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Early taste buds are from Shh⁺ epithelial cells of tongue primordium in distinction from mature taste bud cells which arise from surrounding tissue compartments

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Abstract

Mammalian taste buds emerge perinatally and most become mature 3–4 weeks after birth. Mature taste bud cells in rodents are known to be renewed by the surrounding K14⁺ basal epithelial cells and potentially other progenitor source(s), but the dynamics between initially developed taste buds and surrounding tissue compartments are unclear. Using the K14-Cre and Dermo1-Cre mouse lines to trace epithelial and mesenchymal cell lineages, we found that early taste buds in E18.5 and newborn mouse tongues are not derived from either lineage. At E11.5 when the tongue primordia (i.e., lingual swellings) emerge, the relatively homogeneous sonic hedgehog-expressing (Shh⁺) epithelial cells express Keratin (K) 8, a marker that is widely used to label taste buds. Mapping lineage of E11.0 Shh⁺ epithelium of the tongue rudiment with Shh-CreER^{T2}/RFP mice demonstrated that both the early taste buds and the surrounding lingual epithelium are from the same population of progenitors – Shh⁺ epithelial cells of the tongue primordium. In combination with previous reports, we propose that Shh⁺K8⁺ cells in the homogeneous epithelium of tongue primordium at early embryonic stages are programmed to become taste papilla and taste bud cells. Switching off Shh and K8 expression in the Shh⁺ epithelial cells of the tongue primordium transforms the cells to non-gustatory cells surrounding papillae, including K14⁺ basal epithelial cells which will eventually contribute to the cell renewal of mature taste buds.

Keywords

Taste bud; Progenitors; Sonic hedgehog; Epithelium; Mesenchyme; Lineage tracing

1. Introduction

Taste buds in the mammalian tongue are clusters of specialized epithelial cells that reside in the three types of gustatory papillae-fungiform, circumvallate and foliate. They initially develop perinatally [1], and mostly mature 3–4 weeks after birth [2,3], after which they undergo continuous cell renewal for homeostasis. It has been reported that mature taste bud

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cells are renewed by progenitors in the surrounding tissue compartments. Specifically, taste bud cell progenitors localize to the basal cells of the stratified squamous epithelium [4–10] and potentially the connective tissue [11] in adult mouse tongues. However, the dynamics between the initially developed taste buds and the surrounding tissues are unclear.

The three types of taste papillae, which host taste buds, have unique spatial and morphological features. In rodents, multiple fungiform papillae are distributed on the anterior 2/3 of the oral tongue, foliate papillae on the two lateral sides of the posterior oral tongue, and a single circumvallate located in the midline of the border between the oral and the pharyngeal tongue. Fungiform papillae have distinctive patterned arrays; each papilla hosts a single taste bud in the epithelium of the papilla apex, offering a clear observation of the taste bud in relation to its surrounding tissue compartments. In the anterior tongue, early fungiform papilla placodes emerge at E12.5 as focal epithelial thickenings. The papilla placodes continue to develop, and by E18.5 an early taste bud is clearly defined in the taste papilla (reviewed in Ref. [1]). Previous studies have demonstrated that initial taste buds are pre-patterned, with two out of three differentiated taste bud cell types proven to arise from papilla placodal cells [12–14]. However, questions remain as to whether other tissue compartments give rise to the initial progenitors, and if so, which ones.

In the present study, we tested whether early taste buds are like mature taste buds and have progenitors in the surrounding epithelium and/or the underlying mesenchyme. We discovered that early taste buds are neither derived from surrounding epithelium nor mesenchyme. Instead, the relatively homogeneous epithelial cells of the tongue primordium at E11.0 express sonic hedgehog (Shh) and Keratin 8 (K8), a widely used marker for taste bud cells. This early epithelium gave rise not only to taste buds, but also to the entirety of the lingual epithelium. We propose that these early Shh⁺ and K8⁺ epithelial cells are programmed to be early taste bud cells, persist postnatally, and are then replaced by cells in the surrounding tissues.

2. Materials and methods

2.1. Animals

The use of animals was approved by The University of Georgia Institutional Animal Care and Use Committee and was in compliance with the National Institutes of Health Guidelines for the care and use of animals in research.

Keratin (K)14-Cre mice (B6N.Cg-Tg(KRT14-cre)1Amc/J, #018964) or Dermo1-Cre mice (B6.129X1-*Twist2*^{tm1.1(cre)Dor}/J, #008712) were bred with nuclear tdTomato nuclear EGFP double reporter (hereafter nTnG) (B6; 129S6-Gt-GT(ROSA)26Sor^{tm1(CAG-tdTomato*; -EGFP*)Ees}/J, #023035) mice to generate K14-Cre/nTnG or Dermo1-Cre/nTnG mice. Shh-CreER^{T2} mice (B6.129S6-*Shh*^{tm2(cre/EKrt2)Cjt}/J; #005623) were bred with tdTomato (hereafter RFP) reporter mice (B6.Cg-*Gt(ROSA)26Sor*^{tm14(CAG-tdTomato)Hze}/J; #007914) to generate Shh-CreER^{T2}/RFP mice.

Primers WT-F (CGTCTCAGCTACGCCTTCTC), WT-R (TCACGAGGAGAGATACACTGG) were used with Mut-F

(AACTTCCTCTCCCGGAGACC) and Mut-R (CCGGTTATTCAACTTGCACC) to detect Dermo1-Cre. SRY-F (TTGTCTAGAGAGCATGGAGGGCCATGTCAA) and SRY-R (CCACTCCTCTGTGACACTTTAGCCCTCCGA) were used to detect sex-determining region Y; Cre 200-F (ATTGCTGTCACTTGGTCGTGGC) and Cre 200-R (GGAAAATGCTTCTGTCCGTTTGC) were used to detect Cre. A2-3 (CATGCTAGCAGCTCGGAGAAAC) and A2-5 (ATGCTAGACCTGGGCAGCCATA) were used to detect endogenous type I BMP receptor Alk2 as an internal control.

2.2. Tissue collection

Timed pregnancies were utilized for embryonic tissue collection; noon of the day of vaginal plug detection was designated E0.5. Tongues with mandible were collected from K14-Cre/nTnG embryos at embryonic day (E) 11.5, E12.5, E14.5, and E18.5. Shh-CreER^{T2}/RFP embryos were collected at E18.5 following a single tamoxifen administration at E11.0 (~160 mg/kg body weight; T5648, Sigma) via oral gavage to the dam. Newborn (P1) K14-Cre/nTnG and Dermo1-Cre/nTnG pups were collected on the day of birth. Both male and female mice were collected for each time point.

Embryonic and P1 mouse tongues with mandibles were placed in 4% paraformaldehyde (PFA) in 0.1 M PBS for 2–3 h. Young adult (8 wk) K14-Cre/nTnG mice were transcardially perfused then fixed in 2% PFA in 0.1 M PBS for 2–3 h. After cryoprotection in 30% sucrose, E11.5, E12.5, and E14.5 tongues with mandible were embedded for sagittal sections without further dissection. E18.5, P1, and 8 wk mouse tongues anterior tongues were cut as in Ref. [11] and were embedded for sagittal sections.

2.3. RNA extraction, RT-qPCR, and RNA-Sequencing

Lingual epithelium was collected from E11.5 embryos and P1 pups. Tongues were collected and bathed in (E11.5) or sub-epithelially injected (P1) with collagenase A (1 mg/mL Sigma Aldrich, 10103586001) and dispase II (2.5 mg/mL Sigma Aldrich, 04942078001) for 30 min at 37 °C, then tongue epithelium was separated from mesenchyme. RNA was extracted as previously described [15] and after normalization converted to cDNA using Thermo Fisher's SuperScriptTM First-Strand Synthesis System for RT-PCR.

RT-qPCR was carried out using Genecopoeia's All-in-one qPCR mix with the following primers: K14 CAGCCCCTACTTCAAGACCA and GGCTCTCAATCTGCATCTCC [16]; K8 GGACATCGAGATCACCACT and TGAAGCCAGGGCTAGTGAGT [17]; GAPDH ATGCCAGTGAGCTTCCCGTTTCTAG and CATCACTGCCACCCAGAAGACTG. PCR conditions were as follows: 95 °C for 15min, 40 cycles of 95 °C, 55 °C, and 72 °C for 30 s, 15 s, and 30 s respectively. Bands were visualized on a 3% agarose gel.

RNA-sequencing was carried out and analyzed as previously described [15]. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were utilized to represent gene expression levels.

2.4. Immunohistochemistry

2.4.1. Whole mount—Whole tongues were dissected from E11.5 wild type embryos, fixed for 2 h in 4% PFA in 0.1 M PBS, then stored in methanol at -20°C until use. Tissues were stained for Shh (1:300, R&D Systems, AF464) as previously described [18].

2.4.2. Sections—Frozen tissues were sectioned at 10 μm thickness and placed onto charged slides for immunostaining as previously described [11]. Primary antibodies against E-cadherin (1:500, R&D systems, AF 748) and Keratin (K) 8 (1:500, Developmental Studies Hybridoma Bank, TROMA-1) were used. Alexa Fluor 488- or 647-conjugated secondary antibodies (1:500, Jackson Immunoresearch) were utilized for visualizations. Slides were mounted with Prolong® Diamond antifade mounting medium (Fisher Scientific, P36970).

2.5. Photomicroscopy and data analyses

Whole mount tissues were imaged and analyzed under a stereomicroscope (Olympus SZX16). Tissue sections were thoroughly examined under a fluorescent light microscope (EVOS FL, Life Technologies). Representative images were taken using a laser scanning confocal microscope (Zeiss LSM 710) at UGA's Biomedical Microscopy Core. Adobe Photoshop was used for figure assembly; image editing was minimal.

RNA-Sequencing data was analyzed by comparing the fold change of genes of interest to GAPDH at various time points. RT-qPCR data was analyzed by comparing the genes of interest to GAPDH using the $2^{-\text{CT}}$ method. The average of three biological replicates as well as three technical replicates per sample was utilized for analysis.

3. Results

3.1. Neither K14-Cre nor Dermo1-Cre labels early taste bud cells in fungiform papillae

To test whether early taste bud cells share the same progenitor population as those of adults, which include the surrounding basal epithelial cells [5–7] as well as potentially the underlying (GFP^+) signals was examined at P1 (Fig. 1B). GFP^+ cells were only observed in the E-cadherin-labeled epithelium and were absent from the underlying connective tissue.

Distribution of K14-Cre/nTnG labeled cells were closely examined within taste papillae and early taste buds at multiple developmental stages (Fig. 1C). At E12.5, when taste papilla placodes emerge [1], the placodal epithelium was largely K8^+ . K14-Cre driven GFP^+ cells were sparsely seen in the dorsal tongue connective tissue [11], the cell lineages of these populations were traced using K14-Cre and Dermo1-Cre, respectively. The RNA expression of K14 in tongue epithelium at various time points was confirmed (Fig. 1A). At E12.5, K14 expression was detected at a low level; the expression level progressively increased at E14.5 and P1. Further, we utilized a double Cre reporter, nTnG, to cross with K14-Cre, a transgenic mouse model that labels K14^+ epithelial cells and their derivatives. The specificity and efficiency of K14-Cre-driven epithelium between taste papilla placodes, which was consistent with the low K14 RNA expression observed at E12.5. In contrast, GFP^+ cells were abundant in the ventral surface of the tongue and the oral surface of the mandible demonstrating efficient Cre-mediated DNA recombination at this stage. At E14.5,

when developing taste papillae were evident, K8 immunosignals became more restricted (but not exclusive) to the taste papillae. K14-Cre driven GFP⁺ cells were abundant in the epithelium, but were absent from the developing K8⁺ taste papilla epithelial cells (Fig. 1C, E14.5). By E18.5 and postnatal day (P) 1, K8 immunosignals were restricted to early taste buds in fungiform papillae (Fig. 1C, E18.5 and P1). K14-Cre driven GFP⁺ cells were completely absent from K8⁺ early taste bud regions. This is in contrast to the observation that at 8 wks of age, taste buds were mostly full of GFP⁺ cells labeled by K14-Cre (Fig. 1C, 8 wks), though RFP⁺ cells were occasionally detected within the taste bud (8 wks, not shown). No differences between male and female mice were observed at any of the time points examined.

Given the apparent absence of K14-Cre labeled GFP⁺ cells within the taste papillae and early taste buds at every time point examined, we tested whether early taste papillae/buds could instead be mesenchyme-derived using Dermo1-Cre, which labels mature taste buds in adult mice [11]. The RNA expression of Dermo1 in tongue mesenchyme at various time points was confirmed (Fig. 2A), i.e., at a high level at E12.5, and at progressively decreased levels at E14.5 and P1. At P1, Dermo1-Cre-labeled (GFP⁺) cells were specifically and abundantly distributed in the underlying connective tissue (Fig. 2B and C), and absent from E-cadherin-labeled epithelium (Fig. 2B) and K8-labeled early taste buds (Fig. 2C).

3.2. Upon tamoxifen treatment at E11.0, Shh-CreER labels both early taste buds and surrounding epithelium

In combination with the data that early taste buds were not labeled by K14-Cre or Dermo1-Cre, and the previous reports that the relatively homogeneous epithelial cells of the tongue primordium express Shh which is known to label precursors for taste bud cells at later stages [14], we speculate that the epithelial cells of the tongue primordium are likely programmed early at the first development of the tongue for a taste bud cell fate.

To characterize the early E11.5 epithelium, expression of multiple markers was examined including taste bud cell marker K8, taste bud precursor marker Shh, and K14. At E11.5, K8 mRNA in the epithelium of lingual swellings was detected at a high level compared to P1 (Fig. 3A_{1,3}). K8 immunosignals were also distributed in the entire epithelium (Fig. 3B₁). In contrast, K14 expression was low in the tongue epithelium at E11.5 relative to P1 (Fig. 3A_{2,3}). This was confirmed by the absence of labeling in sections from E11.5 K14-Cre/nTnG mouse tongues, in which K14-Cre driven GFP⁺ cells were not observed in the tongue epithelium (Fig. 3B₂). Similar to K8, Shh immunosignals were homogeneously distributed in the lingual epithelium of the lateral tongue swellings (Fig. 3C).

To map the lineages of Shh⁺ epithelial cells of E11.5 tongue swellings, a single dose of tamoxifen or vehicle was administered to pregnant dams carrying Shh-CreER^{T2}/RFP embryos at E11.0. At E18.5, RFP signals were broadly, though patchily, distributed in the surface of tongues from Shh-CreER^{T2}/RFP embryos with tamoxifen treatment at E11.0 (Fig. 3D₁). This was in sharp contrast to the absence of RFP signals in the tongues of vehicle-treated Shh-CreER^{T2}/RFP embryos (Fig. 3D₂). On sections, RFP⁺ cells were also abundant in the lingual epithelium including the papilla and inter-papilla epithelium, as denoted by

colocalization with E-cadherin (Fig. 3D₃). Importantly, the early taste buds were fully occupied by RFP⁺ cells, as demonstrated by colocalization with K8 (Fig. 3D₄).

4. Discussion

4.1. Unlike mature taste buds, early taste buds are not derived from cells in the surrounding tissue compartments

In postnatal mouse tongues, Keratin (K) 14 expression is restricted to the basal cells of the stratified squamous epithelium. Multitudes of studies show that these K14-expressing (K14⁺) basal epithelial cells can differentiate to become taste cells [4–10]. Another source of progenitors has also been indicated to contribute to adult taste buds – the underlying connective tissue [11]. In this report, our studies using K14-Cre and Dermo1-Cre to trace epithelial and mesenchymal cell lineages provide clear evidence that early taste buds in E18.5 and newborn mouse tongues are not derived from either lineage. The data demonstrate that unlike the taste buds of postnatal mice, initially developed taste buds are not derived from cells of the surrounding tissue compartments.

This raises an interesting question: when do the progenitors in the surrounding tissue compartments start to contribute to taste buds? In a study using K14-CreER with a one week chase after tamoxifen at P2, K14-CreER-labeled cells were observed within taste buds 2 weeks after birth [5]. This indicates that recruitment of new taste bud cells from surrounding K14⁺ basal epithelial cells occurs soon after birth. Further analysis is needed to understand whether the newly recruited taste bud cells are an addition to or a replacement of the existing taste bud cells.

4.2. Shh⁺ cells in the epithelium of tongue primordia constitute a common progenitor for early taste buds and surrounding epithelium

At the first development of the primordial tongue at E11.5, it is covered by a homogeneous epithelial bilayer expressing Shh, a marker that labels developing taste papilla and basal taste bud cells – both of which constitute taste bud progenitors [19,20]. Interestingly, K8, which is widely used as a pan taste bud cell marker, is also ubiquitously expressed in the E11.5 Shh⁺ epithelium of tongue swellings. In combination with the observations and reports below, we believe that at this early time point, the Shh⁺ epithelial cells of tongue swellings are programmed to become early taste buds. First, after tamoxifen treatment at E11.0 Shh-CreER fully labels early taste buds. Second, later in development, Shh expression remains in only the taste papilla placodes [10,13,18,19] (Shh⁺K8⁺ cells) and are known to give rise to primarily the taste bud cells in postnatal mice [14]. It is only the cells that *continue* to express Shh and K8 that are recognized as taste papilla placodes at E12.5.

Interestingly, however, the earlier E11.0 Shh⁺ epithelium of tongue swellings also give rise to inter-papilla and taste bud-surrounding epithelium in addition to papilla and taste bud cells, demonstrating that non-gustatory epithelium and taste buds share a common progenitor, a result which has been speculated but until now lacked support [21]. One of the hallmark characteristics of the differentiation of these early epithelial cells is the transition from K8⁺ to K14⁺ [22–25]. During development, this transition did not obviously occur in

the dorsal surface of the mouse tongue until roughly E12.5, and even then, it only occurred in a few epithelial cells between taste papilla placodes. Considering that at this point, the taste papilla placode has already developed and the placodal cells are known to give rise to the taste bud [14], K14⁺ cells can be ruled out as a potential origin for taste papillae. Furthermore, when taste papillae/early taste buds were examined at later time points in development using the K14-Cre/nTnG mouse model, taste buds remained entirely unlabeled despite robust labeling of the surrounding epithelium. These data indicate that after derivation from Shh⁺K8⁺ cells, K14⁺ cells constitute a separate population from the remaining Shh⁺K8⁺ cells.

A matter of particular interest is the regulatory factors guiding cell fate decisions of epithelial cells in the tongue swellings. Within a short period of time, the epithelium covering the tongue rudiment progresses from ubiquitously expressing Shh and K8, to restricted Shh and K8 expression primarily in the taste papilla placodes [12,14]. The K14⁺ cells in between taste papillae lose Shh and K8 expression. Multiple studies have examined the effects of signaling pathways on papilla patterning [13]. For example, inhibition of Shh signaling [18,26,27] or BMP signaling [28–30], and activation of Wnt/ β -catenin [31–33] signaling induces the formation of many more papillae – even outside regions of normal papilla development. These papillae were capable of developing *after* K14 expression first appears in the dorsal tongue epithelium, indicating that cell fate is reversible, i.e., from Shh⁺K8⁺ to K14⁺ and vice versa. Considering that K14⁺ cells give rise to taste bud cells in postnatal mice, a clear understanding of the signaling pathways determining whether a progenitor will become a taste bud cell or a cell of the surrounding epithelium will provide a significant step towards understanding the signaling pathways guiding postnatal progenitor cell fate decisions.

In summary, we provide evidence that taste buds and non-gustatory tongue epithelium derive from a common precursor population – the early E11.5 Shh⁺K8⁺ epithelium of tongue swellings. In combination with previous reports, we propose a model regarding the development of early taste buds (Fig. 4). At E11.5, the tongue primordium is covered by a homogeneous Shh⁺K8⁺ epithelium, which will either remain Shh⁺K8⁺ to become taste papilla placodes or lose Shh and K8 expression to become K14⁺ epithelium between taste papillae. The original Shh⁺K8⁺ cells will become early taste bud cells and are renewed postnatally by the K14⁺ cells of the surrounding epithelium and potentially the Dermo1⁺ cells of the connective tissue.

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Fig. 1. K14-Cre does not label taste papillae or early taste buds.

A: Histograms show RNA-Seq FPKM-derived fold changes for K14 compared to GAPDH at E12.5, E14.5, and P1. **B:** Single-plane laser scanning confocal images of a P1 K14-Cre/nTnG mouse tongue. Lingual epithelium was stained with E-cadherin (purple) to demonstrate the specificity of K14-Cre labeled cells (GFP⁺) to epithelium. **C:** Single-plane laser scanning confocal images of sagittal sections of K14-Cre/nTnG mouse tongues to illustrate distribution of K14-Cre labeled cells (GFP⁺) in developing fungiform papillae or taste buds at E12.5, E14.5, and E18.5, P1, and 8 wks old. Sections were immunostained with K8 (purple). Dots encircle taste papilla placodes (E12.5), taste papillae (E14.5), or taste buds (E18.5, P1, 8 wks). Arrows in C (E12.5) point to GFP⁺ cells in the dorsal tongue surface; arrowheads (E12.5) point to the ventral surface of the tongue. Scale bars: 50 μ m in B and C.

Fig. 2. Early taste buds were not labeled by Dermo1-Cre.

A: Histograms show RNA-Seq FPKM-derived fold change for Dermo1 compared to GAPDH at E12.5, E14.5, and P1. **B, C:** Single-plane laser scanning confocal images of sagittal sections of fungiform papillae of Dermo1-Cre/nTnG mice at P1. Sections were immunostained with E-cadherin (**B**, purple) or K8 (**C**, purple). Dermo1-Cre labeled cells (GFP⁺) were abundantly and exclusively distributed in the connective tissue under the E-cadherin⁺ epithelium. Dots in **C** encircle a taste bud. Scale bars: 50 μm in **B** and **C**.

Fig. 3. Gene expression and fate mapping of the epithelial cells of E11.5 tongue swellings.
A: RT-qPCR 2^{-CT} values for K8 (**A₁**) and K14 expression (**A₂**) at E11.5 and P1, normalized to GAPDH, and the PCR product bands (**A₃**). **B:** Single-plane laser scanning confocal images of tongue epithelium from an E11.5 K14-Cre/nTnG embryo stained with K8 (purple) (**B₁**) or showing GFP only (**B₂**). No GFP⁺ cells were observed in the tongue swelling's epithelium. **C:** Wild type E11.5 tongue/mandible immunostained for Shh (**C₁**), followed by sectioning (**C₂**). Low and high (inset) magnification images of a section from an E11.5 tongue whole mount immunostained for Shh show abundant Shh expression in the epithelium of the tongue primordium. **D:** E18.5 Shh-CreER^{T2}/RFP mice were provided tamoxifen or vehicle at E11.0. **D_{1,2}**: whole mount images of the tongue/mandible of tamoxifen (**D₁**) or vehicle (**D₂**) treated mice. **D_{3,4}**: Sagittal sections of E18.5 Shh-CreER^{T2}/RFP embryo tongues that received tamoxifen at E11.0 were stained with E-

cadherin (green, **D₃**) or K8 (green, **D₄**). Scale bars: 20 μm in **B₁₋₂** and **D₃₋₄**, 100 μm in **C₁₋₂**, 12.5 μm in **C₂** inset, 200 μm in **D₁₋₂**.

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Fig. 4. A proposed model about cell derivation for early taste bud development (left) and mature taste bud cell renewal (right).

Yellow nuclei denote cells that express Shh and K8 and possess a multipotent lineage. Red denotes cells that express Shh and K8, but are lineage restricted to become taste bud cells, while green nuclei show cells that have stopped expressing Shh and K8, instead express K14, and are lineage restricted to become non-gustatory epithelial cells. Purple cells are cells in the underlying connective tissue of the papilla. Blue and pink cells are mature taste bud cells that are derived from surrounding epithelium and underlying connective tissue, respectively.