Myc regulates embryonic vascular permeability and remodeling

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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 over-expression resulted in severe defects in the embryonic vascular system. Myc expressing embryos suffer from widespread edema formation and multiple hemorrhagic lesions. They die between embryonic day (E) E14.5 and E17.5. 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 up-regulation of the pro-angiogenic factors VEGF-A and angiopoietin-2 (Ang-2). Thus, the net outcome of an excess of VEGF-A and Ang-2 in the face of an elevated cellular turnover appears to be a defect in vascular integrity.

Keywords: Myc, VEGF-A, vascular permeability, angiogenesis
Vascular development of the mouse embryo is governed by complex molecular and cellular processes and involves two distinct mechanisms. Vasculogenesis is responsible for de novo generation of vessels from angioblasts and occurs during the formation of the primitive vascular plexus in extra-embryonic tissues and within the embryo. Angiogenesis subsumes 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 (SMC) 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 prior to 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 two 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 \(^1\). Angiopoietins, the ligands for the Tie2 receptor have different functions during vascular development. Ang-1 acts to stabilize vessels, while 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 \(^3\).

The Myc-protein is known to regulate several aspects of blood vessel formation. Myc contains a basic-helix-loop-helix (bHLH) 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 \(^5\). Myc represses expression of the anti-angiogenic factor thrombospondin-1 \(^6\) and stimulates the pro-angiogenic factor VEGF-A \(^7\). Furthermore, many genes critical for diverse cellular functions like cell cycle progression, apoptosis and metabolism have been identified as Myc-target genes \(^8\).

Homozygous c-myc null mutation leads to lethality at embryonic developmental day E10.5 \(^9\). 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 \(^7\). A recent analysis of endothelial-cell-selective Myc deletion concluded that the vascular phenotype
observed in these mouse embryos may not be due to an endothelial-cell-autonomous effect of Myc\textsuperscript{10}.

In order 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, over-expression 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.

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 11. 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 by unbiased estimators described elsewhere 12. The Wilcoxon signed rank-test was used for statistical calculation. A p-value <0.01 was considered statistically significant.
Results

In order to target c-Myc expression to the endothelium, mice carrying a tet operator-driven human c-Myc transgene and luciferase reporter gene (tetO-Myc) 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). 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). The Myc transgene is expressed from a bi-directional promoter that allows luciferase gene transcription in the opposite direction. We observed high luciferase expression levels exclusively in non doxycycline-treated double transgenic embryos (Figure 1c). Finally, even though endothelial cells represent only a subset of cells in the lung, we were still able to detect increased Myc protein levels in double transgenic embryos as compared to wild type or single transgenic controls (Figure 1d and data not shown).

The analysis of offspring from mothers not treated with doxycycline during pregnancy revealed no life double transgenic animals. However, double transgenic mice were born at the expected Mendelian frequency when the pregnant mothers were treated with doxycycline in the drinking water (Online Table I). Litters of dams not treated
with doxycycline were analyzed at different time points of gestation to determine when embryos die. Double transgenic embryos with a normal appearance were present at normal frequency up to E12.5. The exact time point of death was somewhat variable between individual embryos. Some of the Tie2-tTA/tetO-Myc embryos had already died at E14.5, most of the animals were dead at E16.5, and not a single living double transgenic animal survived to E18.5 (Figure 2a). Double transgenic embryos displayed subcutaneous edema (Figure 2b, upper panel, embryo on the right and Figure 2c) and subsequently developed hemorrhages (Figure 2b, lower panels).

One possible cause of increased vascular permeability could be impaired tight junction formation. Therefore, expression of junctional proteins such as ZO-1, ZO-2, claudin-5, and occludin was assessed in embryonic skin. Immunofluorescence analyses revealed no significant changes in expression of these junctional proteins (Figure 3a, Online Figure II). To test if 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 IIIa). In addition, organization of smooth muscle cells covering dermal arteries was analyzed with an α-SMA antibody and again no differences were observed (Online Figure IIIb). Matrix metalloproteinases (MMPs) are responsible for remodeling of the extracellular matrix during development and disease. As 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 IIIc).

Edema formation could also be due 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, no blood cells were present in lymphatic vessels adjacent to blood vessels (Figure 3c left panels). 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 panels).

Subsequently, ultrastructural analyses of embryonic endothelium at stage E14.5 were performed by transmission electron microscopy (TEM). At least 20 vessels per section of seven 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). About 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-f). Apoptotic cells were found between completely normal
endothelial cells with apparently intact tight junctions between the apoptotic and the normal cell (Figure 4c). 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,e). 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 two neighboring apoptotic cells (Figure 4d,e arrows). In two out of seven 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 1.5-2 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 due 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/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 over-expressing 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 panel). 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 panel) 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.
In order 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. FACS analyses revealed a 92-98% purity of the sorted CD31<sup>high</sup> 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<sup>-</sup>/CD105<sup>-</sup> 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 two-fold up-regulation 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<sup>15, 16</sup>, the increased expression levels of VEGF-A are of relevance. Importantly, we also saw an increase in the levels of angiopoietin-2, a pro-angiogenic factor and potential antagonist of angiopoietin-1. Ang-1 levels were only marginally affected leading to a shift in the net Ang-1/Ang-2 balance toward a prevalence of 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 (CRLR), the mediator of adrenomedullin signaling, or receptor activity modifying protein-2 (RAMP-2) develop severe edema and die at midgestation<sup>17, 18</sup>. 
We therefore assessed the expression of AM pathway signaling molecules in sorted endothelial cells and found a down regulation of RAMP-2 and an up-regulation of AM (Online Figure VIII). Given the increased numbers of sprouts, we analyzed expression of PDGF-B, a marker of sprouting endothelial cells but could not detect any differences (Online Figure VIII).

In order to determine whether the twofold 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).
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 at least in part explain 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\(^\text{19}\). 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 two 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\textsuperscript{20}. The levels of VEGF-A expression during embryogenesis need to be tightly regulated. It has been shown that two- to threefold overexpression of VEGF-A results in severe abnormalities in embryonic heart development, edema formation and embryonic lethality at E12.5-E14\textsuperscript{21, 22}. On the other hand, inactivation of a single VEGF-A allele in mice already resulted in embryonic lethality between E11 and E12\textsuperscript{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\textsuperscript{7}. Our results mirror these earlier reports as we see a comparable increase of VEGF-A levels in c-Myc over-expressing endothelial cells.

In addition to its strong angiogenic effect, VEGF-A overexpression in mouse skin induced enlargement of lymphatic vessels\textsuperscript{23}. Moreover, several studies suggest that VEGF-A/VEGFR-2 signaling may also directly affect the lymphatic endothelium\textsuperscript{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 due to combined defects in both blood and lymphatic vessels\textsuperscript{26}. We found that RAMP-2 is slightly down regulated 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.

Angiopoietin-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 to promote sprouting, proliferation, and migration of endothelial cells. By contrast, in the absence of VEGF, Ang-2 up-regulation promotes vascular destabilization and subsequent vessel regression. 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 over-expression in endothelial cells. Myc-induced expression of the pro-angiogenic 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 \(^{34}\). The Notch-ligand Delta-like-4 (Dll-4) has been shown to mediate these effects. We have analyzed Dll-4 expression in the embryonic endothelial cells but did not find any evidence for alterations. The analysis of PDGF-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 due to 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 \(^{10}\), 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 \(^{7, 10}\). Both loss of Myc as well as its over-expression is 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.
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Disclosures

None.
References


32. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A.* 2007;104:3219-3224


Figure Legends

Figure 1.
Conditional expression of transgenic Myc in endothelial cells.
(a) Constructs for Myc endothelial cell specific expression in transgenic mice. Transgenic mice expressing the transactivator under the control of the Tie2 promoter/enhancer construct (tet-off system). The tetO-Myc mice carry the Myc gene under the control of a bidirectional promoter (co-expressing luciferase). (b) Expression of the human c-Myc transgene was measured in the lungs of E14.5 day embryos by RT-PCR. The lower panel shows a control RT-PCR for β-actin. Genotypes of mouse embryos are indicated at the top. Pregnant mothers were kept with or without doxycycline to control the tet-off system as indicated. (c) Luciferase activities were measured in E14.5 embryonic lung protein extracts. The respective genotypes and doxycycline treatment are indicated. A representative experiment is shown. (d) Western blot analysis of Myc protein expression in E14.5 embryonic lungs of one wild type and two double transgenic embryos. Genotypes are indicated. Expression of β-actin was determined as a control.

Figure 2.
Endothelial cell specific Myc expression results in embryonic lethality.
(a) Survival rate of Tie2-tTA/tetO-Myc transgenic embryos derived from crosses without doxycycline. A total of 66 double transgenic Tie2-tTA/tetO-Myc embryos were scored out of 231 embryos in total at the indicated days of embryonic development. Genotypes were determined both by luciferase measurements as well as PCR analyses. Beating of the heart was used to distinguish between live and dead embryos. Single transgenic or wild type animals were used as controls. (b) Gross
appearance of embryos at E16.5. Note the widespread subcutaneous edema (red arrowhead, upper panel) and multifocal hemorrhages (white arrowheads) on the extremities (upper right and lower right panels) and head (lower left panel). None of these alterations was seen in control littermates (embryo on the left upper panel). Scale bars 2 mm in upper panel, 400 µm in lower left and 350 µm in lower right panel. (c) Hematoxilin and Eosin staining of sagittal sections of E15.5 embryos. Widespread subcutaneous edema is visible. Scale bar 2 mm.

Figure 3.

Characterization of vessel structure in embryonic skin.

(a) Junctional protein analysis in E14.5 embryonic dermis by immunofluorescence. Double labeling for different junctional markers (red), such as ZO-1, ZO-2, occludin, and claudin-5, and nuclear stain by DAPI (blue) is shown for the indicated genotypes. Scale bar 20µm. (b) Pericyte coverage of embryonic blood vessels was visualized in E14.5 embryos by whole-mount immunofluorescence. Endothelial cells were stained with the CD31/PECAM-1 antibody (green), pericytes were stained with the NG2 antibody (red). Scale bar 20 µm. (c) Left panels show LYVE-1 immunohistochemical staining of lymphatic vasculature in E14.5 embryos and CD31/PECAM-1 staining of blood vessels from adjacent sections from wild type and double transgenic embryos. Lymphatic vessels are indicated by white arrows, blood vessels are indicated by black arrows. Scale bar 160 µm. On the middle panels double-immunofluorescence staining was performed with LYVE-1 (red) and CD31/PECAM-1 (green) antibodies. Scale bar 100µm. Quantification of lymphatic vessel area by LYVE-1 staining on embryonic skin and jugular area is shown on the right graphs.
Electron microscopical analyses of dermal blood vessels in Myc over-expressing mice.

A, B: single transgenic, C-F double transgenic. A: a normal vessel with an erythroblast in its lumen. The endothelial cell sheet is continuous and interconnected by tight junctions (arrows). B: a 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.

Endothelial cell proliferation and apoptosis.

Flow cytometry 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 (wt 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 (wt 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.

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 pictures. In all cases, identical anatomical 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 (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. *P<0.01 Wilcoxon test.

Figure 7.
Expression of angiogenic modulators in sorted E14.5 transgenic endothelial cells.

(a) Expression of transgenic human c-Myc mRNA was determined by RT-PCR in sorted endothelial cells from double transgenic and wild type control embryos.
Endothelial cell preparation was 92-98% CD31-positive. (b) Transgene expression is specific for the enriched endothelial cell population. Luciferase measurements were performed on 10,000 sorted cells. The double positive (CD31+/CD105+) endothelial cell fraction and the double negative (CD31-/CD105-) cell fraction from double transgenic Tie2-tTA/tetO-Myc as well as control embryos were analyzed. As expected, Tie2-tTA/tetO-Myc embryos show high luciferase activity. (c) Expression of angiogenic regulators in embryonic endothelial cells. Real-time quantitative PCR analyses of sorted endothelial cells from wild type (gray bars) and double transgenic Tie2-tTA/tetO-Myc embryos (black bars). SEM is shown as error bars. *P < 0.05 (d) VEGF-A expression was determined by ELISA measurements. The VEGF-A concentration was normalized against the total protein in the tissue sample. VEGF-A levels were increased in lungs from double transgenic Tie2-tTA/tetO-Myc embryos from day E13.5 onwards. SEM is shown as error bars. *P<0.05, **P<0.005

Figure 8.
Model illustrating the role of Myc during embryonic vascular development
In wild type embryos, vasculogenesis, angiogenesis, vessel maturation, and stabilization occurs in a highly coordinated manner. These processes are controlled by the balanced expression of angiogenic modulators. In the presence of excess Myc activity in endothelial cells vasculogenesis and angiogenesis occur normally up to E13.5. However, during consecutive blood vessel formation Myc affects expression of angiogenic regulators and endothelial-cell-turnover. Elevated VEGF-A and Ang-2 levels contribute to vascular hyperpermeability and edema. In addition, endothelial cell apoptosis results in a leaky vascular network and finally hemorrhages. Moreover, elevated expression of pro-angiogenic factors VEGF-A and Ang-2 impairs vessel
remodeling and results in aberrant vascular architecture and immature vasculature with ongoing angiogenic activities.
Figure 1

a) Diagram showing the relationship between Tie2-tTA and tetO-Myc under different conditions (+DOX and -DOX).

b) Gel images showing expression levels of Human-Myc and β-Actin under +DOX and -DOX conditions.

c) Graph depicting RLU/µg protein levels for different genotypes under +DOX and -DOX conditions.

d) Western blot images showing expression levels of Myc and β-Actin.
Figure 2

(a) Graph showing changes over time (E) for control and Tie2-tTA/tetO-Myc conditions.

(b) Images comparing control and Tie2-tTA/tetO-Myc conditions.

(c) Images comparing control and Tie2-tTA/tetO-Myc conditions.
Figure 3

a) ZO-1, ZO-2, Occludin, Claudin-5

b) CD31, NG2, Merged

c) LYVE-1, CD31, LYVE-1 / CD31

Graphs showing:
- Lymphoid vessels
- CD31
- LYVE-1 / CD31
Figure 5

(a) Fold Endothelial Cell Apoptosis

(b) Fold Endothelial Cell Proliferation

(c) Relative Endothelial Cell Number
Figure 6

a) 

wt

Tie2-tTA/tetO-Myc

b) 

branching points / mm²

vessel no. / mm²

mean vessel length (µm)

sprout number / image

C

Tie2-tTA/tetO-Myc

C

Tie2-tTA/tetO-Myc

C

Tie2-tTA/tetO-Myc

C

Tie2-tTA/tetO-Myc
Figure 8

a) Wild type

Vasculogenesis

Angiogenesis

Maturation, stabilization

de novo endothelial cell differentiation

Primary vascular plexus

Remodelled vascular network

Mature network

b) C-Myc overexpression in endothelial cells

Vasculogenesis

Angiogenesis

Maturation, stabilization

de novo endothelial cell differentiation

Primary vascular plexus

C-Myc overexpression

Altered EC proliferation and apoptosis

Leaky vascular network

Vascular hyperpermeability

Aberrant vascular architecture

Abortive sprouting activity

EC apoptosis