

# Sonic hedgehog mediates a novel pathway of PDGF-BB-dependent vessel maturation

## SupplementalMaterials

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### Detailed Methods

#### **Mice and mouse cornea pocket angiogenesis assay:**

C3HeB/FeJ mice were obtained from Charles River Laboratories and bred in our animal facility. Mice were handled in accordance with the guidelines established by the National Institute of Medical Research (INSERM) and approved by the local Animal Care and Use Committee N° A501303-A. Mouse cornea angiogenesis model was first described by Kenyon B Met al. <sup>1</sup>. Animals (4-5-week-old) were anesthetized by ketamine-xylazine injection. After surgery, during the 24 hour recovery period, mice were medicated with Vetergesic 0.1 mg/Kg, (Reckitt Benckiser Healthcare Ltd), for analgesia. VEGF pellets were prepared by mixing 5 µg of recombinant VEGF protein diluted in 10 µL sterile PBS with 2.5 mg sucrose octasulfate-aluminium complex (Sigma-Aldrich Co.), in the presence or absence of 20 nmol cyclopamine or imatinib. Next, 6 µL of 12% hydron in ethanol was added and the final suspension was deposited on a 400 µm nylon mesh (Sefar America Inc., Depew NY, USA). Finally, both sides of the mesh were covered with a thin layer of 12% hydron and allowed to dry. Pellets were implanted in the corneas of C3HeB/FeJ mice as previously described<sup>1</sup>. After mice were euthanized by carbon dioxide intoxication, corneas were harvested 3, 5 or 28 days after pellet implantation for immunohistological or PCR analysis.

#### **Cells and cell cultures:**

Rat aortic SMCs (Lonza) were maintained in DMEM:F12 (1:1) medium supplemented with 20% fetal bovine serum, 365.1 mg/mL L-glutamine, 3.151 g/L glucose, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.1 mmol/L HEPES. Cells from passages 2 to 12 were used for all experiments. Quiescent SMCs were obtained by a 24 hour culture in DMEM:F12 (1:1) medium without fetal bovine serum.

Human pericytes were from Promocell and were cultured in the medium proposed by the provider. EC were then made quiescent by 24 hour incubation in pericyte medium supplemented with low concentration of growth factor cocktail (1/10).

### **Migration assays:**

Cell migration was evaluated using the Transwell migration assay with BD Falcon™ inserts, which allows cells to migrate through an 8 µm pore polycarbonate membrane. Briefly, cells were seeded in 6 cm dishes and incubated in serum-free DMEM:F12 medium for 24 hours. After trypsin-EDTA treatment,  $6.5 \times 10^4$  cells were seeded in the insert chamber, placed in 24 well culture plate containing serum-free DMEM:F12 and assayed factors (PDGF-BB(10 ng/mL), Shh(1µg/ml) or vehicle (BSA 0.01%)) were added with or without various inhibitors at appropriate concentrations, for 6 hours. At the end of the experiment, cells remaining on the upper surface of the insert membrane were removed with a cotton swab. Cells under the membrane were fixed with methanol and stained with hemalun. Migration was evaluated as the mean number of migrated cells in 9 fields (10x magnification) per well. Each condition was assayed in triplicate, and each experiment was performed at least three times.

For wound healing assay, SMCs were seeded at 60 000 cells/cm<sup>2</sup> and cultured until confluence is reached. Wounds (2scratches) were performed with a yellow automatic pipette tip. Cells were washed with serum free medium and recombinant Shh (1µg/mL) or vehicle (0.01% BSA) were added. If indicated, inhibitors were also added in the medium at this time. The size of scratches was measured using Sigma Scan Pro software just after wounding and 72 hours later on images captured with a Nikon DXM1200F digital camera.

For random motility assay, SMCs were seeded at 10 000 cells/cm<sup>2</sup> in a glass slide chamber and kept in serum free medium overnight. The slide was install on a time lapse Zeiss microscope at 37°C and recombinant Shh (1µg/mL) or vehicle (1% BSA) were added. Pictures were captured every 15 minutes during 15 hours. More than 60 cells per condition were tracked (Axio Vision software). Data represent means±SD of relative values versus Ctrl from 3 independent experiments performed in triplicate (NS: p>0.05, \*: p<0,05, \*\*: p<0.005 and \*\*\*: p<0.0005).

### **siRNA transfection and adenovirus transduction :**

The Shh-siRNA and control siRNA (Ref: SR-CL000-005, Eurogentec) were purchased from Eurogentec and were constructed as following: siA (5'GGGAAGAUCUCCAGAAACU-3'), siB (5'-GGUGUAAGGACAAGUUGAA-3') and siC (5'-GAACUCCGAGCGAUUUAA-3'). Cells were transfected using Interferin (Polyplustransfection™) according to the manufacturer's instructions. Cells ( $2 \times 10^5$ ) were seeded in 6 well plates in 2 mL medium and transfected for 4 hours with Shh-siRNA (final concentration 30 nmol/L), Interferin (4 µL) and Opti-MEMR serum reduced media (200 µL, GIBCO). Efficacy and specificity of siRNA was verified by determining Shh expression using real-time RT-qPCR in transfected cells. Migration assays were performed 48 hours after transfection of quiescent cells.

Adenovirus encoding for an inactive form of PI3Kγ (KRPI3Kγ) and control adenovirus were from M Laffargue's lab<sup>2</sup>. Cells were transduced at 50 MOI overnight then washed twice with serum free medium. Fresh medium was added for 24 hours before using cells.

**Reverse Transcriptase-Real Time Polymerase Chain Reaction (RT-qPCR):**

Total RNA was extracted from cultured SMCs or mouse corneas, according to the TriReagent manufacturer's instructions (MRC). One  $\mu\text{g}$  of total RNA was converted into cDNA using M-MLV Reverse Transcriptase (Invitrogen). The resulting cDNA was subjected to polymerase chain reaction (PCR) analysis according to the Taq DNA polymerase manufacturer's instructions (Promega) in a final volume of 20  $\mu\text{L}$ . For quantitative PCR, each reaction included cDNA (1  $\mu\text{L}$ ), a mixture of specific forward and reverse primers (1.5  $\mu\text{L}$ , 225  $\mu\text{mol/L}$  final concentration each), IQTM SYBER Green Supermix (7.5  $\mu\text{L}$ , BIO-RAD) in a final volume of 15  $\mu\text{L}$ . Primers were obtained from Eurogentec: human pdgf-b (forward: 5'-TTAAGAAGGCCACGGTGACG-3'; reverse: 5'-TCCAAGGGTCTCCTTCAGTG-3'), rat Shh (forward: 5'-TGGTGGCCCTTGCTTCCTCG-3'; reverse: 5'-CCCTTCATATCGGCCGCTGG-3'), rat Ihh (forward: 5'-CGTGCCGGAGAAGACCCTG-3'; reverse: 5'-CTGGTTCATGACAGAGATGGC-3'), rat Dhh (forward: 5'-AGGATGAGGAGAAGACGCGGC-3' ; reverse: 5'-GGTCAGACGTGGTGATGTCC-3'), and rat  $\beta$ -actin (forward: 5'-CTTCTTGCAGCTCCTCCGTC-3' ; reverse: 5'-CCTTCTGACCCATACCCACC-3'), mouse pdgf-b (forward: 5'-TCTTCTTCTCTCTGCTGCTACC-3'; reverse: 5'-CCCCATCTTCATCTACGGAGTCTC-3'), mouse Shh (forward: 5'-GTCGAGCAGTGGACATCACC-3'; reverse: 5'-GAAACAGCCCGGATTTGG-3'), mouse Ihh (forward: 5'-TGCAAGTCTGAGCATTCCG-3'; reverse: 5'-GTCTCGATGACCTGGAAAGC-3') and mouse Dhh (forward: 5'-CTTGGACATCACCACGTCTG-3'; reverse: 5'-ATGTAGTTCCTCAGCCCCT-3'). Hh signaling elements were detected by classic PCR using the following primers: rat Ptch-1 (forward: 5'-AAGGCTACTGGCCGAAAGC-3'; reverse: 5'-CTTCTCTATCTTCTGGCGGG-3'), rat Smo (forward: 5'-GCAGTTCCTCGCTGCCTC-3'; reverse: 5'-AGCCTCATTAGGTTAGTGCG-3'), rat Gli1 (forward: 5'-CAGCTCAAAGCTCAGCTCCT-3'; reverse: 5'-ATGCAAAGCCAGATCCAAAC-3'), rat Gli2 (forward: 5'-CACACCCGCAACACCAAGC-3'; reverse: 5'-GAGAAGTATGGGGAGATGCC-3') and rat Gli3 (forward: 5'-ATCATGGACTCGACCATTTCC-3'; reverse: 5'-GACTGTGTGCCATTTCTATG-3'). Absolute quantification of cDNA copy number was achieved using plasmid DNA standards in which the corresponding PCR amplification product was cloned. The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to  $\beta$ -actin mRNA expression.

**Human angiogenesis antibody array:**

HUVEC were seeded and cultured in serum-reduced medium for 24 hours. Cells were then treated with VEGF (20 ng/mL) for 24 hours or left untreated. Conditioned medium was collected and centrifuged to remove the cell debris at 3 000g. Fifty-five factors secreted by EC were detected by Proteome Profiler Human Angiogenesis Array Kit (R&D Systems) according to supplier's instructions. Non-conditioned medium was used as negative control.

**Immunological techniques:**

For Enzyme-linked immunosorbent assay (ELISA) SMC conditioned culture media was collected and supplemented with protease inhibitors (10  $\mu\text{g/mL}$  aprotinin, 10  $\mu\text{g/mL}$  leupeptin and pepstatin 10  $\mu\text{g/mL}$ ). Cell debris was removed by centrifugation at 3000 g. The concentration of Shh protein was measured using the ELISA Kit "Mouse Sonic Hedgehog N-Terminus Quantikine" (R & D Systems) as recommended by the supplier. This kit recognizes both mouse and rat Shh proteins.

For immunocytochemistry, cells were fixed with 2% paraformaldehyde for 10 minutes at 4°C. After saturation with horse serum (10% in PBS), immunostaining was performed with mouse anti-Hh antibody 5E1 (Developmental Studies Hybridoma Bank) and secondary antibody Alexa-488 (Invitrogen). An assay without primary antibody was used as negative control. Nuclei were stained with DAPI (Vector Laboratories).

For cornea preparations, C3HeB/FeJ mice were sacrificed and the eyes were enucleated, fixed with 2% paraformaldehyde for 10 minutes at 4°C and dissected under a dissecting microscope. After saturation with BSA (1%) and TritonX40 (0.5%), corneas were incubated with first antibodies at 37°C overnight. Corneas were then incubated with the appropriate secondary antibodies coupled with different fluorochromes (Alexa-488nm and Alexa-568nm) for an hour. Nuclei were stained with DAPI.

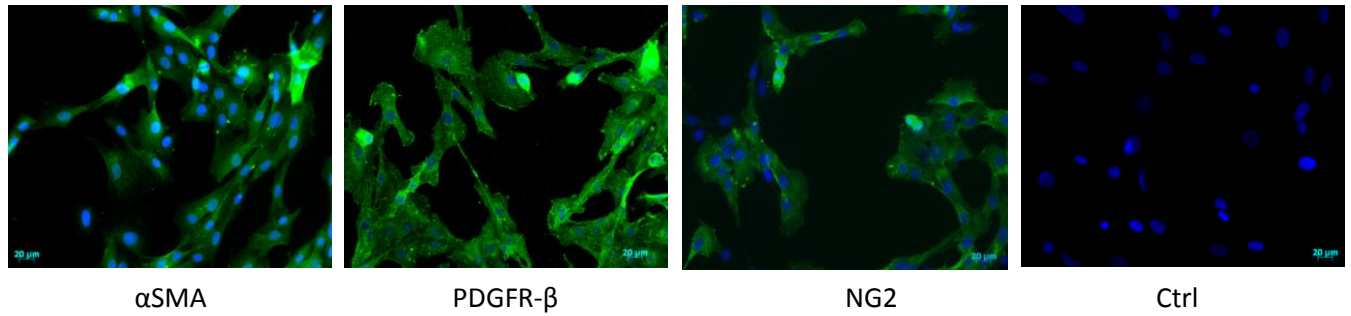
Antibodies are from: anti-PDGF-BB antibody (Santa Cruz.), anti-C terminal Shh antibody (R&D systems). anti-CD31 antibody (BMA), anti-NG2 antibody (Millipore), anti  $\alpha$ SMA antibody (Sigma), anti PDGFR- $\beta$  antibody (R&D system).

For Western blot: SMCs ( $2.5 \times 10^5$ ) were seeded in 6-well plates, and cultured in serum-free medium for 24 hours. After stimulation with appropriate factors, cells were lysed with 200  $\mu$ L Leamli solution, containing 50 mmol/L Tris-HCl (PH: 6.8), 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol and 0.002% bromophenol blue. Phosphorylation of Akt and mitogen-activated protein kinase (MAPK) ERK1/2 was evaluated with anti-Akt, anti-phospho-Akt (Ser473), anti-ERK1/2 and anti-phospho-ERK1/2 (Tyr202/Tyr204) antibodies (Cell Signaling Technology). Equal loading was verified by immunodetection of  $\alpha$ -tubulin with anti- $\alpha$ -tubulin antibody (Sigma). Primary antibodies were then incubated with secondary Alexa-700 nm and Alexa-750 nm coupled antibodies (Invitrogen) and Alexa-800 nm coupled antibodies (LI-COR) and resolved with an infrared system (OdysseyR, LI-COR).

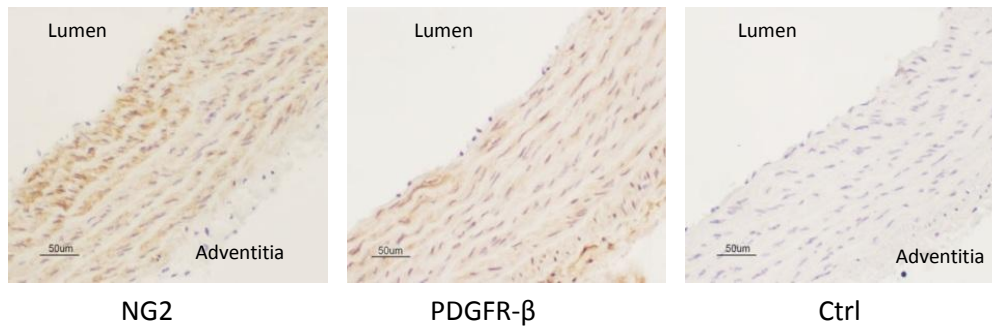
## **References**

1. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci.* 1996;37(8):1625-1632.
2. Fougerat A, Smirnova N, Gayral S, Malet N, Hirsch E, Wymann M, Perret B, Martinez L, Douillon M, Laffargue M. Determinant role of Phosphoinositide 3-kinase gamma in MCP-1-mediated amplification of PDGF-induced aortic smooth muscle cell migration. *Br J Pharmacol.* 2012;166:1643-1653.

## A. Rat aortic SMC

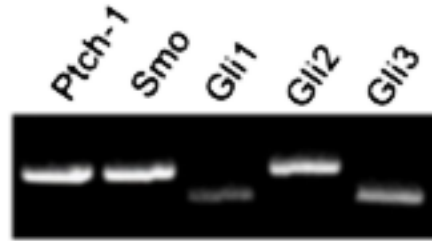


## B. Rat aorta



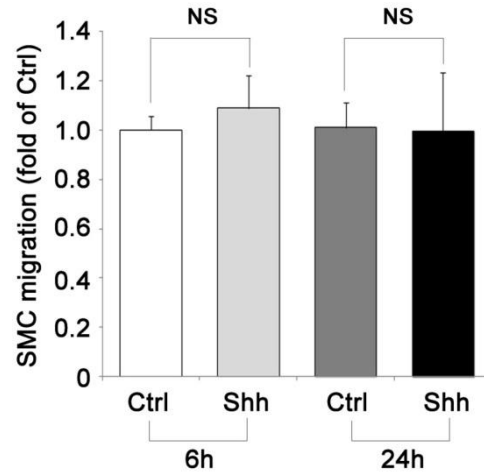
### Figure S1: Rat aortic smooth muscle cell phenotype

(A) Commercial rat aortic SMCs (Lonza) were seeded on labteck and cultured for 24 hours. Cells were fixed in methanol and SMA, PDGFR- $\beta$  and NG2 were detected by immunocytochemistry using specific antibodies and Alexa 488 coupled 2<sup>nd</sup> antibodies. Control (Ctrl) was performed by omitting primary antibody. (B) Rat aorta were harvested from 12 weeks old mice, fixed in formalin and embedded in paraffin. NG2 and PDGFR- $\beta$  were detected by immunohistochemistry using specific antibodies and peroxidase complex coupled 2<sup>nd</sup> antibodies. Control (Ctrl) was performed by omitting primary antibody.



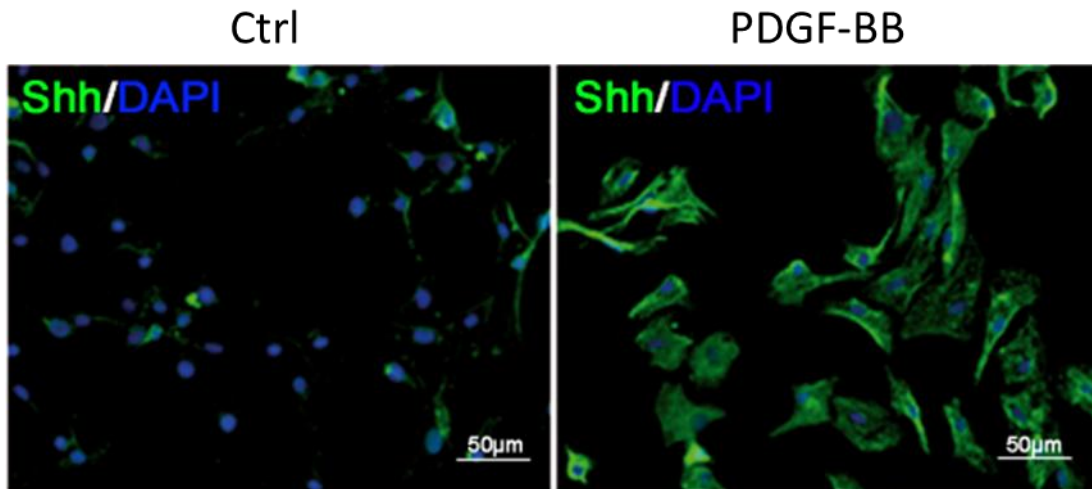
**Figure S2: SMC expressed primary elements of Hh signaling pathway**

SMCs were cultured in 6 well plates and made quiescent by 24 hour culture in serum free medium. Expression of Hh pathway elements was analyzed by classic end point RT-PCR. The amplification products were loaded on to agarose gel and images were captured with a DMK72AUC02 camera. This image is representative of 3 independent experiments.

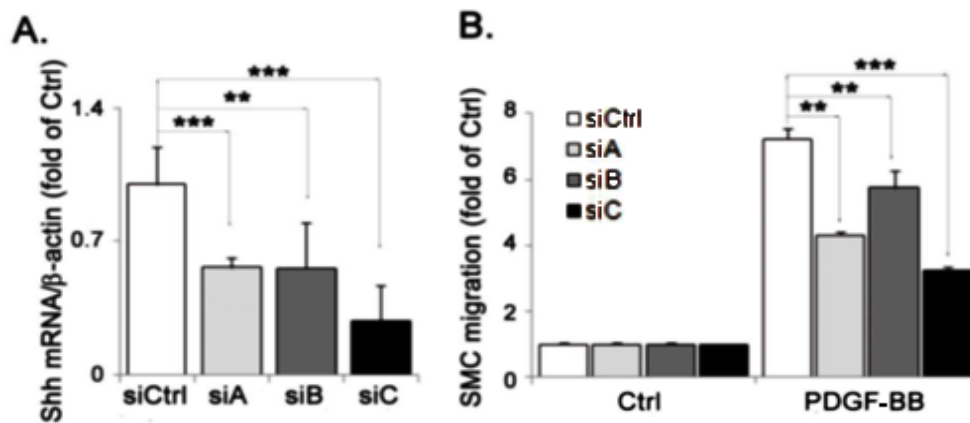


**Figure S3: Shh is not chemotactic for SMCs**

SMC migration was assessed in Transwell chemotactic assay BD Falcon™ inserts in response to Shh (1 µg/mL) during 6 or 24 hours. Data represent means±SD of relative values versus Ctrl from 3 independent experiments performed in triplicate (NS:non significant).



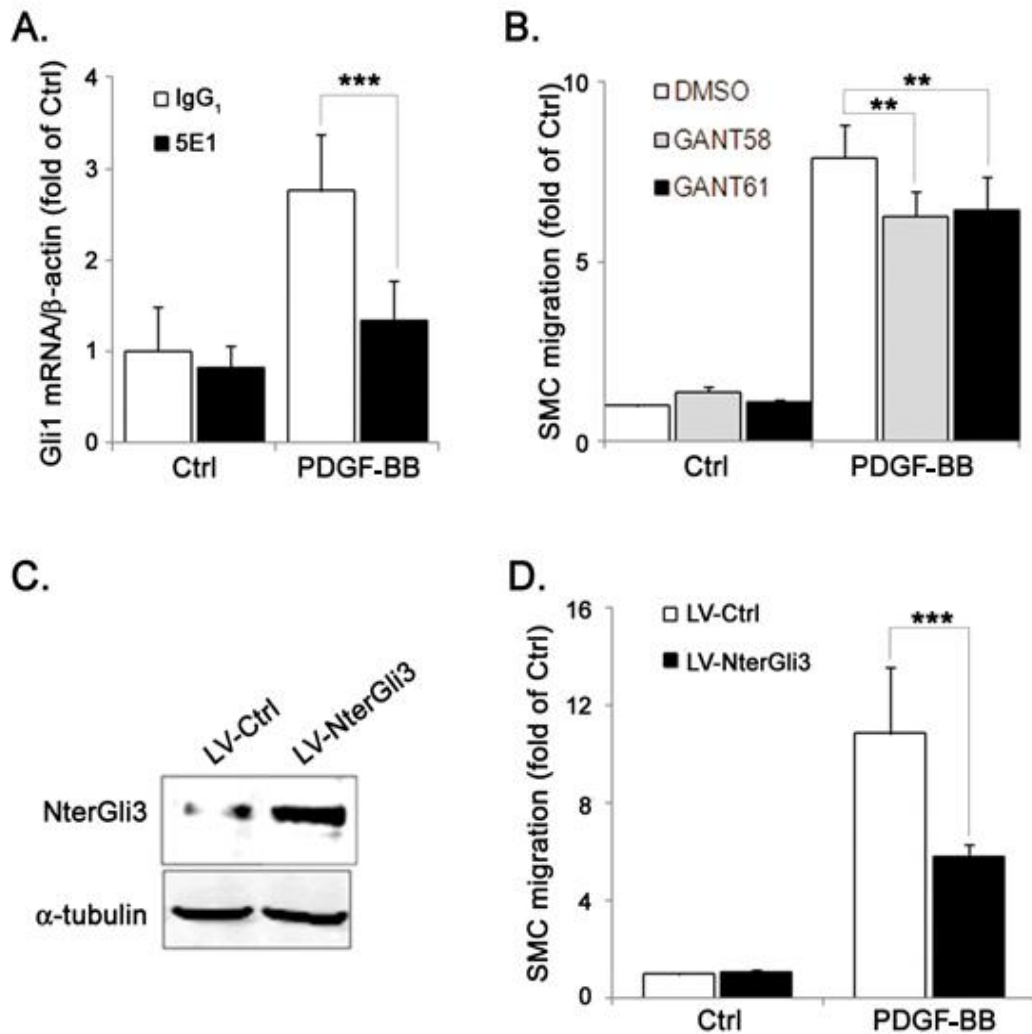
**Figure S4: PDGF-BB-stimulates Shh expression in SMC** Quiescent SMC were treated with 10 ng/mL PDGF-BB for 16 hours or maintained in serum free medium. Cellular expression of Shh in unstimulated and 16 hour-PDGF-BB-stimulated SMC was detected by immunocytochemistry using the anti-Hh antibody 5E1. Nuclei were stain with DAPI.



**Figure S5: Shh mRNA inhibition by different siRNA reduces PDGF-BB-stimulated SMC migration**

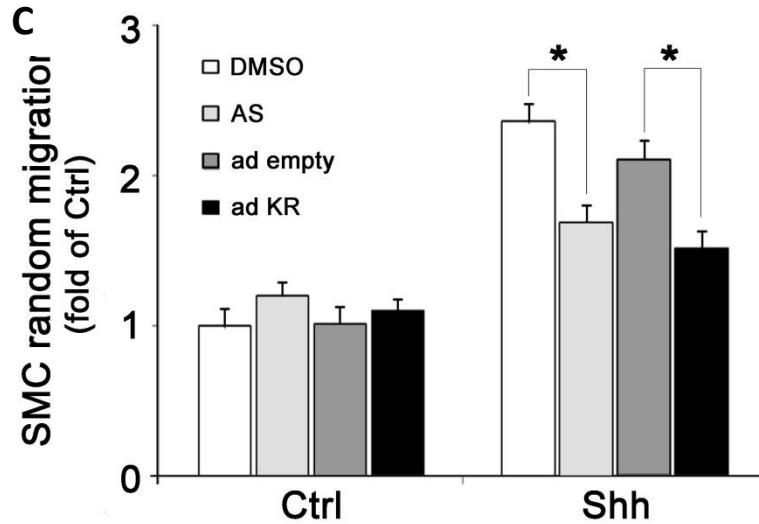
**(A)** Efficiency of 3 different siRNA directed against Shh (siA, siB, siC) were evaluated on Shh mRNA expression by RT-qPCR in quiescent SMCs stimulated by PDGF-BB (10 ng/mL). **(B)** Effect of 3 different Shh-siRNA on control (Ctrl) and PDGF-BB (10 ng/mL)-stimulated SMC migration. Migration assays were performed as described in Materials and Methods. Data represent means  $\pm$  SD of relative values versus Ctrl from 3 independent experiments performed in triplicate (\*\*:  $p < 0.005$  and \*\*\*:  $p < 0.0005$ ).





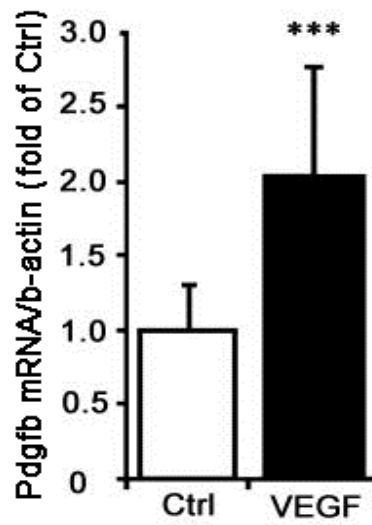
**Figure S6: PDGF-BB-stimulated SMC migration required Hh- Gli1 canonical pathway**

**(A)** Gli1 mRNA expression was evaluated in control (Ctrl) and PDGF-BB-stimulated SMCs by RT-qPCR in presence or not of the Hh blocking antibody, 5E1 (1.5 μg/mL). **(B)** PDGF-BB (10 ng/mL)-stimulated and non-stimulated (Ctrl) SMC migration was assessed in the presence of the Gli1-inhibitors, Gant58, Gant61 (5 μmol/L) or vehicle (DMSO) (1 μmol/L). **(C)** SMCs were transduced by the lentivirus carrying NterGli3 (LV-NterGli3) or its empty control (LV-Ctrl) (MOI=50) and the Gli3 protein expression was assessed by western blot. **(D)** PDGF-BB (10 ng/mL)-stimulated and non-stimulated (Ctrl) SMC migration was assessed in lentivirus (LV-Ctrl or LV-NterGli3) transduced SMCs. Data represent means ± SD of relative values versus Ctrl from 3 independent experiments performed in triplicate (\*\*: p<0.005 and \*\*\*: p<0.0005).



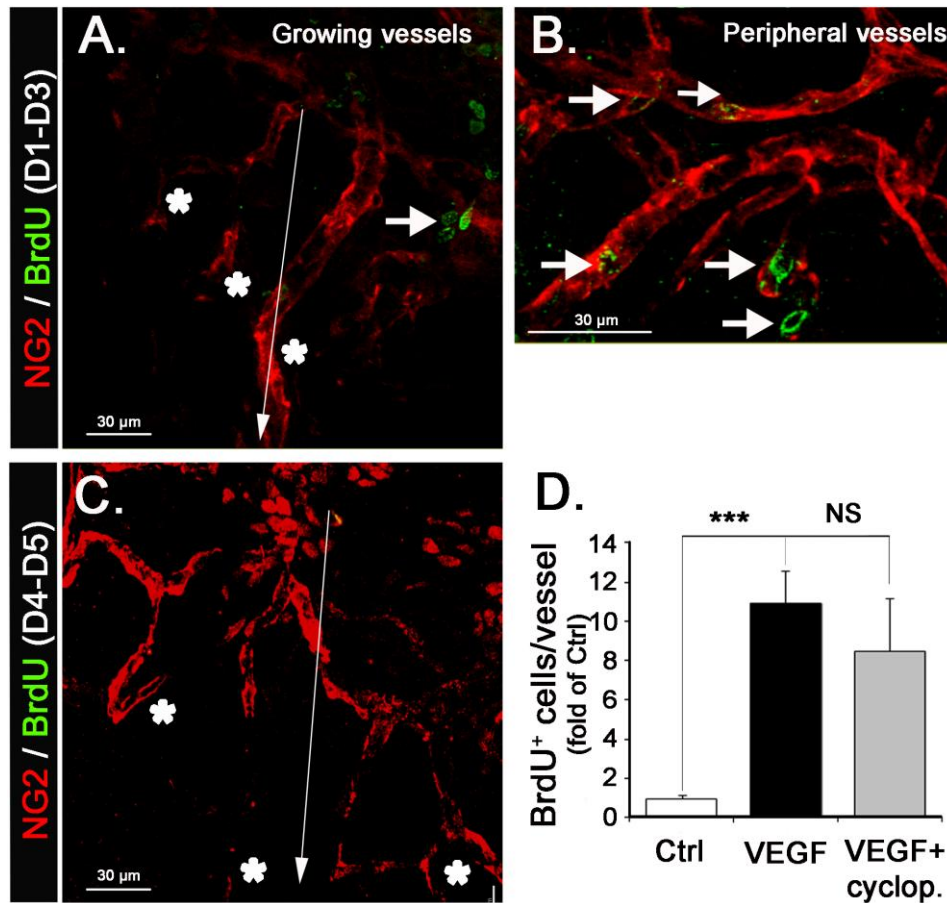
**Figure S7: PI3K $\gamma$  is required for Shh-mediated AKT phosphorylation**

SMC were transduced by the dominant negative PI3K $\gamma$  adenovirus (Ad-KRPI3K $\gamma$ ) or by a control adenovirus). After a 24 hour incubation in serum free medium, quiescent transduced SMC were stimulated for 15 min with PDGF-BB (10 ng/mL) or Shh (1  $\mu$ g/mL). A) Proteins were extracted and processed by western blot before Akt and phosphorylated Akt (p-Akt) detection. B) Histogram represents the means of the ratio of the fluorescence intensity measured by the Licor system, of 3 different experiments. The mean ratio obtained for Ad-Ctrl was taken as 1. C) SMC were proceeded in motility tests for 15 hours for random motility test with or without stimulation by recombinant Shh (1 $\mu$ g/mL) and in presence or absence of PI3K $\gamma$  inhibitor (100 nmol/L) (AS) or after cell transduction with AD-KRPI3K $\gamma$  (ad KR) or control empty adenovirus (ad empty). Data represent means $\pm$ SD from 3 independent experiments performed in triplicate (\*\*\*: p<0.0005, \*\*: p<0.005 and \*: p<0.05).



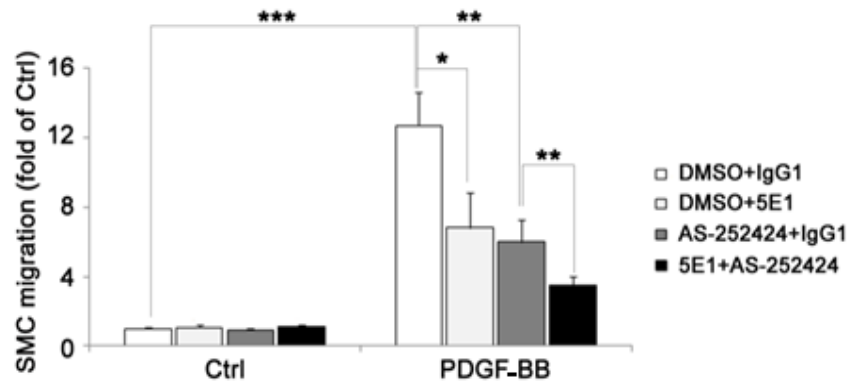
**Figure S8: Pdgfb expression in cornea following VEGF pellet implantation**

VEGF (5  $\mu$ g) containing pellets were implanted into the cornea of C3HeB/FeJ mice (n=8 per group). Five days later, the mRNA levels of Pdgfb were evaluated, in Ctrl and VEGF-treated corneas, by RT-qPCR;  $\beta$ -actin was used as reference of expression of an housekeeping gene.



**Figure S9: Cyclopamine did not reduce early mural cell proliferation in newly growing vessels**

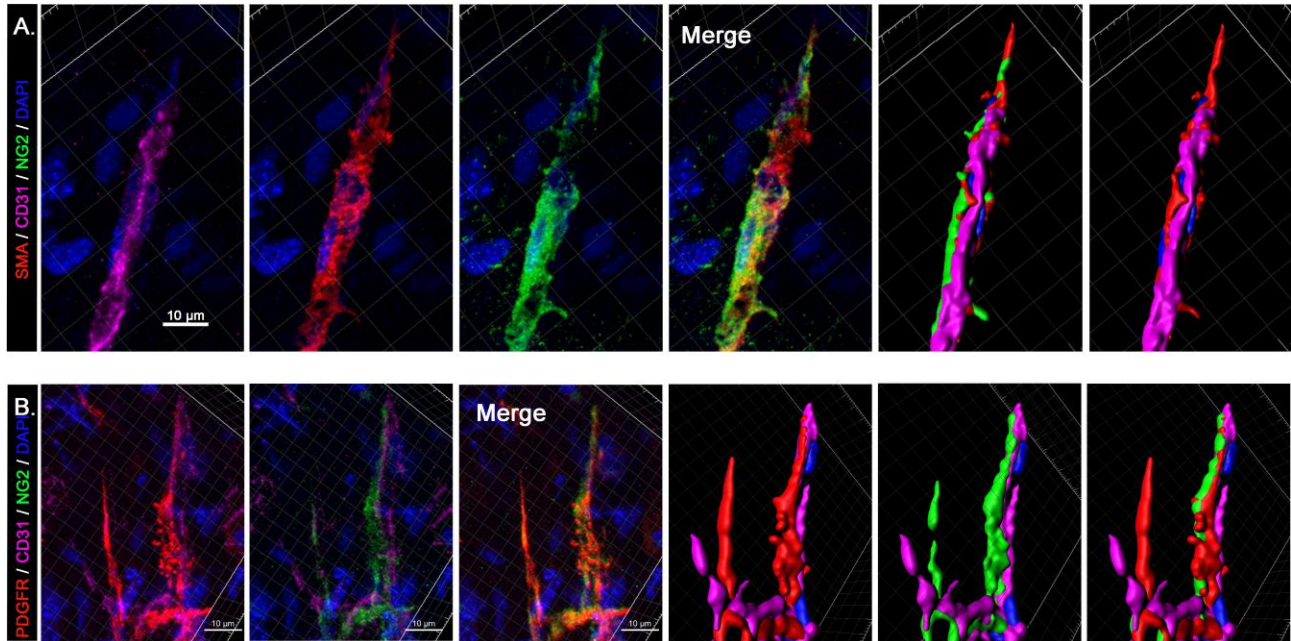
Mouse corneas were implanted with VEGF containing pellet. (*A and B*) Corneas were harvested at day 3. IP injections of BrdU were performed at day 1 and 2. (*C*) Corneas were harvested at day 5. IP injections of BrdU were performed at day 4. Mural cells were stained with anti-NG2 (red) and anti-BrdU (green) antibodies. \* indicates vessel tips. The arrow shows the direction of the pellet. (*A and C*) growing vessels and (*B*) preexisting limbal vessels. \* indicates vessel tips. The arrow shows the direction of the pellet. (*D*). Mouse corneas were implanted with VEGF or VEGF + cyclopamine containing pellet. Corneas were harvested at day 5. IP injections of BrdU were performed at day 1, 2, 3 and 4. BrdU<sup>+</sup> NG2<sup>+</sup> mural cells of limbal vessels were counted under microscope (Zeiss, objective 40).



**Figure S10: Common and complementary role of Hh and PI3K $\gamma$  effects in PDGF-mediated SMC migration**

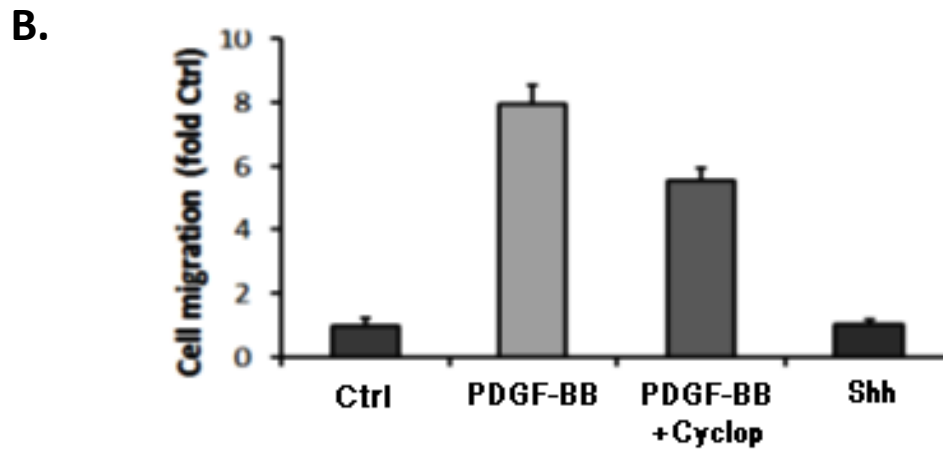
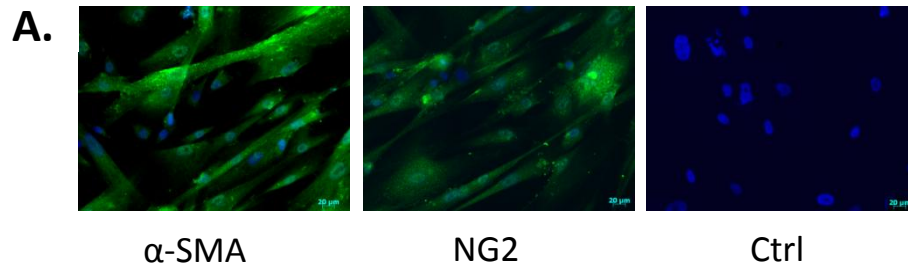
Quiescent SMC were submitted to Transwell chemotactic test. PDGF-BB (10 ng/mL) was added in the lower compartment. Inhibitors 5E1 (1.5  $\mu$ g/mL, anti-Hh antibody) or AS-252424 (100 nmol/L, PI3K $\gamma$  selective inhibitor) or their control (isotypic immunoglobulin IgG1 or DMSO) were added in both compartments. Ctrl indicate the same experiment without addition of PDGF.

Data represent means $\pm$ SD of relative values versus Ctrl from 3 independent experiments performed in triplicate (\*: p<0.05, \*\*: p<0.005 and \*\*\*: p<0.0005).



**Figure S11: Characterization of the cells of the tip of growing vessels.**

VEGF (5  $\mu\text{g}$ ) containing pellets were implanted into the cornea of C3HeB/FeJ mice (n=4 per group). Five day later corneas were collected and (A) Smooth muscle alpha actin (red), EC (CD31, pink), NG2 (green) or (B) PDGFR (red), EC (CD31, pink), NG2 (green) expression was revealed by immunohistology and observed by confocal microscopy. Nuclei were counterstained using DAPI (blue). The two (A) and three (B) right panels show isosurface analysis by ImarisBitplane software.



**Figure S12: Phenotypic and migration properties of human pericytes**

**(A)** Human pericytes were cultured in specific medium spread at 60000 cells/cm<sup>2</sup>. 24 hour after spreading, cells were methanol fixed and processed for immunostaining with anti- $\alpha$ -SMA, PDGFR- $\beta$  or NG2 antibodies or without antibodies and revealed by a second fluorescent antibody complex. **(B)** Quiescent human pericytes (60 000 cells) were spread in different Transwells and migration was assessed 6 hour later. As indicated in the lower compartment were added PDGF-BB (10 ng/ml) (PDGF), Cyclopamine (1  $\mu$ mol/L) (Cyclop), Shh (1 $\mu$ g/mL). Data represent means $\pm$ SD of relative values versus Ctrl from 2 independent experiments performed in triplicate \*\*: p<0.005.