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Supplemental Information

Wnt-Responsive Stem Cell Fates in the Oral Mucosa

Xue Yuan, Quanchen Xu, Xiaohui Zhang, Lauren A. Van Brunt, Pavla Ticha, and Jill A. Helms

Supplemental figures and figure legends

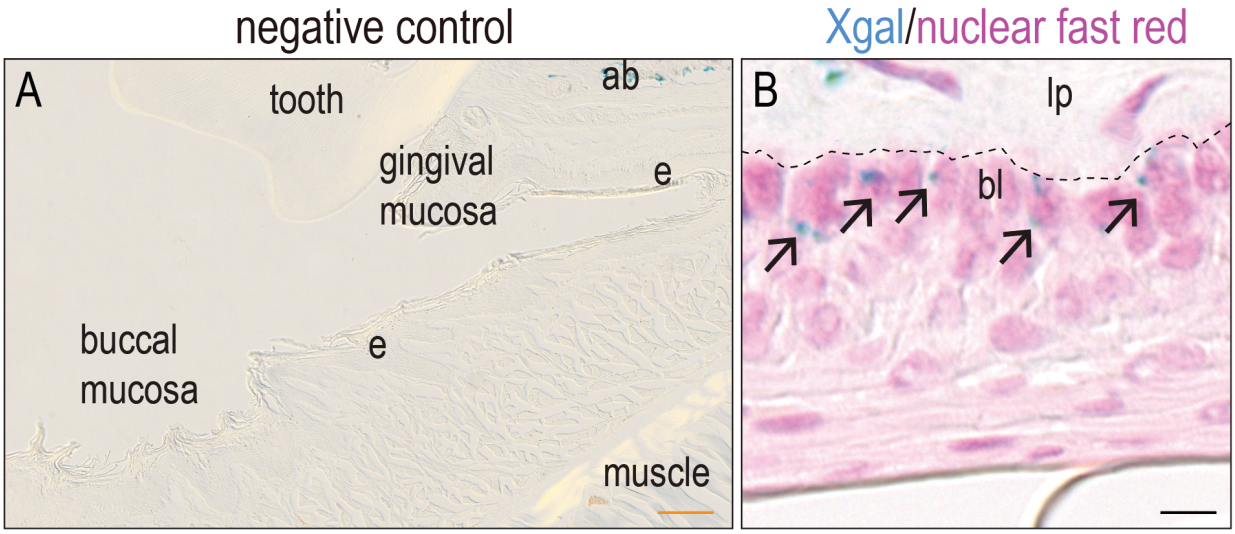

Figure S1. Xgal staining in the oral mucosa. Related to Figure 1. (A) A negative control of Xgal staining in the oral cavity. The section is from the *Axin2*$^{+/+}$ mouse. Non-specific Xgal staining was not evidence in the epithelial layer. Only the alveolar bone area showed non-specific signaling, which is known as osteoclasts. (B) Xgal co-stained with nuclear fast red. Nuclear fast red staining was used to distinguish the epithelial layer from the lamina propria. The dotted line indicates the basement membrane. Arrows indicate the Xgal^{+ve} cells. Abbreviations: ab, alveolar bone; e, epithelial layer; lp, lamina propria, bl, basal layer. Scale bars: orange = 100 μm; black = 10 μm.

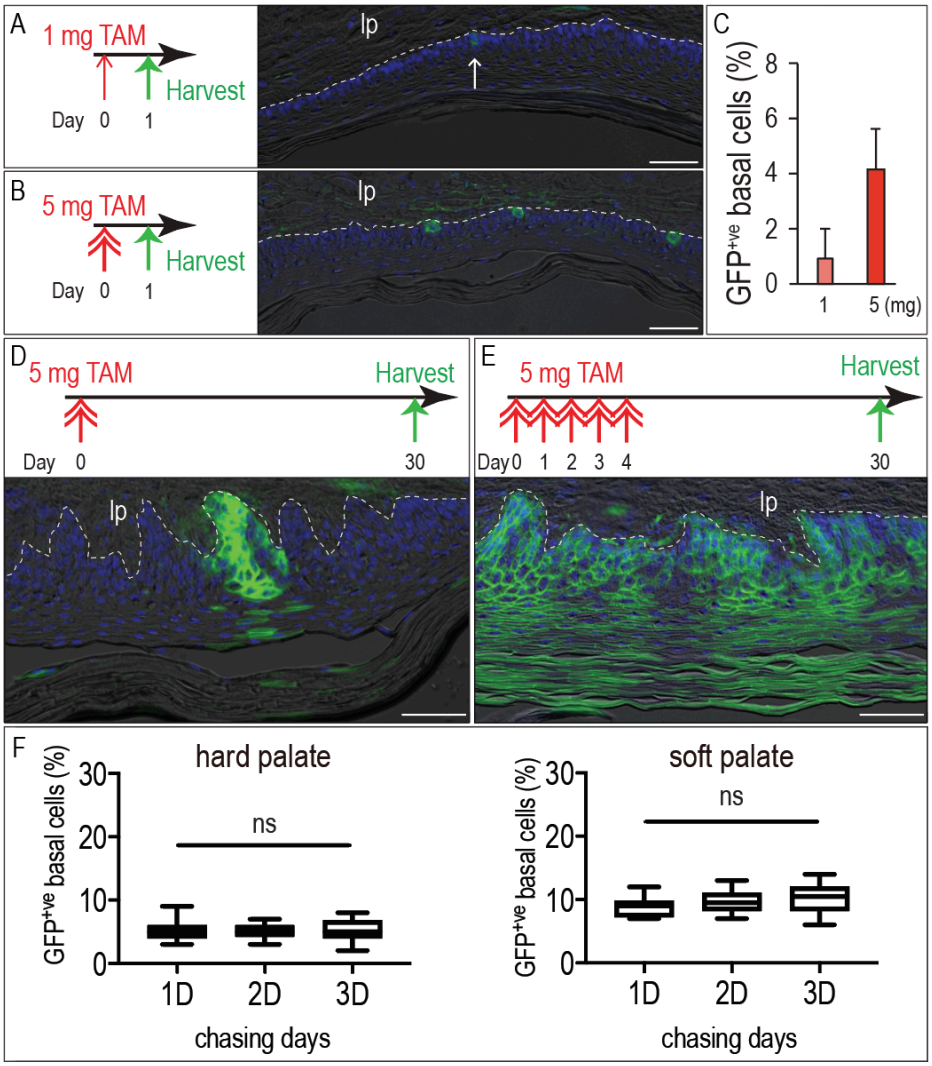

Figure S2. Titration of tamoxifen. Related to Figures 2 and 6. (A) GFP^{+ve} cells in the oral epithelium 1 day (D) after 1mg/25g body weight tamoxifen injection. Arrow indicates the GFP^{+ve} cell. (B) GFP^{+ve} cells in the oral epithelium 1D after 5mg/25g body weight tamoxifen injection. (C) Quantification of GFP^{+ve} basal cells in both groups. (D) GFP^{+ve} cells in the oral epithelium 30D after one-time 5mg/25g body weight tamoxifen injection. (E) GFP^{+ve} cells in the oral epithelium 30D after five-time 5mg/25g body weight tamoxifen injection. This label strategy resulted in a near-complete labeling of all cells in the oral mucosa, which made it impossible to follow the fate of a single clone. (F) Mice were injected with one-time 5mg/25g body weight tamoxifen and the GFP^{+ve} basal cells were quantified on 1D, 2D and 3D chasing from both hard palate and soft palate (n=3, 5 sections per mouse). The GFP^{+ve} basal cells remained the same from 1D to 3D in both tissues, indicating tamoxifen was below the threshold to activate Cre recombination 1D after the injection. Abbreviations: lp, lamina propria; TAM, tamoxifen. Scale bars: 50 μm. Data represent mean ± SD. ns: no significant difference as determined by one-way ANOVA, followed by Tukey's post hoc testing.

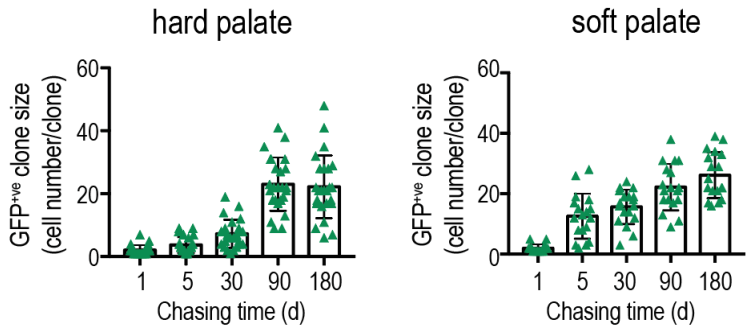

Figure S3. Quantification of GFP^{+ve} cell number in each clone in (A) the hard and (B) soft palates. Related to Figure 2. n=3, 5 sections for each mouse. Data represent mean ± SD.

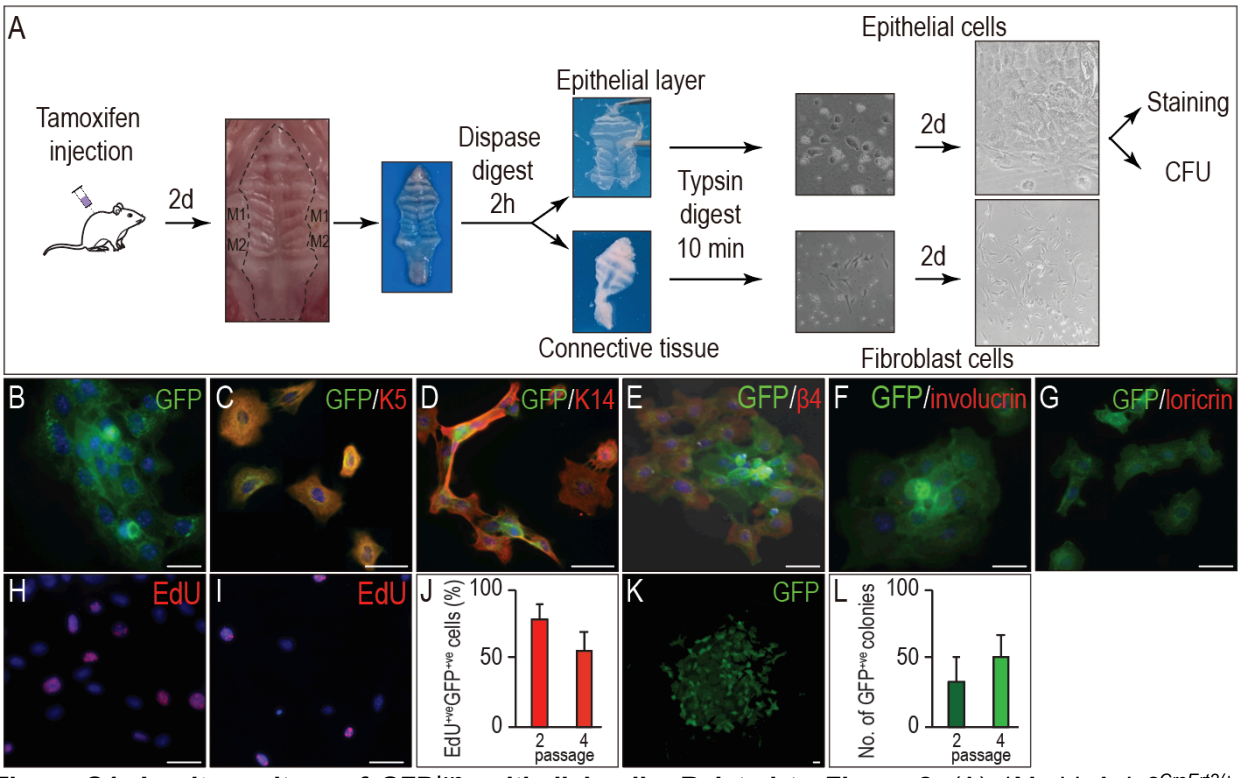

Figure S4. *In vitro* culture of GFP^{+ve} epithelial cells. Related to Figure 2. (A) 1M-old *Axin2$^{CreErt2/+}$; R26$^{mTmG/+}$* mice received tamoxifen two days before sacrifice. The hard and soft palates were isolated and digested with dispase for two hours. After digestion, the epithelial layer and the connective tissue were separated and then digested with trypsin for 10 min. The epithelial cells and the fibroblasts were cultured in plates. The epithelial cells were split once and then subjected for staining and colony-forming unit (CFU) assay. (B) GFP^{+ve} epithelial cells. GFP^{+ve} cells were co-stained with (C) keratin 5, (D) keratin 14, (E) integrin β4, (F) involucrin, (G) loricrin. EdU staining for the GFP^{+ve} cells in (H) 2nd passage and (I) 4th passage. (J) Quantification of the EdU^{+ve} cells. (K) GFP^{+ve} colony in 4th passage. (L) Quantification of colony formed by GFP^{+ve} cells. Scale bars: 25 μm. Data represent mean ± SD.

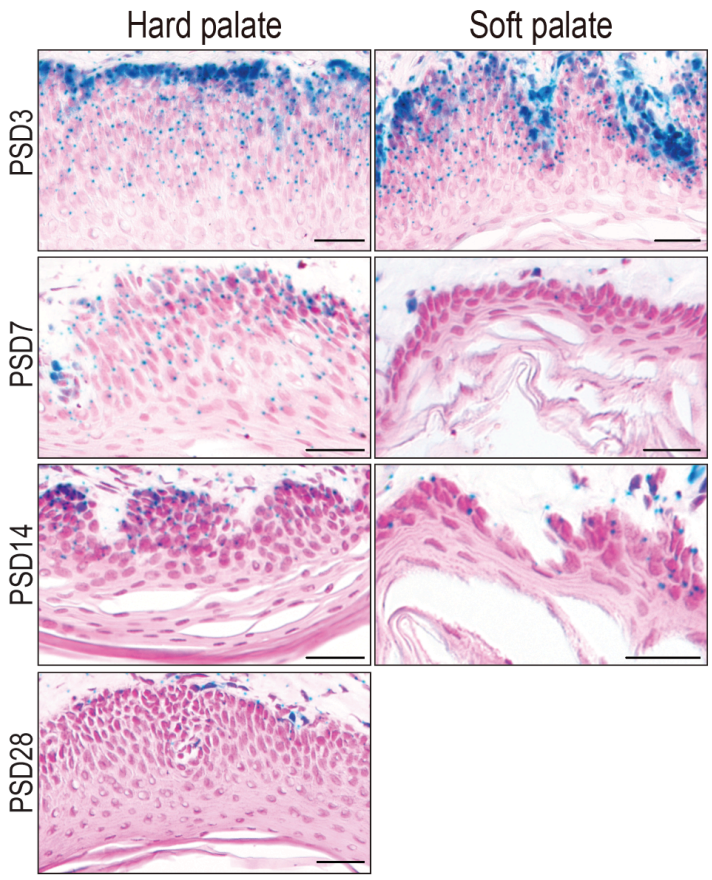

Figure S5. Xgal co-stained with nuclear fast red. Related to Figures 4 and 5. Scale bars: 50 μm.

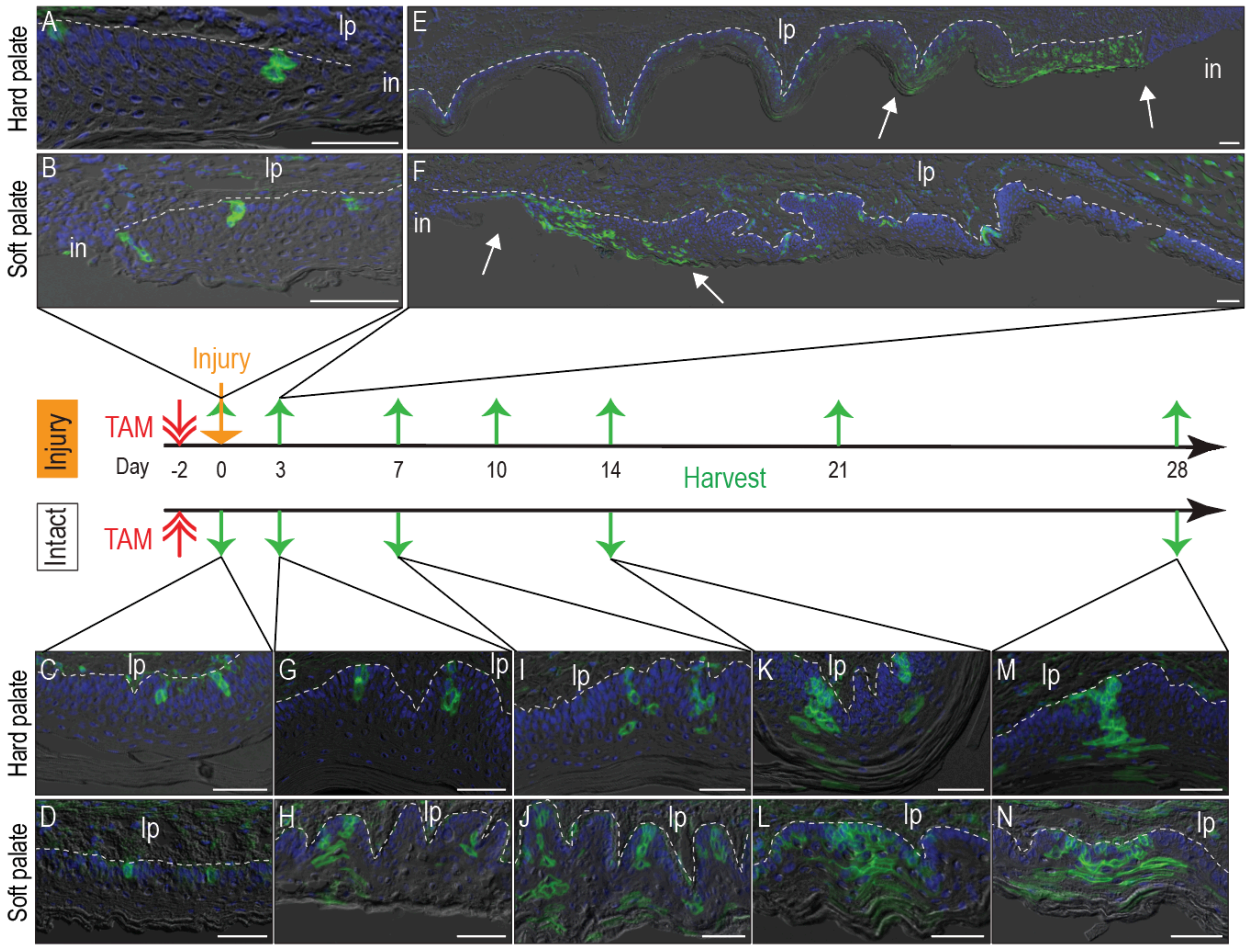

Figure S6. Lineage tracing of GFP^{+ve} Wnt-responsive cells. Related to Figure 6. A schematic diagram showing the lineage tracing strategy. Mice were injected with one-dose of tamoxifen at the age of 3M. For the injury group, a 2 mm full-thickness injury was generated 2D after tamoxifen injection. Wnt-responsive cells were analyzed immediately, 3D, 7D, 10D, 14D and 21D after surgery. After injury (PSD0), GFP^{+ve} Wnt-responsive cells were examined in the remaining (A) hard palate and (B) soft palate. GFP^{+ve} Wnt-responsive cells were also examined in the intact (C) hard palate and (D) soft palate at the same time point. On PSD3, GFP^{+ve} Wnt-responsive cells were examined in the remaining (E) hard palate and (F) soft palate. GFP^{+ve} Wnt-responsive cells were accumulated in the edge as indicated by arrows. Wnt-responsive cells were also examined in the intact (G) hard palate and (H) soft palate at the same time point. For injury group, Wnt-responsive cells on PSD7, 10, 14, 21, and 28 are showing in Figure 6. For the same time point controls, Wnt-responsive cells were shown in I-N. Abbreviations: lp, lamina propria; in, injury. Scale bars: 50 μm.

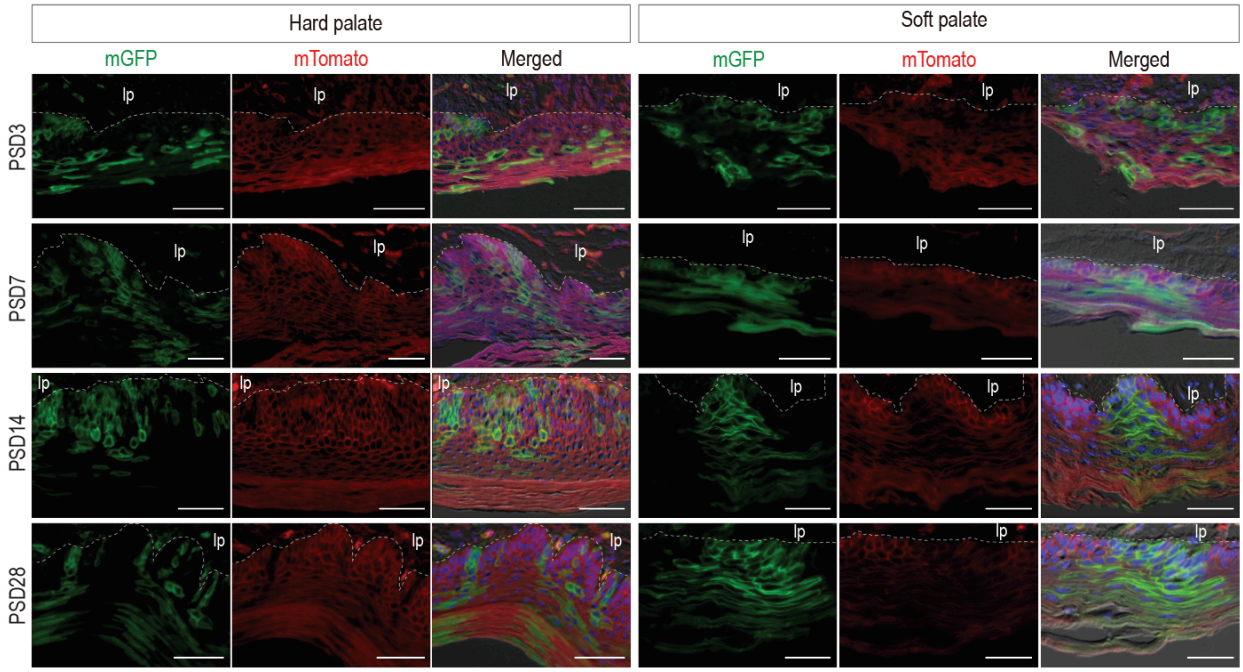

Figure S7. Lineage tracing of Wnt-responsive cells 3D, 7D, 10D, 14D and 28D after injury. Related to Figure 6. In *Axin2*$^{CreERT2/+}$*; R26R*$^{mTmG/+}$ mice, all cells express cell membrane-localized tdTomato (mTomato, red). Cre recombinase expressing cells, which require the present of Wnt signaling and tamoxifen at the same time, have cell membrane localized EGFP (mGFP, green) fluorescence expression replacing the mTomato. Future cell lineages derived from these cells are still positive for mGFP. The dotted line indicates the basement membrane. Abbreviations: lp, lamina propria. Scale bars: 50 μm.

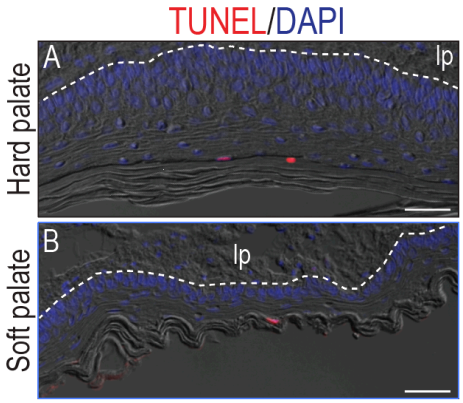

Figure S8. TUNEL staining of intact (A) hard and (B) soft palates. Related to Figure 6. The dotted line indicates the basement membrane. Abbreviations: lp, lamina propria. Scale bars: 50 μm.

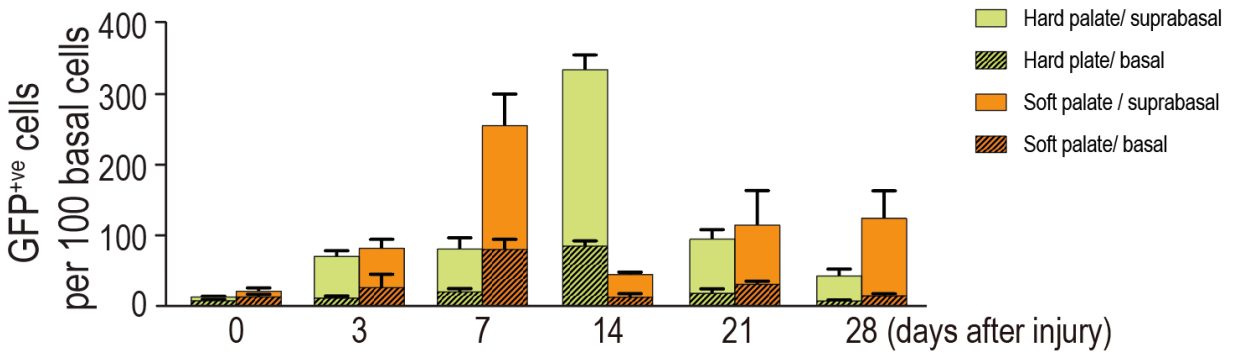

Figure S9. Quantification of GFP^{+ve} cells. Related to Figure 6. For quantification, a region of interest is defined as 100 μm (~100 cells) from the edge of the injury (PSD0) or 200 μm from the center of the injury (PSD3, 7, 14, 21, and 28) for both hard and soft palates. Basal cells were defined as cells that colocalized with the β4 integrin. n=3, 5 sections per mouse. Data represent mean ± SD.

Transparent Methods

Animals

All experimental protocols followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Stanford Committee on Animal Research (#13146), which is an AAALAC-accredited animal care and use program. *Axin2*$^{CreERT2/+}$*; R26R*$^{mTmG/+}$ mice (#018867 and #007576) and *Axin2*$^{LacZ/+}$ mice (#11809809) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a temperature-controlled environment with 12-hour light/dark cycles. All experiments were conducted using both genders. Age of analyzed mice is indicated in each figure legend.

Lineage tracing

Tamoxifen (T5648, Sigma) was dissolved in ethanol first and then diluted with sunflower seed oil to the final concentration of 10 mg/ml. The frequency of Cre recombination was affected by tamoxifen dosage. To find the proper concentration for lineage tracing, we initially tested tamoxifen of 1mg/25g body weight and 5mg/25g body weight. We also tested one injection versus five injections. Analyses of the resulting patterns of GFP^{+ve} supported the conclusion: a one-time, 5mg/25g body weight dose of tamoxifen resulted in GFP^{+ve} colonies that were sufficient to be detectable yet isolated enough to allow separation of each colony (Figure S2). Therefore, in this study, we delivered tamoxifen as a single injection of 5mg/25g body weight.
3-month old *Axin2*$^{CreERT2/+}$*; R26R*$^{mTmG/+}$ mice were used for lineage tracing and mice were sacrificed at indicated time points.

Surgeries

3-month old *Axin2*$^{CreERT2/+}$*; R26R*$^{mTmG/+}$ mice were given tamoxifen before injury to label the existing Wnt-responsive cells. The mice received same dose of tamoxifen but without any injury were used as control. Two days after tamoxifen injection, mice were anesthetized with Ketamine and Xylazine, followed by subcutaneous injections of buprenorphine. A 2 mm full-thickness injury was generated with a biopsy punch (Miltex, Inc., USA). The wound was in the junction: half of the injury in the hard palate and the other half in the soft palate (Figures 3A and 3B). Bleeding was controlled by local pressure. After surgery, mice were supplied with a soft diet for one week.

Sample preparation, histology and cellular assays (Masson's trichrome staining, Movet's Pentachrome staining, Xgal staining, immunohistochemistry staining)

Maxillae were harvested and fixed with 4% paraformaldehyde (PFA) overnight at 4°C. Following 10-day decalcification with 10% ethylenediaminetetraacetic acid (EDTA), specimens were incubated with 30% sucrose in phosphate-buffered saline (PBS) overnight and then embedded with Tissue-Tek OCT embedding medium. Tissues were cut into 8 μm-thick sections and collected on Superfrost-plus slides.
To visualize the morphology of oral mucosa, we performed a Masson's trichrome staining with the staining kit from Electron Microscopy Sciences (#26367) according to the manufacturer's instruction. Movat's pentachrome staining was also performed (Arioka et al., 2019), in which nuclei stain blue to black, cytoplasm stains red, collagen stains yellow to greenish yellow, and fibrous tissue stains an intense red (Movat, 1955).
To detect β-galactosidase activity, we performed an Xgal staining with a staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml Xgal (Thermo Fisher Scientific). Sections were rinsed with PBS, dehydrated with a graded ethanol series, cleared in CitriSolv, and then mounted with Permount (Chai et al., 2000; Kim et al., 2007). For quantification, the sections were stained with nuclear fast red (N3020, Sigma-Aldrich). In *Axin2*$^{LacZ/+}$ mice, Xgal^{+ve} signaling is restricted to the nuclei in Wnt-responsive cells (appeared punctate staining) because of the NLS (nuclear localization signal)-LacZ. To address the possibility that Xgal staining was non-specific, we included a negative control, e.g., *Axin2*$^{+/+}$ littermates that carry no LacZ gene (Zhang et al., 2019). Tissues from these mice were fixed, processed and stained side-by-side with *Axin2*$^{LacZ/+}$ samples. Non-specific staining was only found in the alveolar bone area (Figure S1A) as has been reported previously (Odgren et al., 2006).
Immunostaining was performed as described before (Minear et al., 2010) with slight modifications. In brief, tissue sections were permeabilized with 0.5% Triton X-100 and blocked with R.T.U. Animal-Free Block and Diluent (SP-5035, Vector Laboratories) for 30 min. Samples were incubated with β4 integrin (Clone 346-11A, BD science) antibody or β-catenin (ab6302, Abcam) antibody overnight at 4°C. After washing with PBS, slides were incubated with Cyanine 5 conjugated goat anti-rat secondary antibody (Invitrogen, A10525) or Cyanine 5 conjugated goat anti-rabbit secondary antibody (Invitrogen, A10523) for 30 min, then mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific).

Cell proliferation and apoptosis

EdU incorporation assay was used to examine cell proliferation. Cell proliferation is characterized by DNA replication. 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog, is readily incorporated into DNA during DNA synthesis. Incorporated EdU can be detected by a fluorescent azide through a Cu(I)-catalyzed [3 + 2] cycloaddition reaction (''click'' chemistry) (Salic and Mitchison, 2008). Mice were injected intraperitoneally with EdU (50 mg/kg) in PBS and harvested 2 hours later. Samples were fixed and processed as described above. Tissues were permeabilized with 0.5% Triton X-100. Click-iT reaction cocktail was prepared with EdU in vivo kits (BCK-IV-IM, Base Click, Germany) according to the protocol and was applied to each section for 30 min. The slides were then washed and mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to detect cell apoptosis. The assay was performed with Click-iT Plus TUNEL assay for In Situ Apoptosis Detection kit (C10619, Thermo Fisher) according to the manufacturer's instruction.

Primary cultures of GFP^{+ve} oral epithelial cells

1-month old *Axin2$^{CreERT2/+}$; R26R$^{mTmG/+}$* mice (both males and females) were given tamoxifen 2 days before sacrifice. Oral epithelial cells were isolated as described (Igarashi et al., 2008; Nakamura et al., 2003) with some modifications. In brief, the hard and soft palates were peeled off from the palatal bone and then incubated with 2.4 IU dispase II (Roche, Indianapolis, IN) at 37℃ for 2 h with shaking. After separating the epithelial layer with the connective tissue, both parts were subjected to trypLE Express (ThermoFisher Scientific) digest for 10 min to dissociate cells. The cells were cultured in DMEM (ThermoFisher Scientific) + 10% FBS and maintained in a humidified atmosphere of 5% CO2 at 37℃ (Figure S4A). Plates for epithelial cell culture were coated with type I collagen (10 μg/cm^2, C3867, Sigma).

Immunofluorescence was performed with following primary antibodies: keratin 5 (905504, Biolegend), keratin 14 (905301, Biolegend), β4 integrin (Clone 346-11A, BD science), involucrin (924401, Biolegend), and loricrin (ab24722, Abcam). Cyanine 5 conjugated goat anti-rat antibody (Invitrogen, A10525) or Cyanine 5 conjugated goat anti-rabbit antibody (Invitrogen, A10523) was used as secondary antibodies.

To access the cell proliferation ability, EdU was added to the culture media as a final concentration of 100 μM for 2 hours. The cells were then washed, fixed and performed Click-iT reaction (BCK-IV-IM, Base Click, Germany).

In order to evaluate colony forming efficiency, 1x10^4 cells were seeded on collagen coated 100 mm dish. After 14 days, cells were fixed and imaged. GFP^{+ve} colonies with more than 20 cells were counted (n=3).

Quantification and statistical analyses

To quantify GFP^{+ve} cells, GFP was co-stained with β4 integrin. GFP^{+ve}β4 integrin^{+ve} cells were defined as GFP^{+ve} basal cells. GFP^{+ve}β4 integrin^{-} cells were defined as GFP^{+ve} suprabasal cells. DAPI^{+ve} β4 integrin^{+ve} cells were counted and used as basal cell number. The result was expressed as the number of GFP^{+ve} basal cells or GFP^{+ve} suprabasal cells per 100 basal cells. Each time point included at least 3 mice, and 5 slides from each mouse at each time point were calculated.

To quantify Xgal^{+ve} cells, Xgal was co-stained with nuclear fast red (Figure S1B). The epithelial tissue can be easily distinguished from the connective tissue from its unique cell morphology and higher cell density. The first layer of epithelial cells that facing the connective tissue was defined as the basal layer. Xgal^{+ve} cells and total cells in basal layer were counted. The result was expressed as the number of Xgal^{+ve} cells per 100 basal cells. Each time point included at least 3 mice, and 5 slides from each mouse at each time point were calculated.

For injury response, each time point included at least 6 mice. A region of interest is defined as 1 mm (~100 cells) from the edge of the injury (PSD0) or 2 mm from the center of the injury (PSD3, 7, 14, 21, and 28) for both hard and soft palates. To quantify the wound healing, wound areas in the hard and soft palates were calculated by Image J. The results were expressed as the percentage of the remaining open wound area over the total original wound area (n=6). The repair rate was calculated based on the histology images from PSD1 and PSD3. For each sample, only sections close to the middle line were selected and the length of the repaired hard and soft palates were measured by Image J. The repair rate was expressed as the length of the newly formed tissue (mm) per day (n=6). EdU^{+ve} basal cells in the hard and soft palates were calculated using the same method described above for GFP^{+ve} cells.

Results are presented as the mean ± standard deviation of independent replicates (n≥3). Comparison was based on a one-way ANOVA, followed by Tukey's post hoc testing. P≤0.05 was considered significant. The program GraphPad Prism (GraphPad Software, Inc., San Diego, USA) was used for these analyses.

Supplemental References:

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