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A Genetically Engineered Mouse Model of Venous Anomaly and Retinal Angioma-like Vascular Malformation

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[Abstract] Characterization of key regulators in vein development will advance our understanding of mechanisms underlying venous anomalies and provide therapeutic targets for the treatment of vascular malformations. Here, we provide a detailed protocol for the generation of genetically engineered mouse models targeting the *Tek* gene for the analysis of vein formation and vein-associated vascular diseases at the embryonic and postnatal stages. It includes steps involved in the whole-mount processing of mouse skin, mesentery, and retina for the examination of vascular malformation during embryonic and postnatal development.

Keywords: TIE2, Vein development, Venous anomaly, Angioma-like, Retinal vascular tuft, Disease model

[Background] During embryogenesis in mammals, the blood vascular system is one of the first organs to develop from mesoderm-derived hemangioblasts. The developmental process includes the initial fusion of blood islands to form the primitive vascular plexus, followed by vascular specification to form a network comprised of arteries, veins, and capillaries. Characterization of the mechanisms underlying arteriovenous specification will advance our understanding of venous anomalies and provide therapeutic targets for the treatment of vascular diseases. Among the characterized regulators and pathways, the VEGF-A/VEGFR-2 pathway mediates the activation of RAF1 and ERK1/2 kinases to induce the expression of genes required for arterial development (Lanahan *et al.*, 2013; Deng *et al.*, 2013), including Delta-like 4 (Dll4)-mediated activation of NOTCH signaling (Lawson *et al.*, 2001; Duarte *et al.*, 2004; Wythe *et al.*, 2013). Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII, also known as Nr2f2) is a key regulator of venous identity via the inhibition of NOTCH-mediated signals (You *et al.*, 2005). Akt activation inhibits Raf1-ERK1/2 signaling in endothelial cells (ECs) to favor venous specification (Ren *et al.*, 2010); however, the upstream regulators of vein development are poorly characterized. TIE2 is a receptor tyrosine kinase that mediates angiopoietin signaling for EC survival and vascular remodeling and integrity (Augustin *et al.,* 2009). Patients with venous malformations possess *Tie2* missense point mutations (Vikkula *et al.*, 1996). TIE2 deficiency leads to embryonic lethality (Dumont *et al.*, 1994; Sato *et al.*, 1995). We have recently demonstrated that TIE2 is essential for the specification of venous EC identity via the Akt-mediated regulation of COUP-TFII protein stability

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(Chu *et al.*, 2016).

Here, we aim to describe a detailed protocol for analyzing the process of vein development and vein-associated vascular diseases in skin, mesentery, and retina, employing a genetically modified mouse model targeting the *Tek* gene (Chu *et al.*, 2016).

Materials and Reagents

1. Plastic pipette (disposable, 5 ml, cut the tip for tissue manipulation)
2. Needle (ENTO SPHINX s.r.o., 03.15-Minutens white, 03.20-Minutens white)
3. Insulin syringe (BD, Ultra-Fine®, catalog number: 328421)
4. Coverslips
5. 4% paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: 158127)
6. Skimmed milk powder (Valio)
7. Triton X-100 (Sigma-Aldrich, catalog number: V900502-100ML)
8. Glycerol (Sigma-Aldrich, catalog number: G5516-500ml)
9. Rat anti-mouse PECAM-1 (BD Pharmingen, catalog number: 553370)
10. Goat anti-mouse/rat TIE2 (R&D, catalog number: AF762)
11. Goat anti-mouse EphB4 (R&D, catalog number: AF446)
12. Cy3-conjugated anti-mouse αSMA (Sigma-Aldrich, catalog number: C6198)
13. Alexa Fluor 488-conjugated secondary antibody (Invitrogen, catalog number: A-21208)
14. Cy3-conjugated secondary antibody (Jackson, catalog number: 705-165-147)
15. Cy5-conjugated secondary antibody (Jackson, catalog number: 705-175-147)
16. Tamoxifen (Sigma-Aldrich, catalog number: T5648-5G)
17. Silicone rubber (SYLGARD™ 184 Silicone Elastomer Kit)
18. Phosphate-buffered saline (PBS), pH 7.4 (see Recipes)
19. PBS-TX (see Recipes)
20. 50% glycerol (see Recipes)

Equipment

1. Confocal laser scanning microscope (Olympus, model: FluoView)
2. Fluorescence stereomicroscope (Olympus, model: SZX16)
3. Dissecting scissors (Medical scissors; 66 Vision-Tech Co., catalog number: 54002, straight tip, 100 mm; Vannas capsulotomy scissors: Suzhou Mingren Medical Apparatus and Instruments Co., catalog number: MR-S302A, pointed tips, 16 mm blades)
4. Stereomicroscope (Motic, catalog number: SMZ168 Series, magnification range: 7.5-50×)
5. Micro-forceps (Shanghai Medical Instruments Group Ltd., catalog number: WA3040, 14 cm straight, head width 0.3 mm)

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Software

1. FV-ASW Viewer 3.0 or 4.2a

Procedure

A. Generation of *Tek*-targeted mouse models for vein analysis in skin and mesentery at the embryonic stage. This protocol is based on the paper by Chu *et al.* (2016).
   1. Prepare mouse embryos. Place one male mouse (*Tek*$^{+/-}$; *UBC-CreERT2*, ≥ 2 months old) and two female mice (*Tek*$^{Flox/Flox}$, ≥ 2 months old) into a cage for mating in the late afternoon around 18:00. Check females for vaginal plugs in the early morning of the following day (around 8:00). If the female mouse is pregnant, the embryonic stages are estimated considering midday of the day on which the vaginal plug is present as embryonic day 0.5 (E0.5).
   2. Induce gene deletion. Use sunflower seed oil (COFCO Fortune) as the diluent for the preparation of tamoxifen (tamoxifen free base, 10 mg/ml). Perform intraperitoneal injection of tamoxifen solution (100 µl, 10 mg/ml) from E12.5 to E14.5 (Figure 1A).
   3. Euthanize the pregnant female by cervical dislocation at E17.5 (embryonic day 17.5) and place the mouse on its back on a dissecting board. Use pins to attach the feet of the mouse to the board, and adequately soak the fur with 75% ethanol.
   4. Make a cut in the skin and the abdominal wall with scissors to expose the abdominal cavity and dissect the uterus with scissors.
   5. Separate embryos in ice-cold PBS by removing the uterine muscle layers, pulling the extra-embryonic membranes off with fine forceps, and removing the placenta under a dissecting microscope.
   6. Cut the tip off the tail of the embryos for genotyping.
   7. Peel back the skin with ophthalmic scissors and fix the skin tissues to the 6-well plate containing silicone rubber using fine needles (Figure 1B).
   8. Open the abdomen of the embryo. Cut a fragment of the small intestine long enough to form a ring to keep the mesentery intact for vascular visualization. Fix the intestine to the 6-well plate containing silicone rubber with fine needles (Figure 2A).
   9. Fix the tissues with 4% paraformaldehyde (PFA) at 4°C or on ice for 2 h. Wash 3 times with PBS solution for 5 min each.
   10. Block the tissues with PBS-TX solution containing 3% skimmed milk powder at 4°C for 6-8 h with gentle agitation on an orbital shaker.
   11. Remove the blocking solution and incubate the tissues with primary antibodies (PECAM-1, αSMA, TIE2; dilution according to the manufacturer's recommendation) in blocking solution (total volume: 1 ml) with gentle agitation on an orbital shaker overnight at 4°C.
   12. Wash the tissues 5 times with PBS-TX for 20 min each.
   13. Incubate the tissues with secondary antibodies (Alexa Fluor 488, Cy5-conjugated secondary

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antibodies) in PBS-TX solution (dilution 1:500) with gentle agitation on an orbital shaker overnight at 4°C.

14. Cut off the excess tissues. Transfer the trimmed skin onto a glass slide, add a drop of 50% glycerol, place a coverslip and seal with nail polish at the edges. For the analysis of intestines, transfer the trimmed intestine directly onto a coverslip and keep the mesentery fully stretched under a dissecting microscope. Avoid drying of tissues by adding drops of 50% glycerol.
15. Analyze blood vessels under a confocal laser scanning microscope (Olympus FluoView) and acquire and process images with the FV-ASW Viewer 3.0 /4.2a software (Figures 1C and Figure 2B).

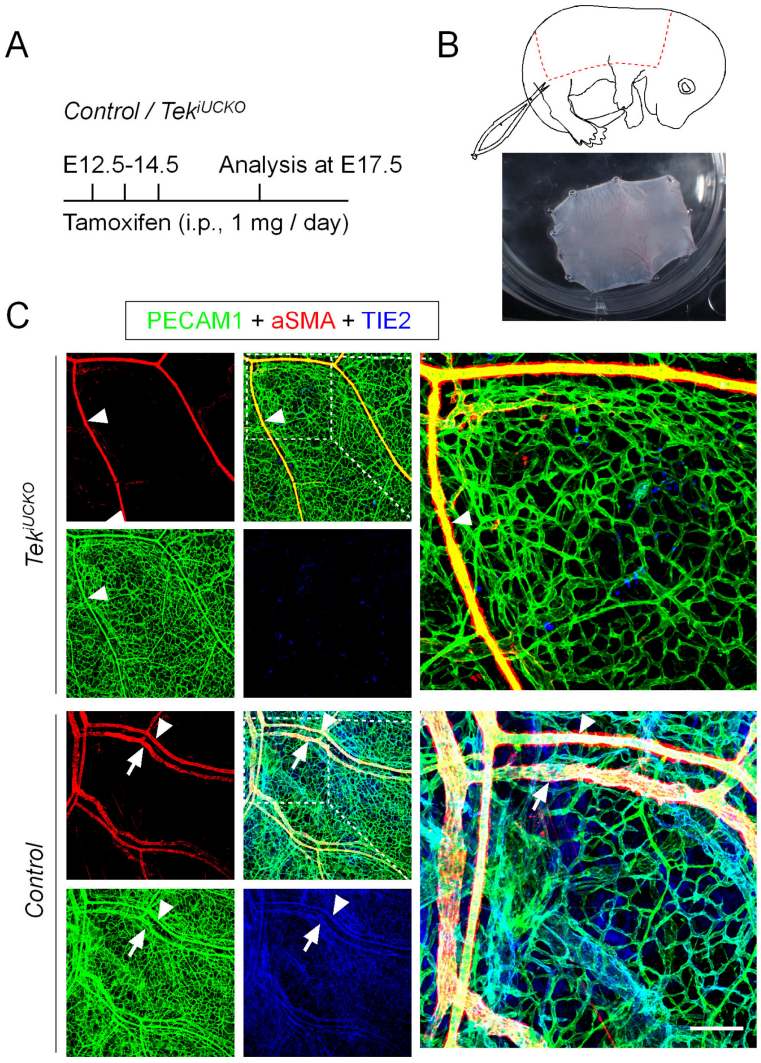

Figure 1. Disruption of cutaneous vein development following *Tek* deletion. (A) Tamoxifen intraperitoneal administration and analysis scheme. (B) Diagram showing the area of dorsal skin of the embryos dissected for analysis (dotted line). (C) Analysis of dorsal skin blood vessels by whole-mount immunostaining of PECAM-1 (green), αSMA (red), and TIE2 (blue) in *Tek*$^{-/iUCKO}$ (*Tek*$^{Flox/-}$*; UBC-CreERT2*, *Tek*^{iUCKO}) and control (*Tek*$^{Flox/+}$*; UBC-CreERT2*) mice. Note that *Tek* deletion was confirmed by immunostaining analysis in *Tek*^{iUCKO} mice. Arrowheads indicate

arteries, and arrows indicate veins. Scale bar: 100 μm in C.

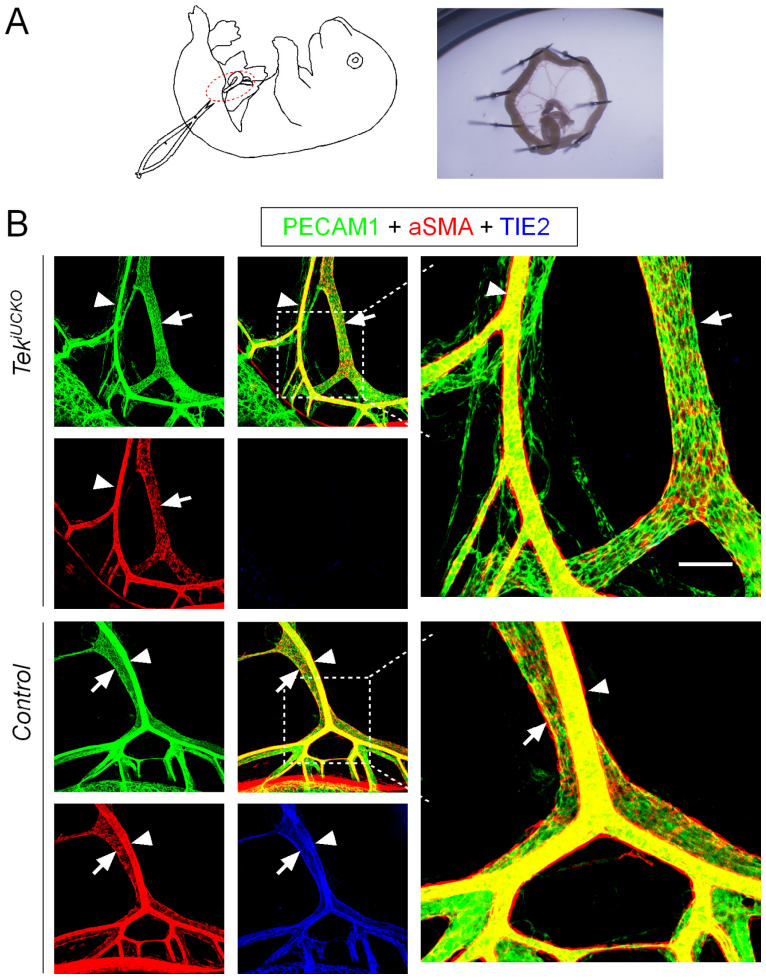

Figure 2. Misalignment of arteries and veins in the mesentery of *Tek* knockout mice. (A) The tamoxifen administration and analysis scheme is described in Figure 1A. Diagram showing the dissection of intestines and preparation for further analysis by fixing in a 6-well plate containing silicone rubber using fine needles. (B) Analysis of mesenteric blood vessels by whole-mount immunostaining of PECAM-1 (green), αSMA (red), and TIE2 (blue) in *Tek*^{iUCKO} and control mice. Arrowheads indicate arteries, and arrows indicate veins. Scale bar: 100 μm in B.

B. Generation of a *Tek*-targeted mouse model for retinal vein analysis at the neonatal stage

Note: Dissection of the retina was performed according to the protocol described by Pitulescu et al. (2010).

1. Administration of 30-50 μl tamoxifen solution (tamoxifen free base, 2 mg/ml in seed oil) by intragastric injection (Insulin syringe, BD Ultra-Fine®) to neonatal mice daily from postnatal day 1 to 4 (P1-4) (Figure 3A).
2. Euthanize the mice by cervical dislocation at P21.
3. Dissect the eyeballs from the mice into a 2-ml tube, and fix eyeballs in 4% PFA on ice for 2 h.
4. Prepare the retina by removing the cornea, sclera, choroid, pigment layer, and lens from the

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eye in cold PBS under a dissecting microscope.

5. Make four radial incisions to divide the retina into four quadrants after detaching the hyaloid vessels (Figure 3B).
6. Transfer the retina into a 1.5-ml tube and wash 3 times with PBS for 15 min each.
7. Remove PBS completely and block the retina with 3% skimmed milk powder in PBS-TX solution with gentle agitation on an orbital shaker at 4°C overnight.
8. Remove the blocking solution and incubate the retina with primary (PECAM-1, EphB4) and secondary antibodies at the appropriate concentrations for 12-16 h at 4°C with gentle agitation on an orbital shaker.

   Note: There is a wash step between the primary and secondary antibody incubation.
9. Wash the retina 5 times with PBS-TX for 20 min each.
10. Transfer the retina onto a glass slide with a few drops of 50% glycerol and cover with a coverslip.
11. Analyze the retinal blood vessels under a confocal laser scanning microscope (Olympus FluoView) and acquire and process the images as described above (Figure 3C).

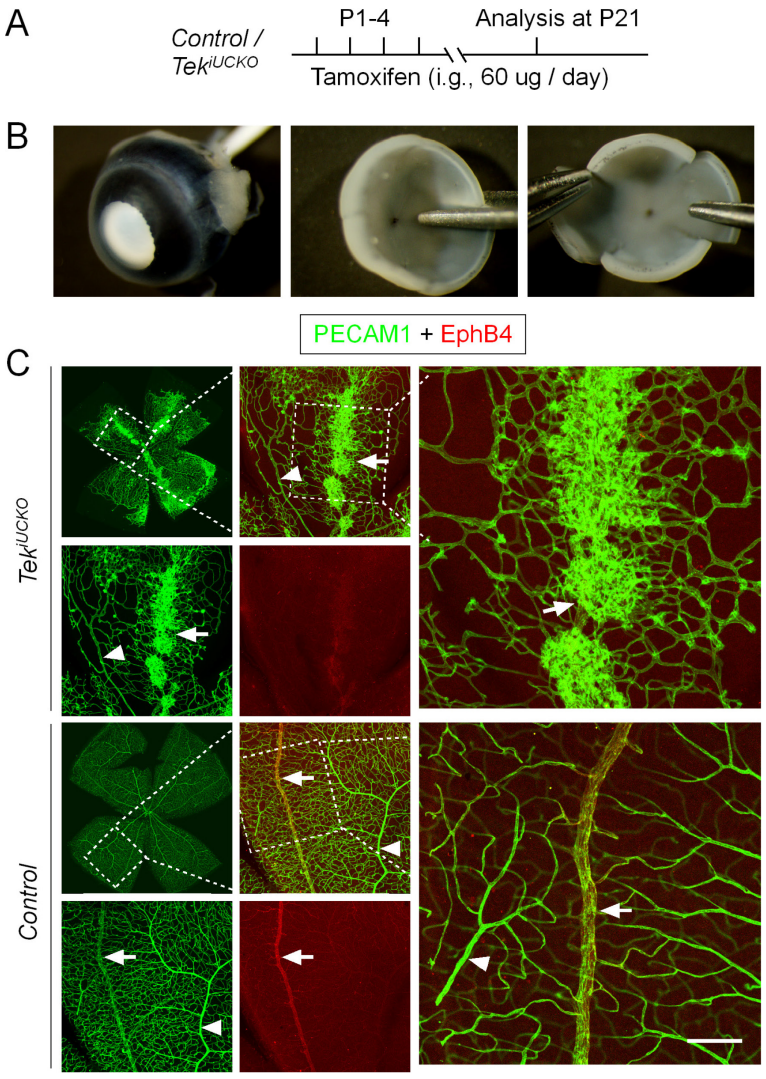

Figure 3. Formation of retinal vein-associated vascular tufts following TIE2 attenuation.

(A) Tamoxifen intragastric administration and analysis scheme. (B) Mouse eyeballs were collected with the cornea, sclera, choroid, and pigment layer removed. Retinas were cut by four radial incisions for further immunohistochemical analysis. (C) Analysis of retinal blood vessels for PECAM-1 (green) and EphB4 (red) in *Tek*^{iUCKO} and control mice at P21. Note that TIE2 insufficiency leads to vascular tuft formation along retinal veins. Arrowheads indicate arteries, and arrows indicate veins. Scale bar: 100 μm in C.

Data analysis

In this protocol, samples were analyzed under a confocal laser scanning microscope (Olympus FluoView), and images were acquired and processed using the FV-ASW Viewer 3.0 or 4.2a software. Quantification of the retinal vascularization area and blood vascular density, which is not included in this protocol, was performed using the Image-Pro Plus 6.0 software as described in the original paper (Chu *et al.*, 2016). We have noticed that the vascular phenotypes of *Tek* mutants vary depending on the Cre deleter used. For example, when VEcad-CreERT2 was used to induce *Tek* deletion in the postnatal studies (Okabe *et al.*, 2014), we found that most of the *Tek* knockout mice (*Tek*$^{Flox/-}$*; VEcad-CreERT2*, *Tek*^{iECKO}) died before P21 (postnatal day 21, data not shown). Although *Tie2* mRNA could still be detected in *Tek*^{iECKO} mice, this may be due to the expression of TIE2 in other cells, including hematopoietic cells, whereas TIE2 in ECs was almost undetectable (data not shown). However, almost all the *Tek*^{iUCKO} mice generated in this study survived even though their body weight was slightly lower than that of their littermates (Chu *et al.*, 2016). The remaining *Tie2* transcripts were analyzed by quantitative PCR. In this study, the level of *Tie2* mRNA was 0.14 ± 0.05 (P7, n = 7) in the lungs of *Tek*^{iUCKO} mice as compared with 1.0 ± 0.16 in those of control mice (P7, n = 3).

In addition, it is noteworthy that the timepoints for tamoxifen administration and tissue analysis should be defined depending on vascular phenotypes following induced gene deletion and the specific questions to be addressed. For example, the administration of tamoxifen to pregnant mice was performed by intraperitoneal injection from E12.5 to E14.5 in this protocol as the earlier induction of *Tek* deletion (*e.g.*, from E10.5) will lead to embryonic lethality at E17.5. Detailed information regarding the selection of timepoints is also described in Chu *et al*. (2016) (Figures 1 and 3).

Notes

1. Tamoxifen is light sensitive. Keep tamoxifen protected from light.
2. All the incubation steps should be carried out with gentle agitation on an orbital shaker.
3. Avoid muscle attachments when separating skin from embryos.
4. Keep the mesentery samples moist during confocal imaging.
5. Isolate eyeballs immediately after the mice have been euthanized. Avoid touching the retina

with the tip of pipettes or other instruments during dissection to prevent tissue damage.

Recipes

1. Phosphate-buffered saline (PBS), pH 7.4
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   137 mM NaCl
   2.7 mM KCl
2. PBS-TX
   0.3% Triton X-100; PBS, pH 7.4
3. 50% glycerol
   50% glycerol; PBS, pH 7.4

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Competing interests

The authors have no financial or non-financial competing interests to declare.

Ethics

All animal experiments were performed in accordance with the institutional guidelines of the Soochow University Animal Center (#IACUC-201611).

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