Pathological Neovascularization Is Reduced by Inactivation of ADAM17 in Endothelial Cells but Not in Pericytes

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Rationale: Pathological neovascularization is a critical component of diseases such as proliferative retinopathies, cancer and rheumatoid arthritis, yet much remains to be learned about the underlying causes. Previous studies showed that vascular endothelial growth factor (VEGF)-A activates the membrane-anchored metalloproteinase ADAM17 (a disintegrin and metalloproteinase 17) in endothelial cells, thereby stimulating crosstalk between VEGF receptor 2 and extracellular signal-regulated kinase. These findings raised interesting questions about the role of ADAM17 in angiogenesis and neovascularization in vivo.

Objective: The objective of this study was to inactivate ADAM17 in endothelial cells or in pericytes to determine how this affects developmental angiogenesis, pathological retinal neovascularization and heterotopic tumor growth.

Methods and Results: We generated animals in which floxed ADAM17 was removed by Tie2-Cre in endothelial cells, or by smooth muscle (sm) Cre in smooth muscle cells and pericytes. There were no evident developmental defects in either conditional knockout strain, but pathological retinal neovascularization and growth of heterotopically injected tumor cells was reduced in Adam17flox/flox/Tie2-Cre mice, although not in Adam17flox/flox/sm-Cre mice. Moreover, lack of ADAM17 in endothelial cells decreased ex vivo chord formation, and this could be largely restored by addition of the ADAM17 substrate HB-EGF (heparin-binding epidermal growth factor–like growth factor). Finally we found that ADAM17 is important for the VEGF receptor 2 stimulated processing of several receptors with known functions in endothelial cell biology.

Conclusions: These results provide the first evidence for a role for ADAM17 in pathological neovascularization in vivo. Because ADAM17 does not appear to be required for normal developmental angiogenesis or vascular homeostasis, it could emerge as a good target for treatment of pathological neovascularization. (Circ Res. 2010; 106:932-940.)

Key Words: ADAMs ■ metalloproteinase-disintegrins ■ TNFα-convertase ■ proliferative retinopathy ■ pathological neovascularization

Pathological neovascularization has a critical role in diseases such as cancer,1,2 rheumatoid arthritis3 and proliferative retinopathies, including retinopathy of prematurity, diabetic retinopathy and the wet form of macular degeneration.4,5 Therefore molecules with roles in pathological neovascularization are considered potential targets for treatment of these conditions. Previous studies have identified a role for the cell surface metalloproteinase ADAM17 (a disintegrin and metalloproteinase 17, also referred to as TACE [tumor necrosis factor α–converting enzyme]) in crosstalk between the VEGFR2 and extracellular signal-regulated kinase 1/2 in endothelial cells, and in processing several receptors with key functions in angiogenesis, including the VEGFR2 and Tie2.6 The goal of the present study was to determine whether ADAM17 has a role in angiogenesis or pathological neovascularization in vivo by subjecting conditional knockout mice carrying floxed alleles of ADAM17 and a Cre-recombinase expressed either in endothelial cells (Tie2-Cre) or in smooth muscle cells and pericytes (α-smooth muscle actin [α-sm] Cre) to mouse models of pathological neovascularization.

ADAM17 was first discovered as the converting enzyme for tumor necrosis factor (TNF) α,8,9 a potent proinflammatory cytokine that is a causative factor in autoimmune diseases such as rheumatoid arthritis and Crohn’s disease as well as in septic shock in mice.10 Once mice lacking ADAM17 were generated, it became clear that ADAM17 is

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also critical for epidermal growth factor (EGF) receptor (EGFR) signaling, via the proteolytic release of several ligands of the EGFR. Mice lacking ADAM17 die shortly after birth with defects resembling those in animals lacking transforming growth factor α (wavy whiskers and open eyes), HB-EGF (thickened and misshapen heart valves), or the EGFR. Moreover, ADAM17-dependent shedding of several of its substrates, including EGFR-ligands, can be stimulated by VEGF-A in endothelial cells. The activation of ADAM17 by VEGF-A is responsible for crosstalk between the VEGFR2 and extracellular signal-regulated kinase 1/2, most likely because EGFR-ligands shed from VEGF-A-stimulated endothelial cells activate the EGFR.

The ability of ADAM17 to release endothelial cell membrane proteins on stimulation with VEGF-A raised questions about what role ADAM17 has during developmental angiogenesis and in pathological neovascularization in adult animals. Although mice lacking ADAM17 die perinatally, most likely as a consequence of their severe heart valve defects, there have been no reports of defects in developmental angiogenesis in these animals. To address whether ADAM17 has a role in angiogenesis or pathological neovascularization or both, we conditionally inactivated ADAM17 in endothelial cells or in smooth muscle cells such as pericytes, and then determined how lack of ADAM17 affects two mouse models for pathological neovascularization, the oxygen induced retinopathy model for retinopathy of prematurity, and growth of heterotopically injected tumor cells. Moreover, we assessed proliferation and chord formation of endothelial cells lacking ADAM17, and evaluated the role of ADAM17 in the proteolytic release of membrane proteins with known roles in angiogenesis and pathological neovascularization.

Methods

Reagents and Cell Lines

Porcine aortic endothelial cells expressing VEGFR2/KDR (PAE/KDR cells) and mouse embryonic fibroblasts (mEFs) lacking ADAM17 have been described previously. Reagents were from Sigma, unless indicated otherwise. VEGF-A and HB-EGF were from R&D Systems, and antibodies against platelet endothelial cell adhesion molecule (PECAM), NG2, endothelial NO synthase, and smooth muscle were from BD Pharmingen. 

Mouse Lines

To generate mice lacking ADAM17 in endothelial cells, we crossed Adam17flox/flox with Tie2-Cre mice (kindly provided by Dr. Tom Sato) or sm-Cre mice (Jackson labs; Tg(TagIn-cre)1Her/J). Expression of Cre was monitored using Rosa26-Lac-Z reporter (R26R) mice (Jackson labs; B6.129S4-Gt(Rosa)26Sortm1Sor/J).

Oxygen-Induced Retinopathy, Heterotopic Tumor Injection, and Evaluation of Retinal Vascular Development

The analysis of postnatal retinal vascular development, the oxygen-induced retinopathy model and heterotopic injection of B16F0 melanoma cells have been described elsewhere. For details, see the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Shedding Assays

Protein ectodomain shedding assays using alkaline phosphatase (AP)-tagged substrates in mouse embryonic fibroblasts and PAE/KDR cells were performed as described.

Endothelial Cell Assays

Primary endothelial cells from lungs and hearts of 9 to 12-day-old mice were prepared as described. Proliferation of primary endothelial cells was measured with the Celltiter proliferation assay from Promega. In vitro endothelial cell chord formation was performed using a kit from Cell Biolabs Inc (San Diego, Calif).

Immunofluorescence, Western Blot, and Fluorescence-Activated Cell-Sorting Analysis

Immunofluorescence analysis for PECAM, isoelectin B4, NG2 and osma, Western blot analysis of retina extracts and fluorescence-activated cell-sorting (FACS) analysis was performed as described.

Results

Characterization of Adam17flox/flox/Tie2-Cre Mice

To assess whether ADAM17 has a role in pathological neovascularization, we generated mice carrying floxed alleles of ADAM17 and the Cre-recombinase expressed in endothelial cells under the Tie-2 promoter (see Methods for details). Matings of Adam17flox/flox/Tie2-Cre with Adam17flox/flox mice gave rise to offspring of the expected Mendelian ratio (48% Adam17flox/flox, 52% Adam17flox/flox/Tie2-Cre, n = 327). The efficient excision of ADAM17 in endothelial cells isolated from Adam17flox/flox/Tie2-Cre mice was confirmed by Western blot analysis (Figure 1A). Adam17flox/flox/Tie2-Cre mice appeared normal during routine handling, and a complete necropsy and histopathologic evaluation did not uncover any evident defects compared to littermate controls (Adam17flox/flox) (see Methods). Moreover, stain-
Conditional Inactivation of ADAM17 in Endothelial Cells Reduces Oxygen-Induced Retinopathy

To assess whether ADAM17 contributes to pathological retinal neovascularization, we subjected Adam17flox/flox/Tie2-Cre mice and Adam17flox/flox littermate controls to a model for retinopathy of prematurity, the oxygen induced retinopathy (OIR) model, (see Methods). At the completion of the OIR experiment at day p17, we found a significantly larger central avascular area in Adam17flox/flox/Tie2-Cre mice compared to controls (Figure 2A and 2B). Moreover, there was a significant decrease in the number of endothelial cells that traversed the internal limiting membrane toward the vitreous body in Adam17flox/flox/Tie2-Cre mice compared to controls (Figure 2C). X-gal staining of retinas from Adam17flox/flox/Tie2-Cre/R26R mice corroborated that Tie2-Cre was effective in endothelial cells throughout the retinal vasculature (Online Figure III, A) and in pathological neovascular tufts (Online Figure III, B). When we subjected mice carrying one wild type and one floxed allele of ADAM17 in the presence or absence of Tie2-Cre to the OIR model (Adam17flox/+Tie2-Cre mice or Adam17flox/+ controls), we found that Tie2-Cre did not significantly affect the outcome of this model when ADAM17 was present. This control corroborates that the decreased response of Adam17flox/flox/Tie2-Cre mice to the OIR model is attributable to deletion of floxed ADAM17, but not the expression of Tie2-Cre (Online Figure III, C). An immunofluorescence analysis of the expression of the endothelial cell marker isoelectin B4 or the pericyte marker NG2 in pathological neovascular tufts showed a similar staining pattern in the tufts that developed in Adam17flox/flox/Tie2-Cre mice compared to Adam17flox/flox controls (Online Figure III, D). Finally, a Western blot analysis of retina extracts from wild type or Adam17flox/flox/Tie2-Cre mice subjected to the OIR model showed expression of ADAM17 at all stages after return to room air at P12 in wild type mice (Online Figure III, E), and comparable expression at P12, 14 and 17 in Adam17flox/flox/Tie2-Cre mice (Online Figure III, F), so deletion of the widely expressed ADAM17 in endothelial cells does not noticeably alter ADAM17 levels in extracts of whole retinas.

Heterotopic Tumor Injection Model

Because the results of the OIR model suggested that ADAM17 in endothelial cells has a role in pathological neovascularization, we subjected Adam17flox/flox/Tie2-Cre/R26R or Adam17flox+/Tie2-Cre/R26R. No difference in the distribution of X-gal–stained cells in the presence or absence of ADAM17 was observed (Online Figure II). Moreover, the presence or absence of Tie2-Cre in Adam17flox/flox mice also did not affect the development of the retinal vascular tree with respect to its size relative to that of the retina as well as the appearance of the vessels at postnatal day 6 (Figure 1B through 1D). Thus conditional inactivation of ADAM17 in endothelial cells did not lead to evident defects in mouse development or adult homeostasis, or in the development of the retinal vasculature.

Figure 1. Evaluation of developmental retinal angiogenesis in Adam17flox/flox/Tie2-Cre mice. A, Western blot analysis shows a strong reduction of ADAM17 protein levels in primary endothelial cells isolated from an Adam17flox/flox/Tie2-Cre mouse compared to an Adam17flox/flox littermate control, with tubulin serving as a loading control (see Methods). B and C, Whole-mount isoelectin B4 staining of the developing retinal vasculature of 6-day-old Adam17flox/flox/Tie2-Cre and Adam17flox/flox littermates. C shows higher magnification photomicrographs of the density of the vascular web (top images) and the leading edge of the vascular tree (lower images). D, Ratio of the surface area of the developing vascular tree over that of the retina showed no significant difference (Wilcoxon–Mann–Whitney [WMW] test, P=0.76) in the extent of developmental retinal angiogenesis in Adam17flox/flox/Tie2-Cre mice (n=11) compared to Adam17flox/flox controls (n=11).
mice and Adam17flox/flox controls to a heterotopic tumor injection model, which provides information on the contribution of host-derived factors and cells, such as endothelial cells, to tumor growth. After subcutaneous injection of B16F0 melanoma cells, tumor growth was monitored for two to three weeks. In three separate experiments, tumor growth was significantly reduced in Adam17flox/flox/Tie2-Cre mice compared to controls (Figure 3A). Sections of tumors from Adam17flox/flox/Tie2-Cre mice and controls did not display significant differences in the distribution or appearance of PECAM-stained tumor vessels (Figure 3B and 3C). When we compared heterotopic tumor growth in mice with one wild type allele of ADAM17 in the presence or absence of Tie2-Cre (Adam17flox/+Tie2-Cre or Adam17flox/+ mice), we found no difference in tumor growth, arguing against an effect of the Tie2-Cre alone on this heterotopic tumor model (Online Figure IV). These experiments are consistent with a role for ADAM17 in pathological neovascularization or in generation of host-derived factors from endothelial cells that contribute to tumor growth.

Inactivation of ADAM17 in αSMA-Expressing Cells Does Not Detectably Affect Pathological Neovascularization

Pericytes represent another major cell type in the vasculature besides endothelial cells. To determine whether ADAM17 in pericytes is important for angiogenesis or pathological neovascularization, we generated mice carrying floxed ADAM17 and a Cre-recombinase expressed under the control of the αsma promoter (Adam17flox/flox/αsma-Cre mice). The Adam17flox/flox/αsma-Cre animals were born at the expected Mendelian ratio (52% Adam17flox/flox/αsma-Cre, 48% Adam17flox/flox, n=101) and developed normally, with no evident pathological changes compared to littermate Adam17flox/flox controls (see Methods). A Western blot analysis of vascular smooth muscle cells cultured from aortae of Adam17flox/flox/αsma-Cre mice and Adam17flox/flox controls by the explant method (see Methods) corroborated the efficient excision of floxed ADAM17 by αsma-Cre in vascular smooth muscle cells (Online Figure V). Moreover, an analysis of several tissues and organs containing αsma-expressing cells (aorta, heart, small intestine) did not uncover any evident defects in Adam17flox/flox/αsma-Cre mice compared to Adam17flox/flox controls (Online Figure VI, A). To determine whether the presence or absence of ADAM17 affected the distribution of αsma-Cre expressing cells, we performed X-gal staining on sections of aortae and hearts of mice carrying αsma-Cre and the Rosa26 lac-Z reporter in the presence of either one or both floxed alleles of ADAM17 (Adam17flox/flox/αsma-Cre/R26R or Adam17flox/+/αsma-Cre/ R26R). No difference in the distribution of X-gal stained cells in the presence or absence of ADAM17 was observed (Online Figure VI, B). In addition, there was no difference in the development of the retinal vascular tree at P6 in Adam17flox/flox mice in the presence or absence of αsma-Cre (Online Figure VII, A and B). After exposure to the OIR model, the size of the central avascular area (Online Figure VIII, A and B) and the number of endothelial cells that crossed the internal limiting membrane were comparable between Adam17flox/flox mice and Adam17flox/flox controls (Online Figure VIII, C; note that because of the relatively low numbers of endothelial cells in Adam17flox/flox controls, we cannot rule out subtle effects of the lack of ADAM17 in αsma-expressing cells in the OIR model). X-gal staining of retinal sections from αsma-Cre/R26R mice corroborated the expression of αsma-Cre in neovascular tufts (Online Figure VIII, D). Finally, heterotopically injected B16F0 melanoma cells gave rise to tumors of similar weight in Adam17flox/flox/αsma-Cre mice and Adam17flox/flox controls (Online Figure IX). Thus, we found no evidence for a contribution of ADAM17 in
ADAM17 Has a Role in Chord Formation of Endothelial Cells

To explore the contribution of ADAM17 to ex vivo endothelial cell assays, we isolated endothelial cells from Adam17flox/flox/Tie2-Cre mice and Adam17flox/flox controls, and assessed proliferation and chord formation in the presence or absence of VEGF-A or HB-EGF. We found no difference in proliferation of Adam17flox/flox/Tie2-Cre endothelial cells compared to controls (Figure 4A). However, there was a substantial decrease in chord formation in endothelial cells from Adam17flox/flox/Tie2-Cre mice that were treated with or without VEGF-A compared to controls (Figure 4B). This defect in chord formation in Adam17flox/flox/Tie2-Cre endothelial cells could be largely rescued by addition of soluble HB-EGF, an EGFR-ligand that is a substrate of ADAM17 (Figure 4B).

ADAM17 Is Involved in Shedding a Variety of Membrane Proteins With Roles in Angiogenesis and Neovascularization

Previous studies have implicated ADAM17 in the proteolytic release of several membrane-anchored proteins, including molecules with known roles in angiogenesis, such as VEGF-A, ICAM-1, Tie2, and CD40.6 To test whether ADAM17 is involved in the shedding of additional membrane-proteins with known functions in endothelial cell biology, we transfected AP-tagged VE-cadherin, vascular cell adhesion molecule (V-CAM), EphB4, extracellular matrix metalloproteinase inducer (EMMPRIN), insulin-like growth factor receptor (IGFRI), or PECAM into wild-type or Adam17−/− mouse embryonic fibroblasts (MEFs). We found a phorbol 12-myristate 13-acetate (PMA)-dependent increase in the shedding of the ectodomains of these membrane proteins in wild type MEFs, which could be prevented by incubation with the hydroxamic acid-type metalloproteinase inhibitor marimastat (Figure 5A). The PMA-stimulated component for each of these substrates was abolished in Adam17−/− MEFs, and for VE-cadherin, V-CAM and EMMPRIN, constitutive shedding was also reduced. Moreover, we found that shedding of the Adam17 substrates VE-cadherin, V-CAM, EphB4, EMMPRIN, IGFRI or PECAM from pig aortic endothelial cells expressing the VEGF-R (PAE-KDR cells) was stimulated by addition of VEGF-A, whereas shedding of the ADAM10 substrates EGF and betacellulin was not (Figure 5B). Finally, FACS analysis showed an approximately 40% increase in PECAM on the surface of endothelial cells from Adam17flox/flox/Tie2-Cre mice compared to Adam17flox/flox controls (Figure 5C). This was further corroborated by Western blot analysis of the sorted cells, where increased levels of PECAM and Tie2 correlated with strongly decreased ADAM17 in Adam17flox/flox/Tie2-Cre endothelial cells compared to Adam17flox/flox controls (Figure 5D). These results confirm that ADAM17 regulates the levels of endogenous PECAM and Tie2 in primary endothelial cells.

Discussion

The main objective of this study was to evaluate the role of the membrane-anchored metalloproteinase ADAM17 in angiogenesis and pathological neovascularization. We found that inactivation of ADAM17 in endothelial cells had no evident effect on developmental angiogenesis, whereas it significantly reduced pathological neovascularization in a mouse model for retinopathy of prematurity, and affected the growth of heterotopically injected tumor cells. Moreover, chord formation in ADAM17-deficient endothelial cells was strongly reduced compared to controls, and could be partially rescued by addition of the EGFR-ligand and ADAM17 substrate HB-EGF, which is expressed on endothelial cells.12,15,21 On the other hand, inactivation of ADAM17 in a sma-expressing cells had no evident effect on retinal angiogenesis, the outcome of the OIR model or on the growth of heterotopically injected tumor cells.
ADAM17 is able to process several receptors with important functions in endothelial cells, and so defects in endothelial cells isolated from Adam17lox/lox/Tie2-Cre mice or Adam17lox/lox controls (see Methods). There was no significant difference in proliferation at day 4 (d4) of the culture plated at comparable density on day 0 (d0) (A), but a clear decrease in chord formation was seen in endothelial cells from Adam17lox/lox/Tie2-Cre mice compared to controls (B), which could be largely rescued by treatment with 5 ng/mL HB-EGF, but not with 5 ng/mL VEGF-A.

The observation that the inactivation of ADAM17 in endothelial cells reduces pathological neovascularization provides the first direct evidence for a role of this cellular sheddase in endothelial cells in vivo. Moreover, the ability of HB-EGF to largely rescue the decreased chord formation of ADAM17-deficient endothelial cells suggests that the underlying mechanism involves EGFR-signaling stimulated by HB-EGF or related EGFR-ligands released by ADAM17 from endothelial cells. This is consistent with previous studies that have implicated HB-EGF and its proteolytic release in angiogenesis, although the identity of the responsible enzyme was not determined. Moreover, our finding that HB-EGF partially rescues chord formation in ADAM17-deficient endothelial cells, whereas VEGF does not, suggests that HB-EGF affects endothelial cells directly instead of via production of VEGF, as proposed by Hollborn et al.27 The apparently normal pericyte ensheathment of endothelial cells in the absence of ADAM17 suggests that release of HB-EGF by ADAM17 is not essential for this process. However, addition of HB-EGF to ADAM17-deficient endothelial cells did not completely restore chord formation, so other substrates of ADAM17 that are important for its role in neovascularization are likely to exist. Indeed, we also show that ADAM17 is able to process several receptors with important functions in endothelial cells, and that their shedding can be activated by VEGF (also see elsewhere6), and so defects in the processing of one or more additional substrates of ADAM17 could also be partially responsible for its role in neovascularization. In principle, processing could inactivate membrane receptors, although ectodomain shedding can also activate receptors such as Notch.28

Given the substantial number of currently known substrates for ADAM17 and the dearth of information on how shedding affects the function of individual receptors, it is currently not possible to predict which additional shedding events besides the release of HB-EGF account for the role of ADAM17 in neovascularization. To address this question, it will be necessary to study how the processing of individual receptors affects their function in cell-based assays and in vivo, for example by “knocking in” mutations that inactivate their cleavage site. Nevertheless, the ability of HB-EGF to largely rescue chord formation in ADAM17-deficient endothelial cells suggests that activation of the EGFR is an important component of the mechanism underlying the role of ADAM17 in neovascularization. So, even though ADAM17 can, in principle, process many membrane proteins on the surface of endothelial cells, our results suggest that HB-EGF and possibly also other EGFR-ligands that are shed by ADAM17 are likely to be the functionally dominant substrates of ADAM17 in the context of pathological neovascularization. Perhaps the increase in surface levels of membrane proteins such as Tie2 and PECAM in ADAM17-deficient endothelial cells is less relevant to angiogenesis and neovascularization than the regulation of the bio-availability of EGFR-ligands, which is also the functionally dominant activity of ADAM17 during mouse development. ADAM17 has also been implicated in processing Notch. However, mice lacking Notch1 and 4 die very early during embryogenesis, and ADAM10-deficient mice resemble mice that lack Notch1 and 4, whereas mice lacking ADAM17 die at birth. Therefore ADAM17 does not appear to be essential for activating Notch during mouse development. Finally, it should be noted that ADAMs are modular proteins that also contain a disintegrin domain, cysteine-rich region and a cytoplasmic domain, so it is conceivable that functions of these ancillary domains that are not related to the catalytic activity of ADAM17 could also be important for its role in pathological neovascularization.13,14

Taken together, these results suggest that ADAM17 could be an attractive target for treatment of proliferative retinopathies and potentially also for preventing other diseases that depend on pathological neovascularization, such as cancer.
and rheumatoid arthritis. An appealing feature of ADAM17 in the context of pathological neovascularization is that it does not have an evident role in normal developmental angiogenesis or in the maintenance of the vasculature in adult mice. ADAM17 is currently considered as a target for angiogenesis or in the maintenance of the vasculature in adult mice. ADAM17 is currently considered as a target for treatment of rheumatoid arthritis because of its role in generating soluble TNFα, and for treatment of ErbB-dependent tumors, because of its critical role in activating EGFR-ligands. Interestingly, TIMP3, which is tightly associated with ADAM17 in extracts from endothelial cells and inhibits ADAM17 and other metalloproteinases, reduces pathological neovascularization in an OIR mouse model. Moreover, abnormal choroidal neovascularization as well as an increased angiogenic response has been observed in
ADAM17 in pathological neovascularization. In summary, the conditional inactivation of ADAM17 in endothelial cells presents the first evidence for a critical role of ADAM17 during pathological neovascularization in mice in vivo. Moreover, the ability of HB-EGF to rescue chord formation in endothelial cells lacking ADAM17 is consistent with the previously established essential role for ADAM17 in activating ligands of the EGFR, including HB-EGF.\textsuperscript{11,13,15,39} Based on these results, it will now be interesting to test how conditional inactivation of the EGFR in endothelial cells or pericytes affects the outcome of the models for pathological neovascularization presented here. Our results raise the possibility that selective inhibition of ADAM17 could be beneficial for the treatment of pathological neovascularization in the context of proliferative retinopathies, rheumatoid arthritis, and cancer.

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Disclosures

None.

References


What Is Known?

- The cell surface metalloproteinase ADAM17 regulates the bioavailability and function of several ligands of the EGF receptor, including HB-EGF, and transforming growth factor α.
- Mice lacking ADAM17 die at birth, with developmental defects that resemble those observed in knockout mice for the EGF receptor, or its ligands transforming growth factor α (open eyes at birth, skin defects) and HB-EGF (heart valve defects).

What New Information Does This Article Contribute?

- This study establishes a role for ADAM17 on the vasculature that could be of significant clinical relevance.
- We show that inactivation of ADAM17 in endothelial cells in mice reduces pathological neovascularization in a model for proliferative retinopathies and impedes the growth of injected tumor cells, without detectably affecting the development of a normal vasculature.
- Studies with isolated endothelial cells lacking ADAM17 uncover defects in chord formation that can be rescued by addition of the EGF receptor ligand HB-EGF.
- Taken together, our results provide the first evidence for a role of ADAM17 in pathological neovascularization, and suggest that this is caused by a defect in the functional activation of ligands of the EGF receptor.

ADAM17 (a disintegrin and metalloproteinase 17, also referred to as TNFα-converting enzyme, TACE) is a cell surface metalloproteinase with critical roles in EGF receptor signaling and processing the proinflammatory cytokine TNFα. Mice lacking ADAM17 die at birth because of severe skin and heart valve defects, so it has not been possible to study the role of ADAM17 in the adult vasculature. The main goal of this study was to evaluate how inactivation of ADAM17 in vascular cells affects physiological and pathological vascular functions. We found that deletion of ADAM17 in endothelial cells offers significant protection from oxygen-induced retinopathy and slows the growth of injected tumor cells in mice. Moreover, cell culture experiments suggest that ADAM17 is important for EGF receptor signaling in endothelial cells. These results show for the first time that ADAM17 has a critical role in pathological neovascularization, even though we found no evidence for a contribution to normal vascular development. The implications of this study for clinical medicine are that inhibitors of ADAM17, currently in development for treatment of cancer and rheumatoid arthritis, could potentially offer previously unanticipated benefits by preventing pathological neovascularization in rapidly growing tumors, rheumatoid arthritis and proliferative retinopathies such as retinopathy of prematurity, diabetic retinopathy and the wet form of macular degeneration.
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Online Supplementary Materials for the manuscript entitled “Pathological neovascularization is reduced by inactivation of ADAM17 in endothelial cells, but not in pericytes”, by Weskamp et al.

Online Figure I. Immunofluorescence analysis of sections of the aorta and a heart vessel from an Adam17flox/flox/Tie2-Cre mouse and an Adam17flox/flox control.

Immunofluorescence analysis with antisera against the endothelial cell marker PECAM (red) or against a-smooth muscle actin (green), which is expressed in smooth muscle cells including pericytes, showed no evident difference in the staining pattern of the aorta or a heart vessel from an Adam17flox/flox/Tie2-Cre mouse (right panels, labeled as “flox/flox,tie2-cre”) and an Adam17flox/flox control (left panels, labeled as “flox/flox”). Scale bar top panel =25µm, middle panel=40µm, lower panel=20µm.
Online Figure II

heart

flox/+, Tie2-Cre, R26R

flox/flox, Tie2-Cre, R26R

aorta

lung
Online Figure II. Staining of sections from Adam17flox/flox/Tie2-Cre/R26R and Adam17flox/+/Tie2-Cre/R26R mice to identify cells expressing Tie2-Cre in the presence or absence of ADAM17.

A reporter mouse strain in which the expression of Lac-Z is activated through Cre-mediated excision of a floxed stop codon between the ubiquitously expressed Rosa26 (R26) promoter and a Lac-Z gene (referred to as R26 reporter mice, or R26R mice) was used to monitor expression of Tie2-Cre in mice carrying either two floxed alleles of ADAM17, or one wild type and one floxed allele. Please note that mice that are germline-heterozygous for ADAM17, so Adam17-/+ animals, have no evident developmental or pathological phenotype 1, 2 (and data not shown), so the presence of one wild type allele of ADAM17 in endothelial cells of Adam17flox/+/Tie2-Cre/R26R mice should also ensure their normal function. No evident difference in the number or distribution of X-gal stained cells expressing Tie2-Cre in the presence of R26R was found in sections of the heart, aorta or lung in the presence of one copy of wild type ADAM17 (Adam17flox/+/Tie2-Cre/R26R) or two floxed alleles of ADAM17 (Adam17flox/flox/Tie2-Cre/R26R). Since the expression of ADAM17 is strongly reduced in a Western blot of endothelial cells isolated from Adam17flox/flox/Tie2-Cre mice (see figures 1A and 5D), this indicates the Tie2-Cre efficiently removes both floxed alleles of Adam17 in endothelial cells. Therefore the comparable distribution of X-gal-stained endothelial cells in sections of heart, aorta and lung of an Adam17flox/flox/Tie2-Cre/R26R and a Adam17flox/+/Tie2-Cre/R26R control suggests that lack of ADAM17 does not affect the distribution of endothelial cells in adult mice. Scale bar for the top panel of heart sections or lung sections =25µm, lower panel heart=10µm, scale bar for aorta=5µm.
Online Figure III

A

flox/flox, R26R
flox/flox, Tie2-Cre, R26R

B

flox/flox, R26R
flox/flox, Tie2-Cre, R26R

C

# of endothelial cells in neovascular tufts

flox/+
flox/+, Tie2-Cre
Online Figure III. Analysis of the expression of the R26R Lac-Z reporter gene in retinas from Adam17flox/flox/Tie2-Cre/R26R mice and Adam17flox/flox/R26R controls, evaluation of the effects of the Tie2-Cre transgene on the OIR model and immunofluorescence and Western blot analysis of mouse retinas.

A) Retinas from Adam17flox/flox/Tie2-Cre/R26R mice and Adam17flox/flox/R26R controls were incubated with X-gal to evaluate the expression of Tie2-Cre. X-gal staining was observed in vessels of retinal whole mounts of Adam17flox/flox/Tie2-Cre/R26R mice, but not in Adam17flox/flox/R26R controls that did not express Tie2-Cre. Scale bar=50µm. B) Staining of sections of neovascular tufts with X-gal corroborated expression of Tie2-Cre in tufts in Adam17flox/flox/Tie2-Cre/R26R mice, whereas no X-gal staining was observed in Adam17flox/flox/R26R controls. Scale bar=20µm. C) Mice carrying one wild type and one floxed allele of ADAM17 in the presence or absence of Tie2-Cre (Adam17flox/+Tie2-Cre mice or Adam17flox/+ controls) were subjected to the OIR model to determine whether Tie2-Cre affects the outcome of this model independently of ADAM17. No significant difference between the number of endothelial cells that crossed the internal limiting membrane was seen in a comparison of sections from Adam17flox/+Tie2-Cre mice, n=13, or Adam17flox/+ controls, n=13 (Wilcoxon-Mann-Whitney test P=0.54). D) Immunofluorescence analysis of pathological neovascular tufts with isolectin B4 to mark endothelial cells, or NG2 to mark pericytes, showed a similar expression pattern of these two markers in whole-mounted stained retinal tufts from Adam17flox/flox/Tie2-Cre mice compared to Adam17flox/+ controls. Scale bar=25µm. E) Western blot analysis of retinal extracts from wild type mice subjected to the OIR model at different time points after removal from 75% oxygen, probed with polyclonal antisera against the cytoplasmic domain of ADAM17 (upper panel). The same blot was re-probed with an antibody against tubulin as a loading control. F) Western blot analysis of extracts of retinas from P12, P14 and P17 Adam17flox/flox/Tie2-Cre mice (right panel) shows comparable levels of ADAM17 as in Adam17flox/flox mice (left panel), suggesting that the deletion of the widely expressed ADAM17 in endothelial cells does not detectably affect the overall levels of ADAM17 in extracts of retinas, which contain many other cell types besides endothelial cells.
Online Figure IV. Evaluation of the effect of Tie2-Cre on the growth of heterotopically injected B16F0 melanoma cells in mice with one wild type and one floxed allele of ADAM17, in the presence or absence of Tie2-Cre. B16F0 melanoma cells were injected into the flanks of mice with one wild type allele and one floxed allele of ADAM17 in the presence or absence of one Tie2-Cre allele (Adam17flox/+;Tie2-Cre and Adam17flox/+ mice), to determine whether Tie2-Cre affects the outcome of this heterotopic tumor model. No significant difference in the size of the resulting tumors was observed in a comparison of Adam17flox/+;Tie2-Cre and Adam17flox/+ controls, suggesting that the presence of Tie2-Cre alone is not critical for the outcome of this tumor model. (n=12, Wilcoxon-Mann-Whitney test p=0.73)
Online Figure V. Western blot analysis of vascular smooth muscle cells from Adam17floxflox/sm-Cre mice and Adam17floxflox controls shows efficient excision of the floxed ADAM17 alleles by sm-Cre.

Vascular smooth muscle cells (VSMCs) were isolated from Adam17floxflox/sm-Cre mice and Adam17floxflox controls as described in materials and methods. Western blots of these cells were probed with an antibody against ADAM17 (Santa Cruz H300). Almost no ADAM17 is detected in VSMCs from Adam17floxflox/sm-Cre mice under conditions where both the pro- and mature form of ADAM17 can be readily detected in VSMCs from Adam17floxflox controls. The asterisk indicates a non-specific band. It should be noted that VSMCs used for the Western blot in this figure were lysed with a different lysis buffer (SDS-sample buffer) as the endothelial cells shown in figure 1A and 5D, and that the blot was probed with a different antibody under conditions that had been optimized for detection of ADAM17 in VSMC. The same blot was probed with anti-GAPDH to confirm equivalent loading of protein samples. The results corroborate that sm-Cre efficiently removes the floxed alleles of Adam17 in VSMCs.
Online Figure VI. Staining of sections of the aorta, heart and intestine from Adam17flox/flox/sm-Cre mice and Adam17flox/flox controls.

A) Sections of the aorta (top two rows of panels), and a vessel in the heart (third row of panels) of Adam17flox/flox/sm-Cre mice or Adam17flox/flox controls stained with anti-PECAM (red) or anti-asma (green) showed a comparable staining pattern for these two markers between the two genotypes. Staining of sections of the small intestine with anti-asma antiserum (green) also did not uncover evident differences in the staining pattern between Adam17flox/flox/sm-Cre mice or Adam17flox/flox controls. Scale bar=20µm except for the lower aorta panel, where the scale bar=40µm. B) To confirm that sm-Cre is expressed in the vasculature of Adam17flox/flox/sm-Cre mice, and to assess how the expression of sm-Cre affects the distribution of cells in Adam17flox/flox/sm-Cre mice, X-gal staining of sections of the aorta and heart of Adam17flox/flox/sm-Cre/R26R mice carrying two floxed alleles of ADAM17 or Adam17flox/+/sm-Cre/R26R controls carrying one wild type allele and one floxed allele of ADAM17 was performed. No difference in the appearance or distribution of X-gal stained cells was observed in sm-Cre mice with one wild type allele and one floxed allele of ADAM17 versus two floxed alleles of ADAM17. Scale bar for the upper panel =25µm, for the lower panel =10µm.
Online Figure VII. Developmental retinal angiogenesis in Adam17flox/flox/sm-Cre mice compared to Adam17flox/flox controls.
A) Isolectin B4 staining of retinal whole mounts of a 6 day-old Adam17flox/flox/sm-Cre mouse and an Adam17flox/flox littermate control showed no differences in the development of the retinal vascular tree between these two samples (please note that pericytes only appear in the retinal vasculature in significant numbers after day 7, so these samples are mainly shown for comparison to the analysis of developmental angiogenesis in Adam17flox/flox/Tie2-Cre mice in figure 1) Scale bar=250µm. B) Quantification of the size of the vascular tree compared to the size of the retina showed no significant difference in developmental retinal angiogenesis between Adam17flox/flox/sm-Cre mice (n=12) or Adam17flox/flox controls (n=12, Wilcoxon-Mann-Whitney test P=0.56).
Online Figure VIII. The response to oxygen induced retinopathy is comparable in Adam17flox/flox/sm-Cre mice and Adam17flox/flox controls.
A) Isolectin B4 staining of retinas from OIR treated mice at p17 (5 days after removal from the 75% oxygen chamber) shows a similar size of the central avascular area relative to the size of the entire retina in Adam17flox/flox/sm-Cre mice compared to Adam17flox/flox controls. The central avascular area is outlined in yellow, whereas the retina is outlined in red. Scale bar=250µm. B) Quantification of the size of the central avascular area in the retinas of Adam17flox/flox/sm-Cre mice (n=17) compared to Adam17flox/flox controls (n=21) showed no significant difference between these two genotypes (Wilcoxon-Mann-Whitney test P=0.62). C) Quantification of endothelial cells that had crossed the internal limiting membrane in sections of retinas from mice subjected to the OIR model showed no significant difference between samples from Adam17flox/flox/sm-Cre mice (n=16) and Adam17flox/flox controls (n=17) (P=0.89). Please note that subtle effects of the lack of ADAM17 in sm-expressing cells in the OIR model cannot be ruled out because of the relatively low numbers of cells in neovascular tufts in Adam17flox/flox controls in panel C. D) Staining of sections of neovascular tufts with X-gal corroborated expression of sm-Cre in tufts in Adam17flox/flox/sm-Cre/R26R mice, whereas no X-gal staining was observed in Adam17flox/flox/R26R controls.
Online Figure IX. Growth of heterotopically injected B16F0 cells in Adam17flox/flox/sm-Cre mice and Adam17flox/flox controls. 10^6 B16F0 melanoma cells were injected into the flanks of Adam17flox/flox/sm-Cre mice (n=10) or Adam17flox/flox controls (n=10). No significant difference in tumor growth was seen in the presence or absence of sm-Cre (Wilcoxon-Mann-Whitney test P=0.82).
Online Supplementary Materials and Methods

Reagents and Cell lines
All reagents were from Sigma, unless indicated otherwise. Isolectin B4 was from Vector Labs (Burlingame, CA), rabbit anti-mouse NG2 from Chemicon International (Temecula, CA), and rat anti-PECAM from BD Biosciences/Pharmentogen (San Diego, CA). Rabbit anti-ADAM17 (H300) used in supplementary figure 5 from Santa Cruz Biotech (Santa Cruz, CA), and the anti-ADAM17 cytoplasmic domain antibodies have been previously described 3. Antibodies for FACS analysis: CD31-FITC (Becton Dickinson), ICAM2-PE (Southern Biotech), eNOS AlexaFluor647 (Becton Dickinson), Tie2-PE (eBioscience). Porcine aortic endothelial cells expressing the KDR receptor were kindly provided by Dr. Shahin Rafii (Weill Cornell, New York, NY) 5, and Adam17−/− mouse embryonic fibroblasts have been previously described 1, 6. Beads for isolation of endothelial cells were prepared according to the manufacturer’s instructions (Dynabead Sheep anti-Rat IgG, Dynal, Oslo, Norway). Briefly, 5µl rat-anti PECAM antibody was bound to 100µl magnetic beads for 5 h at 4°C. Unbound antibody was removed by washing several times in phosphate buffered saline. Recombinant murine VEGF-A and HB-EGF were obtained from R&D Systems (Minneapolis, MN). Transfections were performed using either Lipofectamine (Invitrogen, Carlsbad, CA) for PAE-KDR or Lipofectamine2000 (Invitrogen, Carlsbad, CA) for all mEF cultures, following the manufacturer’s protocols.

Mouse lines used in this study
In order to generate mice lacking ADAM17 in endothelial cells, we initially crossed Adam17flox/flox mice (Adam17tm1Bbl) 1 with a strain of Tie2-Cre mice available from Jackson labs, Bar Harbor, ME (B6.Cg-Tg(Tek-cre)12Flv/J). However, when we genotyped the offspring of these animals, it became clear that this Tie2-Cre promoter is also active in the female germline, since mice lacking ADAM17 in the germline were generated from these crosses (data not shown). We therefore obtained a different Tie2-Cre strain (kindly provided by Dr. Tom Sato, Cornell University, Ithaca, NY) in which the Cre-recombinase is expressed under the control of a well-characterized Tie2 promoter 7 that is highly selective for endothelial cells 8. These were crossed with Adam17flox/flox mice to obtain Adam17flox/flox/Tie2-Cre and Adam17flox/flox controls after several crosses. From these matings, we also generated Adam17flox/+Tie2-Cre mice expressing Tie2-Cre in the presence of one wild type allele and one floxed allele of ADAM17 to have a control for the effect of Tie2-Cre in the presence of wild type ADAM17. No pathological phenotypes were observed in Adam17flox/+Tie2-Cre animals, and there was no difference in the outcome of the OIR models and heterotopic tumor injection model (described below) in Adam17flox/+ mice in the presence or absence of Tie2-Cre. To generate Adam17flox/flox/sm-Cre mice, we mated Adam17flox/flox with sm-Cre mice (also referred to as SM22-Cre mice), obtained from Jackson labs (Tg(Tagln-cre)
Her/J). After the appropriate matings, we obtained Adam17flox/flox/sm-Cre and Adam17flox/flox controls.

In order to monitor the expression of the two Cre-transgenes used in this study during mouse development and in tissues of mice subjected to the OIR model, we obtained the R26R Lac-Z reporter mouse strain, which contains a floxed stop codon between the ubiquitously expressed Rosa 26 promoter and a Lac-Z gene, so that the expression of Lac-Z is activated through Cre-mediated excision of the stop codon (Jackson labs; B6.129S4-Gt(ROSA)26Sortm1Sor/J). These animals were mated with Adam17flox/flox/Tie2-Cre or Adam17flox/flox/sm-Cre mice to ultimately obtain animals carrying the R26R reporter, the respective Cre-transgene and either one or two floxed alleles of ADAM17. R26R was used to monitor expression of Tie2-Cre in mice carrying either two floxed alleles of ADAM17, or one wild type and one floxed allele. By comparing the X-gal staining in various tissues in animals carrying the respective Cre-transgene and the R26R reporter, but only one floxed allele of ADAM17, to animals with the Cre-transgene and the R26R reporter and both floxed alleles of ADAM17, we could evaluate whether there was a defect in the number or localization of the X-gal expressing cells in the presence or absence of ADAM17. An analysis of Lac-Z expression in endothelial cells of mice carrying Tie2-Cre and R26R showed that there was no difference in the appearance of these cells or the X-gal staining pattern in the presence of one wild type and one floxed allele of ADAM17 versus both floxed alleles. Likewise, there was no evident difference in the number or appearance or localization of X-gal stained cells in mice expressing R26R and sm-Cre together with one or two floxed alleles of Adam17.

The genetic background of the Adam17flox/flox mice was (129P2/OlaHsd/C57BL/6) 1, and these mixed background mice have been mated with one another as heterozygotes to maintain the colony for over 10 generations, so they remain of mixed genetic background. One difference between the Adam17flox/flox mice used in the study by Horiuchi et al. 1 and the current study was that the neo selection marker, which was flanked by FRT sites (see the map in reference 1 for details), was removed by crossing these animals with an FRT deleter strain from Jackson labs (B6;SJL-Tg(ACTFLPe)9205Dym/J), which have a C57BL/6J background. The Adam17flox/flox mice used in this study will be available at The Jackson Laboratory with the JAX Stock No. 009597 (http://jaxmice.jax.org/query). The genetic background of the Tie2-Cre mice originated on a C3B6F2 background and had been backcrossed to C57BL/6J for at least seven generations, and the background of the sm-Cre was mixed (129S5/SvEvBrd, C57BL6 and SJL). However, since both Cre-driver lines were mated to the mixed genetic background Adam17flox/flox mice (see above), all animals used in our study were of mixed genetic background.

To control for possible effects of the genetic background in the experiments presented here, we only compared littermates that were offspring of matings of Adam17flox/flox/Cre x Adam17flox/flox mice with one another, so of matings
where one parent carried both floxed alleles of ADAM17 as well as the Cre-transgene was mated with another parent carrying both floxed alleles, but no Cre-transgene. Such matings gave rise to approximately equal numbers of Adam17flox/flox/Tie2-Cre and Adam17flox/flox littermates or Adam17flox/flox/sm-Cre and Adam17flox/flox littermates, as described in the results section, and only these littermates were compared to one another. Since the parentals were of mixed genetic background, the contribution of the background strains could differ somewhat from one littermate to the next due to meiotic crossovers. However, any background specific modifiers should be randomly distributed among the offspring, and not be linked to the targeted ADAM17 alleles, which are present in all animals. Thus the main difference between any experimental (Adam17flox/flox/Cre) and control animals (Adam17flox/flox) used in our study therefore was the presence or absence of one transgenic Cre allele (which was addressed through additional controls, see below), and the presence or absence of the floxed exon 2 of ADAM17. By using this mating scheme, we also avoided the possibility of generating mice carrying 2 alleles of the Cre-transgene, which could potentially affect the efficiency of Cre-mediated recombination in these experiments.

In order to assess the effect of Tie2-Cre alone on the OIR model and heterotopic tumor injection model, we subjected Adam17flox/+Tie2-Cre mice and their Adam17flox/+ littermate controls to both models. In this context it should be noted that animals that are heterozygous for a germline deletion of ADAM17 show no developmental or spontaneous pathological defects. So Adam17flox/+Tie2-Cre mice lacking one copy of ADAM17 in endothelial cells would be expected to be normal, and we did not see any differences in the OIR or heterotopic tumor model in the presence or absence of the Tie2-Cre transgene, arguing against a role of this Cre-transgene that was independent of the excision of ADAM17. The genotyping for the presence of floxed ADAM17 was by Southern blot and PCR, as described previously. The presence or absence of the Cre-transgene was determined by PCR (5’GCGGTCTGGCAGTAAAAACTATC-3’ and 5’-GTGAAACAGCATTGCTGTCACTT-3’). All animals were sacrificed by CO₂ inhalation according to the guidelines of the American Veterinary Association, and all animal experiments were approved by the Internal Animal Care and Use Committee of the Hospital for Special Surgery.

Oxygen induced retinopathy assay for retinopathy of prematurity
In order to trigger oxygen induced retinopathy in mice, 7-day old mice were placed in a plexiglass chamber with an oxygen concentration of 75% (the chamber and oxygen regulator, Proox model 110, were supplied by Biospherix, Redfield, N.Y.), together with their nursing mothers, and kept in the chamber for 5 days. The exposure to high concentrations of oxygen results in a regression of the vasculature in the central portion of the retina, giving rise to a central avascular area. When the animals are returned to normoxic room air (21% oxygen) after 5 days in 75% oxygen, the resulting relative hypoxia triggers
production of VEGF-A, which in turn stimulates a neovascular response. This manifests itself as a re-vascularization of the central avascular area and in the growth of endothelial cells on the vitreal side of the internal limiting membrane and formation of pathological neovascular tufts. The evaluation of the size of the central avascular area relative to the size of the retina, of the number of endothelial cells that crossed the internal limiting membrane and the immunofluorescence and immunohistochemical evaluation of the pathological neovascular tufts were performed 5 days after returning the animals to room air, so in animals that were 17 days old (P17).

After the animals were euthanized at P17, the eyes were removed and fixed in 4% Paraformaldehyde (PFA). One eye was then processed for sectioning, and the other eye was used to analyze neovascular tufts and the size of the central avascular area by staining a whole-mounted retina. For histological evaluation of pathological neovascularization, the eyes were embedded in paraffin, sectioned to generate samples of 6 µm thickness, then stained with hematoxylin and eosin. After preparing about 150 sections per eye, five sections on each side of the optic nerve, 30 to 90 µm apart, were selected and used to assess neovascularization in a double blinded manner by counting endothelial cell nuclei on the vitreal side of the internal limiting membrane, as previously described. The average number of endothelial nuclei per section for each eye was determined, and the Wilcoxon-Mann-Witney test was used to assess the statistical significance of the response to the OIR model in Adam17flox/flox mice expressing Tie2-Cre or sm-Cre compared to littermate controls not expressing the Cre transgene, or in other pairs of genetically modified mice presented here.

The second eye from each animal was used to determine the relative size of the central avascular compared to the size of the retina after the animals were exposed to the OIR model. The eyes were fixed overnight with 4 % PFA, then washed 5x with PBS and incubated overnight in LBB (lectin blocking buffer, PBS, 1% BSA, 0.1 % Triton X-100, 0.1 M glycine). The next day, the retinas were excised, flat mounted on microscope slides and incubated with LBB for 2 hrs. Fluorescein labeled isoelectin B4, diluted 1:200 in 0.2x LBB, was added and incubated overnight at 4°C. The retinas were washed twice in PBS, excess fluid absorbed with filter paper and then mounted in fluorescent mounting medium (DAKO). The samples were photographed using a Nikon Eclipse E600 fluorescent microscope with a 2x objective and a Qimaging Retiga EXi camera. Images were processed with QCapture 2.68.04 software, keeping the exposure and gain constant for all samples. The size of the avascular area and the total retina were determined using the NIH Image J software. There was no significant difference in the size of the retina in littermates with or without the Cre-transgenes used in this study.

**Evaluation of developmental retinal angiogenesis**

To assess developmental retinal angiogenesis we focused on postnatal day P6, because the developing vascular bed covers about 2/3 of the retina at this stage.
Any significant defects in developmental retinal angiogenesis and growth of the vascular bed should therefore be apparent at P6 in a comparison of littermates. For whole mount preparations to evaluate retinal angiogenesis, the hyaloid vessels were first removed, and then the retinas were prepared for staining with fluorescein-labeled isolectin B4 to visualize endothelial cells before they were photographed, as described above. The outline of the whole retina as well as of the area covered by the developing vascular bed was delineated using NIH Image J 1.36b software, and used to calculate the surface area in order to determine the percent of the retinal area covered by the developing retinal vasculature. We did not observe a significant difference in the size of the retina in P6 littermates with or without the Cre-transgenes used here.

Immunohistochemistry and immunofluorescence analysis
Tumors, eyes and other tissues were isolated and fixed in freshly prepared 4% paraformaldehyde at 4°C overnight, then washed 3x with PBS and either stored in 70% ethanol or processed for embedding in paraffin. In order to generate frozen sections, samples were washed in PBS and then placed in 20% sucrose overnight at 4°C. The next day, the samples were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) mixed with 20% sucrose in a 1:1 ratio. Sections of a thickness of 5 µm were cut and mounted on glass slides and then postfixed with ice-cold acetone for 10 min. Paraffin sections were deparaffinized, rehydrated through a graded alcohol series and placed in 10 mM Sodium-citrate (pH 6.0), heated in a microwave until boiling, and then allowed to cool down to room temperature to retrieve antigens. Then the paraffin and frozen sections were preincubated with 5% normal goat serum/2% BSA/PBS for 1h, followed by 1 h incubation with rat-anti-platelet endothelial cell adhesion molecule-1 (PECAM) antibodies 12, which were detected with Cy3 conjugated donkey anti-rat antibodies (Jackson Immunoresearch, West Grove, PA) or 30 min with FITC-labeled anti αsma antibodies (Sigma, St Louis, MO). For an analysis of the vasculature and neovascular tufts in flat-mounted retinas, these were incubated with modified LBB (0.5% Triton X-100) for 2h, then washed 3x with PBS and stained overnight with fluorescein labeled isolectin B4 to mark endothelial cells, washed with PBS and processed as described above.

Shedding assays and construction of alkaline phosphatase-tagged fusion proteins
Protein ectodomain shedding assays were performed in mouse embryonic fibroblasts isolated from Adam17/- embryos or wildtype controls (see above, and references 1, 14), as well as in porcine aortic endothelial cells expressing human VEGFR2/KDR (PAE-KDR cells 5). The expression constructs for wild type ADAM17 and the inactive ADAM17 control containing a Glutamate to Alanine (E>A) mutation in the catalytic site (ADAM17EA) as well as the expression constructs for AP-tagged TNFα, HB-EGF, TGFα, EGF and betacellulin were described previously 6, 15, 16. The expression plasmids for AP-tagged forms of the IGFR1, VE-cadherin, EphB4, PECAM, EMMPRIN and V-CAM were constructed as follows: Full-length cDNAs were obtained from the A.T.C.C. (Manassas, VA)
and used as a template to generate PCR products that included the coding sequences for the carboxyl end of each open reading frame starting with the extracellular domain closest to the transmembrane domain, the juxtamembrane region including the putative sheddase cleavage site (please note that ADAM17 usually processes its substrates at a sequence that is within the first 20 amino acid residues extracellular boundary of the transmembrane domain), the transmembrane domain and the cytoplasmic domain of each substrate protein. The primers used to generate alkaline phosphatase-fusion proteins with CD34, V-CAM, E-Selectin, EMMPRIN, PECAM, IGFR1, EphB4 and VE-cadherin were:

**CD34** (amino acid residues 31 - 385):
5'-ATGAGTCTTTGACAACAACGGTA-3';
5'-TCACAATTCCGGTATCAGCCACCACG-3';

**VCAM-1** (amino acid residues 28 - 739):
5'-GAGATCTCCCCTGAATACA-3';
5'-CTACACTTTGGATTTCAGCC-3';

**E-Selectin** (amino acid residues 31 - 612):
5'-ATGACGTATGATGAAGCCAGTGCA-3';
5'-GTTCCTGATTGTTTTGAACCTAGA-3'.

**EMMPRIN** (amino acid residues 30 - 385):
5'-CCACTCGAGCTGTCCCAGCAGAGGTGGGT-3; 5'-CCACTCGAGTCAGGAAGAGTTCCTCTCTGGGACGCT-3'.

**CD31(PECAM)** (amino acids 172 - 738):
5'-CGCGTTTTATCCTTCCGATGTCAA-3';
5'-ACAAGGGCCCCTAAGTTCCATCAAGGGACGCT-3'.

**IGFR1** (amino acid residues 933 - 1367):
5'-ATCCACAGCTGCAACCACGAGGCT-3'.
5'-TCAGTCTCATAGATATCTCGCGTATACC-3'.

**EphB4** (amino acid residues 377 - 624):
5'-CGAGACCTGGTTGAGGCTGG-3';
5'-GACCGGGGTCAGGAGGTAC-3'.

**VE-cadherin** (amino acid residues 481 - 721):
5'-CCCTACGAACCTAAGTGATGTGAGAAT-3';
5'-ACCGCCGTCATTGTGCTCCTCTCC-3'.

Each PCR-amplified cDNA was cloned in frame with human alkaline phosphatase into the pAP-tag mammalian expression vector (Genehunter, Nashville, TN).
Protein ectodomain shedding assays
For protein ectodomain shedding assays, mouse embryonic fibroblasts (mEFs) or porcine aortic endothelial cells expressing the human VEGFR2/KDR (PAE-KDR cells) were transfected with plasmid cDNA (0.5 µg vector/well of a 6 well plate) encoding for the alkaline phosphatase tagged fusion proteins listed above using lipofectamine2000 or lipofectamine, respectively, and then cultured for 1 day in DMEM with 5% FCS. Then fresh medium was added and collected after 1 hr, followed by addition of another aliquot of fresh medium that contained 25 ng/ml of the phorbol ester PMA (phorbol-12-myristate-13-acetate), or 50 ng/ml of murine VEGF-A in the case of PAE-KDR cells for 1 h, as indicated. The AP assay of culture supernatant or cell lysates (lysis buffer: 100mM Tris, pH 8, 2.5% Triton-X 100 (Sigma), 1mM EDTA, and 1 mM 1,10 Phenanthroline) were developed with the substrate P-NPP (2mg/ml, Thermo Scientific) in Alkaline Phosphatase buffer (100mM Tris, pH 9.5, 100mM NaCl, and 20mM MgCl₂) for 1-4 hours. Calculation of percent shedding equals total shed Alkaline Phosphatase activity in the culture supernatant divided by total Alkaline Phosphatase activity of the combined culture supernatant and cell lysate. In each case, the background Alkaline Phosphatase activity of either supernatants or lysates resulting from untransfected cultures is subtracted from the appropriate sample following the formula:

\[
\frac{\text{O.D. 405nm (Transfected Supernatant) – (Untransfected Supernatant)}}{\text{O.D. 405nm (Transfected Supernatant) + O.D. 405nm (Transfected Lysate) – (Untransfected Lysate)}} \times 100 = \% \text{Shedding}
\]

Endothelial cell assays
Primary mouse endothelial cells were isolated from heart and lungs of 9 – 12-day-old mice (P9-12) as described. Briefly, the heart and lungs were collected in PBS, rinsed and minced into small pieces with a scalpel. These tissue fragments were digested in Collagenase type1 for 1 h at 37°C, then subjected to mechanical dissociation by gently passing the samples twenty times through a 19 gauge needle attached to a syringe. The dissociated tissue was filtered through a 70µm mesh (BD Falcon) and washed 3 times with base medium (DMEM, 10% fetal calf serum, 1% penicillin/streptomycin). The separation of endothelial cells from other cell types was accomplished by affinity purification with anti-PECAM beads for 20 minutes at 4°C. The beads and bound cells were washed 5 times with serum containing ice-cold base medium. After the final wash, the cells with beads were resuspended in complete growth medium and plated on gelatin coated 6-well tissue culture plates. Once the primary cultures reached 90% confluence, they were dissociated and purified a second time with magnetic beads that had previously been incubated with antibodies against ICAM. Over 95% of the cells in the resulting cultures were positive for staining with anti-PECAM- and anti-VE-cadherin by immunofluorescence microscopy and Fluorescence activated cell sorter (FACS), and displayed a cobblestone-like appearance in culture, consistent with the behavior of endothelial cells.
The proliferation rate of the isolated primary endothelial cells was determined with the Celltiter proliferation assay from Promega. Briefly, 5x10^3 cells isolated from Adam17flox/flox/Tie2-Cre mice or Adam17flox/flox controls were plated per well of a 96 well tissue culture plate in growth medium, with a total of at least 3 wells for each genotype per experiment. The cells were allowed to attach for 1 hour, then washed three times with PBS, and cultured for 4 days. The colorimetric assay was carried out following the manufacturer's protocol (Promega). Briefly, 20µl of substrate solution was added to 100µl of cultured cells followed by incubation for 1h at 37°C. Absorbance of the plate was then read at 490nm using an ELISA plate reader. The data represent the means of three independent experiments ± SEM.

For the ex vivo chord formation assay, 50 µl Cultrex matrigel (Trevigen, Gaithersburg, MD) was added to each well of a 96 well plate and incubated at 37°C for 30 min to allow for gel formation. Then 3x10^4 endothelial cells, isolated as described above, were plated per well with or without 5 ng/ml VEGF-A or 5 ng/ml HB-EGF and incubated for 4 h in 5% CO₂ at 37°C. Each condition was tested in duplicate and each experiment was performed at least 3 times. At the end of each experiment, the endothelial cells and tubes were first fixed in 4% Paraformaldehyde-PBS and then photographed at 12x magnification. Formation of endothelial cell tubes was documented using a Zeiss inverted microscope with a 10x objective.

Heterotopic injection of B16F0 mouse melanoma cells
Male and female mice were each injected subcutaneously with 1x10^6 B16F0 mouse melanoma cells in 100µl PBS. All animals in a given experiment were sacrificed at the same time, between 2 to 3 weeks after injection, depending on the tumor burden in animals with the fastest tumor growth in that experiment, and the tumors were removed and weighed. The Wilcoxon-Mann-Whitney test was used for statistical analysis. Immunofluorescence analysis of PECAM expression in frozen tumor sections was performed to evaluate the density and distribution of vessels in tumors from Adam17flox/flox/Tie2-Cre mice compared to Adam17flox/flox controls. Bound antibodies were detected with Cy3-conjugated AffiniPure donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA).

Western Blot analysis
Western blots of whole retinas were prepared by lysing individual freshly isolated retinas in 40 µl cell lysis buffer each (PBS, 1% TX-100, protease inhibitor cocktail, plus 5 mM 1,10 phenanthroline to prevent the post-lysis degradation of the mature form of ADAM17). Following cell lysis, cell and tissue debris as well as nuclei were removed by spinning the samples at 27,000 g for 15 minutes at 4°C in a microcentrifuge. The supernatant was recovered and mixed with Sample loading buffer containing 20 mM DTT, separated by SDS-PAGE on a 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes (Millipore). After blocking the membranes for 1 hr with 3% milk reconstituted from dry milk powder into 1x PBS, the blot was incubated for 1 hr in PBS, 0.1%
Tween 20 and a 1:1000 dilution of a rabbit polyclonal antiserum against the cytoplasmic domain of ADAM17, followed by 3 washes in PBS, 0.1% Tween 20, and incubation with the secondary HRP-coupled anti-rabbit antibodies from Promega at 1:3000 for 30 min. After three additional washes in PBS, 0.1% Tween 20 for 5 minutes each, the membrane was incubated with ECL developers (GE Healthcare, Buckinghamshire, UK) for 1 minute, transferred inside a plastic sheet protector and the resulting chemoluminescence was detected and photographed using a Biorad Molecular Imager Gel Doc XR System. Image processing was performed using Adobe Photoshop version 8.0.

For the Western blot analysis of primary mouse endothelial cells, the cells were lysed in the buffer described above (PBS, 1% TX-100, protease inhibitor cocktail, plus 5 mM 1,10 phenanthroline), then centrifuged at 27,000 g for 15 minutes at 4°C in a microcentrifuge, and then the supernatant was incubated with 40 µl of a 1:1 slurry of Concanavalin A lectin beads to enrich for glycoproteins. After 3 hrs, the beads were washed in cell lysis buffer, and then the bound glycoproteins were recovered by boiling the beads in SDS-sample lysis buffer and subjected to Western blot analysis with the anti-ADAM17-cytoplasmic domain antibody, as described above.

For Western blot analysis of vascular smooth muscle cells isolated from aortas of Adam17flox/flox/sm-Cre mice or Adam17flox/flox controls, cells were prepared by the explant method allowing VSMCs to grow out of the tissue sections and attach to tissue culture plates. Upon subculture, these cells showed more than 99% positive immunostaining of smooth muscle alpha-actin antibody. The cells were then lysed directly in 1xSDS sample buffer and immunoblotting was performed with an anti-ADAM17 antiserum from Santa Cruz (H300) as described.

FACS analysis of the cell surface expression of CD31/PECAM1 on mouse lung endothelial cells from Adam17 flox/flox or Adam17flox/flox/Tie2-Cre mice
Adam17flox/flox/Tie2-Cre mouse were used as a source of Adam17+/− endothelial cells and Adam17flox/flox mice served as control. Cells were purified as described above and used within 4 passages for surface staining of CD31/PECAM1 after removal from the tissue culture plate with collagenase. Endothelial cells were stained with Fc block mABs to reduce non-specific antibody binding. The cell surface was stained with anti-PECAM-FITC and anti-CD102(ICAM2)-PE (Southern Biotech) before fixation with PFA and permeabilization with 0.2% saponin for staining with anti-eNOS AlexaFluor647. All antibodies listed above were first tested on the mouse endothelial cell line bEnd.3 cells (kindly provided by Dr. Heidi Stuhlmann, Weill Cornell University) and on mouse mEFs to establish proper staining conditions. Anti-PECAM and anti-eNOS stained bEnd.3 cells but not mEF cells. The variation in cell surface expression of PECAM on endothelial cells from Adam17flox/flox mice and Adam17flox/flox/Tie2-Cre controls was quantified for the double positive ICAM-
2^eNOS^ populations by cytometry, which was performed with a FACSCalibur (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson).

**Histopathology**
Histopathological analysis of one female and one male *Adam17flox/flox/Tie2-Cre* mice and one age-matched female and male *Adam17flox/flox* littermate control mouse, each 5 months old, as well as of one female and one male *Adam17flox/flox/sm-Cre* mice and one age-matched littermate female and male *Adam17flox/flox* control was performed by the Laboratory of Comparative Pathology at the Memorial Sloan-Kettering Cancer Center, New York, NY. The slides examined included sections of the following tissues: heart, thymus, lungs, kidneys, liver, pancreas, spleen, gall bladder, intestines (small and large), stomach, mesenteric lymph nodes, salivary glands, submandibular lymph node, uterus, skin, urinary bladder, adrenals, pituitary, thyroid, esophagus, trachea, ovaries, bone marrow, spinal cord, vertebral column, knee joint (femur, tibia and surrounding musculature), sternum, coronal sections of head (eyes and nasal cavity), and coronal sections of head (brain). No morphologic lesions that could be specifically attributed to the lack of expression of ADAM 17 in endothelial or smooth muscle cells under physiologic conditions and at the ages examined were identified in this analysis.

**Statistical analysis**
All statistical analyses were performed using the SAS 9.1.3 (SAS Institute, Cary, NC). Wilcoxon-Mann-Whitney test, a non-parametric analog to the independent samples t-test, was used for all comparisons throughout the paper in order to accommodate the relatively small size of the sample and the skewness of the data. A *P*-value<0.05 was considered significant. The results of the statistical analyses were as follows. For figure 1D, we used 11 animals per genotype *P*=0.76. For figure 2B we used 22 (*Adam17flox/flox*) and 26 (*Adam17flox/flox/Tie2-Cre*), *P*<0.001; for figure 2C we used 21 (*Adam17flox/flox*) and 25 (*Adam17flox/flox/Tie2-Cre*) mice, *P*<0.001; for figure 3A we used 24 (*Adam17flox/flox*) and 20 (*Adam17flox/flox/Tie2-Cre*) mice, *P*<0.001; for figure 3C we used 9 mice for each genotype, *P*=0.25; for Supplementary figure IIIC we used 13 mice for each genotype, *P*=0.54; for Supplementary figure IV we used 12 mice for each genotype, *P*=0.73; for Supplementary figure VIIB we used 12 mice for each genotype, *P*=0.56; for Supplementary figure VIIIIB we used 17 (*Adam17flox/flox*) mice and 21 (*Adam17flox/flox/sm-Cre*) mice, *P*=0.62; for Supplementary figure VIIIC we used 17 (*Adam17flox/flox*) mice and 16 (*Adam17flox/flox/sm-Cre*) mice; *P*=0.89; for Supplementary figure IX we used 10 mice per genotype, *P*=0.82.

The *P* values for the experiments presented in figure 5A are as follows. Figure 5A, comparison of shedding in unstimulated- and PMA-stimulated wild type mEF cells: IGFR1, *P*=0.02; VE-cadherin, *P*=0.04; EphB4, *P*=0.02; EMMPRIN, *P*=0.04; V-CAM, *P*=0.04; PECAM, *P*=0.04
Figure 5A, comparison of shedding in unstimulated and PMA-stimulated Adam17-/- mEF cells: IGFR1, P=0.42; VE-cadherin, P=0.66; EphB4, P=0.66; EMMPRIN, P=0.38; V-CAM, P=0.66; PECAM, P=0.66.

Figure 5B, comparison of shedding in unstimulated cells and VEGF-A-stimulated wild type mEF cells: IGFR1, P=0.04; VE-cadherin, P=0.04; EphB4, P=0.04; EMMPRIN, P=0.04; V-CAM, P=0.04; PECAM, P=0.04; EGF, P=0.1; BTC, P=0.5.

References for Figure Legends and Supplementary Materials and Methods


