Supplement Material

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 [1]. The tetO-Myc line contains the human Myc cDNA and a luciferase reporter gene under the control of the tetracycline responsive bidirectional promoter (tetO) [2]. In 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 [5]. 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 [2]. 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:

β-Actin forward 5'-GGTCAGAAGGACTCCTATGTG-3', reverse 5'-AGAGCAACATAGCACAGCTTC-3'

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

Primers used for quantitative real-time RT-PCR:

PBGD forward 5'-GACCTGGTTGTTCACTCCCT-3', reverse 5'-TGGGTGAAAGACAACAGCAT-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'-GTCAACAACTCGCTCCTTCA-3', reverse 5'-GATTTCCGCACAGTCTCTGA-3'

VEGFR-2 forward 5'-CCATTGGAGGAACCAGAAGT-3', reverse 5'-CTCTTCTGATGCAAGGACCA-3'

VEGF-A forward 5'-CACTGGACCCTGGCTTTACT-3', reverse 5'-TCACTTCATGGGACTTCTGC-3'

Nrp-1 forward 5'-AGGACCATACAGGAGATGGC-3', reverse 5'-AATAGACCACAGGGCTCACC-3'

Dll4 forward 5'-GAGGTCCAAGCCGAACCTG-3', reverse 5'-ATCGCTGATGTGCAGTTCACA-3'

PDGF-B forward 5'-GCACCGAAAGTTTAAGCACA-3', reverse 5'-AAATAACCCTGCCCACACTC-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 elsewhere [8] 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 quantitive 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

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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

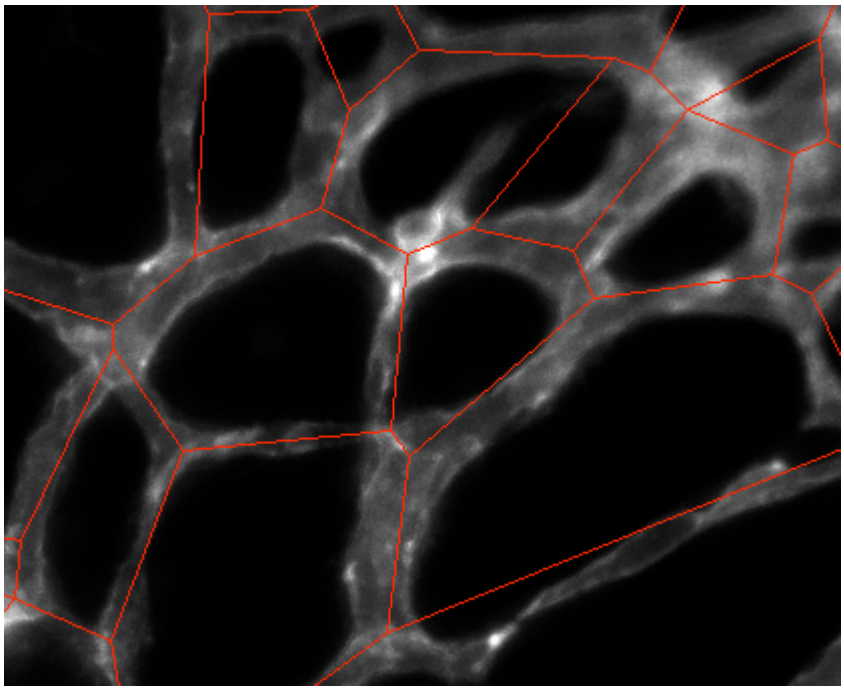
Online Figure VII

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



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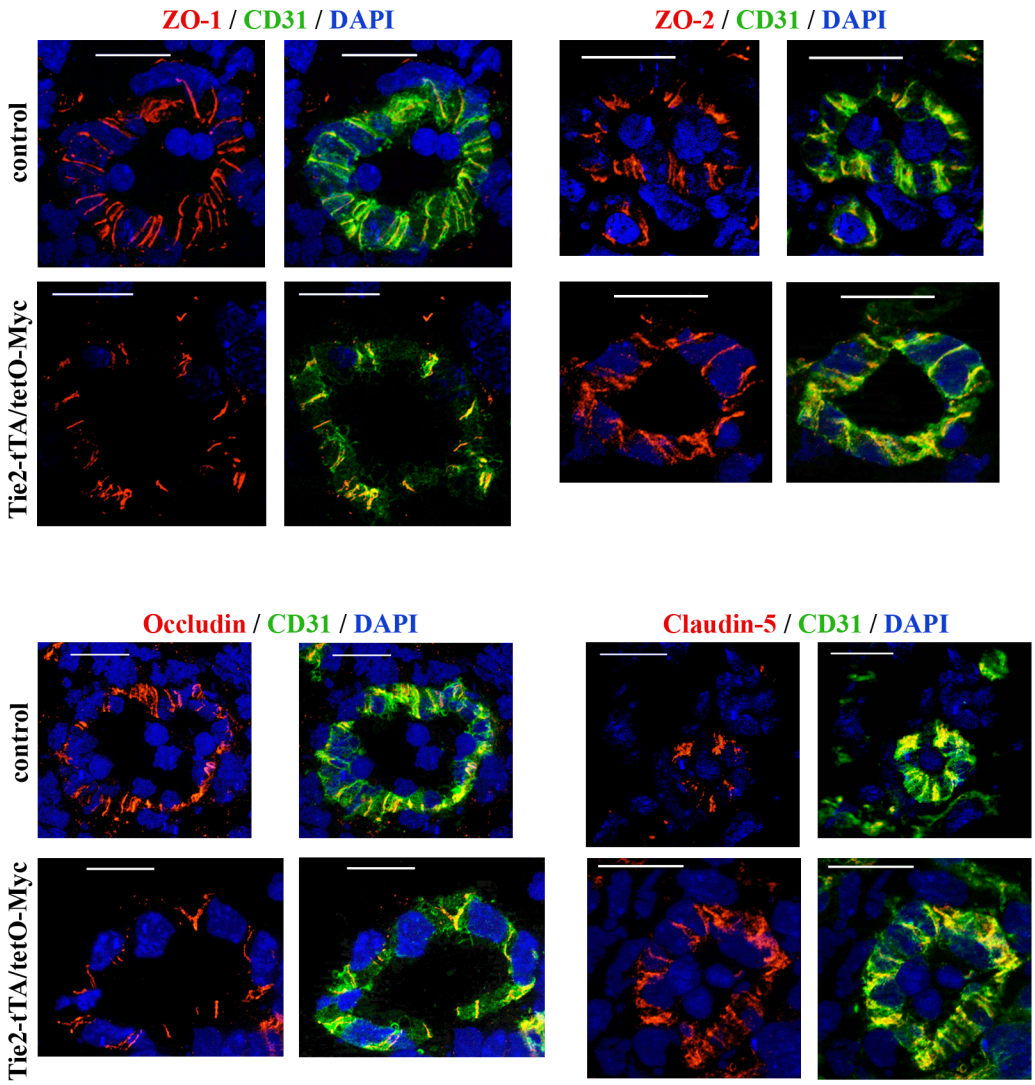

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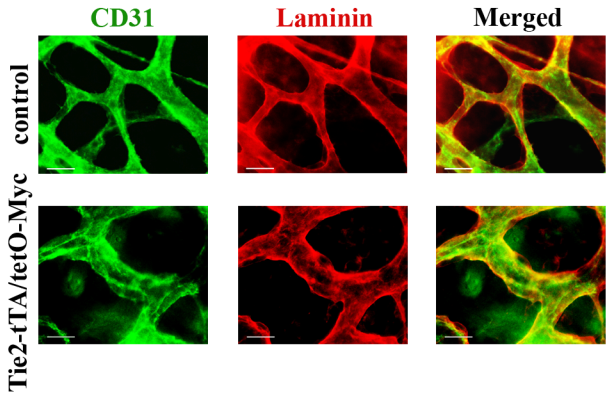

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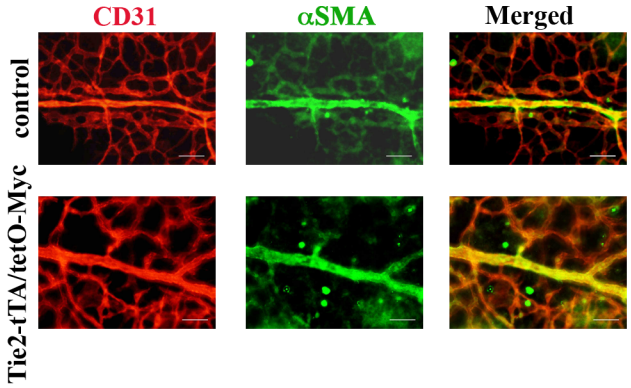

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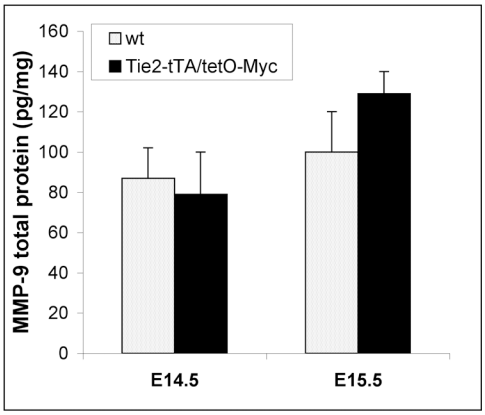

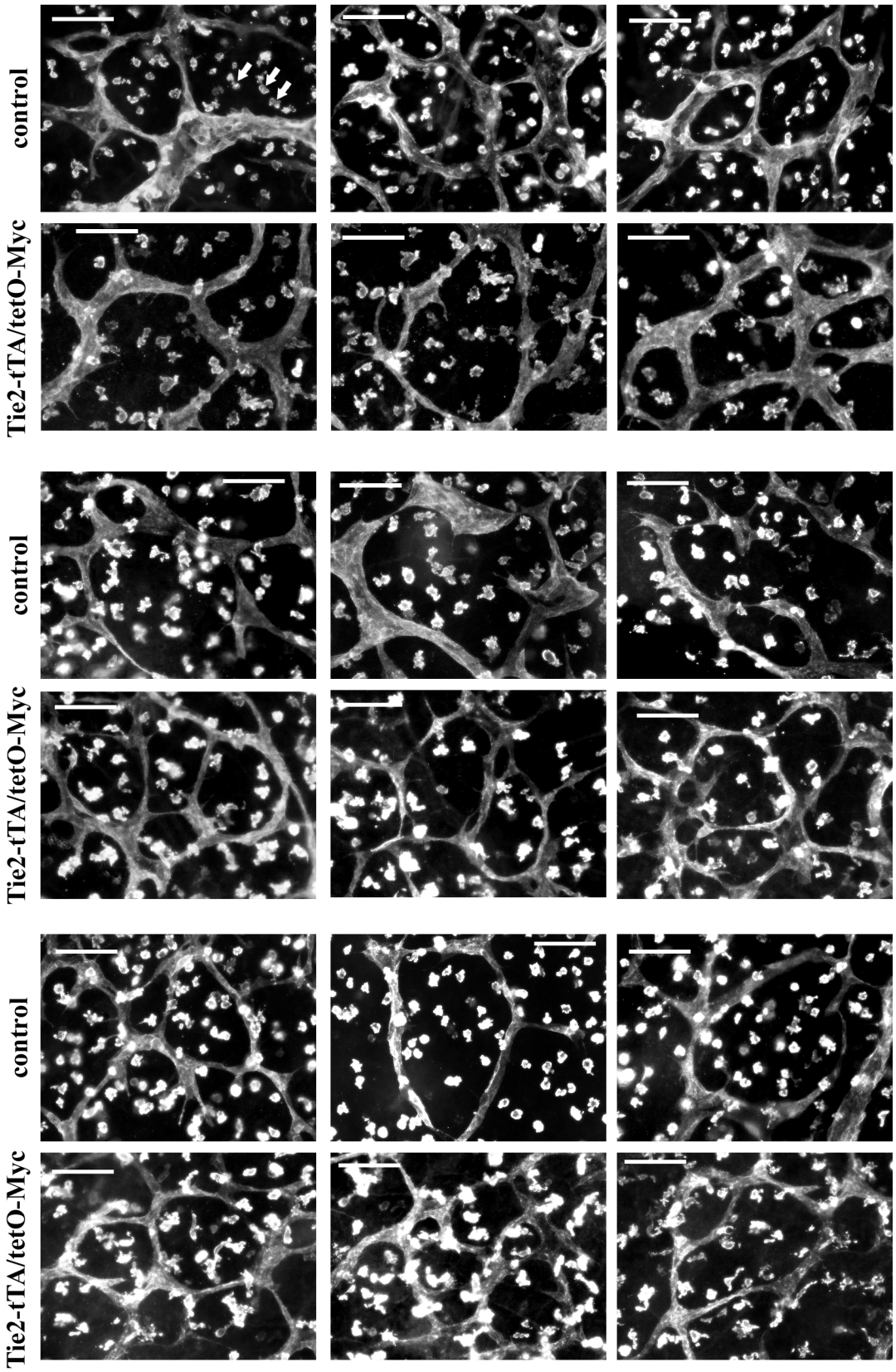
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Tie2-tTA/tetO-Myc
control
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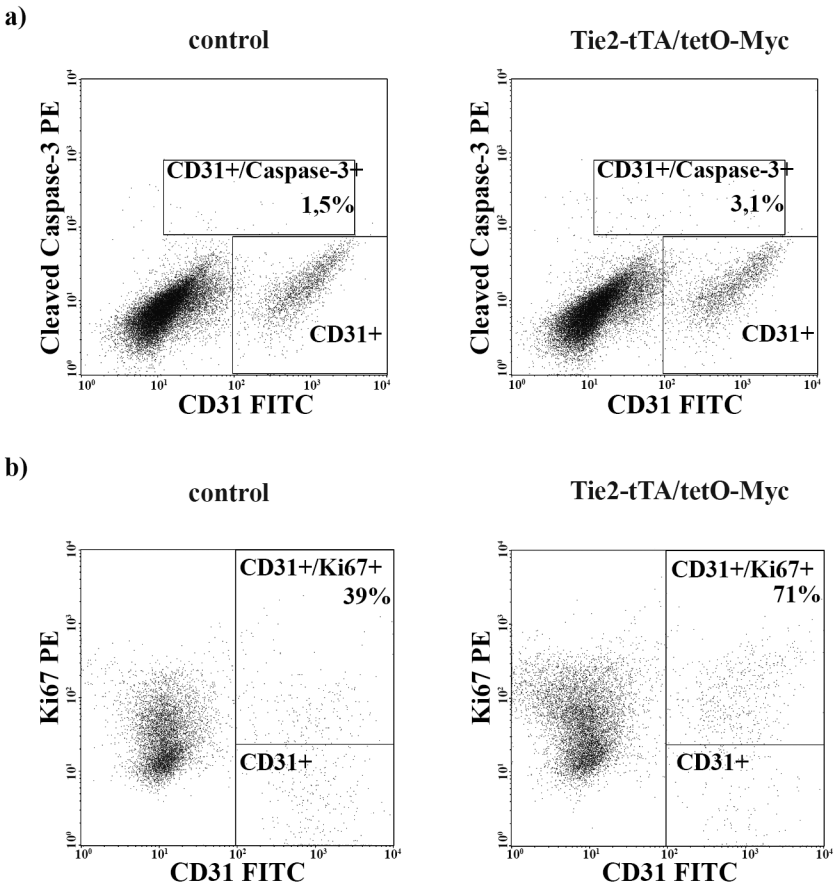
control
Tie2-tTA/tetO-Myc

Online Figure V



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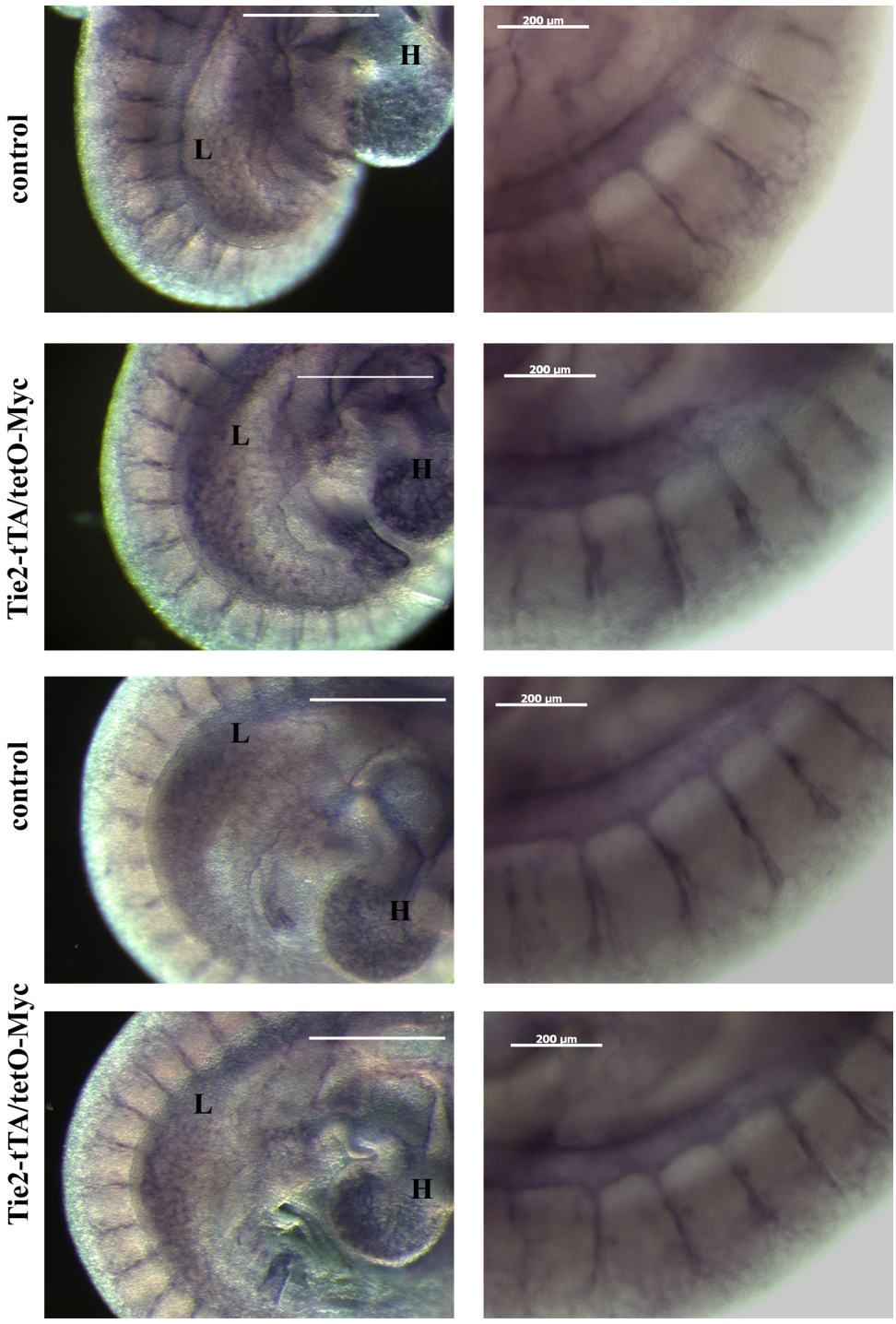

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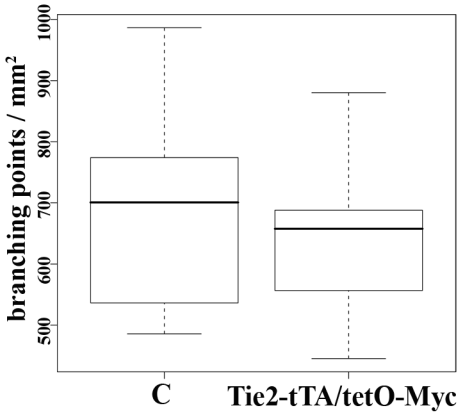
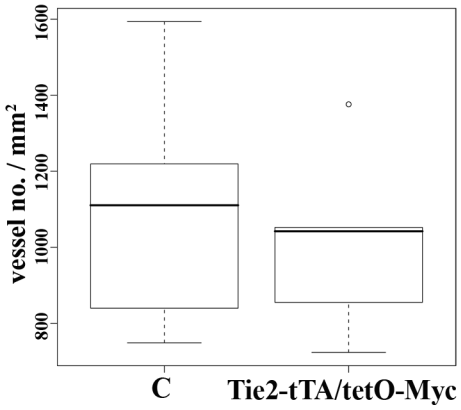
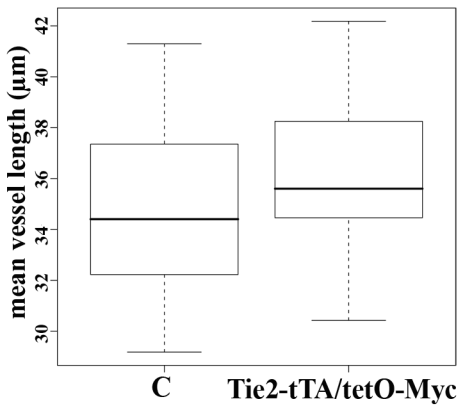
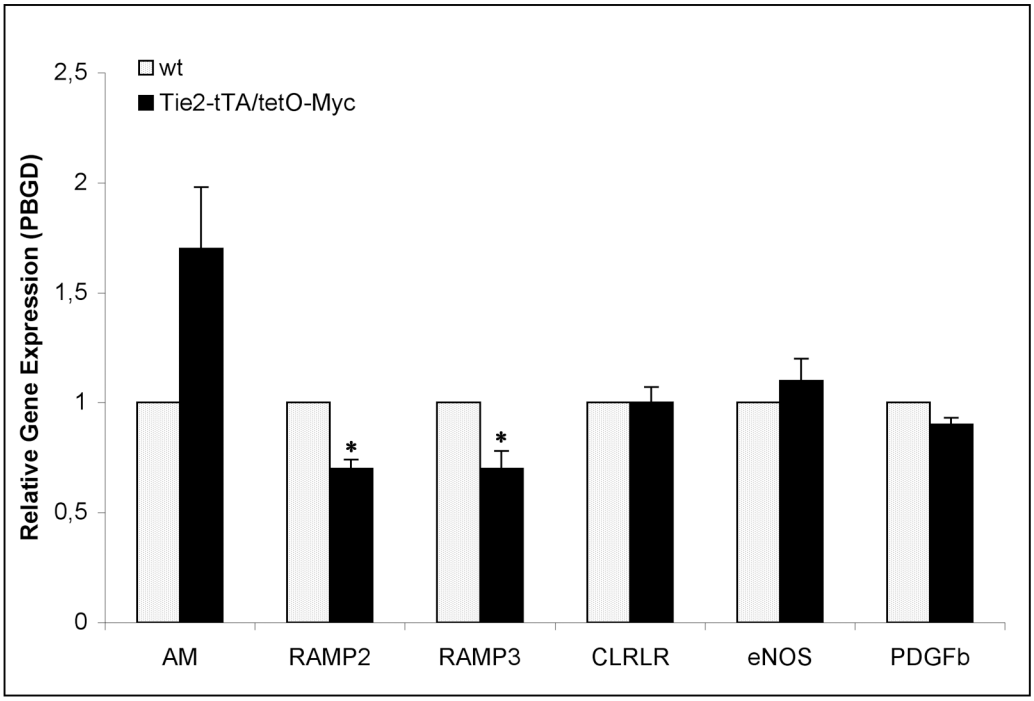






Online Figure VIII



Online Table I

	-DOX	+DOX
wt	**13**	**14**
tetO-Myc	**21**	**17**
Tie2-tTA	**11**	**13**
Tie2-tTA/tetO-Myc	**0**	**17**
Total	**45**	**61**