Four-Dimensional Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation

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**Rationale:** Angiogenesis occurs after ischemic injury to skeletal muscle, and enhancing this response has been a therapeutic goal. However, to appropriately deliver oxygen, a precisely organized and exquisitely responsive microcirculation must form. Whether these network attributes exist in a regenerated microcirculation is unknown, and methodologies for answering this have been lacking.

**Objective:** To develop 4-dimensional methodologies for elucidating microarchitecture and function of the reconstructed microcirculation in skeletal muscle.

**Methods and Results:** We established a model of complete microcirculatory regeneration after ischemia-induced obliteration in the mouse extensor digitorum longus muscle. Dynamic imaging of red blood cells revealed the regeneration of an extensive network of flowing neo-microvessels, which after 14 days structurally resembled that of uninjured muscle. However, the skeletal muscle remained hypoxic. Red blood cell transit analysis revealed slow and stalled flow in the regenerated capillaries and extensive arteriolar-venular shunting. Furthermore, spatial heterogeneity in capillary red cell transit was highly constrained, and red blood cell oxygen saturation was low and inappropriately variable. These abnormalities persisted to 120 days after injury. To determine whether the regenerated microcirculation could regulate flow, the muscle was subjected to local hypoxia using an oxygen-permeable membrane. Hypoxia promptly increased red cell velocity and flux in control capillaries, but in neocapillaries, the response was blunted. Three-dimensional confocal imaging revealed that neoarterioles were aberrantly covered by smooth muscle cells, with increased interprocess spacing and haphazard actin microfilament bundles.

**Conclusions:** Despite robust neovascularization, the microcirculation formed by regenerative angiogenesis in skeletal muscle is profoundly flawed in both structure and function, with no evidence for normalizing over time. This network-level dysfunction must be recognized and overcome to advance regenerative approaches for ischemic disease. (*Circ Res. 2017;120:1453-1465. DOI: 10.1161/CIRCRESAHA.116.310535.*)

**Key Words:** angiogenesis ■ erythrocyte ■ microcirculation ■ microvascular dysfunction ■ smooth muscle

It is well established that a robust angiogenic response can occur after ischemic injury to skeletal muscle. This neovascularization response, together with opening of collateral vessels, can restore blood flow to the otherwise compromised muscle.1,2 The innate capacity to regenerate a vasculature in muscle has also provided an important rationale for efforts to augment angiogenesis for individuals with peripheral vascular disease. However, to date, such strategies have met with little to no success.3,4 There may be several reasons for this lack of success, but a fundamental and unanswered question is the extent to which a regenerated microvasculature in adult muscle can meet the metabolic needs of the tissue.

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
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<td>SM</td>
<td>smooth muscle</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>(V_{RBC})</td>
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What New Information Does This Article Contribute?

- The structure and function of the neomicrovasculature that forms after ischemic injury can be quantified, at a network level, by real-time microvascular imaging in the mouse.
- Despite robust angiogenesis, the regenerated microvasculature is profoundly flawed.
- The microcirculation that forms in muscle after ischemic injury fails to adequately control the delivery and distribution of red blood cells and is ineffective at delivering oxygen in accordance with local needs.

What Is Known?

- Subjecting mice hindlimb skeletal muscle to ischemic injury leads to regenerative angiogenesis and near-complete restoration of blood flow.
- Strategies to augment skeletal muscle angiogenesis in individuals with peripheral vascular disease have met with little to no clinical success.
- Current approaches to evaluating regenerative angiogenesis in muscle lack the combined spatial and temporal resolution required for delineating microcirculatory architecture and function.

What is Known?

- Regenerative angiogenesis is critical for restoring perfusion after ischemic muscle injury. However, strategies that augment angiogenesis have not benefited patients with peripheral vascular disease. Importantly, it is not known whether a regenerated microcirculation, even when extensive, can meet the metabolic needs of muscle. We tested this by developing a real-time imaging strategy with spatial and temporal resolution suitable for probing angiogenesis in the ischemic mouse hindlimb. We found that the formation of a new microvascular network was rapid and robust, but the end result was flawed. Branching architecture was aberrant, red blood cell transit was slow and monotonous, and there were arteriovenous shunts. Moreover, the network did not appropriately respond to hypoxia, and smooth muscle cell processes haphazardly wrapped the terminal arterioles. These findings provide the first network-level indication that the regenerated microcirculation in ischemic muscle does not have the attributes required for effective, local delivery of oxygen. They further imply that (1) capillary density and bulk flow are inadequate indicators of functional angiogenesis after ischemic injury; and (2) therapeutic strategies beyond stimulating angiogenesis in ischemic muscle are required, including normalizing microcirculatory physiology.

Methods

A detailed description of methods and materials is provided in the Online Data Supplement and includes information on histology, immunostaining, hypoxia detection, analysis of network architecture, quantifying RBC velocity, and statistical analysis.

Mouse Hindlimb Ischemia

Experiments were conducted in accordance with the University of Western Ontario’s Animal Care and Use Subcommittee. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) 12 weeks of age were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Hindlimb ischemia was induced by ligating the right femoral artery above and below the profunda femoris branch using 6-0 silk sutures and excising the intervening 5- to 6-mm portion of artery.²

Intravital Video Microscopy

RBC transit in individual microvessels within a 50-µm-deep zone across the entire mouse extensor digitorum longus (EDL) surface was assessed by epifluorescent intravital video microscopy. Briefly, mice were anesthetized with ketamine and xylazine intraperitoneally, and a longitudinal incision was made over the anterior hindlimb. Tibialis anterior and peroneus longus muscles were separated from surrounding fascia and spread apart to reveal the underlying EDL.¹ The EDL was covered with an 8x8-mm glass coverslip and positioned face-down on the stage of an inverted microscope (Olympus IX81), maintaining body temperature at 37°C. After a 20-minute stabilization period, the EDL was epi-illuminated with a 120 W Mercury X-Cite high-pressure bulb light source via a 10x objective (Olympus UPlanSApo). RBC transit was visualized by either ultraviolet light
Video sequences were digitized and stored as uncompressed AVI (avi-
monitor for at least 315 images (15 seconds). For all studies, the en-
tire EDL surface was recorded and analyzed (7–10 fields of view). 
Video sequences were digitized and stored as uncompressed AVI (au-
 dio video interleave) files for postprocessing using custom acquisition 
software (NeoVision) and in-house software written in the MATLAB 
(Mathworks) programming environment. RBC hemoglobin O₂ satu-
ratation in capillaries was assessed using transmission intravital video 
microscopy and dual-camera detection of differential oxy- and deoxy-
hemoglobin white light absorption, as described previously.12

Assessment of Network Responsivity to Hypoxia 
To evaluate the network responsivity to an acute hypoxic challenge, we 
constructed a localized O₂ delivery system. The hindlimb and ex-
posed EDL muscle was positioned in a custom-built frame (Sugru; 
FormFormForm Ltd.) fashioned to stabilize the EDL muscle for an 
extended period (28 days) of imaging, with a 2 cm2 mm opening. 
A custom-built gas flow chamber controlled by computer-modu-
lated flow meters was integrated within the microscope stage.13,14 The 
hindlimb was positioned above the chamber, with the EDL muscle 
contacting either an optically transparent O₂-permeable 100-
µm-thick polydimethylsiloxane polymer membrane or a 50-
µm-thick fluor-
rosisilicone acrylate disk.15 Chamber O₂ levels were measured using a 
fiber-optic PO₂ sensor (Ocean Optics) in the gas outlet. Gas consisting 
of 12% O₂, 5% CO₂, and 83% N₂ at 37°C was passed through the 
chamber for 5 minutes to generate a hypoxic O₂ environment 
across the entire EDL and standardize the baseline O₂ content for both 
normal and regenerated muscle. This %O₂ was selected based on 
a previously established capillary normoxia tension in the 
rat EDL of 6.3% O₂.15 Real-time video microscopy blood flow record-
ing was initiated, and 30 seconds later the EDL muscle was subjected 
to the hypoxia challenge by changing the gas content to 2% O₂, 5% 
CO₂, and 93% N₂, effectively removing O₂ from the EDL muscle. 
RBC transit was continuously recorded before and after the hypoxia 
challenge for a minimum of 3 minutes, and RBC velocity (V_{RBC}) and 
RBC supply rate (No of RBCs per second) were quantified.

Mean V_{RBC} and mean RBC supply rates were determined in a 
total of 255 capillaries from control and regenerated EDL muscles. 
Hypoxia RBC measurements were based on 30 seconds of record-
ing prior to induction of hypoxia. Hypoxia RBC measurements were 
acquired from 120 to 180 seconds of recording after a 20-second transi-
tion phase. A stable hyperemic response to hypoxia was considered 
sustained if the plateau V_{RBC} persisted for at least 120 seconds.

Laser Scanning Confocal Microscopy and 
3-Dimensional Reconstruction of Precapillary 
Arterioles 
For thick-section immunostaining, mice were euthanized by isoflu-
orane overdose and perfused sequentially with PBS and 4% parafor-
maldehyde via left ventricle cannulation at physiological pressure. 
EDL muscles were dissected, immersed in 4% paraformaldehyde for 
2 hours, and cryoprotected with 15% sucrose for 2 hours and 30% su-
crose overnight at 4°C. Tissues were then embedded in optimal cutting 
temperature embedding medium (Tissue-Tek) and stored at −80°C. 
One hundred-micrometer-thick longitudinal cryosections were per-
meabilized with 0.5% Triton-X in PBS and double-immunostained 
using biotinylated rat anti-mouse CD31 (cluster of differentiation 
31) antibody (1:50) and mouse anti–smooth muscle (SM) α-actin 
alkaline phosphatase–conjugated antibody (1:50, Clone 1A4; Sigma 
A5691). Bound antibodies were visualized using Streptavidin-488 
(1:200; Life Technologies). Nuclei were visualized with TO-PRO-3 
(1:100; Sigma) and Alexa Fluor 546–conjugated goat anti-mouse IgG 
(1:200; Life Technologies). Sections were transferred to 
positively charged glass slides, mounted with Permafluor (Thermo
Scientific), and flanked with 100-µm-thick plastic coverslip spacers 
(Thermo Scientific) prior to coverslipping and sealing.

Femoral Artery Excision Induces Widespread 
Infarction and Capillary Obliteration in the EDL 
Muscle Followed by Extensive Regeneration 
To study the regeneration of microvessels after ischemic in-
jury, C57BL/6 mice were subjected to right femoral artery 
excision. We screened all muscles in the hindlimb for the ex-
tent of infarction and identified that the anterior muscle bun-
dle distal to the knee was the most extensively damaged (data 
not shown). Within this bundle is the EDL muscle, which we 
found to be 2 cm long, 2 mm wide, and with well-defined borders, making it well suited to a comprehensive assessment. 
Furthermore, the EDL was accessibly located for live imag-
ing. Histological assessment revealed that 1 day after femo-
ral artery excision, the entire EDL muscle was infarcted and necrotic. Myofibers were shrunken, had lost their nuclei, and 
were stained palely with eosin (Figure 1A and 1B). However, 
10 days after surgery, the muscle had repopulated with int-
ensely staining myocytes with large central nuclei, indicative 
of regenerated skeletal myocytes (Figure 1C).16 By 28 days, 
the regenerated myocytes had matured, with larger diameters 
and more condensed nuclei (Figure 1D).

To evaluate the microvasculature, sections were imm-
unostained using a panel of distinct endothelial markers. 
Immunostaining uninjured EDL muscles for CD31, endomu-
cin, von Willebrand factor, and isoclectin-B4 revealed capill-
aries at the interface between the skeletal myofibers (Figure 
1E). One day after femoral artery excision, immunoreactivity relative to control muscle fell to 14%, 11%, 23%, and 44% 
for each marker, respectively (Figure 1F). Remarkably, 3 days 
after surgery, immunoreactivity for CD31, endomucin, and 
von Willebrand factor was entirely absent, with some residual 
isoclectin-B4 staining (34%; Figure 1E and 1F). Notably, the 
latter stains basement membranes in addition to endothelial 
cells.17 Furthermore, hematoxylin and eosin staining revealed a 
complete absence of capillaries, with only the occasional 
fibrous ghost structure without nuclei (Online Figure I).

Capillary obliteration was evident down to ~150 µm below the 
muscle surface, with only scattered capillaries in the muscle core. 
Interestingly, neocapillaries emerged thereafter throughout the 
EDL muscle, in concert with the skeletal myo-
genesis (Figure 1G). By 28 days, their density exceeded that of 
control EDL muscle by 1.65-fold (P<0.006; Figure 1G and 1H), 
although with a capillary-to-muscle fiber ratio that was similar 
to that of control muscle (1.09±0.06 versus 1.07±0.06;
Collectively, these findings reveal extensive capillary destruction in EDL muscle after ischemic injury, followed by a vigorous microvascular regeneration program that was associated with skeletal muscle regeneration.

**Regenerated EDL Muscle Displays Chronic Hypoxia Despite Robust Angiogenesis**

We next asked whether the regenerated EDL muscle returned to a well-oxygenated state. To assess for hypoxia, tissues were immunostained for pimonidazole adducts after in vivo perfusion with hypoxynprobe-1. Control EDL muscle showed no hypoxia signal (Figure 2A). However, regenerated EDL muscle (28 days) displayed widespread hypoxia signals in the muscle fiber cytoplasm and nuclei, with myofiber to myofiber variability (Figure 2B and 2C). We also identified increased expression of the hypoxia-dependent, HIF (hypoxia-inducible factor)-1α-regulated genes adrenomedullin (P=0.004), lysyl oxidase (P=0.043), and procollagen lysyl hydroxylase (P=0.008; Figure 2D). Interestingly, expression of VEGF-A (vascular endothelial growth factor-A), which can stabilize sooner than that of adrenomedullin,18 was not greater than that in the control muscle (P=0.272) and neither was that of angiopoietin-1 (P=0.370). However, angiopoietin-2 was substantially upregulated (P=0.029) as was the ratio of angiopoietin-2/angiopoietin-1 (P=0.003). These findings suggest that despite having a capillary–myofiber ratio similar to baseline, the regenerated EDL muscle was subjected to a chronic, non-angiogenic level of hypoxia.

**In Vivo Microvascular Imaging Reveals Robust Network Regeneration but With an Aberrant Branching Architecture**

To establish the network architecture of the regenerated microvasculature, we performed real-time microscopy after...
intravenous injection of fluorescein isothiocyanate–labeled high molecular size Dextran. This enabled visualization of flowing microvessels, with RBCs in relief against the fluorescent plasma, within a 50-µm-thick zone along the entire EDL muscle. As we generated RBC transit maps, from 15-second video sequences, which revealed the location and geometry of all flowing microvessels, contrasted against stationary and, thus, nonvisible muscle. Transit maps and live video sequences revealed a highly organized microvasculature in uninjured EDL muscle with hierarchical units of feeder arterioles, capillaries, and postcapillary venules (Figure 3A; Online Movie I). Feeder arterioles penetrated orthogonally through the muscle to the surface zone, where they bifurcated into capillaries. Capillaries could also be seen to bifurcate as they coursed parallel to the EDL surface, ultimately converging and draining into venules.

One day after femoral artery excision, blood flow in the EDL microvasculature had ceased, and there was no RBC transit signal (Figure 3A; Online Movie II). However, 10 days after injury, an extensive but relatively chaotic network of flowing neovessels had regenerated (Figure 3A). Quantitative analysis confirmed a hypervascular network, with a 21% greater length density than in normal EDL muscle (P<0.003; Figure 3B) and a 3-fold increase in the number of branches, normalized to total network length (P<0.0001; Figure 3C). Although microvessels of different lumen caliber were present, there was no evidence for arteriole–capillary–venule hierarchy at this time point (Online Movie III). However, the network subsequently underwent rapid structural maturation. Transit maps indicated that by day 14, there had been substantial branch pruning (Figure 3C), and both network length and branch density stabilized by day 14 to 21. Real-time tracking of RBCs also revealed that the network had acquired arteriole–capillary–venule hierarchy by day 14.

Twenty-eight days after ischemic injury, the network resembled that of the uninjured EDL muscle (Figure 3A; Online Movie IV). Notably, however, there was still a 21% greater vessel length density when compared with the normal network (P=0.019) and a residual 1.6-fold increase in bifurcations (P=0.0001). Remarkably, elevated length and branch densities were still present 56 and 120 days after ischemic injury (Figure 3A–3C; Online Movies V and VI).

We also identified several abnormalities in architecture of the feeder arterioles. First, arterioles were seen to course parallel to the muscle fibers, rather than orthogonal to them, running ≤1 mm before bifurcating (Figure 3D). Second, abnormalities in arterial branch caliber were found. In uninjured muscle, arterioles bifurcated into equal-caliber daughter branches, whereas neoarterioles branched into daughter vessels with unequal luminal diameters (Figure 3D; Online Movie VII). Third, neoarterioles were seen not only to bifurcate, but also to trifurcate and quadrificate (Figure 3D; Online Movie VII). Although only bifurcations were found in control muscle, supernumerary branching was identified in 6.5%, 6.7%, and 7.7% of arterioles within the neovasculature (28-day) networks. This predicted a 66% decline in network resistance, indicating a hemodynamic consequence of the altered network architecture (P=0.032; Online Figure II). Thus, despite a rapid microvascular regeneration cascade, these findings reveal multiple architectural abnormalities, even in the late-stage network, that are predicted to impact hemodynamics within the network.
We next sought to determine whether RBC transit through the regenerated network differed from that of normal EDL muscle. Within the uninjured EDL, RBCs traversed through capillaries in single file with a velocity ($V_{RBC}$) of 526.8 (337.0–727.0) μm/s (median [IQR]; Figure 4A). In contrast, within the early regenerated vasculature (10 days), median $V_{RBC}$ was only 36% of that of native capillaries ($P<0.0001$). RBC transit was chaotic, with frequent direction changes as they passed through the hyperbranched, nonhierarchical network. Furthermore, the smallest caliber neovessels had 2 to 3 RBCs tumbling side-by-side, rather than transiting in single file (Online Movie III).

At 28 days, single file RBC transit in neocapillaries was evident, and arteriolar and venular flow was discernable (Online Movie IV). However, median $V_{RBC}$ remained low, at 52% of that of normal capillaries ($P<0.0001$). Notably, $V_{RBC}$ was even lower on days 56 and 120 (23% of that of normal capillaries; $P<0.0001$; Figure 4A).

We also identified an abundance of neocapillaries in which blood flow was halted altogether. This was evident either as a static column of plasma devoid of RBCs or as channels with stationary RBCs (Figure 4B; Online Movie VIII). On average, 2.0±1.6 capillaries/mm² in the entire control EDL microvasculature displayed stopped flow for a duration of at least 15 seconds, and this increased by over 9-, 19-, and 19-fold in the regenerated EDL on days 28, 56, and 120, respectively (Figure 4C).

**Regenerated Microvasculature Contains Arteriolar–Venular Malformations**

The movement of oxygenated RBCs from arterioles to capillaries is fundamental to efficient oxygen delivery. However, we found that despite the return of single file capillary transit, not all arterioles diverged into a capillary network. Instead, we observed neoarterioles from which one branch diverged into a capillary mesh, while the other branch flowed directly into a venule (Figure 5A and 5B; Online Movies IX–XI). RBC transit within the direct arteriovenous connection was not single file, and flux was demonstrably greater than in the adjacent capillary system, revealing conditions for diverting oxygenated RBCs directly into the venule. Quantitative analysis revealed that 37%, 32%, and 33% of all neoarterioles on days 28, 56, and 120, respectively, branched into an arteriovenous connection, whereas arteriovenous shunts were rarely observed (2%) in control muscle ($P<0.0001$; Figure 5C).
RBC Velocity Dispersion Is Severely Blunted in the Regenerated Microvasculature With an Abnormal Oxygen Delivery Profile

We next assessed for heterogeneity in RBC transit among capillaries within a network. This heterogeneity is fundamental to the skeletal muscle circulation and critical for precisely matching the metabolic demands of each myofiber in the muscle with RBC delivery. To assess network heterogeneity, we compared the frequency distributions of mean \( V_{\text{RBC}} \) among capillaries over the entire EDL zone. The intercapillary \( V_{\text{RBC}} \) histogram derived from the 28-day regenerated network was significantly different than that of the native network, with both a leftward shift and narrower range (K-S stat =0.54; \( P=1.12 \times 10^{-13} \); Figure 6A). In addition, the normal microcirculation was found to contain a hyperemic subpopulation of capillaries with a particularly high \( V_{\text{RBC}} \) (>1000 \( \mu \)m/s). In contrast, there was no hyperemic subpopulation of capillaries in the regenerated network on day 28. These abnormalities did not normalize over time and in fact were worse on days 56 and 120. Thus, the regenerated microcirculation displayed profoundly reduced dispersion in \( V_{\text{RBC}} \), suggesting an inability of the network to tune the delivery of \( O_2 \) to meet the needs of individual myofibers.

To determine whether the homogeneity in \( V_{\text{RBC}} \) was associated with abnormal \( O_2 \) delivery, we directly measured...
hemoglobin O\textsubscript{2} saturation in RBCs transiting through the capillaries. Using dual wavelength absorption microscopy to distinguish oxy- and deoxyhemoglobin, we found that median RBC O\textsubscript{2} saturation in regenerated capillaries (120 days) was 75\% of that in normal capillaries (\textit{P}=0.0003). This reduction was evident not only for RBCs at the downstream end of the capillary (69\%; \textit{P}=0.002) but also at the upstream end (77\%; \textit{P}=0.005; Online Figure III). Furthermore, there was a strikingly wide spread in RBC O\textsubscript{2} saturation in the regenerated capillaries, with O\textsubscript{2} saturations as low as 2\% and as high as 87\%. These findings indicate the variable presence of regions with profoundly unmet O\textsubscript{2} demands, further supporting a failure of local tuning of RBC delivery.

**Regenerated Microvasculature Has Impaired Flow Responsiveness to Hypoxia**

To directly determine whether the regenerated microcirculation could regulate blood flow, we generated a methodology to locally control the O\textsubscript{2} content in the mouse EDL muscle. A defined O\textsubscript{2}-containing gas mixture was delivered to the EDL.
muscle through a gas-permeable membrane. After generating a stable $P_{O2}$ of 12% at the muscle site, we abruptly decreased the ambient oxygen content to 2%, establishing conditions for microremoval of muscle $O_2$. The ability of the microvasculature to respond to this hypoxic challenge was then evaluated by tracking both RBC velocity and RBC supply rate through individual capillaries.

Figure 6B depicts RBC velocity response profiles to local hypoxia. $V_{RBC}$ were acquired every second within a single capillary for each condition before and after hypoxia challenge. C and D, Graphs depicting the $V_{RBC}$ and RBC supply rate responses to local hypoxia. Left, The fold-change response in individual capillaries is shown for day 28. The line depicts the median. Right, The relative median responses (and 45–55 percentile) at the time points indicated are shown. Data are from 164, 65, 27, and 60 capillaries from control (n=6), day 28 (n=3), day 56 (n=3), and day 120 (n=3) EDL muscles, respectively. For $V_{RBC}$: * $P=0.004$, † $P=0.001$, ‡ $P=0.021$ vs control. For RBC supply rate: * $P=0.004$, † $P=0.0004$, ‡ $P=0.005$ vs control.

Figure 6. Red blood cell (RBC) velocity dispersion within the microvasculature and network responsivity to hypoxia. A, Frequency histograms of the average capillary RBC velocity ($V_{RBC}$) within native and regenerated extensor digitorum longus (EDL) muscles 28, 56, and 120 days after injury. The distribution is significantly narrowed on day 28 (K-S stat=0.54; $P=1.12\times10^{-13}$) and remains narrow and left-shifted. B, RBC velocity response profiles to local hypoxia. $V_{RBC}$ were acquired every second within a single capillary for each condition before and after hypoxia challenge. C and D, Graphs depicting the $V_{RBC}$ and RBC supply rate responses to local hypoxia. Left, The fold-change response in individual capillaries is shown for day 28. The line depicts the median. Right, The relative median responses (and 45–55 percentile) at the time points indicated are shown. Data are from 164, 65, 27, and 60 capillaries from control (n=6), day 28 (n=3), day 56 (n=3), and day 120 (n=3) EDL muscles, respectively. For $V_{RBC}$: * $P=0.004$, † $P=0.001$, ‡ $P=0.021$ vs control. For RBC supply rate: * $P=0.004$, † $P=0.0004$, ‡ $P=0.005$ vs control.
supply rates were found to be significantly lower on each of days 28, 56, and 120 after ischemic injury (Figure 6C and 6D). These findings identify that the regenerated microvasculature is impaired in its ability to effectively augment RBC delivery when faced with local hypoxia.

Smooth Muscle Cell Wrapping Around Regenerated Arterioles Is Aberrant
The combination of impaired responsivity to hypoxia and the homogeneity of RBC velocities throughout the network implicated failure of a vasomotor tuning mechanism. We considered

Figure 7. Aberrant wrapping of smooth muscle cells (SMCs) around arterioles in the regenerated microvasculature. A, Confocal micrographs of distal arteriolar trees from normal and regenerated extensor digitorum longus (EDL) muscle, immunostained for CD31 (cluster of differentiation 31; green) and smooth muscle (SM) α-actin (red). Images are projections of 30 optical sections. Arrows indicate sites of discontinuous SMC coverage. B, Confocal micrographs of projected optical sections of arteriolar segments ≈14 to 15 μm in diameter of control and regenerated (day 28) muscle, immunolabeled for SM α-actin (red). C, Projected optical sections of arteriolar segments ≈9 μm in diameter of control and regenerated (day 56 and 120) muscle, immunolabeled for SM α-actin (red) and TO-PRO-3 (blue). Arrows indicate sites of discontinuous SMC coverage, and arrowhead indicates site of aberrant process orientation. D, Graph depicting the SMC interprocess gap widths. Data are from 25 arterioles from native EDL muscles and 23, 19, and 22 neoarterioles from EDL muscles 28, 56, and 120 days, respectively, after ischemic injury. *P<0.0001 vs native. E, Graph depicting surface area coverage of feeder arterioles by SM α-actin-containing processes. *P<0.0001 vs native.
that a unifying basis for this failure might reside in the cellular architecture of the feeder arterioles. To elucidate the microstructure of normal and regenerated arterioles, we generated 3-dimensional confocal reconstructions from 100-μm-thick sections of EDL muscle double-immunolabeled for CD31 and SM α-actin. In normal EDL, arterioles were found to be intimately invested by SMCs. The precise architecture varied with arteriolar caliber and location within the tree. SMCs on arterioles of diameter 12 to 20 μm enveloped the artery with circumferential processes containing SM α-actin, which we could resolve as individual microfilament bundles (Figure 7A and 7B). SMCs on arterioles of 7 to 12 μm diameter also circumferentially wrapped the vessel with SM α-actin cytoplasmic protrusions, but these processes were somewhat thinner and more variably oriented (Figure 7A and 7C). SMC investment and protrusion-based wrapping terminated once the vessel diameter fell to 5 to 8 μm, consistent with transition to a capillary (Figure 7A).

SMCs were also found to layer around arterioles of the regenerated network and send out processes. However, in contrast to native arterioles, the coverage was discontinuous and disordered (Figure 7A–C). In some neoarterioles, there were zones up to 13 μm in length that were largely devoid of SM α-actin bundles. Moreover, even in regions where SMC processes wrapped the vessel, the spacing between adjacent processes was increased compared with that of normal arterioles of similar caliber (Figure 7B and 7C arrows). Quantitative analysis revealed that the median space from one SM α-actin containing process to the next in normal terminal arterioles was 0.74 μm (Figure 7D). These gaps constituted 7% of the arteriolar surface area (Figure 7E). In contrast, 28 days after injury, the median interprocess space was 1.40 μm (P<0.0001), which corresponded to 22% of the neoarteriolar surface area (P<0.0001; Figure 7D and 7E). Increased process spacing was also observed at days 56 and 120, although this improved somewhat. However, prominent gaps in SMC process coverage were still evident, with widely variable process orientations, rendering a haphazard and disordered coverage profile (Figure 7C arrows). These findings strongly implicate arteriolar wall microdisorder as a basis for impaired control over RBC delivery through the regenerated skeletal muscle.

**Discussion**

Through high-resolution mapping of RBC transit, dynamic tracking of RBC velocities, and optical reconstruction of terminal arterioles, we have discovered that the reconstructed microvasculature in EDL muscle is a highly flawled network. The array of abnormalities we uncovered in this regenerated tissue included (1) aberrant arteriolar branching; (2) arteriolar–venular shunting; (3) slow RBC transit through capillaries; (4) a monotony of RBC transit velocities throughout the capillary system; and (5) an impaired vasomotor control system. These abnormalities were still evident 4 months after injury, indicating a failure to normalize. The functional importance of these abnormalities was supported by the presence of hypoxia signals and profoundly abnormal capillary O2 saturations in the EDL muscle, despite a capillary-to-skeletal myocyte ratio that was similar to that of normal EDL muscle. Thus, notwithstanding an extensive regenerative response, the microvascular neonetwork was ill-suited for effective oxygen delivery.

This is the first study to evaluate regenerative angiogenesis in skeletal muscle at a network level and at the site where gas and nutrient exchange occur. The mouse EDL subjected to femoral artery excision proved to be a valuable model for this because the entire EDL muscle was infarcted and the native microcirculation was destroyed, as evidenced by staining with hematoxylin and eosin and a panel of endothelial markers. All subsequently identified microvessels could, thus, reliably be attributed to a regeneration program. This contrasts with other sites in the mouse hindlimb, where infarction is patchy and a mix of preexisting and new microvessels would coexist.1,7 High-resolution mapping of RBC transit in the regenerating EDL revealed that the vasculization response was in many respects impressive. It entailed an early hypervascular phase that covered the entire EDL muscle surface zone. Branching was extensive at this early stage, and the network was not yet hierarchical. However, rapid branch pruning and realignment of microvessels quickly ensued, yielding a network that coursed primarily parallel to long axis of the maturing skeletal myofibers. Moreover, within 14 days of the ischemic insult, these vessels had differentiated into arteriole–capillary–venular circulation units, further indicating the rapid angiogenesis and network remodeling program.

Nevertheless, discordance between the microvascular restoration process and functionality of the RBC delivery system was remarkable. Whether the identified array of microcirculatory defects exists in all models of ischemia, as well as different mouse strains and forms of injury, is unknown. However, femoral artery excision in C57BL/6 mice constitutes a substantial insult and is also a setting where the return of bulk flow is robust, reaching 85% to 100% that of the contralateral limb.1,6,7 The current findings, therefore, highlight the hidden pathophysiology that can exist in the microcirculation. Some differences in capillary network architecture might be expected, given the different size and arrangement of regenerated myofibers. However, the abnormal RBC transit, impaired vasoreactive control, and violations of arteriole–capillary–venule geometry and hierarchy constitute previously unrecognized pathologies in this regenerative setting. Moreover, these defects would not be detectable with currently used techniques for assessing blood flow, such as laser-Doppler, Doppler-ultrasound, and magnetic resonance imaging strategies.

Our findings of asymmetrical arteriolar bifurcations, trifurcations, and quadrifications in the regenerated microvasculature identify a distinctly abnormal arteriolar morphogenesis program. Bifurcation of arterioles into symmetrical caliber branches is fundamental to the microcirculation and ensures ordered dispersion of red cells through the capillary network.23–26 Although the molecular underpinnings of this arteriolar dysgenesis response are unknown, it is noteworthy that both asymmetrical arteriolar branching and supernumerary branching are features of the highly disordered microvasculature of tumors.19,20 The similarities between the tumor and regenerated muscle milieu would only be partial, but it is noteworthy that aberrant arteriole branching in tumors is
could also explain the striking monotony in RBC velocities microvasculature was defective in a fundamental control pro-
response to hypoxic challenge. However, the responses in the rapid means of generating a hypoxic muscle milieu and di-
transit of RBCs through regenerated capillaries was particu-
to hypoxia in skeletal muscle is also supported by mathemati-
proliferation. Three-dimensional reconstruction of confocal optical images revealed how SMCs and their contractile elements are organized around these arterioles. Our finding of circumfer-
transit velocities rose >1000 m/s is also noteworthy because RBCs within capillaries provides im-
velocity of RBCs within capillaries provides imp-
shunts. The persistent increase in the prevalence of capil-
The normal microvasculature does not act as an inert set of microtubes but, instead, as an exquisitely controlled vasomotor system that distributes RBCs in an orchestrated manner to meet the needs of the tissue. Hypoxia is a powerful stimulus for enhancing delivery of RBCs in skeletal muscle. The oxygen exchange strategy we used, coupled with direct tracking of RBC transit in individual capillaries, provided a rapid means of generating a hypoxic muscle milieu and directly linking this to network responsivity. RBC velocity and supply rates in control EDL muscle rapidly increased in response to hypoxic challenge. However, the responses in the regenerated vasculature were blunted. Thus, the regenerated microvasculature was defective in a fundamental control process for modulating RBC delivery. This muted responsivity could also explain the striking monotony in RBC velocities we observed throughout the network. Although it is possible that the demands of the regenerated EDL were more uniform than in the normal EDL, this seems unlikely given the striking variations in capillary RBC O2 saturation that we identified. Together, the findings indicate that the regenerated network does not have the ability to tune the delivery RBCs to match the regional needs.

What might be the mechanism for this relative inertness in the regenerated microcirculation? Distal arterioles constitute the critical effector limb for RBC delivery and the site where hormonal, metabolic, or neural regulatory factors impinge. Three-dimensional reconstruction of confocal optical images revealed how SMCs and their contractile elements are organized around these arterioles. Our finding of circumfer-
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The potential for arteriolar asymmetry to similarly contribute to hypoxia in skeletal muscle is also supported by mathemati-
cal modeling, which has indicated skinning of plasma, disparate local hematocrits, and regional heterogeneity in oxygen tensions unrelated to tissue needs.20,25,28

Our discovery of arteriole–venule shunts in the regenerated microcirculation was striking and reveals another basis for impaired oxygen delivery to the muscle. These aberrant conduits were rarely found in the uninjured muscles, yet constituted the output pattern of 37% of terminal arterioles. Their presence in muscle can be expected to compromise gas exchange by diverting red cells away from nearby single file transit capillaries into the high-flow microshunts. Interestingly, these microshunts were scattered throughout the vascular tree, which differs from the focal arteriovenous malformations seen in conditions, such as hereditary hemorrhagic telangiectasias.29,30 The shunts emerged at the same time as capillaries were forming elsewhere in the network, suggesting a primary, albeit localized, failure in capillary differentiation. This differs from recent studies in the brain where arterio-
venous malformations arise via pathological transformation of preexisting capillaries.31

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Disclosures
None.

References
19. Arpino et al. 4D Analysis of Regenerative Angiogenesis.
Four-Dimensional Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation
John-Michael Arpino, Zengxuan Nong, Fuyan Li, Hao Yin, Nour Ghonaim, Stephanie Milkovich, Brittany Balint, Caroline O’Neil, Graham M. Fraser, Daniel Goldman, Christopher G. Ellis and J. Geoffrey Pickering

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4D Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation

SUPPLEMENTAL MATERIAL

DETAILED METHODS

Histology and Immunostaining

Extensor digitorum longus (EDL) muscles were dissected, fixed in either Tris-buffered zinc or 4% paraformaldehyde (PFA) overnight and embedded in paraffin. Five-μm thick cross-sections of EDL muscle were stained with hematoxylin and eosin. Near adjacent sections were subjected to heat-mediated antigen retrieval with citrate buffer and immunostained for endothelial cells using biotinylated rat monoclonal anti-mouse CD31 antibody (1:100, BD Biosciences 553371) and DAB Substrate (Vector). Nuclei were visualized by counterstaining with hematoxylin. For immunofluorescence detection of endothelial cells, five-μm thick sections of EDL muscle were subjected to antigen retrieval and immunostained using rat monoclonal anti-mouse CD31 antibody (1:20, Dianova, Clone SZ31) or rat monoclonal anti-mouse endomucin antibody (1:100, Santa Cruz, Clone V.7C7, sc-65495) and detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100 for CD31, 1:200 for endomucin, Life Technologies). Endothelial cells were also stained using biotinylated isolectin-b4 (1:100, Sigma, BSI-B4, L2140), detected using Dylight 488-conjugated streptavidin (1:200, Vector) and immunostained for Von Willebrand factor (vWF). In the latter instance, EDL muscles were fixed in 4% PFA at room temperature for two hours, subjected to cryoprotection by immersion in 15% sucrose at room temperature for two hours then 30% sucrose at 4°C overnight, frozen in OCT compound (Tissue-Tek), and stored at -80°C. Ten-μm thick frozen sections were post-fixed with 4% PFA for ten minutes and immunostained using rabbit polyclonal anti-vWF antibody (1:50 in 0.1% Triton-x 100, Millipore AB 7356) and biotinylated goat anti-rabbit IgG (1:100 in 0.1% Triton-X 100, Vector) and Dylight 488-conjugated streptavidin (1:200 in 0.1% Triton-X 100, Vector). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech, 0100-020). Sections were imaged by widefield microscopy (Olympus BX-51) and photomicrographs captured with Northern Eclipse (EMPIX Imaging Inc.) software.

Capillary density and capillary-to-muscle fiber ratio were quantified in 10 equally spaced high-powered (60x objective) fields from a 50-μm thick zone at the muscle periphery using ImageJ (NIH). Muscle fiber density and cross-sectional area were quantified in the mid-zone of the native and regenerated EDL muscle under high-powered view (40x objective) using ImageJ (NIH). For infarct analysis of all muscles in entire the hindlimb, the PFA-fixed hindlimb was decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days and then divided into five blocks that were embedded in paraffin, sectioned, and stained for hematoxylin and eosin.

Detection of Muscle Hypoxia

Muscle hypoxia was assessed by immunohistochemical detection of pimonidazole adducts, following intraperitoneal injection of pimonidazole hydrochloride (60 mg/kg, Hypoxyprobe™-1Kit, Hypoxyprobe, Inc.) into mice 28 days following femoral artery excision. Forty minutes after injection, mice were sacrificed by isoflurane overdose, perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiological pressure, and both injured and contralateral EDL muscles were dissected. Five-μm thick paraffin-embedded cross-sections were immunostained with a monoclonal mouse anti-pimonidazole antibody (Hypoxyprobe-1 mAb, 1:50) and the signal detected according to the manufacturer’s protocol with DAB substrate (Vector). Injured EDL muscles dissected from mice 28 days following femoral artery excision that were not injected with pimonidazole hydrochloride served as a technical control. Sections were counterstained with hematoxylin and hypoxia signal was quantified from entire EDL muscle mid-zone cross-sections using ImageJ, by isolating DAB via the colour deconvolution plugin¹ and measuring the raw integrated signal density following thresholding, using an identical grey
scale (61-255) for all samples.

**Transcript Analysis by RT-PCR**

Total RNA from uninjured and regenerated EDL muscles 28 days following femoral artery excision was extracted with Trizol (Life Technologies) and RNEasy (QIAGEN) and subjected to reverse transcription as described previously.2 The mRNA abundance of adrenomedullin, lysyl oxidase (LOXL2), procollagen lysyl hydroxylase 2 (PLOD2), and VEGF-A was quantified using quantitative RT-PCR and SYBR Green chemistry and a ViiA 7 Real Time PCR System (Life Technologies). Gene expression was normalized to the mouse 18S signal using the \( \Delta \Delta \text{Ct} \) method. Primers pairs (OriGene) employed were: Adrenomedullin, 5'-GCCAGATACCCCTGCAGTTC-3' and 5'-GCGACTACTCTGCTGTAAGGA-3'; LOXL2, 5'-TCTGCTGGAGACACTGAGT-3' and 5'-CGGTCACCTATCTGTAGTGGCT-3'; PLOD2, 5'-CTCCGAGAGGAAGCCAGC-3' and 5'-CATGAGTGGACTCTGAGGGCT-3'; VEGF-A 5'-CTGCTGTAAAGGATGAAAGCTC-3' and 5'-GATCTCTCTGAGAGGATTGTC-3'; ANGPT1 5'-AACCGAGCCTACTCAGTGC-3' and 5'-GCATCCCTTCGTAAGTCG-3'; and ANGPT2 5'-AACTCGCTCTTCCGAGGCAG-3' and 5'-TTCCGACAGTCTGAGGGTG-3'.

**Intravital Video Microscopy**

RBC hemoglobin O2 saturation in capillaries near the EDL surface was assessed by white light transillumination IVVM, as described previously.3,4 For this, a silk suture was attached to the distal EDL tendon. The tendon was severed distal to the ligature, and the EDL was reflected onto the microscope stage (Olympus IX81) and secured at approximately its in situ resting length and orientation. The EDL was kept moist with Plasma-Lyte 148 (Baxter International) at 37°C, and covered with polyvinylidene chloride film (Saran Wrap, Dow Chemical) and a glass coverslip to isolate it from room air. The EDL was transilluminated with a 100-W xenon lamp, and transmitted light was captured with 10x and 20x objectives (Olympus UPlanSApo) and a parfocal beam splitter (DualCam) fitted with 442-nm (an O2 sensitive wavelength for oxy- and deoxyhemoglobin)5 and 454-nm (an isosbestic or O2 insensitive wavelength) band pass filters for dual video cameras. The beam splitter passed 442-nm light to one cooled coupled device camera (Rolera-XR, QImaging) and 454-nm light to a second camera, and dual video recordings (696x520 pixels, 21 images/s) were simultaneously obtained using custom capture software (Neovision). Images from both cameras were registered using the beam splitter and the capture software ensured synchronized frame-by-frame acquisition from both cameras. A minimum of 20 fields of view (10 at the arteriolar end, and 10 and the venular end of a capillary network) were randomly selected and recorded for 60 seconds in each EDL muscle. Video sequences were stored as uncompressed PNG files for post-processing using in-house software written in the MATLAB (Mathworks) programming environment. O2 saturation in capillaries was obtained by analysis of PNG files using custom image analysis software written in Matlab, as described by Ellis et al.6,7 and Japee et al.8,9. Briefly, in-focus capillary segments were selected, and the location of the vessel centerline was used to extract light intensity values from every video frame and generate space-time images for both the 442- and 454-nm wavelengths. The light intensity values were analyzed frame-by-frame to identify the location of each RBC and plasma gap within the capillary. The mean optical density (OD) of each RBC at both wavelengths, was computed as \( \text{OD} = \log(I_o/I_m) \) were \( I_o \) is the measured light intensity of the plasma gaps (intensity of incident light) and \( I_m \) is the measured light intensity values for RBCs. The mean RBC hemoglobin O2 saturation was determined from the ratio of OD(442)/OD(454), which is linearly related to O2 saturation, i.e. \( \text{SO2} = a + b \times \text{ODratio} \). The slope and intercept were determined from an in vivo calibration.

**Analysis of Microvascular Network Architecture**

Length density and bifurcation density of EDL muscle microvascular networks were quantified from RBC transit maps, which effectively displayed a micro-angiogram of all flowing microvessels, using ImageJ. RBC transit maps were generated from IVVM video sequences using custom MATLAB software.
as described. From the videos generated by ultraviolet light epi-illumination, RBC transit maps were generated from “minimum” (MIN) images, which displayed the minimum light intensity value and therefore RBC transit at a given pixel during the duration of the video sequence. From the videos generated by blue light epi-illumination, RBC transit maps were generated from “sum of all differences” (SAD) images, which displayed the cumulative sum of the square of differences in light intensity values at each pixel between consecutive video frames, thus generating a single map of all microvessels perfused with RBCs and plasma. Length density ([μm] / EDL area [μm²]) was quantified at each time-point via manual tracing of all vessel centerlines and normalizing total network length to EDL area. Bifurcation density was determined by manual point counting of all vessel bifurcations and normalizing to total network length. Arteriole-capillary-venule microcirculatory units and AV malformations were identified from blue light epi-illumination videos and corresponding SAD images.

Analysis of Capillary Red Blood Cell Velocity and Distribution

Red blood cell velocities (V_{RBC} [μm/sec]) in individual capillaries exhibiting characteristic single-file RBC transit were quantified from video files using space-time images. Briefly, 1-second (21 frames) interval mean red blood cell velocities were calculated using custom MATLAB software, generating a 2D gray scale plot of RBC location change with time. This enabled an unbiased quantification of RBC transit velocities within capillaries throughout the entire muscle. RBC velocity was determined from a total of 1658 one-second intervals in 115 uninjured capillaries, and from 2269 one-second intervals in 138 neocapillaries, 28 days following femoral artery excision. Histograms of mean V_{RBC} determinations from each uninjured capillary and neocapillary were generated to define the spatial heterogeneity of red blood cell velocity within the microvasculature. The number of non-flowing capillaries and neocapillaries was determined from blue light epi-illumination videos, cross-referencing with corresponding SAD images, evident as either empty channels of plasma or capillaries with stationary RBCs for at least 15 seconds duration.

Analysis of Capillary Network Hemodynamic Resistance

To assess possible changes in capillary network resistance, three control and three regenerated networks (all at day 28) were reconstructed using 10x IVVM data from fields of view acquired to determine RBC flow and network architecture, as described above. Each network was reconstructed into a collection of nodes and connecting cylindrical vessel segments using our previously developed software. Two-phase (RBCs and plasma) steady-state blood flow simulations were performed using an established computational model with a fixed pressure difference (DP) between all inflow and outflow nodes. The resistance for each network was calculated as DP divided by the total flow (Q_{tot}) through (i.e., into or out of) the network, R=DP/Q_{tot}. Flow was mainly unidirectional (e.g., in at bottom and out at top of network), but for two networks flow was bidirectional indicating a major source (arteriole) or sink (venule) within the network. For these networks, resistances were calculated for upward and downward flowing portions separately and added to approximate the resistance of a single network with unidirectional flow. For all networks resistance was normalized by dividing by length (L) in the main flow directional and multiplying by width (W) in the perpendicular direction, so that R_{norm}=W×R/L. This was done to account for variability in network lengths and widths produced by the capture and reconstruction procedure, since resistance is proportional to vessel length and total flow should be proportional to width of a network.

Statistics

All values passing D’Agostino and Pearson omnibus normality testing are presented as mean ± SEM. Those not passing the normality test are presented as median and IQR. Comparisons among normally distributed variables were made by t-test or analysis of variance with Bonferonni’s post hoc test. Comparisons among hypoxia RBC measurements, width of inter-process gaps, and V_{RBC} were made by Mann-Whitney test or Kruskall-Wallis test with Dunn’s post hoc test. Comparisons among percent of arterioles with AV malformations, percent of capillaries depicting a greater than 5-fold hyperemic
hypoxia response, and percent of capillaries sustaining a hyperemic hypoxia response were made by Fisher’s exact contingency test. Mean $V_{RBC}$ histograms were compared using a two-way Kolmogorov-Smirnov test in MATLAB custom software.
Online Figure I  Loss of Capillaries Following Ischemic Injury to the EDL Muscle

**A-B.** Hematoxylin and eosin-stained sections of EDL muscle before (A) and three days following femoral artery excision (B). The native muscle fibers are surrounded by capillaries and discrete endothelial cell nuclei can be seen (A, arrows). Following ischemic injury, the skeletal myocytes have lost their nuclei. Capillaries are no longer evident, with only the occasional fibrous ghost structure without nuclei (B, arrowheads).
Online Figure II  Capillary Network Hemodynamic Resistance

A-B. Three-dimensional microvascular network reconstructions of IVVM data collected from native (A) and regenerated (B, 28 days) EDL muscle. Each reconstruction is a collection of nodes and cylindrical vessel segments. Color coding denotes depth separation of the capillary segments. C. Graph showing capillary network hemodynamic resistance ascertained by simulating two-phase (RBCs and plasma) steady-state blood flow through three-dimensional network reconstructions (n=3 native and regenerated network reconstructions, *p=0.032).
Online Figure III  Capillary RBC Hemoglobin O₂ Saturation

A. Box and whisker (min-max) plot of capillary RBC hemoglobin O₂ saturation in native capillaries in control EDL muscle and regenerated capillaries in EDL muscle 120 days after ischemic injury. Data are from 24 capillaries from 3 native EDL muscles and 56 neocapillaries from 3 regenerated muscles. *p=0.0003. Plots in B and C depict RBC hemoglobin O₂ saturation selectively in post-arteriolar capillary segments (*p=0.005) and in pre-venular capillary segments (*p=0.002).
SUPPLEMENTAL REFERENCES

LEGENDS FOR VIDEO FILES

**Online Video I:** RBC transit in the native EDL muscle microvasculature visualized by blue light epi-illumination real-time video microscopy. RBCs are seen in relief against the bright plasma that has been labeled with high molecular-weight FITC-labeled dextran.

**Online Video II:** Real-time video microscopy sequence showing no RBC transit in the EDL muscle one day following femoral artery excision.

**Online Video III:** Real-time video microscopy sequence showing RBC transit in the early (10 day) regenerated EDL muscle microvasculature.

**Online Video IV:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 28 days after femoral artery excision.

**Online Video V:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 56 days after femoral artery excision.

**Online Video VI:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 120 days after femoral artery excision.

**Online Video VII:** Real-time video microscopy sequence showing a neo-arteriole (top of field) in the late-stage regenerated EDL muscle microvasculature that bifurcates into daughter vessels with strikingly unequal lumen diameters. RBCs are visualized by ultraviolet light epi-illumination.

**Online Video VIII:** Real-time video microscopy sequence of the microvasculature 28 days after femoral artery excision showing halted RBC transit.

**Online Video IX:** Real-time video microscopy sequence showing RBC transit (demarcated by FITC-labeled dextran) through an arteriole-capillary-venule microcirculatory unit in the native EDL muscle. A normal microvascular hierarchy is evident with divergence of an arteriole into discrete capillary meshes that drain into venules.

**Online Video X:** Real-time video microscopy sequence showing RBC transit through an aberrant microcirculatory unit in the regenerated (28 day) EDL muscle microvasculature. An arteriolar-venular malformation (AV-connection) with high RBC flux can be seen to directly connect the neo-arteriole to a venule, bypassing a capillary mesh.

**Online Video XI:** Real-time video microscopy sequence showing RBC transit through an AV-connection in the 28-day regenerated EDL muscle microvasculature, visualized by ultraviolet light epi-illumination. A direct connection between the neo-arteriole and a venule is present.

**Online Video XII:** Real-time video microscopy sequence of a native EDL microvasculature showing an increase in RBC velocity and supply rate in response to a local hypoxia challenge. The video sequence displays the identical field of interest during hyperoxia (12% O₂) and 120 seconds after converting to hypoxia (2% O₂).