Transient Activation of Hedgehog Pathway Rescued Irradiation-Induced Hyposalivation by Preserving Salivary Stem/Progenitor Cells and Parasympathetic Innervation

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Abstract

Purpose: To examine the effects and mechanisms of transient activation of the Hedgehog pathway on rescuing radiotherapy-induced hyposalivation in survivors of head and neck cancer.

Experimental Design: Mouse salivary glands and cultured human salivary epithelial cells were irradiated by a single 15-Gy dose. The Hedgehog pathway was transiently activated in mouse salivary glands, by briefly overexpressing the Sonic hedgehog (Shh) transgene or administrating smoothened agonist, and in human salivary epithelial cells, by infecting with adenovirus encoding Gli1. The activity of Hedgehog signaling was examined by the expression of the Ptch1-lacZ reporter and endogenous Hedgehog target genes. The salivary flow rate was measured following pilocarpine stimulation. Salivary stem/progenitor cells (SSPC), parasympathetic innervation, and expression of related genes were examined by flow cytometry, salisphere assay, immunohistochemistry, quantitative reverse transcription PCR, Western blotting, and ELISA.

Results: Irradiation does not activate Hedgehog signaling in mouse salivary glands. Transient Shh overexpression activated the Hedgehog pathway in ductal epithelia and, after irradiation, rescued salivary function in male mice, which is related with preservation of functional SSPCs and parasympathetic innervation. The preservation of SSPCs was likely mediated by the rescue of signaling activities of the Bmi1 and Chrm1–HB-EGF pathways. The preservation of parasympathetic innervation was associated with the rescue of the expression of neurotrophic factors such as Bdnf and Nrtn. The expression of genes related with maintenance of SSPCs and parasympathetic innervation in female salivary glands and cultured human salivary epithelial cells was similarly affected by irradiation and transient Hedgehog activation.

Conclusions: These findings suggest that transient activation of the Hedgehog pathway has the potential to restore salivary gland function after irradiation-induced dysfunction. Clin Cancer Res; 20(1); 140–50.

Introduction

Head and neck cancers account for approximately 3.2% of all cancers in the United States with approximately 342,550 survivors in 2012 (1, 2). Radiotherapy is the most common form of treatment for head and neck cancers, and nondiseased salivary glands are often exposed to radiotherapy. Due to the exquisite radiosensitivity of salivary glands, irreversible hyposalivation is common in long-term survivors of head and neck cancers treated with radiotherapy even when various new techniques have been applied to minimize the irradiation (IR) damage to salivary glands (3). Hyposalivation exacerbates dental caries and periodontal disease, and severely impairs the quality of life of patients. Irreversible hyposalivation has been attributed to the loss of functional salivary stem/progenitor cells (SSPC) and their functional restoration.

Hedgehog (Hh) signaling is required for salivary branching morphogenesis (6), and is activated during the regeneration of adult salivary glands (7). The Hh receptor Patched...
Translational Relevance

Irreversible hyposalivation is common in survivors of head and neck cancer who were treated with radiotherapy even when various new techniques were applied to minimize the irradiation damage to the salivary glands. Current treatments for irradiation-induced hyposalivation can only temporarily relieve symptoms of xerostomia. The morbidity is caused by the loss of functional epithelial stem/progenitor cells. Recent studies indicated that parasympathetic innervation is also essential for the regeneration of salivary glands and is impaired by radiotherapy. This article reports on a novel finding that the transient activation of Hedgehog pathway after irradiation rescued salivary gland dysfunction by preserving both salivary SSPCs and parasympathetic innervation in a mouse model. Moreover, in cultured human salivary gland epithelial cells, transient Hedgehog activation after irradiation rescued expression of genes essential for the maintenance of SSPCs and parasympathetic innervation similarly as in mice. These findings suggested that targeting the Hedgehog pathway is a promising option to treat irradiation-induced hyposalivation.

(Ptc) acts as a negative regulator of Smoothened (Smo) that blocks production of repressor forms of the Gli transcription factors (GliR) and promotes production of the activator forms (GliA). The binding of Hh ligands to Ptc unleashes Smo activity to tip the balance toward GliA that consequently activates the expression of Hh target genes (8). Sonic Hedgehog (Shh), one major Hh ligand, is expressed in the epithelium of the embryonic salivary gland, and may act within the epithelium in a juxtacrine manner to promote proliferation and differentiation of epithelial cells (9). In submandibular glands (SMG) of the adult mouse, Hh signaling is marginal, but is activated significantly during functional regeneration after duct ligation (7). We report, in this study, that IR in mouse models does not activate the Hh pathway in SMGs similar to occlusive damage, and transient activation of the Hh pathway by Shh overexpression in the basal epithelia of male mice rescued IR-induced hyposalivation, likely through the preservation of SSPCs and parasympathetic innervation. IR and transient Hh activation by other approaches similarly affected the expression of genes essential for the maintenance of SSPCs and parasympathetic innervation in the SMGs of female mice and cultured human salivary epithelial cells. These data indicated that the transient activation of Hh pathway could potentially benefit salivary gland function following radiotherapy of head and neck cancer.

Materials and Methods

Mice

Hh signaling activity was analyzed with B6;129-Ptch1tm1Mps/J (Ptch1-lacZ) mice (The Jackson Laboratory). Mice carrying tetO-Shh (10) and Krt5-rtTA (11) transgenes were placed on doxycycline (Dox) chow (6 g/kg, Bio-serv) to induce Shh expression. Irradiation of mouse and measurement of stimulated saliva flow rate was as previously reported (11). All animal procedures were approved by the Texas A&M Health Science Center (TAMHSC) and the Institutional Animal Care and Use Committee of the Scott and White Hospital.

Quantitative reverse transcription PCR analysis

Quantitative reverse transcription PCR (qRT-PCR) was done as reported previously (7). A qRT-PCR analysis for miRNAs was performed with TaqMan miRNA assays (Applied Biosystems) using U6 snRNA as the reference RNA.

ELISA and Western blot

Fresh SMG samples were homogenized with 40-μl T-PRE reagent containing protease inhibitors (Pierce) per milligram followed by centrifugation at 10,000g for 5 minutes to collect the supernatant for ELISA and Western blot. The concentration of Bdnf and Nrtin in saliva and SMG samples was examined with ELISA kits (Insight Genomics and MyBioSource). Western blot analysis was conducted as reported previously (7) with antibodies for Aqp5 (Abcam 1:5,000), p21Waf1 (Millipore, 1:500), and GFRa2 (Abcam 1:1,000).

Flow cytometry

The antibodies used were against c-kit or Sca-1 (BioLegend, 1:50), Bmi1 (Abcam, 1:100), Gli1 (Thermo, 1:100), or Chrm1 (ABBIOTEC, 1:100). For Bmi1 and Gli1 staining, cells were permeabilized with FIX & PERM reagents (Life Technologies). The stained cells were analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter), and data were processed using the manufacturer’s software (CXP).

Histology and immunofluorescence staining

Frozen SMG sections were stained with an acetylcholinesterase (ACHE) rapid staining kit (MBL) or an antibody against Chrm1 (1:2,000; R&D Abs). The AChE stain was quantified with NIS-Elements AR software (Nikon).

SAG treatment

The small-molecule Hh agonist smoothened agonist (SAG) (EMD) or vehicle were administered to female Ptch1-lacZ mice through SMG cannulation (2 μg/g), then followed by daily intraperitoneal (i.p.) injection of 5 μg/g for 3 days. SMGs samples were collected 1 day after the last injection for X-gal staining and qRT-PCR analysis.

Isolation and treatment of human salivary epithelial cells

Healthy human salivary gland samples from patients (2 males and 2 female) with an age range of 25 to 61 years were provided by the Cooperative Human Tissue Network (CHTN), Southern and Mid-Western Divisions, a National Cancer Institute-supported resource. Human SMG epithelial cells were isolated as reported previously (12) and cultured in mammary epithelial medium CnT-27.
Passage-4 cells were treated with a 15-Gy single-dose IR; then, some cells were infected with adenoviruses encoding human Gli1 or GFP (Applied Biological Materials Inc.; MOI = 10) 3 days later. All cells were collected 7 days after IR for analysis.

**Effects of transient Hh activation in SMGs on SCC VII tumors**

The mouse SCC VII tumor model was established, and some tumors were irradiated as previously reported (13). Adenoviral vectors encoding GFP or rat Shh (AdGFP or AdShh, Applied Biological Materials Inc.) were delivered at 10^8 particles per SMG by retrograde ductal instillation on day 0 (inoculation of 3 x 10^4 SCC VII cells) or day 9 (3 x 10^5 cells). Mice were euthanized when the tumor diameter was approximately 15 mm.

**Statistical analyses**

All quantified data were analyzed using one-way ANOVA followed by Tukey multiple-comparison test. Statistical analysis and graphical generation of data were done with the GraphPad Prism software.

**Results**

**Hh activity in SMGs after irradiation or transient Shh overexpression**

We reported previously that the Hh signaling is activated during functional regeneration of the salivary gland after ligation of the main excretory ducts of SMGs (7). Using *Ptch1-lacZ* Hh reporter transgenic mice, we confirmed that the Hh activity is marginal in SMGs of adult mice, but is remarkably elevated in ductal epithelia after duct ligation (Supplementary Fig. S1). To examine the effect of IR on Hh signaling in the salivary gland, *Ptch1-lacZ* mice were treated with 15-Gy single-dose IR in the head and neck region, and the SMGs were collected 3, 7, or 14 days after IR for qRT-PCR analysis and X-gal staining for lacZ activity. IR did not significantly affect the expression of reporter *Ptch1-lacZ* and endogenous *Ptch1* gene in the SMGs of both male and female mice (Fig. 1A and Supplementary Fig. S1, *P* > 0.05, *n* = 3). The expression of another Hh target gene Gli1 was not significantly affected by IR in male SMGs, but was significantly decreased in female SMGs on days 7 and 14 (Fig. 1A, *P* < 0.05, *n* = 3). These results indicated that IR does not activate the Hh pathway in SMGs.
To modulate the Hh pathway, we generated Krt5-rtTA/tetO-Shh mice that overexpress Shh in basal epithelial cells including those in the ducts of salivary glands upon Dox induction. Seven days of Dox induction significantly upregulated the expression of the Hh target genes Gli1, Ptc1, Hhip, Bmi1, and Jag2 in SMGs of male, but not female, mice (Fig. 1B). The much higher expression level of Ptc1, compared with Ptc2L1, indicated that Ptc1 is the dominant Hh receptor in SMGs of both gender (Fig. 1B). whereas the Ptc1 expression in SMGs from females is much lower than that in males before or after Dox induction (Fig. 1B).

Similarly, although the expression of Gli2 and Smo in SMGs before induction (Supplementary Fig. S6) was comparable between males and females, the expression of Gli1 with dominant activator activity (14) was much lower, whereas the expression of Gli3 with dominant repressor activity (14) was much higher in females (Fig. 1B). In addition, the induction of Shh transgene in SMGs from females is much less efficient, likely due to the lower expression of Krt5 and rtTA (Fig. 1B). All these factors likely contribute to the insufficient Hh activation by Shh in SMGs of females. In the SMGs of male Krt5-rtTA/tetO-Shh/Ptc1-lacZ mice, the expression of lacZ Hh reporter was marginal before Dox induction and was significantly elevated in ductal epithelia after 7 days of Dox induction (Fig. 1C). The upregulation of the Ptc1-lacZ reporter and Hh target genes after transient Shh overexpression was much higher in males as well as females than in females after Dox induction, whereas concurrent Hh activation improved the saliva flow rate only from day 60 after IR to a much less extent than Hh activation from day 3 (Fig. 2A, n = 5). Consistently, the expression of acinar markers Aquaporin5 (Aqp5) and cholinergic receptor muscarinic 3 (Chrm3) in SMGs was significantly downregulated by IR on day 120, but improved by transient Hh activation 0, 3, or 90 days after IR as indicated by qRT-PCR and Western blot, with the highest improvement in the day-3 group (Fig. 2B–D, n = 3). Seven days of Dox treatment in female Krt-rtTA/tetO-Shh mice or male wild-type C57BL/6 mice 3 or 90 days after IR had no significant effects on IR-induced decrease of saliva flow rate and expression of Aqp5 and Chrm3 (Supplementary Fig. S2, P > 0.05, n = 5), indicating that the rescue effects of Dox induction in male Krt-rtTA/tetO-Shh mice was mediated by efficient Hh activation.

Preservation of salivary stem/progenitor cells and Bmi1 signaling pathway after IR by transient Hh activation

SSPCs can be identified by the expression of surface markers c-Kit and Sca-1 (16). Flow cytometric analysis

Figure 2. Transient Hh activation rescued radiation-induced hyposalivation and expression of acinar markers. A, the stimulated whole saliva flow rate in male Krt5-rtTA/tetO-Shh mice irradiated (IR) or IR and induced with Dox for 7 days starting from day 0, 3, or 90 after IR (IR + Dox D0, D3, or D90) were standardized with the body weight and normalized to that of the NT group (n = 5). B–D, 120 days after IR, SMG samples were collected and analyzed with qRT-PCR (B and C) and Western blot (D) for the expression of the acinar markers Aqp5 and Chrm3 normalized to Gapdh (n = 3).

A: *P < 0.05; **P < 0.01.
found that the population of c-Kit\(^+\)/Sca-1\(^+\) cells was very rare in nontreated SMGs, but was significantly expanded after 7 days of Hh activation (Fig. 3A, 0.85% vs. 0.06% in the NT group, mean ± SEM, \(P < 0.05\), \(n = 3\)). However, 60 days after Hh activation, the percentage of c-Kit\(^+\)/Sca-1\(^+\) cell population was not significantly affected compared with control mice (Supplementary Fig. S3), indicating that the effect of Hh activation on expansion of SSPCs is transient. The rarity of c-Kit\(^+\)/Sca-1\(^+\) cells in NT SMGs made it difficult to evaluate the effects of IR and transient Hh activation after IR on this cell population. Therefore, we examined the number of salisphere-forming cells per gland (Fig. 3B, \(n = 3\)) that reflects the number of functional SSPCs, and it was significantly reduced several days after IR (15). Shh overexpression for 7 days in male mice increased the numbers of salisphere-forming cells per SMG significantly (\(P < 0.05\)), consistent with the expansion of the c-Kit\(^+\)/Sca-1\(^+\) cell population. IR decreased salisphere numbers by day 10 as expected, whereas Hh activation 3 days after IR significantly ameliorated IR-induced decrease of salisphere numbers (\(P < 0.05\)). Consistently, as indicated by qRT-PCR analysis of SMG tissues (Fig. 3C, \(n = 3\)), the expression of c-Kit mRNA was significantly upregulated by transient Hh activation, decreased by IR on both days 10 and 120, recovered on days 10 and 120 by transient Hh activation 3 days after IR, and upregulated on day 120 by transient Hh activation 90 days after IR (\(P < 0.05\)). In contrast, the expression of the salivary progenitor cell marker Ascl3 (17) was not significantly affected by Hh activation or by IR on day 10, but was significantly decreased on day 120 after IR, and recovered by transient Hh activation 3 or 90 days after IR (\(P < 0.05\)). Consistent with the reported impairment of proliferation by IR (18), the expression of proliferation-related genes Prc1 and Top2a (19) was significantly decreased by IR (\(P < 0.05\)). Transient Hh activation did not significantly affect the expression of proliferation...
markers Prc1, Top2a, and the proliferative cell nuclear antigen (PCNA) in nonirradiated SMGs (Fig. 3C and Supplementary Fig. S4A, P > 0.05), but significantly induced recovery of the expression of Prc1 and Top2a decreased by IR (Fig. 3C, P < 0.05).

IR-induced apoptosis in salivary glands peaked on days 2 to 3 after IR and decreased significantly thereafter (11, 20), which may have contributed to SSPCs loss. Hh signaling is antiapoptotic in most circumstances (21). However, the Tunel assay (Supplementary Fig. S4B) indicated that, on days 7 and 11 after IR, Hh activation from day 3 did not significantly affect the IR-induced apoptosis, suggesting that the beneficial effects of post-IR transient Hh activation is not likely associated with the inhibition of apoptosis. Interestingly, although concurrent transient Hh activation (days 0–7) significantly inhibited apoptosis on day 3, it significantly increased apoptosis on days 7 and 11, which may contribute to the much less improvement of salivary function in this group. The delayed increase of apoptosis on days 7 and 11 in the concurrent Hh activation group might be associated with increased Ptch1 expression that is proapoptotic in the absence of Hh ligands (22) and decreased expression of the antiapoptosis protein p21Waf1 (23) after Hh activation as shown further.

The Hh target gene Bmi1 is associated with the maintenance of various adult epithelial stem cells through repression of cyclin-dependent kinase (CDK) inhibitors p16Ink4a/p19Arf and p21Waf1 (24–26). As indicated by qRT-PCR analysis (Fig. 3C, n = 3), the expression of Bmi1 mRNA in SMGs was significantly upregulated by transient Hh activation, decreased by IR on day 10 but not on day 120, and recovered by transient Hh activation 3 days after IR (P < 0.05). Meanwhile, the expression of p16Ink4a and p21Waf1 mRNA was regulated conversely to that of Bmi1 by Hh activation and IR, and remained at a high level on day 120 after IR (P < 0.05). Flow cytometric analysis (Fig. 3D, n = 3) confirmed that the Bmi1+/− cell population in SMGs was significantly increased after 7 days of Hh activation (1.95% ± 0.23% vs. 1.02% ± 0.07% in the NT group, P < 0.05), decreased on day 10 after IR (0.22% ± 0.06%, P < 0.05 vs. NT), and was preserved by Hh activation 3 days after IR (0.74% ± 0.22%, P > 0.05 vs. NT). Western blot analysis confirmed that the expression of p21Waf1 in SMGs was significantly increased by IR on day 120 but reversed by transient Hh activation 3 or 90 days after IR (Fig. 3E, P < 0.05, n = 3). In addition, flow cytometry indicated that Bmi1 is expressed in a subpopulation of c-Kit+ SSPCs that is expanded by Hh activation (Supplementary Fig. S5). These data indicated that the rescue of SSPC maintenance after IR by Hh activation is related to the Bmi1 pathway.

Preservation of parasympathetic innervation, Chrm1–HB-EGF signaling and expression of neurotrophic factors after IR by transient Hh activation

Parasympathetic innervation is essential for both organogenesis and regeneration of salivary glands after duct ligation (27, 28), whereas IR reduced parasympathetic innervation in the salivary glands of both human adults and mouse embryos (29). By examining the activity or expression of two markers of parasympathetic nerve, AChE and glial cell line-derived neurotrophic factor family receptor α2 (GFRα2; Fig. 4A and B, n = 3), we confirmed that IR impaired parasympathetic innervation similarly in adult mouse SMGs on day 120 (P < 0.01). The impairment of AChE activity by IR was significantly ameliorated by transient Hh activation 3 or 90 days after IR (P < 0.05), whereas GFRα2 expression was remarkably increased by transient Hh activation alone (P < 0.01) and was not significantly decreased in SMGs that underwent transient Hh activation 3 or 90 days after IR compared with that in the NT group (P > 0.05). These data indicated that the parasympathetic innervation is preserved by transient Hh activation after IR.

Hh signaling is known to promote innervation or regeneration of various peripheral nerves via maintenance of

Figure 4. Transient Hh activation preserved the parasympathetic innervation and the expression of neurotrophic factors impaired by IR. A, AChE staining on SMG sections and the quantification of staining intensity. B, Western blot and relative quantification of GFRα2, C, expression of neurotrophic factors and miR-206 examined by qRT-PCR. D, concentrations of Bdnf in whole saliva or SMG homogenates and that of SMG Ntrn detected by ELISA. n = 3 for all studies.
neural structure (30), induction of angiogenesis (31), or induction of neurotrophic factors such as brain-derived neurotrophic factor (Bdnf) via repression of miR-206 or other unknown mechanisms (32, 33). Bdnf, Nerve growth factor (Ngf), and Neurturin (Nrtn) are expressed in salivary glands (34), and are essential for parasympathetic innervation in embryonic mouse SMGs (29) or other tissues (35, 36). As indicated by qRT-PCR (Fig. 4C, n = 3), transient Hh activation significantly increased miR-206 expression and decreased Bdnf expression, whereas IR significantly increased miR-206 expression and decreased Bdnf expression both on days 10 and 120, and transient Hh activation after IR significantly reversed the effects of IR on expression of miR-206 and Bdnf on day 7 or 30 after Hh activation (P < 0.05). The expression of Ngf and Nrtn was only significantly decreased by IR on day 10 or 120, respectively (P < 0.05). Similar to Bdnf, the expression of Ngf and Nrtn was significantly upregulated by transient Hh activation, and restored or elevated by transient Hh activation after IR on day 10 or 120, respectively (P < 0.05). ELISA confirmed that the Bdnf level in SMG homogenates was significantly increased by transient Hh activation, whereas the levels of Bdnf and Nrtn in SMG homogenates and whole saliva Bdnf were significantly decreased on day 120 after IR and restored by transient Hh activation either 3 or 90 days after IR (Fig. 4D, P < 0.05, n = 3).

During salivary organogenesis, parasympathetic innervation signals via a cholinergic receptor muscarinic 1 (Chrm1) to promote epithelial morphogenesis and proliferation of progenitor cells by transactivating the Heparin-Binding EGF (HB-EGF) pathway (27), which activates the expression of genes associated with cell-cycle progression such as cyclin A2 (Ccna2; ref. 37). Chrm1 expression is repressed by miR-107 (38), which is upregulated by IR (39) but decreased in cells expressing high level of Shh (40). With qRT-PCR analysis (Fig. 5A, n = 3), we found that, in mouse SMGs, the expression of miR-107 was significantly decreased by transient Hh activation and increased by IR as expected and recovered by transient Hh activation after IR within 7 or 30 days (P < 0.05); consistently, the expression of Chrm1 and Ccna2 was significantly increased by transient Hh activation, decreased by IR, and recovered by transient Hh activation after IR (P < 0.05). Flow cytometry assay (Fig. 5B, n = 3) indicated that the proportion of Chrm1+ cells in SMGs was significantly decreased after transient Hh activation (6.79% ± 1.55% vs. 1.63% ± 0.24% in the NT group, P < 0.05), decreased on day 10 after IR (0.07% ± 0.03%, P < 0.05 vs. NT), and was preserved by Hh activation 3 days after

Figure 5. Transient Hh activation preserved the activity of the Chrm1 pathway impaired by IR. A, expression of miR-107, Chrm1, and Ccna2 examined by qRT-PCR (n = 3). B, flow cytometric analysis of Chrm1+ cells in SMGs. C, immunofluorescence staining of Chrm1.
IR (1.88% ± 0.21%, \( P > 0.05 \) vs. NT). Immunofluorescent staining for Chrm1 (Fig. 5C) indicated that, in nontreated SMGs, Chrm1 is expressed weakly in acinar structures and strongly in ductal structures; IR significantly downregulated Chrm1 expression particularly in ductal structures, whereas transient Hh activation after IR restored Chrm1 expression. These data suggested that Chrm1–HB-EGF signaling activity was preserved by transient Hh activation after IR, which may contribute to the preservation of functional SSPCs in combination with preserved parasympathetic innervation.

**Effects of IR and transient Hh activation on SMGs of female mice and human salivary epithelial cells**

Sexual dimorphism is well recognized in the salivary glands of rodents, and the SMGs of male rodents contain a unique structure—granular convoluted tubule (GCT)—that is not present in SMGs of human or female rodents (41). To determine whether the data obtained earlier from male mice are gender-specific, we examined the effects of IR and transient Hh activation with a small-molecule Hh agonist SAG (Smoothened Agonist) in SMGs of female mice on the basis of the comparable expression of Smoothened in SMGs between males and females (Supplementary Fig. S6). As indicated by qRT-PCR (Fig. 6A, \( n = 3 \)), in female SMGs, IR affected the expression of genes related to SSPC maintenance (c-Kit, Bmi1, p16, Chrm1 and Ccn2a) on days 10 and 120 and the gene essential for parasympathetic innervation (Bdnf) on day 10 after IR in a manner similar to that in male SMGs (\( P < 0.05 \)). Local delivery of SAG by cannulation efficiently activated the Hh pathway in ductal SMG epithelial cells of female mice as indicated by induction of the Ptc1-lacZ Hh reporter (Supplementary Fig. S6). Similar to that in male SMGs transiently overexpressing Shh, qRT-PCR indicated that SAG treatment significantly upregulated the expression of c-Kit, Bmi1, Bdnf, Chrm1, and Ccn2a, and decreased p16 expression (\( P < 0.05 \)).

To examine the effects of IR and transient Hh activation on human salivary glands, we isolated human salivary epithelial cells from biopsy samples. These cells showed a typical cobblestone morphology of epithelial cells in culture, and most cells expressed the epithelial marker Krt14 and/or the salivary glandular cell marker Aqp5 (Supplementary Fig. S7). Similar to that in mouse SMGs, qRT-PCR (Fig. 6B, \( n = 4 \)) indicated that, in human salivary epithelial cells collected on day 10 after IR, the mRNA expression of Bmi1, BDNF, CHRM1, and CCNA2 was significantly decreased, whereas that of P16 and P21 was significantly increased (\( P < 0.05 \)). These IR-induced changes of gene expression were not significantly affected by infection with control adenovirus encoding GFP (GFP-Ad; \( P > 0.05 \)), but was significantly reversed by infection with adenovirus encoding Gli1 (Gli1-Ad) 3 days after IR (\( P < 0.05 \)). Western blot assay confirmed that the expression of P21\(^{\text{Waf1}}\) protein was upregulated by IR, and this upregulation was not affected by GFP-Ad infection, but was repressed by Gli1-Ad infection (Fig. 6C, \( P < 0.05 \), \( n = 4 \)).

Taken together, these data indicated that the effects of IR and transient Hh activation on SMGs of female mice and cultured human salivary epithelial cells are similar to that in male mice, and suggested that the transient activation of the Hh pathway might restore the expression of genes related to SSPC maintenance and parasympathetic innervation impaired by IR to rescue IR-induced hyposalivation in humans.

**Effects of Shh gene transfer within SMGs on solid tumor**

Because the activation of the Hh pathway in head and neck cancer is linked to poorer outcomes (42), it is important to determine whether transient Hh activation within the salivary gland can affect Hh activity and the growth of such preexisting cancers. Squamous cell carcinoma is the most common head and neck cancer; therefore, we used the mouse SCC VII tumor model to test this possibility. Infecting SCC VII cells in vitro with adenovirus-encoding rat Shh (AdShh) significantly increased the expression of Hh target genes Gli1 and Ptc1 in 7 days (Supplementary Fig. S8A), indicating that these cells are Hh-responsive. Retrograde delivery of AdShh into SMGs of mice carrying subcutaneous SCC VII tumors significantly increased the expression of the rShh transgene and the Hh target genes Gli1 and Ptc1 in SMGs in 7 days (\( n = 4 \), \( P < 0.05 \)), but had no significant

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**Figure 6.** Effects of IR and transient Hh activation on gene expression in SMGs of female mice and human salivary epithelial cells. A and B, gene expression examined by qRT-PCR in SMGs of female C57BL/6 mice 10 or 120 days after IR or in SMGs of female Ptc1-lacZ mice treated with SAG for 3 days (A, \( n = 3 \)), and in human salivary epithelial cells 10 days after IR with or without infection of Gli1 or GFP adenoviruses (B, \( n = 4 \)). C, Western blot and relative quantification of human P21 (\( n = 4 \)).
effect on the expression of these genes in tumors at this time point or on tumor growth with or without IR of tumor throughout the observation period (Supplementary Fig. S8B–D. P > 0.05). Similar results were found when AdShh was delivered into SMGs right after subcutaneous inoculation of 10 times fewer SCC VII cells (Supplementary Fig. S8E). These data indicated that local Hh activation could be achieved in SMGs without promoting the growth of preexisting tumors outside the salivary glands.

Discussion

The loss of functional SSPCs is believed to be a major cause of IR-induced hyposalivation (4). We reported here that transient Hh activation promotes SSPC expansion without IR and rescues SSPC maintenance after IR. Hh signaling regulates adult stem/progenitor cells upstream of the Bmi1 pathway in other epithelial tissues (24, 43). Bmi1 is a transcriptional repressor that regulates stem cell self-renewal through the repression of important cell–cycle-regulatory genes including p16ink4a, p15ink4b, and p21waf1 (24–26). Our data indicated that, in the salivary glands, the expression of Bmi1 and its target genes is highly associated with changes of the functional SSPC population caused by IR and transient Hh activation, suggesting that the Bmi1 pathway may mediate the effect of Hh activation on SSPC maintenance.

In human salivary glands (29), Hh signaling can protect as well as promote the recovery of various peripheral nerves from injuries (30–32). We showed here that, in adult mouse SMGs, the expression of multiple neurotrophic factors, the parasympathetic innervation, and the Chrm1–HBEGF signaling pathway connecting the parasympathetic innervation to the maintenance of stem/progenitor cells were significantly impaired by IR and rescued by transient Hh activation after IR, which may also contribute to IR-induced hyposalivation and the rescue by Hh activation. The mechanisms of regulation by IR and Hh activation on the expression of Bdnf and Chrm1 were related with miR-206 and -107 respectively, but other mechanisms and those on the expression of Ngn and Ntrn remain to be explored.

Although it was reported that aberrant regulation of Hh signaling is associated with tumorigenesis of head and neck cancer (44), 7-day induction of Shh overexpression in basal epithelia in the present study led to Hh activation comparable with that observed after duct ligation and did not result in any detectable tumor in mice for up to 4 months after induction. Prolonged Hh activation for 15 weeks in salivary glands by GlI1 transgene resulted in hyperplasia, but these lesions regressed after withdrawal of transgene expression and became histologically normalized (45). These data indicated that the probability of inducing cancer in adult salivary glands by transient Hh activation at near-physiologic levels is likely very rare. For potential application in survivors of head and neck cancer, great caution should be exercised to only activate the Hh pathway in the salivary gland to eliminate the risk of prompting growth, relapse, or metastasis of preexisting cancers. Advantageously, salivary glands are well encapsulated, and local gene/protein/drug delivery can be easily achieved by cannulation via the orifices in the mouth. Previous reports and our preliminary data have indicated that transgenes delivered by this method would neither significantly increase levels of transgenes and/or protein products systemically nor the growth of preexisting tumors (13).

Interestingly, in SMGs of female mice, transient Shh overexpression failed to activate the Hh pathway efficiently and rescue the IR-induced hyposalivation, which is related to the lower expression of both endogenous components of Hh pathway and Shh transgene. For future study with female mice and, probably, other animal models, such gender differences should be considered and a higher dose or an alternative agonist may be needed to achieve efficient Hh activation in female salivary glands. In SMGs of female mice and human salivary epithelial cells from female patients, transient Hh activation was achieved with other approaches and similarly regulated expression of genes associated with SSPCs and parasympathetic innervation, suggesting that the transient Hh activation may also rescue IR-induced hypofunction in females. Even in the worst scenario that this strategy only works in males, it will still be of significant importance because approximately two-thirds of the patients with head and neck cancer are male (1, 2).

In summary, we have been able to rescue salivary gland function by transient activation of Hh signaling in a mouse model. The mechanism is associated with the preservation of functional stem/progenitor cells and parasympathetic innervation. We observed similar rescue effects on the expression of genes related to both aspects in cultured human salivary epithelial cells, suggesting the potential of transient Hh activation in treating IR-induced hyposalivation in human patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: B. Hai, F. Liu
Development of methodology: B. Hai, L. Qin, F. Liu
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