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# SHORT COMMUNICATION

# Myoglobin Overexpression Inhibits Reperfusion in the Ischemic Mouse Hindlimb through Impaired Angiogenesis but Not Arteriogenesis

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Adaptive vascular remodeling in response to arterial occlusion takes the form of capillary growth (angiogenesis) and outward remodeling of pre-existing collateral arteries (arteriogenesis). However, the relative contributions of angiogenesis and arteriogenesis toward the overall reperfusion response are both highly debated and poorly understood. Here, we tested the hypothesis that myoglobin overexpressing transgenic mice ($MbTg^+$) exhibit impaired angiogenesis in the setting of normal arteriogenesis in response to femoral artery ligation, and thereby serve as a model for disconnecting these two vascular growth processes. After femoral artery ligation, $MbTg^+$ mice were characterized by delayed distal limb reperfusion (by laser Doppler perfusion imaging), decreased foot use, and impaired distal limb muscle angiogenesis in both glycolytic and oxidative muscle fiber regions at day 7. Substantial arteriogenesis occurred in the primary collaterals supplying the ischemic limb in both wild-type and $MbTg^+$ mice; however, there were no significant differences between groups, indicating that myoglobin overexpression does not affect arteriogenesis. Together, these results uniquely demonstrate that functional collateral arteriogenesis alone is not necessarily sufficient for adequate reperfusion after arterial occlusion. Angiogenesis is a key component of an effective reperfusion response, and clinical strategies that target both angiogenesis and arteriogenesis could yield the most efficacious treatments for peripheral arterial disease. *(Am J Pathol 2013, 183: 1710–1718; http://dx.doi.org/10.1016/j.ajpath.2013.08.005)*

Peripheral arterial disease (PAD) is caused by atherosclerosis and is characterized by the progressive and often complete occlusion of large- and medium-size arteries at sites other than the heart. PAD most often occurs in the lower limbs, with progressive PAD leading to the debilitating consequences of intermittent claudication and critical limb ischemia. Given the high prevalence (>20% of those >65 years of age[1]) and economic impact ($4.4 billion estimated treatment costs[2]) of PAD, along with few therapeutic options, there is a critical need for developing new therapeutic modalities. One promising approach entails stimulating adaptive vascular remodeling to enhance perfusion around occlusions. To date, however, trials using this approach have largely failed. An improper understanding of the balance of angiogenesis versus arteriogenesis has been cited as a reason for many of these failures.[3–5]

Adaptive vascular remodeling to arterial occlusion(s) can be broken down into two aspects. First, in ischemic tissues downstream of an arterial occlusion, capillaries grow from existing vessels via angiogenesis, expanding blood flow distribution throughout the ischemic tissue. In contrast, collateral arteries around the occlusion are stimulated to undergo structural lumenal expansion (ie, arteriogenesis) that allows for greater in-flow into the distal, ischemic tissue. Therapeutic clinical trials have largely focused on only one process (either angiogenesis or arteriogenesis).[3–5] The most direct examples of an unbalanced approach come from the early and prominent failures of

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many large clinical trials using predominantly angiogenic factors (eg, vascular endothelial growth factor and hypoxia inducible factor 1-$\alpha$) to induce angiogenesis.[6–8] However, trials targeting factors chosen specifically for their arteriogenic potential (eg, fibroblast growth factor 2 or granulocyte macrophage-colony stimulating factor) have also reported only marginal success.[9–12] A more fruitful strategy was recently hinted at in a study by West et al,[13] which suggested that even in the presence of increased perfusion pressure to the distal tissue after a percutaneous intervention, revascularization is unable to restore microvascular perfusion in PAD patients. This suggests that microvascular perfusion impairments must be addressed for full functional recovery. Moreover, strategies that do not change large vessel occlusion but alter angiogenesis can be clinically beneficial. In summary, these findings suggest the need to better understand how angiogenesis and arteriogenesis work together to improve reperfusion after arterial occlusion.

Data demonstrating how angiogenesis and arteriogenesis separately contribute to reperfusion after arterial occlusion could outline how targeting both aspects of neovascularization could improve therapy. However, being able to identify how angiogenesis and arteriogenesis each contribute to reperfusion after ischemic injury requires both a condition that does not simultaneously impact arteriogenesis and angiogenesis, and methods for separately quantifying angiogenesis and arteriogenesis. Myoglobin overexpression in skeletal muscle may serve as a unique stimulus that has an effect on tissue reperfusion during ischemia, but has uncoupled effects on arteriogenesis and angiogenesis. Skeletal muscle myoglobin overexpression has been previously documented to impair angiogenesis and reperfusion in a severe hindlimb ischemia model.[14] The impaired angiogenesis was proposed to arise from the excess myoglobin acting as a sink for nitric oxide,[14,15] which resulted in the loss of key trophic and angiogenic factors during ischemia.[16,17] The close physical association of the endothelium with muscle fibers required for this mechanism occurs at the capillary level, but the greater separation of collateral arteries from the parenchymal muscle tissue reduces the potential for this mechanism on arteriogenesis.[18,19] The potential angiogenesis-specific impairment is further supported by the delayed time course of the perfusion deficit.[14,20] However, because the more severe ischemia model was chosen, the ability to assess arteriogenesis was limited.[21] Alternatively, a milder hindlimb ischemia model [ie, femoral artery ligation (FAL) without excision] can be used to produce consistent collateral artery remodeling needed for the precise quantification of arteriogenesis.[21–23] Therefore, we hypothesized that coupling the MbTg$^+$ transgenic model with the milder FAL ischemic stimulus would permit quantification of the degree to which impairment in angiogenesis alone can contribute to the reperfusion response after arterial occlusion. Here, we present a unique dataset demonstrating that angiogenesis is required along with normal arteriogenesis for a more fully effective revascularization response.

## Materials and Methods

### Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Virginia and conformed to all regulations for the ethical use of animals as outlined in the American Heart Association Guidelines for the Use of Animals in Research. The intact myoglobin gene and promoter regions of transgenic mice (MbTg$^+$) produce a moderate level (approximately 200% of normal) of myoglobin overexpression specific to cardiac and skeletal muscle tissue.[14,24] Transgenic mice were backcrossed for nine generations into C57Bl/6 mice before use. MbTg$^+$ and wild-type (WT) littermates were identified using previously described markers.[14,24] Only male mice were used for this study.

### Femoral Artery Ligation Model

FAL was performed similar to that previously described.[21,23,25,26] The protocol produces consistent remodeling of the collateral arterial pathway traversing the gracilis adductor muscle, representative of upper hindlimb arteriogenesis, and produces a moderate level of ischemia in the downstream tissue. Age-matched mice (5.3 $\pm$ 0.9 months of age) were anesthetized (120 mg/kg ketamine i.p., 12 mg/kg xylazine, and 0.08 mg/kg atropine) and prepared for aseptic surgery. On the left leg, a small incision was made to expose the femoral artery, which was then isolated from the femoral vein and nerve. Two ligatures of 6-0 silk suture were placed around the femoral artery (one immediately distal to the epigastric artery and the other proximal to the saphenous-popliteal artery bifurcation). The artery segment between the two ligatures was then severed, and the surgical site was closed. A sham operation was performed on the opposite (ie, right) limb, in which the femoral artery was exposed, but not ligated. Mice received injections of buprenorphine for analgesia immediately after the procedure and 8 to 12 hours later.

### Functional Assessment of Ischemic Injury

To quantify the extent of functional injury induced by FAL, mice were assessed for postoperative weight loss and gain, and for foot use at days 2, 4, and 7 after FAL. Foot use score was assessed similar to that previously described,[26] with each scored as: 0, normal use; 1, no flexion of the toes; 2, no plantar flexion; and 3, dragging of the foot.

### Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging was performed to monitor blood flow recovery in response to FAL as previously described.[23,25] Briefly, mice were anesthetized (120 mg/kg ketamine i.p., 12 mg/kg xylazine, and 0.08 mg/kg atropine) and placed prone on a surgical heating pad for 5 minutes to

minimize temperature variation and allow imaging of the soles of the feet. The lower limbs were scanned (Lisca PIM laser Doppler imager; Perimed AB, Stockholm, Sweden), and mean voltage of foot perfusion was used to calculate relative perfusion ratio (ligated:unligated).

### Tissue Harvesting

Immediately before tissue collection, gracilis muscles of anesthetized mice were exposed and drip perfused with $10^{-4}$ M adenosine in warmed Ringer's physiological saline solution. Mice were then euthanized by an overdose of anesthetic and then flushed with 2% heparinized saline and perfusion fixed with 10% neutral buffered formalin via cardiac cannulation. After 30 minutes of fixation, gracilis muscles were removed for whole mount immunofluorescence analysis, and calf muscles (containing the gastrocnemius and plantaris muscles) were removed and prepared for paraffin embedding by the University of Virginia Research Histology Core.

### Whole Mount Immunofluorescence and Collateral Network Image Analysis

Harvested gracilis muscles were labeled by immunofluorescence for smooth muscle α-actin to visualize the collateral vasculature, similar to that previously described.[23,25] Immediately after harvesting, gracilis muscles were washed, and then incubated with 1:200 anti-smooth muscle α-actin−Cy3 (1A4 clone; Sigma-Aldrich, St. Louis, MO) antibody in blocking buffer (PBS with 0.1% saponin, 2% bovine serum albumin, and 0.2% sodium azide) for 72 hours at 4°C. Gracilis muscles were then washed and cleared in 50% glycerol in PBS overnight at 4°C. Clear tissues were mounted between two coverslips using 500 μm thick spacers (645501; Grace Bio-Labs, Bend, Oregon) to keep constant thickness between muscles. The muscles were imaged (magnification, ×4) on a Nikon (Melville, NY) TE300 fluorescence microscope with a CCD camera (Microfire, Optronics, Goleta, CA). Individual fields of view were montaged together for network analysis (Photoshop CS2; Adobe Systems Inc., San Jose, CA).

Each head of the gracilis muscle (anterior and posterior) contained one primary collateral pathway that connected the muscular branch artery (also known as the profundus femoris or lateral caudal femoral artery) to the saphenous artery. The diameter was measured at nine evenly spaced intervals along the length of each primary collateral using the Fiji image analysis platform (*http://fiji.sc*, last accessed June 1, 2011).[27] The diameter measurements across both heads of the gracilis muscles were pooled to determine mean collateral diameter per muscle.

### Cross Section Immunofluorescence

Sections (5 μm) of formalin-fixed, paraffin-embedded calf muscles underwent immunofluorescence labeling for CD31 (PECAM1). Dewaxed and rehydrated slides underwent heat-mediated antigen retrieval in a 1200 W microwave for 20 minutes in citrate-based antigen retrieval buffer (H-3300; Vector Laboratories, Burlingame, CA). Slides were then blocked and labeled with primary antibody [rat anti-CD31 (1:100, SZ31 clone; HistoBioTec LLC, Miami Beach, FL)] overnight at 4°C. Slides were then washed and incubated with goat-anti rat Alexa Fluor 647 (1:200; Life Technologies, Carlsbad, CA) for 45 minutes at room temperature. Nuclei were counterstained using 10 nmol/L SYTOX green (Life Technologies). Slides were then sealed with Prolong Gold (Life Technologies) to minimize photobleaching.

### Capillary Growth (Angiogenesis) Analysis

Cross sections of calf muscle stained for CD31 were used to determine capillary density metrics. Because there are two largely distinctive regions of the harvested calf muscle with significantly different capillary and muscle fiber composition, analysis was separated into two regions: the superficial region (comprised of the white gastrocnemius muscle) and the deep region (comprised of the plantaris and red gastrocnemius muscle, termed here as the glycolytic and oxidative regions, respectively).[28–30] Two fields of view from each region in each section (two separate sections per muscle) were imaged (magnification, ×20) on a Nikon TE2000 C1 laser scanning confocal microscope. Number of capillaries (CD31+ structures, <7 μm in diameter), mature and regenerating muscle fibers (identified from autofluorescence), and muscle area were counted in each field of view using Fiji image analysis software. For comparison across the calf muscle regions, values were normalized to the mean metric (eg, capillary:fiber ratio) from the unligated limb. The presence of centrally located nuclei was used to identify regenerating fibers. Each field of view yielded >100 and >200 muscle fibers per glycolytic or oxidative region per muscle, respectively.

### Statistics

All results are reported as means ± SE. All images were randomized and de-identified to enable blinded analysis (J.K.M.). All data were first tested for normality. Statistical significance was assessed by one- and two-way analysis of variance, followed by paired comparisons using the Holm-Sidak method for multiple comparisons (SigmaStat 3.5; Systat Inc., Chicago, IL). Significance was assessed at $P < 0.05$.

## Results

### Myoglobin Overexpression Impairs Perfusion and Functional Recovery after FAL

The time course of reperfusion after FAL was assessed by Laser Doppler Perfusion Imaging in myoglobin overexpression mice ($MbTg^+$) and control littermates (WT). Both $MbTg^+$ and WT mice show a perfusion deficit only within

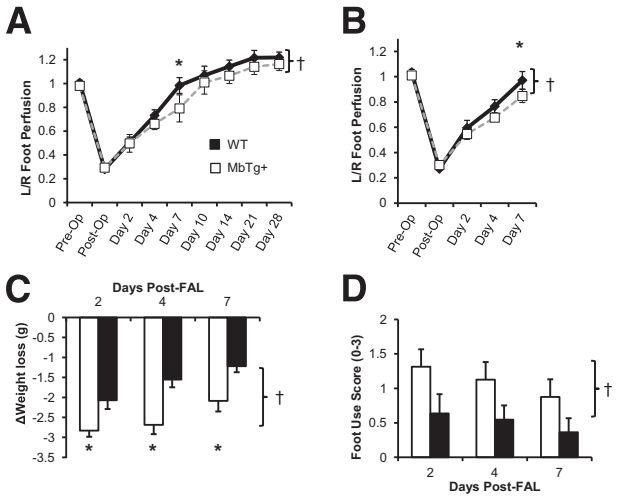


**Figure 1** Myoglobin overexpression impairs perfusion recovery after FAL. **A:** Laser Doppler perfusion recovery curve [ischemic ligated leg (L); normalized to nonischemic sham (R), leg] between myoglobin overexpressing transgenic mice (MbTg$^+$) mice and WT littermate controls out to day 28 ($n$ = 9 and 5, respectively). **B:** Early perfusion recovery within the first 7 days after FAL ($n$ = 14 and 9, respectively). Functional recovery as determined by after FAL weight recovery (**C**) and foot use (**D**) [scaled from 0 (normal) to 3]. *$P < 0.05$ between MbTg$^+$ and WT within the given time point. †$P < 0.05$ between MbTg$^+$ and WT recovery curves across all time points.

the first 7 days after FAL, with a return to baseline perfusion levels by 10 days after FAL (Figure 1A and Supplemental Figure S1). After day 10, perfusion was higher in the ligated limb during ketamine- and xylazine-induced anesthesia. Within the window of reduced perfusion (ie, <7 days post-FAL), there is a significant deficit in perfusion within the MbTg$^+$ mice (Figure 1, A and B). This perfusion recovery deficit is further reflected in a functional deficit in terms of postoperative weight loss and foot use score within the MbTg$^+$ mice (Figure 1, C and D). However, both foot use and weight were fully restored by day 28 after FAL in both groups [no mice had foot use >0 in either group; $-1.89 \pm 0.38$ g and $-1.36 \pm 0.31$ g weight change for MbTg$^+$ ($n$ = 11) and WT ($n$ = 7) mice, respectively; $P = 0.33$].

## Myoglobin Overexpression Does Not Alter Arteriogenesis in Response to FAL

As previously demonstrated, the FAL method used produced consistent arteriogenesis in the collateral pathways within the gracilis adductor muscle (Figure 2A).[21–23] Immunofluorescence labeling of smooth muscle α-actin was used for the identification and measurement of collateral artery diameter along the length of the collateral pathway spanning the gracilis muscle (Figure 2B). Both MbTg$^+$ and WT mice showed a similar degree of outward remodeling (ie, arteriogenesis) within the ligated limb by 7 days after FAL that was maintained to day 28 after FAL, without further expansion (Figure 2C). This matches the reperfusion time course, with limited additional gains in foot

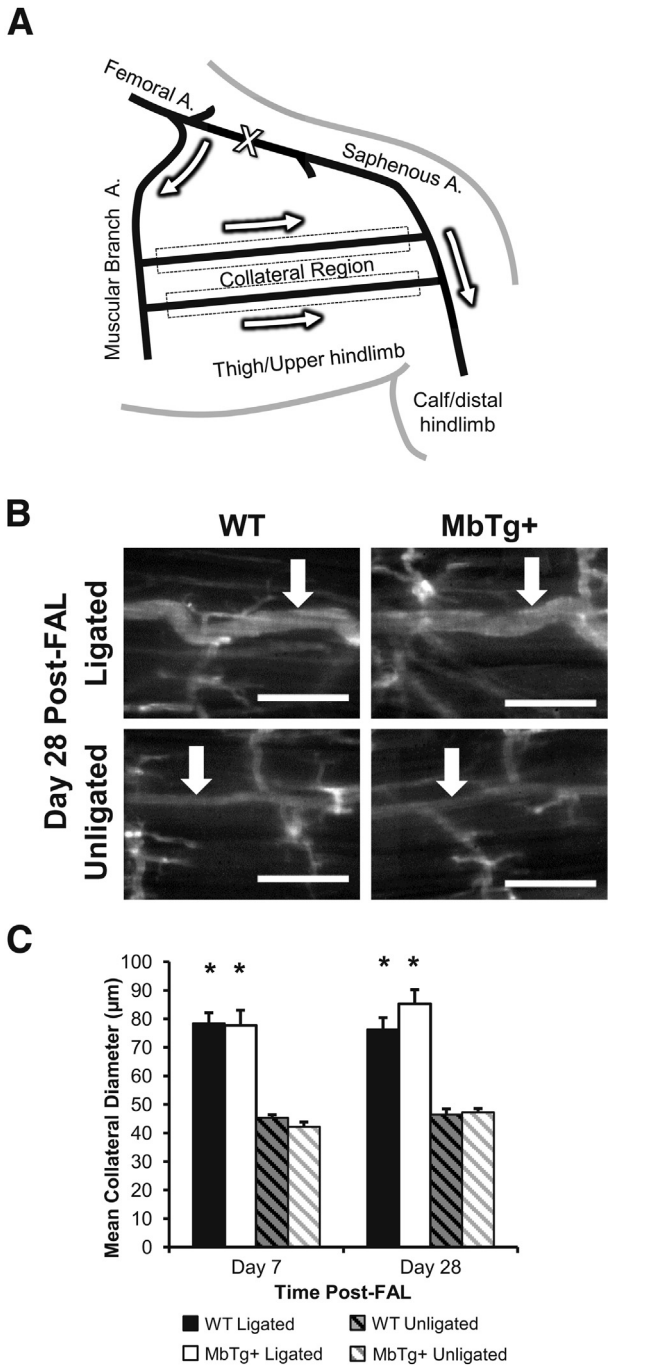


**Figure 2** Collateral artery development in gracilis adductor muscle induced by FAL. **A:** Schematic illustration showing the position of the femoral artery ligation (X) relative to the gracilis collateral arteries (dashed-line boxes) that experience a significant increase in flow and undergo arteriogenesis. Flow directions are denoted with **white arrows. B:** Gracilis muscle whole mount regions from the ligated and unligated limbs of WT and myoglobin overexpressing transgenic mice (MbTg$^+$) mice 28 days after FAL fluorescently labeled for smooth muscle α-actin to identify and quantify collateral artery remodeling (**arrows**). Images were taken from the collateral artery regions shown in **A. C:** Whole mount collateral artery diameters were quantified, showing outward remodeling of collateral arteries within the ligated limb starting at day 7 after FAL, but no additional growth by day 28 after FAL. There were no differences between MbTg$^+$ and WT mice ($n$ = 5 and 4 at day 7 and $n$ = 11 and 7 at day 28, respectively). Scale bars: 500 μm (**A**–**C**). *$P < 0.05$ between ligated versus unligated limbs within MbTg$^+$ and WT mice.

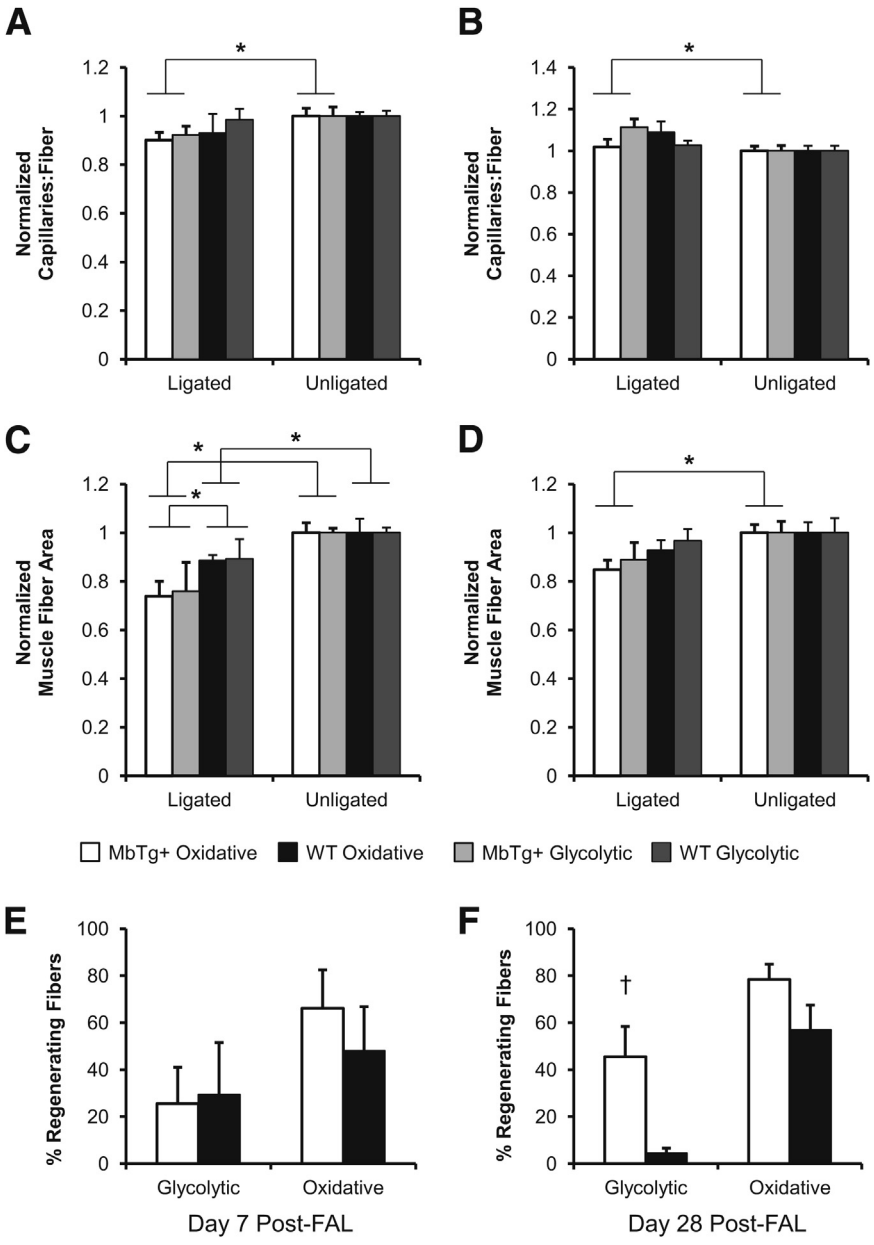


**Figure 3** Morphometric muscle and capillary analysis within distal calf muscle cross sections. Endothelial cells were labeled with CD31 and imaged in the oxidative (plantaris and deep gastrocnemius) and glycolytic (superficial gastrocnemius) regions of the calf muscle (Supplemental Figure S2). **A**–**F**: Quantification of capillary to muscle fiber ratio (**A** and **B**), mean fiber cross-sectional area (**C**–**D**), and percentage of regenerating fibers (**E**–**F**) within the glycolytic and oxidative regions of ligated and unligated limbs in myoglobin overexpressing transgenic mice ($MbTg^+$) and WT control mice at 7 days [$n = 5$ and 4, respectively (**A**, **C**, and **E**)] and at 28 days [$n = 11$ and 7, respectively (**B**, **D**, and **F**)] days after FAL. *$P < 0.05$ between groups denoted by bars. †$P < 0.05$ between $MbTg^+$ and WT within ligated or unligated muscles.

perfusion at 7 days after FAL. At no time did the mean collateral diameters within the ligated or unligated limb significantly differ between $MbTg^+$ and WT groups, showing no indications of differences in baseline or remodeled collateral diameters (Figure 2C).

## Myoglobin Overexpression Inhibits Angiogenesis and Delays Skeletal Muscle Recovery after FAL

To determine whether the impaired perfusion recovery seen in the $MbTg^+$ mice was related to impairments in capillary remodeling in the downstream ischemic tissue, capillary, and muscle fiber structures were analyzed in cross sections of calf muscle at days 7 and 28 after FAL (Supplemental Figure S2). Because of the spatial clustering of muscle fiber type in the muscle[30,31] and the variation in myoglobin expression across fiber types and muscle groups,[24] analysis was divided into glycolytic (superficial, white gastrocnemius muscle, predominantly IIB and IIDB fibers) and oxidative (deep, plantaris, and red gastrocnemius muscle, mixed IID, IIAD, IIA, and I) regions.[29,31] As expected, at baseline (unligated limb), glycolytic regions showed larger average fiber size (1585 ± 53 $\mu m^2$, MbTg+; 1544 ± 55 $\mu m^2$, WT, $n = 16$ and 11, respectively, from pooled day 7 and 28 groups) and lower capillary to muscle fiber ratio (1.160 ± 0.024 capillary:fiber, MbTg+; 1.163 ± 0.021 C:F, WT) than oxidative regions (1203 ± 41 $\mu m^2$ and 1.927 ± 0.045 C:F, MbTg+; 1161 ± 41 $\mu m^2$ and 1.896 ± 0.036 C:F, WT), with no significant differences between WT and $MbTg^+$ mice, as previously seen.[14] To enable

comparisons across muscle regions, the fiber size and capillary to muscle fiber ratio data were normalized to the averages from the unligated limb (Figure 3). At 7 days after FAL, which showed significant perfusion deficits, MbTg+ mice showed a moderate, yet statistically significant, decrease in normalized capillary to muscle fiber ratio across both muscle regions that was not present in WT mice (Figure 3A). Not surprisingly, there were only moderate effects on capillary to muscle fiber ratio in the ischemic limbs of either WT or MbTg+ mice at 28 days after FAL, in which there was a modest, but significant increase in capillary to muscle fiber ratio within the MbTg+ mice (Figure 3B). This increase in capillarity occurred in a setting of delayed regeneration, in which MbTg+ mice showed a larger presence of regenerating fibers than WT mice, which showed few regenerating fibers and a return to baseline fiber size by 28 days after FAL (Figure 3, C–F).

### Myoglobin Overexpression Increases within Ischemic Glycolytic and Oxidative Muscle Regions

To assess the degree of myoglobin overexpression (transgene tagged with a hemaglutinnin-epitope) across fiber regions, cross sections of calf muscle were labeled with anti-hemaglutinnin epitope using immunofluorescence. The specificity of staining was illustrated by comparing an unligated MbTg+ mouse (Figure 4A) to a negative control WT mouse (Figure 4B). As previously demonstrated,[24] baseline production of the myoglobin transgene was higher in the oxidative region of the calf muscle (Figure 4C) with limited production in the glycolytic regions (Figure 4D). However, by day 7 after FAL, the transgene was strongly produced in both glycolytic and oxidative regions, particularly within regenerating muscle fibers (Figure 4, E and F).

## Discussion

The major finding of our study is that the impairment in capillary growth within the distal ischemic hindlimb after FAL is sufficient to impair limb function and reperfusion, even in the setting of normal upstream arteriogenesis. We base this central conclusion on three key pieces of information from the response to FAL in MbTg+ mice. First, we confirmed the deficit in reperfusion capacity of MbTg+ that was previously identified (Figure 1).[14] In the relatively mild ischemia model chosen here, the more distal femoral arterial ligation procedure allowed for full perfusion recovery to baseline levels by 10 days after FAL (Figure 1). Nonetheless, MbTg+ mice still showed decreased reperfusion during this initial ischemic window. Although the milder ischemic stimulus decreased the extent of functional tissue damage and duration of reperfusion deficit, it also allowed for reproducible quantification of potential differences in the arteriogenic capacity within the two strains (Figure 2). Second, when arteriogenic capacity was quantified, it demonstrated that the deficit in perfusion capacity seen in

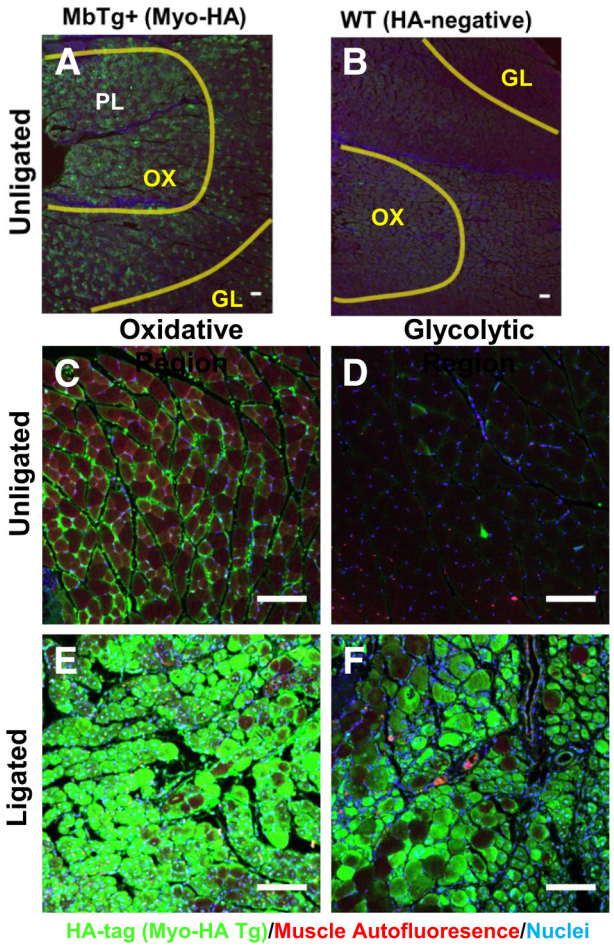


**Figure 4** Myoglobin transgene production increased within ischemic calf muscle. **A** and **B**: Cross sections (low magnification, ×4) of immunolabeled (hemaglutinnin)-tagged myoglobin transgene production using anti-hemaglutinnin epitope antibody (green) in myoglobin overexpressing transgenic mice (MbTg+) (**A**), and WT mice (**B**). Autofluoresence (red) and nuclear counterstain (blue) provide contrast for muscle visualization. **Yellow borders** define the oxidative region (OX), containing the plantaris muscle (PL) and glycolytic regions (GL) of the calf muscle. **C**–**F**: Images of myoglobin transgene production in nonischemic [unligated (**C** and **D**)] and ischemic [ligated (**E** and **F**)] limbs in oxidative (**C** and **E**) and glycolytic (**D** and **F**) regions of the calf muscle (high magnification, ×20). Scale bars: 500 μm (**A**–**F**).

MbTg+ mice could not be attributed to impaired arteriogenesis. Third, MbTg+ mice showed a moderate, yet significant, impairment in their angiogenic capacity, as assessed by capillary to muscle fiber ratio at day 7 (Figure 3C), which corresponds to the period of significant perfusion deficit (Figure 1B). These data are consistent with previous findings, wherein MbTg+ mice showed decreased angiogenesis during active ischemia in the distal muscle at 28 days due to reduced nitric oxide (NO) bioavailability.[14] The degree of angiogenic inhibition seen here in MbTg+ mice was more moderate than previously reported[14]; however, this was not surprising as we used a less severe ischemic stimulus. Although capillary density differences were not present >2 weeks after ischemia resolution, the impact of impaired angiogenesis is likely attributed to the decreased perfusion recovery seen in MbTg+

mice during the first 7 days after FAL, when angiogenesis is more prominent.[32] Together, these data provide evidence that microvascular impairment in capillary growth within the distal ischemic hindlimb is sufficient to impair limb function and reperfusion without any impairment in upstream arteriogenesis.

### Relative Contributions of Arteriogenesis and Angiogenesis to Perfusion Recovery

Understanding how arteriogenesis and angiogenesis contribute to the vascular reperfusion response is critical for determining the optimal approach for therapeutic revascularization. Few studies have made detailed analyses of the relative contribution of the collateral vasculature versus remodeling of the distal microcirculation to the decreased resistance of the vascular tree downstream of an arterial occlusion. However, those that have clearly demonstrated that arteriogenesis and the expansion of the pre-existing collateral circulation are the predominant contributors to lowering the resistance imposed by the occlusion of a major artery (Ziegler et al[33]). A similar observation is present in the current data, whereby the collateral growth seen at 7 days after FAL correlates well with the large increase in perfusion within the first several days. However, there is still a perfusion deficit within the $MbTg^{+}$ mice, and perfusion continues to increase, despite no further evidence of arteriogenesis. Although this does not conflict with the well-founded conclusion that arteriogenesis is the most efficient means of lowering bulk resistance to the distal tissue,[22,32,33] it does not preclude angiogenesis from still playing a significant and critical role in the total revascularization response, as is suggested in the current data.

As such, there is a need to view the total contribution of both elements to the vascular remodeling process (ie, angiogenesis and arteriogenesis) to understand the full impact on the reperfusion response to ischemia. Our results present one such example, but there likely exists multiple instances in which such a view could be beneficial. For example, it was recently demonstrated that $Rac2^{-/-}$ mice have significantly impaired tissue reperfusion after FAL with a significant increase in muscle damage in the downstream ischemic tissue.[21,34] However, through a detailed analysis of arteriogenesis, the investigators showed no effect on collateral remodeling that could sufficiently explain the deficits in perfusion or increased tissue damage.[21] However, incorporating the known deficit in angiogenesis seen in $Rac2^{-/-}$ mice may provide sufficient explanation for the total impairment in reperfusion response.[34] A similar case can be made in the opposite direction. One example is the involvement of CD18 and intercellular adhesion molecule 1. The documented impairment in arteriogenesis seen with a blockade of intercellular adhesion molecule 1[35] or arteriogenic enhancement with additional CD18 signaling[23] offers a strong alternative, or at least synergistic explanation of EPC-mediated effects on angiogenesis alone in response to ischemia.[36–38]

### Clinical Impetus for a Combined Role of Arteriogenesis and Angiogenesis in PAD

The controversy and conflicting conclusions arising from focusing on arteriogenesis in comparison with angiogenesis, and vice versa, may, in part, be explained by the different roles arteriogenesis and angiogenesis play during revascularization. Without sufficient arteriogenic remodeling, upstream resistance at the site of an arterial occlusion will remain high and limit the resolution of ischemia. However, it is important to note that improving bulk flow to the distal, ischemic tissue alone, as occurs during revascularization procedures, is insufficient to yield functional improvements or even tissue perfusion.[13] Furthermore, the functional outcomes induced by supervised exercise, one of the few class IA recommendations for the treatment of PAD,[39] is preceded and correlated with angiogenic expansion within the ischemic muscle.[40,41] Taken together, these findings suggest that, even if microvascular remodeling does not contribute to the bulk flow into the ischemic tissue (Ziegler et al[33]), microvascular function and capillary remodeling can play a significant role in the functional reperfusion within the distal tissue of PAD patients. By demonstrating that a selective impairment in angiogenesis alone can functionally contribute to the reperfusion process during hindlimb ischemia, the most widely used pre-clinical model to study the response to arterial occlusion, we hope to bridge the divide between looking at perfusion solely through the lens of arteriogenesis or angiogenesis. Rather, these data suggest that to reach functional recovery and a full, healthy reperfusion response, it requires both a functional arteriogenesis and angiogenesis capacity. Therefore, clinical strategies that target both elements of vascular remodeling will likely yield the greatest therapeutic benefits for PAD.

## Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.08.005*.

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