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C. Martínez[1,2,3], P.C. Smith[2], J.P. Rodriguez[3], and V. Palma[1]*

[1]Center for Genomics of the Cell (CGC), Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Santiago, Chile; [2]Laboratory of Periodontal Physiology, Dentistry Academic Unit, Faculty of Medicine, Pontificia Universidad Católica de Chile; and [3]Laboratory of Cell Biology, INTA, Universidad de Chile; *corresponding author, vpalma@uchile.cl

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Sonic Hedgehog Stimulates Proliferation of Human Periodontal Ligament Stem Cells

ABSTRACT

Regulation of cell renewal in the periodontium is a critical cell function that has not been clarified. Sonic hedgehog (Shh) is a secreted signaling molecule that plays a key role during development and adult tissue homeostasis. In the present study, we have analyzed the role played by Shh in human periodontal ligament stem cell (HPLSC) proliferation. HPLSC were isolated with anti-STRO-1 antibodies. Shh increased the expression of *GLI1* and *PTC-1* and selectively stimulated cell proliferation in STRO-1(+) derived from adult periodontal ligament. Shh components were localized to primary cilia in STRO-1(+) cells after Shh stimulation. STRO-1(+) also expressed Shh, suggesting an autocrine-regulated phenomenon. Thus, we propose that Shh plays a critical role in the regulation of cell proliferation in STRO-1(+)/HPLSC.

KEY WORDS: human periodontal ligament stem cells, Sonic hedgehog, proliferation, primary cilia.

INTRODUCTION

Human periodontal ligament stem cells (HPLSC) constitute a mesenchymal stem cell population that persists in adults, exhibits features of bone-marrow-derived cells, and is able to differentiate to many cell lineages (Seo *et al*., 2004; Trubiani *et al*., 2005; Lin *et al*., 2008; Huang *et al*., 2009; Lin *et al*., 2009). Considering their role in periodontal regeneration, it is critically important to identify factors that control their differentiation and proliferation (Liu *et al*., 2008; Feng *et al*., 2010; Lin *et al*., 2010). Sonic hedgehog (Shh) is a member of the mammalian Hedgehog (Hh) family that plays a key role during embryogenesis, organogenesis, and adult tissue homeostasis (Karhadkar *et al*., 2004; Fendrich *et al*., 2008; King *et al*., 2008). A key role for Hh signaling has been suggested during tooth development, since genetic removal of Shh activity from the dental epithelium alters tooth growth (Hardcastle *et al*., 1998; Dassule *et al*., 2000; Gritli-Linde *et al*., 2002; Cobourne and Sharpe, 2005). Shh canonical signaling acts through the Patched-1(Ptc-1) and Smoothened (Smo) membrane proteins, inducing the transcriptional activation of 3 target genes in vertebrates: the *GLI* genes (*GLI1, GLI2*, and *GLI3*). In the absence of Shh, Ptc-1 maintains Smo in an inactivated state. After the binding of Shh, Ptc-1 inhibition of Smo is released, and the signal is transmitted to promote the transcription of Shh target genes, such as *PTC-1* and *GLI1* (Jiang and Hui, 2008; Mas and Ruiz i Altaba, 2010; Kiefer, 2010). The role played by Shh/GLI signaling in human periodontal ligament (HPL) growth is so far unknown. However, recent studies have identified the expression of *SHH/GLI* components in Hertwig´s epithelial root sheath and the proliferative dental mesenchyme during root development (Nakatomi *et al*., 2006). Considering that Shh influences stem cell behavior in several tissues (Jiang and Hui, 2008), here we have explored the putative role played by Shh in HPLSC proliferation.

MATERIALS & METHODS

Human Periodontal Ligament Cell Cultures and Immunomagnetic Isolation

Healthy human impacted third molars were collected from 15 young adults (15-24 yrs old) under approved guidelines set by the Ethics Committee of the Faculty of Science, University of Chile. Written informed consent was obtained from all donors.

Explants, obtained from the middle third of the root, were cultured in α-MEM (Invitrogen, Carlsbad, CA, USA) plus 10% Fetal Bovine Serum (FBS, Hyclone-Thermoscientific, Logan, UT, USA), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (SIGMA, St. Louis, MO, USA) according to a previously described protocol (Seo *et al*., 2004).

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After 2 or 3 passages, cells were subjected to magnetic isolation with antibodies to detect STRO-1 antigen (R&D Systems, Minneapolis, MN, USA) and magnetic beads (Dynabeads, Invitrogen) (Seo *et al*., 2004). The resulting 2 cell populations STRO-1(+), identified as HPLSC/Progenitors, and STRO-1(-) were plated and cultured in α-MEM plus 10% FBS, penicillin, and amphotericin B in 5% CO_2.

Immunofluorescence

Cells were grown on coverslips, fixed, blocked, and incubated with primary antibodies overnight. The following antibodies were used: goat polyclonal osteoprotegerin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal 4-prolyl hydroxylase (Dako, Glostrup, Denmark), rabbit polyclonal Runx-2 (AbCam, Cambridge, MA, USA), mouse monoclonal STRO-1 (R&D Systems), rabbit polyclonal MUC18/CD146 (e-BioSciences, San Diego, CA, USA), mouse monoclonal acetylated α-tubulin, rabbit polyclonal flag (SIGMA), mouse monoclonal BrdU (Dako), and rabbit polyclonal Ki67 (Novocastra-Leica, Wetzlar, Germany). Subsequently, cells were incubated with appropriate secondary antibodies and counterstained with either DAPI or TOPRO-3. BrdU immunostaining was performed as previously reported (Marichal *et al*., 2009).

Treatments, RNA Isolation, RT-PCR

After immunomagnetic isolation, cells were treated with recombinant Shh 3.3 μg/mL (R&D Systems), the Smo agonist Purmorphamine 10 μM (Calbiochem, La Jolla, CA), or the Hh inhibitor Cyclopamine (Cyc) 10 μM (Infinity Pharmaceuticals, Inc., Cambridge, MA, USA) for 48 hrs. Total RNA isolation and cDNA and PCR amplification were performed as specified previously (Jo *et al*., 2007). The primer sequence used to amplify *SHH/GLI* pathway components was according to Sanchez *et al*. (2004)

Flow Cytometry

STRO-1 was evaluated with a STRO-1 antibody (R&D Systems) and appropriate secondary antibodies, and was sorted in a Becton Dickinson Flow Cytometer.

Western Blotting

Western blot analyses were carried out as previously reported (Stecca and Ruiz i Altaba, 2009). Protein extracts were separated with 8% and 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with antibodies against: Collagen type I, Osteonectin, Ptc-1, and Parathyroid Hormone Receptor, all from Santa Cruz Biotechnology; Runx-2 (AbCam), Shh (kindly provided by Dr. Juan Larraín), and α-tubulin (SIGMA). Proteins in immunoblots were visualized by ECL (Pierce, Rockford, IL, USA) with horse secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Western blot experiments were quantified through densitometric analysis with Kodak Molecular Imaging Software.

Cell Culture Transfection

HPLSC were transiently transfected with expression plasmids by Lipofectamine 2000 plus (Invitrogen). Expression plasmids with smo-wt and smo mutant sequence have been reported previously (Taipale *et al*., 2000). After 24 hrs of post-transfection, cells were stimulated either with Shh or Purmorphamine (Merck, Darmstadt, Germany).

Alkaline Phosphatase Assay

C3H/10T1/2 (ATCC, CCL-226) cells were grown for 24 hrs in DMEM containing 10% FBS. After this, cells were cultured in the presence of conditioned media (2 days) derived from HPLSC or STRO-1(-) supplemented with 10% FBS. Recombinant Shh or Purmorphamine was used as a positive control. Cyc was used to verify the presence of biologically active Shh in the conditioned media. Cells were fixed and washed twice in PBS. Alkaline phosphatase activity (ALPA) was visualized by the incubation of cells in BCIP/NBT (Roche, Mannheim, Germany) as previously reported (Regl *et al*., 2002).

Scanning Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde and dehydrated in a graded series of ethanol followed by sputter-coating with gold-palladium. Specimens were examined with a scanning electron microscope (FEI QUANTA 200) (Ko *et al*., 2008).

Cell Proliferation Assays

HPLSC were grown in α-MEM supplemented with 10% FBS on coverslips at 5 x 10^3 cells/well. After 3 days, cells were incubated for 48 hrs in the presence of Cyc. We performed gain-of-function experiments by starving cells for 24 hrs after 3 days of plating. Cells were then stimulated with recombinant Shh for 48 hrs. In both types of experiments (gain or loss of function), BrdU (2 μg/mL; SIGMA) was added during the last 20 hrs of culture, followed by immunofluorescence detection.

Statistical Analysis

We quantified cells by counting the number of (+) cells and obtaining the average of DAPI-(+) cells *per* 10 fields using a Leica DFC300 FX camera at 40x (epifluorescenceZeiss microscope, Axiovert 200M) or as the average of TOPRO-3(+) cells (confocal microscope, Zeiss Laser Meta). Images were processed with Photoshop CS3. A minimum of 4 independent samples for each experiment was counted. Results are presented as mean ± standard error. Significance was determined through Student's *t* test and was set at $p < 0.05$.

RESULTS

Characterization of Human Periodontal Ligament Cell Cultures

Primary cultures, derived from adult HPL, expressed the mesenchymal markers 4-prolyl hydroxylase and osteoprotegerin

(Lossdörfer *et al*., 2005). A significant population of cells also expressed the transcription factor Runx-2 (Figs. 1A, 1B, 1E). Consistent with previous reports, the mesenchymal stem cell marker STRO-1 was observed in only 5% of the cells (Figs. 1C-1F) (Seo *et al*., 2004; Ivanovski *et al*., 2006; Xu *et al*., 2009). After immunomagnetic isolation, we were able to obtain enriched cultures of STRO-1(+) cells (up to 70% of total cells), identified as HPLSC/progenitors (Fig. 1G). These cells do self-renew, as evaluated by the generation of single, separated, fibroblastoid colonies, termed 'colony-forming units' (CFU), and were (+) for MUC18/CD146 stem cell markers (Appendix Figs. A-B'). In the absence of osteogenic media, STRO-1(+) cells did not express bone differentiation markers (collagen type-I, PTH-Rc, osteonectin, and Runx2). However, these markers were evident in the STRO-1(-) population (Figs. 1H-1K). We confirmed the differentiation potential of STRO-1(+) cells into the bone/cementum, adipose, and neuronal lineages by culturing cells in the presence of either osteogenic, adipogenic, or neurogenic medium for 21 days. Bone/cementum differentiation was evidenced by calcium deposition by Alizarin red staining, colorimetric quantification, and Energy-dispersive x-ray analysis (Appendix Figs. C-L). Adipose lineage differentiation was detected by oil red stain, and neuronal differentiation by immunostaining against neuronal marker βIII tubulin (Appendix Figs. F-L).

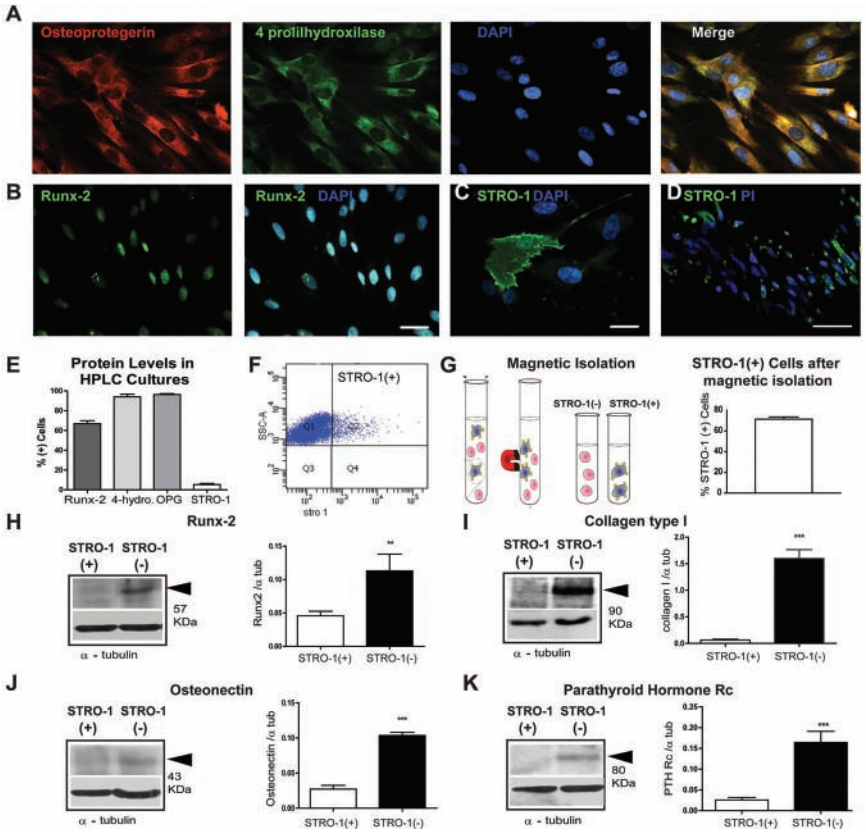

Figure 1. Cells from HPL are mesenchymal and have stem cells/progenitors (HPLSC) capable of being immunomagnetically isolated. Cell cultures were immunoassayed against mesenchymal markers and counterstained with DAPI. Representative epifluorescence images of immunostaining for **(A)** osteoprotegerin (red), prolyl-4hydroxylase (green). **(B)** Runx-2. **(C)** STRO-1. **(D)** Representative confocal image of STRO-1 labeling in explant of PL. **(E)** We quantified images of cells by counting the number of marker-(+) cells as a percentage of DAPI(+) total cells. **(F)** Flow cytometry quantification of STRO-1(+) cells of HPL. **(G)** HPLSC/progenitors were isolated with a STRO-1 antibody and magnetic beads; (+) cells were quantified after isolation. STRO-1(+) and STRO-1(-) populations were characterized by Western blot after 10 culture days in the presence of basal media. Densitometric analysis was done, expressing, in relative units of intensity, a ratio between protein and load control α-tubulin. Majority of the expression of characteristic osteoblast proteins was found in STRO-1(-) cells. **(H)** Runx-2. **(I)** Collagen type I. **(J)** Osteonectin. **(K)** Parathyroid Hormone Receptor. *p < 0.03. **p < 0.003. ***p < 0.0001. (A,B,D) Bar = 50 μm. (C) Bar = 20 μm. PI (propide iodide).

STRO-1(+) Cells Express Shh/Gli Components and Are Able to Respond to Shh

Cells were stimulated with 3.3 μg/mL Shh in serum-free medium or with 10 μM Cyc, in the presence of FBS (10%). As assessed through RT-PCR, after 48 hrs the read-out genes of the Shh pathway *GLI1* and *PTC-1* showed an increase in the presence of Shh and a corresponding decrease in the presence of Cyc (Fig. 2A). Additionally, we observed the expression of the transcription factors *GLI2* and *GLI3* (Fig. 2A). Likewise, protein levels of Ptc-1 were modulated by Cyc treatment in HPLSC, showing a decrease in the presence of the drug (Fig. 2B). Analysis of Shh revealed its expression in both STRO-1(+) and STRO-1(-) populations, although HPLSC/progenitors expressed higher levels of Shh when compared with the STRO-1(-) population (Fig. 2C). To assess the activity of Shh protein, we incubated the Shh reporter cell line (C3H10T1/2), in which Shh induces ALPA (Ingram *et al*., 2002), with conditioned media derived from HPLSC or the STRO-1(-) cells. Notably, only media derived from STRO-1(+) cells were able to induce ALPA (Figs. 2D, 2E). Treatment with Cyc of the preconditioned STRO-1(+) media significantly reduced the positive cell-reporter response, indicating an autocrine source of Shh (Figs. 2D, 2E).

STRO-1(+) Cells Have Primary Cilia: a Signaling Center for Shh/Gli Activation

Considering the prominent role played by the primary cilia in Hedgehog signaling activation (Wong and Reiter, 2008), we evaluated its presence in STRO-1(+) cells. In the absence of Shh, Smo is localized to the cytoplasm of cultured cells.

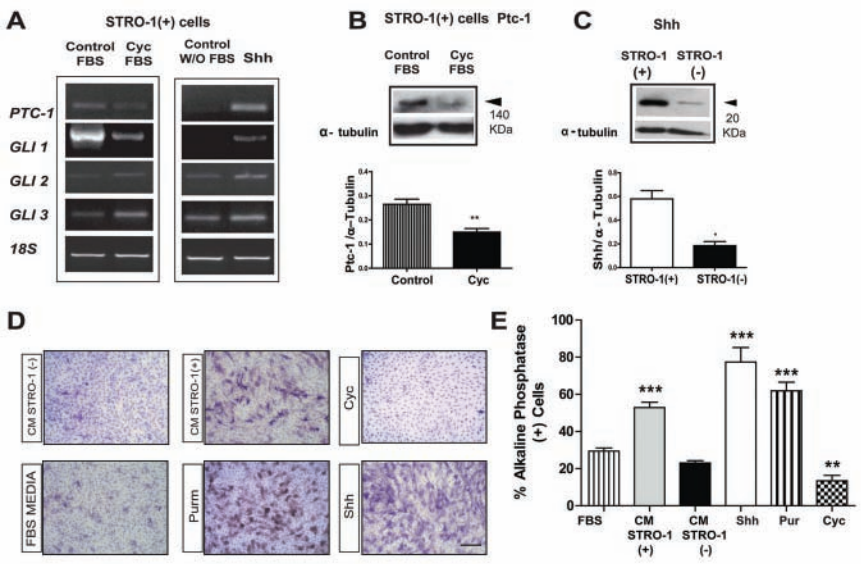

Figure 2. STRO-1(+) cells express Shh/Gli proteins and are able to respond to pathway activation. **(A)** STRO-1(+) cells were treated during 48 hrs in culture with agonist Shh (3.3 μg/mL) without FBS (W/O) or Cyc (10 μM with FBS 10%), followed by RT-PCR analysis. STRO-1(+) cells showed changes of *GLI1* and *PCT -1* expression, the read-out genes of the Shh pathway. **(B, C)** Western blot and densitometric analysis. **(B)** Ptc-1 expression decreased after Cyc treatment. **(C)** Shh protein expression in STRO-1(+) and STRO-1(-) populations revealed higher Shh levels in HPLSC. **(D)** Representative images of ALPA of C3H-10T1/2 cells, treated with conditioned media of STRO-1(+) or STRO-1(-) populations, Shh, Purmorphamine, or conditioned media of STRO-1(+) plus Cyc. **(E)** Percentage of ALPA (+) cells in response to indicated treatments. CM = conditioned media. *p < 0.01. **p < 0.001. ***p < 0.0001. Bar = 200 μm.

Conversely, Smo becomes enriched in the primary cilium in the presence of Shh (Rohatgi *et al*., 2007; Wang *et al*., 2009). Primary cilia were detected in HPLSC cultures by acetylated tubulin expression (Figs. 3A-3A', 3B). After transient transfection with a flag-tag version for Smo (wt-Smo), we observed its localization distributed throughout the cytoplasm in unstimulated conditions (Figs. 3C, 3E). Notably, in cells stimulated with either Shh or the Smo agonist Purmorphamine, Smo localized to the primary cilium (Figs. 3D, 3F, 3G). As a control, we used a mutant activated form of Smo (SmoA1) that localizes exclusively in the primary cilium (Fig. 3H). Based on these observations, we conclude that HPLSC/progenitors actively respond to Shh/Gli stimulation.

Shh Stimulates STRO-1(+) Cell Proliferation

We evaluated the proliferative responses of STRO-1(+) cells after 48 hrs of stimulation with recombinant Shh or inhibition with Cyc. We quantified BrdU and Ki67 (+) cells and found a significant increase after Shh treatment of both markers. Moreover, after Shh stimulation, a two-fold increase in the double (+) cells (BrdU-Ki67) was obtained (Figs. 4A, 4B, 4E-4G), while the opposite result was observed after Cyc treatment, even in the presence of 10% FBS (Figs. 4C, 4D-4G). This effect was found only in HPLSC, since we could not detect any proliferative changes in the STRO-1(-) population (data not shown). Based on these results, we conclude that Shh has a specific mitogenic effect on HPLSC/progenitors.

DISCUSSION

A significant issue in periodontal regeneration is the identification of the molecular mechanisms that control cell proliferation and/or differentiation of HPLSC/progenitors (Huang *et al*., 2009; Feng *et al*., 2010). The present study reports for the first time that HPLSC/progenitors are able to respond to the mitogenic stimuli of Shh. We were able to obtain and characterize STRO-1(+) and STRO-1(-) populations from HPL. STRO-1(+) cells, defined as HPLSC/progenitors, were plastic-adherent under standard culture conditions, had double-positive expression for STRO-1-MUC18/CD146, and did not express bone differentiation markers (Collagen type I, PTH-Rc, Osteonectin, and Runx2). In agreement with previous reports, our STRO-1(+)-enriched cultures preserved their differentiation potential to the bone/cementum, adipogenic, and neuronal lineages (Seo *et al*., 2004; Xu *et al*., 2009; Psaltis *et al*., 2010). In contrast, bone differentiation markers, initially abundant in heterogeneous cultures prior to STRO-1 magnetic isolation, were preserved mainly in the STRO-1(-) population. Our HPLSC/progenitor population expressed components of the Shh pathway, including all *GLI* transcription factors. Moreover, HPLSC/progenitors actively respond to Shh activation, since these cells showed increased expression of *PTC-1* and *GLI1* transcripts upon recombinant Shh treatment or a decrease of these read-out markers after Cyc inhibitor incubation. Likewise, we could observe lower expression of Ptc-1 protein levels upon Cyc treatment. The primary cilium was detected in STRO-1(+) cell populations. The primary cilium is considered as a true signaling antenna used by mammalian cells to sense signals such as Platelet-derived Growth Factor and Wnt and Shh pathways (Christensen and Ott, 2007). Regulated movement of key proteins into and out of the cilium creates a sophisticated switch by which cells can turn this powerful signaling center on and off. The cilium is required for Hh signaling, since no GLI activator is produced in mutants that lack cilia (Wong and Reiter, 2008). In agreement with results from previous studies in other mammalian models, we report that after transient transfection of STRO-1(+) cells with wild-type combined with Shh stimulation or mutant forms of flag-tag Smo, Smo becomes localized to the cilium tip (Rohatgi *et al*., 2007; Wang *et al*., 2009). Hence, our evidence supports the idea that STRO-1(+) cells constitute a new Shh-responsive population.

Shh acts as a mitogen on the STRO-1(+) population, increasing BrdU and Ki67 labeling after recombinant Shh treatment. Strikingly, even in the presence of high concentrations of 10% FBS, Cyc was able to decrease BrdU uptake and Ki67 expression.

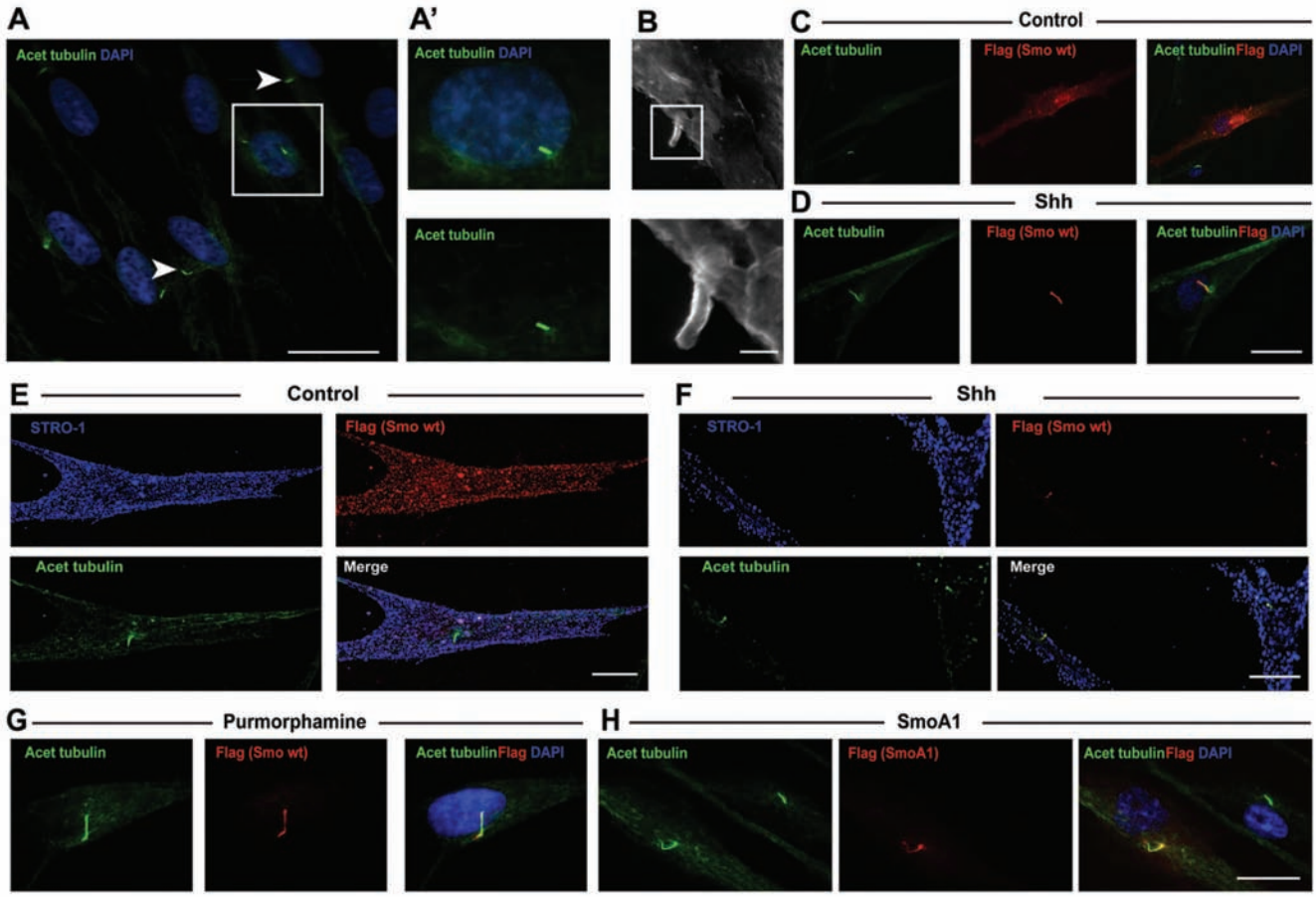

Figure 3. HPLSC have a primary cilium, considered a critical signaling center required for Shh pathway activation. **(A)** Images of immunostaining against acetylated α-tubulin show the presence of primary cilia in HPLSC. **(A')** Higher magnification of a single cell with primary cilia. **(B)** Scanning electron microscope view showing representative image of a primary cilium and a higher magnification inset; bar =5 μm. **(C)** HPLSC were transfected with a Smo-wt flag-tag construct and double-immunostained against acetylated α-tubulin and flag. Representative images in control cells **(D)** treated with Shh (3.3 μg/mL) **(G)** or Purmorphamine (10 μM). Transfected cells were immunostained against acetylated α-tubulin, flag, and STRO-1. **(E)** Representative images in control cells or **(F)** treated with Shh. Smo-flag protein was observed only in the cilia tip upon Shh or agonist treatment. **(H)** STRO-1(+) cells also were transfected with an activator form of Smo (SmoA1). Bar = 20 μm.

These results indicate a potent role for Shh in the modulation of self-renewal of this stem/progenitor cell population in the HPL. Regarding the source of Shh protein, both STRO-1(+) and STRO-1(-) populations express the Shh active protein (N-terminal). However, Shh was detected predominantly in STRO-1(+) cells. The functional activity of Shh secreted by HPL cells was evaluated in the Shh reporter cell line (C3H-10T1/2). This experimental approach showed a significant increase in the ALPA of C3H-10T1/2 cells upon treatment with STRO-1(+) cell-conditioned media. Therefore, we propose that HPLSC/progenitors, through Shh secretion, may modulate self-renewal of its own cell population, acting as a physiological niche factor. In summary, to our knowledge, this is the first report giving evidence of a mechanism by which Shh maintains the self-renewal capacity of the stem cell population in the periodontal ligament. Further studies are needed to determine the effect of Shh on STRO-1(+). We propose that Shh should be explored as a novel molecular niche factor to study regenerative models in HPL and tooth-supporting tissues.

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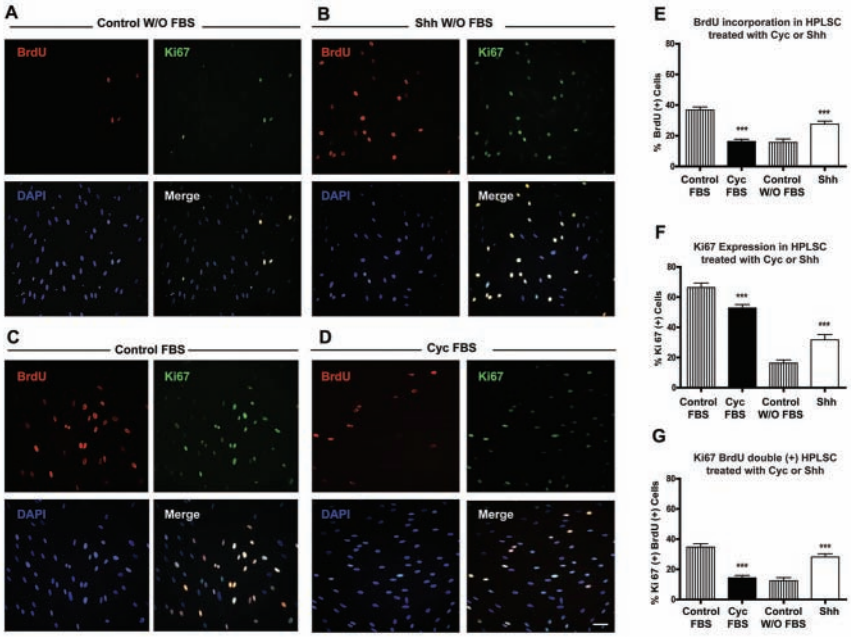

Figure 4. HPLSC/progenitors increased proliferation in the presence of Shh. HPLSC were treated during 48 hrs in culture with agonist Shh (3.3 μg/mL) or Cyc (10 μM), including a BrdU pulse for the last 20 hrs. Immunostaining against BrdU and Ki67 proliferation markers. **(A)** Representative images of marker (+) cells in control media without FBS (W/O). **(B)** Shh treatment in media without FBS. **(C)** Control cultures in media with FBS. **(D)** Cyc treatment in media with FBS. Quantification of **(E)** BrdU (+) cells. **(F)** Ki67 (+). **(G)** Double BrdU-Ki67 (+) cells. All marker (+) cells expressed as a percentage of DAPI (+) cells. Bar = 20 μm ***p < 0.001. Bar = 50 μm.

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