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Sonic Hedgehog Is An Autocrine Viability Factor for Myofibroblastic Hepatic Stellate Cells

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Abstract

Background/Aims—Factors released during liver injury, such as platelet derived growth factor-BB (PDGF) promote accumulation of myofibroblastic hepatic stellate cells (MFB) that drive the pathogenesis of cirrhosis. The Hedgehog (Hh) pathway regulates remodeling of other injured tissues. This study evaluates the hypothesis that autocrine production of Sonic hedgehog (Shh) promotes MFB growth.

Methods—Primary rat HSC were treated without or with PDGF, a pharmacologic inhibitor of PDGF-regulated kinases, adenovirus expressing activated or dominant negative AKT, or Hh signaling inhibitors. Shh production, expression of Hh inhibitors and target genes, and HSC growth were assessed.

Results—HSC expressed Shh, Hh pathway components, and the Hh inhibitor, Hip. During culture Hip expression fell, Shh production increased, and Hh target gene expression was induced. Neutralizing Shh antibodies promoted apoptosis. Adding PDGF increased Shh expression and MFB growth. Both processes followed activation of AKT and were abrogated by AKT inhibitors. Adenoviral delivery of activated AKT up-regulated Shh expression, demonstrating a direct role for AKT in regulating Shh expression. Shh-neutralizing antibodies and other Hh pathway inhibitors blocked the mitogenic effects of PDGF.

Conclusion—These results identify Shh as an autocrine growth factor for MFB and suggest a role for Hh signaling in the pathogenesis of cirrhosis.

Keywords

hepatic stellate cells; Sonic hedgehog; AKT; PDGF

Introduction

Liver injury is often accompanied by transformation of HSC (HSC) to myofibroblastic cells (MFB) that produce copious matrix [1]. Because fibrosis is the hallmark of cirrhosis, MFB are believed to play a central role in the pathogenesis of this condition [1,2]. Cirrhotic livers also exhibit varying degrees of architectural distortion related to changes in hepatic sinusoidal

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structure, parenchymal nodularity, and accumulation of hepatic progenitors. These structural alterations are generally accompanied by some degree of organ dysfunction [3–5]. Cancers of bile ductular cells and hepatocytes also occur most often in the context of cirrhosis [6].

These observations prompted us to hypothesize that MFB might produce Hedgehog (Hh) ligands. Hh ligands are pleiotropic morphogens that mediate mesenchymal-epithelial interactions that regulate the development of many organs [7]. There are three known Hh family ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Hh-target cells express the Hh-receptor, Patched, a transmembrane-spanning cell surface receptor. In the absence of Hh ligands, Patched tonically inhibits Smoothened (Smo) and Hh signaling is silenced. However, interaction of Hh ligands with Patched liberates Smoothened signaling activity, leading to a cascade that ultimately activates members of the Gli transcription factor family (Gli1, Gli2, Gli3) to regulate the transcription of Hh target genes, including cell cycle regulators and anti-apoptotic factors [8–11]. Interestingly, Gli factors regulate the transcription of Ptch, Gli1 and Gli2, permitting the pathway to auto-regulate its activity [12].

Recent evidence demonstrates that the Hh pathway becomes re-activated in many adult organs during injury, helping to orchestrate wound healing responses [13,14]. For example, Shh promotes vasculogenesis [15,16], cardiac repair [17], neural and skin regeneration [18–21], bone remodeling [22], and differentiation of hematopoietic progenitors [23] in adults. Although Hh ligands are not known to regulate liver development during embryogenesis [24], Hh pathway activity has been demonstrated in some cholangiocarcinomas [25] and hepatocellular carcinomas [26–28]. We reported that clonal HSC lines derived from cirrhotic rat liver express mRNA for Hh ligands [29]. However, the significance of this finding is uncertain. The purpose of the present study was to determine if primary HSC produce biologically active Hh ligands and to characterize the mechanisms involved.

Materials and Methods

Cell Isolation and culture

Liver cells were isolated from normal rats [30,31]. Briefly, after *in situ* perfusion of the liver with 20 mg pronase (Boehringer Mannheim, Indianapolis, IN) followed by collagenase (Crescent Chemical, Hauppauge, NY), dispersed cell suspensions were layered on a discontinuous density gradient of 8.2% and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY). The resulting upper layer consisted of > 95% stellate cells. The viability of all cells was verified by phase contrast microscopy as well as the ability to exclude propidium iodide. The viability of all cells utilized for culture was >95%. Isolated stellate cells were seeded at a density of 3×10^2 cells/mm² with DMEM supplemented with 10% fetal bovine serum, 100 units/mL streptomycin and 100 units/mL penicillin.

Adenoviral Transduction of HSCs

Ad5GFP, which contains the GFP gene driven by the cytomegalovirus promoter, was used as a control virus. The Ad5myrAkt and Ad5dnAkt viruses express activated and dominant-negative forms of Akt, respectively [32,33]. Seven days after isolation, cultured HSCs were infected with Ad5dnAkt or Ad5GFP at a multiplicity of infection (m.o.i.) of 100 for 12 h in Dulbecco's modified Eagle's medium containing 0.2% FBS. After 12 h, medium was changed to fresh medium containing 0.2% FBS, and cells were incubated for an additional 24 h before other experiments were done.

Immunoblot

Cells were homogenized in Dignam C buffer [34] containing protease and phosphatase inhibitors [35]. After protein concentrations were determined, lysates were separated by SDS-

PAGE and then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Equal loading was confirmed by staining with Ponceau S. Membranes were incubated with primary antibodies to anti-Shh (1:250 dilution, Santa Cruz, Santa Cruz, CA), smooth muscle α -actin or β -actin (both 1:1000 dilution, Sigma, St. Louis, MO), and pAKT (Thr 308), pAKT (Ser 473), or total AKT (all 1:1000 dilution, Cell Signaling Technology, Danvers, MA). Secondary antibody (horseradish peroxidase – conjugated anti-mouse IgG from Amersham, UK, 1:1000 dilution) was added and antibody complexes were detected with Amersham ECL system. Specific signals were quantitated by scanning densitometry.

mRNA Quantification by Real-Time RT-PCR

mRNAs were quantified by real-time RT-PCR per the manufacturer's specifications (Stratagene, Mx3000P™ Real-Time PCR). The sequences of primers for 18S, Shh, Ptc, Smo, Gli2, and Hip are as in Table 1.

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), reverse-transcribed using T15-oligonucleotide and Superscript RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). Amplification reactions were performed using a SYBR Green PCR Master Mix (Applied Biosystems). cDNA samples (1 to 5 dilution) were used for quantitative two-step PCR as described[36]. Each sample was analyzed in triplicate.

Bromodeoxyuridine Proliferation Assay

After 7 days in culture, HSC were re-seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to 0.2% FBS for 24 hours; cells were treated with LY294002 or DMSO, anti-Shh neutralizing antibody or control IgG, and then incubated in medium containing human PDGF-BB (20 ng/ml) (Roche, Indianapolis, IN) for 48 h with BrdU present during the last 24 h. BrdU incorporation was assessed as per the manufacturer's protocol (Amersham, Little Chalfont, England). Assays were performed in triplicate and experiments repeated three times.

Caspases 3/7 activity

Apoptotic activity was assayed in parallel using the Apo-ONE Homogeneous Caspase 3/7 Apoptosis Assay (Promega, Madison, WI), according to the vendor's instructions[29]. A FLUOstar OPTIMA microplate reader (BMG Labtech; Durham, NC) was used for all absorbance, luminescence and fluorescence measurements.

Immunofluorescence

Hepatic stellate cells were isolated, placed on glass-bottomed plastic culture dishes and allowed to undergo activation as described above. For detection of Shh, the cells were fixed in 50% acetone/methanol, washed, and exposed to anti-Shh antibody (1:1,000) (Santa Cruz, Santa Cruz, CA) in PBS. After washing, primary antibody was detected with Cy3- (Molecular Probes)-conjugated anti-donkey antibody. After washing and mounting, signals were visualized with a Zeiss LSM 510 META confocal microscope. A solution of bovine serum albumin/PBS at 3% and a non-immune donkey IgG were employed as a negative control.

Transient Transfection and Reporter Gene Assay

To determine the bioactivity of Shh in HSC culture medium, NIH 3T3 cells in 12-well plates (70,000 cells/well) were transiently transfected with a 9x-Gli-BS-Luciferase reporter construct that contained nine Gli-binding sites upstream of a luciferase reporter driven by a thymidine kinase promoter (provided by W. Chen, Duke U[29]), using Eugene 6 (Roche, Indianapolis, IN). HSC conditioned medium were added to transfected cells 24 later. At 48 h post-transfection, the luciferase assay system (BD Pharmingen, San Diego, CA) was used to measure Gli1

transcriptional induction according to the manufacturer's protocol. All measurements of luciferase activity (relative light units) were normalized to internal control renilla luciferase to obtain relative luciferase units (RLU).

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Significance was established using the student's t-test and analysis of variance. Differences were considered significant when $p < 0.05$.

Results

Primary rat HSC produce biologically active Shh

To evaluate the effect of HSC activation on induction of Shh expression, we isolated primary HSC from healthy adult rats and plated them on plastic dishes. As expected [37], there was gradual spontaneous activation of the cells during the first week of culture, characterized by loss of lipid droplets, upregulation of alpha smooth muscle actin (α -sma) and acquisition of a myofibroblastic phenotype (Fig 1A–B). Western blot analysis of HSC lysates obtained on days 0, 4, and 7 of culture demonstrated progressive accumulation of both full length (FL) Shh and the N-terminal biologically active fragment of Shh (Fig 1A). The expression of Shh protein was further verified by immunohistochemistry of 7 day HSC cultures (Fig 1B). To determine if culture-activated HSC also released biologically-active Shh, HSC conditioned medium was added to NIH 3T3 cells that had been transfected with a Gli-luciferase reporter construct. Compared to conditioned medium from freshly plated HSC (control), conditioned medium from 7 and 10-day HSC cultures activated reporter gene activity (Fig 1C). Thus, spontaneous, culture-related activation of primary rat HSC increases expression and release of biologically active Shh protein.

Hh activity increases during HSC culture and Shh functions as an autocrine viability factor for primary HSC

To determine the biological significance of these changes in Shh production, we examined the effects of HSC culture on Hh pathway activity. Shh activates Hh signaling by engaging Patched (Ptc), the cell surface receptor for Hh ligands. This interaction de-represses Ptc inhibition of the co-receptor, Smo, and leads to transcription of Hh target genes, such as Gli2[38,39]. HSC cultures were evaluated for expression of Ptc, Smo and Gli2 by QRT-PCR. The cells expressed both the Hh receptor (Fig 2A) and co-receptor (Fig 2B), and culture induced Hh target gene expression (Fig 2C), demonstrating that increases in Shh ligand were accompanied by increased Hh pathway activity. Culture also decreased expression of Hh-interacting protein (Hip) (Fig 2D). Hip inhibits Hh signaling by Shh and prevents Shh from engaging Ptc[40]. Thus, culture promotes two mechanisms for Hh pathway activation in primary HSC, i.e., induction of Hh ligand production and repression of Hh inhibitor expression.

In many cells that express Shh, Shh functions as a viability factor by inhibiting caspase activation [41–43]. Therefore, to further address the functional implications of Hh activity in primary HSC, we treated cultures with neutralizing antibodies to Shh and assessed caspase 3/7 activity. Neutralizing endogenous Shh activity significantly increased caspase 3/7 activity in culture-activated HSC (Fig 2), demonstrating that Shh functions as an autocrine viability factor for these cells.

The HSC growth factor, PDGF-BB, induces HSC expression of Shh

During various types of chronic liver injury, factors released by damaged liver, such as platelet derived growth factor (PDGF)-BB, promote the accumulation of myofibroblastic HSC [1, 44]. Therefore, additional experiments were done to determine whether or not PDGF

influenced HSC production of Shh. PDGF-BB (20 ng/ml) was added to primary cultures of rat HSC and cells were harvested at several time points to obtain RNA for Q-RT PCR analysis of Shh mRNA expression. PDGF-BB rapidly induced Shh mRNA expression, with levels of Shh mRNA peaking at about three times basal values within three hours and remaining elevated for the duration of the nine hour experiment (Fig 3A). To evaluate the effect of PDGF-BB on Shh protein levels, experiments were repeated and cultures were harvested 16 hours after treatment with vehicle (control) or PDGF-BB. Western blot analysis demonstrated increased Shh protein content in HSC that were treated with PDGF-BB (Fig 3B).

PDGF-BB induction of Shh involves AKT-dependent mechanisms

PDGF-BB-activation of AKT mediates PDGF-BB's trophic actions [32,45,46]. Therefore, subsequent studies addressed the effect of AKT activation on HSC expression of Shh. As expected [32,45,46], PDGF-BB elicited rapid phosphorylation of AKT on Thr308 and Ser473, with peak AKT activation occurring within the initial hour after PDGF-BB addition (Fig 4A). The ensuing accumulation of Shh protein was inhibited by addition of either the PI3 kinase inhibitor, Ly294002, which inhibits PDGF-BB activation of AKT [45] (Fig 4B) or adenovirally-mediated delivery of dominant negative AKT (Ad5dnAkt) (Fig 4C). The effects of Ad5dnAkt were specific because treatment with a control adenoviral vector (Ad5GFP) did not inhibit PDGF induction of Shh. Further evidence for AKT-regulated events in induction of Shh expression was demonstrated by treating cultured cells with adenoviral vectors bearing constitutively active AKT. Compared to HSC treated with control adenoviral vector (Ad5GFP), HSC treated with Ad5myrAkt expressed more Shh protein (Fig 4D). Hence, PDGF-BB induces Shh expression in HSC via AKT-dependent mechanisms.

Shh mediates the mitogenic actions of PDGF-BB in HSC

These findings prompted us to evaluate the role of Shh as a possible mediator of the trophic effects of PDGF-BB. HSC proliferation was compared in HSC cultures that were treated with PDGF-BB in the absence or presence of Hedgehog (Hh) pathway inhibitors. In initial experiments, the effects of pharmacologic inhibitors of Hh signaling, cyclopamine and AY9944 [47] were compared to those of the known PI3K/PDGF antagonist, LY294002 (Fig 5A). LY294002 completely abolished PDGF-BB-mediated induction of BrdU incorporation, repressing proliferative activity to below basal levels. Both Hh pathway inhibitors also significantly inhibited the mitogenic actions of PDGF-BB. Cyclopamine decreased PDGF-BB stimulated BrdU incorporation by more than 50%, and AY9944 completely prevented PDGF-BB from increasing HSC BrdU incorporation. However, at the doses tested, neither agent was as effective as LY294002 at blocking HSC proliferation. Subsequent experiments compared BrdU incorporation in HSC cultures treated with PDGF-BB plus nonimmune Ig or neutralizing antibodies to Shh. Antibodies to Shh also significantly inhibited the mitogenic effects of PDGF-BB. Similar to cyclopamine-treated HSC (Fig 5A), HSC treated with anti-Shh antibodies exhibited less than half the induction of BrdU incorporation that occurred in control cultures following treatment with PDGF-BB (Fig 5B). These results identify Shh as an important signaling intermediary for PDGF-BB's trophic actions on HSC.

Discussion

The results of the present study show that HSC isolated from healthy adult rats express Shh and Hh pathway components, as well as the Hh inhibitor, Hip. During "spontaneous" activation to MFB during standard culture, production of Shh increases, expression of Hip falls, and biologically active Shh protein accumulates in the medium. This is accompanied by increased HSC expression of the Hh target gene, Gli2. Shh functions as a morphogen during embryogenesis [7], and promotes remodeling after injury in various adult tissues [13,17,19, 21]. Thus, evidence that MFB derived from adult rat primary HSC produce Shh has potentially

important implications for remodeling of damaged livers. Indeed, the fact that MFB produce a factor that promotes vasculogenesis [15], hematopoiesis [23], and progenitor cell survival [14,41,42] calls into question the wisdom of therapeutic attempts to induce apoptosis in MFB as a strategy for reducing liver fibrosis [48–52]. In other tissues, wound healing is impaired when Shh is inhibited, and accelerates when supplemental Shh is administered [17,21,41,53–56].

This study also demonstrates that PDGF-BB, a potent inducer of HSC transformation to MFB [1,44], doubles expression of Shh mRNA within an hour and subsequently increases expression of Shh protein. Induction of Shh is dependent on PI3K and activation of AKT because the process is inhibited by a pharmacologic inhibitor of PI3K, as well as by adenovirus-mediated delivery of dominant negative AKT. Moreover, it is likely that other factors that activate AKT also up-regulate HSC expression of Shh because adenoviral delivery of active AKT increases Shh expression in the absence of exogenous PDGF. This concept is supported by evidence that epidermal growth factor (EGF) activates AKT-dependent mechanisms that induce Shh expression in some cells [57]. We reported that EGF also increases Shh mRNA levels in primary HSC [58]. Evidence that two different growth factors that activate AKT in HSC induce HSC expression of Shh is intriguing because insulin like growth factor (IGF)1 cooperates with Shh to activate AKT-dependent post-translational mechanisms that stabilize Gli2 in Hh-responsive C3H101/2 cells [59]. Thus, there is growing evidence that Hh signaling modulates growth factor actions (and vice versa) in Hh responsive cells. In such cells, growth factors appear to induce Hh signaling by at least two mechanisms (i.e., increasing Hh ligand expression and prolonging the half life of Hh pathway transcriptional activators). This redundancy suggests that conservation of Hh activity is important for growth factor function in certain cell types.

This concept is supported by evidence that Shh functions as an autocrine viability factor for cultured MFB. Adding Shh neutralizing antibodies to primary rat HSC increased their inherent caspase activity. These findings extend and confirm data derived from earlier studies of clonal HSC lines, in which Shh antibodies increased cellular apoptosis and recombinant Shh stimulated growth [29]. The present study also demonstrates that proliferative actions of PDGF-BB, a major HSC mitogen [45,46], are mediated via the Hh pathway because three distinct approaches that abrogate Hh signaling each significantly inhibited PDGF-related increases in HSC incorporation of BrdU. Both agents that specifically antagonize Hh signaling by preventing activation of discrete Hh pathway signaling components (Anti-Shh antibodies prevent Shh from activating Ptc, and cyclopamine prevents Ptc from activating Smo) [60], exerted comparable effects on HSC DNA synthesis, reducing the stimulatory actions of PDGF-BB by about 50%. LY294002 and AY9944 had greater inhibitory effects on PDGF mitogenicity. Both of the latter agents also influence other signaling mechanisms. LY294002 inhibits PI3K, thereby effecting activation of multiple PI3K targets, including AKT [61]. AY9944 inhibits oxysterol metabolism and is thought to abrogate Hh signaling by interfering with the cholesterol biosynthetic pathway [47]. Therefore, this drug also has potential “off-target” actions that might influence the cellular response to PDGF-BB. Nevertheless, the novel evidence that AY9944 inhibits HSC growth raises the intriguing possibility that statins, which have also been reported to inhibit HSC activation [62], might also function by down-regulating Hh signaling.

In summary, the present study demonstrates that the pleiotrophic morphogen, Shh, is produced by MFB from adult livers. MFB play a major role in liver fibrogenesis [1,52]. The discovery that MFB also produce Hh family ligands that have been shown to regulate vasculogenesis [15], hematopoiesis [63], lymphoid cell differentiation [64], and hepatic progenitor viability [65] has broad implications. From a fundamental perspective, this finding suggests novel mechanisms by which expansion of MFB populations might modulate hepatic blood flow, as

well as hepatic epithelial cell growth and differentiation. The latter have potential diagnostic and therapeutic relevance for patients with various types of chronic liver injury.

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Figure 1. Shh protein expression increased in rat HSC during culture-induced activation
 (A) Stellate cells were isolated and placed in culture. Shh and smooth muscle α -actin were detected by immunoblotting; β -actin was detected in the same membrane to control for protein loading. This immunoblot is representative of 3 other independent experiments. (B) Culture-activated HSC were stained with anti-Shh antibody and Cy-3-conjugated secondary antibody 7 days after isolation. Identical results were obtained in analysis of three other separate HSC preparations. (C) Culture medium was pooled from 3 plates on each of the following days of culture (0, 7 and 10), then added to NIH 3T3 cells which were transfected with Gil-Luciferase reporter constructs. Shh activity was measured by luciferase assay. Results are the mean (SE) of triplicate assays (* $p < 0.05$ vs day 0 (control)).

Figure 2. HSC express Hh pathway components, increase expression of Hh target genes during culture and utilize Shh as a viability factor

Stellate cells were isolated and placed in culture. RNA was obtained from the freshly plated cells (day 0) and day 4 cultures. Expression of the Hh receptor (Patched) (A), co-receptor (Smoothed) (B), target gene (Gli2) (C), and inhibitor (Hip) (D) were evaluated by QRT-PCR as described in the Methods. (E) Culture-activated HSCs (7days in culture) were re-seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to 0.2% FBS and the cells were treated with anti-Shh neutralizing antibody or control IgG for 48 hours. Caspase 3/7 activity was measured. (* $p < 0.05$ compared to control, $n = 3$ experiments),

Figure 3. PDGF induces Shh expression in culture-activated rat HSC

(A) Seven-day culture-activated HSCs were serum-starved for 24 h and then treated with 20ng/ml PDGF-BB for 0, 1, 2, 3, 6 and 9 hours. Total RNA was isolated at each time point, and Shh mRNA levels were assessed by real-time PCR. (B). Parallel cultures of culture-activated HSCs were serum-starved for 24 h and then treated with 20ng/ml PDGF-BB for 16 hours. Total cell protein was harvested, and Western blot analysis was performed using anti-Shh (upper panels) or β -actin (lower panels). * $p < 0.05$ compared to control, $n = 3$ experiments

Figure 4. PDGF induction of Shh expression in activated HSC involves an AKT dependent mechanism

(A) Seven day culture-activated HSCs were serum-starved for 24 h and then 20ng/ml PDGF-BB was added to the cells for 0, 5, 10, 30, 60 and 120 min. Total cell protein was harvested, and Western blot analysis was performed using anti-phospho-Thr308 Akt (upper panels), anti-phospho-Ser473 Akt (medium panels) or anti-total Akt (lower panels) polyclonal antibody. (B) After 24 hour serum starvation, 7day-culture activated HSCs were treated with 25uM ly294002 for 30min, followed by 20ng/ml PDGF-BB for another 16 hours. Shh was detected by immunoblotting; β-actin was detected in the same membrane as a loading control. (C) In parallel, culture-activated HSCs were transduced with Ad5GFP or Ad5dnAkt; and 24 h later cells were treated with vehicle (control) or 20ng/ml PDGF for 16h. Total cell proteins were harvested, and Western blot analysis was performed using anti-Shh antibody. Equal loading was confirmed by western blotting for β-actin. (D) Culture-activated HSCs were transduced with Ad5GFP or Ad5myrAkt; and 24 h later, the culture medium were switched to DMEM supplied with 0.2% FBS for an additional 48h. Total cell proteins were harvested, and Western blot analysis was performed using anti-Shh antibody. β-actin was used as loading control. All results (A–D) are representative of findings from 3 separate experiments. (*p < 0.05 vs PDGF alone (B, C) or ad5GF (D)).

Figure 5. Shh pathway mediates effects of PDGF on HSC growth

(A) Culture-activated HSCs (7days in culture) were re-seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to 0.2% FBS for 24 hours and cells were pre-treated with vehicle, cyclopamine, LY294002, or AY9944 for 30 minutes. Then PDGF-BB was added and cells incubated in medium containing human PDGF-BB (20 ng/ml) for 48 h with BrdU present during the last 24 h. DNA synthesis was assessed by BrdU incorporation. (B). Experiments described in (A) were repeated except cells were pre-treated with vehicle, nonimmune IgG or anti-Shh neutralizing antibody. All data represent 3 independent experiments. # $p < 0.05$ compared to respective controls. * $p < 0.05$ vs cultures treated with PDGF without inhibitors

Table 1

RT-PCR primers for analysis

Primer		Sequence	Product size
18S	Sense	TTGACGGAAGGGCACCACCAG	130
	Antisense	GCACCACCACCCACGGAATCG	
Shh	Sense	CTGGCCAGATGTTTTCTGGT	117
	Antisense	TAAAGGGGTCAGCTTTTGG	
Ptc	Sense	ACGCTCCTTTCCTCTTGAGAC	168
	Antisense	TGAACTGGGCAGCTATGAAGTC	
Smo	Sense	GCCTGGTGCTTATTGTGG	75
	Antisense	GGTGGTTGCTCTTGATGG	
Gli2	Sense	CCATCCATAAGCGGAGCAAG	105
	Antisense	CCAGATCTTCCTTGAGATCAG	
Hip	Sense	TGTGCCGTGGATCGAC	238
	Antisense	GATCTCCGAACACGTAGCTT	