Endothelial Smoothened-dependent Hedgehog Signaling is Not Required for Sonic Hedgehog Induced Angiogenesis or Ischemic Tissue Repair

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Abstract

Sonic Hedgehog (Shh) signaling induces neovascularization and angiogenesis. It is not known whether hedgehog signaling pathway in endothelial cells is essential to angiogenesis. Smoothened (Smo) transduces hedgehog signaling across the cell membrane. This study assessed whether endothelial Smoothened-dependent Shh signaling is required for Shh mediated angiogenesis and ischemic tissue repair. Endothelial specific smoothened knockout mice, eSmo^{Null} were created using Cre-lox recombination system. eSmo^{Null} mice had no observable phenotype at baseline and showed normal cardiac function. Smoothened in CD31+ cells isolated from eSmo^{Null} hearts was significantly reduced compared to CD31+ cells from eSmo^{WT} littermate control hearts. Fluorescence immunostaining of eSmo^{Null} heart sections showed Smo expression in endothelial cells was abolished. The hind-limb ischemia (HLI) model was used to assess the response to ischemic injury. Perfusion ratio, limb motor function, and limb necrosis were not significantly different after HLI between eSmo^{Null} mice and eSmo^{WT}. Capillary densities in the ischemic limb in eSmo^{Null} mice were also similar to eSmo^{WT} at 4 weeks after HLI. Next, response to exogenous Shh was assessed in the corneal angiogenesis model. There was no significant difference in corneal angiogenesis induced by administration of Shh pellets between eSmo^{WT} and eSmo^{Null} mice. Furthermore, *in vitro* experiments demonstrated that direct Shh had limited effects on endothelial cell proliferation and migration. However, conditioned media from Shh-treated fibroblasts had a more potent effect on endothelial cell proliferation and migration than non-treated conditioned media. Furthermore, Shh treatment of fibroblasts dramatically stimulated angiogenic growth factor expression, including PDGF-B, VEGF-A, HGF and IGF. PDGF-B was

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the most upregulated and may contribute to the large neo-vessels associated with Shh induced angiogenesis. Taken together, these data demonstrate that Shh signaling via Smoothened in endothelial cells is not required for angiogenesis and ischemic tissue repair. Shh signaling via stromal cells likely mediates its angiogenic effects.

INTRODUCTION

The Hedgehog (Hh) family of secreted proteins play an important role in cell growth, survival, and fate.[1] They are morphogens in multiple tissues during embryonic development.[2] Sonic Hedgehog (Shh) is the most widely expressed Hedgehog protein. It has a critical role in the development of the neural and vascular systems. [3,4] In addition, Shh mediates key processes in neovascularization in response to injury, angiogenesis, and carcinogenesis in adults.[5 −8] Initial reports of the angiogenic effect of sonic hedgehog implicated an indirect mechanism where Shh stimulated the secretion of angiogenic growth factors.[6] More recent work has implicated a direct effect on the endothelium via a non-canonical signaling pathway involving Rho Kinase.[9,10]

The Shh receptor system consists of two major cell surface proteins, Patched-1 (Ptc-1) and Smoothened (Smo).[4] Ptc-1 is the receptor for Shh, whereas Smo is the signal transducer. Ptc-1 tonically inhibits Smo, but once Shh binds Ptc-1, Smo is disinhibited and downstream signaling occurs, classically through activation of the glioma-associated oncogene homolog (Gli) family of transcription factors.[11]

Smo is a Frizzled-class 7-transmembrane protein composed of a heptahelical transmembrane domain (TMD) and an extracellular cysteine-rich domain (CRD).[12] It has been shown that Shh signaling improves recovery after ischemic injury in mouse models of hind limb ischemia (HLI) and myocardial infarction.[7,13] *In vitro* findings showed direct angiogenic effect of Shh via Rho kinase-dependent signaling on endothelial cells.[9] However, the *in vivo* significance of endothelial Shh signaling and the mechanism by which Shh exerts an angiogenic effect have remained unclear.[6,9,10]

Based on robust evidence for the role of Shh in angiogenesis and vasculogenesis, and the *in vitro* findings of direct angiogenic effect on endothelial cells, we hypothesized that direct hedgehog signaling via endothelial Smo would be necessary for Shh mediated angiogenesis and ischemic tissue repair. Therefore, we generated genetic, tissue-specific mouse knockout models utilizing Cre-lox technology to explore the *in vivo* role of direct endothelial hedgehog signaling via Smo in Shh mediated angiogenesis and ischemic tissue repair. Surprisingly, we found that endothelial hedgehog signaling via Smo is dispensable for Shh-mediated angiogenesis and ischemic tissue repair. Furthermore, *in vitro* studies implicate a critical role of the stromal compartment in mediating the angiogenic response to Shh.

MATERIALS AND METHODS

Animals

Tie2-Cre mice (stock# 008863) and mice with a floxed allele of *Smoothened* (stock# 004526) obtained from Jackson Laboratory (Bar Harbor, ME), were bred to generate Cre

+/Smo $^{flox/flox}$ mice (referred to as "eSmo^{Null}") and Cre- Smo $^{flox/flox}$ (referred to as "eSmo^{WT}"). [14,15] Mice were housed in a sterile barrier facility. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Northwestern University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Littermates were used for controls in all experiments. Both groups of mice were born with expected Mendelian frequencies and appeared normal. Routine PCR genotyping was performed on tail DNA. The following primers were used: Smo: forward, 5′-CTT GGG TGG AGA GGC TAT TC-3′ and reverse, 5′-AGG TGA GAT GAC AGG AGA TC-3′; Cre: forward, 5′TGC CTG CAT TAC CGG TCG ATG C 3′ and reverse, 5′CCA TGA GTG AAC GAA CCT GGT CG 3′.

Mouse Hind-Limb Ischemia Model

Hind-Limb Ischemic (HLI) was performed in 8 to10 week-old male mice as described previously.[16] Briefly, the left femoral artery was exposed 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, and the overlying skin was closed with sutures. Critical limb ischemia was verified postoperatively by Laser Doppler Perfusion Imaging (LDPI, Moor LDI-Sim, Moor Instruments) to ensure the LDPI ratio (ischemic / nonischemic limb) was   0.20. For follow-up, animals underwent reevaluations with LDPI at postoperative day 3, 7, 14, 21, and 28. At each time point, tissue perfusion was measured via LDPI and reported as the ratio of ischemic / nonischemic limb. All LDPI measurements were taken on a 37°C heating pad to control body temperature.

For limb functional assays, limb motor function was scored as follows: 1, no limb use; 2, no foot use, limb use only; 3, restricted foot use; 4, no active toe use (spreading), foot use only; and 5, unrestricted limb use. Tissue salvage score was scored as follows: 1, limb amputation; 2, foot amputation; 3, total toe amputation; 4, partial toe amputation; 5, minor necrosis, nail loss only; 6, full recovery.

On the day of sacrifice, mice were injected with 50 μL fluorescien-BS1-Lectin I (Vector Laboratories Inc.) via tail vein and sacrificed at least 15 minutes later. Tibialis anterior muscles were harvested, fixed in methanol, paraffin-embedded, and cut into 5-μm sections. Capillary density was evaluated in sections stained for the expression of BS-lectin, an EC-specific marker. Perfused capillaries were identified by positive staining for BS-lectin. Sections were viewed at 40x magnification and vessels were counted in 10 high power fields per section.

For HLI specimens, skeletal muscle immunohistochemistry was performed to identify capillaries. Mice were identified by injecting mice with BS-1 lectin (Vector Laboratories) 10 min before sacrifice, and then sections were stained with anti-lectin primary antibodies (Vector Laboratories) and biotinylated anti-goat IgG (Vector Laboratories).

Mouse Corneal Angiogenesis Assay

Shh Pellets were prepared as previously described.[17] Briefly, Shh pellets were prepared by mixing 10 μg of Shh protein (Curis Inc., Cambridge, MA, USA) diluted in 10 μL sterile PBS with 5 mg sucrose octasulfate-aluminum complex (Sigma-Aldrich Co.). 10 μL of 12% hydron in ethanol was added, and the suspension was deposited on a 400 μM nylon mesh (Sefar America Inc., Depew NY, USA), then both sides of the mesh were covered with a thin layer of hydron and allowed to dry. eSmo[Null] and eSmo[WT] mice (8–10 week old) were anesthetized by intraperitoneal injection of 125 mg/kg Avertin, then pellets were implanted in the cornea as previously described.[17,18] Shh containing pellets were implanted in one eye and control pellets without Shh were implanted in the other eye. Mice were excluded from the study if nonspecific angiogenesis was detected in response to the control pellet. Seven days after pellet implantation, mice were injected with 50 μL fluorescien-BS1-Lectin I (Vector Laboratories Inc.) via tail vein and sacrificed at least 15 minutes later, then eyes were harvested and fixed with 1% paraformaldehyde, and corneas were excised and prepared for fluorescent microscopy. Angiogenesis was quantified as previously described.[18]

Echocardiographic Assessment of Cardiac Function

Trans-thoracic two dimensional measurements were performed with a high-resolution echocardiographic system equipped with a 30-MHz mechanical transducer (VEVO700, VisualSonics Inc., Toronto, Canada). Mice were anesthetized with a mixture of 1.5% isoflurane and compressed room air (1L / min). Mice were anchored to a warming platform in a supine position, limbs were taped to the echocardiograph electrodes, and thoraxes cleaned with a chemical hair remover to minimize ultrasound attenuation. Body temperature was monitored with a rectal thermometer probe and maintained at 37°C +/− 1°C. Aquasonic 100 gel (Parker Laboratories), from which all air bubbles had been expelled, was applied to the thorax to optimize the visibility of the cardiac chambers. Two-dimensional, parasternal long-axis and short-axis views were acquired. Fractional shortening (FS, %) was calculated as FS % = (LVEDD – LVESD)/LVEDD × 100%.

Mouse Cardiac Endothelial Cell Isolation

Hearts were harvested from mice and washed with ice cold saline to remove blood. Rinsed hearts were then minced to 1 mm^3 pieces and placed in 5 mL of collagenase (2 mg/mL) for 30 minutes at 37°C. Using a 30 mL syringe attached to a sterile 14-gauge cannula, the suspension was then fully triturated. The triturated suspension was then passed through a 70 μm cell strainer and then centrifuged at 400g for 8 minutes at 4°C to pellet the cells. Cells were then incubated with anti-CD31-magnetic bead complexes at 4°C for 30 minutes. Magnetic beads (Dyanbeads, Invitrogen, Life Technologies, Grand Island, NY) were previously complexed with anti-CD31 antibodies to form antibody-bead complexes. Then, cells were selected with a magnetic separator, and resuspended in rinse buffer. This sequence was repeated six times to ensure a highly selected population of cells. The selected cells were resuspended in 1 mL RNA-STAT-60 (Tel Test Electronic Labs, Inc., Austin, TX) for further use.

Western Blot Analysis

Expression of Smo from mouse cardiac CD31+ cells was evaluated by SDS PAGE using rabbit polyclonal anti-Smo (Abcam, Cat# ab72130) and secondary antibody goat anti-rabbit IgG-horseradish peroxidase (HRP).

Quantitative Real Time PCR (qRT-PCR)

RNA was isolated from cells with RNA STAT-60 according to manufacturer's instructions. Total RNA was reverse transcribed with a Taqman cDNA synthesis kit (Applied Biosystems, Foster City, CA) and amplification was performed with a Taqman 7500 (Applied Biosystems). The relative expression of each mRNA was calculated by the comparative threshold cycle (C_T) method, normalized to endogenouse18S rRNA expression, and expressed as fold change relative to control. Primer and probe sequences are reported in Table 1.

Immunofluorescence Microscopy

Cardiac tissue sections were prepared as previously described. [19] Endothelial cells were stained with rat anti-CD31 antibodies (BD Pharmingen Inc, San Diego, CA, USA) and the presence of Smoothened was assessed with rabbit polyclonal anti-Smo (Abcam, Cat# ab72130). For immunofluorescence microscopy, primary antibodies were resolved with Alexa-Fluor–conjugated secondary antibodies (Invitrogen Corporation, Carlsbad, CA, USA) and nuclei were counterstained with DAPI (1/5000). Slides were imaged using fluorescent microscopy (Zeiss).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA) were cultured in endothelial complete growth medium-2 (EGM™-2) (Lonza, Basel, Switzerland). NIH 3T3 embryonic fibroblasts (ATCC) were maintained in 4.5 g/L glucose containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/mL streptomycin/penicillin.

Cell Proliferation Assay

Subconfluent HUVECs were seeded on 96-well flat-bottomed plates (1×10^4 /well) containing 100 μL EGM-2 growth medium. Before the treatment, cells were starved overnight with EBM-2 basal media. Cells were incubated with 100 μL DMEM with 0.5% FCS, [20] DMEM with 0.5% FCS plus Shh 1μg/mL (Shh), or conditioned media from NIH3T3 fibroblasts treated with 0.5% FCS and Shh (Shh CM) or without Shh (ctrl CM) for 24 hours. Cell proliferation was measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay [21] (Promega, Madison, WI) according to the instruction of manufacturer.

Migration Assay

Cell migration was conducted in a modified Boyden's chamber (Neuro Probe, Inc., Bethesda, MD, USA). Briefly, a polycarbonate filter (5-μm pore size) (GE Infrastructure Fairfield, CN, USA) was coated with a solution containing 2.5 μg/mL fibronectin (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.15% gelatin (Sigma-Aldrich Co.) and inserted

between the upper and lower wells, then 5×10^4 cells per well were seeded in the upper chamber. The lower chamber contained DMEM with 0.5% FCS as negative control, DMEM with 0.5% FCS plus Shh 1 ug/mL (Curis, Lexington, MA), or conditioned media from NIH3T3 fibroblasts cultured in 0.5% FCS for 24 hours with (Shh CM) or without (ctrl CM) the addition of Shh 1 μg/mL. The chamber was incubated at 5% CO2 at 37°C for 6 hours and then disassembled and the membrane was stained. The number of cells that had migrated to the lower chamber was counted in 3 high-power fields (HPFs, 20x magnification) per well. Migration was reported as the mean number of migrated cells per high-power field and normalized to control.

Statistical Analysis

Data are presented as mean ± standard error. Statistical analysis was performed by using SPSS software (IBM, version 21). Student's t test was performed to compare the means of two groups. One-way ANOVA, followed by the Bonferroni's post-hoc test was used to compare the means of multiple groups. Difference was considered statistically significant at $p < 0.05$.

RESULTS

Characterization of endothelial eSmo^{Null} mice

To prove effective endothelial specific Smo knockout, hearts from eSmo^{Null} and eSmo^{WT} mice were isolated, collagenase digested, and triturated to form a cell suspension. Endothelial cells were isolated using a CD31 antibody with magnetic bead conjugation and column based sorting. mRNA and protein expression of Smo from endothelial cells (CD31+ cell fraction) were evaluated by real time RT-PCR and Western blot, respectively. Both mRNA and protein expression of Smo were significant reduced in endothelial cells from eSmo^{Null} mice (Fig. 1A and B). Furthermore, the distribution of Smo was assessed by immunofluorescence staining. Smo was present in cardiomyocytes, but was abolished in vascular endothelial cells from eSmo ^{Null} hearts (Fig. 1C).

eSmo ^{Null} mice exhibited no observable phenotype from birth to age of one year. Echocardiography was performed at months 1, 4, 8, and 12 to determine whether loss of endothelial Shh signaling resulted in impaired cardiac function, There was no significant difference in fractional shortening (FS) between eSmo^{Null} and eSmo^{WT} mice (Fig. 2).

Endothelial Smo-dependent Hedgehog signaling is not required for ischemic tissue repair

Prior work has demonstrated that Shh is upregulated in the ischemic limb in the HLI model and that blocking Shh with a blocking antibody worsens recovery.[7] Ischemia induced upregulation of endogenous Shh was confirmed in the HLI model in eSmo ^{WT} mice (Fig. 3). Tissue repair after HLI was assessed with Laser Doppler Perfusion Imaging (LDPI), capillary density, Limb motor function and tissue salvage scores (necrosis). Perfusion ratio (ischemic / nonischemic limb), limb motor function, or limb necrosis were not altered in eSmo^{Null} mice compared with eSmo ^{WT} mice (Fig. 4). Furthermore, there was no significant alteration in capillary density in ischemic limb between eSmo^{Null} and eSmo ^{WT} mice at day 28 after HLI (Fig. 5).

Endothelial Smo-dependent Hedgehog signaling is not required for angiogenesis

Since the HLI model, which is known to induce endogenous Shh, did not show any difference in recovery between eSmo^{Null} and eSmo^{WT}, we assessed the response to exogenous Shh signaling using the corneal angiogenesis model. Prior work has shown that Shh is an angiogenic factor and induces angiogenesis in the mouse corneal angiogenesis model. Shh or control pellets were implanted into the corneas of eSmo^{Null} and eSmo^{WT} mice. After 7 days, the degrees of Shh-induced angiogenesis were similar between eSmo^{Null} and eSmo^{WT} mice (Fig. 6).

Conditional media from Shh treated fibroblasts promotes endothelial cell proliferation and migration

Next, we assessed the response of endothelial cell proliferation to Shh. As shown in Fig. 7A, direct Shh had a minimal effect on endothelial cell proliferation. In contrast, conditional media from Shh-stimulated NIH 3T3 fibroblasts had more potent effect on cell proliferation, and this effect was superior to both direct Shh and conditional media from fibroblasts that were not treated with Shh. Next, we assessed endothelial cell migration using a modified Boyden chamber. Once again, conditional media from Shh treated fibroblasts had most potent effect on endothelial cell migration compared with direct Shh and conditional media from untreated fibroblasts. (Fig. 7B).

To determine the possible mechanism involved in the angiogenic effects observed with conditioned media from Shh stimulated fibroblasts, we assessed fibroblast gene expression using RT-PCR. Shh significantly stimulated the gene expressions of VEGF-A, PDGF-B, HGF, and IGF-1 in fibroblasts (Fig. 7C–F). PDGF-B was upregulated by about 8 fold, the highest stimulation amongst the angiogenic growth factors assessed.

DISCUSSION

Given the established role of Shh in angiogenesis, vasculogenesis, and ischemic tissue repair, the current study investigated the role of endothelial Smo-dependent Hedgehog signaling in a tissue-specific genetically modified mouse model utilizing the Cre-Lox system. Tie2-Cre animals were bred with Smo flox/flox mice to generate endothelial Smo knockout mice. Surprisingly, we did not detect a deficient phenotype either at baseline or in mouse models of corneal angiogenesis and hind limb ischemia. Although it has been clearly established that inhibition of global hedgehog signaling with a blocking antibody results in deficient recovery from ischemic injury,[7] the current findings demonstrate that endothelial Smo-dependent Hedgehog signaling is not required for recovery from ischemic injury or Shh induced angiogenesis.

Prior findings have demonstrated direct effects of Shh on endothelial cells via non-canonical hedgehog signaling (Ptc1/Smo-dependent/Gli-independent) *in vitro*.[9,10] Conversely, this study indicates that the greater portion of the Hedgehog signaling effect on angiogenesis *in vivo* is mediated through activation of non-endothelial cells. Although endothelial cells indeed express the Sonic Hedgehog receptor, Ptc-1, and the Hedgehog signal transducer,

Smo, an intact Hedgehog signaling system in endothelial cells does not appear to be required for recovery after ischemic injury or for Shh mediated angiogenesis.

Importantly, our work addresses a central concern of hedgehog signaling and endothelial biology, namely that Shh demonstrates robust *in vivo* effects on angiogenesis and ischemic tissue repair, yet direct Shh on endothelial cells in cell culture demonstrates limited effects. In contrast to fibroblasts and other stromal cells, which are known to respond to Shh via the canonical signaling pathway (i.e. Ptc-1/Smo/Gli), prior publications have shown limited effect on Gli signaling when Shh is applied to endothelial cells in vitro. This has led to the postulation of a non-canonical hedgehog signaling pathway operating in endothelial cells. [10,22,23] Our work demonstrates that, *in vivo*, endothelial Smo is dispensable for ischemic tissue repair and Shh-induced angiogenesis.

Intriguingly, in the original report by Pola et al [6] which demonstrates that Shh is an angiogenic agent, it was noted that Shh treatment resulted in larger and more complex neo-vessels when compared with VEGF. Our finding that PDGF-B is significant upregulated by Shh (Fig. 7D) may explain the previous observations, since PDGF-B regulates pericyte recruitment and pericytes are critically involved in the maturation of neo-vessels.[24–26] Furthermore, prior work has demonstrated that fibroblasts respond to hedgehog ligands in a Smo-dependent fashion and can be inhibited by cyclopamine, a pharmacologic Smo inhibitor.[27] These prior findings also demonstrated that fibroblasts respond to hedgehog ligand via the canonical signaling pathway with activation of Gli transcription factors. Based on these prior findings, it is likely that the gene expression response to Shh demonstrated in NIH3T3 fibroblasts (Figure 7C–F) occurs via canonical hedgehog signaling. Taken together, these prior findings with our current work indicate that stromal cells have robust responses to hedgehog signaling and elaborate a secondary signaling cascade to achieve angiogenesis and ischemic tissue repair.

There are a number of limitations in this study. First, Tie2-Cre transgenic mice are a genetic tool for the analyses of endothelial cell lineage gene targeting, however this promoter is not entirely endothelial specific since others have demonstrated myeloid expression of Tie2.[14,28] Nonetheless, we did not find any impairment of angiogenesis or ischemic tissue repair in the Tie2-Cre Smo flox/flox mice. Therefore, the question of myeloid versus endothelial cell type specificity is less relevant. Regarding the experiments demonstrating the endothelial-specific Smo knockout, there was a small amount of residual Smo expression from isolated primary cardiac endothelial cells using a CD31 antibody (Fig. 1A and 1B). We believe this was mainly residual contamination from other cell types during the isolation procedure. Furthermore, immunofluorescence staining of cardiac sections clearly demonstrated Smo was only present in vessels of wild type mice but not knockout mice (Fig. 1C).

Overall, this study demonstrates that the angiogenic effect of Shh signaling is independent of an intact endothelial Hedgehog signaling system *in vivo*. Instead, the results from conditioned media from fibroblasts support the hypothesis that activation of stromal cells and consequent secretion of angiogenic proteins and growth factors by Shh is sufficient to trigger angiogenesis in endothelial cells. Despite the previously established angiogenic effect of Shh, the mechanism of this effect is not via endothelial Smo-dependent Hedgehog

signaling. Along with other reports,[29] these findings suggest that Shh mediates angiogenesis through Shh signaling in stromal cells, and focus future attention on the stromal compartment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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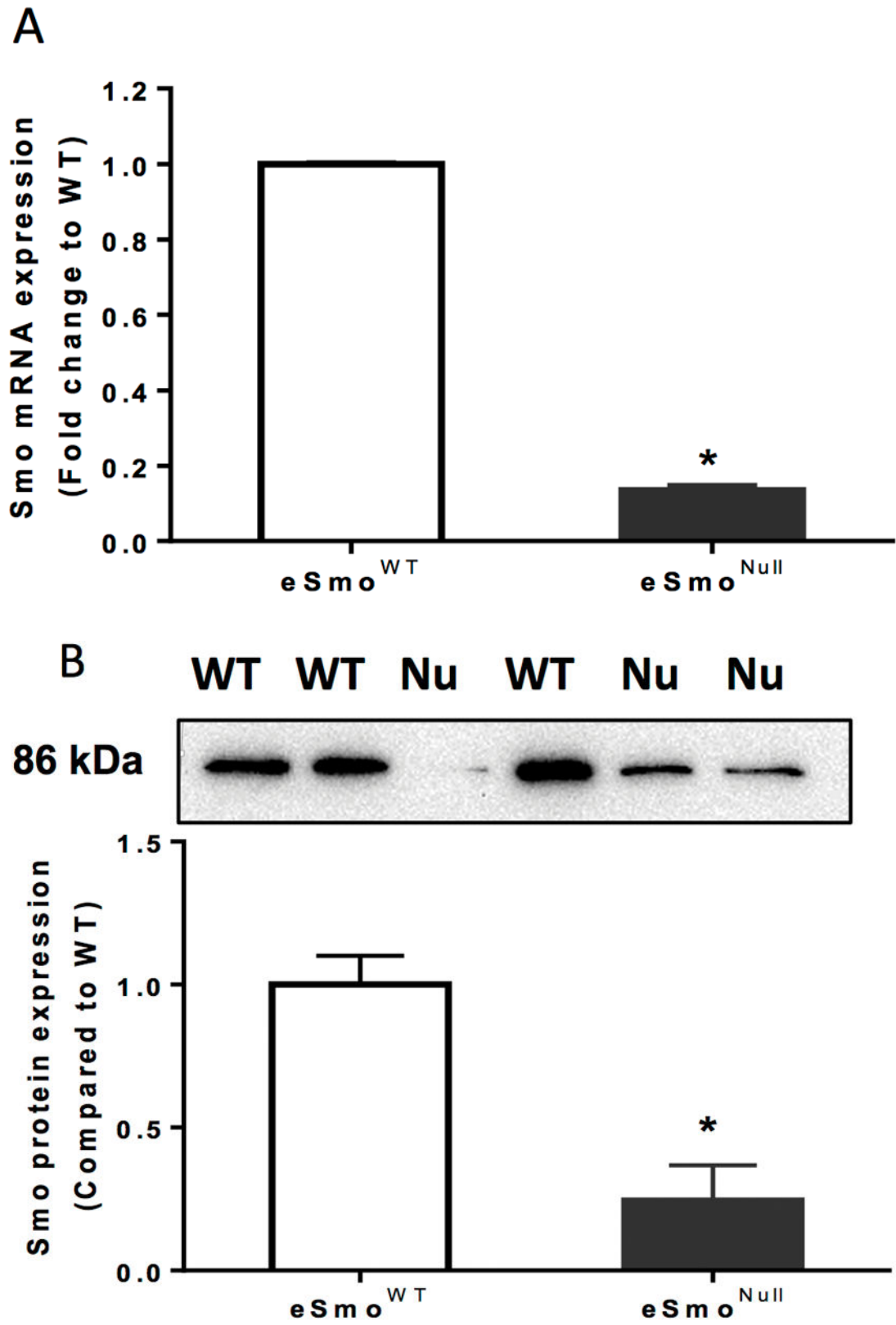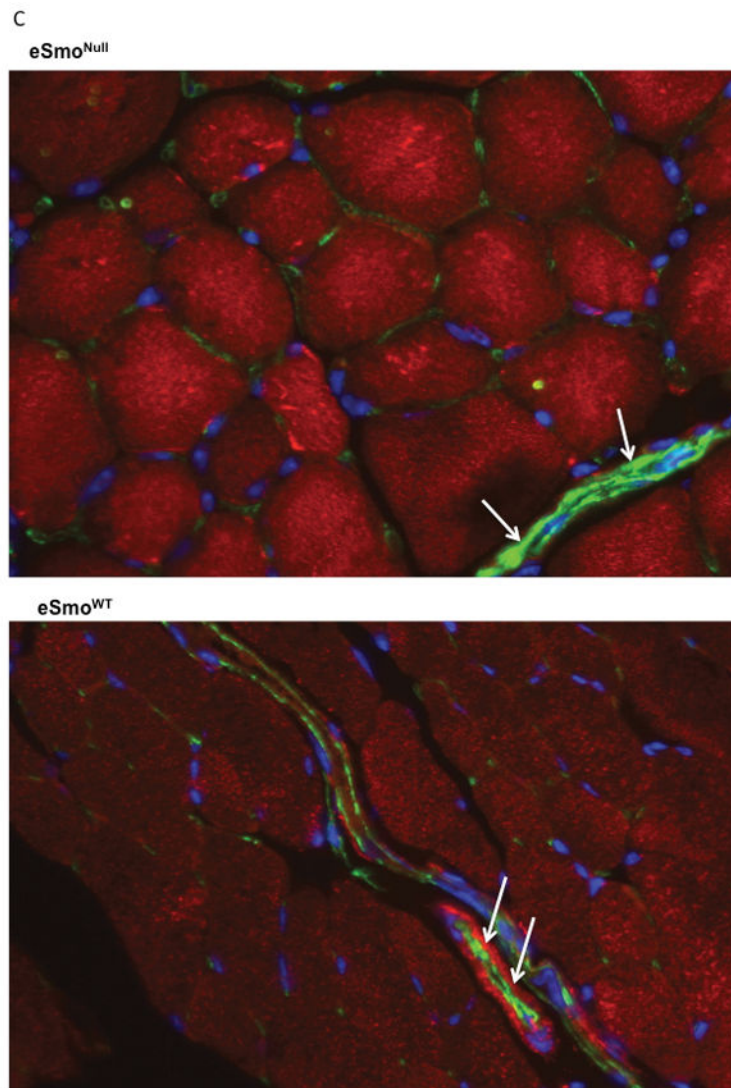
Figure 1. eSmo^{Null} mice exhibit decreased Smo expression in primary endothelial cells

eSmo^{Null} and eSmo^{WT} mice (n=3 per group) were sacrificed and hearts isolated. Hearts were digested with collagenase and CD31+ cells were selected with antibody-magnetic bead complexes. **A.** Smo mRNA expression in primary endothelial cells by RT-PCR. (n=3, *p < 0.01) **B.** Smo protein expression in primary endothelial cells by Western blot. (n=3, *p < 0.01) **C.** Smo protein distribution in heart sections by immunofluorescence staining. Arrows indicate the endothelial cells. Green: CD 31+, Red: Smo, Blue: DAPI (nuclei).
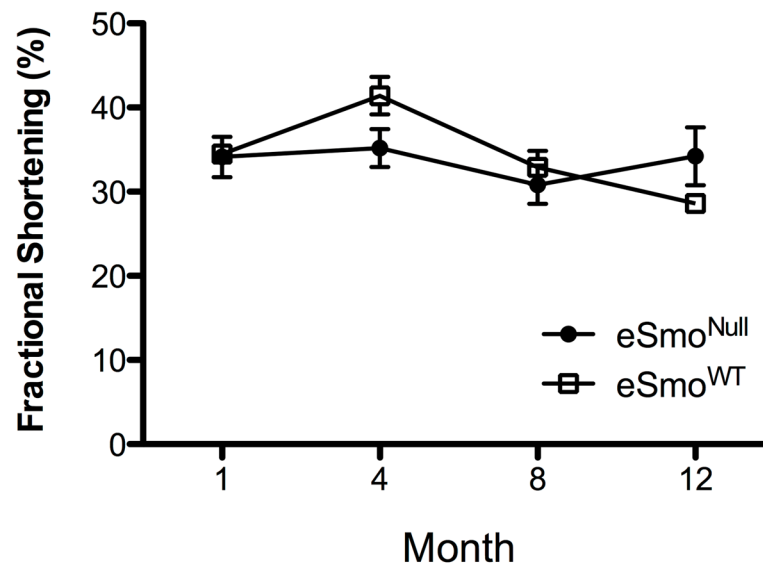
Figure 2. eSmo^Null mice do not show the alteration in cardiac function up to age 12 months

Murine echocardiography was performed at the indicated time points in eSmo^Null and eSmo^WT mice. There was no statistically significant difference in fractional shortening (p >0.05).
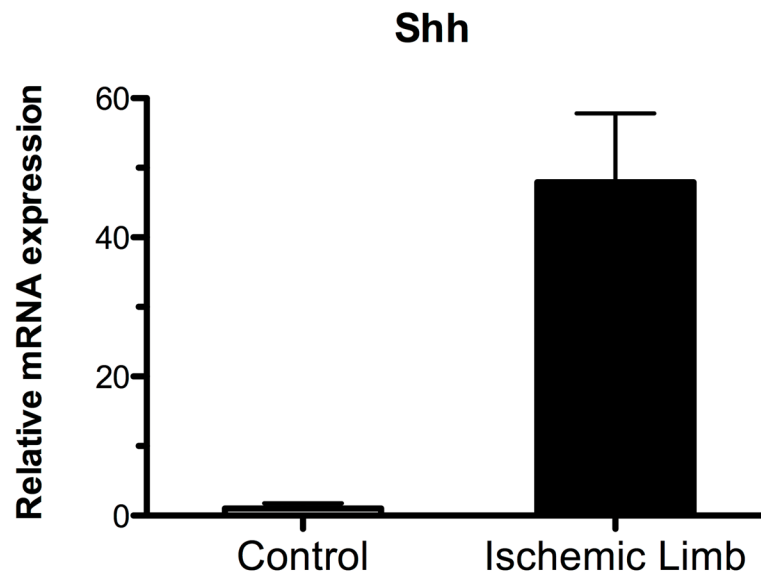
Figure 3. Shh is significantly induced in the Hind Limb Ischemia model
HLI was performed and mice were sacrificed on day 2 after surgery. Tibialis anterior muscle was excised and RNA was isolated. RT-PCR for Shh was performed. Relative mRNA expression is normalized to the control, nonischemic limb. *p<0.05.
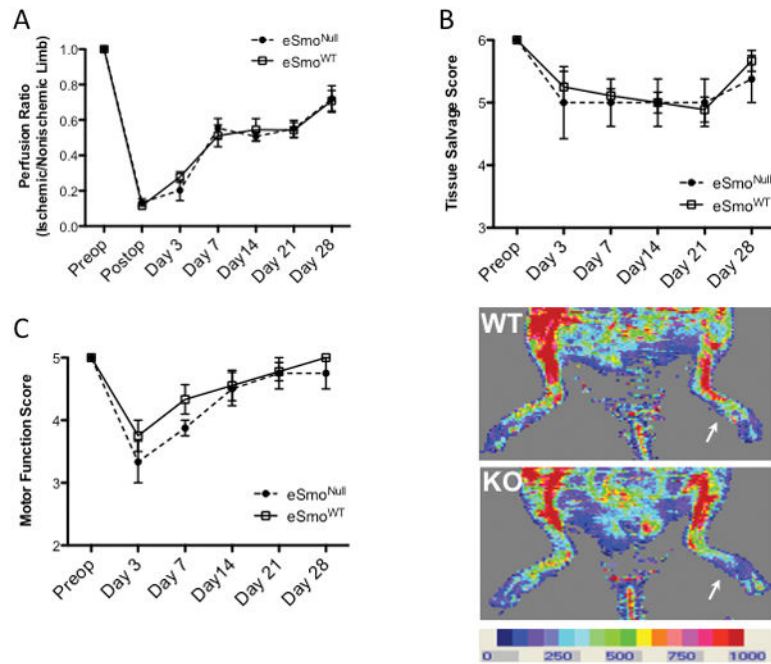
Figure 4. eSmo^Null mice do not demonstrate deficits in the HLI model

Hind Limb Ischemia (HLI) surgery was performed on eSmo^Null and eSmo^WT mice (n = 8–9 per group). (A) Laser Doppler Perfusion Imaging (LDPI); (B) Tissue Salvage Score, and (C) Motor Function Score were assessed at multiple time points. Mice were sacrificed for capillary density measurement at 28 days after HLI. (D) Representative laser doppler perfusion images. Arrows indicate ischemic limb.
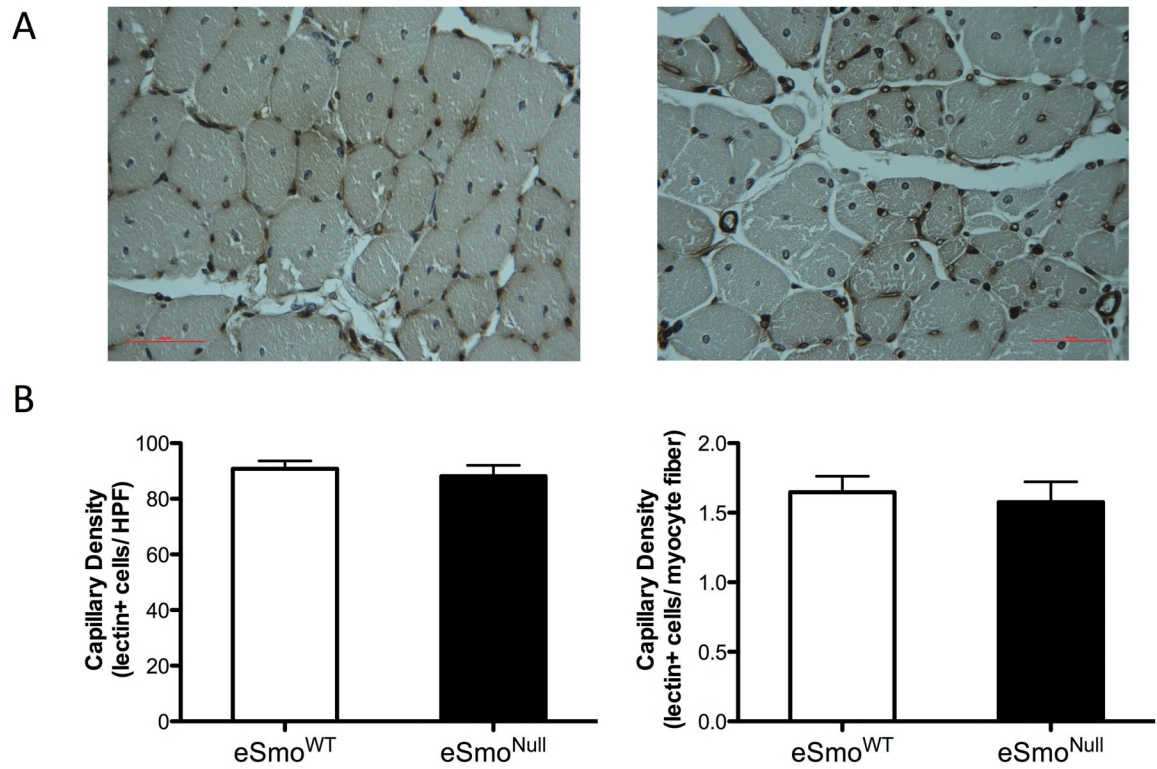
Figure 5. eSmo^Null mice do not show the alteration in capillary density after HLI

Mice were sacrificed for capillary density measurement at 28 days after HLI. (A) Capillaries were identified as Lectin+ cells. Representative images are shown. (B) Capillary density was assessed as Lectin+ cells/HPF and lectin+ cells per myocyte fiber.
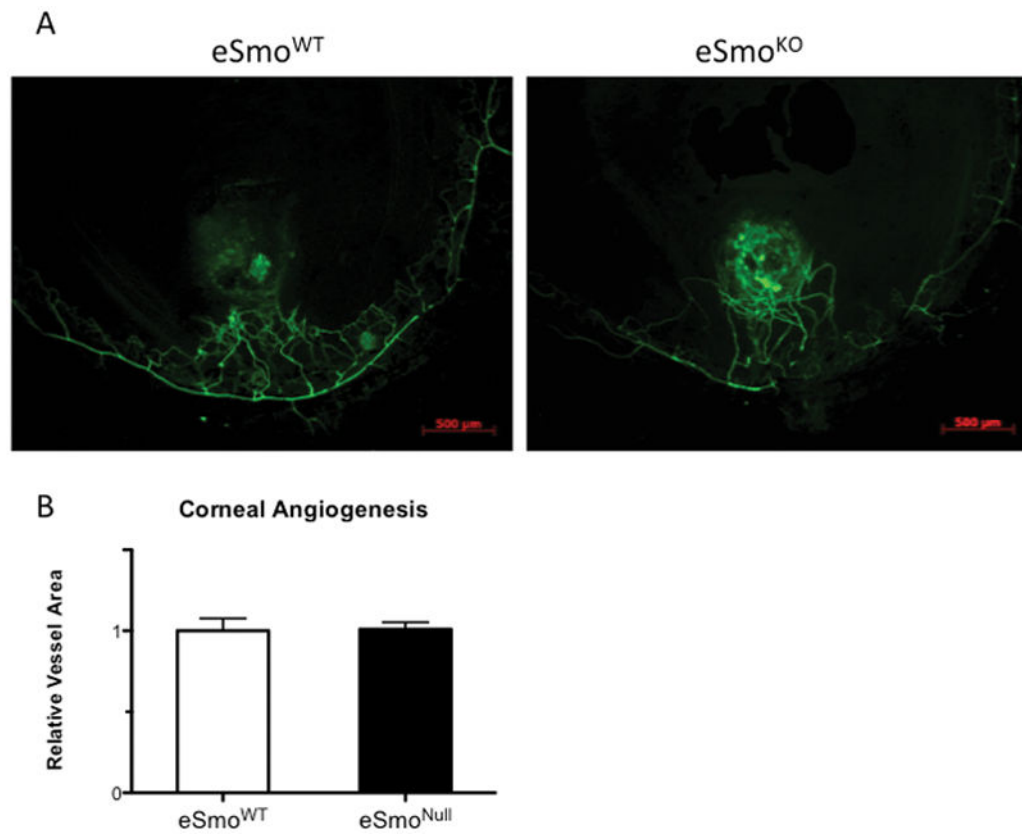
Figure 6. eSmoKO mice do not show the alteration in corneal angiogenesis in response to Shh
(**A**) Corneal pellets containing Shh were implanted in the corneas of eSmo^WT and eSmo^KO mice. Shh-induced angiogenesis was assessed 7 days later by injecting mice with 50 uL fluorescien-BS1-Lectin I 15 minutes prior to euthanasia and then viewing corneas under fluorescence. (**B**) Angiogenesis was quantified as described previously. [16]
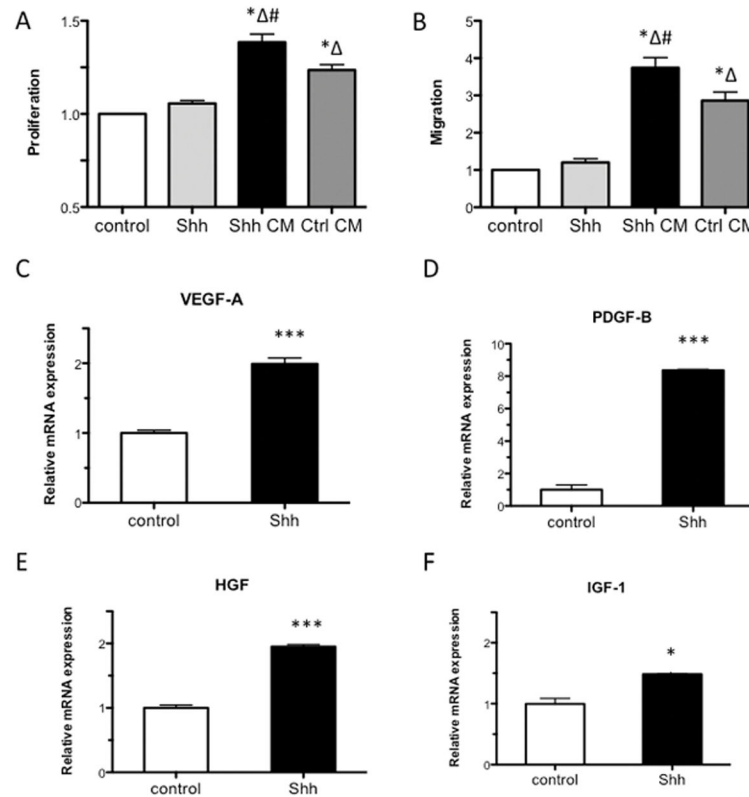
Figure 7. Conditioned Media from Shh treated NIH3T3 fibroblasts (Shh CM) stimulates greater HUVEC proliferation and migration than direct Shh or control conditioned media (Ctrl CM)
Shh conditioned media was created by treating NIH3T3 fibroblasts with Shh 1 ug/mL in DMEM with 0.5% FCS for 24 hrs. Control conditioned media was identical except for the absence of added Shh. **(A)** Serum starved HUVECs were treated for 24 hrs and then HUVEC proliferation was measured with the MTS assay. **(B)** HUVECs were seeded in the upper chamber of a modified Boyden chamber and the bottom chamber was filled with the indicated test conditions. Data are normalized to control. *$p<0.01$ vs. control, $p<0.01$ vs. Shh, #$p<0.05$ vs. Ctrl CM. **(C–F)** NIH3T3 fibroblasts were treated with Shh 1 μg/mL or control media without Shh for 24 hours and then RNA was collected for RT-PCR. Relative mRNA expression is normalized to control. *$p<0.01$ vs. control, ***$p<0.001$ vs. control.

Table 1

Primers and Probes Used for qRT-PCR Analyses

Gene	Primer or Probe	Sequence
18S	Forward primer	5′-CGGGTCGGGAGTGGGT-3′
	Reverse primer	5′-GAAACGGCTACCACATCCAAG-3′
	Probe	5′-Cal Fluor-TTTGCGCGCCTGCTGCCTT-BHQ-3′
PDGF-B (Mouse)	Forward primer	5′-ACCTCGCCTGCAAGTGTGA-3′
	Reverse primer	5′-TGCTCCCTGGATGTCCCA -3′
	Probe	5′-Fam- AGTGACCCCTCGGCCTGTGACTAGAAGTC -BHQ-3′
Shh (mouse)	Forward primer	5′-CAGCGACTTCCTCACCTTCCT-3′
	Reverse primer	5′-AGCGTCTCGATCACGTAGAAGAC-3′
	Probe	5′-Fam-ACCGCGACGAAGGCGCCA-BHQ-3′
HGF (mouse)	Forward primer	5′-CTGACCCAAACATCCGAGTTG-3′
	Reverse primer	5′-TTCCCATTGCCACGATAACAA -3′
	Probe	5′-Fam- TGCTCTCAGATTCCCAAGTGTGACGTGT -BHQ-3′
IGF-1 (mouse)	Forward primer	5′-TGCTTCCGGAGCTGTGATCT -3′
	Reverse primer	5′-CGGGCTGCTTTTGTAGGCT -3′
	Probe	5′-Fam- AGGAGACTGGAGATGTACTGTGCCCCAC -BHQ-3′
Smo (mouse)	Forward primer	5′-TTCTTCGTGGGCAGCATTG-3′
	Reverse primer	5′-CCATCTGCTCGGCAAACAA-3′
	Probe	5′-Fam-CTGGCTGGCCCAGTTCATGGATG-BHQ-3′
VEGFA (mouse)	Forward primer	5′-GCAGGCTGCTGTAACGATGA-3′
	Reverse primer	5′-GCATGATCTGCATGGTGATGTT-3′
	Probe	5′-Fam-CCCTGGAGTGCGTGCCCACG-BHQ-3′