endothelial cell-specific molecule 1 (Esm1) is a secreted protein thought to play a role in angiogenesis and inflammation. However, there is currently no direct in vivo evidence supporting a function of Esm1 in either of these processes.

**Objective:** To determine the role of Esm1 in vivo and the underlying molecular mechanisms.

**Methods and Results:** We generated and analysed Esm1 knockout (Esm1KO) mice to study its role in angiogenesis and inflammation. Esm1 expression is induced by the Vascular Endothelial Growth Factor A (VEGF-A) in endothelial tip cells of the mouse retina. Esm1KO mice showed delayed vascular outgrowth and reduced filopodia extension, which are both VEGF-A-dependent processes. Impairment of Esm1 function led to a decrease in phosphorylated Erk1/2 in sprouting vessels. We also found that Esm1KO mice displayed a 40% decrease in leukocyte transmigration. Moreover, VEGF-induced vascular permeability was decreased by 30% in Esm1KO mice, and specifically upon stimulation with VEGF-A165 but not VEGF-A121. Accordingly, cerebral edema due to ischemic stroke-induced vascular permeability was reduced by 50% in the absence of Esm1. Mechanistically, we show that Esm1 binds directly to fibronectin and thereby displaces fibronectin-bound VEGF-A165 leading to increased bioavailability of VEGF-A165 and subsequently enhanced levels of VEGF-A signaling.

**Conclusions:** Esm1 is simultaneously a target and modulator of VEGF signaling in endothelial cells, playing a role in angiogenesis, inflammation and vascular permeability, which might be of potential interest for therapeutic applications.

**Keywords:** Esm1, VEGF, angiogenesis, vascular permeability, vascular inflammation, cancer and stroke

**Nonstandard Abbreviations and Acronyms:**
- EC: endothelial cell
- ECM: extracellular matrix
- Esm1: Endothelial cell-specific molecule 1
- FN1: fibronectin
- HEK293: Human Embryonic Kidney 293 cells
- HUVEC: Human Umbilical Vein Endothelial Cells
- IP: intraperitoneal
- KO: knockout
- LLC: Lewis Lung Carcinoma Cells
- PHH3: phospho-histone 3
- PMN: polymorphonuclear leukocytes
- tMCAO: transient Middle Cerebral Artery Occlusion
- VEGF-A: Vascular Endothelial Growth Factor A
- VP: Vascular permeability
INTRODUCTION

Endothelial cell-specific molecule 1 (Esm1) is a dermatan sulfate proteoglycan that is expressed and secreted by endothelial cells (ECs) and renal epithelia. Its expression is regulated both by inflammatory cytokines (IL1β, TNFα, IFNγ) and proangiogenic factors (VEGF-A and VEGF-C). Several lines of evidence suggest that Esm1 plays a role in inflammation. For instance, it was shown in vitro that Esm1 interacts with LFA-1 and that this inhibits the adhesion of leukocytes to ICAM1, which suggests a negative role in leukocyte extravasation during inflammation. Moreover, Esm1 plasma levels have been reported to be increased in pathological situations such as sepsis and several cancer types where it is a marker of poor prognosis. It has also been described that Esm1 increases proliferation and survival of tumor cells, which underlies the fact that overexpression of Esm1 in tumorogenic cells is sufficient to induce tumor growth. Concurrently, blocking of Esm1 function through the use of antibodies or siRNA has been described to reduce tumor growth and evasiveness. It has also been suggested that the levels of Esm1 directly correlate with tumor angiogenesis. In vitro experiments showed that Esm1 enhances the promigratory and proliferative effects of VEGF on lymphatic endothelial cells. Esm1 expression is highly enriched in retinal endothelial tip cells and in tumor endothelium, which makes it a useful marker for neoangiogenesis. However, the analysis of angiogenesis through the use of matrigel plugs in vivo concluded that Esm1 had no effect on angiogenesis. Thus, the exact function of Esm1 in vivo and the underlying molecular mechanisms remain elusive.

In this study, we have used Esm1 knockout (Esm1KO) mice to study the function of the gene product in vivo. We show that Esm1 is required for optimal response to VEGF stimuli. Esm1 is strongly and exclusively expressed by endothelial tip cells during retinal angiogenesis. Esm1-deficient sprouts emitted less filopodia and displayed decreased p-Erk1/2 levels, which resulted in reduced vascular progression. Esm1 was also previously implicated in inflammation where it was thought to inhibit leukocyte adhesion to ICAM1 in vitro. Surprisingly, we found that Esm1 does not affect leukocyte rolling and adhesion in vivo, instead it positively regulates leukocyte extravasation at the endothelial transmigration level. In addition, Esm1 is required for optimal VEGF-induced vascular permeability (VP). Mechanistically, we show that Esm1 binds directly to fibronectin (FN1) and thereby displaces FN1-bound VEGF-A165. This leads to increased bioavailability of local VEGF-A165 and increased levels of VEGF signaling. Importantly, Esm1 also modulates pathological angiogenesis and especially VP. Esm1KO mice presented a modest decrease in tumor angiogenesis, while cerebral edema, due to ischemic stroke-induced vascular permeability, was reduced by 50%. Thus, Esm1 is both a target and modulator of VEGF signaling in ECs with roles in the regulation of angiogenesis, inflammation and permeability.

METHODS

An expanded Methods section is available in the Online Supplement.

**Mutant mice, inducible genetic experiments and pharmacological inhibition.**

Endothelial cell-specific deletion of Vegfr2 in the postnatal vasculature was achieved by interbreeding Vegfr2flxed mice with Cdh5(PAC)CreERT2 transgenic mice. 100µg tamoxifen (Sigma, T5648; 2mg/ml) was administered intraperitoneally (IP) in every littermate at P3, P4 and P5.

For the pharmacological inhibition of VEGFR signaling in vivo, we administered subcutaneous injections of the vehicle (DMSO), VEGFR2 inhibitor (SU5416, Sigma; 25µg/g), VEGFR3 inhibitor (MAZ51, Sigma; 10µg/g) or MEK1/2 inhibitor (U0126, Cell Signaling; 25µg/g) at P5 and 8 hours before dissecting the animals. 50mg/kg anti-VEGF-A (Genentech, G6-31) or control IgG antibodies (Jackson ImmunoResearch) were injected IP every day from P4-P6.

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**In vivo permeability and inflammation assays.**
VEGF-A121, VEGF-A165 and histamine-induced VP in the mouse dorsal skin and IL1β-induced peritonitis and neutrophil recruitment in the cremaster muscle were performed as previously described. All animal procedures were carried out in accordance with national and regional laws and regulations, and were approved by animal ethics committees. All efforts were made to minimize suffering of the mice.

**Esm1-FLAG cloning, synthesis and immunoprecipitation.**
C-terminal FLAG-tagged mouse Esm1 was cloned. Human Embryonic Kidney 293 cells (HEK293) were transfected with the cloned construct or empty vector alone. Cells were then incubated in serum free medium for 48hrs. The culture supernatant was then collected, filtered and incubated on a monolayer of Human Umbilical Vein Endothelial Cells (HUVEC) for 45min, at 37°C, after which they were washed and then lysed. Esm1-FLAG bound to HUVEC-derived proteins was immunoprecipitated with anti-Flag M2 agarose. Samples were sent for mass spectrometry analysis.

**Solid phase binding assays.**
Binding of Esm1, VEGF-A165, VEGF-A121 and PDGF-BB to FN1 was shown by solid phase binding assay by ELISA as previously described.

**VEGF-A121, VEGF-A165 and Esm1 ELISA.**
Measurement of VEGF-A121 and VEGF-A165 in coating supernatants was achieved by using an ELISA kit for human VEGF (DVE00, R&D).

For measuring Esm1 levels in mouse serum, blood samples were centrifuged at 2000rpm, 10min at r/t and supernatant was collected. ELISA was performed as previously described. Capture antibody used was goat anti-mouse Esm1 (AF1999 R&D) and the detection antibody was rat anti-mouse Esm1 (LIA0905 Lunginnov).

**RESULTS**

**Esm1 is highly expressed by endothelial cells with high VEGF signaling.**

Esm1 mRNA was recently described to be specifically expressed in endothelial tip cells in the developing mouse retina. Immunostaining of postnatal day 5 (P5) retinas confirmed that the secreted Esm1 protein is indeed marking distal vascular sprouts (Figure 1A). In order to check whether Esm1 is a universal tip cell marker, we analysed the protein expression pattern of Esm1 in LLC tumors. Esm1 immunostaining was detected only in ECs, but its expression was not restricted to sprouting cells in tumor blood vessels. Instead, Esm1 protein was detected throughout the vasculature (Figure 1B).

Esm1 expression is positively regulated by VEGF-A in vitro and in tumor models, and it has also been shown to be upregulated by VEGF-C in lymphatic ECs. In order to confirm the influence of VEGF-A signaling on Esm1 expression under physiological conditions in vivo, we analyzed the lungs of mice in which the Vegfr2/Flk1 gene had been deleted in ECs or of animals treated with VEGF-A blocking antibody. Esm1 transcript levels were prominently decreased in Vegfr2 mutants and after VEGF-A blockade (Figure 1C). Furthermore, P5 pups were treated with chemical compounds that inhibit the kinase domain of VEGFR2 (SU5416) and VEGFR3 (MAZ51). Expression of Esm1 was specifically and profoundly reduced by VEGFR2 inhibition but not by MAZ51 (Figure 1D). These results correlate well with the known expression patterns of VEGF-A and VEGF-C in the retina. VEGF-C, the main VEGFR3 ligand, is expressed by macrophages that are not exclusively localized at the angiogenic front, whereas expression...
of VEGF-A, the main VEGFR2 ligand, is presented by astrocytes in the avascular, peripheral retina. Accordingly, endothelial tip cells in the retina are more exposed to VEGF-A than ECs within the angiogenic plexus, which may account for tip cell-specific expression of Esm1 in the retinal vasculature. On the other hand, Esm1 expression in most, if not all, tumor ECs but not vessels in the surrounding tissue, correlates with higher VEGF-A levels throughout the tumor. Thus, we conclude that Esm1 is not a general endothelial tip cell marker, but that its expression rather reflects high VEGF-A signaling levels (Figure 1E).

Retinal vascular outgrowth and filopodia emission are impaired in Esm1KO mice.

VEGF-A regulates Esm1 expression in vivo (Figure 1C,D) suggesting that it might be implicated in angiogenesis. Therefore we evaluated angiogenesis in control and Esm1KO P5 retinas. The mutant vasculature showed a slight but significant decrease in vascular outgrowth (Figure 2A,D) indicative of impaired angiogenesis in Esm1KO mice. This difference was not caused by general developmental delay because the weight of Esm1-deficient mutants was comparable to littermate controls (Figure 2E). Since Esm1 is secreted and may act on neighbouring stalk cells, we questioned if EC proliferation, which occurs mostly at the stalk cell level, was altered and the cause of the decreased vascular progression. We quantified retinal EC proliferation by immunostaining for phospho-histone-3 (PHH3), which labels mitotic cells. No significant difference was observed between Esm1WT and Esm1KO retinas (Figure 2B, F). Accordingly, no significant differences were observed in the density of control and mutant retinal vasculature (Figure 2G). Vascular outgrowth is not only dependent on endothelial proliferation but also on appropriate sprouting activity of ECs through the sensing of angiogenic factors such as VEGF-A. Therefore, we assessed the number of sprouts and filopodia in Esm1KO mice. Despite its tip cell-specific expression in the retina, Esm1 does not play a role in tip-stalk cell specification since no difference was observed in sprout number between Esm1WT and Esm1KO mice. However, a significant decrease in filopodia number was observed in mutants relatively to control littermates (Figure 2C,H,I), suggesting that the impaired vascular progression in the absence of Esm1 might be due to decreased motility of endothelial sprouts.

To better understand the role of Esm1 in tip cell behavior, we generated a novel transgenic mouse line, Esm1(BAC)iCreERT2, where tamoxifen-inducible iCreERT2 was inserted into a large genomic clone downstream of the Esm1 promoter (Online Figure IA). The resulting mouse line was interbred with R26mT/mG reporter mice to generate Esm1(BAC)iCreERT2,R26mT/mG double transgenics and recombination was induced by administrating 4-hydroxytamoxifen 24hrs before analysis. In the retinas of these animals, recombined GFP-positive cells were mostly restricted to sprouting ECs similarly to the distribution of endogenous Esm1 protein (compare Online Figure IB with Figure 1A).

By combining this novel genetic tool with the Esm1KO mouse line, we labeled retinal sprouts in the presence or absence of Esm1. Recombination was induced at P4 and retinas were analysed at P5. The ratio of GFP-positive sprouts remained unaltered between Esm1WT and Esm1KO retinas, which further supports that Esm1 is not required for tip-stalk cell interconversion (Figure 2J, K). Although the recombination pattern was similar between Esm1WT and Esm1KO retinas, we observed that GFP-positive cells were found in small, contiguous clusters in Esm1KO retinas, whereas recombined cells were scattered and rarely touching each other in Esm1WT samples. Quantification of the GFP-positive areas, which may represent isolated GFP-positive or several GFP-positive cells in direct contact, confirmed that significantly larger patches were found in Esm1KO retinas compared to Esm1WT littermates (Figure 2J, L). Since EC proliferation was not altered in Esm1KO retinas and given the comparable ratio of total GFP-positive cells (GFP+ area) to total ECs (IsolectinB4 area) in Esm1KO and Esm1WT samples (Figure 2J,M), the clustering of GFP-positive cells in Esm1KO retinas suggested reduced migration and/or stronger cell adhesion between Esm1KO endothelial cells.
The absence of Esm1 impairs leukocyte extravasation at the transmigration step.

Suggesting a possible role in inflammation, Esm1 expression is also induced by inflammatory cytokines. In fact, in vitro data indicated that Esm1 inhibits leukocyte extravasation by inhibiting their adhesion to ECs. To investigate this question in vivo, we performed peritonitis assays on Esm1-deficient and control mice. Contrary to the expected, Esm1KO mice showed a significant decrease in leukocyte extravasation when compared to control littermates (Figure 3A). Leukocyte extravasation was also assessed in venules of the cremaster muscle by intravital microscopy, which allows direct visualization and analysis of leukocyte behavior during the rolling, adhesion and transmigration steps. While hemodynamic parameters are similar between control and Esm1KO mice (Online Table I), the decreased leukocyte extravasation observed in Esm1KO mice was solely due to reduced transmigration (Figure 3B-D). These results argue against a prominent role of Esm1 in leukocyte-endothelial cell adhesion in this model and suggested that Esm1 might act on EC junctions. Immunostainings for adherens and tight junctions, however, uncovered no significant structural differences between Esm1WT and Esm1KO mice (Online Figure II).

Esm1 is specifically required for VEGF-A165-induced vascular permeability.

Since leukocyte extravasation was compromised in Esm1KO mice at the transmigration step, we questioned whether Esm1KO mice might present a general defect in vascular permeability (VP). To this end, we performed the Miles assay by injecting different permeability-inducing agents subcutaneously. Both histamine and VEGF-A121 induced a robust and undistinguishable increase in VP both in Esm1WT and Esm1KO mice, while the increase in VEGF-A165-induced permeability was significantly reduced in Esm1KO mice compared to controls (Figure 3E). Co-injection of Esm1 and VEGF-A165 had no effect on the increase in permeability in Esm1WT mice when compared to VEGF-A165 stimulation alone. However, the decreased VEGF-A165-induced permeability response in Esm1KO animals was rescued by co-injection of Esm1 and VEGF-A165 (Figure 3F).

VEGF signaling is decreased in vivo in the absence of Esm1.

To test whether VEGF signaling is compromised in Esm1KO mutants, we performed immunostaining for phospho-Erk1/2 (p-Erk1/2), a well-established downstream effector of VEGF signaling, in P5 retinas. p-Erk1/2 immunostaining was most intense in endothelial sprouts (Figure 4A) in line with high activity of the VEGF pathway in tip cells. To confirm the specificity of the p-Erk1/2 immunostaining, P5 pups were administered a highly selective inhibitor for MEK1/2 (U0126), a kinase that activates Erk1/2 through phosphorylation. U0126-treated pups showed a pronounced decrease in p-Erk1/2 immunostaining in the retinal angiogenic front (Figure 4B). Western blot analysis of lung lysates from the same pups confirmed the decreased Erk1/2 phosphorylation (Figure 4C). In Esm1KO retinas, p-Erk1/2 immunostaining remained stronger in sprouts compared to endothelial cells in the adjacent plexus, but the difference in staining intensity was significantly decreased (Figure 4D-F). Activity of the VEGF pathway was also assessed by injecting VEGF-A165 intraperitoneally and analyzing VEGFR2 phosphorylation in lung lysates. While control pups showed a robust increase in VEGFR2 phosphorylation at 30 minutes after VEGF-A165 injection, only a comparably weak effect was obtained in Esm1KO pups (Figure 4G). Taken together, these results suggested that ECs are less responsive to VEGF-A in the absence of Esm1 in vivo.

Decreased tumor angiogenesis in Esm1KO mice.

Esm1 plasma levels are increased in several pathological conditions including cancer, and inhibition of Esm1 has been described to reduce tumor growth. It is however not known whether this involves alterations in tumor angiogenesis. Esm1 expression in tumors was observed throughout most ECs (Figure 1B and Online Figure IC), suggesting that it may play a prominent role in tumor angiogenesis.
We used syngenic tumor models in \textit{Esm1}^{WT} and \textit{Esm1}^{KO} mice, in which Lewis Lung Carcinoma (LLC) or melanoma (B16F1) cells were inoculated on their flanks. Tumor growth was monitored during two weeks and angiogenesis was evaluated at the end point. With the exception of a transient delay in LLC tumor growth at 12 days post-inoculation, no significant difference in tumor growth was observed when comparing \textit{Esm1}^{KO} with control mice (Figure 5A). To evaluate the effect of Esm1 on tumor angiogenesis, tumor sections were immunostained with an EC marker, CD31 or Endomucin, and vessel density was quantified. \textit{Esm1}^{KO} mice presented a 30% decrease in vessel density in both tumor types when compared with control mice (Figure 5B-D). Thus, Esm1 plays a more prominent role in tumor vascularization than in developmental angiogenesis possibly because of its broader expression pattern in the tumor vasculature.

\textit{The absence of Esm1 leads to decreased brain edema upon ischemic stroke.}

Altered vascular permeability is a hallmark of many pathological situations such as ischemic stroke. It is well established that the breakdown of the blood-brain-barrier is a determining factor in the acute phase of stroke, since it leads to edema and increased neural lesions. We have shown that Esm1 plays a role in VP and therefore we questioned whether this would hold true in the context of ischemic stroke.

As Esm1 plasma levels are increased in several pathological conditions, we quantified Esm1 levels in plasma of mice before and 24 hours after the induction of ischemic stroke by tMCAO (transient Middle Cerebral Artery Occlusion). Circulating Esm1 was increased by 25\% upon ischemic stroke (Figure 5E). Magnetic resonance imaging was performed for each mouse 24 hours after MCAO and edema-corrected lesion volumes (LVc) and the space-occupying effect due to brain edema (\%HSE) were quantified. A striking 50\% decrease in both parameters was observed in \textit{Esm1}^{KO} mice when compared to controls (Figure 5F-H).

\textit{Esm1 modulates VEGF-A\textsubscript{165} bioavailability by competing with it for FN1 binding.}

To uncover the molecular mechanisms by which Esm1 modulates VEGF signaling, we tried to identify endothelial Esm1 binding partners. For this purpose, a murine Esm1 construct carrying a C-terminal FLAG-tag was overexpressed in HEK293 cells in order to produce Esm1-FLAG-enriched supernatant that was added to HUVEC, allowing binding of Esm1 to putative membrane and extracellular binding partners. Esm1-FLAG was then immunoprecipitated (Figure 6A) and samples were analysed by mass spectrometry. Since Esm1 is a secreted protein, only coimmunoprecipitated membrane and extracellular proteins that were found with at least 10 times more abundance in the Esm1-FLAG sample compared to the control (based on MS/MS count differences for each protein) were considered as putative interacting partners. Several extracellular but no membrane proteins were co-immunoprecipitated with Esm1 and detected by mass-spectrometry (Online Table II).

Interestingly, two of the putative interacting partners, FN1 and HSPG2, have been previously described to modulate VEGF-A signaling\textsuperscript{33-36} and are also involved in the regulation of vascular permeability\textsuperscript{37-39}. It is well established that VEGF-A binds heparin and heparan sulfate, including those residues present in HSPG2\textsuperscript{33,40}. Both in vitro and in vivo experiments have shown that HSPG2 positively regulates VEGF-A signaling and helps to establish VEGF gradients that act on angiogenic ECs\textsuperscript{41}. HSPG2 also modulates the binding of VEGF-A to its receptors\textsuperscript{33,42}. The interaction between FN1 and VEGF has also been reported to enhance VEGF signaling in vivo, namely to increase EC migration\textsuperscript{35}. Therefore, we reasoned that the interaction between Esm1 and one or both of these proteins might influence VEGF-A signaling. Starved and confluent HUVEC monolayers were stimulated by adding VEGF-A\textsubscript{165} and/or Esm1 to serum free medium (Figure 6B). Stimulation with Esm1 alone did not lead to appreciable VEGFR2 phosphorylation. Upon VEGF-A\textsubscript{165} stimulation, phospho-VEGFR2 levels were only slightly altered by addition of recombinant Esm1 (Figure 6B). Next, we mimicked an extracellular matrix (ECM)
microenvironment composed of FN1, HSPG2 and VEGF-A in the absence or presence of Esm1 (Figure 6C). Although FN1 is well known to activate integrins, no difference in the levels of phospho-FAK, a key downstream effector of activated integrins, was observed (Online Figure IIIA). Accordingly, no difference was observed between Esm1KO and control littermate retinas immunostained for activated-integrin β1 and phosphor-paxillin (Online Figure IIIB,C). We did however find that HUVEC seeded on wells coated with FN1, HSPG2 and VEGF-A contained consistently higher levels of phospho-VEGFR2 in the absence of Esm1 (Figure 6C). As this result suggested that the presence of Esm1 might negatively regulate VEGF presentation by matrix components, we next tested whether Esm1 might displace ECM-bound VEGF-A165. Measurement of VEGF-A165 levels in the coating supernatant showed that free VEGF-A165 increased in the presence Esm1 in a dose-dependent manner (Figure 6D), indicating that Esm1 inhibits matrix interactions of VEGF-A165. This finding was further supported by strongly increased levels of phospho-VEGFR2 when HUVEC monolayers were stimulated with the supernatant from Esm1-treated wells (Online Figure IVA). When the same experiments were performed with VEGF-A121, which lacks C-terminal matrix-binding motifs, both the levels of VEGF-A121 in the supernatant and of phospho-VEGFR2 in supernatant-treated HUVEC were independent of Esm1 (Online Figure IVA,B).

To elucidate whether the effect of Esm1 on VEGF signaling is mediated by FN1 or HSPG2, each of these components was withdrawn from the coating mixture. The ability of Esm1 to increase the bioavailability of VEGF-A165 5-fold was not significantly changed by the removal of HSPG2. In contrast, removal of FN1 strongly increased the levels of VEGF-A165 in supernatants and reduced the difference between Esm1-treated and untreated samples (Figure 6E). This finding indicates that Esm1 displaces FN1-bound VEGF-A165.

Finally, to confirm Esm1 binding to FN1, a solid phase binding ELISA assay was performed. VEGF-A165 and VEGF-A121 were used as positive and negative controls, respectively. Incubation of increasing concentrations of Esm1 with FN1-coated wells resulted in a binding curve with a clear saturation point, thus confirming the ability of Esm1 to bind directly to FN1 (Figure 6F). Although FN1 has been shown to bind several growth factors including PDGF-BB, we observed a decrease of up to ~20% in FN1-bound VEGF-A165 but not of PDGF-BB when in the presence of Esm1 (Online Figure IVC). Moreover, when assaying the effect of Esm1 on EC migration by performing scratch wound assays on HUVEC stimulated with several growth factors that interact with FN1, we observed that Esm1 only affected VEGF-A165-mediated EC migration (Online Figure IVD,E). This further supports our previous conclusion that Esm1 specifically displaces FN1-bound VEGF-A165.

DISCUSSION

The present study demonstrates that Esm1 modulates VEGF-A165-driven biological processes in vivo, namely angiogenesis and vascular permeability. Esm1 is a dermatan sulfate proteoglycan that is expressed and secreted mostly by ECs. Its regulation both by angiogenic factors and cytokines suggested that it might play a role in both angiogenesis and inflammation. Nevertheless, in vivo evidence for an Esm1 role in either of these biological processes was lacking. In this study, we confirm, through the use of mouse genetics and chemical inhibitors, that Esm1 expression is regulated by VEGF-A but not VEGF-C in vivo. Our findings also establish that Esm1 regulates angiogenesis and inflammation in vivo both in physiological and pathological contexts, albeit to different extents.

Previous in vitro reports established that Esm1 binds to the LFA-1 complex on leukocytes and thereby inhibits the adhesion of leukocytes to ICAM1. Therefore, it was postulated that Esm1 might block leukocyte extravasation. Contrary to this prediction, we now found that Esm1 is necessary for leukocyte extravasation in vivo. Furthermore, visualization and analysis of the distinct steps of leukocyte
extravasation through intravital microscopy establish that Esm1 does not interfere with the adhesion of leukocytes to the endothelium but instead exerts its effect at the transmigration level. Moreover, Esm1KO mice have a specific decrease in VEGF-A165-induced vascular permeability.

Our results point towards the existence of a positive-feedback loop where VEGF-A positively regulates Esm1 expression, which in turn enhances VEGF-A-mediated signaling (Figure 7). Although recent work has suggested the existence of such positive feedback loop for VEGF signaling in vitro47, the underlying mechanisms remained unclear. We now identify FN1 and HSPG2 as potential interactors of Esm1, which are known to regulate VEGF signaling and bind to VEGF-A165 but not VEGF-A121.24, 35, 40, 46. Moreover, FN1 and HSPG2 have also been implicated in the regulation of vessel permeability. FN1 mutants are embryonic lethal and, among several other defects, display hemorrhages27,38. Plasma FN1, which is mostly present postnatally, is a negative regulator of vascular permeability. Likewise, mice deficient in endothelial heparan sulfate also have reduced permeability39. Although heparan sulfate does not bind VEGF-A121, it was shown that these endothelial heparan sulfate deficient mice displayed reduced VEGF-A165- and VEGF-A121-induced VP because heparan sulfate also binds VEGFR2 and thereby modulates ligand-receptor binding affinities. The finding that VEGF-A121-induced vascular permeability is regulated by heparan sulfate and not by Esm1 further supports that HSPG2 is not a relevant interaction partner of Esm1, which is in agreement with our in vitro data. Instead, we propose that Esm1 positively modulates VEGF-A165 signaling by binding to FN1 and preventing the sequestration of VEGF-A165 by FN1. This increases the bioavailability of VEGF-A165 and consequently, to increased levels of VEGFR2 signaling.

Esm1KO mice were born with normal Mendelian ratios and are viable and fertile. Accordingly, we found that Esm1 plays a minor role in sprouting and angiogenesis. However, more prominent and potentially disease-relevant functions of Esm1 have been uncovered in the current study. Esm1 regulates tumor angiogenesis as well as VEGF-induced vascular permeability, which is known to occur in several pathological conditions. For instance, in ischemic stroke, increased levels of VEGF are beneficial for post-ischemic angiogenesis and recovery, however in the acute phase, increased levels of VEGF enhance VP and thereby neuronal damage48-50. Therefore, inhibition of Esm1 might be an attractive therapeutic/preventive strategy, for high stroke risk populations, to modulate the effects of VEGF-A on VP. Moreover, Esm1 blockage in cancer may contribute not only to anti-angiogenic effects but also to decreased cancer cell dissemination, since the hyperpermeable character of tumor vessels are known to facilitate this process.

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DISCLOSURES
None.
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FIGURE LEGENDS

**Figure 1:** Esm1 is a biomarker of high VEGF-A signaling. A, B, Staining for Esm1 in retinal (A) and tumor (B) vasculature (IsolectinB4 or CD31, as indicated). C, D qPCR gene expression analysis of Esm1 in lung tissue of pups with (C) EC-specific deletion of VEGFR2 (R2 dEC) or of pups treated with VEGF-A blocking antibody (VA Ab) and (D) of pups treated with chemical inhibitors for VEGFR2 (SU5416) or VEGFR3 (MAZ51). E, Scheme depicting Esm1 as a biomarker of high VEGF-A (green dots) signaling levels and not as a general endothelial tip cell marker. p<0.05 (*); p<0.01(**), student t-test.

**Figure 2:** Esm1KO mice show decreased vascular outgrowth, filopodia emission and migration. A, Representative low magnification image of IsolectinB4-stained retinas of control and Esm1KO P5 pups. Dashed circle represents the limit of the vascular outgrowth of the control retina. B, IsolectinB4 (IsoB4) and PHH3 staining in control and Esm1KO P5 retinas. C, High resolution picture showing sprouts and filopodia emission in the angiogenic front of control and Esm1KO P5 retinas. D-I, Quantification of the average vascular outgrowth (D), body weight (E), mitotic cell number per area of vascular coverage (F) vessel density (G), sprout (H) and filopodia number (I) per angiogenic front length of control and Esm1KO P5 retinas. J-M, Quantification of the ratio of GFP-positive sprouts (K), of the area of individual GFP-positive patches (L) and extent of EC recombination at the angiogenic front (M) in reporter control and Esm1KO P5 retinas. p<0.05 (*); p<0.001(***), ns (not significant), student t-test.

**Figure 3:** Esm1KO mice show decreased leukocyte extravasation and VP. A, In comparison to control littersmates, Esm1KO mice showed reduced polymorphonuclear leukocytes (PMN) extravasation at both 2 and 4 hours after IL1β-induced inflammation of the peritoneum. B-D, Analysis of leukocyte extravasation through intravital microscopy of the cremaster muscle shows that reduced PMN extravasation observed in Esm1KO mice is due to impaired transmigration (D) and not rolling (B), nor adhesion (C) to the endothelium (n>40 venules from a total of 6 mice per genotype). E, Miles assay performed on dorsal skin shows that Esm1KO mice had reduced VP in response to VEGF-A165 but not histamine or VEGF-A121. F, Reduced VEGF-A165-induced VP in Esm1KO mice was rescued by co-injection of Esm1 and VEGF-A165 (E+V). p<0.05 (*), p<0.01 (**), p<0.001 (***), student t-test (A-D) and one way ANOVA with Dunnett’s post-test (E-F).

**Figure 4:** Reduced VEGF signaling in vivo in the absence of Esm1. A, B, pErk1/2 and IsolectinB4 staining in retinas of P5 pups injected with DMSO (A) or MEK1/2 inhibitor, U0126 (B). C, Western blot analysis of whole lung lysate of pups injected with DMSO or U0126. D, E, pErk1/2 and IsoB4 staining in retinas of P5 control (D) and Esm1KO (E) littersmates. F, Quantification of the ratio of pErk immunostaining in sprouts versus the adjacent stalk ECs in control and Esm1KO P5 retinas. G, Western blot analysis of whole lung lysate of control and Esm1KO pups injected with PBS or 500ng VEGF-A165 30min before dissection. p<0.001 (**), Student t-test.

**Figure 5:** Impact of Esm1 on pathological angiogenesis and vascular permeability. A, Growth curve of LLC and B16F1 tumors in control and Esm1KO mice. B, C, Representative images of endothelial immunostaining for Endomucin (B) or CD31 (C) in LLC and B16F1 tumors, respectively. D, Vessel density quantification in LLC (n>40 sections; 4 mice/genotype) and B16F1 tumors in control and Esm1KO mice (n>60 sections; 6 mice/ genotype). E-H Analysis of control and Esm1KO 24 hours after tMCAO. E, Esm1 protein levels in serum. F, Representative T2-weight MRI images of hyperintense ischemic areas (outlined in red). G, Quantification of edema-corrected lesion volumes (LVc). H, Quantification of the space-occupying effect due to brain edema (%HSE). p<0.05 (*); p<0.001 (**), Student t-test.
**Figure 6:** Esm1 modulates VEGF-A$_{165}$ bioavailability. A, Immunoprecipitation of FLAG-tagged Esm1 that was incubated on confluent HUVECs (asterisk indicates band corresponding to Esm1-FLAG). B, Western blot analysis of phospho-VEGFR2 (p-VEGFR2) and total VEGFR2 levels upon classical stimulation of confluent HUVECs by adding factors to serum free medium. C, Western blot analysis of p-VEGFR2 and total VEGFR2 levels upon stimulation of HUVECs by seeding these onto a matrix containing extracellular molecules and factors. D, Quantification of free VEGF-A$_{165}$ in coating supernatant in the presence of increasing concentrations of Esm1. E, Influence of FN1 and HSPG2 on the levels of free VEGF-A$_{165}$ in coating supernatant in the absence or presence of Esm1. F, Solid phase binding ELISA shows direct interaction between FN1 and Esm1. VEGF-A$_{121}$ and VEGF-A$_{165}$ were used as negative and positive controls, respectively. p<0.05 (*), p<0.001 (**), one-way ANOVA with Dunnett’s post-test (E).

**Figure 7:** Proposed model of Esm1 function. Esm1 competes with VEGF-A$_{165}$ for FN1 binding, which increases VEGF-A$_{165}$ bioavailability and thereby promotes VEGF signaling. The latter increases Esm1 expression, which generates a positive feedback loop. In the absence of Esm1, more VEGF-A$_{165}$ is sequestered to FN1, which decreases VEGF bioavailability and signaling. Thus, loss of Esm1 translates into decreased reduced vascular permeability, filopodia emission and vascular outgrowth.
Novelty and Significance

What Is Known?

- Endothelial cell-specific molecule 1 (Esm1) is secreted by endothelial cells (ECs) and it is regulated both by inflammatory cytokines and proangiogenic factors in vitro.
- Esm1 plasma levels are increased in pathological situations such as sepsis and several cancer types and its overexpression in tumorigenic cancer cells induces tumor growth.
- In vitro evidence points towards a role of Esm1 in inhibiting leukocyte extravasation by blocking LFA-1/ICAM1 interactions.

What New Information Does This Article Contribute?

- Esm1 positively regulates angiogenesis, leukocyte extravasation and vascular permeability (VP) both in physiological and pathological contexts.
- Esm1 interacts directly with fibronectin (FN1), leading to displacement of FN1-bound VEGF-A165, increased bioavailability of VEGF-A165 and consequently, increased VEGF signaling.

For over a decade, Esm1 has been associated to angiogenesis and inflammation. However, in vivo evidence for a role of Esm1 in either of these processes was lacking. We now provide the first in vivo data showing that Esm1 positively regulates angiogenesis, inflammation and vascular permeability. The generation of the Esm1 knockout and Esm1(BAC)CreERT2 reporter mouse lines, have enabled us to show that Esm1 impacts developmental and tumor angiogenesis by regulating VEGF-induced EC migration Contrary to the current belief in the field, Esm1 does not inhibit leukocyte extravasation, instead our in vivo data shows that it promotes leukocyte extravasation at the transmigration step. We also show that in the absence of Esm1 function, VEGF-A165-induced VP is reduced, leading to a 50% decrease in lesion volume upon cerebral ischemia. Mechanistically, we show that Esm1, a target of VEGF-A itself, increases VEGF-A165 bioavailability and consequently VEGF signaling by binding to FN1 and displacing FN1-bound VEGF-A165. Altogether our results render Esm1 as an attractive therapeutic target to modulate the effects of VEGF-A in pathological conditions, namely as a preventive strategy for high stroke risk populations and also in cancer where Esm1 blockage may prevent VEGF-induced angiogenesis and cancer cell dissemination.
Figure 5

A) Tumor Volume (mm$^3$) over Days post-inoculation:
- WT B16F1 (n=14)
- KO B16F1
- WT LLC
- KO LLC (n=8)

B) Immunofluorescence images of LLC Endomucin, CD31, and Esm1$^{K/O}$.

C) Vessel Density (WT vs. KO) for LLC and B16F1:
- LLC
- B16F1

D) Normalized levels of circulating Esm1:
- Before
- After (n=4)

E) LVc (mm$^3$) for WT and KO (n=10)

F) HSE (%) for WT and KO (n=10)

*** indicates statistical significance.
Figure 6

A

Esm1-FLAG  Mock  Esm1-FLAG  Mock

Input  FLAG IP

Blot a-FLAG

B

Esm1  VEGF  VEGF + Esm1

(2ug/mL)  (50ng/mL)

0'  3'  5'  10'  30'

pVEGFR2  VEGFR2

C

20'-60'-90'-20'-60'-90'

pVEGFR2

VEGFR2

+ + + + VEGF (200ng/mL)
- - + + ESM1 (4ug/mL)

D

Relative levels of VEGF165 in supernatant

Esm1 (ug/mL)

E

Relative levels of VEGF165 in supernatant

-FN1  +  +  -

HSPG2  +  -  +

Gelatin  -  -  +

F

OD

VEGF-A_{121}  VEGF-A_{165}  Esm1
Figure 7

Wild type

Esm1 mutant

* Esm1  ○ VEGF165  ○ VEGFR2  ××× FN1  * Vascular leakage

vascular permeability
filopodia
vascular outgrowth

vascular permeability
filopodia
vascular outgrowth
Esm1 Modulates Endothelial Tip Cell Behavior and Vascular Permeability by Enhancing VEGF Bioavailability

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**Supplemental Material**

**Detailed Methods**

**Generation of the Esm1KO mouse line.** LoxP recognition sites for Cre recombinase from bacteriophage P1 were inserted around the second exon of the murine Esm1 gene (Ensembl ID ENSMUSG00000042379). A murine cDNA fragment containing the coding sequence from exons 2 and 3 followed by 2 copies of bovine growth hormone polyadenylation signal sequence was inserted in frame via a unique BstEII restriction site (amino acid sequence at fusion site is -DRTGTR-). Downstream of the loxP-flanked area, the targeting construct also contained a β-galactosidase (lacZ) reporter cassette, which carried an artificial splice acceptor and an internal ribosomal entry site (IRES) at the 5’-end and the SV40 polyadenylation signal sequence at the 3’-end. Furthermore, the targeting construct contained a neomycin resistance cassette surrounded by Frt sites allowing Flp recombinase-mediated removal as well as 5.5kb (5’) long and 1.5kb (3’) short arms for homologous recombination. After electroporation of the linearized targeting construct into F1 embryonic stem cells and selection in G418-containing medium, clones were isolated and characterized by PCR and South-blot hybridization. Injection of ES cell clones into mouse blastocysts first led a conditional mouse lines. Through interbreeding with PGK-Cre deleter mice1, global knockout mice were generated. No β-galactosidase reporter activity was detectable in heterozygous or homozygous mutants, and it was subsequently confirmed that Esm1-lacZ fusion transcript were not detectable by qPCR. Mice were maintained by backcrossing into the C57BL6 background as well as intercrossing of heterozygous mutants.

**Generation of the Esm1iCreERT2 mouse line.** A mouse line expressing the codon-improved Cre recombinase (iCre) fused to a modified estrogen receptor (ERT2) under the control of the Esm1 gene was generated through bacterial artificial chromosome (BAC) recombineering technology. The iCreERT2 was fused to start codon of Esm1 using a PCR-based approach. This was followed by a simian virus 40 (SV40) polyadenylation signal and a bacterial kanamycin selection cassette that was flanked by FRT sites. The 1st exon was deleted upon insertion of the iCreERT2-SV40-polyA-frt-Kanamycin-frt cassette immediately downstream of the translation initiation codon of Esm1. BAC modification was performed in a bacterial system as described previously3. The bacterial antibiotic selection cassettes were removed before microinjection of the constructs into fertilized C57BL/6 eggs. Two founders were obtained of which only one (founder #27) presented transmission of the transgene to offspring. Mice were maintained on a C57BL/6 background. Genotyping was performed by PCR using the following primers: 5’ gagggactacctcctgtacc and 5’ tgcccagagtcatccttggc yielding a 630bp product.

**Genetic labeling of Esm1-expressing cells.** Esm1-iCreERT2 mice were interbred with Rosa26-mTmG reporter mice and the double heterozygous offspring analysed. For the retinal analysis, 50µL of 4-hydroxytamoxifen (0,5mg/mL; H7904 Sigma) were administrated intraperitoneally at P4 and pups were then dissected at P5 (24hrs after induction). For the tumor analysis, mice were injected with 1mg tamoxifen per day at 7 -10 days post-tumor inoculation and then analysed at 14 days post-tumor inoculation.

**In vivo permeability assay**

Gender and age matched mice (8-12 week old) were injected with 100µL of Evans blue solution (1% in PBS; E2129 Sigma) in the tail vein. After 10min, intradermal injections (50µL) of PBS, histamine (4,5µg/mL; H7250 Sigma), human VEGF-A165 (2µg/mL; 300-076 Reliatech), human VEGF-A121 (2µg/mL; HZ-1204 HumanZyme) or mouse Esm1 (10µg/mL; 1999-EC R&D) were administered. Mice were sacrificed after 30 minutes and skin biopsies in the injected regions were harvested and incubated overnight (o/n) in formamide at 55ºC. Formamide was collected after 16hrs and absorbance was measured at 620nm.

**In vivo inflammation models**

**IL-1β-induced peritonitis assay.** Esm1WT and Esm1KO mice, matched for gender and age (7-9 weeks), received 10ng recombinant mouse IL-1β (Biomol) in 1 mL PBS intraperitoneally. At 2h or 4h after stimulation, the mice were sacrificed and the peritoneal cavity was lavaged with 20 mL of PBS
containing 3 mM EDTA. Red blood cells were lysed using BD FACS lysing solution (BD Biosciences). Total cell counts were determined with a cell counter (CASY; Schärfe-System) and the percentage of neutrophils was determined by flow cytometry (FACSCalibur; BD Biosciences) using the monoclonal antibody RB6-8C5 against Ly-6G and Ly-6C (Gr-1). Neutrophils were distinguished from monocytes and macrophages by higher expression of Gr-1.

**Intravital microscopy.** Inflammatory stimulation of the cremaster muscle was achieved by intrascrotal injection of 50ng of recombinant mouse IL-1β (Biomol) in 0.3 mL PBS administered to either Esm1WT or Esm1KO mice (12-16 weeks old) 4 hours before microscopic examination (n = 6 each group). Mice were anesthetized with an intraperitoneal injection of 125mg/kg of ketamine hydrochloride (Ketavet, Pfizer) and 12.5mg/kg xylazine hydrochloride (Rompun, Bayer HealthCare). A tube was inserted into the trachea to facilitate normal breathing and the mice were kept on a thermocontrolled heating pad to maintain body temperature during the experiment. The preparation of the cremaster muscle was essentially done as described previously. The testis and the surrounding cremaster muscle were exteriorized through an incision in the scrotum. Connective tissue was removed, the cremaster muscle was incised longitudinally and stretched across the pedestal of a custom-made intravital microscopy stage fabricated from polymethyl methacrylate. The muscle was fixed by small metal hooks on silicone rubber placed in a groove in the pedestal. The epididymis and the testis were pinned to one side. During the whole experiment, the muscle was superfused with a thermocontrolled (34°C) bicarbonate buffered salt solution (131.9 mM NaCl, 18 mM NaHCO3, 4.7 mM KCL, 2.0 mM CaCl2, and 1.2 mM MgCl2) that was equilibrated with 5% CO2/95% N2. Intravital microscopy was performed on an upright microscope (BX61WI; Olympus; integrated into a LaVision BioTec microscopy system) fitted with a saline immersion objective (XLUMPLFL, 20x, 0.95 numeric aperture; Olympus) and equipped with differential interference contrast (DIC). For each animal, 4-13 single unbranched postcapillary venules with diameters of 20 to 35 μm were observed and recorded through a CCD camera system (CCD camera 1600L, LaVision BioTec). Images were analyzed using ImageJ (National Institutes of Health) and ImSpector (LaVision BioTec) software. Rolling leukocytes passing through a plane perpendicular to the vessel axis were counted and leukocyte rolling flux is expressed as number of leukocytes per millimeter vessel diameter per second. The total number of adherent leukocytes was determined for each vessel segment (100 μm) and is expressed per 10⁴ μm² of vessel surface area. Extravasated leukocytes from venules were determined in an area reaching out 75 μm to each side of a blood vessel over a distance of 100 μm vessel length, representing an area of 1.5 x 10⁴ μm². Venule diameters and lengths were measured by means of the image acquisition- and processing software ImSpector (LaVision BioTec). Centerline red blood cell velocity was measured for each venule using a dual photodiode sensor system (CircuSoft Instrumentation). Centerline velocities were converted into mean blood flow velocities by multiplying with an empirical correction factor of 0.625. Newtonian wall shear rates were estimated as 8 x (v/d), where v is the mean blood flow velocity and d the diameter of the vessel. Interfacial shear rates (referred to here as “wall shear rates”) were estimated as 4.9 x 8 x (v/d), where v is the mean blood flow velocity, d the diameter of the vessel and the constant 4.9 an empirical correction factor. At the end of the experiment blood samples collected by cardiac puncture were used to assess systemic leukocyte counts.

**Animal Flank Tumor Models.** Lewis Carcinoma Cells (LLCs) and B16/F1 were obtained from ATCC cells and cultured in DMEM (Gibco) containing 10% FBS, 1x PenStrep and 2mM Glutamine at 37°C, 5% CO2. Cell medium was changed every other day passaged every 3-4 days. Cells were used for tumor inoculation when they were at 75%-80% confluency. Cells were trysinized and resuspended in Matrigel Solution (Biosciences). Age (8-10 weeks) and gender matched mice were injected subcutaneously on the right flank with 5 x 10⁵ tumor cells suspended in Matrigel. Tumor size was monitored every three days by measuring tumor length (l) and width (w) with a caliper. Tumor volume was calculated in the following way: (l x w²)/2. All experiments had at least 4 mice per group and were repeated at least twice. Mice were sacrificed after 15 days and tumors were extracted and processed for immunostaining.

**Transient Middle Cerebral Artery Occlusion MCAO.** Five month old wild-type and Esm1KO male mice were used throughout the experiments. Animals were anesthetized by intraperitoneal injection of a mixture of 10mg/kg xylazine (Pfizer) and 200 mg/kg ketamine hydrochloride (Bayer). Throughout the whole procedure and during recovery, body temperature was maintained at 37°C via a heating pad. After ligation of the left proximal common carotid artery and external carotid artery, a 7-0-nylon monofilament...
(Doccol Co., NM, USA) with a 0.23mm coated tip was introduced into the distal internal carotid artery via an incision in the ligated common carotid artery. The monofilament was advanced 11 mm distal to the carotid bifurcation to occlude the mid-cerebral artery. After topical application of the local anesthetic lidocainhydrochlorid (Xylocain Gel 2%, AstraZeneca) the neck wound was closed temporarily for a 60 min ischemic period. At reperfusion, the monofilament was withdrawn from the carotid artery and the wound was stitched with 4-0 non-resorbsorbable sutures (Ethibond Excel, Ethicon). The animal was returned to its cage to recover under observation.

**Magnetic Resonance Imaging.** MRI was performed using a 7 Tesla rodent scanner (Pharmascan 70/16 US, Bruker BioSpin, Ettlingen, Germany). For imaging a $^1$H-RF quadratur-volume resonator with an inner diameter of 20mm was used. Data acquisition and image processing were carried out with Bruker software Paravision 5.1. For imaging the mouse brain a T2-weighted 2D turbo spin-echo sequence was used (imaging parameters TR / TE = 4200 / 36 ms, rare factor 8, 4 averages). 20 coronal slices with a slice thickness of 0.5mm, a field of view of (FOV) 2.56 x 2.56 cm and a matrix of 256 x 256. During the examinations mice were placed on a heated circulating water blanket to ensure constant body temperature of 37°C. Anaesthesia (Forene, Abbot, Wiesbaden, Germany) was induced with 2.0% and maintained with 1.3–1.8% isoflurane delivered in a O₂ / N₂O mixture (30/70%) via a facemask under constant ventilation monitoring (Small Animal Monitoring & Gating System, SA Instruments, Stony Brook, New York, USA).

**Image Analysis.** Calculation of lesion volume was carried out with the program Analyze 5.0 (AnalyzeDirect, Inc.; Lenexa USA). The hyperintense ischemic areas in T2-weighted images were assigned with a region of interest tool. This enables a threshold-based segmentation by connecting all pixels within a specified threshold range about the selected seed pixel and results in a 3D object map of the whole stroke region. Further, the whole object map total volume was automatically calculated. The edema-corrected lesion volume (LVc) and the space-occupying effect due to brain edema, expressed as the volume increase of the affected hemisphere (%HSE), was calculated as previously described 5.

**Cell culture and in vitro experiments.** Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Cascades Biologics (C0035C) and cultured on dishes coated with 0,2% Gelatin (Sigma) in Medium 200 supplemented with LSGS (Gibco, Cascades Biologicals) at 37ºC, 5% CO₂. Cell medium was changed every other day and HUVECs were passaged every 4 days. Experiments were conducted with HUVECs between passages 4 and 6.

**Classical stimulation of HUVECS.** Confluent monolayers of HUVECS were incubated in serum free medium for 5hr. The cell medium was then substituted for serum free medium containing human VEGF-A₁₆₅ (50ng/mL; 300-076 Reliatech) and/or human Esm1 (2µg/mL; 1810-EC R&D). After stimulation cells were washed with PBS and lysed with SDS Sample Buffer.

**Stimulation of HUVECs with matrix-bound VEGF.** 12-well dishes were coated o/n at 37ºC with 500µL of 20µg/mL human plasma Fibronectin (FC010, Millipore) or Gelatin (Sigma), 10µg/mL HSPG2 (H4777, Sigma), human VEGF-A₁₂₁ (200ng/mL, HZ-1204 HumanZyme), VEGF-A₁₆₅ (200ng/mL) and/or human Esm1 (4µg/mL). For dose dependent experiments, Esm1 concentrations were varied between 0-10µg/mL as indicated in the results section. Remaining coating supernatant was collected and stored (for ELISA and stimulation of HUVEC monolayers), wells were rinsed with PBS and 4x10⁵ dissociated HUVECs were seeded in each well. Cells were incubated at 37ºC for several timepoints and then rinsed in PBS and lysed with SDS Sample Buffer. In order to preserve maximal membrane receptor integrity, HUVECs were dissociated with Accustase (PAA) instead of trysin.

**Stimulation of HUVEC monolayers with coating supernatant.** Confluent monolayers of HUVECs were incubated in serum free medium for 5hr. The cell medium was then aspirated and cells were stimulated with the different coating supernatants (described above) diluted 1:1 in serum-free medium. After stimulation cells were washed with PBS and lysed with SDS Sample Buffer. All assays were done in at least two independent experiments.

**Scratch wound assay.** Confluent monolayers of HUVECS (in 6-well plates) were incubated in serum free medium for 2hr. The monolayers were then gently scratched with a p200 pipette tip across the center of the well. Wells were washed twice with serum free medium to remove any detached cells and 8 images/well of the scratch were acquired (t=0h) with a phase-contrast microscope. Serum-free medium
was substituted for stimulation medium. Stimulation medium (prepared and incubated at 37°C, 1h30 prior to addition to HUVECs) contained 0.5% serum and distinct combinations of 100ng/mL cytokines, 2µg/mL human Esm1 and 10µg/mL FN1. Cytokines used were human VEGF-A_165 (300-076, Reliatech), human PDGF-BB (220-BB, R&D), human SDF-1 (300-28A, Peprotech) and human TNF-α (300-01A, Peprotech). Images of scratches were acquired again after 5 hours of stimulation. Quantifications were made by defining the initial wound area (t=0) and counting the number of cells that had migrated into this area after the 5 hours of stimulation. 3 independent experiments were performed.

**Esm1-FLAG cloning, synthesis and immunoprecipitation.** In order to identify putative Esm1 interacting partners a C-terminal FLAG-tagged version of mouse Esm1 was cloned. Briefly, mouse Esm1 was amplified from cDNA obtained from mouse lung tissue using the following primers: 5’ctcagcaagacccacgac and 5’ttgcaaatctcagccagg and cloned into pGEM-T Easy vector (Promega). In order to introduce a FLAG tag in the C-terminal region of mouse Esm1, primers were designed as follows: 5’ cacacacagactagacttagctttacttc and 5’ cacacacacttgctttagataattattctc. The PCR product was digested with EcoRI and XhoI and cloned into pDNA3.1 membrane-Tomato-2A. Human Embryonic kidney 293 (HEK293) cells were Purchased from DSMZ (ACC305) and cultured in DMEM (Gibco) containing 10% FBS, 1x PenStrep and 2mM Glutamine at 37°C, 5% CO_2. Cell medium was changed every other day passed every 3-4 days. 10cm dishes with HEK293 cells were transfected with the cloned construct or empty vector alone (mock) by using Lipofectamine 2000 (Invitrogen). Transfection efficiency was monitored 24h hours post-transfection through the expression of the fluorescent Tomato protein. Cells were then incubated in serum free medium (3mL/dish) for a period of 48hrs. After this period, the culture supernatant was collected, filtered and incubated on a monolayer of HUVECs for 45min, 37°C, after which the remaining supernatant was aspirated and HUVECs were washed with PBS. HUVECs were then lysed with lysis buffer (40mM Tris pH7.4, 0.5% NP40, 0.1M NaCl, 1mM EDTA, 1mM DTT, 10% Glycerol, 1mM Na3VO4, 1mM PMSF and protease inhibitor cocktail [P2714, Sigma]). Cell lysates were centrifuged and supernatants were incubated with anti-Flag M2 agarose beads (A2220, Sigma) at 4°C, o/n. Beads were then washed with lysis buffer (without DTT) and immunoprecipitated proteins were eluted with 0.1M Glycine (pH3.5) solution and pH was then neutralized with Tris buffer. Samples were sent for mass spectrometry analysis.

**Mass spectrometry.** Proteins from control as well as ESM1-specific immunoprecipitations were first size fractionated by gel electrophoresis using a 4–20% NUPAGE gel (Invitrogen) and then stained with Colloidal Blue staining Kit (Invitrogen). Both lanes were sliced into 17 pieces each and processed for GeLC–MS/MS. Briefly, proteins within each gel piece were subjected to reduction by 10 mM DTT (45 min at 56 °C) and alkylation using 55 mM jodoacetamide (30 min, RT, in the dark) followed by Trypsin cleavage (Promega) for 16 h at 37 °C. Peptides were then extracted from the gel pieces as described and purified using Stage Tips. Each fraction was analyzed by reversed phase chromatography using a EasyLC nano-flow system (Proxeon) that was online coupled via in-house packed fused silica capillary column emitters (length 15 cm; ID 75 µm; resin ReproSil-Pur C18-AQ, 3 µm) and a nanoelectrospray source (Proxeon) to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Peptides were eluted from the C18 column by applying a multistep linear gradient from 5–7% buffer B (80% acetonitril, 0.5% acetic acid; 2 min) and from 7–40% B over 70 min, followed by a 5 min sanitation step at 98% buffer B (flow rate 250 nl/min). The mass spectrometer was operated in the positive ion mode (source voltage 2.2 kV), automatically switching in a data-dependent fashion between survey scans in the mass range of m/z 300–1650 and MS/MS acquisition. Collision induced MS/MS spectra from the 15 most intense ion peaks in the MS were collected in the ion trap (Target Value of the Orbitrap survey scan: 1000000; resolution R = 60000; Lockmass set to 445.120025; isolation width m/z = 2.0; normalized collision energy 35%; dynamic exclusion enabled with repeat count 1, repeat duration 30.0, exclusion list size 500 and exclusion duration set to 90 s; double charge and higher charges were allowed). Raw data files were processed by MaxQuant software (v 1.2.0.13) in conjunction with the built-in Andromeda search engine. Data were searched against the International Protein Index sequence database (human IPI, version 3.73) concatenated with reversed sequence versions of all entries. Trypsin was set as digesting enzyme, a minimum length of 6 amino acids, a maximum of 2 missed cleavages, carbamidomethylation at cysteine residues set as fixed and oxidation at methionine residues as well as acetylation at the protein N-termini.
as variable modifications were the other analysis parameters. The maximum allowed mass deviation was 20 ppm for MS and 0.5 Da for MS/MS scans. Matching between runs was enabled with a time window of 2 min. Protein groups were regarded as being unequivocally identified with a false discovery rate (FDR) set to 1% for all protein and peptide identifications when there were at least 2 matching peptides, one of which being unique to the protein group.

**Solid phase binding assays.** ELISA plates (Nunc Maxisorp; Thermo Scientific GmBH) were coated with 50uL (at 0.5mg/mL) of human plasma Fibronectin (FC010, Millipore) o/n, 4°C. Wells were then blocked for 1hr at room temperature (r/t) with 2% BSA in PBST (0.05% Tween20 in PBS). Wells were washed with PBST and then incubated with proteins of interest for 30min at r/t. Wells were washed with PBST and then incubated with the appropriate primary antibody for 2hr at r/t. After washing, wells were incubated with the appropriate HRP-conjugated secondary antibody for 30min at r/t. After final washes with PBST and PBS, the antibody was detected with TMB Substrate (Cell Signaling). The optical density (OD) of each well was measured at 450nm with a Synergy2 BioTek microplate reader.

For competition assays between Esm1 and growth factors (VEGF-A165 and PDGF-BB) for fibronectin binding, Esm1 was first incubated at different concentrations in coated wells for 30min, r/t, washed out and then growth factors of interest were incubated for another 30min, r/t. ELISA was then carried out as described above to detect FN-bound growth factor.

Proteins used to evaluate binding to fibronectin were: human VEGF-A165 (300-076 Reliatech), human VEGF-A121 (HZ-1204 HumanZyme), human Esm1 (1810-EC R&D) and human PDGF-BB (220-BB, R&D). Primary antibodies used were: rabbit anti-human VEGF (sc-152, Santa Cruz), goat anti-human Esm1 (AF1810, R&D), and goat anti-human PDGF (AB-23-NA, R&D). Secondary antibodies used were: donkey anti-rabbit-HRP (NA9340V, Amersham) and horse anti-goat-HRP (P1-9500, Vector). Assays were done in duplicate and at least in two independent experiments.

**VEGF-A121, VEGF-A165 and Esm1 ELISA.** Measurement of VEGF-A121 and VEGF-A165 in coating supernatants was achieved by using an ELISA kit for human VEGF (DVE00, R&D).

For measuring Esm1 levels in mouse serum, blood was extracted through the mandibular vein. Samples were centrifuged at 2000rpm, 10min at r/t and supernatant was collected. ELISA plates (Nunc Maxisorp; Thermo Scientific GmBH) were coated with 100µL of capture antibody (goat anti-mouse Esm1, AF1999 R&D) at 1µg/mL and incubated at 4°C, o/n. Wells were incubated with blocking buffer (0.1% BSA, 0.1% Tween20, 5mM EDTA, PBS) for 1hr, r/t. Wells were washed with wash buffer (0.1% Tween20, 5mM EDTA, PBS) and then incubated with 50µL of undiluted plasma for 1h30 at r/t. Wells were washed and then incubated with the detection antibody (rat anti-mouse Esm1, LIA0905 Lunginnov) for 2hr at r/t. After washing, wells were incubated with goat anti-rat-HRP (NA935, Amersham) for 30min at r/t. After final washes with washing buffer and PBS, the antibody was detected with TMB Substrate (Cell Signaling). A secondary antibody was used: goat anti-rabbit-HRP (LMBP005, Amersham) and horse anti-goat-HRP (PI-9500, Vector). Assays were done in duplicate and at least in two independent experiments.

**Western blot of lung lysate.** For the analysis of protein, whole lungs were dissected and immediately snap frozen in liquid nitrogen. In the case of P5 pups stimulated with VEGF-A165, these were injected intraperitoneally with 500ng of human VEGF-A165 30min before being culled. Whole lungs were then extracted and immediately processed for western blot analysis. Lung tissue was lysed in lysis buffer (20mM Tris-HCl pH 8.0, 1mM EDTA, 1mM DTT, phosphatase inhibitor cocktail SetV [Calbiochem, 524629], 1mM Na3VO4, protease inhibitor cocktail (Sigma, P2714, 1:10), 1% TritonX-100, 150mM NaCl) and homogenized with a cylindrical glass and pestle (Potter-Elvehjem). Tissue debris was removed by centrifugation and the supernatant was diluted in loading buffer and analysed by SDS-polyacrylamide gel electrophoresis and western blotting. The following antibodies were used for immunoblotting: rabbit anti-VEGFR2 (Cell Signaling), rabbit anti-phospho-VEGFR2 (Cell Signaling), rabbit phopho-Erk1/2 (Cell Signaling) and rabbit anti-Erk1/2 (Cell Signaling). All the immunoblot images shown represent the average result obtained from a minimum of 2 different lung extracts per group.
qRT-PCR of lung lysates. Relative levels of mRNA expression were determined by isolating total RNA from the inferior left lobe of the lung with the RNAeasy mini kit (Qiagen). 500ng per reaction were used to generate cDNA with the SuperScriptTM III First-Strand Synthesis System (Invitrogen) and oligo(dT) primers. qRT-PCR was performed by using an ABI PRISM 7900HT. Taqman gene expression assays for murine Gapdh, Esm1 and Cdh5 were used in combination with Taqman Gene Expression Master Mix (Applied Biosystems). Gene expression was normalized to Gapdh. The relative expression differences obtained represent the average of the results obtained for at least two independent animals per group from two independent litters.

Immunohistochemistry. For quantification of vessel density and sprout and filopodia number, whole eyes were fixed o/n at 4°C, with freshly made PFA4%. Eyes were then washed in PBS. Retinas were dissected, cut into 4 quadrants and blocked/permeabilized (1% BSA, 0.3% Triton, PBS) o/n at 4°C. On the following day, retinas were washed two times in Pblec buffer (1% Triton X-100, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2 in PBS [pH 6.8]) for 20 minutes and incubated with biotinylated isolectinB4 (1:50, VectorLabs) for 2 hours at r/t. Following five washes in blocking solution, retinas were incubated with Alexa Fluor streptavidin-conjugated antibodies (Molecular Probes, 1:100) for 2 hours, washed three times in blocking solution and flat-mounted on microscope glass slides with Fluoromount-G (SouthernBiotech, 0100-01).

For double immunostaining, retinas were either fixed for 2 hours on ice or at r/t (PFA4%) or in methanol at -20°C. After fixation, retinas were blocked for 1 hour in 1% BSA with 0.3% Triton (or 0.05% Tween20) and incubated overnight or for 2 hours with isolectin B4 (1:50) and the following primary antibodies: goat anti-Esm1 (R&D), rabbit phospho-histone-3 (Upstate), rabbit anti-phospho-Erk1/2 (Cell Signaling), goat anti-Cdh5 (Santa Cruz), rat anti-Cdh5 (Pharmingen), rabbit anti-Clhn5 (Zymed), rat anti-ItgB1 (Becton Dickinson), rabbit anti-phospho Pax (Cell Signaling) and rat anti-CD31 (Pharmingen). For detection suitable species-specific Alexa Fluor–coupled secondary antibodies (1:500) were used.

For immunostaining of tumor tissue, tumors were fixed in 4% PFA, overnight at 4°C, and then embedded in 6.5% low melting agarose. Immunohistochemistry was performed in 100μm vibratome sections. Briefly, sections were permeabilized with 0.3% Triton, 0.1% Tween, PBS and then blocked for with 0.3% Triton, 4% BSA, PBS. Primary antibodies were incubated overnight at 4°C in blocking buffer. Sections were washed 5 times with 0.3% Triton, PBS and incubated with secondary antibodies overnight at 4°C in blocking buffer. Sections were washed 2 times with 0.3% Triton, PBS and mounted. Primary antibodies used: rat anti-CD31 (Pharmingen) and rat anti-endomucin (gift from D. Vestweber). Donkey anti-rat Alexa-488 was used as a secondary antibody.

Quantitative analysis of the retinal vasculature. All the images shown are representative of the retinal vascular phenotype observed in at least 5 individual pups from 2-3 distinct litters per group. Detailed quantifications were performed for retinas belonging to 5 distinct pups of each group. All quantifications were done with Volocity (Improvision) software on high-resolution confocal images or lower resolution stereomicroscope images (vascular outgrowth). The vascular outgrowth was defined as the distance from the center of the retina to the angiogenic front for each retina quadrant. A minimum of 8 quadrants/pup were used for quantification. The vascular density was defined as the ratio of isolectin B4+ area/total area and was calculated in a minimum of 6 fields/pup. Proliferation was determined by quantifying the number of phospho-histone3-positive (PHH3+) endothelial cells in a minimum of 3 fields/pup. The total number of PHH3+ cells was normalized to the endothelial cell area of 1000μm². The number of endothelial sprouts and filopodia extensions were quantified at the retina angiogenic front in a minimum of 8 fields/pup and 4 fields/pups, respectively. The total number of sprouts or filopodia was normalized for a standard endothelial vessel length of 1000μm that was measured and defined according to 10. The ratio of pErk immunostaining between tip and stalk was determined by measuring the mean intensity of pErk staining in endothelial cells contained within sprouts (or in direct contact with the avascular region) and in immediately adjacent endothelial cells that do not contact with the avascular region. The mean intensity of pErk was normalized to the mean intensity of IsolectinB4. A minimum of 2 fields/pup were analysed, with 4 regions were defined per field (each region containing the adjacent tip and stalk domains).
Quantitative analysis of the tumor vasculature. Immunostained tumor sections were imaged with a Leica MZ16F stereomicroscope. Images were processed with Photoshop by thresholding to identify endothelial cell staining. Threshold values were maintained constant throughout all tumor measurements within each independent experiment. Vessel density was determined as the ratio between the CD31+/Endomucin+ area in relation to the total tumor area. The total tumor area was defined to exclude necrotic areas.

Image acquisition and processing. Samples were analysed at lower resolution (flat-mounted retinas) with a Leica Stereomicroscope MZ16F coupled to a digital camera (Hamamatsu C4742-95) or at high resolution (flat-mounted retinas and tumor sections) with a Leica TCS SP5 confocal microscope. Volocity (Improvition), Photoshop CS and Illustrator CS (Adobe) software were used for image acquisition and processing.

Statistical Analysis. Statistical significance was determined by Student t tests (2-tailed) or ANOVA when indicated. All data are shown as mean±SEM. P<0.05 was considered significant.
A, Strategy for modification of the genomic BAC by recombination with a targeting vector containing iCreERT2 and a bacterial Kanamycin resistance cassette (KanR) immediately downstream of the *esm1* promoter. KanR was flanked by FRT sites (open circles) and removed before microinjection of the modified BAC into fertilized eggs. B-C, Analysis of the recombination pattern obtained upon interbreeding of the *Esm1(BAC)iCreERT2* line with the *R26 mT/mG* reporter line. Recombined cells were detected by immunostaining for GFP and ECs were stained for IsolectinB4 (B) or immunostained for CD31 (C). B, GFP-positive cells in the P5 retina are mostly restricted to endothelial sprouts 24hrs after recombination induction by 4-hydroxytamoxifen injection. C, GFP-positive cells in LLC tumors occur throughout the tumor vasculature.
Online Figure II: Adherens and tight junctions are undistinguishable between wild type and Esm1KO mice.

A, Cdh5 and CD31 immunostaining on cryosections of dorsal skin of P5 wild type and Esm1KO mice. Nuclei are stained with DAPI. B, Cdh5 and Cldn5 immunostaining in retinas of P5 wild type and Esm1KO mice. Endothelial cells are stained with IsolectinB4.
Online Figure III: Esm1 does not affect integrin signaling in vitro, nor in vivo conditions.

A, Western blot analysis of phosphorylated FAK, a downstream target of integrin signaling, upon seeding of HUVECs on a FN1/HSPG2/VEGF-A_{165} matrix in the presence or absence of Esm1. B, Activated integrin-b1 immunostaining in retinas of P5 wild type and Esm1\textsuperscript{K0} mice. Endothelial cells are stained with IsolectinB4. C, p-Pax immunostaining in retinas of P5 wild type and Esm1\textsuperscript{K0} mice. Endothelial cells are stained with IsolectinB4.
Online Figure IV: Esm1 interferes specifically with FN1-bound VEGF-A\textsubscript{165} and VEGF-A\textsubscript{165}-induced cell migration.

A, Western blot analysis of p-VEGFR2 and total VEGFR2 levels of starved HUVECs stimulated with coating supernatant containing different VEGF-A isoforms. B, Quantification of free VEGF-A\textsubscript{165} or VEGF-A\textsubscript{121} in coating supernatant in the absence or presence of Esm1. C, Competition assays using solid phase ELISA confirm that Esm1 competes with VEGF-A\textsubscript{165} for FN1 but not with PDGF-BB. (GF, growth factor). D, Scratch wound assays where starved HUVECs are stimulated with factors in the absence or in the presence of FN1. Esm1 alone is not sufficient to induce significant alterations in cell migration independently of the presence or not of FN1. Esm1 affects VEGF-A\textsubscript{165}-mediated migration only when in the presence of FN1. E, Scratch wound assays where starved HUVECs are stimulated with several cytokines (VEGF-A\textsubscript{165}, PDGF-BB, SDF-1 and TNF-\alpha) in the presence of FN1 and with or without Esm1. Only VEGF-A\textsubscript{165}-mediated cell migration is affected by Esm1, albeit in the opposite trend as that expected from \textit{in vivo} results. This is most likely due to the \textit{in vitro} context, where unbound VEGF-A\textsubscript{165} is diluted in the liquid medium and hence presented in lower concentrations to ECs than when bound to FN that binds directly to EC integrins. In an \textit{in vivo} context, unbinding of VEGF-A\textsubscript{165} from FN1 would occur within the matrix surrounding Esm1-expressing ECs, and therefore enhance VEGF-A signaling and consequently EC migration. p<0.05 (*), p<0.01 (**), p<0.001 (***), student t-test and one way ANOVA with Dunnett’s post-test (D,E).
Online Tables

**Online Table I: Hemodynamic parameters (mean ± SEM) of IL-1ß-treated cremaster muscle venules in control and Esm1<sup>ΔKO</sup> mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Esm1&lt;sup&gt;ΔKO&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number mice analyzed</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Number venules analyzed</td>
<td>52</td>
<td>40</td>
</tr>
<tr>
<td>WBC (x 10&lt;sup&gt;6&lt;/sup&gt; cells/ml)</td>
<td>2.32 ± 0.37</td>
<td>2.48 ± 0.16</td>
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<tr>
<td>Diameter (µm)</td>
<td>27.30 ± 0.50</td>
<td>28.97 ± 0.72</td>
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<tr>
<td>Blood velocity (mm/s)</td>
<td>2.45 ± 0.03</td>
<td>2.38 ± 0.04</td>
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<tr>
<td>Wall shear rate (10&lt;sup&gt;3&lt;/sup&gt;/s)</td>
<td>2.24 ± 0.05</td>
<td>2.07 ± 0.07</td>
</tr>
<tr>
<td>Newtonian wall shear rate (10&lt;sup&gt;3&lt;/sup&gt;/s)</td>
<td>4.57 ± 0.01</td>
<td>4.22 ± 0.01</td>
</tr>
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**Online Table II: Mass spectrometry analysis of Esm1-FLAG immunoprecipitation**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Peptides</th>
<th>Intensity CTRL</th>
<th>Intensity ESM1</th>
<th>MS/MS count ratio (ESM1/CTRL)</th>
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</thead>
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<td>32311000</td>
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</table>

Number of peptides differing by sequence, summed up peptide intensities and MS/MS count ratios for each protein identified as ESM1 interactor. Intensity values are given for control as well as ESM1-specific immunoprecipitations. Total protein intensities in the control and the ESM1 experiment were comparable (3.90E10 vs 4.76E10). Extracellular proteins are highlighted in grey.
Supplemental References


