Endothelial Cell–Dependent Regulation of Arteriogenesis

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Running title: Cellular Basis of Arteriogenesis

Subject codes:
[138] Cell signalling/signal transduction
[139] Developmental biology
[147] Growth factors/cytokines
[145] Genetically altered mice

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In June 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.67 days.
ABSTRACT

**Rationale:** Arteriogenesis is the process of formation of arterial conduits. Its promotion is an attractive therapeutic strategy in occlusive atherosclerotic diseases. Despite the functional and clinical importance of arteriogenesis, the biology of the process is poorly understood. Synectin, a gene previously implicated in the regulation of VEGF signalling, offers a unique opportunity to determine relative contributions of various cell types to arteriogenesis.

**Objective:** We investigated the cell autonomous effects of a synectin knockout in arterial morphogenesis.

**Methods and Results:** A “floxed” synectin knock-in mouse line was cross-bred with endothelial (Tie2, Cdh5, Pdg fb) and smooth muscle (Sm-MHC)- specific Cre driver mouse lines to produce cell type specific deletions. Ablation of synectin expression in endothelial but not smooth muscle cells resulted in the presence of developmental arterial morphogenetic defects (smaller size of the arterial tree, reduced number of arterial branches and collaterals) and impaired arteriogenesis in adult mice.

**Conclusions:** Synectin modulates developmental and adult arteriogenesis in an endothelial cell-autonomous fashion. These findings show for the first time that endothelial cells are central to both developmental and adult arteriogenesis and provide a model for future studies of factors involved in this process.

**Keywords:**
Arteriogenesis, synectin, endothelial cells, VEGF, collateral, VEGF receptor, artery

**Nonstandard abbreviations and Acronyms:**

- **EAA1:** Early Endosome Antigen 1
- **EC:** Endothelial cell
- **ECKO:** Endothelial cell conditional knockout
- **FGF:** Fibroblast growth factor
- **HIF:** Hypoxia-inducible factor
- **IGF:** Insulin-like growth factor
- **iEC-SynKO:** Induced Endothelial Synectin KO
- **MCP1:** Monocyte Chemotactic Protein 1
- **Micro-CT:** Micro-Computed Tomography
- **NPY:** Neuropeptide Y
- **PDGF:** Platelet-derived growth factor
- **PDGFR-β:** Platelet-derived growth factor receptor β
target
- **SMC:** Smooth muscle cell
- **SMKO:** Smooth muscle cell conditional knockout
- **Sm-MHC:** Smooth muscle myosin heavy chain
- **VEGF:** Vascular endothelial cell growth factor
- **VEGFR:** Vascular endothelial cell growth factor receptor
- **WT:** Wild type
INTRODUCTION

Arteriogenesis, the process of formation of arterial conduits, is a promising therapeutic approach to the treatment of a number of ischemic vascular diseases. Yet its biology remains poorly understood. New vasculature can form via three distinct processes—vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis refers to formation of the primitive vascular plexus from vascular progenitor cells during embryonic development. Angiogenesis is the process of new capillary formation that occurs by sprouting or by longitudinal splitting (intussusception) of existing vessels. Finally arteriogenesis refers to development of larger vessels such as arterioles and arteries. Collateral formation represents a specific case of arteriogenesis. By definition, collateral vessels provide artery-to-artery connections and are thought to play a protective role by providing alternative routes to blood flow.

While angiogenesis is largely driven by vascular endothelial growth factor (VEGF) production in response to hypoxia or inflammation, little is known about factors controlling arteriogenesis in general and collateral formation in particular. In adult tissues, arteriogenesis can occur at sites of arterial occlusion where ischemia is not prominent and is thought to be triggered by hemodynamic factors such as changes in shear stress forces sensed by endothelial cells. How that happens is the subject of considerable controversy. One possible scenario is that arteriogenesis is the result of remodelling of pre-existing collaterals. Indeed, the existence of small collaterals that expand upon occlusion of the main arterial trunk has been clearly described, both in animals and in humans, and the ability to restore compromised tissue perfusion has been linked to the extent of pre-existing collateral circulation.

Alternatively, adult arteriogenesis has been described as a de novo process that occurs by the expansion and arterialization of the capillary bed. In this scenario, the ability of EC to proliferate and to secrete growth factors, such as PDGF, are crucial for the new vascular network development and subsequent arterialization via recruitment of mural cells. One piece of evidence strongly in favour of this hypothesis is the extent of new arterial growth observed by micro-CT angiography following a large artery occlusion. This is highly unlikely to arise solely from pre-existing collaterals.

Studies over last decade have identified a number of growth factors, including PDGF, FGF and VEGF, cytokines such as MCP1, peptides such as NPY and master regulators such as HIF-1, HIF-2 and PR3 that can promote arteriogenesis. Of these, VEGF-A appears to play the central role. In particular, experimental studies have demonstrated that collateral growth is prevented by anti-VEGF-A neutralizing antibodies, VEGF receptor inhibitors, and soluble VEGFR traps, while genetic approaches further demonstrated the requirement for VEGF-A expression for collateral development in healthy tissue. Disruption of VEGF signalling due to impairment of VEGFR2 trafficking has also been shown to result in decreased arteriogenesis.

Although the role of VEGF-A in arteriogenesis is well established, its cellular site of action remains uncertain. In this study we set out to address the cellular underpinnings of VEGF-dependent arteriogenesis. To this end, we have taken advantage of the recent identification by our laboratory of an arterial morphogenetic defect associated with a global deletion of synectin. Synectin is a widely expressed single PDZ domain protein that interacts with a variety of plasma membrane and cytoplasmic molecules to control intracellular signalling. Homozygous disruption of synectin in mice or a knockdown of its expression in zebrafish result in a selective reduction of arterial morphogenesis including decreased branching and reduced size and diameter of the arterial vasculature. Synectin-null endothelial cells have reduced responsiveness to VEGF stimulation and decreased activation of ERK, while their responses to other growth factors such as FGF and IGF are normal. In addition, synectin null mice display downregulation of PDGF expression in the endothelium, likely accounting for the loss of vascular smooth muscle cells coverage of smaller blood vessels observed in these settings. This gene, therefore, offers a unique ability to help determine relative contributions of various cell types to arteriogenesis.
To study that problem, we generated a mouse line with a floxed synectin gene knocked-in into the synectin (gipc1) locus and cross-bred it with endothelial (Tie2-, Cdh5- and Pdgfb-) and smooth muscle (Sm-MHC-) Cre mouse lines to produce endothelial (EC) and smooth muscle cell (SMC)- specific deletions, respectively. We find that ablation of synectin expression in EC but not SMC mimics the arterial phenotype of global synectin null mutants 31 thereby implicating endothelium as the key cell type regulating arteriogenesis.

METHODS

Full methods are described in the online data supplement.

**Mice.**
Mice homozygous for a conditional allele of synectin (gipc1) were generated by flanking exon 2 with loxP sites. Exon 2 of the synectin (gipc1) was deleted by crossing the gipc1flox/flox mice with either the Sm-MHC-Cre 37 , Tie2-Cre 38, Cdh5-CreERT2 39 or Pdgfb-Cre/ERT240 mice. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University.

**Primary smooth muscle and endothelial cell isolation and culture.**
Primary SMCs were isolated from dorsal aorta, as previously described 41 with minor modifications. Primary ECs were isolated from the heart and lung of adult mice using a previously described protocol. 31

**Hindlimb ischemia model.**
This was done as previously described by our lab. 28, 31 Laser-Doppler flow- imaging was carried out using a Moor Infrared Laser Doppler Imager (LDI; Moor Instruments Ltd) at 36.5°C- 37.5°C under isofluorane anesthesia.

**Micro-CT angiography.**
Microcomputed tomography (mCT) of the cardiac, renal and hindlimb vasculature was done by injecting 0.7ml bismuth contrast solution in the descending aorta and the vasculature was imaged and quantified as described previously. 28, 31, 42

**Spino-trapezius assay.**
Spinotrapezius muscles (both left and right) from euthanized mice, were stripped of fascia and dissected as described previously. 14, 43 Quantification of vessel diameters and collateral arcades was done using ImageJ software. 44

**Retina analysis.**
The eyes were harvested at postnatal day 5 (P5) and 17 (P17) and processed as described in Supplementary Data.

**In vivo Matrigel assay.**
The Matrigel was pre-mixed with heparin (5U) with or without VEGF-A 165 (100ng/ml) and injected subcutaneously. 7 days later the plugs were recovered from the sacrificed mice, embedded in OCT, cryosectioned in 10 μm sections and stained with anti-CD31 antibody.

**Wound healing assay.**
Wound healing assays were done by creating in the skin of the back of 12-14 week-old male mice 6mm punch wounds. The healing process was analyzed over time. 45
RESULTS

Generation of gipc1 floxed mice and smooth muscle and endothelial knockouts.

To evaluate the specific contributions of EC and SMC to arteriogenesis, we generated mice carrying a floxed synectin allele inserted into the endogenous gipc1 gene locus. The targeting strategy (Fig. 1A) resulted in the creation of a gipc1 allele with loxP sites flanking a neomycin resistance cassette and exon 2 of the gipc1 gene. The neomycin resistance gene was subsequently removed using flop-mediated recombination. Screening of the progeny confirmed the presence of the floxed allele (Fig. 1B).

In order to ablate synectin expression in SMCs, gipc1[+/β] mice were crossed with Sm-MHC-Cre strain and the progeny was bred to produce mice homozygous for the loss of smooth muscle synectin expression (SynSMKO). Genotyping of litters containing SynSMKO mice (Sm-MHC-Cre;Syn+/F) revealed the presence of all genotypes with the expected Mendelian frequency.

Carotid arteries and aorta from SynSMKO mice had markedly lower mRNA levels of synectin than littermate controls (Fig. 2A). Western blot analysis of synectin expression in primary SMC and EC, isolated from these mice confirmed nearly complete loss of synectin expression in SMC but not EC (Fig. 2B). Stimulation of SynSMKO SMC with PDGF-BB, one of the key growth factors regulating SMC migration and proliferation, revealed no differences in ERK activation as compared with control SMC, demonstrating that synectin expression in smooth muscle is not required for a normal response to PDGF-BB stimulation (Fig. 2C). These observations are in line with what has been previously shown in SMC from global synectin null mice.36

In order to ablate synectin in EC, homozygous floxed synectin mice were crossed with the Tie2-Cre mouse line and the progeny was bred for endothelial-specific knockout of synectin (SynECKO). Western blot analysis of primary heart and lung EC isolated from these confirmed a marked reduction in synectin expression in these cells (Fig. 2D). Previous studies from our laboratory have demonstrated reduced responsiveness to VEGF stimulation of synectin null arterial EC isolated from synectin global knockout mice.31, 32 To confirm that EC-selective disruption of synectin resulted in the same phenotype at the cellular level, we examined VEGF signaling in ECs isolated from SynECKO mice. Analysis of VEGF receptor 2 (VEGFR2) activation following stimulation with VEGF-A demonstrates an expected reduction in Y1175 site phosphorylation and a decrease in ERK1/2 activation (Fig. 2E).

Impaired arterial development in SynECKO but not in SynSMKO mice.

In order to assess the impact of EC and SMC synectin deletion in the arterial development, we performed micro-computed (mCT) angiography of the heart (Fig. 3A) and renal vasculature (Fig. 3B) of SynSMKO, SynECKO and littermate control mice as described previously.28, 31 Arterial trees in both the heart and kidneys of SynECKO, but not SynSMKO mice, exhibited less branching, had fewer artery-to-artery connections (collateral arteries) and appeared smaller in diameter. Quantitative analysis of coronary circulation showed a significant decrease in the number of smaller arteries (≤64µm in diameter) in SynECKO mice. SynSMKO mice showed a trend towards reduced number of smaller arteries (≤32µm in diameter) that was not statistically significant (Fig. 3C). This reduction in the number of smaller caliber arteries in SynECKO mice is very similar to that observed in the global synectin knockout.31 To our surprise we found aneurysmal dilations of distal coronary arteries (Fig. 3D, arrowheads) and even frank tubular aneurysms in SynECKO mice (arrow) that were not previously seen in global synectin null mice.

To gain a better understanding of arterial microcirculation changes associated with an endothelial-specific synectin knockout, we examined vascular networks in the spino-trapezius...
muscle. Because of its thinness (~150 µm), the entire vascular network of the muscle can be imaged in whole-mount at micron-level resolution (Fig. 4A). The muscle is fed by multiple arterioles, notably lateral and caudal feed vessels (red arrowheads, Fig. 4A) which are connected by an arteriolar arcade network providing multiple collateral pathways for blood flow. In wild type mice, the collateral networks have a ramified arcade structure, with multiple arteriole linkage while in SynECKO mice the network has a dendritic structure with smaller arteries and fewer arteriolar-level connections (Fig. 4A). No abnormalities were observed in SynSMKO mice (images not shown). This finding was confirmed by quantitative analysis of the spino-trapezius circulation in wild type, SynECKO and SynSMKO mice (Fig. 4B, C). The input arterioles are significantly narrower in the SynECKO but not SynSMKO mice compared to littermate controls (Fig. 4B), thus confirming spino-trapezius angiography and coronary mCT observations. In addition, the number of arteriolar arcades (collateral arteries) is significantly lower in SynECKO as compared with SynSMKO or littermate control mice (Fig. 4C).

Postnatal arteriogenesis defects in synectin endothelial knockout mice.

To investigate the effect of SMC- and EC-specific synectin knockout on arterial morphogenesis in adult tissues, we next analysed arteriogenesis in SynSMKO and SynECKO mice using a hindlimb ischemia model. In this model, arteriogenesis is induced by ligation and excision of a segment of the common femoral artery of 10-12 week old mice. Following artery ligation, blood flow in the distal limb is assessed by a deep penetrating Laser Doppler and expressed as a ratio of flow in the foot of operated to non-manipulated limb. Immediately after surgery, there was a dramatic but equal reduction in perfusion in all three groups (Fig. 5A, D). Blood flow in the distal limb of control and SynSMKO mice recovered to the same extent over the next 2 weeks (Fig. 5A). As growth of new arterial vasculature is the principal event responsible for blood flow restoration in this model, we next performed mCT analysis to visualize and quantify the extent of hindlimb vasculature, 14 days after surgery. In agreement with the finding of unimpaired distal blood flow recovery, mCT of the arterial vasculature confirmed comparable arteriogenesis in SynSMKO and wild type mice (Fig. 5B, C). Interestingly, there was a non-significant trend towards larger artery sizes in SynSMKO than control mice (Fig. 5C). On the other hand, blood flow recovery was significantly impaired in SynECKO mice, which was apparent one week after surgery (Fig. 5D) and was similar to that previously observed in synectin global knockout mice. As expected, mCT analysis of the arterial circulation demonstrated a significant reduction in the number of smaller arterial vessels in the tight muscle (Fig. 5E, F).

Inducible ablation of synectin expression in endothelial cells.

Since the Tie2 promoter has been shown to activate Cre expression not only in endothelial lineage but also in hematopoietic lineages during development, this may result in deletion of synectin expression in monocytes and monocyte-derived macrophages. Indeed, these cells play an important role in postnatal arteriogenesis, particularly, in the hind limb ischemia model. To exclude the possibility that decreased synectin expression in cells other than the endothelium was responsible for the impaired arteriogenesis following common femoral artery ligation in adult mice, we employed Cdh5-CreERT2 transgenic mouse line. Unlike Tie2, Cdh5 expression is much more restricted to the endothelium and very little, if any, expression occurs in cells of hematopoietic lineages postnatally.

Analysis of the arteriogenesis in the hindlimb ischemia model was done in 10 weeks gender matched iEC-SynKO mice and controls (n=6/group). Similar to SynECKO (Tie2-Cre; SynFF), iEC-SynKO (Cdh5-CreERT2;SynFF) mice demonstrated a significant decline in blood flow recovery following the common femoral artery ligation (Fig. 6A, B). Quantitative PCR analysis of synectin mRNA expression in lung EC isolated from iEC-SynKO mice (n=3) showed markedly decreased expression compared with controls (Fig. 6C).
Analysis of the spino-trapezius vascular networks in iEC-SynKO mice again demonstrated a significant reduction in the number of collateral arcade structures compared with control littermates (Fig 6D, E). In addition, diameters of the input arterioles were significantly less in iEC-SynKO mice compared with control littermates (Fig. 6E), similarly to what was observed in SynECKO mice.

To assess the impact of reduced endothelial synectin expression in angiogenesis, we analysed P5 and P17 retinas from Tie2-Cre;SynFF (SynECKO) mice (Fig 7A) and P5 retinas from Pdgfb-iCre/ERT2;SynFF (iEC-ECKO) mice (Fig 7B) induced on the first postnatal day. Retinal angiogenesis, included the extent of vascular coverage and the number of vascular branchpoints was the same in both synectin mutant lines and in their littermate controls.

To address the effect of synectin ablation on adult angiogenesis, we first utilized a puncture wound model in Cdh5Cre-ERT2; SynFF mice (iEC-ECKO). The rate of wound healing in this model reflects the underlying angiogenesis and angiogenic defects impair the rate of wound closure. Visual inspection and quantitative analysis demonstrated no difference in wound closure rate between synectin deletants and littermate controls (Fig 7A, B). Finally, iEC-SynKO mice were utilized for an in vivo Matrigel assay. Analysis of Matrigel plugs impregnated with VEGF extracted from control and iEC-SynKO mice demonstrated no differences (Fig 7C, D).

DISCUSSION

The results of this study demonstrate that synectin modulates developmental and adult arteriogenesis in an endothelial cell-autonomous fashion. In addition, we demonstrate that endothelial synectin is not required for adult angiogenesis. Specifically, synectin knockout in EC results in a number of developmental and adult arterial morphogenetic defects including smaller size of the arterial tree, reduced numbers of arterial branches and collaterals, and impaired blood flow recovery after common femoral artery ligation. At the same time, SMC-specific deletion of synectin does not affect the size of the arterial tree, the number of collaterals, or blood flow recovery in adult animals.

We focused on cell-type-specific synectin knockout because of its critical role in the regulation of VEGF signalling, which is central to the process of arteriogenesis. Synectin accomplishes this by facilitating, in complex with myosin-VI, trafficking of newly endocytosed VEGFR2-containing vesicles away from the plasma membrane to EEA1+ endosomes. This allows prolonged phosphorylation of the VEGFR2 Y1175 site involved in activation of PLCγ/ERK signalling which is essential for normal arteriogenesis. While this mechanism has been shown to operate in EC, given the ubiquitous nature of synectin expression and the central role of synectin/myosin-VI complex in trafficking intracellular vesicles formed during clathrin-dependent endocytosis, it is possible that the same holds true in all cells expressing VEGF receptors.

To address, therefore, the cell autonomous effects of synectin, we used SMC- and EC-specific Cre driver lines crossed with floxed synectin knock-in mice to generate synectin deletions in these cell types. Analysis of arterial circulation in hearts, kidneys and skeletal muscles of endothelial cell-specific synectin mice demonstrated reduced numbers of arteries, reduced branching and decreased arterial diameter, while no significant differences in these parameters were seen in smooth muscle-specific synectin knockout mice. These results were confirmed by analysis of arteriolar networks in the spino-trapezius muscle where we also observed reduced arteriolar size and fewer artery-to-artery connections in EC-but not in SMC-specific synectin knockouts. Adult arteriogenesis, assessed as the ability to form new arteries in response to common femoral artery ligation, was also abnormal in SynECKO but not SynSMKO mice. This was demonstrated both by the impaired blood flow recovery to the distal limb in SynECKO mice and by the reduced numbers of small arteries forming above the knee.

DOI: 10.1161/CIRCRESAHA.113.301340
All of these studies were done using Tie2-Cre driver line which, in addition to EC, is also expressed in a small number of hematopoietic precursor cells which differentiate into a number of cell types, including monocytes/macrophages that play an important role in arterial morphogenesis. To exclude the possibility that Tie2 expression in cells other than EC was responsible for the observed arteriogenesis defects, floxed synectin mice were also crossed with Cdh5-CreERT2 transgenic mouse line that has a much more endothelium-restricted expression. Activation of Cdh5-driven Cre (iEC-SynKO) either during development or postnatally reproduced the arterial morphogenesis defects observed in global synectin null and in SynECKO mice while angiogenesis (developmental and adult) was not affected.

As already alluded to, synectin regulates VEGFR2 signaling and, specifically, activation of ERK, by ensuring, in partnership with neuropilin-1 and myosin-VI, a rapid transit of VEGFR2 through the “phosphatase zone” thus preventing its deactivation by PTP1b. Any reduction in endothelial ERK activation, as seen in synectin or myosin-VI knockouts or in Nrp1cyto knock-in mice, results in arterial morphogenic defects. Interestingly, in all of these cases, as well as in the present study of endothelial-specific synectin deletion, angiogenesis remains normal.

The availability of synectin floxed mice has allowed us to address another critical point in arteriogenesis biology- namely the identification of the cell type critical to this process. The fact that endothelial disruption of synectin expression impairs all forms of arterial morphogenesis- developmental and adult as well as collateral development, clearly states that endothelial activation of ERK signaling, a process that synectin regulates, is central to arteriogenesis.

The nature of arteriogenic process, especially in adult tissues, has longed remained poorly understood. The two most frequently advanced hypotheses are the remodelling of pre-existing arterial vasculature into larger size arteries and \textit{de novo} growth of new arteries. The remodelling of pre-existing arteries (usually invoked in terms of collateral development, a special case of arteriogenesis) implies the expansion of the lumen and increased size of the vessel wall, a process that requires SMC proliferation. In contrast, the \textit{de novo} growth of new arteries suggests formation of new arterial conduits via arterialization of the capillary network. Such a process would be expected to be heavily dependent on EC proliferation since it requires capillary bed expansion.

Defective arteriogenesis in EC-specific synectin mutants strongly argues for the second hypothesis. The decreased VEGF responsiveness of synectin-deficient endothelium likely affects arteriogenesis by affecting two critical process- formation and expansion of vascular lumen and recruitment of mural cells. With regard to the former it is interesting to note that all cases of reduced endothelial ERK activation, including the present study of EC-specific synectin deletion, arterial diameter for comparable arteries (e.g. proximal coronary vs. proximal coronary) is smaller than in control mice. Furthermore, in vitro assay demonstrate that reduced ERK activation leads to reduced lumen formation. At the same time, excessive endothelial activation of ERK signaling leads to formation of larger than normal diameter arteries.

With regard to the latter, decreased endothelial synectin expression leads to reduced secretion of PDGF-BB, growth factor most associated with mural cell recruitment. Combined, these two defects would lead to decreased capillary bed arterialization, a process that requires an increase in capillary lumen diameter and mural cell recruitment.

The role of synectin and endothelial VEGF resistance is supported by two other studies: 1) rescue of synectin null arteriogenic defect by treatment with PTP1b inhibitor; which abolishes the delayed VEGFR2 trafficking thereby activating VEGFR2/ERK signaling, and 2) rescue of synectin null and hypercholesterolemic mice (which are VEGF resistant) by treatment with a PI3K inhibitor, which activates ERK signaling independently of VEGFR2. While these studies were done with global knockouts or
globally impaired mice (hypercholesterolemia), the present study, with a selective endothelial knockout, closes the loop. Impaired VEGF signalling only in endothelial cells is enough to affect arteriogenesis. This supports the de novo arteriogenesis rather than remodelling of pre-existing collaterals, as the dominant process.

In addition, we employed a severe model of hindlimb ischemia that effectively eliminates any protective role of pre-existing collaterals. In fact, measurements of blood flow immediately after surgery demonstrate no difference between WT and synectin EC-null mice (iEC-SynKO, SynECKO), supporting this argument. Also, the difference in blood flow recovery does not appear until day 7, a time course that is consistent with capillary bed arterialization which is largely endothelium-driven, while pre-existing collateral growth typically peaks by day 3. Therefore, all subsequent differences are largely due to de novo arteriogenesis, not to pre-existing collateral remodelling.

More broadly, endothelial resistance to VEGF signalling, whether due to defective VEGFR2 trafficking or to other mechanism(s), would be expected to result in arteriogenic defects. This concept is supported by a number of previous studies that addressed the role of VEGF signalling in the process of collateral formation. In conclusion, understanding how new arteries form in response to ischemia is crucial to developing effective treatments in ischemic vascular diseases. This study identifies endothelial cells and endothelial VEGF responsiveness as key elements in growth and development of new arteries. We suggest that the focus of future therapeutics efforts should be on overcoming endothelial VEGF resistance frequently observed in patients with underlying pathologies such as atherosclerosis and diabetes and that simply providing additional VEGF either directly or via stimulation of mononuclear cell recruitment, is unlikely to result in a therapeutic benefit.

ACKNOWLEDGMENTS
We thank Steve N. Fiering (Dartmouth knockout core lab) for his help with generation of gipc1 flox/flox mice, Rita Webber, Nicole Copeland and Wayne D. Evangelisti (YCVRC) for mouse colony management, Jiang Yifeng for mCT angiography, John Bent for help with image editing and Roa Harb for assistance with statistical analysis.

SOURCES OF FUNDING
Supported, in part, by Brown-Coxe Foundation postdoctoral fellowship (FM), NIH grant HL62289 (MS), Leducq ARTEMIS Trans-Atlantic network grant (FM, MS) and American Heart Association Beginning Grant-in-Aid 12BGIA12060154 (FMG).

DISCLOSURES
None.

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FIGURE LEGENDS

**Figure 1**: Generation of a floxed allele of the mouse Synectin gene. (A) The targeting vector contains: a loxP site (black triangle) into BsrB I restriction site upstream of exon 2, exon 2 cDNA, a fragment with Neo selection cassette (grey arrow) and a loxP site into the PshAI site downstream of exon 2. The Frt-flanked Neo-cassette was removed by Flp-mediated, site-specific recombination (Frt recombination elements are white triangles). (B) Genomic mouse DNA was genotyped with the oligonucleotides indicated in A (black arrows).

**Figure 2**: Validation of Syn$^{SMKO}$ and Syn$^{ECKO}$ mouse lines. (A-C) In vivo ablation of synectin expression in smooth muscle cells (Syn$^{SMKO}$). (A) Decreased synectin RNA levels in carotid (CA) and aorta (Ao) from Syn$^{SMKO}$ mice compared with control littermates. (B) Western blot of total cell lysates of aortic smooth muscle (SMC) and heart endothelial cells (EC) from Syn$^{SMKO}$ mice. Note a reduction in synectin protein levels in SMC but not in EC. (C) ERK activation: Western blot analysis of total cell lysate of aortic SMC. Confluent, serum starved cells were stimulated for 5 min with 50ng/ml PDGF-BB. Phosphorylation of ERK (P-ERK) in response to PDGF-BB is not altered in synectin null smooth muscle cells (Syn$^{SMKO}$) relative to wild type smooth muscle cells (Syn$^{WT}$). T-ERK: total ERK. SM-α-actin: SMC α-actin. (D, E) In vivo ablation of synectin expression in endothelial cells (Syn$^{ECKO}$). (D) Decreased synectin protein levels in endothelial cells isolated from Syn$^{ECKO}$ mice compared with Syn$^{WT}$ mice. (E) Western blotting of total cell lysates isolated from heart endothelial cells (EC). Confluent, serum starved EC were stimulated for the times indicated with 50 ng/ml VEGF-A165. Note reduced phosphorylation of ERK (P-ERK) and in VEGFR2 Y1175 site in synectin null endothelial cells (Syn$^{ECKO}$).

**Figure 3**: Developmental arteriogenesis defects are present in Syn$^{ECKO}$ but not in Syn$^{SMKO}$ mice. Representative reconstructed mCT images of whole hearts (A) and kidneys (B) arterial vasculature (16µm resolution; n=4) from age- and gender-matched Syn$^{WT}$, Syn$^{SMKO}$ and Syn$^{ECKO}$ mice. Note marked reduction in branching in Syn$^{ECKO}$ mice compared with Syn$^{WT}$ or Syn$^{SMKO}$ control mice. (C) Quantitative analysis of mCT images of whole hearts (grey bars, Syn$^{SMKO}$ and black bars Syn$^{ECKO}$). Note a marked decrease in total number of <64µm diameter arterial vessels in Syn$^{ECKO}$ mice relative to control littermates. F = 3.962, p = 0.003; post-hoc Tukey’s HSD test: *, p<0.01; ** p<0.001. (D) Reconstructed mCT images of the heart vasculature from Syn$^{ECKO}$ mice. Note the presence of aneurism-like structures (arrow) and increased diameters in the distal part of some arteries compared with their proximal partly (arrowheads). Scale bar 1mm.

**Figure 4**: Quantification of arterial networks in mouse skeletal muscle. (A) Fluorescent image of the caudal half of the mouse spinotrapezius. Pseudocoloring is used to indicate arteries (red) and veins (blue). Scale bar, 500 µm. Input arterioles entering the muscle are denoted by red arrowheads. Note extensive arcading arterial networks providing collateral pathways between the lateral (top left) and caudal (bottom left) input arterioles. The networks of spinotrapezius muscle vasculature in controls (Syn$^{WT}$) have a ramified arcade structure, with multiple arteriole arteriole linkage. Note that networks in Syn$^{ECKO}$ mice have a predominatly dendritic structure with few arteriolar-level connections. (B) Diameter of the input arterioles as they enter the spinotrapezius. (C) Number of arcades in the caudal collateral spinotrapezius arterial network. Wild type mice, n=10; Syn$^{SMKO}$ and Syn$^{ECKO}$ mice, n=6. ANOVA with Tukey’s HSD post-hoc test analysis: *, p<0.05; **, p<0.01.

**Figure 5**: Impaired blood flow recovery in Syn$^{ECKO}$ mice following ligation of femoral artery. (A, D) Laser Doppler analysis of blood flow perfusion: Syn$^{SMKO}$ (A) and Syn$^{ECKO}$ (D) mice were subjected to common femoral artery ligation. The graph shows blood flow in the ischemic foot (right) expressed as a ratio to flow in the normal foot (left) (R/L) at various time points after femoral artery ligation. Note the absence of flow recovery in Syn$^{ECKO}$ mice at 7 and 14 days. Mean±SD, *p<0.05, †p=0.07, n=10 per group. (B, E) Representative mCT images of reconstructed limb arterial vasculature from Syn$^{SMKO}$ (B) and...
Syn<sup>ECKO</sup> (E) compared with control littermates 14 days after surgery (C, F) Quantitative mCT analysis in Syn<sup>SMKO</sup> (C) and Syn<sup>ECKO</sup> (F) compared with control littermates. Note a significant decrease in the amount of smaller size arteries in Syn<sup>ECKO</sup> mice compared with control group (C) *p<0.05, n=5 mice/group.

**Figure 6.** Arteriogenesis defects in iEC-SynKO mice. (A, B) Laser Doppler analysis of blood flow perfusion in iEC-SynKO (Cdh5-CreERT2;Syn<sup>FP</sup>) and control mice subjected to common femoral artery ligation. (B) Laser-Doppler analysis of blood flow recovery after surgery expressed as a ratio of flow in ischemic (R) to normal (L) foot (R/L ratio) at various time points. Note a significant decrease in flow recovery in iEC-SynKO mice at 7 and 14 days compared to controls. Mean±SD, (*p≤ 0.05, **p≤ 0.01, n=6 per group). (C) Decreased synectin RNA levels in lung EC from iEC-SynKO mice compared with control. (D) Image of the caudal half of the mouse spinotrapezius. Staining for smooth muscle α-actin has been pseudo-colored to indicate arteries (red) and veins (blue). Scale bar, 500 μm. Input arterioles entering the muscle are denoted by red arrowheads. (E) Quantitative analysis of the number of collateral arcades in the spinotrapezius muscle and diameter of the input arterioles as they enter the muscle in iEC-SynKO and WT mice (n=3 each); p≤0.05, two-tailed t-test.

**Figure 7.** Retinal angiogenesis is not affected in Syn<sup>ECKO</sup> and iEC-SynKO mouse lines. (A) Whole mounts of P5 and P17 retinas from Syn<sup>ECKO</sup> mice labelled with isolectin B4 (IB4) and stained with smooth muscle α-actin (SMA) antibody. Representative images for P5 (top) and P17 (two, bottom) are shown. There were no visual differences in vessel area and SMC coverage between Syn<sup>ECKO</sup> mutants and controls. Scale bar 0.5mm. n=4 mice/group. (B) Quantitative analysis of P5 whole mount retinas from iEC-SynKO mice generated with Pdgfb-iCre. Expression of EGFP in mutant pups demonstrates the efficiency of Cre induction after tamoxifen administration (top, scale bar 200 μm). Retinal vasculature and quantitative analysis of vascular area and vessel branchpoints are shown (bottom, scale bar 50 μm, N=3 mice/group.

**Figure 8.** Adult angiogenesis is not affected in iEC-SynKO mouse. (A,B) Wounds were introduced in iEC-SynKO (Cdh5-Cre-ERT2) and littermates controls and imaged at the indicated times. Representative images of healing wounds are shown in (A) and quantification of the area of wound closure (mean ±SEM) are shown in (B). n=4 mice/group. (C,D) Matrigel implants from iEC-SynKO (Cdh5-Cre-ERT2) and littermates controls. Representative images (C) and quantification (D) from CD31 immunolabelled Matrigel plug sections, with and without VEGF-A165 treatment. Scale bar 100um. n=3 mice/group.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Arterial occlusion results in the failure of blood delivery, leading to tissue ischemia.
- In some circumstances blood flow can be re-established via the development of collateral vessels.
- Formation and growth of new arteries and collateral vessels are poorly understood processes.

What New Information Does This Article Contribute?

- Partial disruption of vascular endothelial growth factor receptor 2 (VEGFR2) signaling in endothelial cells impairs arteriogenesis, indicating that endothelial cells play a central role in arteriogenesis.
- These findings suggest a model of the de novo arteriogenesis, in which arteries are formed by arterialization of the capillary bed, expansion of the vascular lumen, and recruitment of mural cells to form new arterial structures. This process is driven by endothelial cell responses to VEGF.

Adult arteriogenesis is a physiologically important but poorly understood process. We examined the role of the endothelium in regulating arteriogenesis by specifically deleting synectin (gipc1), a protein involved in the regulation of VEGFR2 trafficking in endothelial and smooth muscle cells. We found that endothelium-specific deletion of synectin decreased arterial formation during embryonic development and reduced collateral formation in adult tissues. Together with prior studies that have demonstrated synectin’s involvement in VEGFR2 trafficking and activation of ERK signalling, our observations point to the key role of endothelium and, specifically, endothelial ERK signalling, in regulating arteriogenesis. These results suggest that impaired endothelial responsiveness to VEGF, during conditions such as diabetes or atherosclerosis, would attenuate arteriogenesis.
Figure 1

**A**

**WT gipc1 allele**

![Diagram of WT gipc1 allele with genotyping strategy and PCR milestones.](image)

**B**

**Targeting Vector**

![Diagram of targeting vector with genotyping strategy and PCR milestones.](image)

**Targeted allele**

![Diagram of targeted allele with genotyping strategy and PCR milestones.](image)

**gipc1<sup>flox</sup>**

![Diagram of floxed allele with genotyping strategy and PCR milestones.](image)

**Genotyping strategy**

- PCR (WT allele 500bp)
- PCR (floxed allele 600bp)

**FLP-mediated recombination**

- exons
- LoxP sites
- Frt
- Neo selection cassette
- Primers
Figure 2

A

B

C

D

E

Syn\textsuperscript{WT}

Syn\textsuperscript{EKO}

Heart ECs

Aorta SMCs

Syn\textsuperscript{WT}

Syn\textsuperscript{EKO}

0

5'

0

5'

Synectin

VE-cadherin

SM\textalpha; actin

Synectin

P-ERK

T-ERK

VE-Cadherin

P-VEGF-R2 (Y1175)

T-VEGFR-2

Lung ECs

Heart ECs

Synectin

VE-Cadherin

Syn\textsuperscript{WT}

Syn\textsuperscript{EKO}

Syn\textsuperscript{WT}

Syn\textsuperscript{EKO}

Syn\textsuperscript{WT}

Syn\textsuperscript{EKO}

Synectin

P-ERK

T-ERK

VE-Cadherin

P-VEGF-R2 (Y1175)

T-VEGFR-2

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Figure 5

A

Laser Doppler RL Ratio

pre | post | day 3 | day 7 | day 14

Syn WT | Syn SMKO

B

Syn WT | Syn SMKO

C

Vessel Number

THIGH

Syn WT | Syn SMKO

D

Laser Doppler RL Ratio

pre | post | day 3 | day 7 | day 14

Syn WT | Syn ECKO

E

Syn WT | Syn ECKO

F

Vessel Number

THIGH

Syn WT | Syn ECKO
Figure 8

(A) Images showing wound closure over time in Control and iEC-SynKO groups.

(B) Bar graph illustrating % Wound Closure with error bars.

(C) Images showing Matrigel angiogenesis with and without VEGF treatment for control and iEC-SynKO groups.

(D) Bar graph showing Matrigel plug angiogenesis with error bars for WT and iEC-Syn KO groups under no treatment and VEGF treatment conditions.
Endothelial Cell-Dependent Regulation of Arteriogenesis
Filipa Moraes, Julie Paye, Feilim Mac Gabhann, Zhen W Zhuang, Jiasheng Zhang, Anthony Lanahan and Michael Simons

_Circ Res._ published online July 29, 2013;
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2013/07/29/CIRCRESAHA.113.301340

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ENDOTHELIAL CELL-DEPENDENT REGULATION OF ARTERIOGENESIS

SUPPLEMENTAL MATERIAL

Mice

Mice homozygous for a conditional allele of synectin (gipc1) were generated by flanking exon 2 with loxP sites. Exon 2 contains the start codon for the synectin gene. A bacterial artificial chromosome (BAC) clone, containing the synectin genomic region, was obtained by screening the RPCI-22 129/SvEvTacBr BAC library (Genome Resource Facility at the Hospital for Sick Children). A 10kb BstB I-Mfe I fragment containing exon 2 was subcloned into pSMART LC Kan (Lucigen) plasmid vector for the construction of the targeting vector. A replacement targeting vector was constructed with the insertion of a loxP site into BsrB I restriction site upstream of exon 2, and the insertion of an Frt/loxP flanked neomycin cassette positioned 5’ of exon 2, into the PshAI site downstream of exon2. The integrity of the construct was confirmed by restriction digestion and DNA sequencing. Using standard techniques previously described 1, targeted 129SvIMJ embryonic stem (ES) cells were generated and C57BL/6/129 SvIMJ mouse lines were developed from four positive ES cell clones. The presence of the targeted allele was identified by PCR and Southern blot. Three chimeras were born and bred to C57BL/6 mice, and F1 agouti offspring mice were genotyped by PCR to validate germline transmission.

The Frt-flanked neo gene was deleted in vivo by breeding gipc1\textsuperscript{flax/+} with the FLPeR (flipper) mice 2. Offspring were backcrossed seven times into C57/Bl6 and genotyped by PCR. The gipc1\textsuperscript{flax} allele was genotyped with primer pair Flox-BsrBI-Fwd: 5’AAGCAAAAGGACAGTGCCAGT3’, BsrBI Rev: 5’GGACCCACATACTAGACTGC3’. Exon 2 was deleted by crossing the gipc1\textsuperscript{flax/flax} mice with the Sm-MHC-Cre 3, Tie2-Cre 4, Cdh5(PAC)-CreERT2 5 and Pdgfbicre/ERT2 6 mice. Gipc1\textsuperscript{flax/flax} animals were crossed with Sm-MHC-Cre mouse line and the progeny was bred to produce mice without synectin expression in smooth muscle cells (Syn\textsuperscript{SMKO}). To produce mice with endothelial-specific knockout of synectin, homozygous floxed synectin mice were crossed with the constitutive active Cre mouse line, Tie2-Cre (Syn\textsuperscript{ECKO}) and with the inducible Cre mouse lines (iEC-SynKO), Cdh5-CreERT2 5 and Pdgf-
icre/ERT2<sup>±</sup>. After established the different mouse lines, the genotyping was performed by Transnetyx (Cordova) using a quantitative real-time PCR-based (qPCR-based) system to detect the presence or absence of a target sequence within each sample. The animals were maintained in the Animal Research Center at Yale University. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University.

**Tamoxifen injection**

Induction of Cre expression in pups from was done by consecutive intragastric injections (P1-P3) of 100 ug of tamoxifen per pup. Induction of Cre expression in adult mice was done by 5 alternate day intragastric injections (P1-P9) and by other 5 alternate subcutaneous injections from (P11-P20) of 50ul/pup of tamoxifen (2mg/ml in corn oil).

**Primary smooth muscle and endothelial cell isolation and culture**

Primary smooth muscle cells (SMCs) were isolated as previously described<sup>7</sup> with minor modifications. In summary, the dorsal aorta was removed from adult mice and submerged in sterile PBS containing penicilin-streptomycin (100 I.U./ml penicillin:100 ug/ml streptomycin) and amphotericin B (2.5 µg/mL). The vessels were incubated in enzymatic digestion solution (Collagenase - 1mg/mL, Elastase-0.5mg/mL in media without serum) for 10min at 37ºC. After the first enzymatic digestion the vessels were stripped of adventitia and placed into new enzymatic solution for 1h at 37ºC, centrifuged and plated in culture medium (DMEM with 20% Fetal bovine serum, nonessential amino acids, sodium pyruvate, L-glutamine, and antibiotics at standard concentrations). After 7–10 days, the cells began to migrate out from the tissue sections, reaching confluence in 15-20 days. The cells were harvested and protein lysates extracted to undergo western blotting in order to check synectin protein levels. Primary endothelial cells were isolated from the heart and lung of adult mice using a previously described protocol<sup>8,9</sup>. In summary, the tissues (heart and lung) of 4 mice/group were harvested, minced finely with scissors and then digested in 25ml collagenase 0.2% (w/v) at 37ºC for 45 min. The cells were pelleted and resuspended in DMEM-
20% FBS. The cell suspension was incubated, with rotation, with PECAM-1-coated beads (Invitrogen) at room temperature for 20min. Using a magnetic separator, the bead-bound cells were recovered, washed with DMEM-20%FBS, suspended in 10ml complete culture medium (same as described above used for smooth muscle cell culture but complemented with 100ug/ml heparin, 100ug/ml ECGF growth supplement [ECGF: Biomedical Technologies, Stoughton, MA]) , and finally plated in a gelatin-coated 10cm tissue culture dish.

**Western blotting**

Cells were washed with PBS and lysed in RIPA buffer with protease (Sigma) and phosphatase (Bioproducts) inhibitors. The cell lysate was used for western blot. Equal amounts of protein were loaded in 12% ReadyGels (Bio-Rad), separated and transferred at constant voltage.

**Reagents and Antibodies**

The following antibodies were used in the study: mouse monoclonal αSMA clone 1A4 (#A2547, Sigma), goat polyclonal anti-VE-cadherin (#SC6458, Santa Cruz), rabbit polyclonal anti-synectin (courtesy Dr. A. Horowitz), anti- phospho-VEGF Receptor 2 (Tyr1175 #2478, Cell Signaling), anti-total VEGFR-2 (#2479, Cell Signaling) anti-phospho p44/42 MAP Kinase (phospho-ERK, #9106, Cell Signaling), anti-p44/42 MAP Kinase (total ERK, #9102, Cell Signaling). The growth factors, PDGF-BB (220-BB) and VEGF-A165 (293-VE) were obtained from R&D Systems.

**RNA isolation and RT-PCR**

Total RNA was extracted from Syn^{SMKO} carotid artery and aortas and from iEC-SynKO lungs, using RNeasy plus Mini Kit (Qiagen), cDNA synthesis was performed with iScript cDNA Synthesis kit (BioRad) and PCR amplification (RT-PCR) was performed with synectin specific primers:

*LSyn* (1-2): 5’CAGGTCTCCAGCCAGAGT3’

*RSyn* (1-2): 5’GTAGTCGGAAGGCCTCAGC 3’
Quantitative real time PCR (qRT-PCR) was used to measure synectin expression in iEC-SynKO lungs and in Syn<sup>ECKO</sup> endothelial cells (data not shown). SYBR GREEN Mastermix (BioRAD) and Bio-Rad CFX94 detection system were used. Data were normalized to endogenous VE-Cadh primers shown in the table below and confirmed with commercial available primers from Qiagen (VE-Cadh #QT01052044).

### List of primers used for qPCR:

<table>
<thead>
<tr>
<th></th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(VeCadh)q</td>
<td>ATTGAGACAGACCCCCAAACG</td>
</tr>
<tr>
<td>R(VeCadh)q</td>
<td>TTCTGGTTTTCTGGCAGCTT</td>
</tr>
<tr>
<td>Lsyn(ex1-2)q</td>
<td>AGTTTCGAGAGGACCGAGCA</td>
</tr>
<tr>
<td>Rsyn(ex1-2)q</td>
<td>CCTCCTCATTTCCACCAGA</td>
</tr>
<tr>
<td>LSyn (ex2)q</td>
<td>GCAGCAGGAGAATCCCAGAT</td>
</tr>
<tr>
<td>RSyn (ex2)q</td>
<td>GCCGTACAGCTCTTGACAT</td>
</tr>
</tbody>
</table>

### Hindlimb Ischemia Model

#### Surgical hindlimb ischemia

This was done as previously described by our lab<sup>8, 10</sup>. Briefly, surgical procedures were performed in mice under anesthesia and sterile conditions. A vertical longitudinal incision was made in the right hindlimb (10 mm long). The right femoral artery and its side branches were dissected and ligated with 6–0 silk sutures spaced 5 mm apart, and the arterial segment between the ligatures was excised.

#### Assessment of Blood Perfusion by Laser-Doppler flow-Imaging (LDI)

Measurement of blood flow was done by scanning both rear paws with a LDI analyzer (Moor Infrared Laser Doppler Imager Instrument, Wilmington, Delaware) before and after the surgical procedure (days 0, 3, 7, and 14). The animal was kept under 1% isoflurane anesthesia and its body temperature was strictly maintained between 36.5°C- 37.5°C as previously described<sup>8, 10, 11</sup>. Low or no perfusion is displayed as dark blue, whereas the highest degree of perfusion is displayed as red. The images obtained were quantitatively converted into histograms with Moor LDI processing software V3.09. Data were reported as the ratio of flow in the right/left (R/L) hindlimb and calf regions (not shown). Measurement of blood
flow was done before and after the surgical procedure (days 0, 3, 7, and 14). For Syn^{SMKO} and Syn^{ECKO} mouse lines, 10 mutant and 9 control 10-12 week old females were used. For iEC-SynKO mouse line, 6 mutant and 6 control 10 week old males were used.

**Micro-CT Angiography**

For microcomputed tomography (mCT) of the cardiac, renal and hindlimb vasculature, euthanized mice were injected with 0.7ml solution (bismuth contrast solution) in the descending aorta. The mice were immediately chilled in ice and immersion fixed in 2% paraformaldehyde overnight. The vasculature was imaged and quantified as described previously and in detail as follows: 2D mCT scans were acquired with a *GE* eXplore Micro-CT System (GE Healthcare), using a 400 cone-beam filtered back projection algorithm, set to an 8-27-μm micron slice thickness. Micro-CT quantification was done as previously described. In brief, data were acquired in an axial mode, covering a volume of 2.0 cm in the z direction with a 1.04-cm field of view. During post-processing, a 40 000 gray-scale value was set as a threshold to eliminate noise (air, water, and bone signals) with minimal sacrifice of vessel visualization. The mCT data were processed using real time 3D volume rendering software (version 3.1, Vital Images, Inc. Plymouth, MN) and microview (version 1.15, GE medical system) software to reconstruct three 2D maximum-intensity projection images (x, y, and z axes) from raw data. Quantification was performed using a modified Image ProPlus 5.0 algorithm (Media Cybernatics). The data are expressed as vessel number, representing total number of vessels, of specified diameter counted in 200 z sections from thigh and kidney or in 350-400 z sections from heart images. For analysis of heart and kidney, and hindlimb vasculature, 4-5 mutant mice and 4 gender and age matched controls were used.

**Harvest and staining of whole mount tissues**

**Spinotrapezius staining and analysis**

Mouse spinotrapezius tissues were extracted as described previously. Briefly, the dorsal skin was
opened and the dorsal shoulder fat pad bisected to expose the spinotrapezius muscle. The superficial muscle was separated from the deeper-lying latissimus dorsi, stripped of fascia and cut at the cranial and medial edges for removal. Tissues were washed in PBS, permeabilized in blocking solution (1% BSA, 0.5% TWEEN-20 in PBS) for 4 hours at room temperature (RT) and labeled with smooth muscle \( \alpha \)-actin antibody (1A4-Cy3, 1:200) and lectin (IB4-Alexa488, 1:100) at RT for one hour and overnight at 4°C. The samples were washed in PBS and whole-mounted on slides for imaging.

Pseudocoloring of arterioles was performed using intensity filters, and confirmed by inspection. Complete maps of the spinotrapezius arterial networks were acquired at micron-resolution by stitching together 20-25 images (each image with 1.85 um/pixel), acquired by fluorescent microscopy (Nikon 80i Microscope). Quantification of vessel diameters was done using ImageJ software. Diameters were measured at (a) the points where the feed arteries entered the spinotrapezius muscle ("input arterioles") and (b) the set points of narrowest diameter ("bottlenecks"- not shown) in the parallel collateral arteriole pathways between the input arterioles. The data are expressed as the diameters of the input arterioles and the number of collateral arcades in these muscles.

**Retina Staining**

The eyes were removed from neonates at postnatal day 5 (P5) and 17 (P17) and prefixed in 4% paraformaldehyde (4%PFA) for 15 min at room temperature. The transgenic mouse line Pdgfb-icre/ERT2-IRES-EGFP allows detection of Cre recombination activity by assessing GFP expression. Therefore, dissected retinas from Pdgf-icre/ERT2;Syn\(^{FF}\) neonates were immediately inspected for GFP expression and iEC-SynKO were screened from the control littermates.

The dissected retinas were blocked overnight at 4°C in TNBT (0.1M Tris-HCl, 150 mM NaCl, 0.2% blocking reagent [PerkinElmer] supplemented with 0.5% TritonX-100). After washing, the retinas were incubated with IsolectinB4, Alexa Fluor® 488 Conjugate (Molecular Probes CatN#I21411) in Pblec (1 mM MgCl2, 1 mM CaCl2, 0.1 mM MnCl2, 1% Triton X-100 in PBS) for 2 hours at RT, P17 retinas were also stained with smooth muscle \( \alpha \)-actin (SMA, IA4-Cy3 #C6198, Sigma). The retinas were washed 6
times, for 10min in PBS, fixed briefly for 5min in 4%PFA, washed twice in PBS and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Three mutant iEC-SynKO (Pdgf-icre/ERT2) mice and 3 control littermates as well as 4 mutant SynECKO (Tie2-cre) mice and 4 control littermates were used for phenotypic analysis under a fluorescent microscope. Low and high magnification images were acquired using fluorescent (Nikon 80i Nikon Ti-E Eclipse inverted microscope) and confocal (ZEISS LSM710 laser scanning confocal) microscopes. Quantification was performed in iEC-SynKO neonates; 20-25 images per group were acquired and Biological CMM Analyzer software\textsuperscript{16} was used to quantify vascular area and number of vessel branch points per image.

**Adult angiogenesis models**

*In vivo* matrigel assay

The growth factor reduced Matrigel (BD Bioscience) was thawed on ice, one day before procedure. The matrigel was pre-mixed with heparin (5U) with or without VEGF-A 165 (100ng/ml) and injected into subcutaneous tissues of 12-week old male mice from iEC-SynKO mouse line (Cdh5-CreERT2). After 7 days from injection, matrigel plugs were recovered from the sacrificed mice, embedded in OCT and cryosectioned in 10um sections.

To identify infiltrating endothelial cells, the sections were stained with anti-CD31 antibody (BD pharmingen TM) and random images from each plug were acquired by fluorescent microscopy. CD31-positive vessels were quantified using ImageJ v1.47g software and expressed as a read out of angiogenesis. Three mutant mice and 3 control littermates were used for the analysis.

Wound healing model

Wound healing assays were performed by the back punch model. In this model a wound is created in the back skin (6-mm punch) and the healing process is analyzed over time. The rate of wound closure is dependent on angiogenesis.\textsuperscript{17} Before surgery, mice were anaesthetized by intraperitoneal injection of ketamine (100mg/kg)/xylazine (10mg/Kg) solution. The fur was removed from the surgical site and the skin was cleaned with ethanol. Full thickness wounds were made with a sterile 6mm biopsy punch in the
back skin (Miltex, # 33-36), keeping the underlying muscle intact. Images of the wounds were acquired with a LEICA M125 microscope with a HC80 HD camera (Leica Germany) and ImageJ v1.47g software was used to quantify wound sizes. Measure of the wound diameter was done at 0, 1, 3, 5 and 7 days after procedure and is shown as a percentage of the original wound. Four mutant mice and 4 control littermates were used for the analysis (12-14 wks, males).

**Statistical analysis**

Data are presented as mean ±SEM. Comparisons between 2 independent groups were performed with a 2-sample t-test. Differences were considered statistically significant if p ≤ 0.05. Differences between multiple groups were assessed with 2-way ANOVA followed by the post-hoc Tukey's HSD multiple comparisons test.

9. Li J, Shworak NW, Simons M. Increased responsiveness of hypoxic endothelial cells to fgf2 is


