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Injury mediated vascular regeneration requires endothelial ER71/ETV2

Changwon Park[#†], **Tae-Jin Lee**[#], **Suk Ho Bhang**, **Fang Liu**, **Rei Nakamura**, **Sunday S. Oladipupo**, **Ian Pitha-Rowe**, **Benjamin Capoccia**, **Hong Seo Choi**, **Tae Min Kim**, **Norifumi Urao**, **Masuko Ushio-Fukai**, **Dong Jun Lee**, **Hiroyuki Miyoshi**, **Byung-Soo Kim**, **Dae-Sik Lim**, **Rajendra S. Apte**, **David M. Ornitz**, and **Kyunghee Choi**[†]

Department of Pediatrics (C.P., H.S.C.), Children's Heart Research and Outcomes Center (C.P.), Molecular and Systems Pharmacology Program (C.P.), Emory University School of Medicine, Atlanta; Department of Pharmacology, College of Medicine, University of Illinois at Chicago, IL (T.M.K., N.U., M.U-F.); School of Chemical Engineering, Sungkyunkwan University, Korea (S.H.B.); School of Chemical and Biological Engineering, Seoul National University, Seoul, Korea (B-S.K.); Korea Advanced Institute of Science and Technology, Korea (D.J.L., D-S.L.); RIKEN BioResource Center, Japan (H.M.); the Departments of Pathology and Immunology (T-J.L., F.L., K.C.), Ophthalmology and Visual Sciences (R.N., I.P-R., R.S.A.), Developmental Biology (S.S.O., D.M.O.), Biochemistry and Molecular Biophysics (B. C.), Developmental, Regenerative, and Stem cell Biology Program (D.M.O., R.S.A., K.C.), Washington University School of Medicine, MO

[#] These authors contributed equally to this work.

Abstract

Objective—Comprehensive understanding of the mechanisms regulating angiogenesis might provide new strategies for angiogenic therapies for treating diverse physiologic and pathologic ischemic conditions. The ETS factor ETV2 (aka ER71) is essential for the formation of hematopoietic and vascular systems. Despite its indispensable function in vessel development, ETV2 role in adult angiogenesis has not yet been addressed. We have therefore investigated the role of ETV2 in vascular regeneration.

Approach and Results—We utilized endothelial *Etv2* conditional knockout (CKO) mice and ischemic injury models to assess the role of ETV2 in vascular regeneration. While *Etv2* expression was not detectable under steady state conditions, its expression was readily observed in endothelial cells following injury. Mice lacking endothelial *Etv2* displayed impaired neovascularization in response to eye injury, wounding, or hindlimb ischemic injury. Lentiviral *Etv2* expression in ischemic hindlimbs led to improved recovery of blood flow with enhanced vessel formation. Following injury, *Flk1* expression and neovascularization were significantly upregulated by *Etv2*, while *Flk1* expression and VEGF response were significantly blunted in *Etv2* deficient endothelial cells. Conversely, enforced *Etv2* expression enhanced VEGF mediated

[†]Correspondence to Changwon Park, PhD, Emory University School of Medicine, 2015 Uppergate Dr. Atlanta, GA 30322. Tel 404-727-7143, cpark23@emory.edu; or Kyunghee Choi, PhD, Washington University School of Medicine, 660 S. Euclid Ave. St. Louis, MO 63110. Tel 314-362-8716, kchoi@wustl.edu.

Disclosures

None

endothelial sprouting from embryoid bodies. Lentiviral *Flk1* expression rescued angiogenesis defects in endothelial *Etv2* CKO mice following hindlimb ischemic injury. Furthermore, *Etv2*$^{+/-}$; *Flk1*$^{+/-}$ double heterozygous mice displayed a more severe hindlimb ischemic injury response compared to *Etv2*$^{+/-}$ or *Flk1*$^{+/-}$ heterozygous mice, revealing an epistatic interaction between ETV2 and FLK1 in vascular regeneration.

Conclusion—Our study demonstrates a novel obligatory role for the ETV2 in postnatal vascular repair and regeneration.

Keywords

ETV2/ER71; FLK1/VEGFR2; Tie2-Cre; VEcadherin-Cre; injury-induced neovascularization; vascular regeneration

Introduction

Angiogenesis is an important process for successful embryogenesis and injury-mediated tissue repair and regeneration. ETS transcription factors have emerged as critical regulators of angiogenesis [1, 2]. A winged helix-turn-helix motif formed by the ETS domain can bind a consensus sequence (GGAA/T) to regulate target gene expression [3]. ETS factors such as *Fli1*, *Erg*, *Ets1* and *Ets2*, are abundantly expressed in blood and endothelial cells. Consistently, mice or zebrafish deficient in these *Ets* factors display differing levels of hematopoietic and vascular defects [4-7]. Importantly, there is functional redundancy in these ETS factors in angiogenesis [5, 8, 9]. Distinct from these ETS factors, *Etv2 (*aka *Er71, etsrp)* is transiently expressed in the primitive streak, yolk sac blood islands and large vessels including the dorsal aorta during embryogenesis [10-12]. Remarkably, *Etv2* deficient animals die early in gestation due to complete block in blood and blood vessel formation [10-12] . ETV2 positively activates genes critical for hematopoietic and endothelial cell specification [10-14]. ETV2 additionally activates other Ets genes and potentiates VEGF signaling through ETV2-FLK1 positive feedback mechanism [14]. Studies in zebrafish and Xenopus have also demonstrated the critical function of *er71* in blood and vessel formation [15-17]. As such, ETV2 performs a non-redundant and indispensable function in hematopoietic and vessel development during embryogenesis [10-17].

Despite the importance of ETV2 in early embryonic hematopoietic and vascular development, *Etv2* inactivation after hematopoietic and endothelial cell specification using *Tie2-Cre* leads to normal development [18], indicating that ETV2 has a transient role in the specification of hematopoietic and endothelial lineages. However, potential functions of ETV2 in adult vascular homeostasis and pathophysiological angiogenesis have not yet been addressed. To directly investigate a role for ETV2 in postnatal vascular regeneration, we generated and characterized conditional knockout (CKO) mice lacking endothelial *Etv2* by targeting the floxed allele of *Etv2* with *Tie2-Cre* [19-21] or *VECadherin-Cre* [22]. Consistent with the observations of Kataoka et al. (2013) [18], we found that endothelial *Etv2* was not required for embryonic development or maintenance of the adult vasculature; however, mice lacking endothelial *Etv2* exhibited significantly impaired new vessel formation in response to tissue injuries including laser-induced eye injury [23], wounding [24], or hindlimb ischemic injury [25, 26], which are models for age-related macular degeneration, wound healing, and

peripheral arterial disease, respectively. Moreover, lentiviral delivery of *Etv2* into ischemic hindlimbs led to improved recovery of blood flow, augmented angiogenic gene expression and enhanced vascular regeneration. We also identify the FLK1 pathway as a major downstream effector of ETV2 in injury-induced neovascularization. These results highlight a requisite postnatal function of ETV2 in injury-induced neovascularization.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement

Results

Etv2 is upregulated in response to injury and required for neovascularization

To investigate the role of *Etv2* after the hematopoietic and endothelial cell lineage specification, we generated *Tie2-Cre; Etv2* (Etv2$^{f/f}$ *or Etv2*$^{f/-}$) conditional knockout (CKO) mice. Consistent with the previous report [18], *Tie2-Cre; Etv2* CKO mice were born at near expected Mendelian ratios and were viable, healthy, and fertile. Successful deletion of the floxed *Etv2* allele was verified in purified lung endothelial cells (CD31^{+}VECADHERIN^{+}) as well as in whole bone marrow (BM) hematopoietic cells from *Tie2-Cre; Etv2* CKO mice (Figure IA and IB in the online-only Data Supplement). *Etv2* message was not detected in CD31^{+}CD45^{-} endothelial cells isolated from *Tie2-Cre; Etv2* CKO adductor muscle (Figure IC in the online-only Data Supplement). In adult *Tie2-Cre; Etv2* CKO mice, established vasculature and BM hematopoiesis appeared similar to those of controls as shown by MECA32 or CD31/PECAM1 staining (Figure 1A; Figure II in the online-only Data Supplement) and hematopoietic cell analysis using FACS (Figure 1B and 1C), respectively.

In adult tissue, *Etv2* expression is extremely low or undetectable, even in isolated endothelial cells [27] (Figure III in the online-only Data Supplement). However, following hindlimb ischemic injury endothelial *Etv2* expression was readily upregulated (Figure 2A; Figure III in the online-only Data Supplement), suggesting its role in neoangiogenesis. To directly determine if *Etv2* was required for injury-induced vascular regeneration, we subjected *Tie2-Cre; Etv2* CKO mice to hindlimb ischemia [25, 26, 28] and assessed their vascular regeneration ability. As expected, *Etv2* expression was almost undetectable in the endothelial cells from *Tie2-Cre; Etv2* CKO mice after injury (Figure 2B). Laser Doppler Perfusion Image (LDPI) analysis revealed significantly impaired blood perfusion in *Tie2-Cre; Etv2* CKO hindlimbs, compared to control mice (Figure 2C and 2D). Capillary density as determined by CD31 (PECAM1) or VECADHERIN staining was lower in the CKO mice after injury (Figure 2E and 2F). The impaired blood perfusion observed in *Tie2-Cre; Etv2* CKO mice was due to *Etv2* deficiency as demonstrated by the rescue experiment with lentiviral *Etv2* injection (Figure IV in the online-only Data Supplement). To confirm that endothelial *Etv2* is responsible for the results of *Tie2-Cre; Etv2* CKO mice, we additionally generated *VEcadherin-Cre; Etv2* (Etv2$^{f/f}$ *or Etv2*$^{f/-}$) CKO mice to specifically delete *Etv2* in endothelial cells [22]. *VEcadherin-Cre; Etv2* CKO mice were also obtained at expected Mendelian ratios and were healthy. Importantly, *VECadherin-Cre; Etv2* CKO mice displayed similar angiogenesis defects in hindlimb ischemic injury response as observed in *Tie2-Cre; Etv2* CKO mice (Figure 2G-2J). It is well appreciated that both hematopoietic and

endothelial cells are required for optimal recovery from hindlimb ischemic injury [29, 30]. Thus, we generated and performed hindlimb ischemia studies using *Vav-Cre; Etv2* CKO mice (hematopoietic *Etv2* deletion) [31, 32] to determine hematopoietic *Etv2* contribution to vascular regeneration. As shown in Figure V in the online-only Data Supplement, *Vav-Cre; Etv2* CKO mice did not show deficiency in blood perfusion recovery at early time points, day 7 and 14. Only at a later time, day 21, blood perfusion was decreased, compared to controls. These data suggest a key contribution of endothelial *Etv2* in ischemia-induced angiogenesis.

In addition to reduced vessel repair ability following hindlimb ischemic injury, *Tie2-Cre; Etv2* CKO mice also displayed reduced vascular regeneration ability in a mouse model of choroidal neovascularization (CNV), the major cause of blindness of age-related macular degeneration (AMD) [23, 33]. Particularly, there was a significant reduction in the CNV volume in *Tie2-Cre; Etv2* CKO mice, compared to controls ($5.1\times10^3 \pm 7.5\times10^2$ μm^3 vs $2.3\times10^3 \pm 3.7\times10^2$ μm^3, p=0.001) when mice were analyzed 7 days after the laser injury (Figure 3A and 3B). In an excisional wound healing model [24], new vessel formation, as measured by a MECA32$^+$ vascular area in the wounded back-skin, was impaired in *Tie2-Cre; Etv2* CKO mice compared to controls (Figure 3C and 3D). Collectively, these results demonstrate that endothelial ETV2 is required for neovascularization in response to injury.

Lentiviral *Etv2* delivery into ischemic hindlimbs results in enhanced blood perfusion recovery accompanying augmented capillary formation and reduced tissue fibrosis

As *Etv2* was required for vascular regeneration, we tested if *Etv2* gene delivery could augment neovascularization process following injury. Thus, we subjected athymic nude mice to hindlimb ischemic injury followed by lentiviral *Etv2* delivery into the adductor muscle. Due to decreased angiogenic recovery in response to injury, athymic nude mice have been instrumental for investigating the efficacy of expressing genes for injury repair [34]. Lentivirus expressing *Venus/Gfp* or *Etv2-ires-Venus/Gfp* efficiently infected endothelial cells, as shown by CD31$^+$GFP$^+$ cells in the injected areas of the hindlimb (Figure VIA-VIC in the online-only Data Supplement). *Etv2* expression was observed at day 4 after the injection, although it was no longer detected after about a month (Figure VID and VIE in the online-only Data Supplement). Whereas control *Venus*-lentivirus injection had no effect on limb perfusion recovery, *Etv2*-lentivirus injection protected mice considerably from limb loss or foot necrosis when examined 28 days later (Figure 4A and 4B). Consistently, LDPI analysis showed enhanced blood perfusion recovery in mice injected with *Etv2*-lentivirus, compared to mice injected with control *Venus*-lentivirus (Figure 4C and 4D). Similar results were also obtained in C57BL/6J mice, which have strong recovery ability of blood perfusion following ischemic injury (Figure VII in the online-only Data Supplement). Immunohistochemical analysis revealed that *Etv2*-lentiviral injection led to a greater increase in new vessel formation (Figure 5A-5C). Vascular endothelial cells were proliferating, as determined by the presence of increased number of CD31(PECAM1)$^+$BrdU$^+$ cells, compared to controls (Figure 5C). We additionally found vessels that were 50-100 μm in diameter and augmented numbers of SMα-ACTIN$^+$BrdU$^+$ cells in *Etv2*-lentivirus injected hindlimbs (Figure 5B and 5C), suggesting that arteriogenesis was also induced. Importantly, *Etv2*-lentivirus injected muscle were protected from necrotic

damage caused by ischemia (Figure 5D, upper panels). Fibrosis, measured by Masson's trichrome staining, was also markedly attenuated in these animals, compared to controls (Figure 5D, lower panels, D').

Etv2-mediated activation of angiogenic program in vascular regeneration

To understand mechanisms by which ETV2 regulates vascular regeneration, we first examined changes in cell population in ischemic hindlimbs. Notably, fewer endothelial cells were present in the ischemic hindlimbs shortly after the injury (4 days later), probably reflecting apoptotic response of endothelial cells to ischemia[35]. In contrast, there was a significant increase in $CD45^+$ blood cells at this time in the injured hindlimbs, presumably indicating an elevated inflammatory response to injury (Figure 6A and 6B). To further understand molecular events occurring following injury, we examined gene expression profiles in the mouse hindlimb tissues collected 7 days after the ischemic injury. We chose this time point, as vessel remodeling had initiated and inflammation was beginning to subside at this time [35, 36]. Angiogenesis PCR array analysis showed that expression of the inflammation-related cytokines, including *Il6*, *Il1β*, *Cxcl2* and *Cxcl5*, was highly upregulated at this time point in the injured tissues (Figure 6C; Table I in the online-only Data Supplement). On the other hand, angiogenesis-related genes were generally downregulated. In particular, *Vegfs* and their receptors, *Flk1* and *Flt1* expression levels were lower in the injured tissues, which could be partly due to decreased numbers of endothelial cells in the injured tissue. To investigate molecular changes induced by ETV2, we additionally performed an angiogenesis PCR array using RNA obtained from hindlimb tissues that received *Etv2* and compared to that of the controls (collected 7 days after injury) (Figure 6D; Table II in the online-only Data Supplement). Upon *Etv2*-lentivirus injection, inflammatory and chemokine gene expression was down regulated. Conversely, a majority of the angiogenic genes that were downregulated in the ischemic tissue became upregulated by lentiviral *Etv2*. Notably, *Vegfs, Flk1* and *Flt1* expression was increased. Western blot analysis confirmed increased CD31, SMα-ACTIN, FLK1 and VEGF protein levels without significant changes in FGF2 production in *Etv2*-injected hindlimbs (Figure 6E). Collectively, these results suggest that *Etv2* expression in ischemic hindlimbs leads to an augmented angiogenic program with concomitant reduction of inflammatory reaction, resulting in endothelial and smooth muscle cell proliferation, neovascularization and tissue repair.

ETV2 regulates VEGF response in vascular regeneration

As the VEGF-FLK1 pathway was highly upregulated by lentiviral *Etv2* delivery during vessel regeneration, we examined whether the FLK1 pathway, the key downstream target of ETV2 in embryonic vascular development [12-14], could also be a target of ETV2 in neovascularization in the adult. Notably, *Flk1* expression levels in endothelial cells isolated from *Tie2Cre; Etv2* CKO ischemic hindlimbs were much lower, compared to controls (Figure 7A). To determine if endothelial cells lacking *Etv2* show a diminished response to VEGF as a result of blunted *Flk1* upregulation, we performed a choroidal explant culture assay, an *in vitro* quantitative measurement of angiogenesis [37]. *Tie2-Cre; Etv2* CKO choroidal explants showed impaired endothelial sprouting in the presence of VEGF compared to controls (Figure 7B, left, column 1 vs. 3; Figure VIII in the online-only Data

Supplement). While VEGF-mediated sprouting from control explants was suppressed by the VEGF inhibitor Bevacizumab treatment [38] (Figure 7B, left, columns 1 vs. 2), the *Tie2-Cre; Etv2* CKO explants were unaffected by Bevacizumab (Figure 7B, left, columns 3 vs. 4), suggesting that impaired sprouting in *Etv2* deficient endothelial cells is partly due to a compromised VEGF-FLK1 response. Notably, *Tie2-Cre; Etv2* CKO and control choroidal explants showed a similar response to FGF2 (Figure 7B, right, columns 1 vs. 3). Additionally, Bevacizumab did not affect FGF2 responsive vascular sprouting (Figure 7B, right, columns 1 vs. 2, columns 3 vs. 4).

To further validate the ETV2-FLK1 pathway in vascular regeneration, we utilized in vitro differentiated ES cells (embryoid bodies, EBs), which exhibit robust endothelial differentiation and sprouting angiogenesis on collagen I matrix or matrigel [39-41]. Specifically, doxycyclin (DOX) inducible *Etv2* EBs [12] were subjected to sprouting angiogenesis on a solidified collagen I matrix in the presence of VEGF and/or DOX. As previously reported, EB sprouting angiogenesis was induced by VEGF [39, 41]. Notably, EB sprouting angiogenesis was significantly increased in the presence of both VEGF and DOX (Figure 7C and 7D; Figure IX in the online-only Data Supplement).

If indeed the FLK1 pathway functions downstream of ETV2 in vascular regeneration, it was expected that *Flk1* expression would rescue the neovascularization defects observed in *Tie2-Cre; Etv2* CKO mice. To test this model, *Tie2-Cre; Etv2* CKO mice were subjected to hindlimb ischemic injury followed by lentiviral *Flk1* delivery into the adductor muscle. Whereas control lentiviral injection had no effect on limb perfusion recovery, lentiviral *Flk1* injection into *Tie2-Cre; Etv2* CKO ischemic hindlimbs restored the impaired blood perfusion (Figure 7E and 7F; Figure X in the online-only Data Supplement). Moreover, vessel density of the CKO hindlimbs was augmented by *Flk1* lentivirus injection, compared to controls (Figure 7G and 7H).

To further validate that ETV2 mediated vascular regeneration is facilitated through activating the FLK1 pathway, we subjected wild type, $Etv2^{+/-}$ and $Flk1^{+/-}$ single heterozygous mice and $Etv2^{+/-}$; $Flk1^{+/-}$ double heterozygous mice to hindlimb ischemic injury (Figure 7I). $Etv2^{+/-}$ or $Flk1^{+/-}$ single heterozygous mice showed a trend (not statistically significant) towards impaired recovery in limb perfusion compared to wild type controls, suggesting the potential of a haploinsufficient role for *Etv2* or *Flk1* in vascular regeneration. Importantly, $Etv2^{+/-}$; $Flk1^{+/-}$ double heterozygous mice showed significantly ($p<0.05$) worse blood perfusion recovery compared to either single heterozygous mouse. These data support a role for *Etv2* and *Flk1* within a common genetic pathway in vessel regeneration.

Discussion

Angiogenesis is fundamental for successful tissue repair and regeneration. It is widely accepted that genetic programs regulating developmental angiogenesis also drive postnatal angiogenesis. For example, VEGF and FLK1 have been extensively studied in the context of embryonic vessel development, vascular regeneration, and pathologic angiogenesis. Notably, *Vegfa* or *Flk1* deficiency leads to embryonic lethality due to defects in vascular and

hematopoietic formation [42-44]. Importantly, neovascularization in ischemic limbs or myocardial ischemia can be enhanced by VEGF administration, suggesting that a proangiogenic approach may be beneficial for ischemic vascular occlusion [45]. Moreover, VEGF targeted therapy is commonly used to correct pathologic angiogenesis such as in AMD and tumors. Tissue injuries or tumors create a hypoxic environment and hypoxia is a key upstream inducer of *Vegf* activation, which may explain how the VEGF pathway can be activated in various pathologic conditions. However, the upstream events leading to FLK1 activation or inhibition in endothelial cells under pathophysiologic conditions have not been well characterized.

ETV2 function is indispensable for vessel development, as *Etv2* deficiency, similar to *Vegf* or *Flk1* deficiency, leads to an absolute block in blood and vessel formation and embryonic lethality. However, *Etv2* deletion using *Tie2-Cre*, *VECadherin-Cre or Vav-Cre* does not affect normal embryonic development, indicating that *Etv2* is only transiently required during vessel and blood specification. Consistently, *Etv2* expression becomes silent once the circulatory system is established and is not detectable beyond E11.5 especially in endothelial cells [10-12]. These studies raise an important question as to whether *Etv2* is dispensable in postnatal life. As mice lacking endothelial *Etv2* displayed seemingly normal homeostatic vessel maintenance, we determined in this study whether ETV2 could be involved in injury response. A major unexpected finding of this study is that although ETV2 is not required for steady-state vessel maintenance, *Etv2* is necessary for efficient vessel regeneration in response to injury. There was noticeable upregulation of *Etv2* in endothelial cells isolated from ischemic tissues. Endothelial *Etv2* deficiency led to a poor vascular regeneration capacity. Thus, our findings are consistent with the notion that embryonic signaling either through reactivation or dysregulation can become an important etiology in the pathophysiological events in adults [46-48]. As lentiviral *Etv2* expression in ischemic hindlimbs led to overall angiogenic gene upregulation including *Flk1*, we envision that *Etv2* reactivation upon injury-mediated signaling may turn on the angiogenic program to facilitate vessel regeneration. Of the angiogenic pathway, we identify the VEGF-FLK1 pathway, as in development [12-14], to be a major target of ETV2 in neovascularization. Specifically, *Etv2* deficient endothelial cells displayed blunted *Flk1* upregulation and defective VEGF responsive capillary sprouting upon injury. Conversely, ETV2 augmented VEGF response in angiogenic sprouting from EBs. Blood perfusion and vascular regeneration in mice lacking endothelial *Etv2* was restored by lentiviral *Flk1* delivery. A significant impairment of blood perfusion in $Etv2^{+/-}$; $Flk1^{+/-}$ mice further suggests a linear relationship between ETV2 and FLK1 in vascular regeneration. While ETV2 functions upstream of Flk1 in development and in injury mediated response as we showed in this study, several papers reported that ETV2 could be induced by VEGF [11, 49]. Notably, VEGF is readily induced in hypoxic conditions. It has recently been suggested that there is a positive feedback mechanism between ETV2 and FLK1 signaling in embryogenesis [14]. Thus, while injury signals that can lead to *Etv2* reactivation remain to be elucidated, it is possible that ETV2-FLK1 feed-forward mechanism might amplify the VEGF response in vascular regeneration.

A single lentiviral *Etv2* injection was sufficient to protect mice from limb loss and enhanced blood perfusion in ischemic hindlimbs. Further, *Etv2*-lentivirus injected muscle was

protected from necrotic damage caused by ischemic injury. One simple explanation for the potent function of ETV2 in this process would be that endothelial cells infected with ETV2 proliferate and activate angiogenic program and thus generate more blood vessels, leading to tissue protection. Indeed, we found that endothelial cells were readily infected by lentiviral *Etv2* in the injured tissues. Since ETV2 delivery can also induce arteriogenesis, it might be possible that endothelial ETV2 upregulates the expression of paracrine factors for smooth muscle cell recruitment and proliferation. Intriguingly, recent studies show that ETV2 can directly reprogram somatic cells into functional endothelial cells *in vitro* [50-52]. Additionally, ectopic *etv2* expression in zebrafish was able to convert skeletal muscle cells into endothelial cells [53]. Thus, it is also possible that reprogrammed endothelial cells in the ischemic hindlimbs from other cell types such as skeletal muscle cells, fibroblasts or hematopoietic cells, if any, could also have contributed to the observed protected phenomenon. In agreement with this, we found that hematopoietic cells and presumably skeletal muscles were also infected with lentiviral *Etv2* in ischemic hindlimbs. Future studies are warranted whether *Etv2* expression could indeed cause in vivo conversion of non-endothelial cells into endothelial cells and if so, then the magnitude of contribution of such in vivo reprogrammed endothelial cells to vascular regeneration. Intriguingly, *Etv2* expression was no longer detected once the vascular regeneration was completed in the mouse hindlimb ischemia model. A transient, but not persistent, upregulation of *Etv2* in the initial stages of vascular regeneration might therefore be critical. Reminiscent to the transient *Etv2* expression pattern during embryogenesis [10-12], there might be a strong pressure to keep this gene off in established vasculature. Indeed, sustained expression of *Etv2* in *Tie2*$^{+}$ cells leads to abnormal vessel development as manifested by dilated yolk sac vessels [54]. Collectively, our findings suggest that *Etv2* delivery into injured tissues could be used for therapeutic purpose for conditions requiring vessel regeneration.

A recent study has demonstrated that FGF2, probably through FGFR1/2, can activate *Flk1* expression via ERK-ETS [55]. However, we found that *Tie2-Cre; Etv2*CKO and control choroidal explants showed similar vascular sprouting in response to FGF2, suggesting that FGF2 could regulate *Flk1* expression by ETV2 independent manner. In the absence of ETV2, other ETS factors such as ETS1 could function downstream of FGF2 in regulating *Flk1* expression. Additionally, the observed discrepancy might be due to 1) FGF2 treatment to choroidal explants vs adenoviral delivery of FGFR1 DN to HUVECs or 2) different genetic models; *Tie2-Cre; Etv2* CKO vs *FGFR1* DN transgenic mice. We propose that ETV2 is critical for regulating VEGF/FLK1 signaling, but dispensable for FGF2 signaling in neoangiogenesis. Combined FGFRs and ETV2 inactivation in endothelial cells is warranted in the future to further understand the interplay between FGF and VEGF/FLK1 signaling with regard to ETV2 in vascular regeneration.

In conclusion, we demonstrated that endothelial *Etv2* is critical for vessel regeneration and tissue repair following injury. Additionally, ETV2 has potent efficacy in therapeutic angiogenesis, providing a novel research platform for vascular regeneration therapies. Current therapeutic angiogenic targets such as VEGF have presented severe limitations in specifically targeting vessels undergoing neoangiogenesis, as they are also required for vessel homeostasis. In contrast, *Etv2* is activated in endothelial cells upon injury, thus

aiming the ETV2 pathway could provide unique opportunity for specifically targeting the activated endothelial cells, while sparing homeostatic vessel integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations and Acronyms

ER71/ETV2	Ets related protein 71/ Ets variant 2
Flk1/VEGFR2	Fetal liver kinase 1/vascular endothelial growth factor receptor 2
CKO	conditional knockout
CNV	choroidal neovascularization
VEGF	vascular endothelial growth factor
FGF	fibroblast growth factor

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Significance

Angiogenesis is an important process for successful embryogenesis and injury-mediated tissue repair and regeneration. ETV2, an ETS factor, is an indispensable regulator of embryonic vessel development. Here, we for the first time reveal a novel and unexpected function of ETV2 in injury-induced vascular regeneration. Specifically, we show that while *Etv2* expression in endothelial cells is not detectable under steady state conditions, it becomes reactivated and upregulates genes critical for angiogenesis upon injury. Mice lacking endothelial *Etv2* display impaired vascular regeneration in response to injuries. Furthermore, enforced *Etv2* expression is sufficient to regenerate vessels, as lentiviral *Etv2* delivery leads to enhanced endothelial cell proliferation, angiogenesis, vascular regeneration and tissue repair in the mouse hindlimb ischemia model. As such, our study provides compelling necessary and sufficient role of ETV2 in vascular regeneration in adult. Our findings could provide a new research platform for the development of novel therapeutic strategies requiring angiogenic intervention.

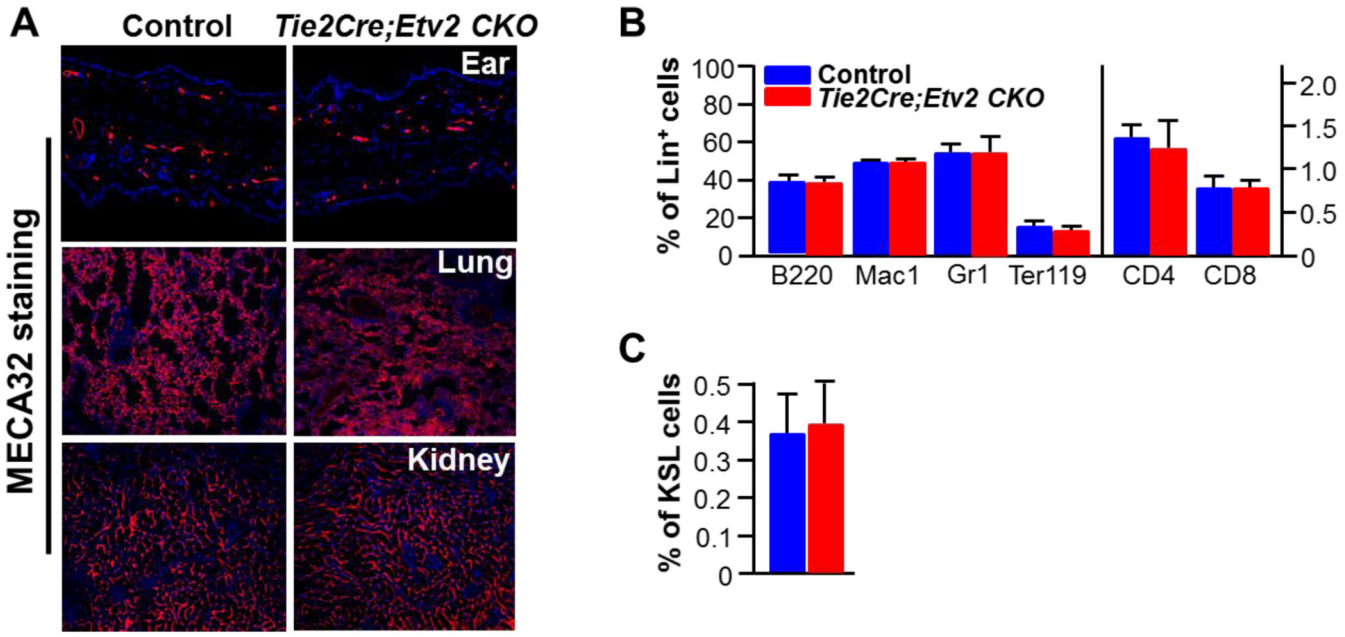
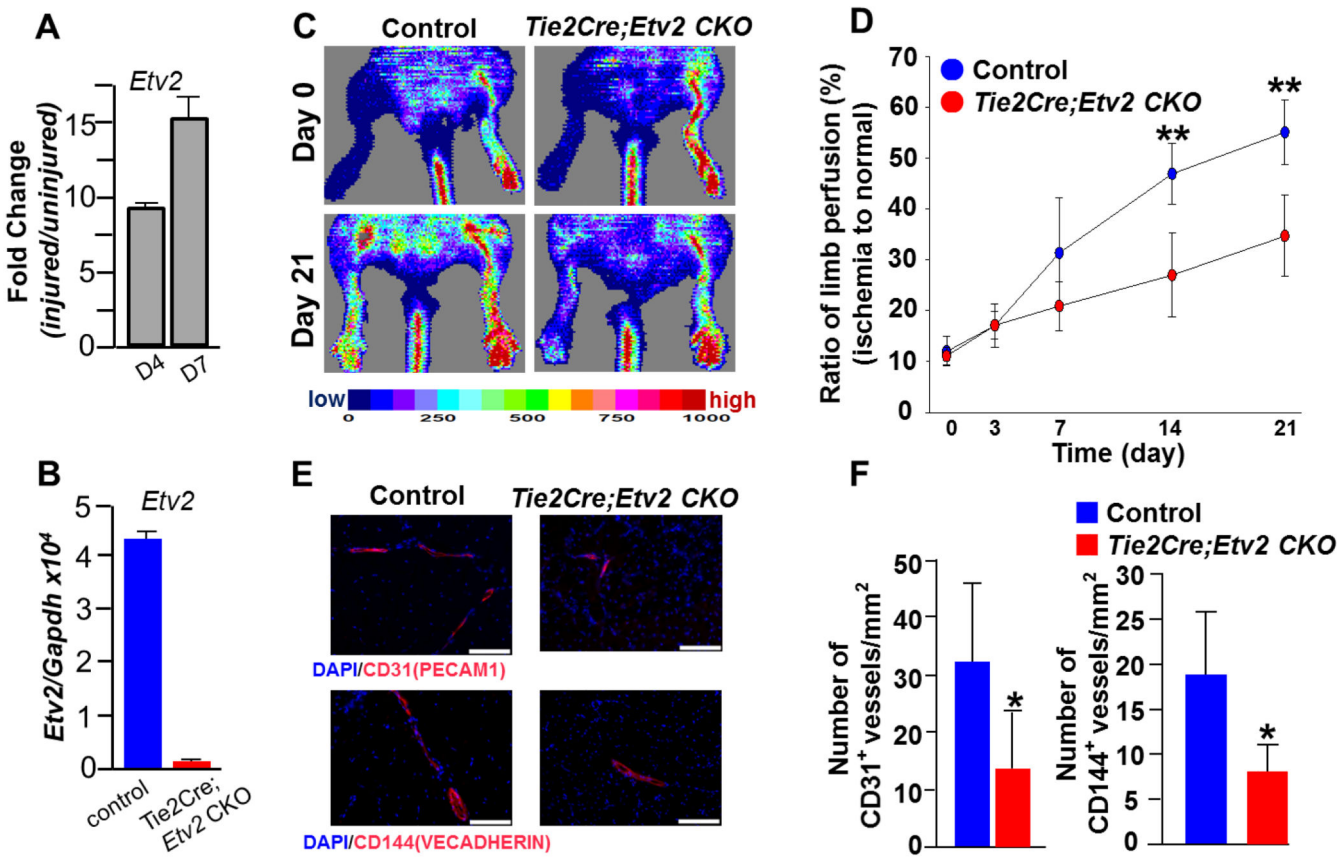


Figure 1. ***Tie2-Cre; Etv2*** **CKO mice show no pheotypic defects in vascular and blood development**

A, Control and *Tie2-Cre; Etv2* CKO mice were subjected to MECA32 staining on ear, lung and kidney. MECA32 (red) for endothelial cells and DAPI (blue) for nuclear staining. n=3, magnifications: ear = 20 ×, lung and kidney = 10×

B, BM analysis with hematopoietic lineage markers. n=3. B220 for B cells; Mac1 and Gr1 for monocyte and granulocytes; Ter119 for erythrocyte; CD4 and CD8 for T cells.

C, BM analysis with hematopoietic stem cell markers. n=3. KSL indicates c-KIT^{+}Sca1^{+}Lin^{-} cell population enriched for BM hematopoietic stem cells. Error bars indicate standard deviations.



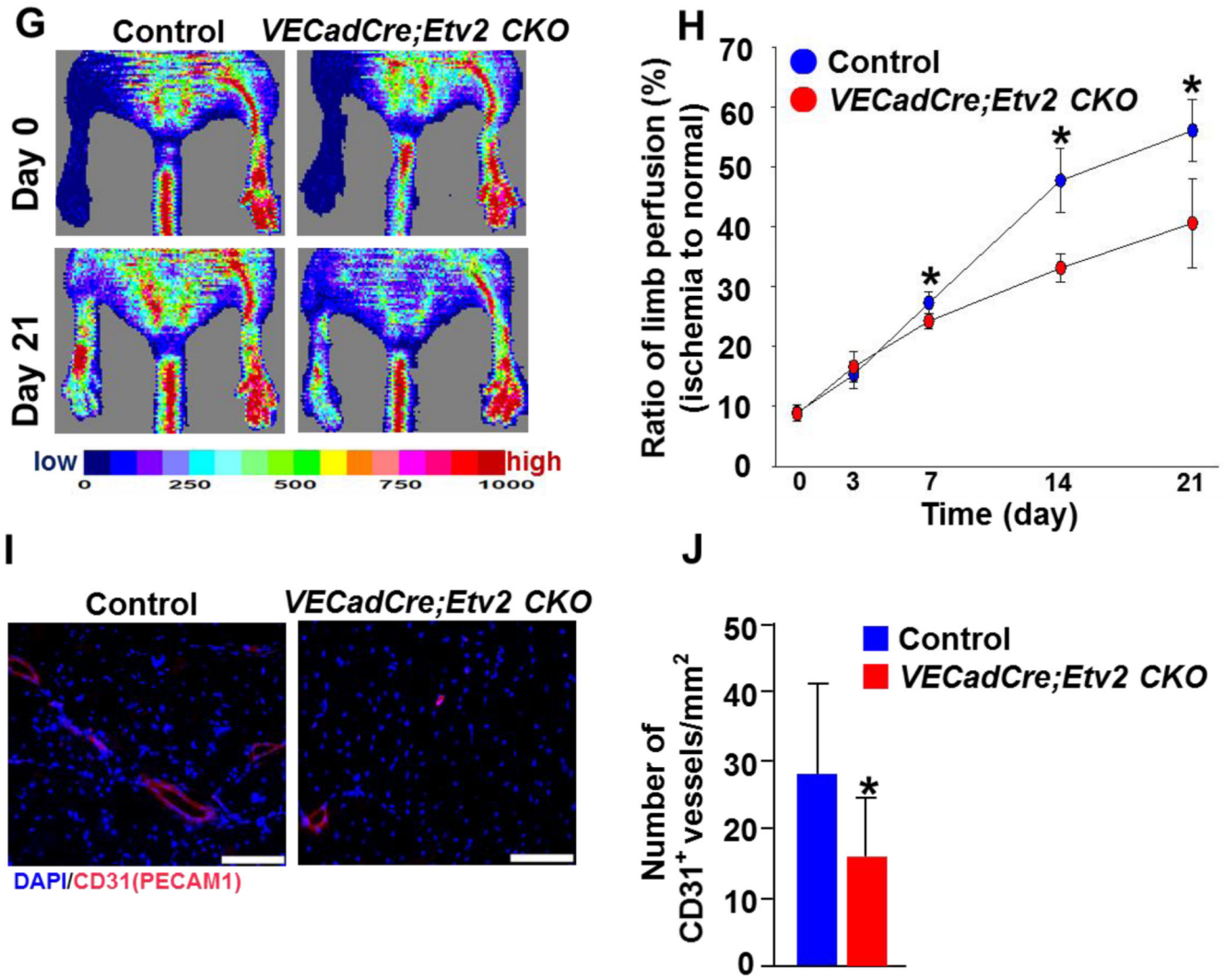


Figure 2. Impaired neovascularization in *Tie2-Cre; Etv2* CKO and *VECadehrin-Cre; Etv2* CKO mice after hindlimb ishcemic injury

A, qRT-PCR analysis on FACS-sorted $CD31^{+}CD45^{-}$ cells from ischemic hindlimbs. Cells were sorted 4 and 7 dyas after injury. Sorted cells from 4 mice/group were combined and used for gene expression analysis.

B, qRT-PCR analysis on *Etv2* expression using FACS-sorted $VECADHERIN(VECAD)^{+}$ cells from adductor muscle. Cells were sorted from control and *Tie2-Cre; Etv2* CKO mice 7 days after ishcemic injury. Sorted cells from 4 mice/group were combined and used for gene expression analysis.

C, Representative images showing physiological status and blood perfusion measured by LDPI of control and *Tie2-Cre; Etv2* CKO ischemic hindlimbs.

D, Blood perfusion ratio of ischemic limbs of control and *Tie2-Cre; Etv2* CKO mice imaged on day 0, 3, 7, 14 and 21 after ischemic injury is shown. n=5/group, $^{**}P < 0.01$

E, Representative images of immunohistochemistry. Adductor muscle from control and *Tie2-Cre; Etv2* CKO mice were harvested 21 days after injury and subjected to

immunohistochemistry using anti-CD31(PECAM1) or CD144 (VECADHERIN) antibody. DAPI (blue) for nuclear staining, Scale bars: 100 μm.

F, Quantification of CD31 (PECAM1) or CD144 (VECADHERIN) positive vessel density in the ischemic region is shown. n=5/group, *P < 0.05

G, Representative images of physiological status and blood perfusion measured by LDPI of control and *VECadherin-Cre; Etv2* CKO ischemic hindlimbs.

H, Blood perfusion ratio of ischemic limbs of control and *VECadherin-Cre; Etv2* CKO mice imaged on day 0, 3, 7, 14 and 21 after ischemic injury is shown. n=5/group, *P < 0.05

I, Representative photographs of immunohistochemistry are shown. Adductor muscle from control and *VECadherin-Cre; Etv2* CKO mice were harvested 21 days after injury and subjected to immunohistochemistry with anti- CD31(PECAM1) antibody. DAPI (blue) for nuclear staining, Scale bars: 100 μm.

J, Quantification of CD31(PECAM1)-positive vessel density in the ischemic region is shown. n=5/group, *P < 0.05. Error bars indicate standard deviations.

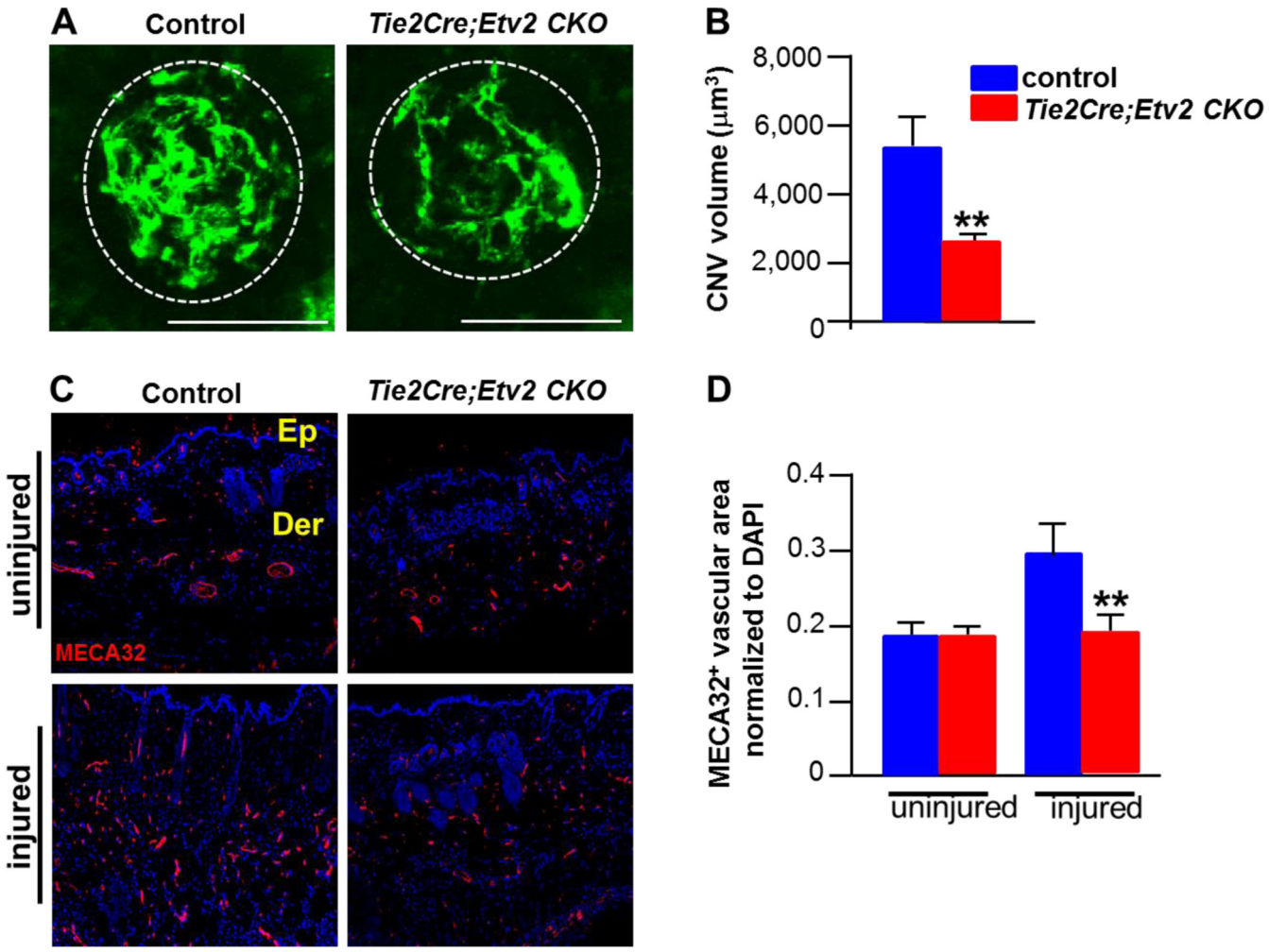


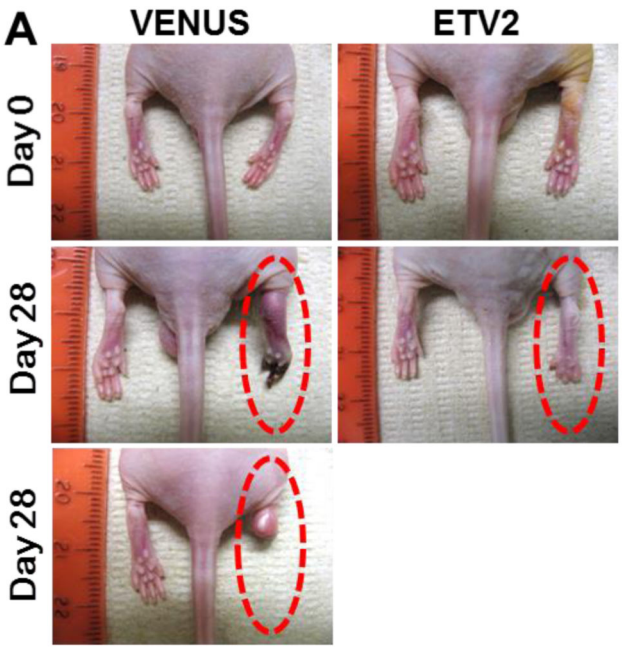
Figure 3. *Tie2-Cre; Etv2* CKO mice display significantly reduced neovascularization in laser-induced eye injury and wounding

A, Representative images of CNV lesions visualized by FITC-dextran labeling from control and *Tie2-Cre; Etv2* CKO mice. Scale bars = 100 μm.

B, CNV lesion volume (expressed as volume of fluorescence) was quantified 7 days after laser treatment. n=5/group. **P <0.01

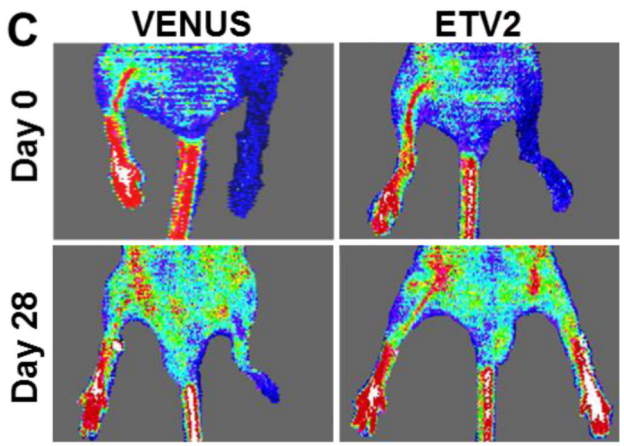
C, Representative images of MECA32 staining from control and *Tie2-Cre; Etv2* CKO back skin obtained 5 days after skin excisional wound (injured). Magnifications = 10 ×, DAPI (blue) for nuclear staining, Ep:Epidermis, Der:Dermis.

D, MECA32 positive area was quantified 5 days after the injury. n=8/group. **P < 0.01. Error bars indicate standard deviations.



B

(N=7)	Limb salvage	Toe necrosis	Foot necrosis	Limb loss
VENUS	0 / 7	1 / 7	4 / 7	2 / 7
ETV2	5 / 7	1 / 7	1 / 7	0 / 7



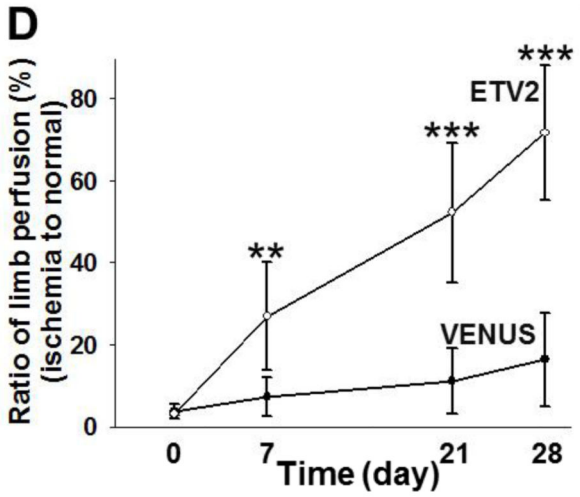


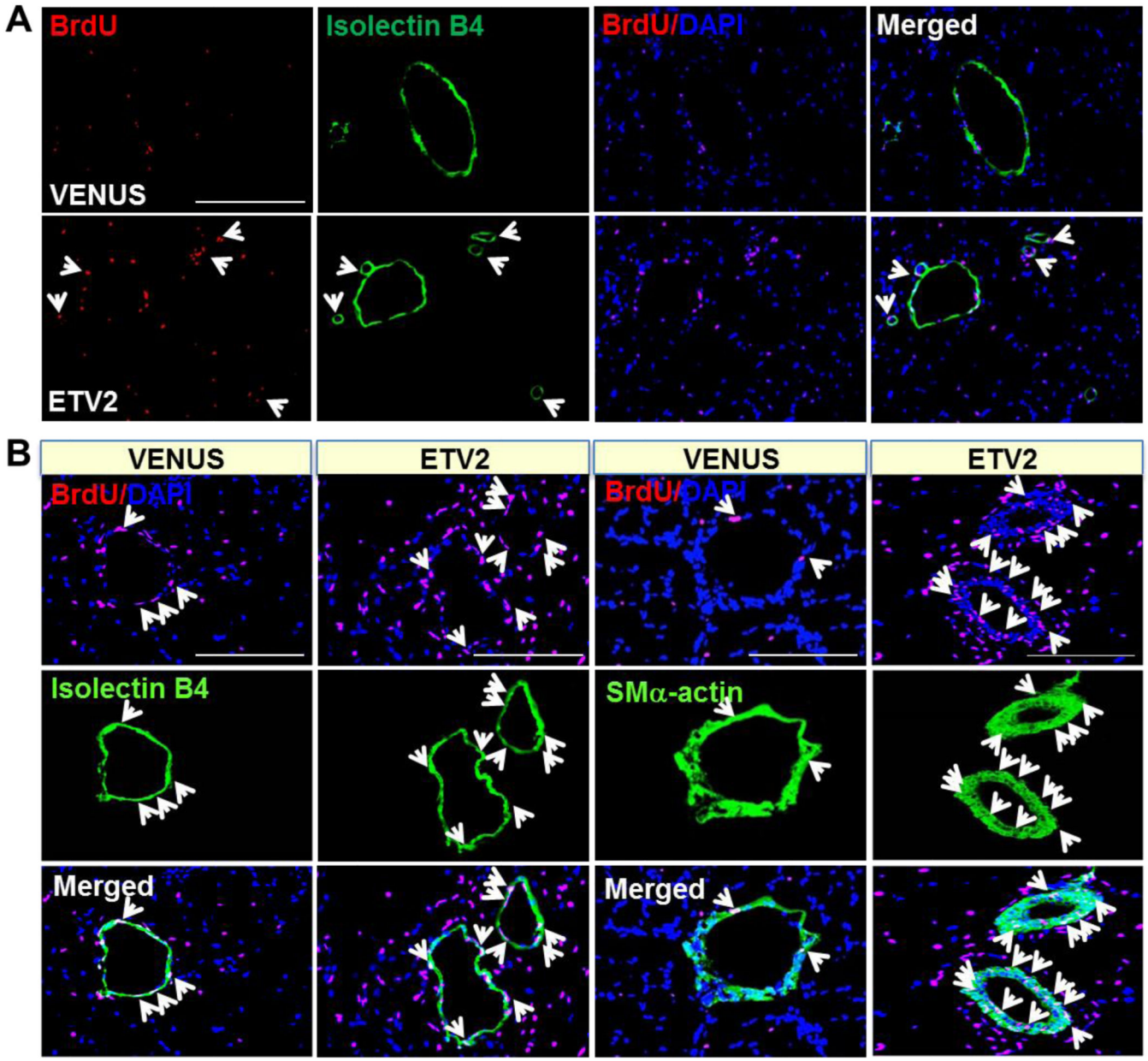
Figure 4. Lentiviral *Etv2* expression in ischemic hindlimbs enhances blood perfusion recovery

A, Representative images of lentiviral *Venus* or *Etv2*-injected hindlimbs. Pictures were taken on day 0 and 28 after ischemic injury.

B, Physiological status of ischemic limbs 28 days after the treatment. n=7

C, Representative images of LDPI analysis performed on day 0 and 28 after treatment.

D, Blood perfusion ratio of ischemic limbs measured by laser Doppler imaging on 0, 7, 21, and 28 day after treatment. n=7, **P < 0.01, ***P < 0.001 compared with VENUS injection group. Error bars indicate standard deviations.



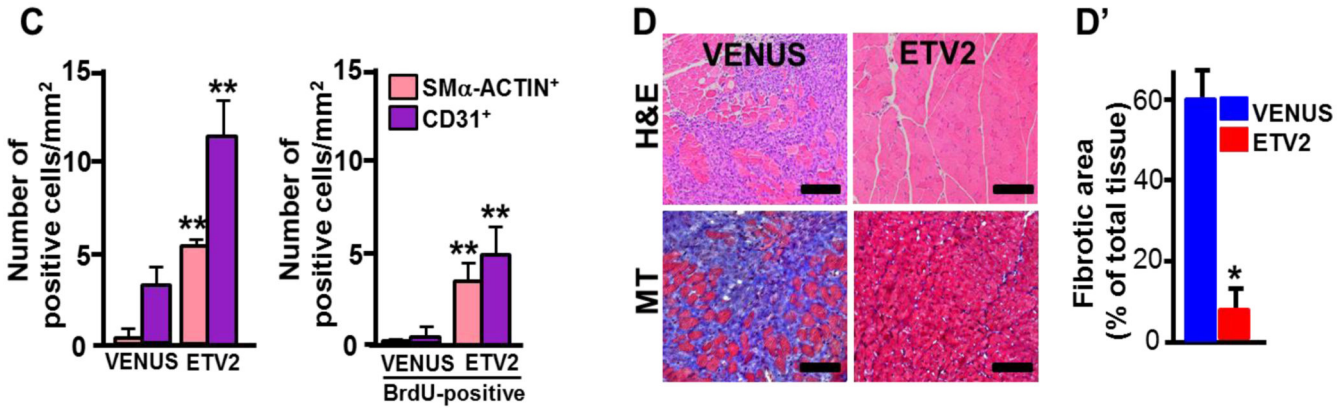


Figure 5. lentiviral *Etv2* expression in ischemic hindlimbs leads to increased vessel formation and reduced tissue fibrosis

A, Increased capillary formation in *Etv2* injected athymic nude mice after hindlimb ischemia. Twenty eight days after ischemic injury and viral particle injection (VENUS and ETV2), the mice were injected with BrdU (red) and Isolectin B4 (green), and adductor muscles were harvested for immunohistochemistry. Arrows indicate colocalization of cells positive for Isolectin B4 with BrdU and DAPI (blue). Scale bars: 345 μm.

B, Increased arteriogenesis by *Etv2*. Adductor muscles from the mice (A) were subjected to immunocytochemistry. Arrows indicate colocalization of cells positive for Isolectin B4 or SMα-actin with BrdU and DAPI (blue). Scale bars: 170 μm.

C, Quantification of CD31-positive or SMα-actin-positive vessel density in the ischemic region of the mice is shown. All $CD31^+$ or SMα-$actin^+$ cells regardless of BrdU (left) and $CD31^+BrdU^+$ or SMα-$actin^+BrdU^+$ cells (right) were counted, respectively. **P < 0.01 compared to VENUS injection group.

D, H & E and Masson's trichrome staining (MT) staining. Adductor muscles obtained 28 days after ischemic injury were subjected to H & E (upper) and MT staining (lower). Blue region indicates fibrosis. (D') Quantification of MT staining. n=7/group, Scale bars = 100 μm, *P < 0.05 compared to VENUS injection group. Error bars indicate standard deviations.

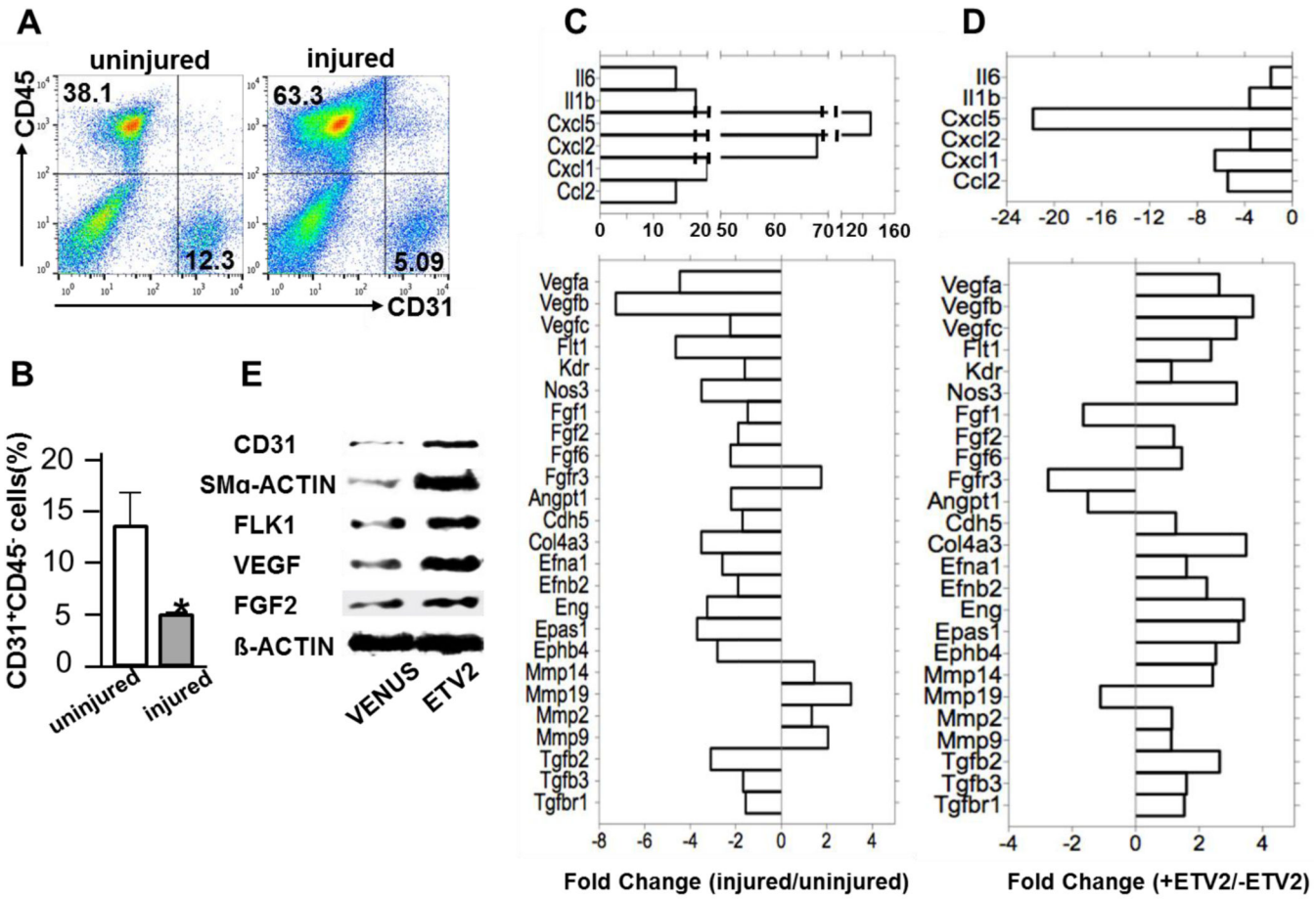


Figure 6. ETV2 Activates angiogenic program in ischemic hindlimbs

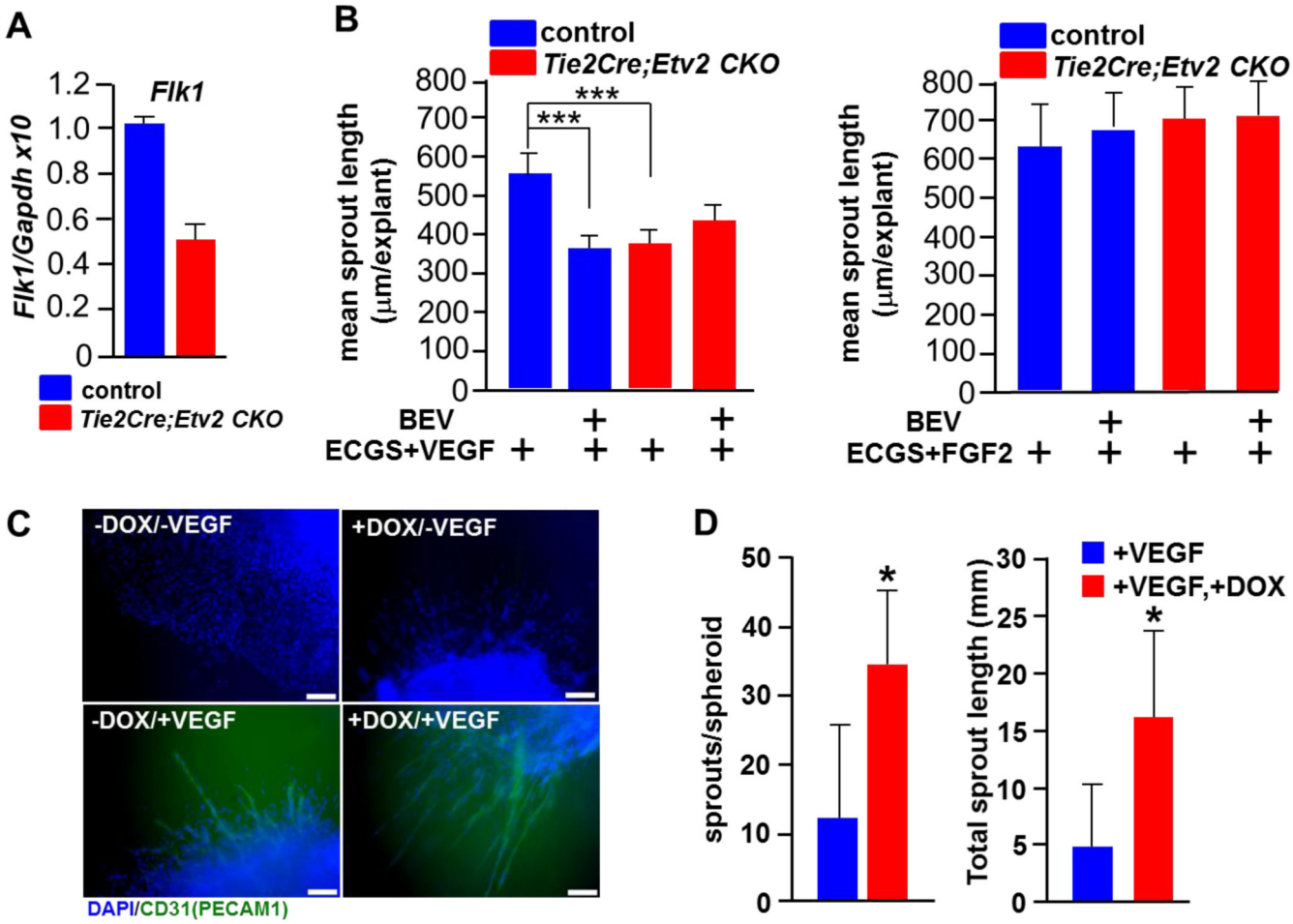
A, FACS analysis using anti-CD45 and anti-CD31(PECAM1) antibody on adductor muscle harvested 7 days after the injury is shown.

B, Quantification of the flow cytometry analysis. n=3/group, *P < 0.05

C, Angiogenesis PCR array using adductor muscle RNA obtained 7 days after ischemic injury is shown. Data is shown as fold change between injured (n=4) and uninjured (n=5) groups.

D, Angiogenesis PCR array using adductor muscle RNA obtained 7 days after ischemic injury of the mice is shown. Data is shown as fold change between *Etv2*-lentivirus injected and injured control group. n=4/group.

E, Western blot analysis with indicated antibodies on adductor muscles harvested on day 28 after the injection. A representative data from four independent experiments is shown. Error bars indicate standard deviations.



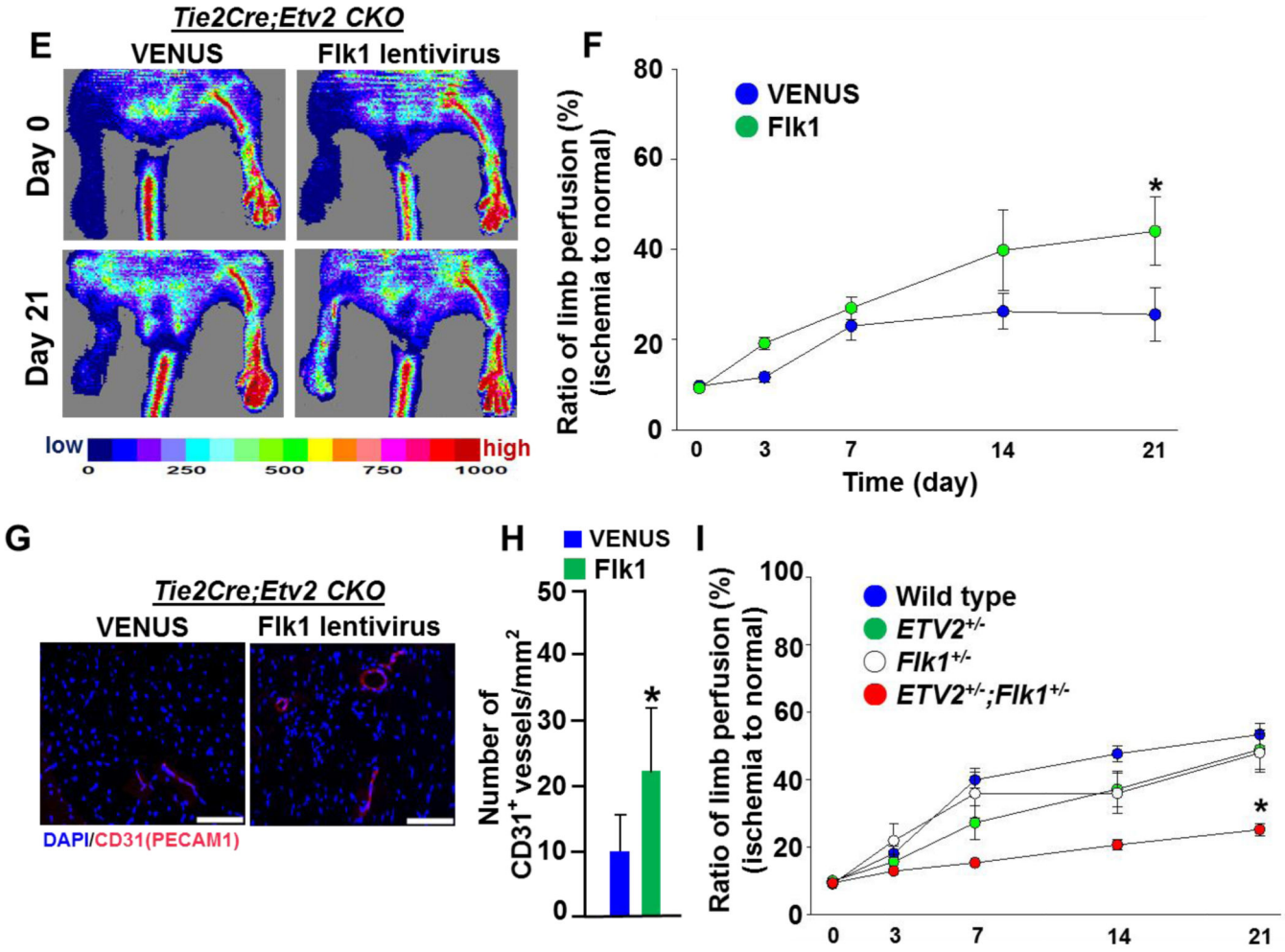


Figure 7. ETV2 regualtes VEGF response in angiogenesis

A, qRT-PCR analysis of *Flk1* expression on FACS-sorted VECADHERIN(VECAD)$^{+}$ cells from adductor muscle of control and *Tie2-Cre; Etv2* CKO mice. Cells were sorted 7 days after ishcemic injury. Sorted cells from 4 mice/group were combined and used for gene expression analysis.

B, Angiogenic sprouting in *Tie2-Cre; Etv2 CKO* choroidal explants. Choroidal explants of the controls and *Tie2-Cre; Etv2* CKO mice were subjected to angiogenic sprouting assay and the mean sprout length was measured 9 days later. BEV; bevacizumab, ECGS; endothelial cell growth supplement, n=4/group, ***P < 0.001.

C, Representative CD31 staining of EB sprouts are shown. DAPI (blue) staining for nucleus is shown. Scale bar: 100 μm.

D, Quantification of the number of sprouts per EB (left) and the total sprout length (right) is shown. n=5/group, *P < 0.05. Error bars indicate standard deviations.

E, Representative images of blood perfusion of *Tie2-Cre; Etv2* CKO mice injected with lentiviral *Venus* or *Flk1*. Images were obtained on day 0 and 21 after ischemic injury.

F, Blood perfusion ratio of ischemic hindlimbs measured by LDPI in *Tie2-Cre; Etv2* CKO injected with lentiviral *Venus* or *Flk1* is shown. n=4/group, *P < 0.05

G, Representative images of CD31 immunohistochemistry. Adductor muscle from *Tie2-Cre; Etv2* CKO and *Tie2-Cre; Etv2* CKO mice injected with lentiviral *Flk1* were harvested 21 days after injury and subjected to immunohistochemistry with anti-CD31(PECAM1) antibody. DAPI (blue) for nuclear staining, Scale bars: 100 μm.

H, Quantification of CD31(PECAM1)-positive vessel density in the ischemic region. n=4/group, *P < 0.05

I, A genetic interation between ETV2 and FLLK in ischemia-induced angiogenesis. Wild type (n=9), $Etv2^{+/-}$ (n=16), $Flk1^{+/-}$(n=14) and $Etv2^{+/-}$; $Flk1^{+/-}$ (n=11) mice were subjected to hindlimb ischemia and blood perfusion ratio of the ischemic limbs was measured on day 0, 3, 7, 14 and 21 after the injury using LDPI. *P < 0.05, wt, $Etv2^{+/-}$, $Flk1^{+/-}$ vs. $Etv2^{+/-}$; $Flk1^{+/-}$. Error bars indicate standard deviations.