Genetic Reduction of Vascular Endothelial Growth Factor Receptor 2 Rescues Aberrant Angiogenesis Caused by Epsin Deficiency

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Objective—We previously showed that endothelial epsin deficiency caused elevated vascular endothelial growth factor receptor 2 (VEGFR2) and enhanced VEGF signaling, resulting in aberrant tumor angiogenesis and reduced tumor growth in adult mice. However, direct evidence demonstrating that endothelial epsins regulate angiogenesis specifically through VEGFR2 downregulation is still lacking. In addition, whether the lack of epsins causes abnormal angiogenesis during embryonic development remains unclear.

Approach and Results—A novel strain of endothelial epsin-deleted mice that are heterozygous for VEGFR2 (Epn1\(^{+/}\); Epn2\(^{-/-}\); Flk\(^{+/}\); iCDH5 Cre mice) was created. Analysis of embryos at different developmental stages showed that deletion of epsins caused defective embryonic angiogenesis and retarded embryo development. In vitro angiogenesis assays using isolated primary endothelial cells (ECs) from Epn1\(^{+/}\); Epn2\(^{-/-}\); iCDH5 Cre (EC-iDKO) and Epn1\(^{+/}\); Epn2\(^{-/-}\); Flk\(^{+/}\); iCDH5 Cre (EC-iDKO-Flk\(^{+/}\)) mice demonstrated that VEGFR2 reduction in epsin-depleted cells was sufficient to restore normal VEGF signaling, EC proliferation, EC migration, and EC network formation. These findings were complemented by in vivo wound healing, inflammatory angiogenesis, and tumor angiogenesis assays in which reduction of VEGFR2 was sufficient to rescue abnormal angiogenesis in endothelial epsin-deleted mice.

Conclusions—Our results provide the first genetic demonstration that epsins function specifically to downregulate VEGFR2 by mediating activated VEGFR2 internalization and degradation and that genetic reduction of VEGFR2 level protects against excessive angiogenesis caused by epsin loss. Our findings indicate that epsins may be a potential therapeutic target in conditions in which tightly regulated angiogenesis is crucial, such as in diabetic wound healing and tumors. (Arterioscler Thromb Vasc Biol. 2014;34:331-337.)

Key Words: angiogenesis ■ epsin ■ receptors, vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) signaling is essential for angiogenesis during development and postnatal organ growth, as well as in pathological conditions such as tumor. VEGF binds to its receptor, VEGFR2 (Flk-1; hereafter referred to as Flk), on endothelial cells (ECs), inducing VEGFR2 homodimerization and autophosphorylation at several tyrosine residues to initiate signaling cascades required for EC proliferation and migration. The amount of VEGFR2 on the cell surface controls the magnitude of signal transmission. Its abundance is negatively regulated by internalization and degradation. These tightly regulated processes are vital for the development and maintenance of a normal vascular system. Prolonged VEGF signaling causes vascular leakage and inflammatory responses.

Epsins are a family of endocytic adaptor proteins required for internalization and degradation of ubiquitinated receptors. Mammals express 3 distinct epsin genes (Epn1, Epn2, and Epn3). Although epsin 3 expression is localized primarily to the stomach and epidermis, epsins 1 and 2 are widely expressed and have redundant functions. Specifically, global deletion of epsins 1 and 2 results in embryonic lethality, largely attributable to epsins role in promoting Notch signaling in all cell types. In addition to the endocytic adaptor function, the multivalent properties of epsins facilitate additional functions such as the regulation of GTPases involved in actin remodeling. However, the specific role epsins play in different cell types, or their implications in normal physiology and disease, remains elusive.

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Endothelial-specific deletion of epsins 1 and 2 specifically increases VEGF signaling, but not signaling of other angiogenic factors including fibroblast growth factor, platelet derived growth factor, epidermal growth factor, and transforming growth factor-β. Furthermore, mice lacking endothelial epsins 1 and 2 exhibited excessive tumor angiogenesis with highly disorganized vessels and significantly increased vascular permeability. Epsin interacts with VEGFR2 through its ubiquitin-interacting motifs, leading to the VEGF-dependent downregulation of VEGFR2, thus acting as a negative regulator of VEGF signaling. However, the role of epsins in regulating tumor angiogenesis, in which surrounding tumor cells secrete exaggerated amounts of VEGF, may not represent the role of epsins during embryonic/developmental angiogenic conditions. Furthermore, although our previous data established a correlation between epsin loss and augmented VEGF signaling, presumably attributable to increased VEGFR2 levels in the absence of epsin-mediated internalization and degradation, direct evidence that epsin loss causes augmented VEGFR2 levels is missing. In other words, it remains to be determined whether epsins directly and specifically downregulate VEGFR2 to control developmental and pathological angiogenesis in vivo.

Here, we report that endothelial epsin deletion during development caused defective embryonic angiogenesis and lethality. Using a novel strain of endothelial epsin-deleted mice that are also heterozygous for VEGFR2, we found that reduced VEGFR2 expression in vivo significantly improved the defective wound healing and pathological angiogenesis produced by the loss of endothelial epsins. Furthermore, reducing VEGFR2 expression in primary mouse ECs (MECs) suppressed heightened VEGF signaling and angiogenic responses including EC proliferation and migration. Our findings provide the first direct evidence that endothelial epsins function to control angiogenesis by specifically downregulating VEGFR2 to modulate the VEGF signaling fundamental for developmental or pathological angiogenesis.

Materials and Methods
Materials and methods are available in the online-only Supplement.

Results
Endothelial Epsins Are Essential for Embryonic Angiogenesis
To determine the role of endothelial epsins in regulating angiogenesis, we first engineered mice constitutively lacking endothelial epsins 1 and 2 (EC-DKO) by crossing Epn1fl/fl; Epn2−/− mice with the EC-specific Cre recombinase expressing Tie2 Cre deleter mice (Figure 1B in the online-only Data Supplement). To rule out any undesirable effects of the Cre expression, we also crossed wild-type (WT) and Epn1fl/fl; Epn2−/− mice with the Tie2 Cre deleter mice. These mice exhibited similar phenotypes to WT (data not shown). After several litters in which no EC-DKO pups were born, we used timed mating of the Epn1fl/fl; Epn2−/− and Tie2 Cre deleter mice to determine whether loss of endothelial epsins resulted in embryonic lethality. Similar to previously reported global DKO embryos, E11 EC-DKO embryos were significantly smaller than WT with striking vascular defects (Figure 1A), suggesting that loss of endothelial epsins is a cause of the defective angiogenesis resulting in embryonic lethality. Immunostaining with CD31, a vascular endothelial marker, revealed major vascular developmental defects, including increased vascular density and disorganized vascular networks, in E10 EC-DKO embryos (Figure 1B). Further immunofluorescent staining analyses of cross sections from isolated embryonic midbrain, hindbrain, skin, and intestine revealed much denser and highly disorganized vascular networks in the EC-DKO embryonic tissues compared with WT (Figure 1C–1J; Figure II in the online-only Data Supplement). In addition, detailed analysis of hindbrain cross sections revealed that loss of endothelial epsins promoted formation of a more elaborate subventricular vascular plexus (Figure 1G). Collectively, our findings demonstrated that endothelial epsins are critical for the regulation of embryonic angiogenesis.

Epsins Negatively Regulate VEGF-Induced Angiogenic Responses in ECs
To determine whether epsins regulate embryonic angiogenesis through modulating EC proliferation, migration, or network formation, we used in vitro angiogenesis assays using isolated primary MECs. We measured EC proliferation by culturing WT or DKO MECs with or without VEGF stimulation in the presence of 5-ethynyl-2′-deoxyuridine (EdU), which labels cells actively undergoing S-phase DNA replication. The small VEGF-dependent increase in WT MEC EdU incorporation (Figure 2A) is consistent with previously established roles for VEGF signaling in EC proliferation. Depletion of epsins exaggerated VEGF-dependent proliferation in the DKO MECs (Figure 2A). We next used an in vitro scratch assay and matrigel tube formation assay to measure EC migration and network formation, respectively. In the scratch assay, confluent WT or DKO MEC monolayers were evenly scarred and then stimulated with VEGF to induce migration. MEC migration was determined by measuring scratch width reduction. Unlike the proliferation assay, VEGF stimulation did not affect WT MEC migration but did significantly increase DKO MEC migration (Figure 2B and 2C). Similarly, the in vitro tube formation assay revealed a more robust VEGF-dependent endothelial tube network in DKO MECs (Figure 2D and 2E). In summary, our in vitro functional assays support an antiangiogenic function for epsins in the regulation of angiogenesis through limiting VEGF-dependent EC proliferation, migration, and tube formation.

We also examined whether VEGF can stimulate the phosphorylation of accumulated cell surface VEGFR2 in DKO MECs. Five minutes of VEGF stimulation was sufficient to induce phosphorylation of VEGFR2 in both WT and DKO MECs with heightened VEGFR2 phosphorylation in DKO MECs (Figure 2F). Prolonged stimulation of WT MECs resulted in the loss of both phosphorylated and cell surface VEGFR2, indicating receptor degradation. In contrast, phosphorylated and cell surface VEGFR2 remained elevated in the DKO MECs.
(Figure 2F). To directly visualize the VEGF-induced phosphorylation of VEGFR2, WT or DKO MECs were immunostained using total and phospho-VEGFR2 antibodies. Consistently, VEGF stimulation rapidly induced the phosphorylation of VEGFR2 at the plasma membrane of both WT and DKO MECs (Figure III in the online-only Data Supplement). In WT MECs, 10 minutes of VEGF stimulation resulted in the internalization and colocalization of phosphorylated VEGFR2 with Early Endosome Antigen 1 (EEA1) (Figure III in the online-only Data Supplement). In contrast, a significant portion of the phosphorylated VEGFR2 in DKO MECs remained localized at the plasma membrane and failed to colocalize with EEA1 (Figure III in the online-only Data Supplement). In summary, our biochemical and cell biology approaches revealed that endothelial epsin depletion significantly impaired VEGFR2 internalization and degradation, resulting in prolonged VEGFR2 phosphorylation. It is important, however, to note that the cell surface level of a housekeeping protein, transferrin receptor, did not change in response to epsin depletion, indicating the specificity of epsins for regulating VEGFR2 (Figure 2F).

Figure 1. Aberrant embryonic angiogenesis caused by endothelial epsin deletion. A, Whole-mount E10 wild-type (WT) or constitutive endothelial epsin-deleted (EC–DKO) embryos. B, Telencephalic region of E10 embryos immunostained by CD31. Arrows indicate regions of disorganized vasculature. C, E, and I, CD31 immunostaining of midbrain (C), hindbrain (E), and skin (I) of E10 WT or EC–DKO embryos. G, CD31 immunostaining of hindbrain cross sections of E10 WT or EC–DKO embryos showing the subventricular vascular plexus. D, F, H, and J, CD31-positive surface area in C, E, G, and I were quantified by SlideBook software. Error bars indicate mean±SEM. n>5; *P<0.05. Scale bars: A, 500 μm; B, 225 μm; C and G; 50 μm; E and I, 100 μm.

Genetic Reduction of VEGFR2 Expression Suppresses Upregulated In Vitro Angiogenesis Caused by Epsin Deficiency

To directly test whether the defective angiogenesis caused by endothelial epsin deletion is a result of heightened VEGFR2 and enhanced VEGF signaling, we crossed Epn1fl/fl; Epn2+/+; Flkfl/+ mice with icDH5 Cre deleter and Flkfl/+ mice (Figure IC–IE in the online-only Data Supplement). The icDH5 Cre deleter mice specifically express Cre in ECs after tamoxifen treatment, resulting in a VEGFR2 heterozygote on an inducible EC-specific epsin 1 and 2 deleted background (EC–iDKO–Flkfl/+). Loss of a single Flk allele was sufficiently reduced VEGFR2 expression by ≈50% in the liver, lungs, and heart extracted from EC–iDKO–Flkfl/+ mice (Figure 3A). It is important to note that postnatal deletion of epsins did not affect quiescent postnatal angiogenesis. Furthermore, to ensure the tamoxifen treatment, the Flkfl/+ genotype, or the Cre expression did not produce undesirable effects, we also created WT; iCDH5 Cre deleter, WT; Flkfl/+, Epn1fl/fl; Epn2+/+; Flkfl/+ and Epn1fl/fl; Epn2+/+; iCDH5 Cre deleter mice, all of which produce phenotypes consistent

Figure 2. Epsin deficiency promotes vascular endothelial growth factor (VEGF)-dependent in vitro angiogenesis. A, Quantification of 5-ethyl-2′-deoxyuridine (EdU) incorporation into wild-type (WT) or epsin-depleted (DKO) mouse endothelial cells (MECs) after labeling in the absence or presence of VEGF-A (50 ng/mL) for 24 hours. B, WT or DKO MEC monolayers were subjected to a scratch assay in the absence or presence of VEGF-A (50 ng/mL) for 12 hours. C, Wound distance in B at 12 hours was quantified using National Institutes of Health ImageJ software. D, WT or DKO MECs were subjected to a tube formation assay by culturing on matrigel for 16 hours in the absence or presence of VEGF-A (50 ng/mL). E, Tube formation in D at 16 hours was quantified as in C.

fied as in C. F. Plasma membrane fractions from WT or DKO MECs stimulated with 50 ng/mL VEGF-A for 0, 5, and 15 minutes were immunoblotted using the indicated antibodies. A, C, and E, Error bars indicate mean±SEM. n>5; *P<0.05. Scale bars: B and D, 50 μm. p-VEGFR2 indicates phosphorylated VEGF receptor 2.
with WT (data not shown). We also isolated and treated MECs with tamoxifen ex vivo, resulting in sufficient epsin 1 deletion and an ～50% reduction of the VEGFR2 in DKO-Flkfl/+ MECs (Figure 3B). We measured EC proliferation via both in vitro EdU incorporation and in vivo 5-bromo-2-deoxyuridine incorporation. In the in vitro study, WT, DKO, or DKO-Flkfl/+ MECs were treated with VEGF in the presence of EdU as described above. Immunofluorescent detection revealed reduced EdU incorporation in the DKO-Flkfl/+ MECs to levels similar to WT MECs. Next, we compared γ downstream activation of phospholipase C gamma (PLCγ) during angiogenesis, revealed by both gross appearance and CD31 immunostaining. Scale bars: 100 μm.

Figure 3. Genetic reduction of vascular endothelial growth factor receptor 2 (VEGFR2) expression rescues increased proliferation caused by epsin deletion. A, Liver, lung, and heart tissue isolated from wild-type (WT) mice, tamoxifen-inducible endothelial epsin-deleted mice (EC-iDKO), or tamoxifen-inducible epsin-deleted mice with reduced VEGFR2 (EC-iDKO-Flkfl/+1) analyzed by immunoblot using the specified antibodies. B, Mouse endothelial cells (MECs) isolated from WT, EC-iDKO, or EC-iDKO-Flkfl/+1 mice were treated ex vivo with 4-hydroxytamoxifen for 2 days, and then whole cell lysates were analyzed by immunoblot using the specified antibodies. C, MEC monolayers isolated from WT, EC-iDKO, or EC-iDKO-Flkfl/+1 mice were processed for in vivo CD31 and BrdU immunostaining. Scale bars: C and E, 50 μm.

Genetic Reduction of VEGFR2 Levels Restores Normal In Vivo Angiogenesis During Wound Healing
To test whether genetic reduction of VEGFR2 is sufficient to rescue abnormal angiogenesis in epsin deficiency in vivo, we used a wound healing assay that measures angiogenesis after postnatal dorsal wounds are generated by biopsy punches.26 We found that EC-iDKO mice exhibited disorganized wound vasculature and a significantly delayed wound healing, suggesting defective angiogenesis (Figure 5A–5C). Importantly, the EC-iDKO-Flkfl/+1 mice exhibited a wound healing curve and wound vasculature comparable with that of WT mice (Figure 5B and 5C). In addition, we used a streptozotocin (STZ)–induced diabetic mouse model, in which angiogenesis is greatly compromised, to investigate the effects of epsin deficiency and modulating VEGFR2 expression in wound healing. Compared with STZ-WT mice, STZ-EC-iDKO mice exhibited more rapid wound healing (Figure 5D), suggesting that boosting angiogenesis by depleting epsins plays a beneficial role in promoting diabetic wound healing. These results demonstrated the importance of epsins for balanced VEGFR2 protein levels during angiogenesis and suggested that epsins are a potential novel therapeutic target in treating diabetic ulcer.

Genetic Reduction of VEGFR2 Levels Rescues Defective Angiogenesis Under Pathological Conditions In Vivo
To determine whether VEGFR2 reduction rescues abnormal pathological angiogenesis in the absence of epsins, we first subcutaneously implanted VEGF-containing matrigel plugs in WT, EC-iDKO, or EC-iDKO-Flkfl/+1 mice.7 VEGF-dependent angiogenesis, revealed by both gross appearance and CD31
immunofluorescent staining of the matrigel plugs, was dramatically increased in the EC-iDKO mice, compared with WT (Figure 5E and 5F). Reduced VEGFR2 expression normalized the excessive angiogenic phenotype in EC-iDKO-Flk14 mice to levels comparable with WT (Figure 5F). Aberrant angiogenesis in the EC-iDKO matrigel plugs was reminiscent of our previously established phenotype in which endothelial epsin deletion significantly enhanced the formation of dysfunctional, leaky vasculature in tumors.26 However, it was unknown whether reducing VEGFR2 expression in the tumor is sufficient to rescue this epsin-deleted phenotype. To test this, we used a Lewis lung carcinoma xenograft model in the WT, EC-iDKO, and EC-iDKO-FlkFl/+ mice. Consistent with our previous findings, epsin deletion significantly reduced tumor growth (Figure 5G and 5H) as a result of the enhanced formation of nonproductive vasculature within the tumor (Figure 5I and 5J). Interestingly, reduced VEGFR2 expression promoted tumor growth in the EC-iDKO-FlkFl/+ mice to sizes comparable with WT (Figure 5G and 5H). Furthermore, the increased tumor growth correlated with normalized vasculature in the tumor (Figure 5I and 5J). Cumulatively, these findings support our model that endothelial epsin deletion results in aberrant embryonic and pathological angiogenesis by prolonging activation of VEGFR2. These results indicated that endothelial epsins specifically downregulate VEGFR2 as a negative feedback regulator for VEGFR2 activation and angiogenesis.

**Discussion**

In this study, we demonstrate a role for endothelial epsins as negative regulators to limit unwanted embryonic angiogenesis. Most importantly, by crossing endothelial epsin-deleted mice with mice heterozygous for VEGFR2, we provided the
first direct evidence that endothelial epsin deficiency promotes angiogenesis by specifically increasing the total amount of cell surface VEGFR2 during angiogenesis (Figure 6A).

We used complementary constitutive and inducible endothelial epsin deletion models to address the specific and temporal role of endothelial epsins. First, using the constitutive Tie2 Cre deleter mice allowed us to identify, for the first time, an important role for endothelial epsins in embryonic angiogenesis. Importantly, the Tie2 Cre deleter mice exhibited delayed lethality and reduced phenotype severity than global epsins 1 and 2 deletion, consistent with the hypothesis that EC-specific epsin deletion specifically altered VEGFR2 signaling and developmental angiogenesis. Although the Tie2 Cre deleter mice also express Cre in hematopoietic cells, we did not observe any gross defects in blood cells, suggesting epsin deletion in hematopoietic cells did not contribute to defective embryonic angiogenesis and lethality. Second, the inducible iCDH5 Cre deleter mice allowed us to study a specific role of endothelial epsins in regulating VEGFR2 in adult mice. Importantly, postnatal epsin deletion does not affect quiescent angiogenesis.

Our findings support a model in which inhibiting activated VEGFR2 internalization and degradation through loss of epsins accumulates VEGFR2 on cell surface, prolongs VEGF signaling, and promotes angiogenesis. Reduction of VEGFR2 by genetic means suppressed the enhanced VEGF signaling and restored normal angiogenesis. Although receptor internalization is a recognized mechanism of signal modification, it is still unclear whether VEGFR2 internalization promotes or hinders VEGF signaling to modulate angiogenesis. Impaired internalization or intracellular trafficking of phosphorylated VEGFR2 reportedly inhibited certain intracellular signals, such as Ras homology gene family of GTPases, member A (RhoA) activation, but enhanced others, such as Akt activation. For example, loss of the endocytic adaptor protein, Dab2, impaired VEGFR2 internalization resulting in reduced downstream signaling to ERK and impaired neovascular angiogenesis. Similarly, depletion of the class II PI3K, C2r, impaired VEGFR2 intracellular trafficking resulting in impaired RhoA activation and formation of endothelial focal adhesions. Given the implicated roles of class II PI3K and Dab2 in receptor endocytosis and trafficking to the endosomes, it is tempting to speculate that impaired internalization of phosphorylated VEGFR2 prevents VEGFR2 traveling to or affects the formation of signaling endosomes responsible for signal enhancement. In contrast, epsins interacted with ubiquitinated VEGFR2, presumably after prolonged activation, and, as suggested by our findings, targeted ubiquitinated VEGFR2 for lysosomal degradation, thus effectively diminishing VEGF signaling. Loss of epsins prevented targeted VEGFR2 degradation, which may, in turn, have promoted VEGFR2 recycling and prolonged signaling, resulting in enhanced EC proliferation, migration, and network formation responsible for aberrant angiogenesis. However, we cannot discount that, through impaired internalization of phosphorylated VEGFR2, epsins may also be inhibiting RhoA activation resulting in impaired focal adhesion formation in vivo, thus contributing to our leaky vascular phenotype. Our findings, in combination with those from Dr Takuwa’s group, strongly suggest that tight modulation of VEGF signaling through VEGFR2 internalization and differential downstream signal regulation is necessary to ensure proper angiogenesis.

Our previous findings support epsins as potential anticancer therapeutic targets that promoted aberrant tumor angiogenesis and retarded tumor growth. We speculated that altering epsins may prove favorable in angiogenic-dependent conditions such as wound healing. Although we demonstrated that endothelial epsin deletion impaired, rather than promoted, wound healing under normal conditions, interestingly, in conditions in which angiogenesis is compromised, such as diabetic ulcers, we found that epsin deletion promoted wound healing in STZ-induced diabetic models, suggesting that modulating endothelial epsins may be beneficial. Cumulatively, these findings establish a role for epsins in developmental and pathological angiogenesis and further fortify that epsins modulate VEGF signaling by specifically downregulating VEGFR2 levels.

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Disclosures

None.

References


Significance

Vascular endothelial growth factor (VEGF) signaling is essential for physiological and pathological angiogenesis. VEGF activates its receptor, VEGFR2, on endothelial cells to initiate angiogenic signaling cascades. Cell surface VEGFR2 levels control the magnitude of signal transmission. VEGFR2 abundance is negatively regulated by endocytosis and degradation. We found that epsins, a family of endocytic adaptor proteins, function in the regulation of tumor angiogenesis by downregulating VEGF signaling. However, whether epsins regulate embryonic angiogenesis is unclear. Here, we report for the first time an important role for endothelial epsins in embryonic angiogenesis. Furthermore, by engineering a novel genetic model with epsin deletion and reduced VEGFR2 expression in endothelial cells, we provided direct evidence that epsins regulate angiogenesis by specifically modulating total VEGFR2. Our findings suggest that modulating endothelial epsins may be a useful therapeutic strategy in diabetic ulcers and tumors.
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In the article by Tessneer et al, which appeared in the February 2014 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2014;34:331–337. DOI: 10.1161/ATVBAHA.113.302586), figure 6 was incorrect. The corrected figure is below.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/34/2/331.
Materials and Methods

Genetic Reduction of VEGFR2 Rescues Aberrant Angiogenesis Caused by Epsins Deficiency

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Materials and Methods

Antibodies and reagents:
Mouse anti-EEA1 and anti-GAPDH were from Santa Cruz (sc-6415; sc-166545). Rat anti-CD31 was from BD Pharmlingen (550274). Rabbit anti-VEGFR2, anti-phospho-VEGFR2 (pY1175), rabbit anti-PLCγ, rabbit anti-phospho-PLCγ, rabbit anti-Akt, rabbit anti-phospho-Akt, mouse anti-ERK and mouse anti-phospho-ERK antibodies were from Cell Signaling Technology. Rabbit anti-epsin 1 was obtained as previously described.1,2 Secondary antibodies were all obtained from Invitrogen. VEGF-A was from R&D systems (293-VE/CF). EdU was from Invitrogen (A10044). Alexa Fluor 594 Azide was from Invitrogen (C10339). BrdU and 4-hydroxytamoxifen were from Sigma (B5002; H6278). Matrigel was from BD Biosciences (354230). Basic laboratory reagents were all from Sigma.

Generation of the constitutive EC-DKO and conditional EC-iDKO mice:
Epn2−/− mice were obtained as described.3 Conditional Epn1+/− mice were obtained as described (Figure IA in the online Data Supplement).4 Epn1+/− mice were mated with Epn2−/− mice to generate Epn1+/−; Epn2−/− mice. Constitutive endothelial cell-specific DKO (EC-DKO) mice were obtained by crossing Epn1+/−; Epn2−/− mice with Tie2-Cre deleter obtained from Jackson Laboratories (8863) (Figure IB in the online Data Supplement).5 Tamoxifen-inducible endothelial cell-specific DKO mice (EC-iDKO) were obtained by crossing Epn1+/−; Epn2−/− mice with iCDH5 Cre deleter obtained as described (Figure IC in the online Data Supplement).6,7 To induce postnatal deletion, we administered 4-hydroxytamoxifen (150 μg per 30 g of body weight) by i.p. injection into ten-week-old mice. Injections were performed once per day for 5-7 consecutive days, followed by a 5-7 day resting period. All mice were bred on C57BL/6J background.

Generation of the EC-iDKO-Flk/+/− mice:
Flk/+/− mice were obtained from Jackson Laboratories (18977).8 Tamoxifen-inducible endothelial cell-specific epsin DKO-Flk/+/− mice (EC-iDKO- Flk/+/−) were obtained by crossing Epn1+/−; Epn2−/− mice with the Flk/+/− mice generating Epn1+/−;Epn2−/−; Flk/+/− mice (Figure 1D in the online Data Supplement), then subsequently crossing with iCDH5 deleter Cre mice (Figure 1E in the online Data Supplement). Tamoxifen-induced deletion was done as described above. All mice were bred on C57BL/6J background.

Generation of the Streptozotocin (STZ)-induced Diabetic mice:
Male mice, aged 2-3 months, were injected i.p. with 50 mg/kg STZ in citrate buffer, pH 4.5 for 5 consecutive days. One week post-injection, blood glucose was measured by applying tail blood to a glucometer as described previously.9 Mice with blood glucose levels >300 mg/dL were considered diabetic and used for the wound healing assays.

Primary Mouse Endothelial Cell Isolation and Culture:
Primary mouse endothelial cells (MEC) isolation from brains was performed as described previously.10 Specifically, meninges of three week-old brains isolated from WT, EC-iDKO or EC-iDKO-Flk/+/− mice were carefully removed and the gray matter minced and then digested with 1 mg/mL collagenase (Gibco; 17100-017) in DMEM (Mediatech; 10-013-CM) for 1 h at 37°C. Microvessels were separated by centrifugation through 20% BSA/DMEM (1000 x g, 20 min). Microvessels from the pellet were further digested with 1 mg/mL collagenase-dispase (Roche) in DMEM for 15 min at 37°C. Microvessel MEC clusters were washed twice with DMEM before planting on 0.2% gelatin-coated plates. Cultures were maintained in DMEM supplemented with 20% FBS (Biowest; S01520HI) and 1 ng/mL bFGF (Roche). MECs were treated for 48 h at 37°C with 5 μM of 4-hydroxytamoxifen (dissolved in ethanol) diluted culture medium followed by incubation for an additional two days without 4-hydroxytamoxifen. Freshly isolated primary MEC were used for all experiments without further passages.

Immunohistochemistry and immunofluorescence:
Embryos, tissue samples, and ECs were processed for immunostaining as described below. All samples were imaged using an Olympus IX81 Spinning Disc Confocal Microscope and Hamamatsu Orca-R2 Monochrome Digital Camera C1D600. Immunostaining was quantified using the companion Slidebook 5.0 software.\textsuperscript{4,11}

**Embryo Whole Mount Staining:** Embryos were harvested at E10 and fixed overnight in cold 4\% paraformaldehyde in PBS. After washing with ice-cold PBS, embryos were whole-mounted or paraffin embedded, sectioned, and processed for staining.\textsuperscript{1,12} For blocking and permeabilization, sections were incubated in PBS containing 1\% BSA, 0.5\% Triton X-100 overnight at 4\(^\circ\)C. Samples were stained with anti-CD31 overnight at 4\(^\circ\)C. Biotinylated goat anti-rat IgG was absorbed to remove anti-murine IgG, and then streptavidin-conjugated to fluorescein isothiocyanate was used for secondary staining. Samples were mounted with Vectashield and analyzed as described above.

**E10 Hind Brain:** Embryos were harvested at E10 and fixed in cold 4\% paraformaldehyde. Hindbrain was harvested and processed for immunofluorescent staining. For blocking and permeabilization, sections were incubated in PBS containing 1\% BSA, 0.5\% Triton X-100 overnight at 4\(^\circ\)C. Samples were stained with anti-CD31 overnight at 4\(^\circ\)C. After washing, donkey anti-rat Alex Fluor 488 secondary antibody was used for secondary staining. Samples were mounted and analyzed as described above.

**Embryonic Skin:** Embryos were harvested at E10 and fixed in 4\% paraformaldehyde. Skin was harvested and processed for staining with anti-CD31 and donkey anti-rat Alex Fluor 488 secondary antibody as described above.

**Embryonic Intestine:** Embryos were harvested at E10 and fixed in 4\% paraformaldehyde. Small intestines was harvested and processed for staining with anti-CD31 and donkey anti-rat Alex Fluor 488 secondary antibody as described above.

**In vitro EC staining:** ECs cultured on gelatin-coated coverslips were stimulated with 50 ng/mL of VEGF-A at 37\(^\circ\)C for 0, 1, or 5 min. Cells were fixed in 4\% paraformaldehyde then permeabilized and blocked in PBS containing 5\% donkey serum, 3\% BSA and 0.3\% Triton X-100. Cells were immunostained with rabbit anti-VEGFR2, rabbit anti-phosphorylated VEGFR2, and mouse anti-EEA1 for 2 h at room temperature. Cells were washed then incubated with respective fluorescent secondary antibodies for 1 h at room temperature. Cells were mounted using PermaFluor (ThermoScientific; TA-030-FM) and analyzed as described above.

**In vitro EdU staining:** Primary ECs were grown on gelatin-coated coverslips until they reached 50\% confluence. EdU was added to the culture media at 10 \(\mu\)M for 16 h.\textsuperscript{13} After labeling, cells were washed with PBS, fixed in 4\% paraformaldehyde and permeabilized in 0.1\% Triton X-100. EdU incorporation was detected by incubating cells with 100 \(\mu\)M fluorescent azide diluted in 100 mM Tris, 0.5 mM CuSO\(_4\) and 50 mM ascorbic acid. Cells were counterstained with 0.2 \(\mu\)g/mL DAPI then processed for imaging as described above.

**Scratch and network/tube formation assays:**

**Monolayer EC scratch assay:** Monolayer EC wound assays were performed as described.\textsuperscript{10} ECs were cultured in 0.5\% FBS/DMEM overnight and subjected to “wound injury” assay with a plastic pipette tip. Cells were then plated with fresh media supplemented with or without 50 ng/mL VEGF-A and further cultured for 12 h. Quantification of wound distance at 12 h was performed using NIH Image J software.
EC network/tube formation. EC network/tube formation in Matrigel was performed as described.  

First, 0.2 mL matrigel was added to each well of 24-well plate and incubated at 37°C for 1 h. ECs were serum starved overnight then plated on Matrigel (2 x 10^3 cells). After 30 min, fresh medium supplemented with or without 50 ng/mL VEGF-A was added and cells were incubated for additional 16 h. Quantification of tube formation at 16 h was performed using NIH ImageJ software.

Plasmal Membrane isolation from MECs:
Isolated and cultured MECs were stimulated with 50 ng/mL of VEGF-A at 37°C for the time indicated followed by homogenization in TES Buffer (255 mM TES, 10 mM NaF, 10 mM Na_4P_2O_7, 2 mM Na_3VO_4) and centrifuged at 16,000 x g for 15 min. Pellets were further homogenized in TES Buffer, layered on a 1.15 M sucrose cushion, and centrifuged at 100,000 x g for 70 min. Plasma membrane interface was isolated and pelleted by centrifugation at 48,000 x g for 45 min. Plasma membrane pellets were resuspended and subjected to SDS-PAGE and immunoblot according to standard protocols.

In vivo BrdU staining:
WT, EC-iDKO or EC-iDKO-FLK^fl/+ pups were injected i.p. with 5 mg/kg (body weight) of 4-hydroxytamoxifen (10 mg/ml of 4-hydroxytamoxifen resuspended in 20% of ethanol and 80% of DMSO) per day from postnatal day 1 (P1) to P3. Pups were euthanized at P6 after i.p. injection of BrdU (100 mg/kg body weight) for 3 hr. Small intestines were harvested. Small intestines were embedded and paraffin sections were stained with anti-CD31 and anti-BrdU antibodies (Invitrogen).

In vivo Wound Healing Assay:
Under anesthesia, mice were wounded using a 4-mm-diameter full-thickness dorsal skin punch biopsy. Wounds were then photographed daily over 9 days after injury, and wound area was measured using NIH ImageJ software. The wounds were biopsied to include the margin of normal surrounding skin and snap-frozen in Tissue-Tek OCT (Sakura) and fixed in cold 4% paraformaldehyde overnight. Cryosections were processed for immunofluorescent staining as described above.

In vivo Matrigel Angiogenesis Assay:
Matrigel plug assay was performed as described previously. Briefly, mice were injected s.c. with 500 μL of un polymerized growth factor-reduced Matrigel mixed with either PBS or VEGF (200 ng/mL). Mice were euthanized after 5 days and the plugs removed. Plugs were photographed, formalin-fixed, paraffin embedded and processed for immunostaining with anti-CD31 as described above.

In vivo Tumor Xenograft Assay:
Lewis Lung Carcinoma (LLC) cells obtained from ATCC were injected s.c. (1x10^5 cells/tumor) in twelve-week-old WT, EC-iDKO, or EC-iDKO-FLK^fl/+ mice. Time of tumor appearance was estimated and tumor growth was monitored in three groups of mice by measuring tumor size with digital calipers. Tumors more than 2 mm in diameter were recognized as positive. Tumor volumes were calculated based on the formula: 0.5326 (length [mm] x width [mm]^2). Tumors were harvested, photographed, paraffin embedded and processed for immunofluorescence.

Miscellaneous Procedures:
SDS/PAGE and immunoblotting were performed according to standard procedures.

Study Approval:
All animal studies were performed in compliance with institutional guidelines and were approved by Institutional Animal Care and Use Committee (IACUC), Oklahoma Medical Research Foundation, Oklahoma City, OK.

Statistical Analysis:
Data was shown as ± S.E.M. Data were analyzed by the two-tailed student’s t test or ANOVA, where appropriate. The Wilcoxon signed-rank test was used to compare data that did not satisfy the student’s t test or ANOVA. P value ≤ 0.05 was considered significant.
References


Supplemental Material

Genetic Reduction of VEGFR2 Rescues Aberrant Angiogenesis Caused by Epsin Deficiency

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Supplemental Figure Legend

**Supplemental Figure I. Generation of EC-DKO, EC-iDKO and EC-iDKO-Flk^{fl/+} mice.**

A, Diagram shows homologous recombination of the floxed gene-targeting vector at the *Epn1* locus. B, Strategy to generate constitutive endothelial cell-specific epsin DKO (EC-DKO) mice by crossing *Epn1^{fl/fl}; Epn2^{-/-}* mice with *Tie2 Cre deleter* mice. C, Strategy to generate tamoxifen inducible endothelial cell-specific epsin DKO (EC-iDKO) mice by crossing *Epn1^{fl/fl}; Epn2^{-/-}*; *Flk^{fl/+}* mice with *iCDH5 Cre deleter* mice. D, Strategy to generate *Epn1^{fl/fl}; Epn2^{-/-}; Flk^{fl/+}* mice by crossing *Epn1^{fl/fl}; Epn2^{-/-}* mice with *Flk^{fl/+}* mice. E, Strategy to generate inducible endothelial cell-specific epsin DKO, VEGFR2 heterozygous (EC-DKO-Flk^{fl/+}) mice by crossing the *Epn1^{fl/fl}; Epn2^{-/-}; Flk^{fl/+}* mice with *iCDH5 Cre deleter* mice.

**Supplemental Figure II. Epsin deficiency promotes VEGF-dependent *in vivo* angiogenesis.**

A, CD31 immunostaining of intestine of E18 WT or EC-iDKO embryos. Scale bars: 100 μm. B, CD31-positive surface area in A was quantified by SlideBook software. Error bars indicate the mean ± s.e.m. n > 5, *p < 0.05.

**Supplemental Figure III. Epsin deficiency impairs internalization and colocalization of phosphorylated VEGFR2 to EEA1 endosomes.**

WT or DKO MECs stimulated with 50 ng/mL VEGF-A for 0, 1 and 10 min were fixed and stained using the specified antibodies. Scale bar: 10 μm.

**Supplemental Figure IV. Genetic reduction of VEGFR2 expression rescues proliferation caused by epsin deletion.**

Quantification of *in vivo* BrdU incorporation in intestines isolated from BrdU injected WT, EC-iDKO, or EC-iDKO-Flk^{fl/+} mice shown in Figure 3E. Error bars indicate the mean ± s.e.m. n > 5, *p < 0.05.

**Supplemental Figure V. Genetic reduction of VEGFR2 expression rescues *in vitro* angiogenesis caused by epsin deletion.**

Quantification of specified immunoblots of whole cell lysates from WT, DKO or DKO-Flk^{fl/+} MECs stimulated with 50 ng/mL VEGF-A shown in Figure 4A. Error bars indicate the mean ± s.e.m. n > 5, *p < 0.05.
Supplemental Figure I
Supplemental Figure II

Intestine

CD31

A

WT  EC-iDKO

B

CD31 area (mm²)

0.4

0.3

0.2

0.1

0

Supplemental Figure II
Supplemental Figure III
Supplemental Figure IV

BrdU labelled cells

Fold Change

WT

EC-iDKO

EC-iDKO-Fik

*
Supplemental Figure V

VEGFR2 level (AU)

VEGF (min)

WT  DKO  DKO-Flk

0  0  0

5  5  5

*  *  *

Supplemental Figure V