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Functionally distinctive Ptch receptors establish multimodal Hedgehog signaling in the tooth epithelial stem cell niche

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Abstract

Continuous growth of the mouse incisor teeth is due to the life-long maintenance of epithelial stem cells (SCs) in their niche called cervical loop (CL). Several signaling factors regulate SC maintenance and/or their differentiation to achieve organ homeostasis. Previous studies indicated that Hedgehog signaling is crucial for both the maintenance of the SCs in the niche, as well as for their differentiation. How Hedgehog signaling regulates these two opposing cellular behaviors within the confinement of the CL remains elusive. In this study we used *in vitro* organ and cell cultures to pharmacologically attenuate Hedgehog signaling. We analyzed expression of various genes expressed in the SC niche to determine the effect of altered Hedgehog signaling on the cellular hierarchy within the niche. These genes include markers of SCs (Sox2 and Lgr5) and transit-amplifying cells (TACs, P-cadherin, Shh and Yap). Our results show that Hedgehog signaling is a critical survival factor for SCs in the niche, and that the architecture and the diversity of the SC niche are regulated by multiple Hedgehog ligands. We demonstrated the presence of an additional Hedgehog ligand, nerve-derived Dhh, secreted in the proximity of the CL. In addition, we provide evidence that Hedgehog receptors Ptch1 and Ptch2 elicit independent responses which enable multimodal Hedgehog signaling to simultaneously regulate SC maintenance and differentiation. Our study indicates that the cellular hierarchy in the continuously growing incisor is a result of complex interplay of two Hedgehog ligands with functionally distinct Ptch receptors.

Introduction

Postnatally, stem cells (SC) remain within SC niches and provide the source of reparative and regenerative potential. The niches provide an environment which maintains the number of SCs and regulates their recruitment and differentiation necessary for organ homeostasis. Continuously-growing rodent incisors contain epithelial and mesenchymal SC niches, which unceasingly produce the cells that deposit the mineralized constituents of the adult tooth: enamel and dentin, respectively (reviewed in (1)). The epithelial SC niche resides in a structure known as the cervical loop (CL), which is located on the labial side of the incisor

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epithelium, hence the name labial cervical loop (LaCL; Fig. 1). Dynamic balance between stemness and commitment/differentiation ensures incisor tooth homeostasis. However, the complex signaling networks which coordinate self-renewal and differentiation are not fully elucidated. Epithelial SCs are identified by expression of *Sox2*, among other genes, and their differentiation is marked by increased expression of secreted frizzled-related protein 5 (*Sfrp5)* (2). Sfrp5+ early progeny simultaneously downregulate the expression of *E-cadherin* and upregulate the expression of *P-cadherin,* thus becoming transit-amplifying cells (TACs) (2, 3). TACs also express Yap and TAZ, and at their final stages acquire Sonic hedgehog (*Shh)* expression, which continues after their commitment to the ameloblast lineage (4, 5).

In the continuously growing incisor, Hedgehog (Hh) signaling pathway simultaneously regulates Sox2-expressing SCs (6) and their differentiation into ameloblasts (7, 8). However, the molecular mechanism enabling these two contrasting roles remains obscure. Shh is the only Hh ligand detected in the tooth. It is secreted not only by TACs and differentiating ameloblasts (5), but also by the neurovascular bundle (9). Hh ligands bind to receptors Ptch1 and 2, which releases Smoothened (Smo) and activates transcription of downstream targets *Gli1* and *Ptch1* (10, 11). Hence, Gli1 and Ptch1 expression defines Hh responsive cells, which in the continuously growing incisor reside within the epithelial SC population (12). Furthermore, maintenance of the SC niche and Sox2 expression depend on high Hh activity (5, 6). Contrary to this, high Hh activity was found necessary for the ameloblast differentiation from TACs, as well (5). TACs are not Hh-responsive cells, as they lack Gli1 and Ptch1 expression (5, 6). Furthermore, TAC region is characterized by high activity of Hh antagonist, BMP4 (6). Yet, *in vivo* pharmacological inhibition of Hh signaling arrested ameloblast differentiation, while it had no effect on the SCs or TACs (5).

Therefore, we decided to analyze the molecular mechanism behind the multimodal Hh signaling in the SC niche of continuously growing incisors that could explain these contradicting findings. In this study we demonstrate the presence of a second Hh ligand, Dhh (desert hedgehog), in the vicinity of the SC niche and show that Hh signaling in the incisor epithelial SC niche is receptor-dependent. This study shows functionally distinct roles of Ptch1 and Ptch2, as well as receptor-dependent specificity in response to Hh ligand. Our results indicate that the Sox2+ SC population is maintained by Hh signaling transduced through Ptch1, while *P-cadherin* expression is negatively regulated through Dhh - Ptch2 signaling. We also demonstrated that Hh signaling regulates cell adhesion and migration, which contribute to the balance between the maintenance of the SC niche and differentiation. We provide a model that describes the molecular mechanism behind the simultaneous regulation of these distinct cellular processes, and provides a novel concept of Hh regulation which may be applicable in other SC systems and can have great impact on future therapeutic treatments and strategies.

Materials and Methods

Animals.

Sox2-GFP reporter mice (13) and mice lacking Ptch2 (Ptch2KO) (14) were previously described. All experimental procedures in this study involving mice were approved by

Ethical Committees on the Use and Care of Animals and the Animal Facility at the University of Helsinki.

Cell cultures.

Mouse embryonic fibroblasts (MEFs) were obtained from wild type, Ptch1 −/− (15) (kind gift of Dr. Benjamin Allen), and Ptch2−/− embryos (14). Confluent cultures were incubated with 100 ng/ml Dhh or Shh for 6h and lysed for RNA isolation.

Incisor clipping.

The erupted part of the lower incisors of the adult mice was clipped above the gumline. Prior to clipping mice were injected intraperitoneally with Xylenol Orange (90 mg/kg) to detect newly made mineralized matrix, as previously described (16).

Tissue isolation and culture.

The proximal ends of the incisors were dissected from lower jaws of 2-3 weeks-old postnatal mice in Dulbecco's phosphate buffered saline (PBS), pH 7.4 and cultured in Trowell-type organ culture as previously described (17). Cultures were treated with 100 ng/ml of Shh or 10 μM Cyclopamine and media was changed every other day.

Histological analysis.

Harvested mandibles were fixed in 10% formalin for overnight at 4°C, washed with PBS and placed in 30% sucrose solution for 72h. The non-decalcified mandibles were then freeze embedded using SCEM embedding medium (Section-Lab Co. Ltd, Hiroshima, Japan). Tissue was sectioned to 7-10μm thick sections using Cryofilm type 2C(9) from the same vendor, and following the established protocol (18).

Immunostaining.

Immunostaining was conducted on whole explants following a modified version of the established protocol (19). The modifications refer to the shortened incubations with primary and secondary antibodies, which were 24h and 12h, respectively. Frozen sections were washed with PBS and incubated with blocking solution (1%BSA, 1% donkey serum in PBS) for 1h, followed by the 1h incubation with primary antibody and 30 min incubation with the conjugated secondary antibody. All incubations were at room temperature. Antibodies used are listed in Table 1. Samples were visualized using a Zeiss LSM 700 confocal microscope and images analyzed with Zen 2012 (Carl Zeiss, Germany).

EdU labeling.

To detect proliferating cells in cultured explants, samples were incubated with 10μM EdU for 2h and then fixed with 4% paraformaldehyde overnight at 4°C. The following day, samples were processed with Click-iT EdU Alexa Fluor Imaging Kit (Invitrogen/Molecular Probes) according to the manufacturer's protocol.

To detect label retaining cells, freshly isolated explants were first incubated with 10 μM EdU for 24h at 37°C, after which the culture media was removed and the explants were cultured

in control media or media supplemented with Shh or cyclopamine. After 48h chase period, explants were fixed with 4% paraformaldehyde and processed with Click-iT EdU Alexa Fluor Imaging Kit. Samples were subsequently mounted with Vectashield (Vector Laboratories) and visualized using a Zeiss LSM700 confocal microscope.

RNA Isolation and RT-qPCR.

Total RNA was isolated using RNeasy Plus Micro Kit (Qiagen), according to the manufacturer's instructions and followed by cDNA synthesis using Quantitect Reverse Transcription Kit (Qiagen). Multiplex RT-qPCR was performed, enabling simultaneous detection of multiple targets in a single reaction well, utilizing a different probe for each target. All probes were calibrated singly and in combination on control cDNA to ensure that they can be distinguished from each other and simultaneously detect different targets. Each sample was analyzed in triplicate and data were normalized to GAPDH and Hprt expression. Probes used are listed in Table 2.

Statistical Analysis.

Student's T-test or Mann-Whitney non-parametric tests were used for statistical analysis of all data. The results are presented as mean ± SEM and p value <0.05 was used as the criterion for statistical significance.

RESULTS

SC population in the CLs is regulated by Shh signaling

Previous studies showed that Hh signaling is important for the maintenance of Sox2 expression and the incisor SC niche (6), but is also necessary for the generation of ameloblasts from TACs (5). Inhibition of Hh signaling halted ameloblast differentiation, but, surprisingly, it had no effect on the SC population, cell death or cell proliferation (5). Activation of Hh signaling, caused by deletion of Smad4 in the dental epithelium, induced ectopic Sox2 expression and cell proliferation in the ameloblast layer, which collectively inhibited ameloblast differentiation (6). We decided to analyze the effect of Hh signaling modulation on cell proliferation and cell death in the incisor SC niche, using a previously published *ex vivo* explant culture system (17), in which the proximal ends of the incisors are cultured in the presence of Shh, or a known Hh pathway inhibitor, cyclopamine, for 2 days (20, 21).

Inhibition of Shh signaling reduced cellular proliferation and increased cell death within the first 12h of treatment (Fig 2B and E). Expectedly, excess of Shh protein increased cell proliferation and completely ablated cell death in and around the CL (Fig 2C and F). Increased proliferation, which was also observed in the mesenchyme, resulted in an overall increase in the size of the explant when compared to control. Increased cell death in the cyclopamine-treated samples was observed throughout the CL and resulted in a reduction in the size of the CLs and in the overall explant size.

We next analyzed whether these changes were due to the loss of a specific cell population, in particular the Sox2+ SCs. Analysis of Sox2 expression in control explants after 2 days of

culture demonstrated that Sox2 is expressed in the SCs (asterisk in Fig 2G and J), but it was also detected in the early progeny cells, including a portion of TACs which extends into the stratum intermedium (SI) (arrow in Fig 2G). Inhibition of Shh signaling decreased the number of Sox2+ SCs, as well as the intensity of the Sox2-GFP (asterisk in Fig 2H and K), which was opposite to the effect of Shh addition (Fig 2I and L). The positive effect of Shh on the number of Sox2-GFP+ cells and *Sox2* expression was confirmed by flow cytometry and RT-qPCR analysis (Fig 2M and N). The Sox2 and Sox2-GFP expression domains in the TAC region expanded in cyclopamine-treated samples (arrows in Fig 2H and K). We also observed absence of Lgr5, another SC marker, in the CLs of cyclopamine-treated explants (Fig 2P), which supported the importance of Hh pathway in maintaining the SC population.

Inhibition of Shh signaling increases the number of TACs

The TAC region preceding the ameloblasts in the inner enamel epithelium is characterized by exclusive *P-cadherin* expression (3). Our analyses, however, demonstrated that the P-cadherin expression domain extends beyond the previously reported area (3) and was also observed at high levels in the outer enamel epithelium (OEE) (arrow in Fig 3A) extending from the Sox2 expression domain. Inhibition of Hh signaling by cyclopamine upregulated P-cadherin protein and mRNA expression through most of the CL (Fig 3B and D), which was accompanied by a decrease in *E-cadherin* expression (Fig 3E). Interestingly, in the presence of cyclopamine, P-cadherin expression was absent from the TAC region, which instead contained label retaining cells (LRC) (white arrow in Fig 3B) and increased numbers of Sox2+ cells (arrow in Fig 2H). This suggested that the SC population in the cyclopamine-treated samples shifted toward the inner enamel epithelium at the lingual side of the CL. Expression of other markers of TACs, Shh and YAP, was also changed in the presence of cyclopamine. In our control samples, Shh was expressed in the population of TACs at the onset of their differentiation into ameloblasts and it continued into early stages of ameloblast differentiation (Fig 3F), as previously reported (5). However, in the cyclopamine-treated samples, Shh expression was only present in the small number of cells at the neck of the CL, which corresponds to the distal portion of TACs (Fig 3G), and it was absent from the presumptive ameloblast region (arrowhead in Fig 3G). Thus, the published effect of Shh on ameloblast differentiation (5) was recapitulated in our explant system. YAP protein was located in the nuclei of the TACs and early preameloblasts in the control samples (Fig 3I), as previously reported (4). Cyclopamine administration upregulated nuclear YAP in the majority of the CL, including the region containing SCs (yellow arrow in Fig 3J), which was also P-cadherin positive (yellow arrow in Fig 3B). Only the lingual portion of the LaCL, which contained LRCs, was void of YAP staining in these samples (compare LRCs in Fig. 3B with population indicated by green asterisk in Fig 3J). In addition, YAP staining indicated absence of polarized (pre)ameloblast cells (white arrowhead in Fig. 3J), which correlated with absence of Shh protein, and confirmed previously reported absence of ameloblast differentiation upon Hedgehog signaling inhibition. Shh treatment, on the other hand, decreased the intensity but not the domain of endogenous Shh protein (Fig 3H), and had little effect on YAP in the LaCL. It did, however, increase YAP nuclear staining in the mesenchyme (asterisk in Fig 3K).

Hedgehog signaling components in the CLs

Our data demonstrate that Hh signaling regulates survival of tooth epithelial SCs, and consequently their number. Inhibition of Hedgehog signaling induced cell death, which depleted the SC population, but at the same time the number of cells expressing markers of TACs was increased, which indicated accelerated differentiation of the remaining SCs. Hence, we can postulate that Hedgehog pathway regulates the rate of SC differentiation into TACs. However, our data, as well as those published by others (5), indicate that the further differentiation of TACs is blocked by Hh signaling inhibition.

How Hh signaling simultaneously regulates SC survival and ameloblast differentiation within the spatial confinement of the SC niche is not known. We therefore decided to further investigate the molecular mechanism behind the multimodal Hh signaling which determines the cellular hierarchy within the tooth epithelial SC niche. First we analyzed the expression of the signaling components in the cervical loop. Hh signaling is activated by Shh, the only ligand detected in the adult mouse incisor (5) and is mainly produced by the cells exiting the TAC region and those at early stages of ameloblast differentiation (Fig 4A). The activity of the hedgehog signaling pathway, measured by *Gli1* expression, is high in the entire cervical loop, except in the TAC region (6), where low levels of *Gli1* (area outlined by yellow dashed line in Fig 4B) correlate with cytoplasmic localization of Gli1 protein (arrows in Fig 4F), as opposed to nuclear localization of Gli1 in the rest of the LaCL (Fig 4F). Hh ligand binds to Patched receptors distributed in the exclusive expression domains, with *Ptch1* predominantly expressed in the mesenchyme and *Ptch2* in the epithelium (Fig 4C and D). As previously reported, Ptch1 was also expressed in the narrow domain within the Sox2+ SC region (yellow asterisk in Fig 4C and F).

Published data suggest that Shh is distributed from the peripheral nerves (22), including the (mandibular) nerve, which together with the associated vasculature constitutes the neurovascular bundle (9). This finding is surprising considering that peripheral nerves are abundant with Dhh (23, 24). Indeed, our immunostaining analysis demonstrated that also the mandibular nerve identified by myelin binding protein (MBP) (green arrows in Fig 4I-K) is positive for Dhh (Fig 4I-K), and that Dhh protein is released in the vicinity of the myelin fibers of the mandibular nerve (yellow arrowheads in Fig 4I-K). This finding was confirmed by Western blot analysis which showed high amounts of Dhh in the cell lysates from three individual incisor neurovascular bundles (Fig 4L), while the levels of Shh were low to undetectable. Proximal end of the incisor, containing dental pulp and LaCL, in contrast, was abundant with Shh ligand (Fig 4L), as reported (5, 9), but no Dhh protein was detected in these samples. Together these data suggest that the nerve is a source of an additional Hh ligand, Dhh, which could participate in the regulation of cellular complexity of the LaCLs.

Shh signaling regulates the cellular hierarchy of the incisor CLs through a Ptch receptor dependent mechanism

Analysis of the expression of Hh receptors Ptch1 and Ptch2 in the incisor epithelium demonstrated striking and exclusive expression patterns for these two receptors, in which the whole epithelium expresses Ptch2 (Fig 4D), while only a small subset of cells located in the putative SC location expresses Ptch1 (Fig 4C and 4E). The molecular significance of the

distinct expression pattern of Ptch receptors in the SC niche is not known. One of the possibilities is that it reflects different roles of these receptors in the regulation of the cellular hierarchy in the niche. Therefore, we analyzed the expression and regulation of molecular markers of TACs and stem cells in Hh-responsive cells expressing either both or just one of the receptors. Mouse embryonic fibroblasts (MEFs) are used extensively to study Hh signaling and pathway components. We obtained wild type MEFs, which express both Ptch1 and Ptch2 receptors, as well as MEFs lacking either Ptch1 or Ptch2, which provided a suitable model to study gene regulation in the context of the individual receptors. We incubated confluent wild type, Ptch1−/−, and Ptch2−/− MEFs with or without Dhh or Shh ligands for 6h and analyzed the expression levels of genes associated with tooth epithelial SCs or TACs. *Gli1,* a Hh target gene expressed by tooth epithelial SCs, demonstrated Hh ligand-dependent increase of expression, with Shh being the stronger inducer (Fig 5A). Expectedly, *Gli1* expression was increased in Ptch1−/− and Ptch2−/− MEFs, albeit at significantly different levels (Fig 5A). Similarly to wt MEFs, addition of Hh ligands to Ptch2−/− MEFs further increased *Gli1* expression (Fig 5A). Expression of Sox2, another marker of SCs which is also regulated by Hh signaling (6, 25) was absent in Ptch1−/− MEFs, while its levels were significantly upregulated in Ptch2−/− MEFs (Fig 5B), suggesting that Ptch2 plays an inhibitory role in Hh regulation of *Sox2*.

Analysis of the expression of *P-cadherin* (marker of TACs) in wt MEFs demonstrated a negative effect of Dhh on *P-cadherin* expression (Fig 5C). Furthermore, Ptch1−/− MEFs demonstrated significantly reduced *P-cadherin* levels, while no effect was seen in Ptch2−/− MEFs (Fig 4C). Concurrently, expression of *E-cadherin,* a gene expressed in a mutually exclusive manner with *P-cadherin* in LaCL in vivo (3), was upregulated in Ptch1−/− MEFs (Fig 5D). In line with our findings in MEFs, addition of Dhh and Shh proteins had a positive effect on Sox2 protein (Fig. 5F, 5G) and mRNA (Fig. 5H) expression in organ cultures. Like in wild-type MEFs, addition of Dhh was able to downregulate P-cadherin expression (Fig. 5I).

Lack of Ptch2 correlates with accelerated tooth growth and repair

P-cadherin is expressed in LaCLs of adult mouse incisors (Fig 6A), despite the Ptch2 expression present in the entire LaCL. P-cadherin is restricted to the TAC region (Fig 5A), which is also characterized by nuclear pSMAD1/5/8 (Fig 6C), and cytoplasmic Gli1 staining (Fig 4F and 4H). This indicates that increased activity of Hh antagonist BMP4 enables P-cadherin expression. Lack of Ptch2 in the tooth epithelium expectedly expanded P-cadherin expression beyond the TAC region (Fig 6B). Interestingly, nuclear pSMAD1/5/8 staining was similarly expanded (Fig 6D), which is most likely a response to the Hh signaling activation caused by the loss of Ptch2.

Importantly, previous studies have demonstrated that odontoblast-derived BMP4 is a major inducer of ameloblast differentiation (26). Hence, increased number of P-cadherin expressing TACs in Ptch2−/− mice together with increased pSMAD1/5/8 domains suggests increased and/or accelerated ameloblast differentiation in these mice. Analysis of the gross phenotype of the Ptch2−/− mandibles demonstrated that these animals generate longer incisors, which contain more enamel (Fig 6F and 6G). In order to assess the speed at which

the enamel is deposited, we clipped the incisors of wild type and Ptch2−/− mice injected with chelating agent Xylenol Orange to detect newly deposited mineralized matrix. Our results demonstrated accelerated production of mineralized matrices in the incisors of Ptch2−/− mice (Fig 6H), which achieved tooth occlusion at earlier point, when compared to wild type control.

Discussion

In this study we unravel the molecular mechanism behind the multimodal Hh signaling that simultaneously regulates SC self-renewal and differentiation in the SC niche of the continuously growing incisors. Our results demonstrate that Hh signaling regulates the number of SCs in the tooth SC niche and the rate of their differentiation through a Ptch receptor-dependent response to Hh ligands. We provide evidence for the presence of the second Hh ligand in the vicinity of an epithelial SC niche, namely Dhh secreted from peripheral nerves. Collectively, our study indicates that the cellular hierarchy in the continuously growing incisor is a result of complex interplay of two Hedgehog ligands with functionally distinct Ptch receptors. Most importantly, the Ptch receptor-dependent Hh signaling regulation of the cellular hierarchy in the tooth epithelial SC niche may be applicable to other organ systems and SC niches.

Our study demonstrated that inhibition of Hh signaling in the incisor SC niche decreased the number of Sox2+ SCs and that this was a direct consequence of increased cell death, which occurred within the first 24h of treatment. Similarly to published data (5), we observed no cell death at later days of treatment, which indicates that changes observed during pharmacological Hh inhibition *in vivo* are a consequence of rapid cell death and the loss of a distinct cell population very early during the treatment. Our studies on tooth explants show comparable locations of apoptotic cells and Gli1, Ptch1, and Sox2 expression domains, which suggest partial, if not complete loss of SCs. This is supported by observed reduction of Sox2+ SCs and loss of Lgr5+ SCs in the explants, while previous studies. demonstrated decreased numbers of Gli1-derived cells 5 days after treatment with Hh antagonists, which could be a result of the loss of Gli1+ SCs, and not necessarily an indication of their inability to differentiate, as was suggested (5). Collectively, our results indicate that Hh signaling is a major survival signal responsible for maintenance of the tooth epithelial SCs in their niche, which is in accordance with previously published data showing that Hh signaling regulates survival of the embryonic tooth germ (27), as well as embryonic neuroepithelium, and neural progenitors in the postnatal brain (28, 29). The complete absence of cell death we observed in Shh-treated explant cultures was most likely caused by the excess of Hh ligand and inability of Ptch1 receptor to induce apoptosis (28, 30).

Hh signaling is mediated through Ptch1 and Ptch2 receptors, which are also Hh signaling targets. Previous studies have proposed redundant roles for these receptors, and that Ptch2 could mediate Hh-induced response in Ptch1−/− cells (31, 32). The striking contrast between the embryonic lethal Ptch1−/− phenotype, and the mild Ptch2−/− phenotype have led to the notion that Ptch1 plays a more important role in Hh signaling (14, 15, 33). However, non-overlapping expression domains of Ptch1 and 2 in the tooth, as well as their structural differences suggest potentially different functions for these proteins (12, 34-36). Our studies

demonstrated that high Hh activity and presence of Ptch1 are necessary for the maintenance of *Sox2* expression. *Sox2* expression was completely absent in Ptch1−/− MEFs, regardless of the significantly increased Hh activity indicated by increased *Gli1* mRNA expression.

The inability of Ptch2 to induce *Sox2* in Ptch1−/− MEFs suggests that Ptch2 cannot compensate for the loss of Ptch1. However, increased *Gli1* expression in Ptch2−/− MEFs indicates upregulation of Hh signaling in these cells, which should positively regulate *Sox2* expression (REF). Hence, it is plausible that Ptch2 negatively regulates *Sox2* expression. A modest increase of *Sox2* expression in wild type MEFs in comparison to more than 100-fold increase in Ptch2−/− MEFs upon Hh ligand addition further supports this possibility. Interestingly, in many tissues, besides LaCL, Sox2 and Ptch1 expression domains are co-localized. One of them is the developing skin, specifically dermal condensate, where Sox2 and Ptch1 are co-localized, while Ptch2 is found at significantly lower levels compared to Ptch1 (12, 37-39).

Another surprising finding was the negative effect of Hh signaling on the expression of *P-cadherin*. Our *in vitro* studies on MEFs indicated that P-cadherin is a Hh signaling target and that the negative regulation of *P-cadherin* is dependent on the presence of Ptch2, while Hh signaling mediated only by Ptch1 had no effect on *P-cadherin* expression. Conflictingly, increase in the P-cadherin expression was also observed in the explants treated with Hh inhibitor cyclopamine. Moreover, P-cadherin is expressed in the wild type LaCLs despite Ptch2 expression (3). One explanation is that in the presence of Ptch2 receptor P-cadherin expression is restricted to regions of inhibited Hh signaling. Global inhibition of Hh signaling in the explants results in the expansion of P-cadherin in most of the LaCL. Conversely, the TAC region in wild type LaCL is characterized by active BMP4 signaling indicated by nuclear pSMAD1/5/8 staining(6). As a result, Hh signaling is inhibited in TAC region, as indicated by cytoplasmic localization of Gli1 in the TACs, previously shown to be characteristic for inactive Hh signaling (40, 41). Nuclear pSMAD1/5/8 is extended in the Ptch2KO mice, which correlates with the expansion of P-cadherin expression domain, and suggests that P-cadherin expression might be regulated by antagonistic interactions between BMP4 and Hh signaling pathways. Besides the TAC region, we also detected P-cadherin in the region adjacent to SC niche from which the OEE arises. Interestingly, this P-cadherin+ population was not affected by Hh signaling inhibition, suggesting it is a separate population of TACs, most likely a precursor of OEE, which is not regulated by Hh signaling. This is supported by published data, which demonstrated that *in vivo* pharmacological Hh inhibition did not have a negative effect on OEE, but, in contrast, the OEE seemed expanded in these animals (5).

Interestingly, Dhh, but not Shh ligand had a negative effect on *P-cadherin* in wild type MEFs. Previous studies have implied that Dhh has a preference for the Ptch2 receptor, and that Dhh-Ptch2 axis regulates the integrity of peripheral nerves (24). It can, therefore, be postulated that it is the Dhh-Ptch2 interaction which negatively regulates *P-cadherin* expression in LaCLs. Dhh is abundant in peripheral nerves (22), including the mandibular nerve analyzed in this study. It was reported earlier that the mandibular nerve, which enters the mesenchyme of the continuously growing incisors, secretes Shh (9). We, however, detected very low levels of Shh in mandibular nerve. The discrepancy in the observed results

is most likely due to the differences in methodology, where in previous studies lineage tracing in Shh-CreERT mice was used to demonstrate the secretion of Shh ligand, while we performed western blot analysis of lysed mandibular nerve, using antibodies specific for each ligand.

Together, our results suggest that Ptch1 and Ptch2 mediate separate and distinct functions of Hh signaling, providing means for a simultaneous dual role of Hh signaling in the regulation of SCs and their differentiation into TACs. Tooth is not the only tissue in which Hh signaling independently regulates two distinct cellular mechanisms. Previous studies have provided evidence that Shh controls neural tube morphogenesis through coordinated and mechanistically distinct regulation of the adhesion/motility and differentiation of the neuroepithelial cells (42). Our results also showed that cellular migration/motility was Ptch receptor dependent and that MEFs which expressed only Ptch1 moved faster from the wild type controls. We believe that these findings correlate with the Ptch2-dependent regulation of E-cadherin and P-cadherin expression. In the LaCL E-cadherin is expressed in the mainly stationary cells that do not express P-cadherin. Loss of E-cadherin coincides with the appearance of P-cadherin, and induction of cell proliferation and cell movement (3). Our *in vitro* studies demonstrate that Ptch2 inhibits cellular migration/mobility, which most likely occurs through antagonistic regulation of E-cadherin and P-cadherin levels.

Based on this study, we propose a model (Fig 7) which describes the LaCL, the SC niche of the mouse incisor, as a structure of highly active Hh signaling. This was demonstrated by nuclear Gli1 expression in the LaCLs with the exception of the TAC region, where Gli1 in the cytoplasm indicated inactive Hh signaling. This is most likely due to high signaling activity of the Hh antagonist; BMP in this region (6). Our data demonstrate that active Hh signaling maintains *Sox2* expression, and consequently the SC population, through Ptch1. Downregulation of Ptch1 expression marks the SC commitment (5) and their differentiation into the Sfrp5+ progeny (2), which does not express P-cadherin (3). Further, our data suggest that negative regulation of P-cadherin in these cells is due to high Hh activity. P-cadherin is inhibited until the cells enter the region of low Hh signaling, a hallmark of their transition into TACs. Further commitment of these cells to ameloblast lineage is marked by a loss of P-cadherin and induction of E-cadherin (3). Our data show that Hh signaling inhibition significantly downregulates *E-cadherin*, providing a possible mechanism for the lack of ameloblast differentiation in mice treated with Hh antagonists (3) However, our studies in MEFs do not provide sufficient evidence to conclude whether *E-cadherin* is regulated only by Shh (8, 43) or by another signaling pathway, such as Fgf (3). Collectively, the simultaneous Hh pathway regulation of SCs, cell adhesion, and differentiation we uncovered in this study provide novel insights into functions of Shh signaling in a SC niche, which can have great impact on the future therapeutic treatments and strategies in various tissues.

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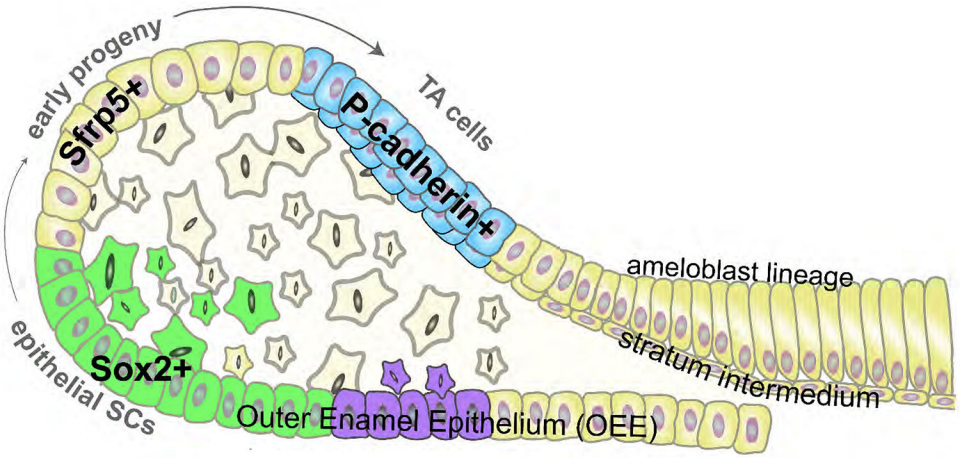

Fig. 1. Schematic of the labial cervical loop.

Sox2 expressing tooth epithelial stem cells (green) and their conventional differentiation pathway, marked by production of early progeny which express Sfrp5. The Sfrp5+ cells become transit-amplifying cells (TACs, blue), which express high levels of P-cadherin, and at the ultimate stage also express Sonic Hedgehog. TACs give rise to ameloblasts and stratum intermedium cells, which is marked by loss of P-cadherin expression. A second cell population, marked by P-cadherin expression (purple), arises from Sox2+ stem cells and extends toward the outer enamel epithelium.

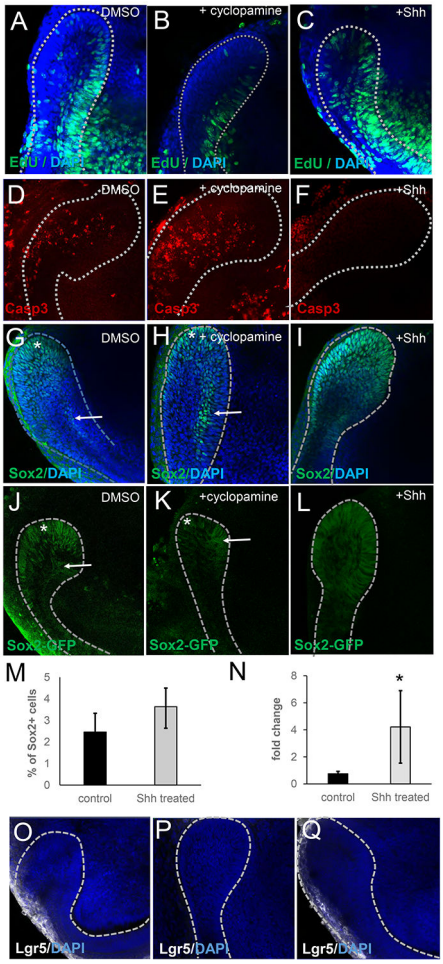

Fig. 2. Shh regulates stem cell population *ex vivo*.

Proximal ends of the incisors were isolated and cultured in the presence of 10 M cyclopamine or 100ng/ml of Shh. (A-C) EdU staining was used to detect cell proliferation, which was analyzed 24h after the initiation of treatments, which include DMSO treated control (A), cyclopamine (B) and Shh (C). (D-F) Caspase3 staining was performed to analyze cell death 12h after the initiation of treatments, which include DMSO treated control (D), cyclopamine (E) and Shh (F). (G-H) Immunostaining for Sox2 to determine the number of Sox2+ cells in DMSO control (G), cyclopamine (H) and Shh (I) treated cultures, 48h after the treatment was initiated. (J-L) Sox-GFP expression analyzed in live cultures 24h after the initiation of DMSO (J), cyclopamine (K), and Shh (L) treatments. Asterisk marks the Sox2+ SCs which are significantly reduced in cyclopamine treated samples, while white arrow points to the Sox2+ domain which appears in cyclopamine treated samples, but in controls only few Sox2+ cells are detected. (M) Flow cytometry analysis of Sox2-GFP + cells in control and cultures treated with Shh for 48h. (N) RT-qPCR analysis of Sox2 expression in control and cultures treated with Shh for 48h. (O-Q) Immunostaining for Lgr5 in DMSO control (G), cyclopamine (H) and Shh (I) treated cultures, 48h after the treatment was initiated. *p value < 0.05, n=7. Scale bar 100 m. Scale bar 100um.

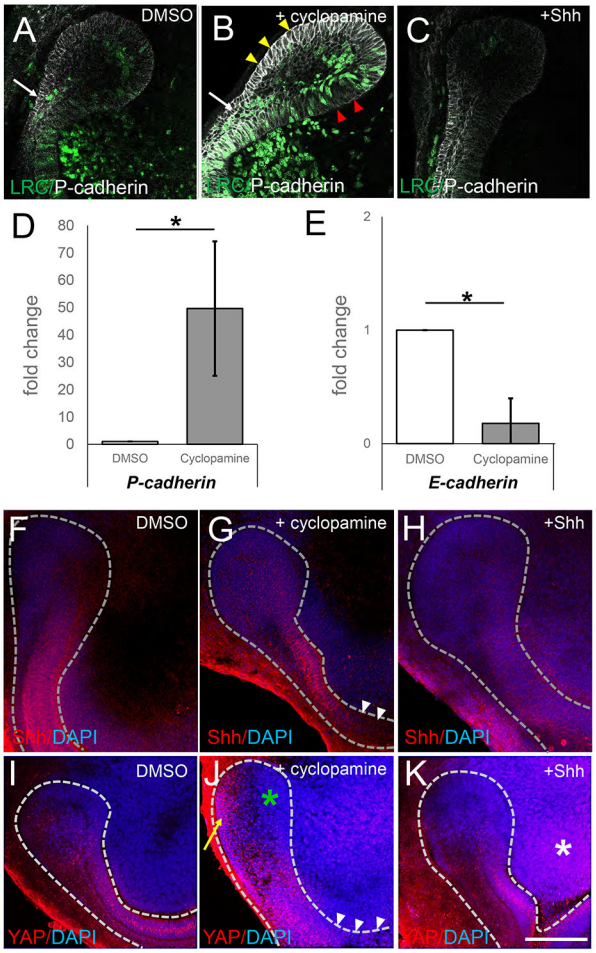

Fig. 3. Shh inhibition increases the number of TACs *ex vivo*

(A-C) Immunolabeling for P-cadherin (white) and label retaining cells (LRC, green) in explants treated with cyclopamine or Shh for 2 days. White arrow in A-C indicates P-cadherin expression in the outer enamel epithelium (OEE). Yellow arrowheads in B points to the region which contains Sox2+ SCs, while red arrowheads in B points to the region where TACs reside. (D, E) RT-qPCR analysis of P-cadherin and E-cadherin expression. N=3. (F, H) Immunostaining for Shh and (I-K) YAP proteins. White arrowheads in (G) indicate lack of Shh signal in the presumptive ameloblast region. White arrowheads in (J) indicate lack of polarized preameloblast and ameloblast cells, and green asterisk indicates location which correlates with the location of LRCs (B) in the cyclopamine treated sample. The yellow arrow in (J) points to a region which corresponds to the region of upregulated P-cadherin (yellow arrow in (B). Scale bar 100um.

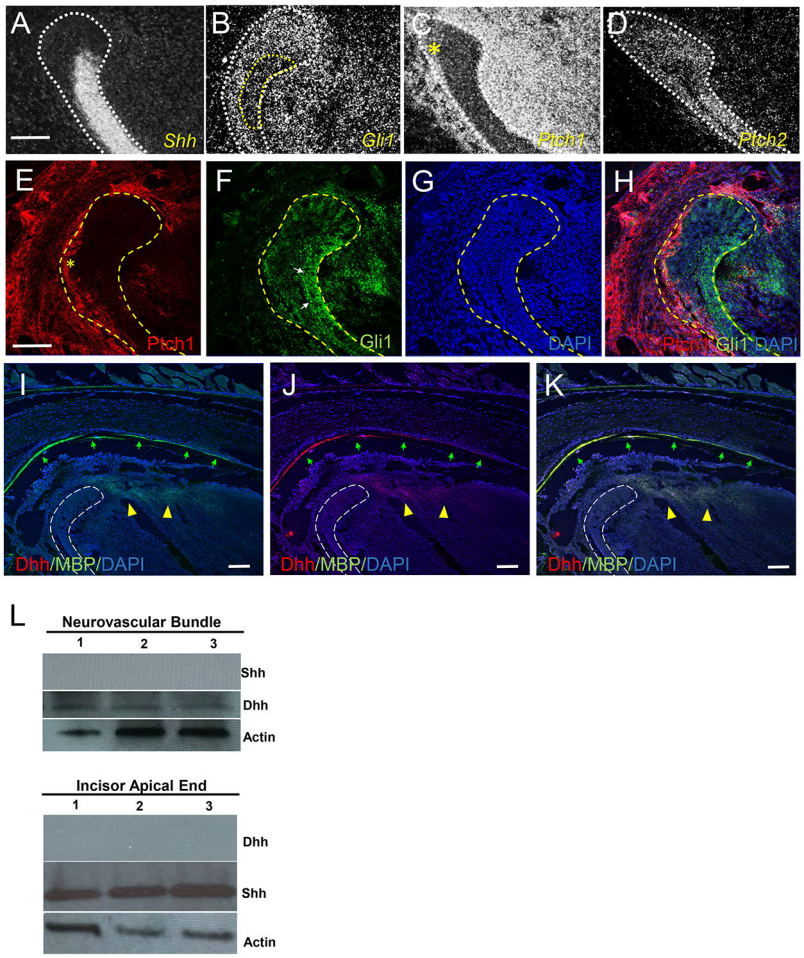

Fig. 4. Hedgehog pathway components in the cervical loop

(A-D) Radioactive in situ hybridization for Shh, Gli1, Ptch1 and Ptch2. (E-H) Immunostaining for Ptch1 and Gli1 on frozen sections of non-decalcified mandibles. Yellow asterisk marks the presumptive Sox2+ SC domain. Arrows in F point to cytoplasmic expression of Gli1 in TAC region. (I, J, K) Immunolabeling of the peripheral nerve in the close proximity to the labial cervical loop using myelin binding protein (MBP) (green, I) and Dhh antibody (red, J). Arrows point to the Dhh expression in the mandibular nerve, while the yellow arrowheads indicate Dhh protein detected at the entry of the neurovascular bundle (NVB) into tooth mesenchyme. (L) Western blot analysis of the neurovascular bundle and proximal portion of the incisor for Dhh and Shh expression. Scale bar 100um.

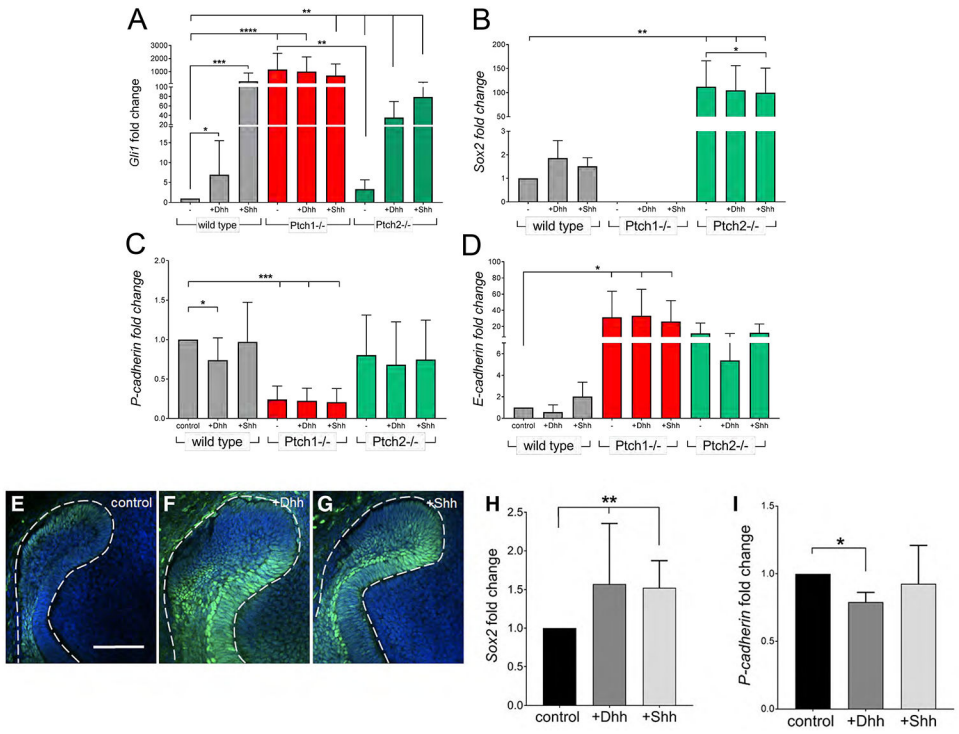

Fig. 5. Hedgehog pathway regulates cellular hierarchy of labial cervical loops in a receptor specific manner

(A-D): RT-qPCR analysis of Gli1,Sox2, P-cadherin, and E-cadherin in mouse embryonic fibroblast cultures. n ≥ 4; *, p < .01; **, p < .005; ****, p < .0001 were determined by Mann–Whitney's nonparametric test. (E–G): Immunolabeling for Sox2 in organ cultures treated with 100 ng/ml of Desert Hedgehog (Dhh; F) or Sonic Hedgehog (Shh; G) for 24 hours. (H, I): RT-qPCR analysis of Sox2 (n ≥ 3) and P-cadherin (n ≥ 4) expression in organ cultures treated with Dhh and Shh for 24 hours. *, p = .015; **, p = .009 were determined by Mann–Whitney's nonparametric test. Scale bar: 100 μm.

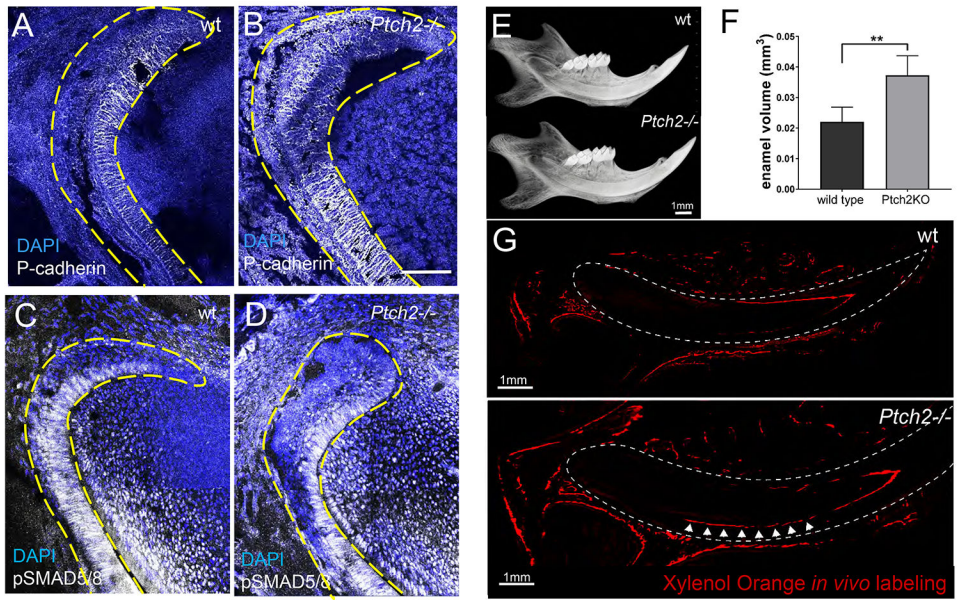

Figure 6. Accelerated growth and repair in Ptch2−/− mice

(A-D) Frozen sections of non-decalcified mandibles from wild type (A, C) and Ptch2−/− (B, D) mice immunostained against P-cadherin (A,B) and pSMAD5/8 (C,D). (E) CT of wild type (upper) and Ptch2−/− (lower) mandibles obtained from 4 weeks old mice. (F) Quantification of the total enamel volume in the wild type and Ptch2−/− mandibles. N=3, **p value < 0.01 was determined by Student's tTest. (G) Visualization of in vivo Xylenol Orange labeling of newly deposited mineralized matrix in frozen sections of non-decalcified mandibles obtained from wild type (upper) and Ptch2−/− (lower) mice, which were injected with Xylenol Orange and their incisors clipped 24h earlier.

Scale bar 100um.

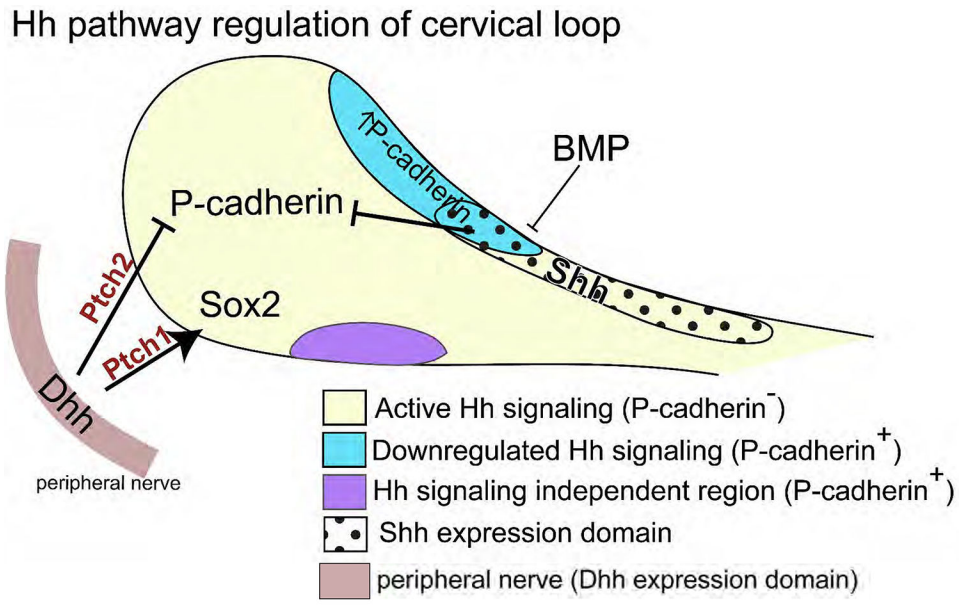

Fig. 7. Model

(A) Schematic of the Hh regulation of the cervical loop hierarchy. Majority of the cervical loop cells are P-cadherin negative (yellow) due to active Hh signaling potentiated by Dhh from peripheral nerve and by Shh. In the TAC region (blue) increased BMP activity inhibits Hh signaling which results in increase in P-cadherin expression, which is a hallmark of TAC region. Abbreviations: OEE = outer enamel epithelium, SCs = stem cells, TACs = transit amplifying cells.

Table 1.

List of antibodies used

Antibody	Vendor and catalogue number	Dilution
Sox2	Santa Cruz, sc-17320	1:250
Sox2	Millipore, ab5603	1:250
YAP	Cell Signaling, 14074S	1:200
P-cadherin	R&D Systems, AF761	1:100
Shh	Santa Cruz, sc9024	1:250
Shh	R&D Systems AF464	1:250
Gli1	Santa Cruz, sc-20687	1:100
Ptch1	Santa Cruz, sc-6149	1:100
Dhh	R&D Systems, MAB733-SP	1:500
Lgr5	Santa Cruz, SC-68580	1:250
pSMAD5/8	Santa Cruz, SC-12353-R	1:250
Casp3	Cell Signaling, 96615	1:500

Abbreviations: Dhh, Desert Hedgehog; Shh, Sonic Hedgehog.

Table 2.

List of PCR probes used

Probe	Catalogue number
Sox2	10031225, qMmuCEPLgr50060283
GAPDH	10031231, qMmuCIP0039581
Hprt	10021228, qMmuCEP0054164
P-cadherin	10031231, qMmuCIP0030319
E-cadherin	10031237, qMmuCEP0052623
Gli1	10031234, qMmuCEP0054131