

Ablation of NTPDase2⁺ cells inhibits the formation of filiform papillae in tongue tip

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Abstract

Background: Lingual epithelia in the tongue tip are among the most rapidly regenerating tissues, but the mechanism of cell genesis in this tissue is still unknown. Previous study has suggested the existence of multiple stem cell pools in lingual epithelia and papillae. Like K14⁺ and Sox2⁺ cells, NTPDase2⁺ cells have characteristics of stem cells.

Methods: We employed a system using doxycycline to conditionally ablate NTPDase2⁺ cells in lingual epithelia and papillae by regulated expression of the diphtheria toxin A (DTA) gene. Transgenic lines, which expressed the rtTA gene in NTPDase2⁺ cells, were produced by pronuclear injection of zygotes from C57BL/6 mice using the BAC clone RP23-47P18. The NTPDase2-rtTA transgenic mice were crossed with the TetO-DTA transgenic animals. The double transgenic mice were treated with doxycycline. Doxycycline (Dox) was diluted in 5% sucrose in water to a final concentration of 0.3-0.5 mg/mL and supplied as drinking water.

Results: After 15 days of Dox induction, the expression of NTPDase2, Sox2 and K14 was ablated from lingual epithelia. DTA expression in NTPDase2⁺ cells did not inhibit the turnover of GNAT3⁺ or PLCβ2⁺ cells in taste buds, nor the expression of S100β beneath lingual epithelia and papillae. After 35 days ablation of NTPDase2⁺ cells, the basic structure of lingual epithelia and papillae remained intact. However, the ratio of cell to total tissue area was decreased in lingual epithelia and circumvallate (CV) papillae. DTA expression also inhibited the regeneration of filiform papillae on the dorsal surface of the tongue tip.

Conclusions: These studies provide important insights into the understanding of dynamic equilibrium among the multiple stem cell populations present in the lingual epithelia and papillae.

KEYWORDS

cell ablation, lingual epithelia, NTPDase2, papillae, tissue regeneration

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1 | INTRODUCTION

It has been established that there are four papillae in lingual epithelia, namely filiform, fungiform, circumvallate and foliate papillae.^{1,2} Comparing current reported tissue turnover rates, lingual epithelia in the tongue tip are among the most rapidly regenerating tissues.³⁻⁶ In a previous study we showed that cell development varies, depending on position on the tongue and type of papillae.⁵ Other studies showed that mutation of the EGF,⁷ TGF- β ⁸ and KLF4⁹ signaling pathways in K14+ cells resulted in significant pathological changes in lingual epithelia, and overexpression of Gli1 in K5+ cells led to basal cell carcinomas in skin epithelia.¹⁰ In addition, disruption of BMP signaling pathways in K14+ cells has been shown to change the shape of spines in filiform papillae.¹¹ While there have been many reports of epithelia stem cell and hair follicle development,^{3,4,12-15} few have focused on lingual epithelia and lingual papillae.¹⁶⁻²¹

In mouse tongue, continual turnover is observed in both the lingual papillae and epithelia. Previous studies have suggested a model in which long-term, self-renewing stem cells reside in the base of the papillae. The transit-amplifying (TA) and differentiating descendants move up along the basal lamina.²² In another study, the basal progenitor cells consist of a common K14+K5+Trp63+Sox2+ population of bipotential progenitor cells, localized at the base of filiform and fungiform papillae.¹⁶ The keratinocytes of the filiform and fungiform papillae originate from these populations.²³ Recently, based on our previous results using mutation of TGF- β signaling in K14+ and NTPDase2+ cells, a developmental model has been suggested for lingual epithelia and papillae. There are multiple stem cell pools in lingual epithelia and papillae consisting of stem cells pools, TA cell pools and differentiated cell pools.^{5,24} Like K14+ cells, NTPDase2+ cells are also a type of stem cell, responsible for generating lingual epithelia and papillae (including fungiform papillae, filiform papillae and taste bud cells).^{24,25}

In order to further investigate the cellular identity of NTPDase2+ cells directly in vivo and provide more evidence to support the cell-genesis model occurred in lingual epithelia and papillae, three strategies were employed in our study. One strategy combined the targeted disruption of the TGF- β signaling pathway with delivery of doxycycline to achieve a specific and inducible mutation of NTPDase2-expressing cells.⁵ The second strategy allowed fate mapping of progeny cells derived from NTPDase2-expressing cells by using the targeted expression of Cre recombinase (Cre) to excise a loxp-flanked stop signal and activate reporter gene expression (GFP/LacZ).⁵ The third strategy used in the current study was to target suicide genes to control cell ablation (Figure S1A and S1B). The most common approach for conditional cell ablation is based on the targeted expression of herpes simplex virus thymidine kinase (HSV-TK), in which the injection of ganciclovir results in damage to cell division.^{26,27} An alternative approach employs the cell-specific expression of the diphtheria toxin-A (DTA) subunit, which is highly dependent on the tissue specificity of the promoter.^{28,29} The toxicity of DTA is

so high that the cell can be killed when one molecular DTA is expressed in the cytosol.³⁰

As previously mentioned, a genetic mapping model has shown that NTPDase2+ cells contribute to the formation of filiform papillae, generate the taste bud cells, and produce lingual epithelia.^{5,24} Given that NTPDase2+ cells are involved in the formation of stem cell pools in lingual epithelia and papillae, the next question is what kind of pathological changes will be observed after ablation of NTPDase2+ cells. The genetic mapping model also offers new opportunities to study epithelial regeneration, and expand our understanding of stem cell plasticity.

2 | METHODS

2.1 | Generation of NTPDase2-rtTA transgenic mice and doxycycline treatment

The expression construct was generated by cloning the rtTA gene and its polyadenylation signal into RP23-47P18 (<http://bacpac.chori.org>), replacing the coding sequence of NTPDase2, using the Red recombination system. Transgenic lines were produced by pronuclear injection of zygotes from C57BL/6 mice using the modified RP23-47P18.²⁴ TetO-DTA animals (No 008468) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mouse lines used in the current experiment were bred and maintained at the Monell Chemical Senses Center animal facility. All procedures involving animals were approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

Doxycycline (Dox; Sigma, St Louis, MO, USA) was diluted in 5% sucrose in water to a final concentration of 0.3-0.5 mg/mL and supplied as drinking water. Access to the Dox-containing water was unlimited and the water was changed every 2-3 days. A single intraperitoneal injection of Dox (10 mg/kg body weight) was also administered when the mice began receiving Dox-containing water.

2.2 | Histology and immunostaining

Mice were sacrificed by inhalation of an overdose of isoflurane. For immunocytochemistry, mice were perfused transcardially with 2%-4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.2-7.4). The tongue tissues were dissected, post-fixed in PFA for 2-12 hours and cryoprotected in 30% sucrose in PBS at 4°C overnight. After sectioning on a cryostat, 10-12 μ m sections were collected onto Superfrost Plus Microscope slides (Fisher Scientific). Monoclonal primary antibodies were raised against Anti-cytokeratin 14 (Chemicon, MAB3232). Polyclonal primary antibodies were raised against NTPDase2,³¹ Sox2 (rabbit, Abcam, ab-59776), PLC- β 2 (rabbit, Santa Cruz Biotechnology, sc-206) and GNAT3 (rabbit, Santa Cruz Biotechnology, sc-395), Tgfbr2 (Goat, Santa Cruz Biotechnology, sc-400), S100 β (Rabbit, Sigma S-2644) and PGP9.5 (Rabbit, Millipore AB1761).

Single antibody immunostaining was performed as follows. Cryosections were washed in PBS (3 \times 10 minutes), placed into blocking solution (1% bovine serum albumin [BSA], 1% normal horse

serum, and 0.3% Triton X-100 in PBS) for 1-2 hours, and then incubated in a mixture of the polyclonal, primary antisera: rabbit anti- GNAT3, PLC β 2, PGP9.5, S100 β (1:200-500 dilution) in blocking solution. Primary antibody incubation lasted for 36-48 hours at 4°C, and then sections were washed in PBS (3 \times 10 minutes) and incubated for 2-18 hours in a mixture of secondary antibodies: Alexa568 goat anti-Rb (1:400; Molecular Probes, USA). The slides were then washed once for 10 minutes in 0.1 M PBS and twice for 10 minutes in 0.1 M PBS before cover-slipping the slides with Fluormount G (Southern Biotechnology Associates, USA). Fluorescent images were captured with a Leica TCS SP2 Spectral Confocal Microscope (Leica Microsystems Inc., Mannheim, Germany).

Standard double immunofluorescence protocols were used for anti-NTPDase2 and anti-Tgfr2. Briefly, oven-dried frozen sections were rehydrated with 0.1 M PBS at pH 7.0. Tissues were then blocked in 1% bovine serum albumin (BSA), 1% normal horse serum, and 0.3% Triton X-100 in PBS for 1 hour at room temperature and incubated with primary antibody for 2 days at 4°C. All double immunolabeling was done sequentially with appropriate second primary antibodies (Alexa488 donkey anti-rabbit, and Alexa555 donkey anti-goat, Molecular Probes, Eugene, OR, USA), and DAPI (dilution 1:1000, Molecular Probes) was used to label cell nuclei. Non-specific immunolabeling was tested by incubating with no primary antibody.

Staining against NTPDase2 and Sox2 was performed using standard immunocytochemical procedures according to the manufacturer's instructions (VECTASTAIN Elite ABC Kits). The standard immunocytochemical procedure used an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine (DAB, Sigma). Cryosections were washed (4 \times 4 minutes) in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100. The slides were subsequently incubated in blocking solution (1% bovine serum albumin [BSA], 1% normal horse serum, and 0.3% Triton X-100 in PBS) for 1-2 hours, followed by a 24 hour incubation with the primary antibody at 4°C. The slides were then washed with PBS four times followed by a 45-minute application of the biotin-conjugated secondary antibody. Three additional washes in PBS preceded both the 30-minute application of ABC and the 10-minute incubation with a PBS solution containing 0.5 mg/mL DAB, and 0.01% H₂O₂ to tint the reaction product blue.

Counterstaining was carried out with standard hematoxylin staining and bright field images of the sections were captured digitally. Cell numbers were counted in the serial sections with ImageJ software. In order to measure the ratio of cell to total tissue area in lingual epithelia (basal/suprabasal cell layers), we drew an irregular frame over a section of lingual epithelium that contained basal/suprabasal cell layers and excluded the cells forming filiform papillae. Then the area of basal/suprabasal cells within the irregular frame was calculated (area of basal-suprabasal cells/area of lingual epithelia). In order to measure the ratio of cell to total tissue area in connective tissues, we drew an irregular frame over a section of connective and muscle tissue. The area of cells within the irregular frame was calculated (area of cells/area of connective tissue). These

data were analyzed by one-way analysis of variance using SPSS 11.5 software. Differences were considered to be significant when $P < .05$.

3 | RESULTS

3.1 | Fifteen days of Dox induction ablated NTPDase2 expression in lingual epithelia

In a previous study, we generated NTPDase2-rtTA transgenic mice. Using double- and triple-crossed mice, NTPDase2 was shown to be expressed in lingual epithelia and papillae.²⁴ In order to study further the characteristics of NTPDase2+ cells, we crossed the NTPDase2-rtTA transgenic mice with TeTO-DTA transgenic mice²⁸ to create a double transgenic NTPDase2-rtTA-TeTO-DTA mice (Figure 1A).

In agreement with previous reports,^{24,32} confocal analysis revealed that NTPDase2 expression was widely observed in lingual epithelia and papillae. Tgfr2 expression was also detected in lingual epithelia and papillae, as suggested in a previous study.³³ NTPDase2 was co-expressed with Tgfr2 in lingual epithelia and papillae in control mice (Figure 1C-F). After 15 days of Dox induction (Figure 1B), NTPDase2+ cells were completely ablated in lingual epithelia and filiform papillae, but Tgfr2 expression was still observed throughout the lingual epithelia and papillae (including filiform papillae and fungiform papillae) (Figure 1G-J).

3.2 | During the 35 days of Dox induction, S100- β expression is observed over time

In order to check the effect of DTA expression in NTPDase2+ cells on taste bud cell genesis and the lingual nerve, we checked the expression of molecular markers related to peripheral nerve (S100 β) and taste bud cells (GNAT3 and PLC- β 2) using immunostaining.

In control mice, S100 β expression was observed in taste bud cells of CV papillae and connective tissues around the CV papillae (Figures 2A and S1A). After 15 days (Figures 2B and S1C) or 35 days (Figures 2C and S1E) of Dox induction, we still detected S100 β expression in taste bud cells of CV papillae and connective tissues around the CV papillae. In control mice, S100 β expression was also observed in connective tissue of fungiform papillae and filiform papillae (Figures 2D and S1B). After 15 days (Figures 2E and S1D) or 35 days (Figures 2F and S1F) of Dox induction, S100 β expression was still detected in connective tissue of fungiform papillae and filiform papillae.

GNAT3 and PLC β 2 are established molecular markers of mature type II cells in taste buds.^{34,35} After 15 days (Figure S2A and S2B) or 35 days (Figure S2C and S2D) of Dox induction, we observed the expression of PLC β 2. In addition, GNAT3 expression was also detected in taste bud cells after 35 days (Figure 2E and 2F) of Dox induction.

In summary, the current results collectively reveal that the ablation of NTPDase2+ cells did not inhibit the turnover of GNAT3+ or PLC β 2+ cells in taste bud cells, and also did not inhibit the expression of S100 β .

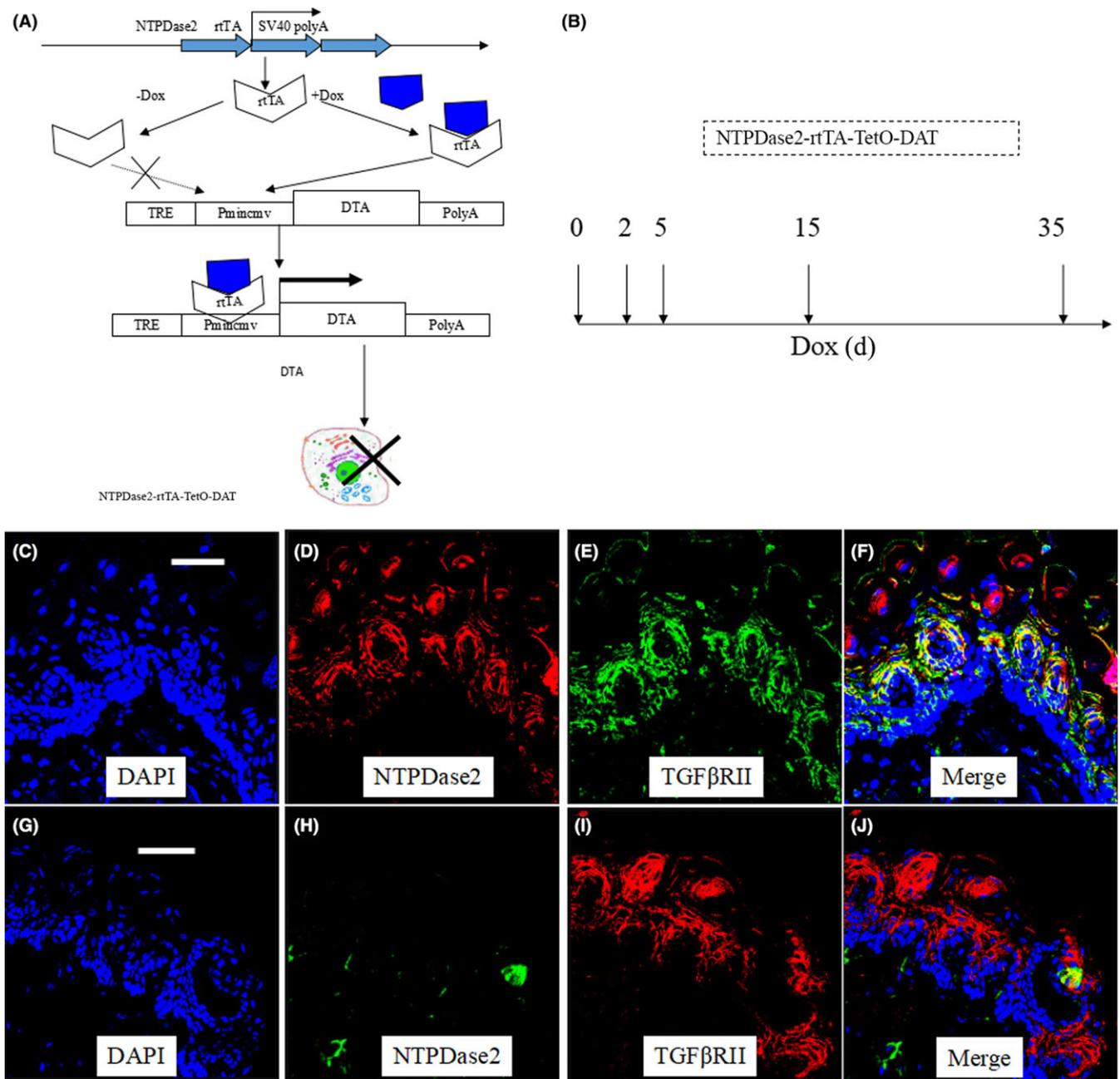


FIGURE 1 DTA expression ablates the NTPDase2+ cells in lingual epithelia after 15 days of Dox induction. A, The NTPDase2-rtTA transgene directs expression of the reverse tetracycline transcriptional activator (rtTA) to the epithelia. The binding of rtTA to the tetracycline responsive element (TetRE) and the induction of the transgene DTA should only occur in the presence of doxycycline (Dox). DTA expression inhibits protein synthesis in NTPDase2+ cells and leads to cell death. B, The double transgenic mouse is induced with Dox for 15 d. C-F, NTPDase2 is co-expressed with Tgfr2 in lingual epithelia and papillae. G-J, After 15 days of Dox induction, NTPDase2 expression is not detected in lingual epithelia and filiform papillae. Scale bars, 50 μ m

3.3 | After 15 days of Dox induction, the expression of NTPDase2, Sox2 and K14 are ablated in lingual epithelia

In previous studies, NTPDase2+,⁵ Sox2+ and K14+ cells^{16,23} have been suggested to form stem cell pools in lingual epithelia. In order to investigate the effect of DTA expression in NTPDase2+ cells on multiple stem cell populations in lingual epithelia, we investigated the expression pattern of K14 and Sox2 over time after Dox induction.

The expression over time of Sox2 was analyzed in lingual epithelia and papillae using immunohistochemistry. In control mice, the expression of Sox2 was undetectable in lingual epithelia under current experimental conditions (Figure S3A). After 2 (Figure S3B) or 5 days (Figure S3C) of Dox induction, expression of Sox2 was observed in lingual epithelia. Curiously, the expression of Sox2 was undetectable in the lingual epithelia under current experimental conditions after 15 days of Dox induction (Figure S3D). However, Sox2 expression was again observed in lingual epithelia after 35 days of Dox induction (Figure S3E).

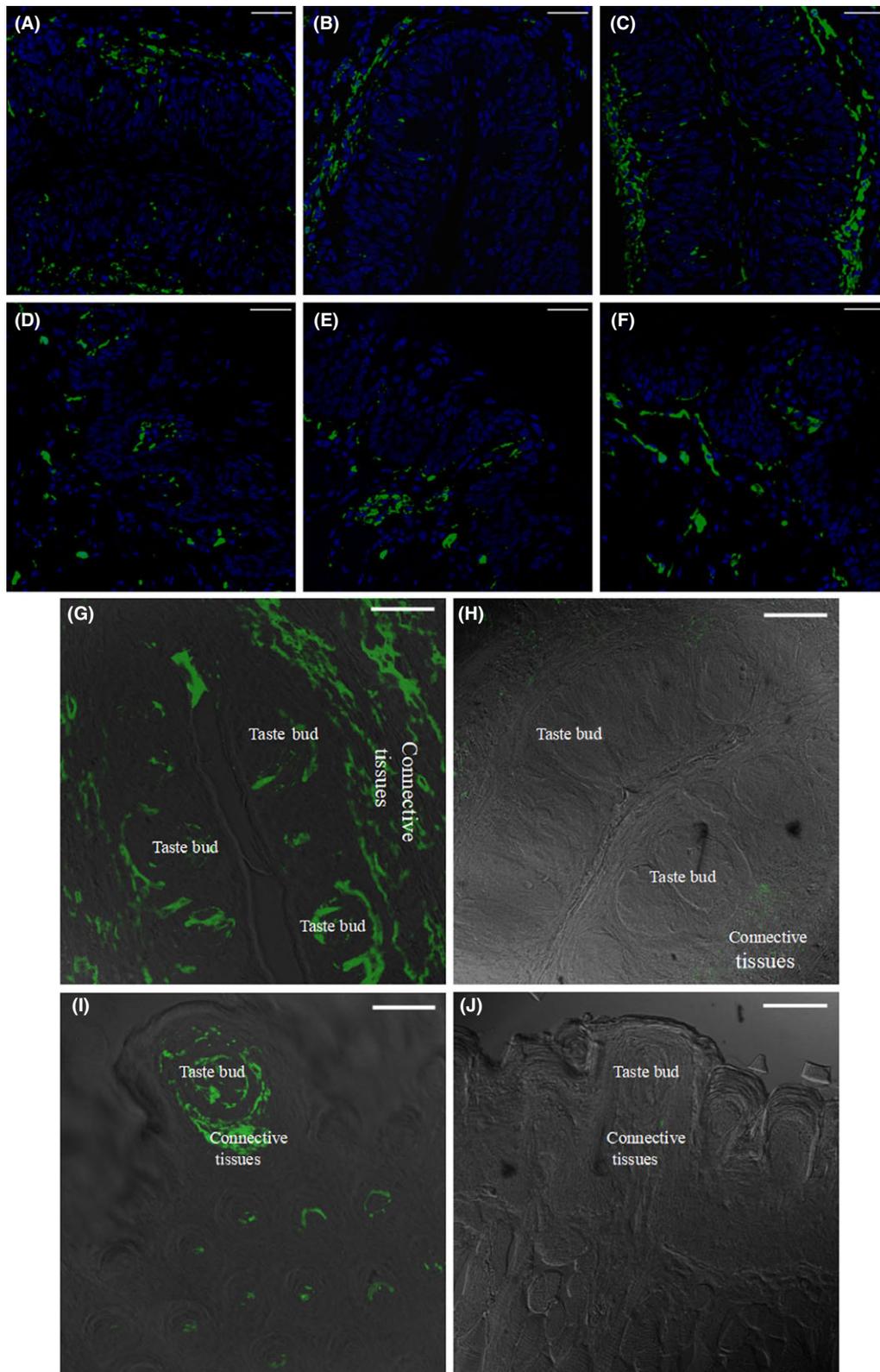


FIGURE 2 S100 β expression over time in lingual epithelia and papillae after DTA expression in NTPDase2⁺ cells. A, In control mice, S100 β expression is observed in CV papillae and connective tissue. In NTPDase2-rtTA-DTA mice, S100 β expression is still observed in CV papillae and connective tissues after 15 days (B) and 35 d of Dox induction (C). (D), S100 β expression is detected in connective tissues of fungiform papillae and beneath the basal membrane in control mice. After 15 days (E) or 35 days (F) of Dox induction, S100 β expression is still detected in connective tissue of fungiform papillae and beneath the basal membrane. K14 expression is detected in CV papillae (G) and fungiform papillae (I). After 15 d DTA expression in NTPDase2⁺ cells, K14 expression is undetectable in CV papillae (H) and fungiform papillae (J). CT, connective tissue; TB, taste bud. Scale bars, 50 μ m

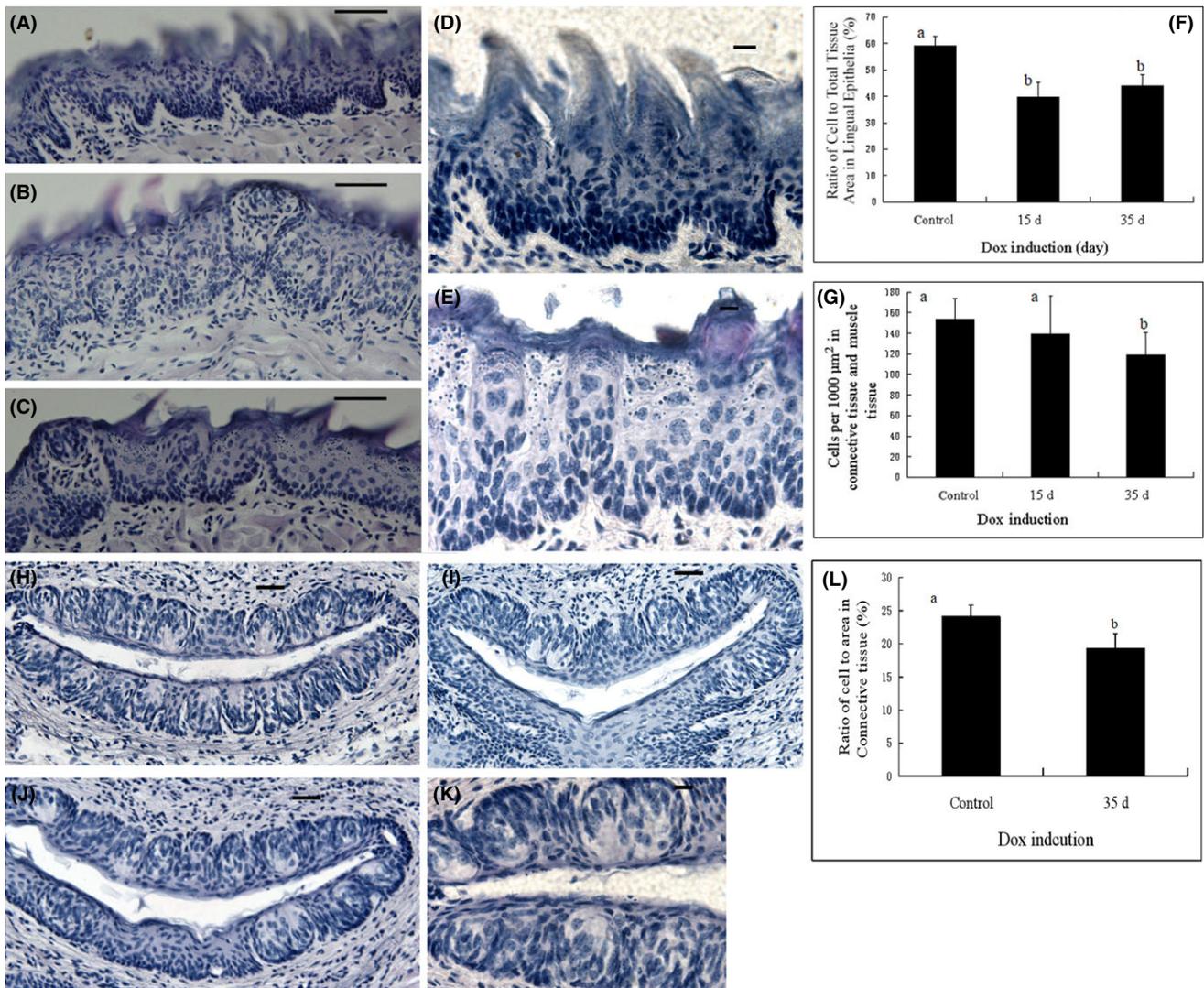


FIGURE 3 The formation of filiform papillae is inhibited in the tongue tip after 35 d of Dox induction. A, Many filiform papillae cover the dorsal surface of the tongue tip in control mice. B, After 15 d of Dox induction, there are lots of filiform papillae covering the dorsal surface of tip tongue. C, After 35 d of Dox induction, abnormal filiform papillae are observed on the dorsal surface. A taste bud is observed on the fungiform papillae. D, High magnification image of lingual epithelia in control mouse. E, High magnification of lingual epithelia in the double transgenic mice after 35 d of Dox induction. Spines on the filiform papillae on the dorsal surface of tongue tip have become frangible. F, Ratio of cell to total tissue area is significantly decrease in lingual epithelia after 15 or 35 days of Dox induction (a, b; $P < .05$). G, ratio of cell to total tissue area is significantly decrease in connective tissue and muscle tissue after 35 days of Dox induction (a, b; $P < .05$). A taste bud is observed in CV papillae after Dox induction over time. H, CV papillae in control mouse. I, CV papillae in transgenic mouse after 15 d of Dox induction. J, CV papillae in transgenic mouse after 35 d of Dox induction. K, High magnification of CV papillae from (J). L, Compared with control mice, the ratio of cell to total tissue area in connective tissues around CV papillae is significantly decreased (a, b; $P < .05$). Scale bars: A-C, 50 μm ; D and E, 12 μm ; H-J, 25 μm ; K, 12 μm

K14 is known to be present in immature taste bud cells and intragemmal epithelial cells, and K14⁺ cells are believed to generate taste bud cells and epithelial cells.^{16,36} Confocal laser scanning microscopy images showed that K14 expression was detected in CV papillae (Figure 2G) and fungiform papillae (Figure 2I). In CV papillae, K14 was mostly detected in the intragemmal epithelial cells, but also in the taste bud cells (Figure 2G). After 15 days DTA expression in NTPDase2⁺ cells, K14 expression was undetectable in CV papillae (Figure 2H) and fungiform papillae (Figure 2J).

To summarize, 15 days of DTA expression in the NTPDase2⁺ cells ablates the expression of Sox2 and K14 in the lingual epithelia. DTA

expression in NTPDase2⁺ cells would thus be detrimental for maintaining the homeostasis of multiple stem cell populations.

3.4 | After 35 days of Dox induction, ablation of the NTPDase2⁺ cells blocks the regeneration of filiform papillae, and decreases the ratio of cell to total tissue area in lingual epithelia and connective tissues

We further observed the pathological changes of lingual epithelia and papillae in the tongue tip after Dox induction, over time.

In control mice, the frontal sections showed that filiform papillae are covered at the dorsal surface of the tongue tip, and muscle forms an orderly layer beneath the lingual epithelium (Figure 3A and 3D). After 15 days of Dox induction, filiform papillae with a seemingly normal shape were observed at the dorsal surface of the tongue tip (Figure 3B). After 35 days of Dox induction, the spines of filiform papillae appeared to be frangible, and to have thinner shapes (Figure 3C and 3E). We then counted cell numbers in lingual epithelia and connective tissue, including the muscle tissue. Compared to that in control mice, the ratio of cell to total tissue area was significantly decreased in lingual epithelia (Figure 3F) and connective tissue (Figure 3G) after 35 days of Dox induction.

In addition, we also observed the CV papillae. Compared to that in control mouse (Figure 3H), the shape of CV papillae was unchanged after 15 days (Figure 3I) or 35 days (Figure 3J and 3K) of Dox induction. After analyzing the cell area in the connective tissue around CV papillae, it was found that the ratio of cell to total tissue area was significantly decreased after 35 days of Dox induction (Figure 3L).

In summary, the current results suggest that 35 days of DTA expression in NTPDase2+ cells lead to the formation of filiform papillae with sharp spines in the dorsal surface of the tongue tip, and decrease the ratio of cell to total tissue area in lingual epithelia and connective tissues.

4 | DISCUSSION

Taste bud cells are morphologically classified based on ultrastructural criteria.^{1,37,38} For many years it has been believed that there are (at least) four subtypes of cells in taste buds, termed types I-IV.¹ Until now, identifying molecular markers that can distinctly distinguish between the four cell subtypes of the taste buds has been difficult.^{17,34,35,39,40} GLAST and NTPDase2 have been considered as the molecular markers of type I cells,^{32,41} GNAT3^{6,42} and PLCβ2^{6,43} are thought to be markers of type II cells, and NACM is the proposed marker of type III cell. In our study, the original goal of our model was to investigate what happens to taste buds after ablation of NTPDase2+ cells. The study has revealed that conditional DTA expression in NTPDase2+ cells fails to ablate not only those positive cells, but also GNAT3+, PLCβ2+, NACM+ (not shown), and PGP9.5+ (not shown) cells in taste buds. As shown in a previous study, regardless of genetic ablation of mT1R2+, mT1R3+, mT2R5 or mPKD2L1+, the cell number and shape of taste buds is not changed.⁴⁴

In a previous study, we have shown that disruption of TGF-β signalling in NTPDase2+ cells leads to site-dependent pathology along the tongue: filiform papillae were initially inhibited but their regeneration occurred over time in the tongue tip, and TGF-β signalling disruption led to thickened epithelia (acanthotic epithelia) along the dorsal surface in the middle-posterior tongue.^{25,45} The current study further reveals that continuous expression of DTA in NTPDase2+ cells inhibits cell genesis in lingual epithelia and connective tissues, leading to the generation of filiform papillae with a frangible and thinner shape at the tongue tip.

On the other hand, the current study also revealed that Sox2+ and K14+ cells were ablated from the lingual epithelia after 15 days of DTA expression in NTPDase2+ cells, indicating the existence of multiple stem cell populations in lingual epithelia, including NTPDase2+, Sox2+, K14+, K5+ and p63 cells.^{16,24} Although there is evidence that reciprocal conversion can occur among undifferentiated epithelial cells,⁴⁶ the mechanism for DTA expression-dependent ablation of Sox2+ and K14+ cells in NTPDase2+ cells is still very unclear.

Subsequently we observed the renewed expression of Sox2 in lingual epithelia after 35 days of Dox induction. A previous study has shown that Sox2 plays an important role in lingual epithelia. Overexpression of Sox2 in K5+ cells changed the tip shape of filiform papillae from a pointed spine to a flattened dome.²³ Combined with the current results, this may suggest that overexpression of Sox2 is related to the morphological changes of lingual epithelia and papillae.

It has been reported that NTPDase2 is expressed in the peripheral nervous system.⁴⁷ Theoretically, DTA expression in NTPDase2+ cells could damage the distribution of the peripheral nervous system in the tongue. The presence/distribution of the nervous system in this tissue is thought to link to the maintenance or regeneration of taste buds.⁴⁸⁻⁵⁰ Our results indicate that S100β,a marker for Schwann cells of the peripheral nervous system,^{51,52} was still observed in lingual epithelia and papillae after Dox induction, indicating the presence of the peripheral nerve system in papillae. This excludes the possibility that damage to the peripheral nerve system may contribute to the pathological changes to filiform papillae. In our previous study, it was shown that NTPDase2+ cells have the characteristics of stem cells, and are able to generate lingual epithelia and lingual papillae. Meanwhile, a model of cell genesis in papillae and lingual epithelia has been suggested. Based on this model, the presence of multiple stem cell populations can be observed in lingual epithelia and papillae.^{5,24,25,45} We speculate that DTA expression in NTPDase2+ cells may interrupt homeostasis of the stem cell populations in lingual epithelia and papillae, which contributes to pathological changes in these tissues.

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CONFLICT OF INTEREST

None.

AUHTOR CONTRIBUTIONS

All listed authors meet the requirements for authorship. FL conceived and designed the experiments. FL wrote the main manuscript text. BWN and MMZ performed the genotyping and management of

the transgenic mice. FL, BWN and MMZ performed the IHC analysis. All authors have read and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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