Polymerase delta-Interacting Protein 2 Promotes Postischemic Neovascularization of the Mouse Hindlimb

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Abstract

Objective—Collateral vessel formation can functionally compensate for obstructive vascular lesions in patients with atherosclerosis. Neovascularization processes are triggered by fluid shear stress, hypoxia, growth factors, chemokines, proteases and inflammation, as well as reactive oxygen species (ROS) in response to ischemia. Poldip2 is a multifunctional protein that regulates focal adhesion turnover and vascular smooth muscle cell migration and modifies extracellular matrix composition. We therefore tested the hypothesis that loss of Poldip2 impairs collateral formation.

Approach and Results—The mouse hindlimb ischemia model has been used to understand mechanisms involved in postnatal blood vessel formation. Poldip2$^{+/-}$ mice were subjected to femoral artery excision, and functional and morphological analysis of blood vessel formation was performed after injury. Heterozygous deletion of Poldip2 decreased the blood flow recovery and spontaneous running activity at 21 days after injury. H_2O_2 production, as well as the activity of matrix metalloproteinases-2 and -9, was reduced in these animals compared with Poldip2$^{+/+}$ mice. Infiltration of macrophages in the peri-injury muscle was also decreased; however, macrophage phenotype was similar between genotypes. In addition, the formation of capillaries and arterioles was impaired, as was angiogenesis, in agreement with a decrease in proliferation observed in endothelial cells treated with siRNA against Poldip2. Finally, regression of newly formed vessels and apoptosis was more pronounced in Poldip2$^{+/-}$ mice.

Conclusions—Together, these results suggest that Poldip2 promotes ischemia-induced collateral vessel formation via multiple mechanisms that likely involve ROS-dependent activation of matrix metalloproteinase activity as well as enhanced vascular cell growth and survival.

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Keywords

Poldip2; ischemia; vascularization; metalloproteinases; apoptosis

Introduction

Postnatal vascularization is an inherent and endogenous compensatory mechanism to restore obstructive vascular lesions.[1] Patients with peripheral arterial obstructive disease (PAOD), mainly caused by atherosclerosis, are at high risk for cardiovascular morbidity and mortality. Promoting vascular regrowth as an adaptive response to limb ischemia to treat PAOD is a major challenge in cardiovascular research. Revascularization occurs via three mechanisms: arteriogenesis, angiogenesis and vasculogenesis.[2-6] These processes are triggered by shear stress and hypoxia and occur simultaneously at different levels, and involve not only endothelial cell activation and upregulation of cell adhesion molecules, but also recruitment of inflammatory cells, expression of growth factors, activation of matrix metalloproteinases, as well as proliferation, migration and apoptosis of vascular cells. The events leading to revascularization are thus complex and multicellular, but share some common cellular signaling pathways.

One such mechanism is the production of reactive oxygen species (ROS) and activation of their downstream targets. It has been shown that overexpression of catalase in vascular smooth muscle cells (VSMCs) impairs blood flow recovery after femoral artery ligation,[7] indicating that H_2O_2 is necessary for collateral formation. However, excess ROS impairs collateral growth in a model of repetitive ischemia in the heart, suggesting that there is an optimal level of ROS required for neovascularization.[8] Several studies have suggested that ROS derived from NADPH oxidases are responsible for this requirement.[9] Tojo et al.[10] and Urao et al.[11] showed that NADPH oxidase 2 (Nox2) in bone marrow-derived cells is essential for ischemia-induced neovascularization. However, others have found that the suppression of hindlimb perfusion in $Rac2^{-/-}$ and $Nox2^{-/-}$ mice does not result from impaired collateral growth.[12] Recently, several groups have suggested that the NADPH oxidase 4 (Nox4) homologue also plays a role in ischemia-induced neovascularization. Transgenic mice with endothelial-specific Nox4 overexpression showed accelerated recovery of blood flow after hindlimb ischemia and enhanced aortic capillary sprouting.[13] Conversely, knockout of Nox4 led to attenuated angiogenesis in response to femoral artery ligation[14] and after pressure overload-induced cardiac hypertrophy.[15]

We previously demonstrated that polymerase delta interacting protein 2 (Poldip2) increases Nox4 activity in VSMCs.[16] The Nox4/Poldip2 complex activates RhoA in VSMCs, leading to focal adhesion turnover regulating migration. In a recent study, we showed that Poldip2 knockdown reduces H_2O_2 production in vivo, leading to increases in extracellular matrix deposition, greater vascular stiffness, and impaired agonist-mediated contraction. Thus, Poldip2 is necessary for vascular integrity and function.[17] However, in addition to regulating Nox4, Poldip2 has a number of other reported functions, including roles in organizing the mitotic spindle, DNA repair, and cellular adhesion[18,19-21] Many of these processes can potentially contribute to collateralogenesis. Moreover, Poldip2 has a variably excised N-

terminal mitochondrial signal peptide and has been implicated in the in the mitochondrial fusion that occurs during cell cycle. In this regard, we recently found that loss of Poldip2 impairs cell cycle progression in mouse embryonic fibroblasts, most likely independent of Nox4.[22] Thus, it appears that as a consequence of its multiple binding partners, Poldip2 potentially alters many cellular functions intrinsic to collateral formation. Based on these observations and our previous work implicating Poldip2 in vascular integrity, we hypothesized that loss of Poldip2 would impair collateral formation. Poldip2 heterozygous mice were subjected to femoral artery excision, and functional recovery was assessed by Laser Doppler Perfusion Imaging (LDPI) of blood flow, running test, and histological analysis of blood vessel formation. We found that loss of Poldip2 does indeed impair neovascularization, apparently via reduced endothelial proliferation, excessive regression of newly formed vessels and inhibition of matrix metalloproteinases (MMPs).

Methods

A detailed Materials and Methods section can be found in the online supplement. Please see http://atvb.ahajournals.org.

Results

Blood flow recovery in Poldip2 $^{+/-}$ mice after femoral artery ligation

To examine the effect of heterozygous deletion of Poldip2 on blood flow recovery after hindlimb ischemia, Poldip2$^{+/-}$ and WT mice were submitted to femoral artery ligation and excision, causing hypoperfusion of the lower leg and foot. Functional blood flow recovery was assessed using LDPI at 7-day intervals up to 3 weeks. LDPI revealed impaired perfusion recovery in Poldip2$^{+/-}$ mice beginning at 14 days with significant impairment at 21 days after surgery (Fig 1).

Morphological assessment of vascularity after ischemia

To determine if impaired perfusion recovery results from a decrease in vessel number, we assessed capillary density using I-isolectin B4 staining in muscle immediately distal to the site the injury. As expected, WT animals showed a ~50% increase in capillary density in the ischemic hindlimb at 7 and 14 days post surgery; however, capillary density did not increase in Poldip2$^{+/-}$ animals and was significantly less than that in WT animals at 7 and 14 days (Fig 2A). Capillary formation normalized by day 21 in both groups. The density of mature arterioles was assessed by staining smooth muscle alpha-actin (Fig 3A). Ischemic hindlimbs from WT mice had a 6-fold increase in alpha-actin staining compared with the non-ischemic leg. The response in Poldip2$^{+/-}$ mice was much less (1.9±0.4 fold). Of interest, Poldip2$^{+/-}$animals showed a regression of the vasculature by 50±5% compared to WT at 21 days after ischemia, suggesting that Poldip2 may have dual roles in formation of new collaterals and maintenance of the structure of new vascular networks. To obtain a more detailed morphological assessment of the collateral development after induction of hindlimb ischemia, legs of WT and Poldip2$^{+/-}$ mice were analyzed by microCT 21 days after surgery. 3D histomorphometric analysis showed that the connectivity ratio of IL to NIL was 2.3-fold higher in Poldip2$^{+/-}$ animals (1.31±0.28 for WT and 3.07±0.10 for Poldip$^{+/-}$) compared to

WT. No other differences were observed. Taken together, these results strongly suggest that Poldip2^{+/-} mice have impaired neovascularization.

In vivo assessment of Poldip2 role in angiogenesis processes

The early decrease in capillary density in Poldip2^{+/-} animals suggests an impairment of angiogenesis. To assess the role of Poldip2 in angiogenic processes *in vivo*, we used the SIS (porcine small intestine submucosa) implant model of angiogenesis. As shown in Fig 2B, Poldip^{+/-} mice had 45±9% less endothelial invasion into the matrix compared with WT animals, suggesting that Poldip2 downregulation impairs the physiological angiogenesis process.

Role of Poldip2 in HUVEC proliferation

During angiogenesis, endothelial cells are induced to proliferate and migrate out of an existing vessel to form new branches.[2] To determinate if Poldip2 can affect proliferation of endothelial cells, human umbilical vein endothelial cells (HUVECs) were transfected with siPoldip2 and proliferation was assessed for 4 consecutive days (Fig 2C). Poldip2 protein expression was reduced after siRNA transfection by 62±3% (Fig SI). As shown in Fig 2C, the rate of proliferation was significantly decreased in cells with Poldip2 downregulation. However, VEGF signaling and HIF1α stabilization seem not to be affected by Poldip2 downregulation. No differences were detected between WT and Poldip2 [+/-] mice in VEGFR2 (VEGF receptor 2) phosphorylation or accumulation of HIF1α in the ischemic muscle 7 days after surgery (data not shown), suggesting that Poldip2 directly affects endothelial progression through the cell cycle, as we have previously shown in mouse embryonic fibroblasts.[22]

Apoptosis in the proximal muscle of the ischemic limb

The reduced density of arterioles observed in Poldip2^{+/-} mice at 21 days together with the impaired blood flow recovery suggest that loss of Poldip2 might lead to inadequate regression of non-functional vessels. To test the role of Poldip2 in vessel regression, we measured vascular apoptosis *in vivo* using TUNEL staining. As shown in Fig 3B, apoptosis of vessels surrounding muscle fibers immediately distal to the site of injury was reduced by 82±22% in Poldip2^{+/-} mice compared to WT mice 21 days after surgery. This result suggests that Poldip2 can affect vessel homeostasis.

Inflammatory response of Poldip2^{+/-} after hindlimb ischemia

Infiltration of inflammatory cells is also an important early event in collateral vessel formation.[7, 23] To determine if inflammatory cell infiltration is impaired in Poldip2^{+/-} mice, histological analysis of the ischemic limbs was performed. Immunostaining for MAC3, a macrophage marker, showed that Poldip2^{+/-} mice had 40±10% less macrophages per section compared with WT mice 7 days after surgery in the proximal muscle of the ischemic limb (Fig 4A). Loss of Poldip2 does not appear to affect macrophage polarization, as WT and Poldip2^{+/-} mice showed similar expression of both M1 and M2 markers (Fig 4B).

Poldip2 regulates matrix metalloproteinase activity in the proximal muscle of the ischemic limb

Macrophages are an important source of MMPs in response to ischemia,[24] which promote matrix degradation and endothelial and smooth muscle cell migration. To assess MMP activity, two methodologies were used. First, total gelatinase activity was assessed using an assay to measure degradation of a fluorescently labeled substrate (Fig 5A). At both 14 and 21 days post surgery, Poldip2^{+/-} mice had less gelatinase activity than WT mice. Because MMP2 and MMP9 have been implicated in the response to hindlimb ischemia,[24-26] we further analyzed the activity of each of these enzymes using gelatin zymography. As shown in Fig 5A, MMP2 and MMP9 activity were increased throughout the recovery period in both genotypes; however, both MMP2 ($74\pm20\%$ decrease) and MMP9 ($82\pm9\%$ decrease) were reduced in Poldip2^{+/-} mice compared to WT mice at 21 days after surgery. To determine if this reduction in activity was due to decreased expression, we measured mRNA levels of MMP2, MMP9 and their corresponding regulators Tissue Inhibitor of Metalloproteinase TIMP2 and TIMP1. As shown in Fig SII, the ratio of MMP2/TIMP2 and MMP9/TIMP1 mRNA was similar at all time points between WT mice and Poldip2^{+/-} mice. These results suggest that Poldip2 regulates activity, but not expression, of MMP2 and MMP9.

H_2O_2 production in the proximal muscle of the ischemic limb

Because Poldip2 has been shown to regulate Nox4, which has been implicated in angiogenesis,[13, 16] we measured total H_2O_2 production in muscle immediately distal to the ligation (Fig 6A). In agreement with previous studies from our group,[17] H_2O_2 production in Poldip2^{+/-} mice was decreased $44\pm7\%$ compared with WT in the proximal muscle at 21 days after surgery, consistent with reduced Nox4 activity.

Spontaneous running activity of Poldip2^{+/-} mice after hindlimb ischemia

Finally, to evaluate the extent to which impaired recovery of perfusion and vascular remodeling affected physiological function, we measured motor activity. WT and Poldip2^{+/-} animals were placed in a voluntary running wheel activity system at 7 and 21 days post surgery and distance traveled was recorded for 7 days (Fig 6B). At baseline conditions (day 0, not shown) and seven days after surgery, WT and Poldip2^{+/-} mice run similar distances. However, by 21 days after surgery, a time when blood flow recovery and vessel formation are impaired, Poldip2^{+/-} mice run 25% less than WT mice. These data indicate that impaired neovascularization in Poldip2 mice impacts the physiological function of the limb muscles.

Discussion

Neovascularization in response to ischemia is a key adaptive response to preserve functional integrity of tissues; however, therapy to improve vascularization remains elusive.[1, 27, 28] In the present study, we report that Poldip2 downregulation impairs the revascularization process after ischemic insult in the adult mouse femoral artery ligation model. We noted only a partial recovery of perfusion, resulting from reduced capillary density and fewer small caliber vessels. These morphological changes resulted in impaired physiological function as assessed by voluntary running.

Collateral formation and remodeling are complex processes involving recruitment, migration, proliferation and apoptosis of vascular cells. [3-6] One of the most intriguing aspects of our results is the finding that Poldip2^{+/-} mice exhibited impaired collateral formation at early time points and also an enhanced loss of collaterals at later time points. These findings suggest that both formation and regression of newly formed vessels is altered in these animals. The complexity of angiogenesis involves not only growth but also maturation and regression of the blood vessel network. While regulated regression is an important aspect of neovascularization and formation of intact networks, the exact endogenous anti-angiogenic factors and the mechanisms responsible for blood vessel regression following robust vessel sprouting are not well understood.[29] In general, one week after injury, the density of blood vessels in the wound bed is over three times higher than that of the uninjured tissue.[5] After a peak in vessel density, some newly sprouted vessels that have integrated in the existing perfused network undergo maturation. Vessels that are not perfused and functional are targeted for elimination. The most accepted mechanism for this process is apoptosis of endothelial cells. Analysis of apoptosis in the proximal muscle of the hindlimb ischemia revealed that Poldip2^{+/-} mice had more vascular cell death compared with WT (Fig 3B). This vascular rarefaction in Poldip2^{+/-} mice suggests that Poldip2 is required for cell survival, which is compatible with other pro-survival roles of Poldip2 reported in the literature.[30] Impaired collateral formation and angiogenesis are also highly dependent upon proliferation of both endothelial cells and smooth muscle cells. Depletion of Poldip2 had a profound inhibitory effect on endothelial proliferation (Fig 2C), consistent with other work in our laboratory showing that growth of vascular smooth muscle cells and mouse embryonic fibroblasts is also adversely affected by loss of Poldip2.[22] This was reflected in impaired angiogenesis (Fig 2A). These results clearly show that Poldip2 has multiple roles in the response to ischemia.

Extracellular matrix also has an important role in collateral formation. Matrix proteins provide not only a supportive scaffold for cells, but also serve as crucial effectors of cellular function by sequestering and releasing growth factors and cytokines, including vascular endothelial growth factor-A (VEGF-A), tumor necrosis factor-alpha (TNF-α) and interleukins (ILs). [24, 31, 32] These vital proangiogenic factors both initiate and maintain vascular remodeling.[29] On the other hand, degradation of extracellular matrix is required in order for cells to migrate and form new vessels. For these reasons, MMP activity is a major regulator of vasculogenesis. In the hindlimb ischemia model, MMP9 activity has been shown to increase in the gastrocnemius muscle tissue beginning at 3 days after injury and remain elevated until perfusion is restored.[25] In the present study, we saw elevated but similar MMP activity in WT and Poldip2^{+/-} mice 7 days after injury; however, at 21 days the gelatinase/collagenase activity, as well as the activity of MMP2 and MMP9, was decreased in Poldip2^{+/-} compared to WT. This suggests that one mechanism for the impaired vasculogenesis seen in Poldip2^{+/-} mice is a failure to adequately degrade extracellular matrix. This deficiency may also contribute to the increase in extracellular collagen that we previously reported in aortas from these animals.[17]

An important source of MMPs is inflammatory cells such as macrophages and neutrophils that infiltrate vascular tissue.[24] We found that macrophage infiltration in Poldip2^{+/-} mice is diminished compared to that in WT mice at 7 days post surgery. Limited inflammation

would result in compromised blood vessel growth, as we observed here, because it has been shown that inflammation is required for collateralogenesis.[23, 33, 34] A reduction in inflammatory cells also means that MMP and growth factor release from the matrix is altered, and may not be sufficient to sustain newly formed vessels.[24, 25, 35, 36] Such an explanation would be consistent with the enhanced regression of newly formed vasculature that we observed in Poldip2$^{+/-}$ mice. However, we observed no difference in MMP activity between WT and Poldip2$^{+/-}$ mice at 7 days after induction of ischemia, suggesting that other cell types contribute to the reduced MMP activity observed at later times.

There is a strong link between MMP activation, oxidative stress and collateral formation. ROS production via Nox enzymes during mechanical stretch enhances MMP2 mRNA expression and pro-MMP2 release.[37] In atherosclerosis, monocytes exhibit increased ROS production via Nox enzymes that leads to enhanced secretion and activity of MMP9.[38] Similarly, Schroeter et al.[39] showed that leptin promotes neovascularization by Nox2-mediated activation of MMP9. ROS can have a direct effect on MMP activation via "the cysteine switch" or by altering the interaction between TIMPs and MMPs, as well as by increasing expression of certain MMPs.[16, 40-42][43, 44] We observed a difference in MMP activity but no change in expression, suggesting that one of the two former mechanisms is active in this model. In the context of hindlimb ischemia, neovascularization occurs via Nox2-derived ROS, increasing MT1-MMP expression and MMP9 activity.[11] We previously showed that Poldip2 increases Nox4 activity in VSMCs, and that it inhibits the secretion of collagen in a ROS-dependent manner. [17] Because Nox4 has been associated with angiogenesis,[14, 15, 45] these data suggest that the reduction in MMP activity seen in Poldip2$^{+/-}$ mice (Fig 5) is a consequence of reduced Nox4-derived H_2O_2 (Fig 6A). This would preserve the extracellular matrix and impair ischemia-induced neovascularization by limiting the infiltration of inflammatory cells as well as the liberation of circulating growth factors from extracellular matrix that would maintain the new vascular network.

It is likely, however, that Poldip2 has additional Nox4-independent effects on cellular processes that contribute to collateral formation. Several reports indicate that Nox4 has a protective function in distinct pathological conditions, for example obesity, pulmonary arterial hypertension, kidney fibrosis and myocardial infarction [14, 46-49]. Conversely, other articles suggest that Nox4 induces apoptosis, especially in cancer cells, endothelial cells and cardiac myocytes[45, 48, 50, 51]. Nox4 exerts these effects by inactivating the protein tyrosine phosphatase-1B (PTP1B) and enhancing vascular endothelial growth factor receptor-2 (VEGFR2) and mTOR signaling, as well as by regulating endothelial nitric oxide synthase (eNOS) expression, phosphorylation of members of the Bcl-2 family and mitochondrial oxidative stress. In contrast, while Poldip2 regulates cytoskeletal dynamics and matrix deposition in a Nox4 and ROS-dependent manner,[16, 17] it has additional functions potentially unrelated to Nox4. For example Poldip2 interacts with DNA polymerase delta and proliferating cell nuclear antigen (PCNA),[52] suggesting an important role in the processes of DNA replication and repair. In addition, as a mitochondrial protein, it has been shown that Poldip2 might regulate viral DNA replication.[53] Klaile et al.[20] showed that Poldip2 is a binding partner for carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and serves to regulate its trafficking from the cell periphery to the nucleus. Finally, a recent publication from our group showed that Poldip2 affects mouse embryonic

fibroblast proliferation by regulating Cdk1 and CyclinA2.[22] While the Nox4 dependence of these roles of Poldip2 has not been studied in detail, at least some are likely to be Nox4 independent because deletion of Poldip2 is embryonic lethal, but deletion of Nox4 is not. Because of the potential role of Poldip2 in so many fundamental cellular processes, it will be important to explore in detail how this novel protein influences cardiovascular physiology and pathophysiology.

In summary, we have shown that heterozygous deletion of Poldip2 results in reduced H_2O_2 production during hindlimb ischemia, which is associated with reduced MMP2 and MMP9 activity, decreased formation of arterioles but increased connectivity between larger vessels, increased vascular rarefaction and apoptosis, and ultimately decreased blood flow and impaired functional recovery. In addition, MMP activity, but not expression, as well as endothelial cell proliferation, are significantly decreased compared to WT. Given the complex cellular and molecular interactions that contribute to neovascularization, as well as the multifunctional nature of Poldip2, much work remains to be done to dissect the potential molecular pathways regulated by this intriguing protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ARG	arginase-1
B2M	beta-2 microglobulin
H$_2$O$_2$	hydrogen peroxide
HIF1α	hypoxia-inducible 1α
LDPI	LASER Doppler perfusion imaging
MMP	metalloproteinase
Micro-CT	Micro computed tomography
MRC	mannose receptor
Poldip2	polymerase delta interacting protein 2
ROS	reactive oxygen species
VEGF	vascular endothelial growth factor

VSMCs vascular smooth muscle cells

Significance

Peripheral arterial obstructive disease (PAOD) leading to limb ischemia is a major challenge in cardiovascular research. Here we demonstrate that Poldip2 regulates a number of cellular functions integral to ischemia-induced collateral vessel formation, including matrix metalloproteinase activity, angiogenesis, endothelial proliferation, and vascular regression. Fully understanding how Poldip2 promotes neovascularization may ultimately allow for the development of new therapeutics to compensate for obstructive vascular lesions and consequently to preserve tissue function in patients with PAOD.
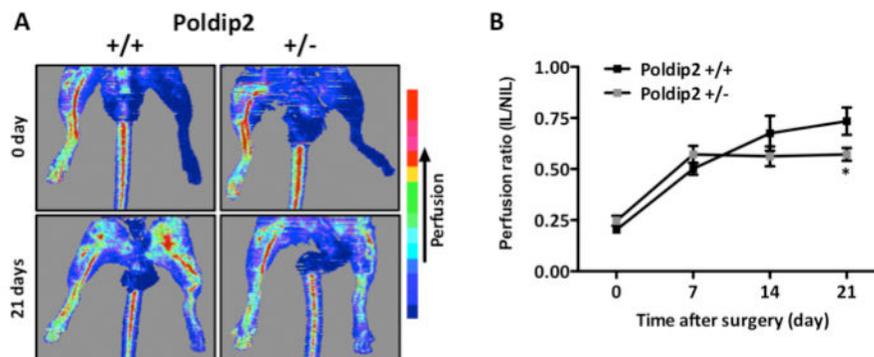
Figure 1. Poldip2$^{+/-}$ mice exhibit reduced blood flow recovery after ischemic injury
Femoral artery ligation was performed on Poldip2$^{+/+}$ and Poldip2$^{+/-}$ mice and blood flow
was assessed in the adductor muscles of the nonischemic (NIL) and ischemic limbs (IL) by
LASER Doppler Perfusion Imaging (LDPI) at the indicated time points. A) Representative
images from LDPI analysis immediately after the surgery and at the 21 day time point. B)
Quantitative analysis presented as perfusion ratios (ischemic leg (IL) / nonischemic leg
(NIL)). Data represent mean±SEM (n = 15-20 per genotype). * P<0.05 vs. Poldip2$^{+/+}$
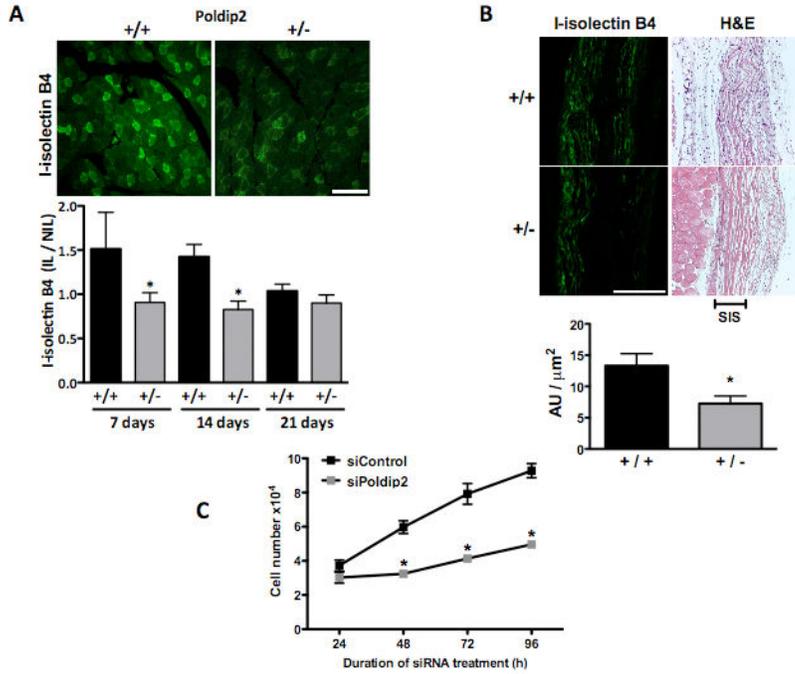
Figure 2. Poldip2$^{+/-}$ mice exhibit reduced angiogenesis and endothelial cell proliferation
A) Representative photomicrographs and quantification of capillary density in ischemic gastrocnemius muscle stained with antibody against I-isolectin B4 (n = 3-6 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2 $^{+/+}$. B) Representative photomicrographs and quantification of endothelial cell infiltration around SIS (small intestine submucosa) area at day 12 after implantation. Endothelial cells were assessed by staining with antibody against I-isolectin B4. C) Time course of serum-induced proliferation in HUVEC treated with siControl or siPoldip2 (n = 3 per condition). Bars are means ± SEM. * P<0.05 vs. siControl. Bar scale = 200 μm.
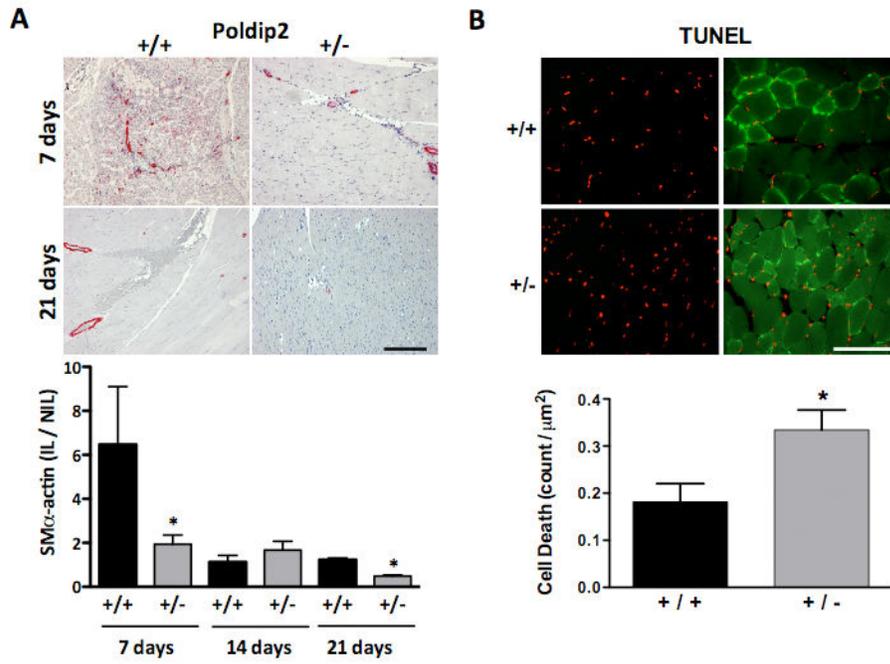
Figure 3. Poldip2 regulates arteriogenesis and apoptosis
A) Representative photomicrographs and quantification of arterioles in ischemic gastrocnemius muscle stained with antibody against smooth muscle α-actin. (n = 3-6 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2^{+/+}. Scale bar = 200 μm. B) Representative photomicrographs visualized by autofluorescence (green) and quantification of apoptotic cells in ischemic gastrocnemius muscle stained by TUNEL (red) 21 days after surgery. (n = 3 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2^{+/+}. Scale bar = 100 μm.
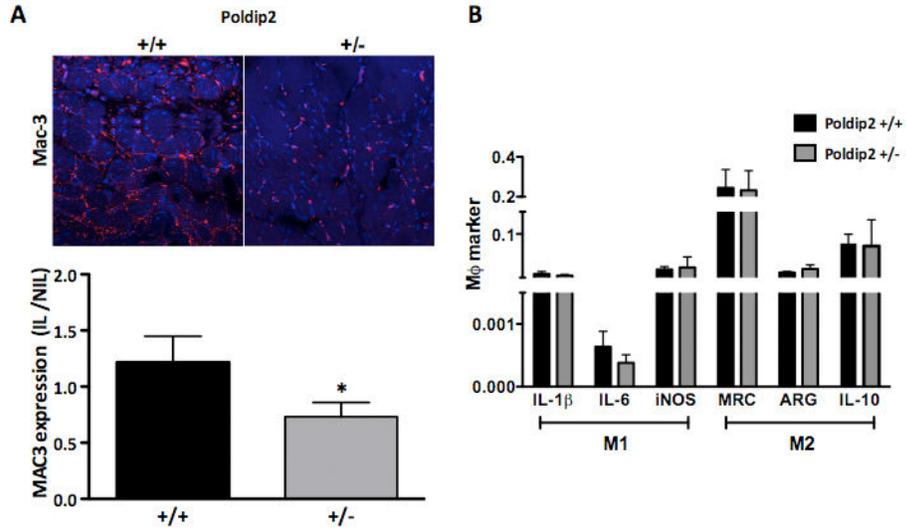
Figure 4. Poldip2 downregulation reduces macrophage infiltration, but does not alter macrophage phenotype

Inflammatory cell infiltration in ischemic tissue was assessed by staining of macrophage cells at 7 days with an antibody against Mac-3. Representative photomicrographs of total macrophage content are shown. Quantitative analyses are presented as ratios (ischemic leg (IL) / nonischemic leg (NIL)) after analysis of total fluorescence using ImageJ software. (n = 3-4 per genotype). Bars are means ± SEM. * $P<0.05$ vs. Poldip2$^{+/+}$. B) mRNA levels of IL-1β, IL-6, iNOS, Mannose Receptor (MRC), Arginase (ARG) and IL-10 were measured in ischemic muscle at 7 days after surgery. B2M (beta-2 microglobulin) rRNA was used to normalize each sample. (n = 3-5 per genotype). Bars are means ± SEM.
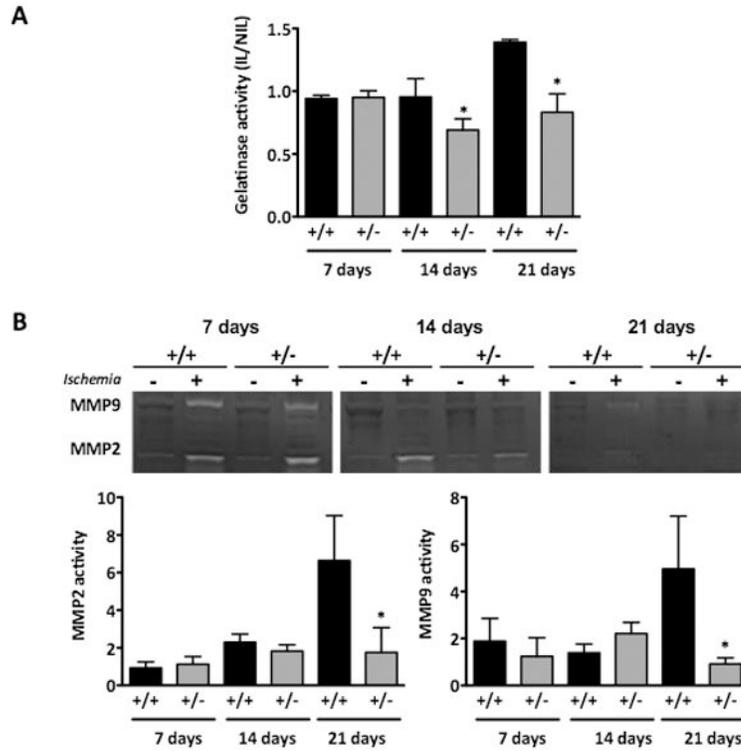
Figure 5. MMP2 and MMP9 activity is reduced in Poldip2$^{+/-}$ mice

A) Representative gelatin zymography images and quantification of MMP2 and MMP9 activity from muscle immediately downstream of the femoral artery ligation in Poldip2$^{+/-}$ and Poldip2$^{+/+}$ mice. (n = 3-5 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2$^{+/+}$ B) Gelatinase activity from ischemic and nonischemic muscle was measured using the EnzChek gelatinase assay kit. (n = 3-4 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2$^{+/+}$.
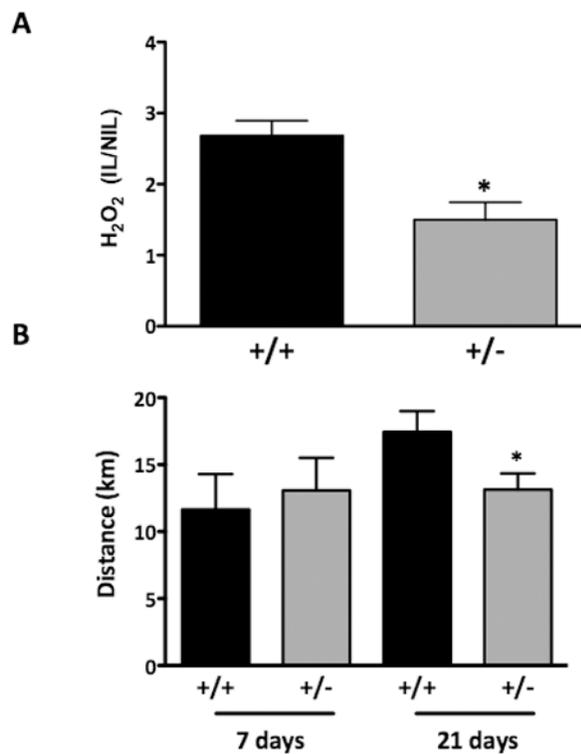
Figure 6. Reduced hydrogen peroxide production and running distance in Poldip2$^{+/-}$ mice
A) H$_2$O$_2$ production from ischemic and nonischemic gastrocnemius muscle was assessed by
Amplex Red assay. (n = 3 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2 $^{+/+}$.
B) Spontaneous running activity recorded over a 7-day period and expressed as total
distance run. (n = 4-10 per genotype). Bars are means ± SEM. * P<0.05 vs. Poldip2 $^{+/+}$.