Desert Hedgehog Promotes Ischemia-Induced Angiogenesis
by Ensuring Peripheral Nerve Survival

Marie-Ange Renault¹,², Candice Chapouly¹,²,³, Qinyu Yao¹,², Frédéric Larrieu-Lahargue¹,², Soizic Vandierdonck¹,²,³, Annabel Reynaud¹,², Myriam Petit¹,², Béatrice Jaspar-Vinassa²,², Isabelle Belloc¹,², Elisabeth Traiffort⁵, Martial Ruat¹, Cécile Duplâa¹,², Thierry Couffinhal¹,²,⁴, Claude Desgranges¹,², Alain-Pierre Gadeau¹,²

¹Univ. Bordeaux, Adaptation cardiovasculaire à l'ischémie, U1034, F-33600 Pessac, France; ²INSERM, Adaptation cardiovasculaire à l'ischémie, U1034, F-33600 Pessac, France; ³CHU de Bordeaux, Pharmacie de l'Hôpital Haut-Lévêque, F-33000 Bordeaux, France; ⁴CHU de Bordeaux, Service des Maladies Cardiaques et Vasculaires, F-33000 Bordeaux, France; ⁵CNRS, UPR-3294, Laboratoire de Neurobiologie et Développement, Institut de Neurobiologie Alfred Fessard IFR2118, Gif-sur-Yvette, France

M-A.R. and C.C. contributed equally to this study.

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Address correspondence to:
Dr. Marie-Ange Renault
INSERM U1034
125, avenue du Haut Lévêque
33600 Pessac
France
Tel : +33 5 57 89 19 79
Fax : (33) 5 56 36 89 79
marie-ange.renault@inserm.fr

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ABSTRACT

Rationale: Blood vessel growth and patterning have been shown to be regulated by nerve-derived signals. Desert Hedgehog (Dhh), one of the Hedgehog (Hh) family members, is expressed by Schwann cells of peripheral nerves.

Objective: The purpose of this study is to investigate the contribution of Dhh to angiogenesis in the setting of ischemia.

Methods and Results: We induced hind limb ischemia (HLI) in WT and Dhh-/- mice. First, we found that limb perfusion is significantly impaired in the absence of Dhh. This effect is associated with a significant decrease in capillary and artery density in Dhh-/- . By using mice in which the Hh signaling pathway effector Smoothened was specifically invalidated in endothelial cells (ECs), we demonstrated that Dhh does not promote angiogenesis by a direct activation of ECs. On the contrary, we found that Dhh promotes peripheral nerve survival in the ischemic muscle and by doing so, maintains the pool of nerve derived-proangiogenic factors. Consistently we found that denervation of the leg, immediately after the onset of ischemia, severely impairs ischemia-induced angiogenesis and decreases expression of VEGFA, Angiopoietin-1 and Neurotrophin-3 in the ischemic muscle.

Conclusions: This study demonstrates the crucial role of nerves and factors regulating nerve physiology in the setting of ischemia-induced angiogenesis.

Keywords: Desert hedgehog, angiogenesis, peripheral nerves, ischemia, neuroprotection

Nonstandard Abbreviations:

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INTRODUCTION

Blood vessels and nerves have a similar organization pattern in the human body. Recent investigations revealed that they share many common mechanisms and signals for growth, patterning and survival\(^1\)-\(^4\). Moreover, studies performed in mice suggest that angiogenesis is at least in part regulated by guidance signals derived from nerves. In the skin, blood vessel branching and arterial differentiation is determined by Schwann cell-derived signals\(^5\). Similarly, in the mouse retina, astrocyte-derived VEGFA has been shown to guide endothelial tip cells\(^6\). In the skeletal muscle, nerves were shown to be necessary to maintain blood vessels in adults\(^7\). Conversely, vascular cells and more particularly smooth muscle cells have been shown to produce Artemin, a neurotrophic factor for sympathetic nerve axons\(^8\). In the brain, activated endothelial cells (ECs) produce brain-derived neurotrophic factor (BDNF) that promote migration and recruitment of neurons\(^9\).

Evidence suggesting modulation of angiogenesis and vasculogenesis by Hedgehog (Hh) signaling emerged in studies of vascularization of embryonic tissues. Because each of the three Hh proteins has a unique expression pattern and because their expression does not overlap\(^10\), Sonic hedgehog (Shh) and Indian hedgehog (Ihh) were shown to regulate angiogenesis in specific tissues during embryogenesis\(^11,12\). On the contrary, Desert hedgehog (Dhh) has never been involved in angiogenesis.

Dhh is expressed by Schwann cells of peripheral nerves and controls structural and functional integrity of adult nerves\(^13\). As a consequence Dhh deficiency in both mice and human has been associated with peripheral neuropathy\(^14,15\). In this tissue, Dhh was shown to regulate endothelial cell function by controlling vessel permeability\(^14\). In adults, previous investigations indicated that Hh signaling is necessary to maintain coronary vasculature in physiologic conditions\(^16\). Moreover, inhibition of Hh protein activity by the neutralizing 5E1 antibody administration impairs ischemia-induced angiogenesis\(^16,17\). Since 5E1 antibody blocks the activity of the three Hh ligands\(^18\) the specific role of each Hh protein in the regulation of blood vessel homeostasis has not been established. We hypothesized that Dhh which is specifically expressed by peripheral nerves is critical for ischemia-induced angiogenesis and performed a series of experiments to further characterize the role of Dhh during angiogenesis and ischemic tissue repair. We found that Dhh is necessary for angiogenesis, however it does not modulate EC function; on the contrary, it promotes peripheral nerve survival in the setting of ischemia. In parallel, we demonstrated that peripheral nerves express proangiogenic factors and are necessary for ischemia-induced angiogenesis demonstrating that Dhh regulates angiogenesis by promoting peripheral nerve survival in the setting of ischemia.

METHODS

**Mice.**

C57BL/6 mice were obtained from Charles River Laboratories and bred in our animal facility. Dhh heterozygote mice\(^19\) under CD1-C57BL/6 genetic background were provided by M. Wijgerde (Erasmus University Medical Center, Rotterdam, The Netherlands). Dhh\(^+/-\) mice were bred together to obtain Dhh\(^+/-\) mice and WT control mice. Pups were genotyped as described previously\(^20\). Smo\(^\text{Flox}\)\(^21\), Rosa26\(^R\) and Ptc1-LacZ mice\(^22\) were obtained from the Jackson laboratory. Pdgfb-Cre\(^\text{ERT2}\) mice\(^23\) were kindly given by M. Fruttiger and bred with Smo\(^\text{Flox}\) mice. Gli1-Cre\(^\text{ERT2}\) mice\(^24\)
were kindly provided by AL Joyner and bred with Rosa26R mice. Mice were handled in accordance with the guidelines established by the National Institute of Medical Research (Inserm) and approved by the local Institutional Animal Care and Use Committee. Animals were anesthetized by 2.5-4% isoflurane inhalation or by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Mice were administrated 1 mg/kg buprenomorphine 30 minutes prior to surgical procedures. Cre recombinase was activated by intraperitoneal injection of 1 mg tamoxifen (Sigma) for 5 consecutive days.

**HLI model and assessments.**

HLI was performed as previously described in 8-12 week old mice. Briefly, the left femoral artery and all side-branches were dissected and excised from the proximal end of the femoral artery to the distal portion of the saphenous vein.

For histological assessment and gene expression analysis, mice were euthanized and tibialis anterior muscles were harvested then cut in half, the lower half was fixed in methanol, paraffin-embedded, and cut into 6-µm sections and the upper half was snap frozen in liquid nitrogen. Each group included 3-15 animals. Capillary density was evaluated in sections stained for the expression of CD31.

For micro-CT vasculature imaging, mice were perfused with a mixture containing 80% neoprene latex (Neoprene Latex Dispersion 671 A, Dupont, France) and barium sulphate powdered to 1 µm (3 g/mL, MicrOpaque® oral solution, Guerbet, France) as previously described. The vasculature was imaged using a high-resolution micro-CT imaging system (eXplore Locus SP, General Electric Healthcare®). Image acquisitions and reconstructions were performed using Scan Control® and Reconstruction Utility® programs. Data were acquired in an axial mode covering a single hindlimb as previously described. The quantification parameters were obtained using the Microview ABA® program.

For X-gal staining, mice were perfused with LacZ fix solution (PBS containing 0.2% Glutaraldehyde, 5 mmol/L EGTA and 100 mmol/L MgCl₂) before tibialis anterior muscles were harvested. Ten µm thick cryosections were prepared and post-fixed for 10 min in 0.2% glutaraldehyde and then stained overnight at 37°C in X-gal staining solution (1 mg/ml X-gal (Sigma)). Tissue sections were counterstained with nuclear fast red solution (Sigma).

**Plasmids/gene therapy.**

The cDNA encoding the N terminal part of Dhh (i.e. the signal sequence + the entire sequence of the processed form of Dhh (NDhh) was amplified from MS1 cells cDNA with the following primers: 5’-AGGACAAGAAGCGCCCTTC-3’ and 5’ GAGCGGATCCATTTCCCCGAAAATCGCCT-3’. NDhh cDNA was subsequently digested with NcoI and BamHI and cloned into pIRES2-EGFP plasmid (Clontech) digested with Smal and BamHI.

Gene therapy was performed in 12 week old mice. Four days after HLI surgery was performed, mice were randomly assigned to receive 200 µg pIRES-EGFP or 200 µg pIRES-NDhh together with 0.05% pluronic as previously described. The DNA/pluronic mix was injected intramuscularly in the tibialis anterior muscle. Mice were sacrificed 10 days after HLI surgery was performed.
**Limb denervation.**
The dorsal skin of the thigh was cut and the posterior muscles divided to show the sciatic nerve. Denervation of the left leg was obtained by cutting and removing 5-mm of the sciatic nerve.

**Immunostaining.**
ECs were identified with rat anti-CD31 antibodies (BD Pharmingen Inc). Dhh was stained with goat anti-C-terminal Dhh (R&D Systems) antibodies. Schwann cells were identified with rabbit anti-S100 antibodies (Dako) or rat anti-Myelin Basic Protein (MBP) antibodies (Abcam). NTF3, VEGFA, and Angpt-1 were stained using rabbit anti-NTF3 antibodies (Abbiotec), rabbit anti-VEGFA antibodies (Abcam) and goat anti-Angpt1 antibodies (Santa-Cruz) respectively. For immunofluorescence analyses, primary antibodies were resolved with Alexa-Fluor–conjugated secondary antibodies (Invitrogen) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin–conjugated secondary antibodies (Vector) and streptavidin-HRP complex (Amersham), then the stain was developed with a DAB substrate kit (Vector Laboratories); tissues were counterstained with hematoxylin.

**Quantitative RT-PCR.**
RNAs were isolated by using Tri Reagent® (Molecular Research Center Inc) as instructed by the manufacturer from 3x10⁵ cells or from skeletal muscle that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). Primer sequences are reported in Supplementary table I.

Absolute quantification of cDNA copy number was achieved using plasmid DNA standards (i.e. Hh encoding plasmids described above or pGEM-T plasmids (Promega) in which we cloned the corresponding PCR amplicon).

The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to HPRT mRNA expression.

**Isolation of nerve cells.**
Mice were sacrificed by cervical dislocation and their sciatic nerves were harvested. Adjacent connective tissues and perineurium were strip off under a dissecting microscope. Nerves were digested in an enzymatic solution containing 0.2% collagenase NB4 (Worthington) for 1 hour at 37°C. An equal volume of 0.05% tryspin-EDTA was then added and nerve segments were mechanically disaggregated by vigorous pipetting for 3 to 5 minutes. After centrifugation, Schwann cells were plated in poly-L-lysine (Sigma) plus laminin (Sigma)-coated dishes and cultured in DMEM/F12 culture medium containing 10% foetal bovine serum, 2 µmol/L Forskolin (Sigma) and 10 ng/mL Heregulin- 1 (Promocell).

To isolated perineurial fibroblasts, nerve were harvested, digested and cultured following the same protocol expect that perineurium was not stripped off and cells were passaged at least once before being used, to get rid of most Schwann cells.

**Cell viability assay using propidium iodide.**
40 000 cells were seeded in each well of a 24-well plate. The day after, cell death was induced by a 24 hour incubation with 100 μM to 1 mM H₂O₂. To identify dead cells, culture medium was
removed and replaced by PBS containing 2 μg/mL propidium iodide (PI). Cells were incubated with PI for at least 5 minutes on ice. PI positive cells were identified by fluorescent microscopy.

**Statistics.**
Results are reported as mean±SEM. Comparisons between groups were analyzed for significance with the non parametric Mann-Whitney test. Differences between groups were considered to be significant when p≤0.05. *: p≤0.05; **: p≤0.01; ***: p≤0.001.

**RESULTS**

*Dhh is the main Hh expressed during the angiogenic phase of ischemic muscle repair.*

To investigate whether Dhh expression is detected and modulated during skeletal muscle ischemia-induced angiogenesis, HLI was surgically induced in mice. Tibialis anterior muscle tissue was harvested before and 2, 5, 7, 10, 14 and 21 days after HLI surgery. Consistently with previous investigations, we found that Shh is strongly upregulated (by 80 fold versus control) 2 days after HLI surgery was performed but its expression returns rapidly to baseline from day 5 after surgery. Interestingly, Dhh mRNA expression which is about 10 times higher than that of Shh and Ihh in healthy skeletal muscle, is quite stable until day 7, increases by about 2 fold from day 10 after HLI (p=0.036) and remains high until muscle repair is completed (Figure 1A). Ihh expression remains very low during the entire process of ischemic muscle repair (Figure 1A). In conclusion, Dhh, the role of which in angiogenesis has never been investigated before, is the most expressed Hh during the angiogenesis phase of ischemic muscle repair (i.e. from day 5 to day 10).

According to previous investigations, we found Dhh to be expressed by S100 positive peripheral nerves both in the healthy muscle and in the regenerating ischemic muscle (2, 5 and 10 days after HLI surgery was performed), (Figure 1B). We thus hypothesized that Schwann cell-derived Dhh regulates ischemia-induced angiogenesis.

*Dhh promotes ischemia-induced angiogenesis.*

The role of Dhh in ischemia-induced angiogenesis was assessed by using the mouse HLI model. HLI was surgically induced in Dhh−/− mice and in their WT littermates. The leg recovery was evaluated 10 days after surgery was performed. The mean clinical score of necrosis of the ischemic foot of Dhh−/− mice was 2.18±0.26 while it was 0 for all of the WT mice (Figure 2A-B). Increased foot necrosis in Dhh−/− mice was correlated with significantly decreased tissue perfusion (Figure 2C-D) and delayed muscle repair (Figure 2E-F and Online Figure I) demonstrating an important role of Dhh in skeletal muscle repair.

Revascularization of the ischemic leg was first evaluated by micro-CT imaging (Figure 3A-D). Artery density was dramatically lower in Dhh−/− mice when compared to WT mice (the number of arteries/μm³ in Dhh−/− was 0.026±0.005 versus 0.111±0.010, p=0.042 in WT mice) (Figure 3B). The mean diameter of vessels was also significantly lower in Dhh−/− mice (33±1 μm versus 50±3 μm in WT mice, p=0.042) (Figure 3C). Moreover, organization of the vessel network was strongly impaired in Dhh−/− mice since vessel connectivity was only 53±11 in Dhh−/− while it was 227±19 in WT mice, p=0.042 (Figure 3D). Those results were confirmed by CD31 staining of tibialis anterior
muscle sections (Figure 3E). The number of CD31+ vessels/mm² was 66±6 in Dhh-/- mice versus 164±11 in WT mice, p=0.002 (Figure 3F).

Altogether those data demonstrate for the first time that Dhh is critical for ischemia-induced angiogenesis in the setting of skeletal muscle tissue repair.

To confirm the specific role of Dhh in angiogenesis in adults, Dhh expression was rescued via gene therapy in Dhh KO mice. Unilateral HLI was surgically-induced in WT and Dhh-/- mice. Plasmids encoding either GFP alone (pIRES-EGFP) or Dhh together with GFP (pIRES-NDhh) were administered in the tibialis anterior muscle 4 days after surgery. This time point was chosen because it comes immediately before the angiogenesis phase of ischemic muscle repair. Dhh overexpression was detected from 5 to 10 days after surgery (data not shown). Dhh gene therapy significantly enhances ischemia-induced angiogenesis in Dhh KO mice since capillary density in the muscle of Dhh-/- mice treated by Dhh was comparable to that of WT mice (162±9 CD31+ vessels/mm² in Dhh-/- mice versus 172±7 in WT mice). These later data then confirm the role of Dhh during muscle repair.

ECs do not mediate Hh dependent angiogenesis in the setting of ischemia.

With the aim to investigate the direct role of Dhh on ECs in the setting of ischemic muscle repair, unilateral HLI was induced in Pdgfb-CreERT2; SmoFlox/Flox mice and in their SmoFlox/Flox control littermates. Smoothened (Smo) recombination was induced by tamoxifen injections 1 week before HLI surgery was performed. The activity of the Pdgfb promoter-driven Cre recombinase in ECs of both healthy and ischemic skeletal muscles was verified using Rosa26R mice (Online Figure II). Revascularization of the ischemic leg was evaluated 5 and 10 days after HLI surgery was performed. Neither feet perfusion measured after laser Doppler perfusion imaging (LDPI) imaging (Figure 4A-C) nor capillary density in the tibialis anterior muscle (Figure 4D, E) were different in Pdgfb-CreERT2; SmoFlox/Flox and in their control SmoFlox/Flox littermates. Consistently, skeletal muscle repair was not delayed in Pdgfb-CreERT2; SmoFlox/Flox mice and the clinical score of necrosis of their ischemic foot was equal to 0.

Dhh does not promote ischemia-induced angiogenesis by regulating EC function directly.

Dhh does not modulate proangiogenic factor expression in nerve fibroblasts.

To identify cells in which Hh signaling is active in the ischemic skeletal muscle, we used Gli1-CreERT2; Rosa26R and Ptc1-LacZ reporter mice. Hh proteins are known to interact with their specific receptor Patched-1 (Ptc1) which de-represses the transmembrane protein Smo and activates Gli transcription factors. Gli activation induces expression of downstream target genes including Ptc1 and Gli1. As shown Online figure IIIA, Hh canonical pathway activity (i.e. Gli1 and Ptc1 expression) is almost exclusively detected in perineurial cells, which indicates that Dhh expressed by Schwann cells mainly regulates perineurial fibroblast physiology.

We thus verify whether Dhh regulates proangiogenic factor expression in perineurial fibroblasts. As shown Online figure IIIB-E, even though Dhh significantly activates Gli1 mRNA expression in nerve-derived fibroblasts, it does not modulate vascular endothelial growth factor A (VEGFA), angiopoietin 1 (Angpt1) nor neurotrophin 3 (NTF3) mRNA expression. Those results suggest that, on the contrary to Shh, Dhh does not promote angiogenesis by upregulating VEGFA, Angpt1 or NTF3.
Peripheral nerve-derived signals are necessary to drive ischemia-induced angiogenesis.

Because Dhh was shown to be necessary in peripheral nerve integrity and regeneration in adults \(^1\), \(^2\), we hypothesized that Dhh signaling to perineurial fibroblasts promotes nerve survival in the setting of ischemia and by doing so maintains the pool of proangiogenic factor expressed by nerves.

To test this hypothesis, we first verified whether peripheral nerves within the regenerating skeletal muscle express proangiogenic factors. To this aim, we performed immunostaining of sections of ischemic skeletal muscles harvested 5 days after HLI was induced. We found that nerves within the skeletal muscle indeed produce VEGFA, Angpt1 and NTF3 (Online Figure IV).

We then verified whether nerve-derived signals are necessary to drive ischemia-induced angiogenesis. To this aim, mice were denervated by removing 5 mm of the left sciatic nerve immediately after HLI was performed. Mice were sacrificed 10 days after HLI surgery. First we verified that nerve density was indeed significantly reduced in the regenerating muscle of denervated mice compared to undenervated mice (Online figure V). Consistently with our hypothesis, CD31 staining of capillaries demonstrated that angiogenesis is significantly reduced in denervated mice compared to the sham operated (undenervated) control (capillary density was 87±6 vessels/mm\(^2\) in denervated mice versus 153±14 in sham operated mice) (Figure 5A, B). Moreover, delayed angiogenesis was correlated with a significant decrease of VEGFA, Angpt1 and NTF3 (Figure 5C-D). Similarly to what we found in Dhh-/- mice, impaired angiogenesis observed in denervated mice induced a significant reduction of foot perfusion (Figure 6A, B), and delayed skeletal muscle regeneration (Figure 6C, D). As a consequence, the clinical score of necrosis was 2.44±0.18 in denervated mice while it was mainly 0 in undenervated mice (Figure 6E, F).

Dhh promotes peripheral nerve survival in the setting of ischemia.

We then tested whether nerve survival was impaired in the absence of Dhh. We measured S100+ peripheral nerve density in the tibialis anterior muscle of Dhh-/- mice and of WT mice 0, 2, 5 and 10 days after HLI was performed. As shown Figures 7A and 6B, in physiologic conditions (i.e. before HLI surgery was performed) nerve density was not different in Dhh-/- and in WT mice. On the contrary, 2 days after surgery, while the number of nerves/mm\(^2\) only slightly decreases (from 6.44±2.03 to 5.64±0.17 nerves/mm\(^2\) in WT mice, it significantly drops down in Dhh-/- mice (from 6.41±1.99 to 3.18±0.18 nerves/mm\(^2\)) showing that Dhh promotes peripheral nerve survival after an ischemic injury. To confirm this result, we co-cultured Schwann cells and fibroblasts from the sciatic nerves of Dhh-/- and WT mice (Online Figure VIA). Schwann cells were used here as the source (or not) of Dhh (Online Figure VIB) and as shown Online figure VIC, Schwann cell-derived Dhh stimulated Hh signalling pathway in fibroblasts. Among Dhh-/- cells, the number of propidium iodide positive dying fibroblasts was significantly higher than among WT cells when cell death was induced by 0.5 mmol/L or 0.8 mmol/L H\(_2\)O\(_2\) (Figure 7C and 7D). This result demonstrates that Dhh promotes nerve fibroblast survival.

DISCUSSION

The present paper demonstrates for the first time the crucial role of Dhh in the setting of skeletal muscle ischemia-induced angiogenesis. Moreover, this study shows that Dhh promotes...
angiogenesis by ensuring peripheral nerve survival and indirectly growth factor production, thus identifying an original regulation of ischemia-induced angiogenesis.

The embryonic Hh signaling pathway was shown to be reactivated in adult injured tissues about 10 years ago 31. More particularly, Shh expression have been shown to be strongly increased by skeletal muscle injury including surgically-induced ischemia, mechanical crush and cardiotoxin injection 16, 17, 28. This pathway was shown to be necessary for ischemia-induced angiogenesis by using the Hh ligand blocking antibody 5E1 17. In the present paper we show, for the first time, that except for the early neutrophil infiltration step of muscle repair where Shh is transiently expressed, the main Hh ligand expressed in the limb skeletal muscle is Dhh. Moreover, Dhh expression is increased during the delayed phase of muscle repair corresponding to the regeneration of the vascular network suggesting a possible role for this protein in vessel growth, which is consistent with the anti-angiogenic effect of 5E1 anti-Hh antibody observed in the mouse model of HLI 17.

In this study, we used Dhh deficient mice to specifically investigate the role of Dhh. Our data reveals, for the first time, that Dhh has a major role in ischemia-induced angiogenesis and muscle repair. This effect of Dhh is different from that of Shh since Shh overexpression observed 2 days after HLI surgery in WT mice (Figure 1) occurs at the same level in Dhh deficient mice (data not shown).

Hh signaling was proposed to promote angiogenesis via at least in part upregulation of several pro-angiogenic factors including VEGFA, Angpt1 and stromal cell-derived factor 1 in mesenchymal fibroblasts or in myocytes 31-33. We found that, on the contrary to Shh, Dhh does not stimulate VEGFA, Angpt1 nor NTF3 expression in fibroblasts. Consistently to what has been observed in embryos 34 and suggested in adults 31, we demonstrated that Dhh does not promote angiogenesis via a direct activation of ECs. On the contrary, the present study reveals that Dhh promotes peripheral nerve survival in the ischemic muscle demonstrating a new mechanism of action of Hh proteins to promote angiogenesis and muscle repair.

Mechanisms underlying tissue regeneration that have been widely studied in amphibians, such as urodeles, have revealed the essential role of peripheral nerves in this process 35. More particularly Schwann cells were shown to produce secreted signals named “AG” for anterior gradient necessary for limb regeneration. In mammals, denervation has been shown to impair ear lobe regeneration after injury induced by an ear punch 35. Accordingly, we found that denervation of the leg, immediately after ischemia was induced, severely impairs ischemia-induced angiogenesis and muscle regeneration.

Interestingly, peripheral nerves were shown to produce proangiogenic factors in the developing skin and signals from Schwann cells have been shown to determine blood vessel network organization 5. Neurotrophins were shown to promote ischemia-induced angiogenesis 36, 37. As a consequence we and others found that pro-angiogenic factor expression including Angpt1 and VEGFA is diminished in denervated muscles 38, 39.

In conclusion, the present study, supported by data from others, demonstrates the essential role of peripheral nerve as a source of proangiogenic factors in the setting of ischemia-induced angiogenesis. As a consequence, factors regulating peripheral nerve physiology and ensuring their survival such as Dhh might be interesting targets to consider for therapeutic angiogenesis at least in the setting of ischemia.
Finally, such concept is supported by observations, made in patients with neuropathy. One study reported that severity of neuropathy was the most important factor associated with the development and recurrence of foot ulcers in diabetic patients, and another one that the somatic and autonomic nerve alterations may play a relevant role in the progression of the disease toward critical limb ischemia.

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DISCLOSURES

None.

REFERENCES


FIGURE LEGENDS

**Figure 1**: Dhh is the main Hh expressed during the angiogenesis phase of ischemic muscle repair. (A) Unilateral HLI was induced in 8-week old C57BL/6 mice (n=6 in each group) and the expression of Dhh (red line), Shh (blue line) and Ihh (green line) in the ischemic hind limb was measured 0-21 days later by real-time RT-PCR. Expression is presented as the cDNA copy number per µg of total RNA. Sections of ischemic muscle were immunostained for expression of the EC marker CD31, and capillary density was quantified as the number of CD31+ vessels per mm² at the indicated time points (broken line). (B) Unilateral HLI was induced in mice. Cross sections of tibialis anterior skeletal muscle were immunostained for the expression of Dhh (green) and marker for Schwann cells (S100, red) and DAPI (blue) as indicated on the pictures. Pictures were taken under a Zeiss Observer.Z1 microscope using a 40x objective and captured with a Zeiss AxioCam Camera. *: p≤0.05; **p≤0.01; NS: not significant.

**Figure 2**: Ischemia-induced muscle repair is impaired in Dhh-/- mice. HLI was induced in 8 week old Dhh-/- mice and their WT littermates. Mice were sacrificed 10 days after HLI was performed. (A) Representative pictures of the legs of WT and Dhh-/- mice. (B) The clinical score of necrosis for each mouse was reported. (C) Perfusion in both hind limbs was measured 10 days after HLI by laser Doppler perfusion imaging (LDPI) (D). Tissue perfusion was quantified as the ratio of blood flow in the ischemic leg versus the non ischemic leg. (E) Sections of tibialis anterior muscle were stained with hematoxylin and eosin. (F) Not repaired, under repair and repaired areas were measured using Sigma Scan Pro software as described Online Figure I. Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 4x objective and captured with a Nikon DXM1200F digital camera *: p≤0.05; ***p≤0.001

**Figure 3**: Ischemia-induced angiogenesis is impaired in Dhh-/- mice. HLI was induced in 8 week old Dhh-/- mice and their WT littermates. Mice were sacrificed 10 days after HLI was performed. (A) The vascular network in the limb of Dhh-/- mice and their WT littermates was imaged by micro-CT. Micro-CT images were used to evaluate (B) the density of more than 7 µm arteries, (C) the mean diameter of vessels and (D) the connectivity of vessels (n=3 in each groups). (E) Capillary density was evaluated after CD31 staining of ischemic tibialis anterior muscle sections and (F) quantified by counting CD31+ vessels per mm² (n=6 and 8 respectively in the WT and Dhh-/- groups). Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 20x objective and captured with a Nikon DXM1200F digital camera *: p≤0.05; **p≤0.01

**Figure 4**: Hh signaling to ECs is not necessary for ischemia-induced angiogenesis. Unilateral HLI was induced in Pdgfb-CreERT2; SmoFlox/Flox mice and in their SmoFlox/Flox control littermates. Mice were sacrificed 5 (n=6 and 8) or 10 (n=5 and 7) days later. (A-B) Perfusion in both hind limbs was measured via LDPI (C). Tissue perfusion was quantified as the ratio of blood flow in the ischemic leg versus the non ischemic leg. (D) Capillary density was evaluated by staining muscle sections for CD31 expression (brown) and reported as the number of CD31+ vessels per mm² (E). Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 20x objective and captured with a Nikon DXM1200F digital camera. NS: not significant.

**Figure 5**: Ischemia-induced angiogenesis is impaired in denervated tissues. HLI was surgically induced in 8 week old WT mice. Immediately after HLI surgery was performed, the leg in which ischemia was induced was denervated or sham operated (n=9 in each group). Mice were sacrificed 10 days later. (A) Capillary density was evaluated by staining muscle sections for CD31 expression (brown) and (B) reported as the number of CD31+ vessels per mm². Pictures were taken under a
Nikon MICROPHOT-FXA microscope using a 20x objective and captured with a Nikon DXM1200F digital camera. The expression of (C) NTF3, (D) VEGFA and (E) Angpt1 mRNA in the ischemic muscle was measured via real-time RT-PCR. *: p≤0.05; **p≤0.01

**Figure 6:** HLI was surgically induced in 8 week old WT mice. Immediately after HLI surgery was performed, the leg in which ischemia was induced was denervated or sham operated (n=9 in each group). Mice were sacrificed 10 days later. (A) Perfusion in both hind limbs was measured 10 days after HLI via laser Doppler perfusion imaging (B). Tissue perfusion was quantified as the ratio of blood flow in the ischemic foot versus the non ischemic foot. (C) Sections of tibialis anterior muscle were stained with hematoxylin and eosin. Pictures were taken under a Nikon SMZ800 instrument and captured with a Nikon DXM1200 digital camera. (D) Not repaired, under repair and repaired surface areas were measured using Sigma Scan Pro software. (E) Representative pictures of the legs of denervated or sham operated mice. (F) The clinical score of necrosis for each mouse was reported. *: p≤0.05.

**Figure 7:** Dhh promotes peripheral nerve survival. (A-B) HLI was induced in 8 week old Dhh-/- mice and their WT littermates. Mice were sacrificed 2, 5 or 10 days after HLI was performed. (A) Peripheral nerve density was evaluated after S100 staining (in brown) of tibialis anterior muscle cross sections and (B) quantified as the number of S100+ nerves/mm² (healthy muscle: n=8 and 13; D2 post HLI: n=7 and 10; D5 post HLI: n=7 and 8; D10 post HLI: n=15 and 11). Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 10x objective and captured with a Nikon DXM1200F digital camera (C-D) Cell death was induced by increasing concentration of H2O2 for 24 hours and quantified as the number of PI+ cells/high power fields (HPF) (orange nuclear staining). Pictures were taken under a Zeiss Observer.Z1 microscope using a 10x objective and captured with a Zeiss AxioCam Camera. *: p≤0.05; **p≤0.01; ***p≤0.001.
Novelty and Significance

**What Is Known?**

- Embryonic signaling pathways participate in ischemic tissue repair in adults.
- Sonic Hedgehog (Shh) promotes angiogenesis by upregulating pro-angiogenic factors including vascular endothelial growth factor A (VEGFA).
- Peripheral nerves drive tissue regeneration in amphibians and regulate angiogenesis and blood vessel network architecture during development.

**What New Information Does This Article Contribute?**

- Peripheral nerves contribute to ischemia-induced angiogenesis
- Desert Hedgehog, a Schwann cell-derived protein, is essential for structural and functional integrity of peripheral nerves. It is also crucial for ischemia-induced angiogenesis by promoting peripheral nerve survival in ischemic conditions.

This study shows that nerve survival maintains pro-angiogenic nerve-derived factor levels and thereby promotes muscle repair. Our findings suggest factors regulating peripheral nerve physiology and survival such as Desert Hedgehog are important regulators of ischemia-induced angiogenesis and might be important targets to consider for therapeutic angiogenesis.
Figure 2

(A) WT and Dhh-/− mice. (B) Clinical score of necrosis: 0 = no sign of necrosis, 1 = 1 necrotic toe, 2 = 2 or more necrotic toes, 3 = loss of substance. (C) perfusion ratio ischemic leg/control. (D) Representative images of WT and Dhh-/− hearts. (E) Representative images showing WT and Dhh hearts. (F) Surface area (% of total) of WT and Dhh hearts.
Figure 3

A) Comparison of WT and Dhh-/- arteries. B) Artery density (≥7 μm) in WT and Dhh-/-. C) Mean vessel diameter in WT and Dhh-/-. D) Connectivity in WT and Dhh-/-. E) Immunohistochemistry for CD31+ vessels in WT and Dhh-/-. F) CD31+ vessels/mm² in WT and Dhh-/-.
Figure 6

Panel A: Immunohistochemical staining of S100+ nerves in WT and Dhh-/- mice.

Panel B: Graph showing S100+ nerves/mm² over time after HLI. WT and Dhh-/- mice are compared with significant differences indicated by ** and ***.

Panel C: Micrographs of cultured cells treated with H₂O₂ concentrations of 0.5 mmol/L and 0.8 mmol/L. WT and Dhh-/- mice show differences in cell morphology.

Panel D: Graph showing cell viability assessed by propidium iodide staining in response to different H₂O₂ concentrations. WT and Dhh-/- mice show significant differences indicated by * and **.
Desert Hedgehog Promotes Ischemia-Induced Angiogenesis by Ensuring Peripheral Nerve Survival

Marie-Ange Renault, Candice Chapouly, Qinyu Yao, Frédéric Larrieu-Lahargue, Soizic Vandierdonck, Annabel Reynaud, Myriam Petit, Béatrice Jaspard-Vinassa, Isabelle Belloc, Elisabeth Traiffort, Martial Ruat, Cécile Duplàa, Thierry Couffinhal, Claude Desgranges and Alain-Pierre Gadeau

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Supplemental Material

Expanded methods section

**Mice**

C57BL/6 mice were obtained from Charles River Laboratories and bred in our animal facility. Dhh heterozygote mice \(^1\) under CD1-C57BL/6 genetic background were provided by M. Wijgerde (Erasmus University Medical Center, Rotterdam, The Netherlands). Dhh+/- mice were bred together to obtain Dhh-/- mice and WT control mice. Smo\(^{\text{Flox}}\) \(^2\), Rosa26R and Ptc1-LacZ mice \(^3\) were obtained from the Jackson laboratory. Pdgfb-Cre\(^{ER\text{T2}}\) mice \(^4\) were kindly given by M. Fruttiger and bred with Smo\(^{\text{Flox}}\) mice. Gli1-Cre\(^{\text{ERT2}}\) mice \(^5\) were kindly provided by AL Joyner and bred with Rosa26R mice. Mice were handled in accordance with the guidelines established by the National Institute of Medical Research (Inserm) and approved by the local Institutional Animal Care and Use Committee. Animals were anesthetized by 2.5-4% isoflurane inhalation or by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Mice were administrated 1 mg/kg buprenorphine 30 minutes prior to surgical procedures. Cre recombinase was activated by intraperitoneal injection of 1 mg tamoxifen (Sigma) for 5 consecutives days. Mice were genotyped using primers described in Online Table I.

**H91I model and assessments**

For hind limb ischemia surgery, Mice were anesthetized by inhaled isoflurane delivered at approximately 2.5-4%. Mice were aseptically prepared and a 5 mm incision was made in the left thigh region. Two ligations were made around the femoral artery (one at the proximal end of the femoral artery and the other one at the distal portion of the saphenous vein. The femoral artery and all side-branches were then dissected and excised. The connective tissues of the sub cutis were then closed with interrupted 6-0 absorbable suture.

For histological assessment and gene expression analysis, mice were euthanized and tibialis anterior muscles were harvested then cut in half, the lower half was fixed in methanol, paraffin-embedded, and cut into 6-μm sections and the upper half was snap frozen in liquid nitrogen. Each group included 3-15 animals. Capillary density was evaluated in sections stained for the expression of CD31.

For micro-CT vasculature imaging, mice euthanized by a lethal injection of pentobarbital, were perfused via the brachiocephalic arterial trunk first with a heparinized isotonic solution and then with a 1% paraformaldehyde solution, followed by a mixture of 80% Neoprene latex (Neoprene Latex Dispersion 671 A, Dupont, France) and barium sulphate powdered to 1 μm (3 g/mL, Micropaque® oral solution, Guerbet, France). At the end, the mouse was put into acid solution for the latex to harden, then, after dissection, the organs were fixed overnight in 4% PFA at 4°C. To study the progression of the contrast medium through the arterioles and capillaries during histology, an inert blue dye was mixed with the Neoprene latex and barium sulphate.

For mCT scanner data processing and analysis, we used the eXplore Locus Micro-CT scanner from General Electric Healthcare® with spatial resolutions of 36 to 7 μm, with the Scan Control® and Reconstruction Utility® programs. Voltage parameters were 40 kVp with a mean current of 80 μA. Our acquisition protocol consisted of 360 views and 4 images per position, with a voxel volume of 16 μm³. The quantification parameters were obtained using the Microview ABA® program after bones of the hind limb were delimited and digitally deleted.
For X-gal staining, mice were perfused with LacZ fix solution (PBS containing 0.2% Glutaraldehyde, 5 mmol/L EGTA and 100 mmol/L MgCl₂) before tibialis anterior muscles were harvested. Ten μm thick cryosections were prepared and post-fixed for 10 min in 0.2% glutaraldehyde and then stained overnight at 37°C in X-gal staining solution (1 mg/ml X-gal (Sigma)). Tissue sections were counterstained with nuclear fast red solution (Sigma).

**Plasmids/Gene Therapy**

The cDNA encoding the N terminal part of Dhh (NDhh) was amplified from MS1 cells cDNA with the following primers: 5'-AGGACAAGAACGCTCCCTTC-3' and 5'-GAGCGGATCCATTTCGCCGGAAGCTAGCTCTC-3’. NDhh cDNA was subsequently digested with NcoI and BamHI and cloned into pIRES2-EGFP plasmid (Clontech) digested with SmaI and BamHI.

Gene therapy was performed in 12 week old mice. Four days after HLI surgery was performed, mice were randomly assigned to receive 200 μg pIRES-EGFP or 200 μg pIRES-NDhh together with 0.05% pluronic. The DNA/pluronic mix was injected intramuscularly at 4 injection points in the tibialis anterior muscle. Mice were sacrificed 10 days after HLI surgery was performed.

**Limb Denervation**

Mice were anesthetized by inhaled isoflurane delivered at approximately 2.5-4%. The dorsal skin of the thigh was cut and the posterior muscles divided to show the sciatic nerve. Denervation of the left leg was obtained by cutting and removing 5-mm of the sciatic nerve.

**Immunostaining**

Tissues were fixed overnight in 100% in methanol, paraffin-embedded, and cut into 6-μm sections. For immunohistochemistry staining, endogenous peroxidases were inhibited by a 20 minutes incubation in PBS containing 3% H₂O₂. Tissue section were then blocked with PBS containing 10% donkey serum and incubated with the primary antibody for 2 hours at 37°C. ECs were identified with rat anti-CD31 antibodies (BD Pharmingen Inc). Dhh was stained with goat anti-C-terminal Dhh (R&D Systems) antibodies. Schwann cells were identified with rabbit anti-S100 antibodies (Dako) or rat anti-Myelin Basic Protein (MBP) antibodies (Abcam). NTF3, VEGFA, and Angpt-1 were stained using rabbit anti-NTF3 antibodies (Abbiotec), rabbit anti-VEGFA antibodies (Abcam) and goat anti-Angpt1 antibodies (Santa-Cruz) respectively. For immunofluorescence analyses, primary antibodies were resolved with Alexa-Fluor–conjugated secondary antibodies (Invitrogen) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (Vector) and streptavidin-HRP complex (Amersham), then the stain was developed with a DAB substrate kit (Vector Laboratories); tissues were counterstained with hematoxylin.

**Quantitative RT-PCR**

RNAs were isolated by using Tri Reagent® (Molecular Research Center Inc) as instructed by the manufacturer from 3x10⁵ cells or from skeletal muscle that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). Primer sequences are reported in online table II.

Absolute quantification of cDNA copy number was achieved using plasmid DNA standards (i.e. Hh encoding plasmids described above or pGEM-T plasmids (Promega) in which we cloned the corresponding PCR amplicon).

The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to HPRT mRNA expression.
Isolation of nerve cells

Mice were sacrificed by cervical dislocation and their sciatic nerves were harvested. Adjacent connective tissues and perineurium were stripped off under a dissecting microscope. Nerves were digested in an enzymatic solution containing 0.2% collagenase NB4 (Worthington) for 1 hour at 37°C. An equal volume of 0.05% trypsin-EDTA was then added and nerve segments were mechanically disaggregated by vigorous pipetting for 3 to 5 minutes. After centrifugation, Schwann cells were plated in 5 μg/mL poly-L-lysine (Sigma) plus 10 μg/mL laminin (Sigma)-coated dishes and cultured in DMEM/F12 culture medium containing 10% foetal bovine serum, 2 μmol/L Forskolin (Sigma) and 10 ng/mL Heregulin-β1 (Promocell).

To isolate perineurial fibroblasts, nerves were harvested, digested and cultured following the same protocol except that perineurium was not stripped off and cells were passaged at least once before being used, to get rid of most Schwann cells.

Cell viability assay using propidium iodide:

40,000 cells were seeded in each well of a 24-well plate. The day after, cell death was induced by a 24 hour incubation with 100 μM to 1 mM H2O2. To identify dead cells, culture medium was removed and replaced by PBS containing 2 μg/mL propidium iodide (PI). Cells were incubated with PI for at least 5 minutes on ice. PI positive cells were identified by fluorescent microscopy.

References

**Online Table I**: Primers used to genotype mice

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**Online Table II: PCR primer list**

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**Online Figure I: Muscle repair assessment.** Unilateral HLI was induced in 8-week-old mice. (A) A representative image of a hematoxylin and eosin stained cross sections of tibialis anterior skeletal muscle. Not repaired area (i.e., necrotic muscle fibers), under repair area (characterized by cell infiltration) and repaired area (newly generated myocytes), can be easily identified after H&E staining. The repaired area is surrounded with a black line. (B) Necrotic myocytes are characterized by their rounded shape, their eosinophilic cytoplasm and the absence of nuclei. (C) The area under repair is characterized by a massive cell infiltration and finally (D) newly generated muscle fibers are characterized by their central nuclei. Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 4x (Panel A) and 40 x objective (panels B-C) and captured with a Nikon DXM1200F digital camera.
Online Figure II: 8 week old Pdgfb-Cre^{ERT2}; Rosa26R mice were injected intraperitoneally with 100 μg tamoxifen for 5 consecutive days. The week after, mice were submitted to unilateral HLI surgery. Mice were sacrificed 10 days later. Tibialis anterior muscle sections for both legs were stained with X-gal. Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 40 x objective and captured with a Nikon DXM1200F digital camera.
Online figure III: (A) Cross sections of Ptc1-LacZ and Gli1-Cre\textsuperscript{ERT2}\textsubscript;Rosa26R mice were stained with X-gal. Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 40 x objective and captured with a Nikon DXM1200F digital camera. (B-E) Fibroblasts were isolated from the sciatic nerve of WT C57BL/6 mice. Fibroblasts were serum-starved for 24 hours in 0.5% FBS containing medium before they were treated or not with 1 μg/mL Dhh for another 24 hours. (B) Gli1, (C) NTF3, (D) Angpt1 and (E) VEGFA mRNA expression were quantified by real time RT-PCR and normalized to HPRT mRNA expression. ***p<0.001; NS: not significant.
Online figure IV: Peripheral nerves express NTF3, VEGFA and Angpt1. HLI was surgically induced in WT mice. Mice were sacrificed 5 days later. Cross sections of tibialis anterior muscle were immuno-stained (A) for NTF3 by immunohistochemistry in brown or triple-stained for NFT3 (in green) and the Schwann cell marker MBP (in red) and DAPI (in blue), (B) for VEGFA by immunohistochemistry in brown or triple-stained for VEGFA (in green) and the Schwann cell marker MBP (in red) and DAPI (in blue), and (C) for Angpt1 by immunohistochemistry in brown or triple-stained for Angpt1 (in green) and the Schwann cell marker S100 (in orange) and DAPI (in blue). Immunohistochemistry pictures were taken under a Nikon MICROPHOT-FXA microscope using a 20 x objective and captured with a Nikon DXM1200F digital camera. Immunofluorescence pictures were taken under a Zeiss Observer.Z1 microscope using a 40x objective and captured with a Zeiss AxioCam Camera.
Online Figure V: HLI was surgically induced in 8 week old WT C57BL/6 mice. Immediately after HLI surgery was performed, the leg in which ischemia was induced was denervated or sham operated (n=9 in each group). Mice were sacrificed 10 days later. (A) Nerve density was evaluated by staining muscle sections for S100 expression (brown) and (B) reported as the number of S100+ nerves per mm². Pictures were taken under a Nikon MICROPHOT-FXA microscope using a 10 x objective and captured with a Nikon DXM1200F digital camera. **: p<0.01.
Online Figure VI: Schwann cells and Fibroblasts were isolated from WT and Dhh-/- sciatic nerves. (A) 40000 fibroblasts were co-cultured together with 1500 Schwann cells. Cells were immuno-stained for the Schwann cell marker S100 (in green) and DAPI –in (Blue). Pictures were taken under a Zeiss Observer.Z1 microscope using a 40x objective and captured with a Zeiss AxioCam Camera. (B) Dhh mRNA expression was measured by real time RT-PCR in WT Schwann cells alone, WT fibroblasts alone in Dhh+/+ fibroblasts co-cultured with Dhh+/+ Schwann cells and in Dhh-/- fibroblasts co-cultured with Dhh-/- Schwann cells. (C) Gli1 mRNA expression was measured by real time RT-PCR in Dhh+/+ fibroblasts co-cultured with Dhh+/+ Schwann cells and in Dhh-/- fibroblasts co-cultured with Dhh-/- Schwann cells.