Myc Regulates Embryonic Vascular Permeability and Remodeling

Enikő Kokai, Florian Voss, Frank Fleischer, Sybille Kempe, Dragan Marinkovic, Hartwig Wolburg, Frank Leithäuser, Volker Schmidt, Urban Deutsch, Thomas Wirth

Abstract—Previous work has shown that c-Myc is required for adequate vasculogenesis and angiogenesis. To further investigate the contribution of Myc to these processes, we conditionally expressed c-Myc in embryonic endothelial cells using a tetracycline-regulated system. Endothelial Myc overexpression resulted in severe defects in the embryonic vascular system. Myc-expressing embryos undergo widespread edema formation and multiple hemorrhagic lesions. They die between embryonic days 14.5 and 17.5. The changes in vascular permeability are not caused by deficiencies in vascular basement membrane composition or pericyte coverage. However, the overall turnover of endothelial cells is elevated as is revealed by increased levels of both proliferation and apoptosis. Whole-mount immunohistochemical analysis revealed alterations in the architecture of capillary networks. The dermal vasculature of Myc-expressing embryos is characterized by a reduction in vessel branching, which occurs despite upregulation of the proangiogenic factors vascular endothelial growth factor-A and angiopoietin-2. Thus, the net outcome of an excess of vascular endothelial growth factor-A and angiopoietin-2 in the face of an elevated cellular turnover appears to be a defect in vascular integrity. (Circ Res. 2009;104:1151-1159.)

Key Words: Myc ■ VEGF-A ■ vascular permeability ■ angiogenesis

Vascular development of the mouse embryo is governed by complex molecular and cellular processes and involves 2 distinct mechanisms. Vasculogenesis is responsible for de novo generation of vessels from angioblasts and occurs during the formation of the primitive vascular plexus in extraembryonic tissues and within the embryo. Angiogenesis subserves formation of new vascular structures from preexisting vessels by sprouting and intussusception. The immature vascular tree adjusts to changes in blood flow and oxygen demand by remodeling. This involves both new vessel growths by sprouting or intussusception, as well as vessel regression, a process called pruning. These processes result in a hierarchically organized vascular pattern that facilitates directional blood flow. Later phases of vascular development involve the differential recruitment of associated support cells, such as smooth muscle cells and pericytes and the formation of vascular basement membranes.

Key molecular regulators of early vascular development are vascular endothelial growth factor (VEGF)-A and its cognate receptors VEGFR-1 and VEGFR-2. Mice with mutations in either VEGF-A or the receptors show severe defects in blood vessel formation and die early in embryogenesis before the establishment of blood circulation. Heterozygous VEGF-A embryos also show a lethal phenotype suggesting that fine-tuned regulation of components of the VEGF/VEGFR system is essential for correct vascular differentiation.

Vascular remodeling and vessel stabilization depend on the angiopoietin (Ang)/Tie pathway. The 2 Tie receptors, Tie1 and Tie2/Tek, encode receptor tyrosine kinases predominantly expressed in endothelial cells. Tie2 expression is detectable starting in angioblasts and throughout development with reduced expression in quiescent endothelial cells. Angiopoietins, the ligands for the Tie2 receptor have different functions during vascular development. Ang-1 acts to stabilize vessels, whereas Ang-2 can have antagonistic as well as agonistic activity depending on the tissue context and environmental conditions. Ang-2 can locally antagonize Ang-1-mediated Tie2 activation and therefore cause destabilization of vessels. In the presence of both Ang-2 and VEGF-A, angiogenesis can occur; in the absence of VEGF-A, Ang-2 destabilizes vessels, leading to endothelial cell apoptosis and, thereby, to vessel regression.

The Myc protein is known to regulate several aspects of blood vessel formation. Myc contains a bHLH (basic helix-loop–helix) leucine zipper motif and binds to conserved DNA motives (E-boxes) after dimerization with its partner protein Max. Myc activity is tightly regulated and it is only expressed when cells actively divide. The Myc/Max heterodimer both activates transcription but can also mediate...
gene repression.\textsuperscript{5} Myc represses expression of the antiangiogenic factor thrombospondin-1\textsuperscript{1}\textsuperscript{-} and stimulates the proangiogenic factor VEGF-A.\textsuperscript{7} Furthermore, many genes critical for diverse cellular functions like cell cycle progression, apoptosis, and metabolism have been identified as Myc target genes.\textsuperscript{8}

Homozygous c-myc--null mutation leads to lethality at embryonic developmental day (E)10.5.\textsuperscript{9} Mutant embryos were growth-retarded and showed defects in cardiac and neuronal development, as well as in primitive erythropoiesis. Moreover, both vasculogenesis and angiogenesis were distorted and this was manifested in an altered expression of direct and indirect Myc target genes.\textsuperscript{7} A recent analysis of endothelial cell--selective Myc deletion concluded that the vascular phenotype observed in these mouse embryos may not be attributable to an endothelial cell--autonomous effect of Myc.\textsuperscript{10}

To determine the consequence of Myc upregulation in endothelial cells for their development, proliferation, and survival, we used a model of endothelial cell--specific conditional expression of Myc. Remarkably, overexpression results in embryonic lethality and embryos show severe edema and hemorrhages. Our results demonstrate that fine-tuned regulation of Myc target genes is essential for proper vascular remodeling and the maintenance of vascular integrity.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Quantitative Analysis of Dermal Vascular Architecture
For the quantitative analysis of vessel morphology, the blood vessels were regarded as a random network of line segments neglecting their width and curvature. Gray scale images of blood vessels were skeletonized using different image processing techniques and then transformed into a network of line segments as described previously.\textsuperscript{11} Based on the network of line segments, different statistical characteristics describing the spatial geometric structure, such as the mean number of branching points and line segments, both measured per unit area, and, in addition, the mean length of the segments, were estimated by unbiased estimators described elsewhere.\textsuperscript{12} The Wilcoxon signed rank test was used for statistical calculation. A probability value of <0.01 was considered statistically significant.

Results
To target c-Myc expression to the endothelium, mice carrying a tet operator--driven human c-Myc transgene and luciferase reporter gene (tetO-Myc)\textsuperscript{8} were crossed with a second transgenic mouse line (Tie2-tTA) that expresses the tetracycline-controlled transactivator under the control of the murine endothelial cell--specific Tie2 promoter/enhancer elements (Figure 1a).\textsuperscript{13} In the absence of tetracycline/doxycycline, this gene expression system is turned on. Addition of doxycycline shuts off Myc transgene expression. During pregnancy, mice were treated either with doxycycline, added to the drinking water, or left untreated. To assess tTA-mediated Myc expression, RNA was isolated from the lungs of E14.5 embryos. RT-PCR analysis revealed that Myc expression was only detectable in Tie2-tTA/tetO-Myc (double-transgenic) embryos from non--doxycycline-treated mothers (Figure 1b).
Immunofluorescence analyses revealed no significant changes in expression of these junctional proteins (Figure 3a and Online Figure II). To test whether edema formation was associated with an immature vascular phenotype, we analyzed the distribution of pericytes along the vasculature, which are required for stabilization of maturing vessels. Pericyte staining with an NG2-specific antibody did not reveal obvious differences between control and subcutaneous edematous tissues of double-transgenic embryos (Figure 3b). We also investigated the integrity of the basement membrane in the dermal vasculature. Laminin expression and organization was normal in double-transgenic animals (Online Figure III, a). In addition, organization of smooth muscle cells covering dermal arteries was analyzed with an α-smooth muscle actin antibody, and again no differences were observed (Online Figure III, b). Matrix metalloproteinases (MMPs) are responsible for remodeling of the extracellular matrix during development and disease.14 Because Myc was shown to induce MMP-9 expression in endothelial cells, we assessed MMP-9 protein levels. Slightly increased MMP-9 protein expression levels were noticed in the lungs of double-transgenic embryos at E15.5 (Online Figure III, c).

Edema formation could also be attributable to lymphatic dysfunction. Therefore, the lymphatic vasculature of E14.5 embryos was assessed with LYVE-1–specific antibodies (Online Figure IV). This analysis revealed no differences between lymphatic vessels of control and Tie2-tTA/tetO-Myc embryos. Further immunohistological analysis showed that lymphatic vessels were intact, and no blood cells were present in lymphatic vessels adjacent to blood vessels (Figure 3c, left images). Examination of both blood and lymphatic vessels revealed that Tie2-tTA/tetO-Myc embryos have unaltered numbers of normally sized lymphatic vessels in the dermis and in the jugular area (Figure 3c, middle and right images). Subsequently, ultrastructural analyses of embryonic endothelium at stage E14.5 were performed by transmission electron microscopy. At least 20 vessels per section of 7 Tie2-tTA/tetO-Myc or control embryonic skins were investigated. The majority of vessels in wild-type and single-transgenic control embryos were intact (Figure 4a). However, in some cases, the surface of endothelial cells was enlarged (Figure 4b) or endothelial cells were necrotic or the circumference of the endothelial lining was discontinuous, leading to local diapedesis of erythroblasts (data not shown). Approximately 300 vessel profiles were inspected on multiple sections without any indication of apoptosis. In contrast, the blood vessels of double-transgenic mice frequently revealed individual apoptotic cells interspersed within apparently normal endothelium (Figure 4c through 4f). Apoptotic cells were found between completely normal endothelial cells with apparently intact tight junctions between the apoptotic and the normal cell (Figure 4c). A total of 80% of vessel profiles showed single apoptotic figures. Apoptosis of individual endothelial cells could well result in a breach of the vascular barrier and thus in a strongly increased permeability and an accumulation of fluid in the interstitium. In other cases, 40% of the vessel profiles showed apoptotic figures and the endothelial lining was completely destroyed, resulting in a loss of contacts between individual cells (Figure 4d and 4e). It is noteworthy that endothelial tight junctions, which constitute the structural and functional elements of the vascular...
permeability barrier, remained unchanged in the vasculature of double-transgenic embryos, except between 2 neighboring apoptotic cells (Figure 4d and 4e, arrows). In 2 of 7 Tie2-tTA/tetO-Myc embryos, an extreme condition was reached, when endothelial cells were completely dissolved in apoptotic bodies (Figure 4f); such defects were never found in wild-type or single-transgenic embryos.

Previous experiments have documented a critical role of Myc in regulating both cell proliferation and apoptosis. To determine whether Myc affected these processes, apoptosis and proliferation of embryonic endothelial cells were measured in control and double-transgenic embryos (Online Figure V). This analysis revealed a 1.5- to 2-fold increase of apoptotic endothelial cells in double-transgenic embryos at E14.5 and E15.5, respectively (Figure 5a). Interestingly, there is a compensatory increase in the number of proliferating (Ki67-positive) cells (Figure 5b) at E15.5. The net result of increased apoptosis and proliferation is a largely unaltered absolute number of endothelial cells (Figure 5c), apparently at the expense of blood vessel integrity.

Myc-deficient mice die at E10.5 with defects in vasculogenesis and angiogenesis; however, a recent analysis suggests that this is largely attributable to Myc activity in hematopoietic cells. We therefore analyzed the embryonic vasculature in our model in more detail. Whole-mount stainings were performed using antibodies specific for the endothelial cell marker CD31/platelet endothelial cell adhesion molecule (PECAM)-1. No obvious differences were found in E9.5 embryos suggesting that vasculogenesis occurred normally (Online Figure VI). However, the vascular network in E14.5 Myc-overexpressing embryos was significantly different. Fewer branches were formed, indicating defective angiogenesis or enhanced pruning of vessels (Figure 6a). To quantify these alterations, statistical analyses were performed on distinct parameters that define the vascular network. The degree of branching was determined as the number of branching points per area, as well as the number and mean length of vessels (for details on the statistical analysis, see Materials and Methods). These analyses revealed a striking reduction in branching points and correspondingly fewer vessels in the double-transgenic animals (Figure 6b, upper panels). Consistent with this sparsity of segments, the mean length of a vessel in between branching points was increased (Figure 6b, lower left graph). In addition, we also counted vessel sprouts that had not successfully connected to another vessel. The number of such sprouts was clearly increased in double-transgenic animals as compared to controls (Figure 6b, lower right graph), indicating an increase in ongoing but not yet completed angiogenic activity. Statistical analysis of E13.5 embryonic back skin vascular architecture revealed no significant differences in the number of branching points, number of vessels, and the mean length of vessels between the control and double-transgenic embryos (Online Figure VII). These data suggest that blood vessels are formed normally up to E13.5 in Tie2-tTA/tetO-Myc embryos.
To elucidate the underlying molecular mechanisms, quantitative RT-PCR analysis of candidate target gene expression was performed in isolated embryonic endothelial cells. Endothelial cells were purified as CD31/PECAM-1 and CD105/endoglin double-positive cells from double-transgenic and control embryos at E14.5. Fluorescence-activated cell-sorting analyses revealed a 92% to 98% purity of the sorted CD31high endothelial cells. Myc transgene expression was analyzed in the enriched endothelial cells (Figure 7a) and detected specifically in the double-transgenic endothelial cells only. Only low levels of luciferase expression were detected in the CD31−/CD105− double-negative cell fractions of Tie2-tTA/tetO-Myc embryos (Figure 7b). Earlier experiments in Myc-deficient mice had revealed that VEGF-A expression requires Myc activity. Consistently, in endothelial cells derived from double-transgenic embryos, we saw a more than 2-fold upregulation of VEGF-A expression (Figure 7c). Given that levels of VEGF-A are tightly regulated and that mutation of a single allele of VEGF-A results in early embryonic lethality,15,16 the increased expression levels of VEGF-A are of relevance. Importantly, we also saw an increase in the levels of Ang-2, a proangiogenic factor and potential antagonist of Ang-1. Ang-1 levels were only marginally affected leading to a shift in the net Ang-1/Ang-2 balance toward a prevalence of

![Figure 4](image1.png)

**Figure 4.** Electron microscopic analyses of dermal blood vessels in Myc-overexpressing mice. a and b, Single-transgenic mice. c through f, Double-transgenic mice. a, Normal vessel with an erythroblast in its lumen. The endothelial cell sheet is continuous and interconnected by tight junctions (arrows). b, Blood vessel with apparently obliterated lumen (asterisks label the rest of the lumen) and increased surface. Endothelial cells are neither necrotic nor apoptotic and interconnected by tight junctions (arrows). c, Blood vessel with normal shape and continuous lining with a single isolated apoptotic endothelial cell (thick arrow). d, Blood vessel with an almost completely dissociated lining. Tight junctions between endothelial cells are still found (thin arrows). Most endothelial cells are dissociated from each other leaving gaps between them (thick arrows). Apoptotic endothelial cells are found (A). e, Higher degree of endothelial apoptosis (A) in a blood vessel revealed by the typical segregation and condensation of nuclear chromatin. Some endothelial tight junctions are maintained, confirming the presence of tight junction proteins. f, Culmination of endothelial apoptosis (A) evident by the blebbing of both nuclear and cytoplasmic apoptotic bodies.

![Figure 5](image2.png)

**Figure 5.** Endothelial cell proliferation and apoptosis. Flow cytometric analysis was performed on cells from E14.5 and E15.5 embryos. a, Quantification of endothelial cell apoptosis using double staining for cleaved caspase-3 and CD31/PECAM-1. In total, 26 control animals (wild-type or single-transgenic, gray bars) and 11 double-transgenic Tie2-tTA/tetO-Myc animals (black bars) were analyzed. Elevated apoptosis was found in Tie2-TTA/tetO-Myc embryos. **P<0.005. b, Quantification of endothelial cell proliferation using staining for the Ki67 antigen as proliferation marker and CD31/PECAM-1 staining to identify endothelial cells. Proliferation was measured in a total of 21 control embryos (wild-type or single-transgenic, gray bars) and 15 Tie2-tTA/tetO-Myc double-transgenic embryos (black bars). The proliferation rate is substantially higher in E15.5 Tie2-tTA/tetO-Myc embryos. *P<0.05. c, Overall endothelial cell number did not change significantly. In total, 47 controls (gray bars) and 26 Tie2-tTA/tetO-Myc embryos (black bars) were analyzed.
Ang-2. Expression of several other candidate genes that could explain the observed phenotype in vascular architecture and permeability, including the notch ligand Delta-like 4, was not altered.

Recent publications report the important role of adrenomedullin (AM) signaling during vascular development. Embryos lacking either AM, or calcitonin-receptor like receptor, the mediator of AM signaling, or receptor activity modifying protein (RAMP)-2 develop severe edema and die at mid-gestation. We therefore assessed the expression of AM pathway signaling molecules in sorted endothelial cells and found a down regulation of RAMP-2 and an upregulation of AM (Online Figure VIII). Given the increased numbers of sprouts, we analyzed expression of platelet-derived growth factor-B, a marker of sprouting endothelial cells, but could not detect any differences (Online Figure VIII).

To determine whether the 2-fold increase in VEGF-A RNA level was also reflected by an increased protein concentration, we measured VEGF-A protein level in wild-type and Tie2-tTA/tetO-Myc embryos by a VEGF-A–specific ELISA. This analysis revealed increased VEGF-A levels in Myc-expressing embryos at all time points of embryonic development analyzed (Figure 7d).

Figure 6. Morphometric analyses of back skin vascular architecture from control and Tie2-tTA/tetO-Myc embryos at E14.5. a, Representative images of CD31/PECAM-1 whole-mount immunofluorescence stainings used for morphometric analyses. Identical magnification was used for both images. In all cases, identical anatomic areas from the back of the embryos were analyzed. The obvious morphological differences between control and Tie2-tTA/tetO-Myc embryos are quantified below. Scale bars: 100 μm. b, Branching points per area, vessel number per area, mean vessel length, and number of incomplete sprouts per image were quantified in control (wild-type or single-transgenic) and Tie2-tTA/tetO-Myc embryos. In both groups, a total of 6 animals and 2 images per animal were analyzed. *P<0.01 (Wilcoxon test).

Discussion

In Tie2-tTA/tetO-Myc embryos, the vasculature established during vasculogenesis and angiogenesis was normal until E13.5. Blood vessels in edematous areas of the embryonic skin at E14.5 still had normal vessel morphology with fully developed basement membranes, extracellular matrix, and an intact pericyte and smooth muscle cell cover. However, electron microscopic investigations revealed accumulations of interspersed apoptotic endothelial cells, which could explain, at least in part, the interruption of vascular integrity and increased vessel permeability. Importantly, tight junctions were intact between endothelial cells, indicating that tight junctional failures did not critically contribute to vascular leakage.

The mitogenic and proapoptotic properties of Myc are genetically inseparable. Ectopic expression of Myc is sufficient to drive many cells into the cell cycle even in the absence of external mitogens. However, Myc also promotes apoptosis, although the precise mechanisms by which this occurs have not been completely elucidated. Therefore, it is not unexpected that endothelial cell–specific expression of c-Myc results in elevated cell cycle progression and at the same time increased levels of apoptosis. Although these 2
effects are balanced with no overall loss of endothelial cells, these processes could lead to transient impairment of endothelial integrity that might contribute to the observed leakiness.

In addition, our analyses revealed a second mechanism by which c-Myc expression might affect vascular permeability. Expression levels of VEGF-A were found to be elevated significantly both by RNA and protein analyses in double-transgenic endothelial cells. VEGF-A induces vascular leakage and is also known as vascular permeability factor.20 The levels of VEGF-A expression during embryogenesis need to be tightly regulated. It has been shown that 2- to 3-fold overexpression of VEGF-A results in severe abnormalities in embryonic heart development, edema formation, and embryonic lethality at E12.5 to E14.21,22 On the other hand, inactivation of a single VEGF-A allele in mice already resulted in embryonic lethality between E11 and E12.15,16 Previously, it has been reported that VEGF-A expression is reduced in Myc-deficient mice, and an indirect mechanism of gene regulation by c-Myc was proposed.7 Our results mirror these earlier reports, because we saw a comparable increase of VEGF-A levels in c-Myc–overexpressing endothelial cells.

In addition to its strong angiogenic effect, VEGF-A overexpression in mouse skin induced enlargement of lymphatic vessels.23 Moreover, several studies suggest that VEGF-A/VEGFR-2 signaling may also directly affect the lymphatic endothelium.3,24,25 We therefore analyzed the gross structure of lymphatic vessels in double-transgenic mice but did not detect any obvious alterations. Thus, the edema observed in Tie2-tTA/tetO-Myc double-transgenic embryos apparently is not the result of compromised lymph vessel function.

Mouse embryos lacking AM signaling have also been shown to develop massive edema resulting from combined defects in both blood and lymphatic vessels.26 We found that RAMP-2 is slightly downregulated in Tie2-tTA/tetO-Myc double-transgenic embryos, and AM expression is enhanced, most likely to compensate for the deficiency in functional AM receptors. Importantly, Tie2-tTA/tetO-Myc embryos show a normally developed lymphatic vasculature, suggesting that this reduced AM signaling did not affect lymphatic development.

Ang-2 plays an important role in the regulation of vascular remodeling. It can destabilize the endothelium and thereby prime it to acquire responsiveness to other growth factors. In the presence of VEGF-A, Ang-2 cooperates with VEGF-A to promote sprouting, proliferation, and migration of endothelial cells. By contrast, in the absence of VEGF-A, Ang-2 upregulation promotes vascular destabilization and subsequent vessel regression.27 Although Tie2-tTA/tetO-Myc embryos have elevated amounts of VEGF-A and an even further elevated expression of Ang-2, they do not show an increase in angiogenesis as might be expected. Instead, elevated expression of VEGF-A and Ang-2 in endothelial cells is accompanied by inadequate vascular remodeling, which results in the establishment of an aberrant blood vessel architecture. Despite an increased number of apparently abortive vascular sprouts, the deficiency in vessel density is not successfully compensated. We propose that these vascular phenotypes are
the consequence of a perturbed ratio of proliferation and apoptosis induced by Myc overexpression in endothelial cells. Myc-induced expression of the proangiogenic factors VEGF-A and Ang-2 is not sufficient to compensate for the dysregulation of proliferation and cell death induced in Myc-expressing endothelial cells (Figure 8).

Several recently published studies have demonstrated that Notch signaling regulates the sprouting and branching behavior in vessels by influencing the differentiation, migration, and proliferation of vascular tip cells. Reduced Notch signaling results in increased numbers of tip cells, filopodia extension, and vessel branching. The Notch ligand Delta-like-4 has been shown to mediate these effects. We have analyzed Delta-like-4 expression in the embryonic endothelial cells but did not find any evidence for alterations. The analysis of platelet-derived growth factor-B expression as sprout marker revealed no differences between Tie2-tTA/tetO-Myc and control embryos. Therefore, sprouts could be a result of an elevated angiogenic activity triggered because of elevated VEGF-A expression. Alternatively, sprout-like figures could arise as result of vessel involution caused by endothelial cell apoptosis.

The role of Myc in vasculogenesis and angiogenesis in different cell types has been addressed in a recent study, where the c-myc gene was deleted cell type-specifically in 80% of endothelial cells. This still allowed the development of a largely normal vascular system. However, when Myc is also deleted in the hematopoietic system, angiogenesis is severely affected. Our results on conditional endothelium-specific expression of c-Myc extend these earlier studies. Both loss of Myc and its overexpression are incompatible with normal vessel development. These data imply that fine-tuned expression of c-myc during vasculogenesis and angiogenesis is a prerequisite for the establishment and/or maintenance of functional vascular structures, and they identify at least some of the Myc-regulated mediators in these processes.

Combining the results of the c-Myc knockout studies with our analyses, it is evident that c-Myc is required for proper vascular development. Myc plays a role both in endothelial cells as well as in interacting compartments. The studies reveal that Myc coordinates the expression of angiogenic factors required for normal and pathological vascular development.

Acknowledgments
We thank Ralf Marienfeld and Balazs Hauser for critical reading of the manuscript and helpful suggestions; Tatiana Petrova for methodical help; and Gabi Frommer-Kästle for technical assistance with electron microscopical preparations.

Sources of Funding
This work was supported by German Science Foundation grant DFG-SFB451 (to T.W.).

Disclosures
None.
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_Circ Res._ 2009;104:1151-1159; originally published online April 30, 2009;
doi: 10.1161/CIRCRESAHA.108.191460

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/10/1151

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Expanded Materials and Methods

Animal models

In order to activate conditional expression of Myc in embryonic endothelial cells, two transgenic mouse lines were combined. The Tie2-tTA mouse line expresses the tetracycline-transactivator under the control of the Tie2 endothelial-cell-specific promoter. The tetO-Myc line contains the human Myc cDNA and a luciferase reporter gene. In the Tie2-tTA/tetO-Myc double transgenic animals, tTA mediates the transcription of the luciferase reporter gene and simultaneously the Myc proto-oncogene specifically in endothelial cells. Both genes are repressed in the presence of doxycycline (Tet-Off system).

F1 embryos were used for experimental investigations deriving from intercrosses between tetO-Myc (NMRI outbred) and Tie2-tTA (C57BL/6 background) mouse lines. The age of embryos subject to analysis was determined via vaginal plug control on the E0.5 day. To suppress expression of Myc transgene during embryonic development, doxycycline (ICN Biomedicals, Inc.) was given in the drinking water of pregnant mothers at a concentration of 100 µg/ml. Animal studies were approved by the Institutional Animal Care and Use Committee of the Ulm University.

Immunohistochemistry

For immunohistochemistry tissues were stained with CD31/PECAM-1 (clone MEC 13.3; BD Biosciences Pharmingen), NG2 (Chemicon), Laminin and α-SMA FITC (Sigma-
Aldrich), LYVE-1 (R&D Systems), LYVE-1 (ReliaTech GmbH), ZO-1, ZO-2, Claudin-5 and Occludin (Zymed) antibodies and Alexa 488-, 555- and 594-conjugated secondary antibodies (Molecular Probes).

Whole-mount immunohistochemistry was performed on 4% paraformaldehyde fixed tissues and stained with the appropriate antibodies. Tissues for light microscopy images were embedded in Tissue-Tek O.C.T. Compound (Sakura) and cryostat sections of 6 µm size were prepared.

All fluorescently labeled samples were analyzed using a fluorescence microscope (Axiovert 200M; Carl Zeiss) equipped with a digital camera (AxioCam MR3, Carl Zeiss). Leica DMIRB light microscope equipped with Jenoptik (ProgRes C14) camera and OpenLab software (version 4.0.4) was used for visualization of LYVE-1 stained samples. Whole embryo photographs were taken with Leica MZ7.5 stereomicroscope.

**LYVE-1 staining quantification**

Lymphatic vasculature in the skin and jugular lymph sac area was analyzed from double fluorescence immunostained sagittal embryonic cryosections by E14.5 animals. Fluorescence images were taken from sections stained with LYVE-1 (green) and CD31/PECAM-1 (red) antibodies. The single color images were converted to 8-bit grayscale using Adobe PhotoShop software (San Jose, CA). Then images were transformed into binary images by automatic thresholding using ImageJ software in order to classify LYVE-1 and CD31/PECAM-1 positive area, respectively 3. As a quantification of LYVE-1 staining, the LYVE-1 positive area divided by the CD31/PECAM-1 area was estimated 4.
Flow cytometry

Isolation of endothelial cells from murine tissue was performed according to the protocol described elsewhere. For sorting, cells were stained with CD31/Pecam-1 - FITC (BD Biosciences Pharmingen), CD105/Endoglin - bio (clone MJ7/18) and PE-conjugated Streptavidin (eBioscience) antibodies. For detection of apoptosis and proliferation, following kits were used: PE-conjugated monoclonal active caspase-3 antibody apoptosis kit and PE-conjugated mouse anti-human Ki67 monoclonal antibody set (both from BD Biosciences Pharmingen). Cells were analyzed, or sorted using FACSCalibur flow cytometer or FACSaria cell sorter instruments (BD Biosciences Pharmingen). The two-tailed paired Student's t test was used for statistical calculation. A \( P \) value <0.05 was considered statistically significant.

RNA Analyses

Total RNA was isolated from sorted endothelial cells using PicoPure RNA Isolation Kit (Arcturus Bioscience) and from embryonic lungs using High Pure RNA Isolation Kit (Roche). Further cDNA preparation protocol was described elsewhere. Real-time quantitative PCR was performed using FastStart SYBR Green PCR Kit (Roche) following manufacturer protocol. A \( P \) value <0.05 was considered statistically significant.

Primers sequences for RT-PCR:

\( \beta \)-Actin forward 5'-'GGTCAGAGGACTCTATGTG-3', reverse 5'-AGAGCAACATAGCAGGCTTCTC-3'

Human-Myc forward 5'-TTCCCCTACCCTCTCAACGACAG-3', reverse 5'-TCCTTACTTTTCTCTACGCACAA-3'

Primers used for quantitative real-time RT-PCR:
PBGD forward 5'-GACCTGGTTGTTCACTCCCT-3', reverse 5'-TGGGTGAAAGACACAGCAT-3'
TSP-1 forward 5'-GTGCTGCAGAATGTGAGGTT-3', reverse 5'-AAGAAGGACGTTGGTAGCTGA-3'
Ang-1 forward 5'-GCTAACAGGAGGTTGGTGGT-3', reverse 5'-GGTGGTGGAACGTAAGGAGT-3'
Ang-2 forward 5'-GTGCTGCAGAATGTGAGGTT-3', reverse 5'-AAGAAGGACGTTGGTAGCTGA-3'
Ang-1 forward 5'-GCTAACAGGAGGTTGGTGGT-3', reverse 5'-GGTGGTGGAACGTAAGGAGT-3'
Ang-2 forward 5'-GTGCTGCAGAATGTGAGGTT-3', reverse 5'-AAGAAGGACGTTGGTAGCTGA-3'
VEGFR-2 forward 5'-CCATTGGAGGAACCAGAAGT-3', reverse 5'-CTCTTCTGATGCAAGGACCA-3'
VEGF-A forward 5'-CACTGGACCCTGGCTTTACT-3', reverse 5'-TCACTTCCCATGGACTTCCTGC-3'
Nrp-1 forward 5'-AGGACCATACAGGAGATGGC-3', reverse 5'-AATAGACCACAGGGCTCACC-3'
DIl4 forward 5'-GAGGTCCAAGCCGAACCTG-3', reverse 5'-ATCGCTGATGTGCAGTTCACA-3'
PDGF-B forward 5'-GCACCGAAAGTTTAAGCACA-3', reverse 5'-AAATAACCCTGGCCACACTC-3'.

Quantitative real-time RT-PCR primers for AM, CRLR, RAMP-2, RAMP-3 and eNOS were described elsewhere.6,7

**VEGF-A and MMP-9 ELISA Measurements**

VEGF-A measurement was performed according to the protocol described elsewhere8 using VEGF-A ELISA detection kit (MMV00; R&D Systems). MMP-9 ELISA
measurement was performed with total MMP-9 protein detection kit (MMPT90; R&D Systems) according to the protocol provided by the company. The plates were read in a SpectraMax 190 microplate reader (Molecular Devices). The VEGF-A and MMP-9 concentration was normalized against the total amount of protein in the sample using Bradford protein assay.

**Reporter Detection / Western blot**

Luciferase activity measurements were previously described 2. Embryonic lung extracts were prepared using a 2 x concentrated sample buffer (70 mM Tris [tris(hydroxymethyl)aminomethane]; 11.15% vol/vol glycerol; 0.0015% bromphenol blue; 3% sodium dodecyl sulfate [SDS]; and 5% vol/vol 2-mercaptoethanol, pH 6.8, 6M Urea). Subsequent steps were performed as described elsewhere 9.

**Electron Microscopy**

For electron microscopy, different organs of embryonic stages of single or double-transgenic mice (such as heart, intestine, liver, kidney, skin of different locations) were fixed for 4 hours in 2.5% glutaraldehyde (Paesel and Lorei, Hanau, Germany) in 0.1 M cacodylate buffer (pH 7.4). Specimens were washed in pure cacodylate buffer, postfixed overnight in 1% OsO4 in cacodylate buffer for 1h, dehydrated in ascending series of ethanol and propyleneoxide, bloc-stained in uranyl-acetate for 4 h and flat-embedded in Araldite (Serva, Germany). Using an ultramicrotome (Ultracut, Leica, Bensheim, Germany),semi-(1µm) and ultrathin sections (50 nm) were cut. Ultrathin sections were stained with lead citrate, mounted on copper grids and finally analysed with a Zeiss EM 10 (Oberkochen, Germany) electron microscope.
Quantitative analysis of dermal vascular architecture

For the quantitative analysis of vascular architecture the blood vessels were regarded as a random network of line segments neglecting their width and curvature.

In order to transform the grayscale images of the blood vessels into a network of line segments, the images were first skeletonized. Therefore the varying brightness of the images was corrected and the images were smoothed by anisotropic diffusion. Then the smoothed images were transformed into binary images by thresholding. In the next step a distance transformation was applied and finally the transformed images were skeletonized using the watershed transformation. The used image processing techniques are described in detail elsewhere 10.

In the last step the skeleton was transformed into a network consisting of line segments as described previously 11. An image together with the network of line segments is displayed in Online Figure I.

Based on the network of line segments different statistical characteristics describing the spatial-geometric structure like the mean number of branching points and line segments both measured per unit area and in addition the mean length of the segments were estimated.

For the estimation procedure a sampling window $W$ with area $|W|$ was chosen. Then the number of branching points and segments in $W$ was counted and divided by $|W|$. A segment was counted if its lexicographically smallest point was inside $W$. Furthermore, the mean length of the segments in each images was estimated using again the line segments whose lexicographically smallest point was inside $W$. All these estimators are unbiased 12.
Supplemental References


Online Figure Legends

Online Figure I
A grayscale image of blood vessels together with the constructed network of line segments.

Online Figure II
Analysis of junctional connections between endothelial cells of dermal blood vessels at E14.5
Immunofluorescence staining shows adherens and tight junctional proteins expression in embryonic dermis for the indicated genotypes. Endothelial cells were stained with CD31/PECAM-1 (green). Junctional proteins were stained with ZO-1, ZO-2, occludin, or claudin-5 (red), and cell nuclei were visualized with DAPI (blue). Scale bars 20 µm.

Online Figure III
(a) Basement membrane examination on E14.5 embryos by whole-mount immunofluorescence. Double labeling for CD31/PECAM-1 (green) and Laminin (red) for the indicated genotypes is shown. Scale bar 20 µm.
(b) Smooth muscle cell examination on E14.5 embryonic back skin tissue by whole-mount immunofluorescence. Double labeling for CD31/PECAM-1 (red) and α-SMA (green) for the indicated genotypes is shown. Scale bar 63 µm.
(c) Total MMP-9 expression was determined by ELISA measurements. The MMP-9 concentration was normalized against the total protein in the tissue sample. MMP-9 levels were increased in lungs from double transgenic Tie2-tTA/tetO-Myc embryos at E15.5. SEM is shown as error bars.

**Online Figure IV**

Lymphatic vasculature was visualized with LYVE-1 specific antibodies by whole-mount immunofluorescence staining of embryonic back skin by E14.5 animals. Three pairs of representative embryos from the indicated genotypes are shown. Three different images of the skin lymphatic vessels from each animal are shown. No differences could be detected in the lymphatic vessel system of control and Tie2-tTA/tetO-Myc embryos with respect to vessel architecture, complexity and sprouting. In addition to lymphatic endothelial cells also on macrophages stains positive the LYVE-1 antibody (arrows). Scale bar 100µm.

**Online Figure V**

(a) FACS analysis of apoptotic endothelial cells with double immunostaining for CD31/PECAM-1 and Cleaved Caspase-3. A representative experiment is shown.

(b) FACS analysis of proliferating endothelial cells with double immunostaining for CD31/PECAM-1 and Ki67. Genotypes are indicated. A representative experiment is shown.
Online Figure VI

The vascular system of E9.5 embryos was visualized by whole-mount staining for CD31/PECAM-1. Representative images were taken from middle of the embryo (left) and a higher magnification from somites is shown at the right side. Scale bar 500µm (left) and 200µm (right). L: hind limb, H: heart

Online Figure VII

Morphometric analyses of back skin vascular architecture from control and Tie2-tTA/tetO-Myc embryos at E13.5. Branching points per area, vessel number per area, and mean vessel length per image were quantified in control (wt or single transgenic) and Tie2-tTA/tetO-Myc embryos. In both groups a total of n=6 animals and two images per each animal were analyzed. The differences between the control and Tie2-tTA/tetO-Myc embryos in the analyzed parameters are not significant (Wilcoxon test).

Online Figure VIII

Expression analysis of adrenomedullin signaling pathway molecules, eNOS, and PDGFβ in embryonic endothelial cells. Quantitative RT-PCR analyses of sorted endothelial cells from E14.5 wild type (gray bars) and double transgenic Tie2-tTA/tetO-Myc embryos (black bars). SEM is shown as error bars. * $P < 0.05$
Online Figure I
Online Figure V

a) control vs Tie2-tTA/tetO-Myc

B) control vs Tie2-tTA/tetO-Myc
Online Figure VIII

![Gene Expression Graph]

- AM
- RAMP2
- RAMP3
- CLRLR
- eNOS
- PDGFβ

Relative Gene Expression (PBGD)

wt
Tie2-tTA/tetO-Myc

* indicates statistical significance.
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