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Concurrent Transient Activation of Wnt/ β -catenin Pathway Prevents Radiation Damage to Salivary Glands

Bo Hai, D.D.S.¹, Zhenhua Yang, D.D.S.¹, Lei Shangguan, M.D.¹, Yanqiu Zhao, M.D.¹, Arthur Boyer, Ph.D.², and Fei Liu, Ph.D.^{1,*}

¹Institute for Regenerative Medicine at Scott & White, Molecular and Cellular Medicine Department, Texas A&M Health Science Center, Temple, Texas 76502, USA

²Departments of Radiology, Scott and White Hospital, Temple, Texas 76502, USA

Abstract

Purpose—Many head-and-neck cancer survivors treated with radiotherapy suffer from permanent impairment of their salivary gland function, for which few effective prevention or treatment options are available. This study explores the potential of transient activation of Wnt/ β -catenin signaling in preventing radiation damage to salivary glands in a preclinical model.

Methods and Materials—Wnt reporter transgenic mice were exposed to 15 Gy single dose radiation in head and neck area to evaluate the effects of radiation on Wnt activity in salivary gland. Transient Wnt1 over-expression in basal epithelia was induced in inducible Wnt1 transgenic mice before, in together with, after or without local radiation, then saliva flow rate, histology, apoptosis, proliferation, stem cell activity and mRNA expression were evaluated.

Results—Radiation damage did not significantly affect activity of Wnt/ β -catenin pathway as physical damage did. Transient expression of Wnt1 in basal epithelia significantly activated Wnt/ β -catenin pathway in submandibular glands of male mice but not in those of females. Concurrent transient activation of Wnt pathway prevented chronic salivary gland dysfunction following radiation by suppressing apoptosis and preserving functional salivary stem/progenitor cells. In contrast, Wnt activation 3 days before or after irradiation did not show significant beneficial effects mainly due to failure to inhibit acute apoptosis after radiation. Excessive Wnt activation before radiation failed to inhibit apoptosis likely due to extensive induction of mitosis and up-regulation of proapoptosis gene *Puma*, while that after radiation might miss the critical treatment window.

Conclusion—These results suggest that concurrent transient activation of Wnt/ β -catenin pathway could prevent radiation-induced salivary gland dysfunction.

Keywords

Salivary gland; Xerostomia; Radiation; Wnt/ β -catenin pathway; Stem cells

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*Corresponding author. Address: 5701 Airport Rd., Module C, Temple, Texas 76502, USA. Tel: +001 254 771 6813, Fax: +001 254 771 6839, fliu@medicine.tamhsc.edu.

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Introduction

Head and neck cancer (HNC) is the fifth most common cancer with 49,260 estimated new cases in 2010 in USA. Radiation therapy is the most common form of treatment for HNC, and nondiseased salivary glands are often exposed to radiotherapy. Due to the exquisite radiosensitivity of salivary glands, irreversible hyposalivation is common (60%–90%) in HNC survivors treated with radiotherapy (1). Hyposalivation exacerbates dental caries and periodontal disease, and causes mastication, swallowing problems, a burning sensation of the mouth, and dysgeusia, which severely impair the quality of life of patients. The irreversible hyposalivation is caused by loss or impairment of serous acinar cells and replacement by connective tissue and fibrosis, which has been attributed to loss of functional glandular stem/progenitor cells that normally continuously replenish aged saliva producing cells (2). Recent research in mouse models indicated that apoptosis of epithelial cells is important in radiation-induced acute salivary gland hypofunction (3), and may contribute to the loss of functional salivary stem/progenitor cells.

Current treatments for dry mouth, such as artificial saliva and saliva secretion stimulators, can only temporarily relieve these symptoms. Several new approaches, including gene transfer and stem cell infusion, showed promises to restore salivary gland function by protection or regeneration of saliva-producing cells (4). Meanwhile, despite decades of preclinical and clinical studies, no safe and effective drug or other approach is available to prevent radiation-induced xerostomia.

Wnt/ β -catenin signaling is essential for maintenance and activation of various adult stem cells. We found recently that this pathway is activated during functional regeneration of submandibular gland (SMG) after ligation-deliigation of main excretory duct, and its forced activation in basal epithelia promoted expansion of salivary stem/progenitor cells (5). On the other hand, Wnt/ β -catenin signaling can promote radiation resistance of mouse mammary gland progenitor cells by activating expression of antiapoptosis gene *survivin* (6). We report in this study that radiation damage does not activate Wnt/ β -catenin pathway in SMG as physical damage does, and concurrent transient activation of Wnt/ β -catenin pathway in basal epithelia prevents both acute and chronic hyposalivation through inhibition of apoptosis and perseveration of functional salivary stem/progenitor cells. These data support the hypothesis that transient activation of Wnt/ β -catenin pathway can significantly benefit salivary gland function following radiotherapy of head-and-neck cancer.

Materials and Methods

Mice

Wnt activity was analyzed using *B6.Cg-Tg(BAT-lacZ)3Picc/J(BAT-gal)* Wnt reporter as reported (5). Mice carrying *tetO-Wnt1* and *KRT5-rtTA* transgenes (7) were placed on doxycycline chow (1g/kg, Bio-serv, Laurel, MD) to induce Wnt1 expression. In each experiment, more than 5 animals are used for each group. All animal procedures were performed under a protocol approved by Texas A&M Health Science Center and Scott & White Hospital IACUC committee.

Radiation

6–10 weeks old BAT-gal or Krt5-rtTA/tetO-Wnt1 mice were anesthetized with Xylazine/Ketamine mixture (4mg/ml Xylazine mixed with 30mg/ml Ketamine, 4ml/kg, intraperitoneally), and the head-and-neck region was exposed to a 6 MeV electron beam produced by a medical linear accelerator (Clinac 2100C, Varian Medical Systems, Palo Alto, California) and collimated and calibrated for irradiation of mice (See details in Supplementary materials).

Saliva collection

At 30, 60, and 90 days after irradiation or doxycycline induction, the stimulated saliva flow rate was determined as reported (8) and standardized with the body weight, then normalized to that of gender-matched non-treated (NT) littermates.

Histology, immunofluorescence and TUNEL assay

SMG paraffin sections were stained with Periodic acid-Schiff (PAS), or with antibodies against PCNA (1:1000, Abcam, MA) then visualized with Texas-Red labeled second antibodies. Apoptosis was examined with Fluorescein In Situ Cell Death Detection Kit (Roche) based on TUNEL method. Surface areas occupied by acinar cells were quantified as reported (5). TUNEL or PCNA positive cells were quantified as percentage of positive areas in DAPI positive areas with NIS-Elements AR software.

Quantitative RT-PCR Analysis

Quantitative RT-PCR (qPCR) were done as reported (5) with primers for *GADPH*, *Axin2*, *lacZ* (5), *Puma* (3), *Survivin* (6), *Aqp5*, *Prc1*, *Top2a* and *Bax* (<http://pga.mgh.harvard.edu/primerbank>).

Determination of functional Stem/Progenitor Cell Numbers

7 days after IR with or without doxycycline induction, SMGs were extirpated and processed for spherical culture and salisphere counting as reported (8).

Statistics

All quantified data were analyzed using one-way ANOVA followed by Tukey's multiple-comparison test. Statistical analysis and graphical generation of data were done with GraphPad Prism software (San Diego, CA).

Results

Radiation damage does not activate Wnt/ β -catenin signaling in submandibular gland of both male and female mice

Our previous study demonstrated that Wnt/ β -catenin signaling activity was marginal in adult submandibular gland (SMG), but was remarkably elevated during regeneration of SMG after ligation of main excretory duct, and forced activation of Wnt/ β -catenin pathway promoted expansion of salivary stem/progenitor cells (5). In this study, apoptosis in SMG was significantly increased by 15Gy single dose irradiation in head and neck region (IR) in males on Day3, but was only slightly increased on other time points examined and in females (Fig. 1A–I). The apoptosis in female parotid after IR peaked on Day2 and decreased significantly from Day3 (3), it's likely that the apoptosis in female SMG mainly happens in a similar pattern. Despite the significant apoptosis after IR, the mRNA expression of Wnt target gene *Axin2* and the reporter gene *lacZ* in SMGs of both male and female BAT-gal Wnt reporter mice was not significantly affected by IR except a significantly decrease in males by Day14 (Fig. 1J,K). These results indicated that Wnt/ β -catenin pathway is not activated by radiation damage of salivary glands despite remarkable induction of apoptosis.

Wnt/ β -catenin signaling is activated in ductal cells by transient Wnt1 expression in male but not female SMG

To test whether activation of Wnt/ β -catenin pathway can facilitate functional recovery of salivary gland after radiation, we used transgenic mice based on a tet-on system for inducible expression of Wnt1, a canonical Wnt ligand. The expression of Wnt1 is controlled

by a tetO (tetracycline-Operator) promoter, and is only activated in the presence of both reverse tetracycline-controlled Transcriptional Activator (rtTA) fusion protein and doxycycline (Dox). We used a Keratin5 (KRT5) promoter to drive expression of rtTA in the basal layer of all stratified epithelia including that in SMG (5). After Dox induction, expression of *Axin2* mRNA in SMG was significantly elevated in male KRT5-rtTA/tetO-Wnt1 mice, but not in females (Fig. 2A), indicating that transient Wnt1 expression in basal epithelia only efficiently activated Wnt/ β -catenin pathway in SMGs of male mice. Consistently, cellular proliferation, a major consequence of Wnt activation, was only significantly increased in SMGs after induction in males but not females as shown by PCNA staining and expression of proliferation associated genes, *Prc1* and *Top2a* (9) (Fig. 2B–D). To identify cell populations responding to Wnt signaling in SMG, *Axin2*-lacZ Wnt reporter were introduced into Krt5-rtTA/tetO-Wnt1 mice. X-gal staining indicated that in SMG without Dox induction, lacZ-positive Wnt-responsive cells were very rare and only occasionally found in large ducts (Fig 2E), consistent with previous finding in BAT-gal mice (5); after 3 days of Wnt1 induction, numerous ductal cells but only a few acinar cells were lacZ-positive in SMG of male mice (Fig 2F), while there was no significant changes of lacZ expression in SMG of females (data not shown). Consistently, in induced male SMGs, PCNA⁺ proliferating cells increased remarkably in ductal cells and to a much less extent in Aqp5⁺ acinar cells (Fig 2G,H), and some ductal PCNA⁺ cells were also positive for Sca-1, a putative maker of salivary progenitor cells (10), while no proliferating Sca-1⁺ cell was observed in control SMGs (Fig 2I,J).

Concurrent transient activation of Wnt/ β -catenin pathway prevents radiation-induced hyposalivation in male mice

To evaluate the potential of transient activation of Wnt/ β -catenin pathway in ameliorating radiation-induced hyposalivation, 6–10 weeks old male and female KRT5-rtTA/tetO-Wnt1 mice were induced with Dox for 7 days either in together with, 3 days before or after, or without 15 Gy single dose irradiation in head and neck region (IR). For concurrent Wnt1 induction, dox food was given 8–10 hours before radiation to allow expression of Wnt1 protein and avoid interference of anesthesia on food intake after IR. IR decreased saliva flow rate significantly on Day30, 60 or 90 after IR and mRNA expression of acini marker *Aqp5* on Day90 after IR in SMGs of both gender (Fig. 3A,B,D,E, $p < 0.05$). Only concurrent transient Wnt activation (IR+Dox D0) in males significantly improved saliva flow rate and *Aqp5* expression after IR (Fig. 3A,B, $p < 0.05$); while no significant improvement of saliva flow rate or *Aqp5* expression after IR were found in other male IR+Dox groups and in all female IR+Dox groups compared with corresponding IR only group (Fig. 3A,B,D,E, $p > 0.05$). The body-weights of all mice were measured before saliva collection to standardize the saliva flow rate. The increases of body-weight in male KRT5-rtTA/tetO-Wnt1 mice appears to be stopped by radiation, while concurrent Wnt activation allowed continuous body weight increase, but the differences were not statistically significant (Fig. 3C, $p > 0.05$). The failure of concurrent Wnt1 expression in females to protect salivary function is consistent with the insufficient Wnt activation in SMGs of females (Fig. 2), and excluded the possibilities that other functions of Wnt1 protein such as non-canonical Wnt signaling or Dox food itself may prevent radiation-induced hyposalivation. To further exclude the effect of Dox food in male mice, we similarly radiated non-transgenic C57BL/6 mice, the genetic background of KRT5-rtTA/tetO-Wnt1 mice, with or without concurrent Dox treatment for 7 days, and no significant improvement in saliva flow rate or *Aqp5* expression was found in Dox treated group (Fig. 3F,G).

Concurrent Wnt activation prevents radiation-induced apoptosis in male mice

Radiation-induced apoptosis correlates with parotid dysfunction in female mouse models (3). In SMGs of male mice, radiation induced significant apoptosis (Fig. 1A–D, I), activated

expression of pro-apoptosis genes *Bax* and *Puma*, and down-regulated expression of *Survivin*, an apoptosis inhibitor (Fig. 4A–D). Concurrent Wnt activation efficiently suppressed IR-induced apoptosis, remarkably inhibited activation of *Bax* and *Puma* by IR, and significantly upregulated *Survivin* expression (Fig. 4A–D, $p < 0.01$). In contrast, Wnt activation 3 days before irradiation did not inhibit apoptosis (Fig. 4A $p > 0.05$); likely due to elevated mitosis of salivary cells including some ductal cells expressing putative progenitor marker Sca-1 (Fig. 2B–J). In addition, although pre-IR Wnt activation inhibited radiation-induced *Bax* expression, it failed to upregulate *Survivin* and remarkably up-regulated expression of *Puma*. In male KRT5-rtTA/tetO-Wnt1 mice, 3 days of Dox induction alone significantly upregulated *Survivin* expression without significant effects on apoptosis and expression of *Bax* or *Puma* (Fig. 4A–D), but Dox treatment itself didn't affect *Survivin* expression in non-transgenic C57BL/6 mice or female KRT5-rtTA/tetO-Wnt1 mice in which Wnt/ β -catenin signaling was not significantly activated (data not shown), consistent with the report that *Survivin* is a direct target gene of Wnt/ β -catenin pathway (11).

Concurrent Wnt activation preserves salisphere-forming cells and putative stem cell marker expression after radiation in male mice

We found previously that induced mutation of β -catenin into a stabilized form promoted expansion of salivary stem/progenitor cells (5). Consistently, Wnt1 over-expression for 7 days in SMGs of male mice expanded functional salivary stem/progenitor cells indicated by numbers of salispheres formed, and significantly upregulated expression of putative stem cell marker *Lgr5* in SMGs (Fig. 5A–B, $p < 0.01$). Radiation decreased salisphere numbers by Day7 (Fig. 5A, $p < 0.01$), consistent with the reported significant decrease of the number of salisphere-forming cells on Day4 after IR (8). Concurrent Wnt activation significantly ameliorated IR-induced decrease of salisphere numbers ($p < 0.01$), and up-regulated *Lgr5* expression; while Wnt activation 3 days before or after IR did not significantly improve either indexes (Fig. 5A–B, $p > 0.05$).

Concurrent Wnt activation prevent chronic impairment of homeostasis of acinar cells after radiation in male mice

Radiation decreases proliferation rate of parotid acinar cells chronically (12). Consistently, PCNA positive proliferating cells in SMG acinar cells were also significantly decreased by 90 days after radiation, while concurrent Wnt activation significantly improved proliferation (Fig. 6A, $p < 0.05$). *Ascl3* is a marker for active proliferating progenitors in salivary glands (13). By 90 days after radiation, expression of *Ascl3* mRNA in SMG was significantly down-regulated by radiation alone, but up-regulated by concurrent transient Wnt activation (Fig. 6B, $p < 0.01$), consistent with the change of proliferation. Wnt activation 3 days before or after IR did not significantly affect either indexes compared with that of IR alone (Fig. 6A–B, $p > 0.05$). These data suggest that concurrent transient Wnt activation may influence the overall tissue homeostasis after IR by expanding active progenitor cells to prevent chronic loss of function.

Discussion

We report here that concurrent transient activation of Wnt/ β -catenin pathway significantly prevents radiation-induced hyposalivation. The underlying mechanisms of this beneficial effects include inhibition of apoptosis and preservation of functional stem/progenitor cells. Inhibition of radiation-induced acute apoptosis seems essential for beneficial effects of Wnt activation, since Wnt activation 3 days after radiation does not prevent hyposalivation. Besides up-regulation of *Survivin* expression, Wnt/ β -catenin signaling may also inhibit apoptosis in SMG by direct inhibition of Glycogen synthase kinase 3β (GSK- 3β) activity via phosphorylated Wnt co-receptor LPR6 (14). GSK 3β promote radiation-induced apoptosis by

inhibiting pro-survival transcription factors and facilitating proapoptotic transcription factors, and GSK-3 β inhibitors were shown to protect irradiated small intestine epithelium and hippocampal neurons from apoptosis (15). Other mechanisms may also contribute to the beneficial effects of Wnt activation: radiation damages to blood supply, innervation and other systemic effects are all potential causes of hyposalivation, and Wnt activation is known to promote angiogenesis and innervation during development. In our experiment, lower part of head is also radiated and the damages to oral mucosal and incisors may also contribute to deterioration of the salivary function by affecting chewing activities. Hence the prevention of radiation-induced oral mucositis by Wnt activation (16) may also contribute to the prevention of radiation-induced hyposalivation. Only about 30% of radiated mice lost their lower incisors around 60 days after radiation and were fed on soft diet thereafter, and Wnt activation didn't affect the incidence of incisor loss significantly, which in-together indicated that the prevention of radiation-induced hyposalivation by Wnt activation is not dependent on its effects on teeth loss and consequent diet change.

The failure of pre-IR Wnt activation in inhibiting apoptosis seems due to extensive induction of proliferation and up-regulation of *PUMA* before radiation. The expression of *PUMA*, but not *Bax*, can be selectively activated by interaction between Axin, Daxx and p53 (17). Although Axin expression is not significantly elevated by pre-IR Wnt activation (data not shown), it's likely that Axin2 protein upregulated by Wnt activation may function similarly as Axin to activate *PUMA* expression following radiation-induced p53 activation.

Salivary stem/progenitor cells can be isolated by spherical culture or their abundant expression of general stem cell markers such as c-Kit and Sca-1 (18), but no specific surface marker has been identified. Concurrent Wnt activation preserves functional salivary stem/progenitor cells capable of forming salispheres, consistent with our previous findings that Wnt/ β -catenin signaling promoted expansion of salivary stem/progenitor cells (5). Interestingly, the mRNA expression of *Lgr5*, a marker of intestine epithelial stem cells activated by Wnt signaling (19), is significantly up-regulated in SMG by transient Wnt activation or ligation of main excretory duct (data not shown), suggesting *Lgr5* may be a more specific marker for salivary stem/progenitor cells.

We only examined effects of Wnt activation on radiation-induced hyposalivation in male mice since Wnt1 over-expression failed to activate Wnt/ β -catenin pathway in SMG of female mice. Due to the sexual dimorphism of SMG, the effects of Wnt activation in SMG of female mice need to be examined and we are planning to do so using R-Spondin1 or small molecule Wnt agonists. Other limitations of this study include the utilization of anesthesia for mouse radiation which is not used clinically; and that submandibular glands are responsible for resting saliva flow rate while this study only looked at stimulated flow; and use of single dose radiation instead of fractionated doses in patients, but for effects on salivary function 15 Gy single does is equivalent to 16×2 Gy used clinically (20).

In summary, our data demonstrated that concurrent transient activation of Wnt/ β -catenin pathway preserves salivary gland function after single dose radiation, which is mediated by inhibition of apoptosis and perseveration of functional stem/progenitor cells. To make our findings applicable, further works are needed to confirm this protective effect in fractionated radiation similar to that in clinical settings and to optimize the approach, treatment window and dosage of Wnt activation for this purpose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Wnt/ β -catenin pathway is not activated by radiation damage. Apoptosis of SMG cells before (D0) or 3, 7 or 14 days after 15Gy single dose neck radiation (D3, D7 or D14) was examined by TUNEL staining, and quantified (A-I). Total RNAs were isolated from these SMG samples and relative expression of *Axin2* (J) and *lacZ* (K) was examined by quantitative RT-PCR and normalized to that of male D0 mice. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 2.

Transient Wnt1 expression activates Wnt/β-catenin pathway in salivary glands of male mice but not in those of females. Krt5-rtTA/tetO-Wnt1 mice were induced with Dox for 0, 3 or 7 days, then sacrificed to collect SMGs for qPCR and PCNA staining. Compared with those of Day 0, expression of *Axin2* (A), *Prc1* (B) and *Top2a* (C) mRNA, and fraction of PCNA+ cells (D) were all significantly up-regulated by Dox induction in males, but not significantly (ns, $p > 0.05$) affected in females. Male Krt5-rtTA/tetO-Wnt1/*Axin2-lacZ* mice were induced with Dox for 0 or 3 days and their SMGs were sectioned for X-gal staining (E,F), or double immunofluorescent staining for PCNA and Aqp5 (G,H), or PCNA and Sca-1 (I,J).

Figure 3.

Concurrent transient activation of Wnt/ β -catenin pathway prevents radiation-induced hyposalivation. 30, 60 and 90 days after IR, stimulated whole saliva flow rate in Krt5-rtTA/tetOWnt1 mice with no treatment (NT), radiation only (IR), 7 days doxycycline induction alone (Dox) or plus radiation 3 days later (IR+Dox D-3), concurrently (IR+Dox D0) or 3 days before (IR+Dox D3) was examined, standardized with body weights and normalized to that of NT mice (A,D). Body weights of male Krt5-rtTA/tetO-Wnt1 mice right before or 30, 60 and 90 days after IR were shown in (C). On Day 90, SMG samples were collected for qPCR analysis on relative expression of acinar marker *Aqp5* (B,E). Male wild-type C57BL/6 mice with no treatment (NT), radiation only (IR), or concurrent Dox treatment (IR+Dox D0) were similarly examined for stimulated whole saliva flow rate (F) and *Aqp5* expression (G).

Figure 4. Concurrent Wnt activation represses radiation-induced apoptosis. Krt5-rtTA/tetO-Wnt1 mice were sacrificed on day 3 after IR (IR, IR+Dox D-3 or D0 groups) or Dox induction (Dox group). SMG samples were collected for TUNEL staining (A) and qPCR analysis on relative expression of pro-apoptosis genes *Bax* (B) and *Puma* (C), and anti-apoptosis gene *Survivin* (D).

Figure 5.

Concurrent Wnt activation prevents decrease of salisphere numbers and putative stem cell markers shortly after radiation. Krt5-rtTA/tetO-Wnt1 mice were sacrificed on day 7 after IR (IR, IR+Dox D-3 D0 or D3 groups) or Dox induction (Dox group). SMG cells were cultured for 4 days to count numbers of formed salispheres (A). Relative expression of putative stem cell markers *Lgr5* (B) was analyzed with qPCR.

Figure 6. Concurrent Wnt activation prevents chronic proliferation defects of acinar cells by radiation. Krt5-rtTA/tetO-Wnt1 mice were sacrificed on day 90 after IR or Dox induction. Proliferation in SMG samples was evaluated by PCNA immunofluorescence staining (A). Relative expression of proliferative progenitor cell markers *Ascl3* (B) was analyzed with qPCR.