Hedgehog-Dependent Regulation of Angiogenesis and Myogenesis Is Impaired in Aged Mice

Marie-Ange Renault, Fanny Robbesyn, Candice Chapouly, Qinyu Yao, Soizic Vandierdonck, Annabel Reynaud, Isabelle Belloc, Elisabeth Traiffort, Martial Ruat, Claude Desgranges, Alain-Pierre Gadeau

Objective—The purpose of this study is to further document alteration of signal transduction pathways, more particularly of hedgehog (Hh) signaling, causing impaired ischemic muscle repair in old mice.

Approach and Results—We used 12-week-old (young mice) and 20- to 24-month-old C57BL/6 mice (old mice) to investigate the activity of Hh signaling in the setting of hindlimb ischemia–induced angiogenesis and skeletal muscle repair. In this model, delayed ischemic muscle repair observed in old mice was associated with an impaired upregulation of Gli1. Sonic Hh expression was not different in old mice compared with young mice, whereas desert Hh (Dhh) expression was downregulated in the skeletal muscle of old mice both in healthy and ischemic conditions. The rescue of Dhh expression by gene therapy in old mice promoted ischemia-induced angiogenesis and increased nerve density; nevertheless, it failed to promote myogenesis or to increase Gli1 mRNA expression. After further investigation, we found that, in addition to Dhh, smoothened expression was significantly downregulated in old mice. We used smoothened haploinsufficient mice to demonstrate that smoothened knockdown by 50% is sufficient to impair activation of Hh signaling and ischemia-induced muscle repair.

Conclusions—The present study demonstrates that Hh signaling is impaired in aged mice because of Dhh and smoothened downregulation. Moreover, it shows that hedgehog-dependent regulation of angiogenesis and myogenesis involves distinct mechanisms. (Arterioscler Thromb Vasc Biol. 2013;33:2858-2866.)

Key Words: aging ■ angiogenesis effect ■ angiogenesis, pathological ■ hedgehogs ■ peripheral arterial disease ■ regeneration

Peripheral arterial diseases are characterized by a decreased limb perfusion because of stenosis or obliteration of upper- or lower-extremity arteries. It affects >27 million people in Europe and in the United States alone. Manifestations are varied and range from asymptomatic disease detected on physical examination and noninvasive testing to symptomatic disease presenting as intermittent claudication or critical limb ischemia.1 Aging is one of the well-established cardiovascular risk factors of atherosclerosis together with tobacco use, diabetes mellitus, hypercholesterolemia, and hypertension. The incidence of symptomatic peripheral arterial diseases thus increases with age, from ≈10% among men aged 65 to 69 years to ≈20% for men aged >80 years.2 Moreover, increased cardiovascular risk in elderly is associated with decreased regenerating properties of most tissues, including skeletal muscle.3 Several studies have indeed reported that angiogenesis and muscle repair after hindlimb ischemia (HLI) are severely impaired in old mice. Altogether those data make the elderly the largest and the more challenging population of patients with peripheral arterial diseases to treat.

Characterizing alterations in the functionality of signaling pathways involved in angiogenesis and ischemic muscle repair, in the setting of aging, is then necessary to design the most adapted therapeutic strategies to treat elderly patients. For instance, the expression of the proangiogenic factor, vascular endothelial growth factor A (VEGFA),4 has been shown to be downregulated in aged animals. Moreover, downregulation of VEGF receptor 25 observed in old mice in the absence of endothelial nitric oxide synthase has been suggested to be responsible for impaired VEGFA-induced angiogenesis in the ischemic limb of 2-year-old mice.6 Other signaling pathways have been shown to be altered in old mice: the Notch Ligand Delta is downregulated in old satellite cells,7 whereas Wnt canonical signaling is hyper activated.8

The hedgehog (Hh) signaling pathway is reactivated after ischemic injury, including HLI9,10 and myocardial infarction11;
Activation of Hedgehog Signaling Is Impaired in Old Mice

We first compared the histology of the tibialis anterior muscle of old (20–24 months) and young (12 weeks) mice in physiological conditions. Skeletal muscle sections were stained with hematoxylin and eosin (Figure 1A in the online-only Data Supplement), capillaries and nerves were stained with anti-CD31 and anti-S100 antibodies, respectively (Figure 1B in the online-only Data Supplement; data not shown). The hematoxylin and eosin stain did not show any alteration of muscle fibers in old mice (Figure 1A in the online-only Data Supplement). Both capillary and nerve densities were identical in old and young mice (Figure 1C and ID in the online-only Data Supplement).

Nevertheless, as previously described, Figure 1A and 1B show that muscle repair after surgically induced HLI is significantly delayed in old mice compared with young mice. Ten days after HLI was performed, 82.1±4.2% of the total surface area of a tibialis anterior muscle cross-section has been repaired in young mice, whereas only 35.1±6.7% of it has been repaired in old mice. The skeletal muscle of old mice can still get repaired as shown by the surface areas measured at day 28 (Figure 1B).

Consistently with previous investigations, 16 we found that the Hh target genes Gli1 and Gli2, but not Ptch1, are overexpressed in the setting of ischemic skeletal muscle repair (Figure IIA–IIC in the online-only Data Supplement), more particularly 10 days after HLI surgery was performed, which correspond to the under repair step of skeletal muscle as shown by the kinetics of desmin staining of skeletal muscle sections. The desmin staining of skeletal muscle section indeed shows a complete loss of myocytes at day 2 (not repaired step), an active myogenesis at day 10, and a complete muscle regeneration at day 28 (repaired step; Figure IID in the online-only Data Supplement). Gli1 overexpression in the regenerating ischemic skeletal muscle was assessed by immunostaining of muscle sections, which confirms that Gli1 is mainly expressed during the under repair step 10 days after HLI was performed in young mice (Figure IIE in the online-only Data Supplement).

Gli1 mRNA expression was then evaluated and compared in the ischemic and control tibialis anterior muscles of young and old mice 2, 10, and 28 days after HLI was performed. In healthy conditions, Gli1 mRNA expression was equivalent in old and young mice. In the setting of HLI, it significantly decreases in the not repaired ischemic skeletal muscle of old mice, whereas it barely changes in young mice. In young mice, Gli1 mRNA expression was increased by 9.4±2.7-fold in the ischemic muscle under repair compared with the healthy muscle, whereas it was only increased by 3.5±0.9-fold in old mice (Figure 1C). This result was confirmed by Gli1 immunostaining of under repair muscle sections of old and young mice. Indeed, Gli1 expression by desmin-positive muscle cells was dramatically decreased in old mice (Figure 1D).

Altogether, these results confirm that Hh signaling is activated during ischemic tissue repair and strongly suggests that this activation is impaired in old mice especially in skeletal myocytes.

Dhh Expression Is Lower in Old Mice

With the aim to investigate the reason why activation of Hh signaling is impaired in old mice, we first measured the expression of Shh and Dhh, the 2 main Hh ligands detected in the skeletal muscle. 5,10 The third member of Hh family, Indian hedgehog, is barely expressed in the muscle. 10 As shown in Figure 2A, Shh is strongly overexpressed (by >200-fold) 2 days after HLI surgery (ie, in the not repaired ischemic skeletal muscle) in both old and young mice. Shh expression level was not significantly different between young and old animals. Figure 2A also shows that Shh mRNA level is significantly higher in the healthy skeletal muscle of old mice compared with young mice. On the contrary, Dhh mRNA level is significantly diminished in the skeletal muscle of old mice compared with young mice in healthy conditions, and its expression remained low in the setting of ischemic skeletal muscle repair (Figure 2B). Those results were confirmed by immunostaining of ischemic muscle sections; Shh-expressing cells were found in the skeletal muscle of both young and old mice 2 days after

Materials and Methods

Materials and Methods are available in the online-only Supplement.
HLI surgery was performed and became undetectable at day 10 in both mice (Figure 2C). As previously described, Dhh is mainly expressed by nerves and consistently with mRNA expression analysis, Dhh staining was weaker on muscle cross-section from old mice (Figure 2D). This first set of data suggests that impaired activation of Hh signaling in old mice is, at least in part, because of the decrease in Dhh expression.

**Dhh Gene Therapy Rescued Nerve Density and Angiogenesis in Old Mice**

To rescue Dhh action in old mice, we administered Dhh via gene therapy. HLI was performed in both young and old mice, and Dhh expressing or empty plasmids (Figure III in the online-only Data Supplement) were administered locally in the ischemic tibialis anterior muscle of old mice 4 days after HLI was performed. As previously described, Dhh is mainly expressed by nerves and consistently with mRNA expression analysis, Dhh staining was weaker on muscle cross-section from old mice (Figure 2D). This first set of data suggests that impaired activation of Hh signaling in old mice is, at least in part, because of the decrease in Dhh expression.

**Figure 1.** Activation of hedgehog signaling is impaired in old mice. Hindlimb ischemia (HLI) was surgically induced in 12-week-old and in 20- to 24-month-old mice (n=10 and n=5 for day 2; n=11 and n=9 for D10; n=6 and n=5 for day 18). A, Hematoxylin and eosin staining was performed on tibialis anterior muscle sections harvested 10 days after HLI was performed. The repaired area is delimited by a red line; the not repaired area is surrounded by a blue line and the under repair area is in between the blue line and the red line. B, Not repair, under repair, and repaired surface areas were measured on hematoxylin and eosin–stained muscle cross-sections at the indicated time points. C, Ischemic tibialis anterior muscles were harvested 2, 10, and 28 days later to prepare not repaired, under repair, and repaired muscle samples, respectively. Gli1 mRNA expression was evaluated by quantitative reverse transcription-polymerase chain reaction and normalized to hypoxanthine-guanine phosphoribosyltransferase. D, Muscle sections were triple stained with anti-Gli1 antibodies (green), anti-desmin antibodies (red), and DAPI. ***P<0.001; and **P<0.01.

**Figure 2.** Desert hedgehog (Dhh) is downregulated in aged mice. Hindlimb ischemia (HLI) was surgically induced in 12-week-old and in 20- to 24-month-old mice. Ischemic tibialis anterior muscles were harvested 2, 10, and 28 days later to prepare not repaired, under repair, and repaired muscle samples, respectively (n=10 and n=5 for day 2; n=11 and n=9 for D10; n=6 and n=5 for day 18). A, Sonic hedgehog (Shh) and (B) Dhh mRNA expression was evaluated by real-time reverse transcription-polymerase chain reaction and normalized to hypoxanthine-guanine phosphoribosyltransferase mRNA. C and D, Ischemic muscle cross-section of young and old mice was stained with (C) anti-Shh antibodies or (D) anti-Dhh antibodies (brown staining). ***P<0.001; *P<0.05; NS, not significant.
after HLI was performed. As a reference, control young mice were administered with control empty plasmids. Capillary density was evaluated after CD31 staining of tibialis anterior muscle sections processed 10 days after HLI was performed (Figure 3A). Dhh gene therapy promoted angiogenesis in old animal (capillary density was 118±10 CD31+ vessels/mm² in Dhh-treated old mice versus 69±5 in empty plasmid–treated old mice; Figure 3B) and capillary density in old mice treated with Dhh was equivalent to that of young mice. Consistently, the ischemic foot perfusion, which was significantly lower in empty plasmid–treated old mice compared with empty plasmid–treated young mice, was increased by Dhh gene therapy to reach young mice perfusion (Figure 3C and 3D) demonstrating that Dhh gene therapy rescued impaired angiogenesis observed in old mice. Because we recently showed that Dhh-induced angiogenesis is dependent on the density of peripheral nerves, we quantified nerve density in the regenerating skeletal muscle of mice after S100 staining (Figure 3E). As shown in Figure 3F, the number of nerves was significantly diminished in old mice compared with young mice 10 days after HLI was performed (nerve density was 5±1 nerves/mm² in old mice versus 11±1 nerves/mm² in young mice). Similar to capillary density, Dhh gene therapy increased nerve density in old mice and the density of nerves in Dhh-treated old mice was equivalent to that observed in young mice.

![Image](image_url)

**Figure 3.** Desert hedgehog (Dhh) gene therapy promoted angiogenesis and increased nerve density in aged mice. Hindlimb ischemia (HLI) was surgically induced in young and in old mice. Four days later, old mice were randomly assigned to be treated by Dhh gene therapy or empty plasmids (vehicle), young mice only received empty plasmids. Tibialis anterior muscles were harvested 10 days later. A, Representative images of CD31 staining (brown) of muscle sections are shown. B, Capillary density was quantified as the number of CD31 positive vessels per mm². C, Foot perfusion was measured via laser doppler perfusion imaging (D) and quantified as the ratio of ischemic foot vs nonischemic foot. E, Representative images of S100 staining (brown) of muscle sections are shown. F, Nerve density was quantified as the number of S100 positive nerves per mm². ***P≤0.001; **P≤0.01; and *P≤0.05; NS, not significant.

### Myogenesis

Myogenesis was assessed by evaluating skeletal myosin heavy chain isoform 4 (Myh4) mRNA expression, which is indicative of the number of living myocyte in the muscle. Dhh gene therapy failed to rescue myosin heavy chain mRNA expression (Myh4) in the regenerating skeletal muscle of old mice (Figure 4A). Consistently, the difference in the surface area of muscle already repaired remained insignificant between old mice treated with Dhh compared with empty plasmid–treated old mice (Figure 4B and 4C). Moreover, the surface area of repaired muscle in Dhh-treated old mice stayed significantly different from that measured in young mice. After further investigation, we found that Dhh gene therapy significantly induced Gli1 expression in young mice (Gli1 expression was increased by 2.2±0.5-fold in Dhh-treated mice), whereas Gli1 mRNA expression remained low in old mice and was not significantly different in Dhh-treated old mice compared with empty plasmid–treated old mice (Figure 4D), suggesting that, in addition to the decrease in Dhh expression, Hh signaling pathway may not be fully functioning in old mice.

Altogether, these results demonstrate that Dhh gene therapy partially rescues impaired muscle repair observed in old mice. Indeed, Dhh gene therapy increased nerve density and rescued angiogenesis in old mice but did not promote myogenesis.
Boc and Smoothened Are Downregulated in Old Mice

We then hypothesized that impaired activation of Hh signaling in aged mice was not only because of Dhh knockdown. Consequently, we first measured the expression of Hh receptors and coreceptors; Ptch1 mRNA expression was not different both in the healthy skeletal muscle and in the regenerating ischemic muscle of old mice compared with young mice (Figure IV A in the online-only Data Supplement). Similarly, the expression of cell adhesion molecule–related/downregulated by oncogenes and growth arrest–specific 1 (Gas1), 2 positive regulators of Hh signaling, was the same in the healthy muscle of old and young mice (Figure IVB and IVC in the online-only Data Supplement). Conversely, the expression of brother of cell adhesion molecule–related/downregulated by oncogenes (Boc), another positive regulator of Hh signaling, was significantly downregulated both in the healthy and in the regenerating ischemic muscle of old mice compared with young mice (Figure IVD in the online-only Data Supplement).

We pursued our investigation by measuring Ptch1 downstream signaling elements, including the G-coupled receptor smoothened (Smo), suppressor of fused, and the transcription factors Gli2 and Gli3. Interestingly, we found that the mRNA expression of Smo, the crucial positive trigger of Hh signaling, was significantly downregulated both in the healthy and in the ischemic regenerating skeletal muscle of old mice compared with young mice (Figure 1VA in the online-only Data Supplement). We then verified that Smo was also downregulated, at protein level, in old mice by western blot analysis (Figure 5B and 5C).

In conclusion, these latter data indicate that impaired Hh signaling observed in old mice may be also because of Boc and Smo downregulation.

Smo Knockdown Is Sufficient to Impair Ischemia-Induced Muscle Repair

We used Smo haploinsufficient mice to test whether Smo knockdown (Figure 6A and 6B) is sufficient to impair muscle repair. HLI surgery was performed in Smo+/− and in their wild-type (WT) littermates. Mice were euthanized 10 days later. We quantified myogenesis by measuring Myh4 mRNA expression and found that ischemia-induced myogenesis is also delayed in Smo+/− mice compared with their WT littermate (Figure 6C).

Consistently, we found that skeletal muscle repair is significantly delayed in Smo+/− mice (Figure 6D and 6E). Smo knockdown is then sufficient to impair myogenesis and ischemic muscle repair. As a consequence, impaired muscle repair observed in old mice is also, at least in part, a result of Smo downregulation.

Smo Knockdown Is Sufficient to Impair Shh and Dhh-Induced Gli1 Overexpression

Finally, we investigated whether Smo knockdown affects Hh ligand–induced activation of Hh signaling. To this aim, we isolated skeletal muscle–derived fibroblasts from Smo+/− and WT mice, which are the best characterized cell in the muscle...
to be responding to Hedgehog ligands and more particularly in the setting of ischemic skeletal muscle regeneration. After a 24-hour incubation in 0.5% FBS-containing culture medium, cells were treated with 1 μg/mL Shh or 1 μg/mL Dhh for 24 hours. As shown in Figure VI in the online-only Data Supplement, Shh and Dhh were still able to induce Gli1 mRNA in Smo−/− fibroblasts. Nevertheless, although Gli1 mRNA was increased by 184-fold in Shh-treated WT fibroblasts, it was only increased by 33-fold in Smo−/− Shh-treated Smo−/− fibroblasts, similarly Gli1 mRNA was increased by ~6-fold in Dhh-treated WT fibroblasts, whereas it was only induced by 1.6-fold in Dhh-treated Smo−/− fibroblasts (Figure VIA in the online-only Data Supplement). Impaired activation of Gli1 in old mice treated by Dhh gene therapy is also because of Smo downregulation.

Smo knockdown also resulted in impaired Shh-induced Gli2, Ptc, and Ptc2 mRNA expression and impaired Dhh-induced Ptc1 mRNA expression. On the contrary to Shh, Dhh did not induce Gli2 and Ptc2 mRNA expression in skeletal muscle–derived fibroblasts (Figure VIB–VIE in the online-only Data Supplement).

**Discussion**

Impaired regenerative properties of muscle, associated with aging, have been shown to be both the result of a decrease in the myogenic progenitor cell number and of a decreased production of secreted chemokines/growth factor, including VEGF. Several studies now suggest that molecules that are involved in embryonic development and more particularly in vasculogenesis and muscle differentiation regulate angiogenesis and ischemic muscle repair in adults. Accordingly, Notch signaling has been involved in satellite cell activation and cell fate determination in postnatal myogenesis, and canonical Wnt signaling has been shown to be activated in the vascular endothelium during neovascularization after myocardial infarction. As a consequence, deregulation of such pathways, including increased Wnt signaling or impaired activation of Notch, has been reported to contribute to impaired muscle regeneration associated with aging.

Similarly, the Hh signaling was also shown to be reactivated in adult ischemic tissues and to be necessary for angiogenesis and ischemic muscle repair. A recent study has suggested that activation of this pathway, in the setting of HLI, is impaired in middle-aged mice (ie, 1 year old). The present study confirms and extends those observations in 2-year-old mice and further investigates the reason why Hh signaling is not fully functional in the setting of aging. We first confirmed the data of Palladino et al and found that, contrary to young (12-week-old) mice, Gli1 mRNA is not as increased in the regenerating ischemic muscle of 2-year-old mice (Figure 1). Moreover, we found that 2 crucial Hh pathway elements, the ligand Dhh and the G-coupled receptor Smo, are downregulated in aged mice (Figures 2A and 5). We recently showed that Dhh, by promoting peripheral nerve survival, is crucial for ischemia-induced angiogenesis. Smo is an essential element of Hh canonical signaling; moreover, the present study shows that Smo downregulation by 50% impairs Shh-induced Gli1 expression (Figure VI in the online-only Data Supplement). Together, these sets of data thus demonstrate for the first time that impaired Hh signaling observed in aged mice is because of both Dhh and Smo downregulation. As previously suggested, upregulation of Shh occurs normally in old mice.

Regulation of the Hh signaling pathway is complex and involves several already identified factors able to positively or negatively modulate the activity of this pathway. Expression of some of them including Ptc1, suppressor of fused, Gli2, and Gli3 were not different in old mice when compared with young mice. However, we observed that Boc, a positive regulator, is also downregulated in old mice. The impact of Boc knockdown needs to be further investigated as Boc was shown to positively regulate myogenesis.

Most importantly, the present study represents a big step in the understanding of mechanisms involved in Hh-dependent regulation of skeletal muscle regeneration. To date, gain-of-function studies have shown that activation of Hh signaling by Shh administration increases capillary density and promotes ischemic limb perfusion and consistently, loss of function studies have shown that the inhibition of the 3 Hh ligands with SE1 blocking antibodies or cyclopamine impairs.
angiogenesis. In parallel, inhibition of Hh signaling was shown to impair satellite cells activation and myogenesis. Nevertheless, the origin and the identity of Hh ligands responsible for those effects are still not fully characterized and the same is true for the Hh-responding cells. The data presented in this article extend our recent study that identifies the crucial role of Dhh in the regulation of ischemia-induced angiogenesis and demonstrates that Dhh, which is the main Hh ligand expressed in the skeletal muscle, regulates angiogenesis by promoting peripheral nerve survival. It demonstrates that our previous finding that is only experimental is relevant in the setting of aging, a physiological/pathological condition.

Moreover, our data show that Hh regulation of myogenesis requires Smo full expression and more likely the activation of Gli1 (Figures 5 and 6). Gli transcription factors have indeed been shown to regulate myogenic transcription factors, including MyoD and Myf5. Contrary to myogenesis, Dhh-induced angiogenesis does not seem to require Gli1 upregulation. This latter result is consistent with a previous study demonstrating that Shh-induced angiogenesis in the mouse cornea is independent of Gli1.

Together these data suggest that Hh-dependent regulation of angiogenesis and myogenesis involves distinct mechanisms. Moreover, these data imply that restoring nerve density or promoting angiogenesis is not sufficient to promote myogenesis at least in old animals.

Nevertheless, the present study also reveals that Hh regulation of ischemic muscle repair is complex and far from being fully understood. Indeed, the specific role of Shh is still not known, and the identity of the Hh ligand involved in the regulation of myogenesis remains to be characterized. We found that, contrary to Shh and Indian hedgehog, Dhh is not able to induce Gli1 overexpression in cultured myoblasts (data not shown). In embryos, somite determination is dependent on neural tube-derived Shh, whereas secondary myogenesis in the limb is dependent on bone-derived Indian hedgehog. Recently, Shh gene therapy was shown to be able to restore myogenesis in aged mice; nevertheless, in this setting, Shh was suggested to promote myogenesis indirectly via insulin-like growth factor-I upregulation.

Proangiogenic gene or cell therapy received much attention for their potential to regenerate ischemic organs, but initial clinical trials in aged patients fail to show significant efficacy. Embryonic signaling pathways, including Hh signaling pathway, are particularly promising new targets for regenerative medicine because of their wide range of action and more particularly their action on stem cells. Nevertheless, studies need to be designed to improve our understanding of age-related changes in the response to ischemic injury and the regenerative capacity of aged patients. Indeed, this study shows that rescuing the expression of Hh ligands by gene therapy in elderly may not be a sufficient strategy because other elements of the Hh signaling pathway, including Smo, are also downregulated and that Smo knockdown is sufficient to impair Shh or Dhh-induced activation of Hh canonical signaling. Finally, this study demonstrates that Dhh gene therapy increased angiogenesis and nerve density in old mice but failed to promote myogenesis. Similarly, Shh therapy was shown to increase capillary density and to promote ischemic limb perfusion in 2-year-old mice.
This result thus implies that combining a promyogenic therapy together with Hh therapy might be helpful to successfully treat aged patient with peripheral arterial diseases. This finding is in accordance with a study that has demonstrated the benefits of simultaneously targeting angiogenesis and myogenesis, with VEGFA and insulin-like growth factor-1, respectively, to promote ischemic limb regeneration in mice.38

Acknowledgments
We thank Jérôme Guignard (INSERM U1034, Pessac) for his excellent technical assistance in the animal facility and Christelle Bouillé for administrative assistance.

Sources of Funding
This study was supported by grants from the Fondation de la Recherche Médicale (FRM), program on cardiovascular aging (DCV20070409258); the Conseil Régional d’Aquitaine (action inter-recherche médicale (FRM), program on cardiovascular aging (DCV20070409258); the Communauté de Travail des Pyrénées and the ANR program (ANR-07-PHYSIO-010-02 to A-P. Gadeau); and The National League against Cancer (M.-A. Renault), C. Chapouly, and S. Vanden-donck are supported by fellowships from the CHU de Bordeaux.

Disclosures
None.

References
Characterizing alteration of signal transduction pathways involved in ischemic muscle repair in aged mice is necessary to design adapted therapeutic strategies to treat elderly patients with peripheral artery disease. Hedgehog signaling is an interesting therapeutic target because of its wide range of action; nevertheless, it was suggested not to be fully functional in aged mice. The present study confirms this result and further demonstrates that impaired Hh signaling is because of desert hedgehog but not of sonic hedgehog knockdown and to the down-regulation of the hedgehog signaling pathway element, smoothened. Moreover, it showed that desert hedgehog gene therapy in aged mice promotes angiogenesis and increased nerve density but fails to rescue myogenesis, thus demonstrating that hedgehog-dependent regulation of angiogenesis and myogenesis involves distinct mechanisms. Indeed, Hh-induced angiogenesis was shown to be independent of Gli1, whereas impaired myogenesis in aged mice was associated with an impaired Gli1 upregulation.
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Arterioscler Thromb Vasc Biol. 2013;33:2858-2866; originally published online October 17, 2013;
doi: 10.1161/ATVBAHA.113.302494
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Hedgehog-dependent regulation of angiogenesis and myogenesis is impaired in aged mice

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Supplemental Material

Supplemental Table I

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Supplemental Figure I: (A) Tibialis anterior muscle sections from young and old mice were stained with Hematoxylin and Eosin. (B) Tibialis anterior muscle sections from young and old mice were stained with anti-CD31 antibodies to identify blood vessels. (C) The number of CD31 positive vessels per mm² was counted. (D) The number of S100 positive nerve per mm² was counted. NS: not significant.
Supplemental Figure II: HLI was surgically induced in young C57BL/6 mice. Ischemic tibialis anterior muscle was harvested 2, 10 and 28 days later to prepare not repaired, under repair and repaired muscle section respectively. The expression of (A) Gli1, (B) Gli2, and (C) Ptc1 mRNA was measured via real time RT-PCR and normalized to HPRT. (D, E) Skeletal muscle sections were stained with (D) anti-Desmin antibodies to identify muscle cells, and with (E) anti-Gli1 antibodies to identify Hh responding cells. ***: p≤0.001; NS: not significant.
Supplemental Figure III: (A) COS7 cells were transfected with pIRES-NDhh or the empty vector pIRES-EGFP. Dhh expression was assessed by western blot analysis. (B) serum-starved primary cultured fibroblasts were treated with the conditioned medium of transfected COS7 for 24 hours. Gli1 mRNA expression was measured by quantitative RT-PCR. **: p≤0.01.
Supplemental Figure IV: HLI was surgically induced in 12 week old and in 18 to 24 month old mice. Tibialis anterior muscles (ischemic and control) were harvested 10 days later (n=8 in each group). (A) Ptch1, (B) Cdon, (C) Gas1, and (D) Boc mRNA expression was evaluated by real time RT-PCR and normalized to HPRT mRNA. ***: p≤0.001; NS: not significant.
Supplemental Figure V: HLI was surgically induced in 12 week old and in 18 to 24 month old mice. Tibialis anterior muscles (ischemic and control) were harvested 10 days later (n=8 in each group). (A) Sufu, (B) Gli3, and (C) Gli2 mRNA expression was evaluated by real time RT-PCR and normalized to HPRT mRNA. **: p≤0.01; NS: not significant.
Supplemental Figure VI: Skeletal muscle-derived fibroblasts were isolated from Smo<sup>+/−</sup> and WT mice. After a 24 hour-incubation in 0.5% FBS containing culture medium, cells were treated or not with 1 µg/mL Shh or 1 µg/mL Dhh for 24 hours. (A) Gli1, (B) Gli2, (C) Gli3, (D) Ptc1, and (E) Ptc2 mRNA expression was evaluated by quantitative RT-PCR and normalized to HPRT. **: p≤0.01; NS: not significant.
Material and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories and bred in our animal facility. Smo^{+/−} mice were obtained after germ line recombination of Smo^{Flox} allele. Smo^{Flox} mice were obtained from the Jackson laboratory. 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. Mice were administrated 1 mg/kg buprenorphine 30 minutes prior to surgical procedures.

HLI model and assessments

HLI was performed as previously described \(^1\) in C57BL/6 male mice. The “young” group of animals included 12 week old mice while the “aged” group included 20 to 24 month old mice; each experimental group included 5 to 12 animals. Briefly, the left femoral artery was resected from the proximal end of the femoral artery to the distal portion of the saphenous vein, then the femoral artery and all side-branches were dissected and excised. Tibialis anterior muscles were harvested from mice sacrificed at the indicated time points, fixed in methanol, paraffin-embedded, and cut into 6-µm sections for histological assessment or snap frozen in liquid nitrogen for gene expression analysis.

Repaired, not repaired and under repair areas were measured after haematoxylin and eosin staining as previously described \(^2\).

Capillary density was evaluated in sections stained for the expression of CD31. Sections were viewed at 40× magnification, and vessels were counted in 20 high-power fields per section. 1 section was quantified per muscle (mice). 5 to 12 mice were included in each group. S100 positive nerve density was measured on 10x magnification pictures. 10 pictures were counted/sections. 1 section was quantified per muscle (mice). 5 to 12 mice were included in each group.

Foot perfusion was measured using a MoorLDI2-IR apparatus after mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and reported as the ratio of blood perfusion in the ischemic vs non-ischemic limb.

Plasmids/Gene Therapy

Gene therapy was performed in 12 week old mice. Four days after HLI surgery was performed, old were randomly assigned to receive 200 µg pIRES-EGFP or 200 µg pIRES-NDhh \(^2\) together with 0.05% pluronic. Young mice were only injected with pIRES-EGFP/pluronic. The DNA/pluronic mix was injected intramuscularly in the tibialis anterior muscle as previously described \(^2\). Mice were sacrificed 10 days after HLI surgery was performed.

Immunostaining

Gli1 was stained using rabbit anti-Gli1 antibodies (Santa-Cruz). Myocytes were identified using either mouse monoclonal anti-Desmin antibodies (Sigma) or rabbit anti-
Desmin antibodies (Abcam). Dhh and Shh were identified using goat polyclonal anti-C-terminal Dhh and anti C-terminal anti Shh respectively (R&D systems).

ECs were identified with rat anti-CD31 antibodies (BD Pharmingen Inc). Nerves were identified using rabbit anti-S100 antibodies (Dako). For immunofluorescent analyses, primary antibodies were resolved with Alexa-Fluor–conjugated secondary antibodies (Invitrogen Corporation) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (Amersham) 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 3x10^5 cells or from skeletal muscle that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, 1 µg 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) from 1/20 of the cDNA obtained from 1 µg RNA. Primer sequences are reported in Supplemental table I.

The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to HPRT mRNA expression. Practically, histograms corresponds to “Relative expression = 2^{-\Delta \text{Ct}} \times 100000”. The efficacy of each PCR primer pair was >98% using a plasmid DNA standards (i.e. pGEM-T plasmids (Promega) in which we cloned the corresponding PCR amplicon).

**Cell Culture-Isolation of skeletal muscle-derived fibroblasts**

Mouse limb skeletal muscle from 6 to 12 week old mice, was dissociated in 2.4 U/mL dispase (Sigma) and 1.5 mg/mL collagenase 2 (Worthington) containing culture medium for 20 minutes at 37°C. Muscle-derived cells were then seeded on 50 µg/mL type collagen coated dishes (Sigma) and cultured 10% FBS containing DMEM. Fibroblasts were used from passage 1 to passage 3.

**COS-7 transfection and preparation of Hh-enriched conditioned medium**

COS-7 cells (CRL-1651™; ATCC) were cultured in 10% FBS-containing DMEM. To produce Hh-enriched conditioned medium, 10^6 COS-7 cells were seeded in a 100 mm-tissue culture dish (Falcon). The day after, cells were transfected with 7.5 µg pIRES-NDhh<sup>2</sup> or with the empty vector using JetPRIME (Polyplus Transfection) according to the manufacturer’s instructions. 24 hours later, culture medium was replaced by 8 mL 0.5% FBS containing DMEM which was harvested 24 hours later. This Hh-enriched conditioned medium, was centrifuged for 10 minutes at 2000 rpm then stored at -80°C before being used in cell culture assays.

Expression and secretion of Dhh protein in COS-7 culture medium was verified by western blot analysis using anti-Shh H160 antibodies (Santa-Cruz) that recognize N-terminal sequence of the all three Hh proteins (Supplemental Figure IIIA).

**Western blot analysis**

Expression of Smo was evaluated by SDS PAGE using a specific polyclonal rabbit antiserum (31Ab)<sup>3</sup>. Equal protein loading was controlled after Amido-Black staining of the membrane on which proteins were transferred for western blot analysis.

**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.
References