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Sonic hedgehog-expressing basal cells are general post-mitotic precursors of functional taste receptor cells.

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Abbreviated title: Shh+ basal cells differentiate into all taste cell types

Key words: Mouse, molecular genetics, lineage tracing, tamoxifen, Cre recombinase, Shh, taste bud, cell lineage, regeneration

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ABSTRACT

Background: Taste buds contain ~60 elongate cells and several basal cells. Elongate cells comprise three functional taste cell types: I -- glial cells, II -- bitter/sweet/umami receptor cells, and III -- sour detectors. Although taste cells are continuously renewed, lineage relationships among cell types are ill-defined. Basal cells have been proposed as taste bud stem cells, a subset of which express Sonic hedgehog (Shh). However, Shh+ basal cells turnover rapidly suggesting that Shh+ cells are precursors of some or all taste cell types.

Results: To fate map Shh-expressing cells, mice carrying ShhCreER\textsuperscript{T2} and a high (CAG-CAT-EGFP) or low (R26RLacZ) efficiency reporter were given tamoxifen to activate Cre in Shh+ cells. Using R26RLacZ, lineage-labeled cells occur singly within buds, supporting a post-mitotic state for Shh+ cells. Using either reporter, we show that Shh+ cells differentiate into all three taste cell types, in proportions reflecting cell type ratios in taste buds (I > II > III).

Conclusions: Shh+ cells are not stem cells, but are post-mitotic, immediate precursors of taste cells. Shh+ cells differentiate into each of the three taste cell types, and the choice of a specific taste cell fate is regulated to maintain the proper ratio within buds.
INTRODUCTION

Taste buds, the sensory endorgans for gustation, are a heterogeneous assemblage of 30-100 elongate cells with a small number of ovoid cells situated in the basal compartment. In the oral cavity of rodents, taste buds are housed in three distinct lingual taste papillae, the fungiform, foliate and circumvallate, and are embedded in the epithelium of the soft palate. Within oral taste buds, elongate cells are classified into 3 main types, based on their morphology and expression of specific molecular markers (Chaudhari and Roper, 2010; Finger and Simon, 2000; Murray, 1973). Type II cells are responsive to sweet, umami or bitter tastants, and express taste receptor proteins for these tastants (DeFazio et al., 2006), as well as elements of the common downstream transduction cascade, including PLCβ2 (Clapp et al., 2004), IP3R3 (Clapp et al., 2001; Miyoshi et al., 2001) and Trpm5 (Pérez et al., 2002; Zhang et al., 2003). Type II cells release ATP as a neurotransmitter in response to taste stimuli (Finger et al., 2005), and secreted ATP is assumed to be catalyzed by NTPDase2, an ecto-ATPase, distributed selectively on the plasma membranes of glial-like Type I cells (Bartel et al., 2006; Vandenbeuch et al., 2013). In addition to clearing neurotransmitter, Type I cells exhibit another glial-like property in that these cells have extensive cellular processes which tightly wrap neighboring taste cells (Pumplin et al., 1997). Lastly, type III cells are responsive to sour taste, and express proteins associated with sour transduction, including PKD2L1 and Carbonic anhydrase IV (Chandrashekar et al., 2009; Kataoka et al., 2008). Further, Type III cells are the only taste cell type to form conventional synapses on gustatory afferent nerves, and thus express numerous proteins associated with differentiated neurons, such as NCAM and Snap25, and accumulate the neurotransmitter, serotonin (Nelson and Finger, 1993; Yang et al., 2000; Yee et al., 2001).

Despite these neural characteristics, taste buds are epithelial in origin (Barlow and Northcutt, 1995; Stone et al., 1995), and in adults, taste cells are continuously renewed throughout life with an average cell lifespan of 10-14 days (Beidler and Smallman, 1965; Farbman, 1980). The current model of taste bud cell turnover holds that taste bud stem cells located outside of taste buds
self-renew and generate transit amplifying (TA) cells. TA cells ultimately exit the cell cycle, enter buds as post-mitotic taste precursor cells, which in turn differentiate into mature taste receptor cells (Mistretta and Hill, 1995; Miura and Barlow, 2010). In support of this model, Okubo et al. (2009) used genetic lineage tracing in mice to show that Keratin (K)14-positive basal epithelial cells located outside of taste buds gives rise to cells which populate taste buds and taste papillae. However, K14+ cells do not generate taste bud cells exclusively; these basal keratinocytes also produce the non-taste epithelial cells of the tongue. In sum, while the progenitors for taste buds have been broadly identified, little is known of progressive steps in the cell lineage which leads to differentiated taste buds.

Basal cells represent a distinct subset of cells within taste buds. They have an ovoid morphology and lack processes, and most are thought to selectively express Sonic hedgehog (Shh) (Miura et al., 2001). However, the precise disposition of basal cells has not been defined. In fact, basal cells, based upon their location in the basal taste bud compartment, have been proposed to function as taste bud stem cells or proliferating progenitors, based on marker expression and incorporation of thymidine analogs to monitor DNA synthesis during S phase (Asano-Miyoshi et al., 2008; Perea-Martinez et al., 2012; Sullivan et al., 2010). By contrast, using Shh expression as a marker of basal cells, Miura and colleagues have shown that Shh-expressing basal cells within taste buds are predominantly, albeit not exclusively, post-mitotic and display a rapid turnover rate (Miura et al., 2004; Miura et al., 2005). Based on these findings, Shh+ basal cells have been proposed as post-mitotic precursors for all or a subset of differentiated taste bud cell types (Miura and Barlow, 2010; Miura et al., 2006).

Here, we use genetic lineage tracing in adult mice to test this hypothesis. We show, using 2 different reporter alleles (CAG-CAT-EGFP – Kawamoto et al., 2000, and R26RLacZ – Soriano, 1999), that Shh-expressing basal cells differentiate into cells located exclusively within taste buds, and that Shh-descendent cells comprise all 3 taste cell types in each of the 3 taste fields examined (fungiform papillae FP; circumvallate papilla CV; soft palate SP). Further, we find that the
proportions of lineage-labeled Type I, II and III taste cells reflect the relative abundance of each cell type in control taste buds, i.e., Type I cell are most frequently labeled, Type II have an intermediate labeling frequency, while lineage-labeled Type III are least common. Finally, leveraging the sparse labeling obtained with the Rosa26LacZ reporter allele (Soriano, 1999), we find that the distribution of lineage-labeled cells in taste buds reinforces a post-mitotic state for Shh-expressing basal cells. Thus, we conclude that these cells are not a stem population, but rather represent a potentially obligate precursor step for differentiation of taste cells. In sum, our study firmly establishes Shh-expressing basal cells as a population of post-mitotic precursors of each of the 3 functional taste receptor cell types, and identifies an important step in cell lineage progression of the primary receptor organs of the sense of taste.

RESULTS
To define the fate of the Shh-expressing basal cells of taste buds in adult mice, tamoxifen treatment was used to activate Cre recombinase in mice carrying the ShhcreER<sub>T2</sub> allele. Both low- and high-efficiency Cre-inducible reporter alleles, R26RLacZ (Soriano, 1999), and CAG-CAT-EGFP (Kawamoto et al., 2000), respectively, were employed to trace the fate of cells descendent from Shh+ basal cells. While both reporters provided qualitatively similar results, the R26RLacZ reporter resulted in significantly fewer lineage-labeled taste bud cells than the CAG-CAT-EGFP allele (see below).

In tongues of ShhcreER<sub>T2</sub>:R26RLacZ adult mice treated with tamoxifen, cells expressing the β-galactosidase lineage label were always detected within taste buds and never in the surrounding epithelium; this pattern was observed in each taste field examined including taste buds in the soft palate (SP), and in the fungiform (FF) and circumvallate(CV) papillae on the tongue. The morphology and distribution of labeled cells within taste buds shifted with the duration of the treatment protocol. Shorter protocols (2 daily tamoxifen doses with a 2 day chase) resulted in labeling of round cells in the basal compartment of taste buds, consistent with the morphology and
location of $Shh^+$ basal cells (Fig. 1A; Miura et al., 2001). By contrast, in tongues from mice given
tamoxifen over more days and harvested after longer chase periods, e.g., 4 daily doses with a 1
week chase, lineage-labeled cells were predominantly elongate, a morphology consistent with that
of differentiated taste cells (Fig. 1B).

In initial experiments with the R26RLacZ reporter allele, mice were given 4 daily injections
for a total tamoxifen dose of 16 mg / 20 g mouse. After 1 week, most labeled taste buds contained
only a single lineage-tagged taste cell, and moreover, across all 3 taste fields, only ~10-20% of buds
contained a $\beta$-galactosidase$^+$ cell (Fig 1C,D; gray bars). This labeling efficacy represented a smaller
proportion of $Shh^+$ cells than expected, as $Shh$ is typically expressed by 2 or more basal cells in
each taste bud in the CV papilla (Miura et al., 2001).

We next explored if providing daily tamoxifen over a longer period would improve labeling
efficiency. Specifically, newly generated basal cells begin to express $Shh$ within 12 hours of their last
cell division, and then cease $Shh$ expression 12-36 hrs later (Miura et al., 2001). We reasoned that if
the level of CreER expression driven by the Shh promoter was only briefly sufficient to activate the
R26RLacZ reporter allele, then supplying tamoxifen over a longer experimental period would
increase the probability of lineage labeling more newly expressing $Shh^+$ cells. Hence,

$Shh$CreER$^{12}$;R26RLacZ mice were treated daily with tamoxifen via oral gavage (to reduce the stress
of repeated dosing; Kiernayer et al., 2007) with 2 mg / 20 g mouse for 8 days (total dose = 16 mg /20 g mouse as above), and tongues examined after 1 or 10 days. Regardless of the duration of the
chase, or the taste field examined, again on average, only single lineage-labeled taste cells were
found in labeled taste buds (Fig. 1C). However, the proportion of taste buds with labeled cells was
significantly greater in mice that received the same total tamoxifen dose spread over 8 days
compared to 4 days of tamoxifen induction (Fig. 1D).

A substantially higher labeling efficiency was achieved with the CAG-CAT-EGFP reporter
allele. For example, using an identical tamoxifen dosing schedule (4 daily injections with a 1 week
chase), the mean number of labeled cells per bud was significantly higher in the 3 taste fields -- SP,
FF, and CV, compared with single cells found using the R26RLacZ reporter (Fig. 2A, gray bars, compare to gray bars in Fig. 1C). The proportion of taste buds with EGFP-labeled cells was also significantly increased, especially in the SP, where almost 90% of taste buds possessed at least one lineage-tagged taste cell (Fig. 2B). Hence, lineage labeling with the high efficiency CAG-CAT-EGFP reporter allele better reflected the number of Shh+ cells in adult mouse taste buds.

As roughly one Shh+ cell per taste bud was lineage labeled per day, e.g. ~3 cells per SP taste bud after 4 daily tamoxifen injections (Fig. 2A), and spreading the same dose across 8 days increased the proportion of taste buds with labeled cells (see Fig 1D), we reasoned that lineage labeling would increase in proportion to the period over which bigenic mice were exposed to tamoxifen, and, assuming labeling efficiency was constant over time, all cells within buds ultimately would be labeled with long term tamoxifen treatment. Thus, ShhcreERT2:CAG-CAT-EGFP mice were given tamoxifen continuously for 3 weeks as a suspension in the drinking water, such that each mouse consumed 36.6 ±1.2 mg / 20g bw of tamoxifen over the 21 day labeling period (roughly 5 times as long as the previous injection protocol). While this approach resulted in more labeled cells per labeled bud, as well as a higher proportion of labeled taste buds in both the SP and FF papillae, the increase in labeled cells per bud was far less than the 5 fold predicted. Rather, in the SP and FF, the number of lineage labeled cells per taste bud encountered after 21 days was less than double the labeling results obtained following 4 days of tamoxifen (Fig. 2A, black bars). Further, in the CV, labeling efficiency was unchanged in terms of the number of lineage-labeled cells per bud compared with 4 days of tamoxifen injection; while the percentage of EGFP-labeled buds within the CV trenches showed a tendency to decline with prolonged tamoxifen treatment (Fig. 2B, black bars).

**Shh-expressing basal cells give rise to Type II and Type III taste cells.**

To identify the specific taste cell types arising from Shh-expressing basal cells, taste buds with reporter-labeled cells were assessed with immunomarkers of Type II (Trpm5, PLCβ2, IP3R3) and Type III (Snap25, NCAM) taste cells (Fig. 3 and data not shown). Reporter-labeled cells of both
taste cell types were readily observed using either the R26RLacZ (Fig. 3A,B) or CAG-CAT-EGFP (Fig. 3C,D) allele. With the R26RLacZ reporter, we generally observed single reporter-labeled Type II or III cells within a bud (Fig. 3A,B), whereas more differentiated Type II and III cells were lineage-labeled with the CAG-CAT-EGFP allele under prolonged CreER induction (Fig. 3C,D). Still, after 3 weeks of tamoxifen induction, many Type II and III cells were unlabeled.

Regardless of reporter allele employed, far more lineage labeled cells were identified as Type II than Type III cells in each taste field (Table 1). This difference was greater for SP and FF taste buds, where Type II cells represented as many as ~30% of lineage-labeled cells, while Type III cells made up ~1.0-5.0% of cells newly arisen from Shh-expressing basal cells. In the CV, Type III cells made up a slightly higher proportion of lineage-labeled cells, yet still far fewer than Type II cells. Importantly, however, the majority of the reporter-labeled cells in each taste field were negative for both Type II and III cell-specific immunomarkers (see Fig 3C,D), suggesting that most of the remaining population likely comprised the abundant Type I glial-like cells.

**Shh-expressing basal cells give rise to Type I taste cells.**

NTPDase2, an ecto-ATPase, is selectively expressed by Type I cells and has become a conventional tool to identify the presence of this cell type in taste buds (Bartel et al., 2006). However, it is extremely difficult to identify individual Type I cells based on NTPDase2 immunoreactivity, because: 1) Type I cells have elaborate cellular processes that tightly wrap adjacent taste cells, as well as overlap with one another (Pumplin et al., 1997); 2) NTPDase2 localizes to the membranes of these complexly structured cells (Bartel et al., 2006); and 3) Type I cells are the most common cell type within buds (Murray, 1993). This problem is illustrated in Figure 4A, where taste buds from the SP, FF and CV are each shown, with Shh+ descendent cells with cytoplasmic EGFP (green cytosol) and NTPDase2-immunoreactivity (red membranes) in close proximity but not clearly coinciding. Based on the caveats above, it is not possible to determine if individual lineage-labeled cells are NTPDase2-immunopositive, or instead are wrapped closely by the processes of NTPDase2-ir Type I cells that lack lineage label.
Nonetheless, we did encounter candidate lineage-labeled cells with a morphology consistent with that of Type I cells (Fig 4B), i.e., irregularly shaped nuclei (red asterisk in Fig. 3B) and elaborate, sheet-like cellular processes (Fig 4B, green arrowhead). Additionally, these Type I-like lineage-labeled cells had cellular processes that closely wrapped other taste cells, such as PLCβ2+ Type II cells (Fig. 4B, PLCβ2-ir is red; white arrowhead Type II cell nucleus, white arrow Type II cell process). These putative Type I cells were always immunonegative for Type II or III cell markers.

To demonstrate unequivocally that Type I cells descend from Shh-expressing cells, we moved to a dispersed taste bud preparation. Taste buds from the CV and FF papillae, and the SP were taken from tamoxifen-treated ShhcreER\textsuperscript{T2};CAG-CAT-EGFP mice and dissociated into individual or small groups of cells which were then immunostained for NTPDase2. Individual EGFP-expressing cells were clearly NTPDase2-ir (Fig. 5, arrows), indicating that Shh-positive basal cells differentiate into Type I taste cells. Consistent with their majority representation in taste buds, NTPDase2-ir cells made up roughly half of all lineage labeled cells in dissociated taste bud preparations (SP: 58.4+/−1.8% of 278 EGFP+ cells; FF: 52.8+/−5.0% of 96 EGFP+ cells, CV: 44.0+/−6.6% of 275 EGFP+ cells, n=3 mice).

**DISCUSSION**

Using both low (R26RLacZ) and high (CAG-CAT-EGFP) efficiency Cre recombinase reporter alleles and unique markers of taste cell identity, we show systematically that Shh-expressing basal cells within buds are immediate precursors of all 3 differentiated taste cell types. Importantly, we obtained qualitatively similar results with both reporters. Moreover, our results with low efficiency genetic labeling strongly support previous findings that Shh-expressing basal cells are not proliferative or stem cells for taste buds, but rather represent a transient post-mitotic population (Miura et al., 2004). Recently, another group also reported that Shh-positive cells give rise to Type III taste cells immunoreactive for carbonic anhydrase IV in CV taste buds, and confirmed the turnover of these basal cells (Takeda et al., 2013). However, our study represents a comprehensive and quantitative
analysis of Shh-expressing basal cell lineage, revealing their contribution to each taste cell type in each of 3 major taste fields, the FF, CV and SP.

Two reporter alleles provide confirmatory results but with different labeling efficiencies

In our fate mapping studies of Shh+ basal cells, R26RLacZ and CAG-CAT-EGFP reporter alleles provided qualitatively identical results in response to CreER activation, consistent with published reports of lineage tracing using both reporters in other tissues (Bi et al., 2011; Schüller et al., 2008; Zhang et al., 2010). However, our quantitative analysis revealed significant differences in reporter efficacy, most likely due to differences in the molecular structure of the reporters. First, Cre recombinase is less efficient as the distance between targeted loxp sites increase, and thus higher levels of Cre enzyme are required with increased inter-loxp distance (Vooijs et al., 2001). As the sequence between loxp sites in the R26RLacZ allele (~2.5kb) (Soriano, 1999) is greater than that of the CAG-CAT-EGFP allele (~1.1 kb) (Kawamoto et al., 2000), higher Cre levels are required for recombination of the R26RLacZ reporter. Second, the CAG promoter is roughly 10 fold more robust than the Rosa26 promoter in transiently transfected or stably integrated mouse ES cells (Chen et al., 2011). Third, the R26RLacZ allele contains coding sequence for one copy of the LacZ gene knocked into the Rosa26 locus, while the CAG-CAT-EGFP transgene contains multiple copies of the EGFP reporter in a head-to-tail arrangement. Thus the CAG-CAT-EGFP reporter provides significant signal amplification over the R26RLacZ allele, and likely contributes the much greater labeling efficiency of the CAG-CAT-EGFP.

One additional experimental permutation may contribute to reporter efficiency in our study; different vehicles were used to dilute tamoxifen -- mineral oil for R26RLacZ and corn oil for CAG-CAT-EGFP. However, it is unlikely that the large differences in reporter allele expression observed are attributable to these different vehicles, as we have used corn and mineral oil interchangeably to activate reporter allele expression in ShhcreERT2 cell fate mapping studies in embryos, and see no differences in labeling efficiency (Thirumangalathu and Barlow, unpub.). Similarly no differences were detected in direct comparison of these oils in tamoxifen-inducible
Cre-lox fate mapping studies of intestinal epithelium (D. Breault pers comm., in prep.). Nonetheless, we cannot rule out that use of metabolically inert mineral oil as a vehicle may have contributed to the more limited labeling seen in the experiments with the R26RLacZ reporter.

**Shh+ basal cells are not stem cells, but are immediate post-mitotic precursors of taste cells.**

Taste cells are renewed from dividing keratinocytes outside of taste buds (Okubo et al., 2009), while cells within taste buds are generally thought to be post-mitotic (Beidler and Smallman, 1965; Harrison et al., 2011; Nguyen et al., 2012). However, occasional proliferative cells within buds have been reported (Hirot a et al., 2001; Perea-Martinez et al., 2012; Sullivan et al., 2010), leading to the proposal that taste buds house a resident proliferative basal cell population. *Shh* is expressed by many of these basal cells, and Miura and colleagues have shown, using BrdU birthdating, that the large majority of *Shh*+ basal cells are post-mitotic (Miura et al., 2006). Consistent with this result, in our low efficiency mapping studies with R26RLacZ, we find that *Shh*+ descendent cells occur singly, while labeled doublets were not encountered (data not shown), regardless of the duration of tamoxifen induction, or the taste field examined. Moreover, with a short chase, we found single lineage-labeled, ovoid cells situated in the basal compartment where *Shh*+ basal cells reside, and with increased chase times, individual lineage-tagged cells had the elongate morphology of differentiated taste receptor cells. The timing of this transition in cell shape is consistent with the known duration of taste cell differentiation. For Type II and Type III cells, differentiation, including expression of appropriate cell type immunomarkers and attainment of a fusiform morphology, takes 3-6 days following the last cell division (Cho et al., 1998; Hamamichi et al., 2006; Perea-Martinez et al., 2012). Finally, it is unlikely that single descendent cells predominate because apoptotic processes within taste buds regularly eliminate sibling cells, as cell death within taste buds is extraordinarily rare (Cohn et al., 2010; Nguyen et al., 2012; Zeng and Oakley, 1999). Thus our data are consistent with a post-mitotic status for *Shh*+ basal cells although we cannot rule out that additional *Shh*-negative basal cells may reside within buds, and retain the ability to undergo cell divisions.
Shh+ cells differentiate into each of the three taste cell types and do so in proportion to the abundance of each type in mature buds.

Using a panel of immunomarkers of each differentiated taste cell type, we show that Shh+ basal cells give rise to Type I, II and III cells, and that the proportions of Shh+ descendent cells differentiating into each type is roughly in agreement with their relative abundance in mature taste buds.

Type I cells are thought to comprise approximately half of the differentiated cells within taste buds (Bartel et al., 2006; Murray, 1993), although the precise proportion has been hard to discern in sectioned material because of the intricate and intertwined morphology of these cells. However, via anti-NTPDase2 immunostaining of dispersed taste bud cell preparations, we found that ~50% of Shh-descendent cells are indeed Type I cells. Type II cells represent ~20% of fusiform cells in each taste bud in all 3 taste fields (CV, Ma et al. 2007; FF and SP: Ohtubo and Yoshii, 2011). In our lineage tracing studies, the proportion of Shh-descendent Type II cells was 10-20% with the R26RLacZ reporter, whereas with the CAG-CAT-EGFP allele, Type II cells comprise 20-30% of Shh-descendent cells, again numbers roughly consistent with the prevalence of this cell type in mature taste buds. Type III cells are the least common, especially in FF and SP, where these Snap25+ cells represent 6 and 5%, respectively, of cells in each bud (Ohtubo and Yoshii, 2011). Type III cells are more common in CV taste buds, as assessed by serotonin immunoreactivity (Kim and Roper, 1995), where they comprise 15% of cells per taste buds (Ma et al., 2007). Consistent with their less frequent occurrence, overall fewer Shh+ descendent cells differentiated into Type III cells, however, far fewer lineage-labeled Type III cells were encountered than expected; no more than 4% of FF or SP, or 8% of CV Type III cells exhibited reporter expression (and see Table 1).

Why is Type III labeling so much more infrequent than expected? First, Type III cells have been shown recently to be significantly longer lived than Type II cells, with half lives of 22 versus 8 days, respectively (Perea-Martinez et al., 2012). Thus, Type III cells are renewed ~3 times less frequently than Type II cells, which could contribute to the limited labeling of Type III cells in our
study. Second, Shh may be expressed at lower levels or over a shorter period in basal cells destined to become Type III cells, such that levels of Cre recombinase driven by the Shh promoter are not always sufficient to activate reporter gene expression in cells destined to differentiate as Type III cells. Third, in lineage tracing studies in mouse embryos, Shh-expressing taste placodes give rise to differentiated Type I and II cells in postnatal mice; Type III cells were not encountered (Thirumangalathu et al., 2009). Thus, as in embryos, Shh-negative precursors for Type III cells may be present in adult taste buds. One plausible candidate is the population of Mash1-expressing basal cells found in adult taste buds (Kusakabe et al., 2002; Miura et al., 2003; Nakayama et al., 2008). The Mash1 bHLH transcription factor is also selectively expressed in NCAM-immunoreactive Type III cells (Miura et al., 2006; Seta et al., 1999; Seta et al., 2006), further implicating Mash1 in the differentiation of this cell type. Interestingly, some but not all Shh-expressing basal cells co-express Mash1, while some basal cells appear to express Mash1 and not Shh (Miura et al., 2006). It remains to be tested whether these expression patterns mark distinct populations of basal cells fated to give rise to different taste cell types.

**Prolonged tamoxifen induction does not increase reporter activation as expected.**

Providing tamoxifen ad libitum over 3 weeks did not dramatically increase lineage labeling rates as expected. In fact, in the CV, this experimental paradigm appeared to lessen labeling efficacy. There are several possible explanations for these observations. First, CreER activity alone can be deleterious, as this has been shown to cause increased apoptosis in developing embryos (Naiche and Papaioannou, 2007). Continual CreER nuclear localization caused by tamoxifen may likewise cause cell death of Shh+ cells and their descendents in taste buds. Additionally, accumulation of tamoxifen during continued dosing may result in cytotoxicity. Recently, Zhu and colleagues have shown that a subset of intestinal stem cells are unexpectedly sensitive to tamoxifen, as they undergo apoptosis when mice are treated with conventional doses for lineage tracing (Zhu et al., 2013). Certainly prolonged tamoxifen dosing via chow causes significant, albeit reversible, loss of body weight (Kiermayer et al., 2007) indicating tamoxifen may broadly influence overall
homeostasis in mice. A third possibility is that tamoxifen, functioning as an estrogen antagonist, may impact taste cell renewal somewhat more specifically. Both the acinar and ductal cells of the von Ebner’s salivary glands of the CV express estrogen receptors, in particular ERβ (Välimaa et al., 2004), as do minor salivary glands of the oral cavity (Tsinti et al., 2009). As salivary glands secrete cytokines, which have been shown to influence taste bud cell renewal (Morris-Wiman et al., 2000), altering estrogen responsiveness of von Ebner’s and other salivary glands may influence secretions important for taste bud maintenance.

How and when does a Shh+ basal cell commit to a specific taste cell fate and what is the role of SHH in this process?
Transit amplifying cells outside of taste buds generate taste precursor cells whose fate may already be committed before or as they complete their terminal division, enter the bud and commence Shh expression. Alternatively, taste cell type fate may be determined only once Shh+ precursors are situated within taste buds. Regardless of the timing of cell fate specification, local signals likely influence cell fate choice. Based on expression patterns in and around mature taste buds, a number of signaling pathways are likely candidates, including Wnt/β-catenin, BMP and SHH (Feng et al, 2014; Gaillard and Barlow, 2011, Nguyen and Barlow, 2010; Miura et al., 2001).
Specifically, Shh+ basal cells are thought to signal to adjacent stem and TA cells, as the latter express both the SHH receptor Ptc, and Gli1, a SHH target gene (Miura et al., 2001); however, the precise function of SHH in taste cell differentiation remains to be determined.

These well-known morphogens, as well as other local signals likely regulate expression of specific transcriptional regulators for each taste cell type. For example, Pou2F3 (Skn-1a), a homeodomain transcription factor, is selectively expressed in Type II cells and is required for their differentiation and/or maintenance (Matsumoto et al., 2011). Pou2F3 is also expressed in a small population of basal cells within taste buds, suggesting that Pou2F3 may assign Type II cell fate to post-mitotic, Shh-expressing precursors. Similarly, Mash1, a Type III cell-specific transcription factor
is also expressed in a subset of Shh-expressing basal cells (Miura et al., 2006), and is required for
development of Type III cells in embryos (Seta et al., 2011). Importantly, although the majority of
Shh-expressing cells differentiate into Type I cells, genes associated with Type I cell fate are
completely unknown.

In conclusion, our results establish Shh-expressing basal cells as general precursors for all 3
morphological taste cell types, but do not exclude the existence of additional Shh-negative
precursors which could give rise to distinct proportions or subsets of taste cells. Specifically, all taste
buds contain at least 5 functional categories of taste receptor cells, which detect sweet, bitter,
umami, sour and/or salt, as well as Type I support cells, and whether all of these functional cell
subtypes arise from Shh+ precursors remains to be tested.
EXPERIMENTAL PROCEDURES

Mice. ShhcreER<sup>T2</sup> mice (Harfe et al., 2004) were a gift from Clifford Tabin, Harvard Medical School or obtained from The Jackson Laboratory. R26RLacZ mice (Soriano, 1999) were obtained from Trevor Williams (University of Colorado School of Dental Medicine, USA). CAG-CAT-EGFP mice (Kawamoto et al., 2000) were obtained from the Center for Animal Resources and Development (CARD), Kumamoto University. ShhcreER<sup>T2</sup> and CAG-CAT-EGFP mice are maintained on the C57BL/6 background; the background of R26RLacZ mice is mixed. Mice were genotyped as described (Harfe et al., 2004; Kawamoto et al., 2000; Soriano, 1999), and maintained and sacrificed in accordance with protocols approved by the Institutional Animal Care and Use committee at the University of Colorado School of Medicine and Kagoshima University. ShhcreER<sup>T2</sup> males were crossed with R26RLacZ or CAG-CAT-EGFP females, and adult mice (5-10 weeks of age) possessing ShhcreER<sup>T2</sup> allele and either one of two reporter alleles were used for analysis.

Tamoxifen administration. Several methods of administration were employed, including i.p. injection, oral gavage, and in the drinking water provided in the home cage. Tamoxifen (Sigma, T-5648) was dissolved in corn oil (CAG-CAT-EGFP) or mineral oil (R26RLacZ) mixed with ethanol (9:1 b.v.) to a stock concentration of 20 mg/ml. For each i.p. injection, mice were dosed with 4 mg/20 g body weight. Tamoxifen in mineral oil was also administrated by oral gavage at a dose of 2 mg/20 g body weight. For administration in the drinking water, tamoxifen solution (20 mg/ml) was diluted 1:200 in water containing 1% (W/V) glucose, resulting in 0.1 mg/ml tamoxifen suspension containing 0.45% corn oil and 0.05% ethanol. Tamoxifen suspension was agitated by inverting the bottle several times each day. A fresh bottle of tamoxifen was provided every 3 days, and the volume of tamoxifen suspension consumed and the body weight of mice were measured at each bottle change. Likewise, body weight of mice given tamoxifen via i.p. injection or oral gavage was monitored daily.

Tissue sections. Dissected tissues of circumvallate papilla (CV), anterior tongue and soft palate were fixed in 4% paraformaldehyde in 0.1 M PBS for 3 hours, followed by immersion in sucrose (20% in 0.1 M PBS) overnight at 4°C. Cryoprotected tissues were embedded in OCT compound.
(Tissue-Tek) and cryosectioned at 6 µm. Sections were stored at -20 or -80°C.

**Dispersed taste bud cells.** Ringer’s solution containing 2.5 mg/ml collagenase (Worthington Biochemical) and 2.0 mg/ml elastase (Worthington Biochemical) was injected beneath the epithelium of the CV, anterior tongue and soft palate. After 25 minutes at room temperature, the epithelium was peeled off, immersed in collagenase/elastase-containing Ringer’s solution and incubated for 20 minutes at 37°C. The epithelium was then incubated in Ca\(^{2+}\)-free Ringer’s solution containing 2 mM EDTA for 15 minutes at room temperature, and fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min at 4°C. The epithelium was washed with deionized water and pinned on a silicon rubber plate with the basement membrane facing up. Taste bud cells were collected by sucking them into a tapered glass capillary of ~100 µm (inner diameter), dispensing them onto glass slides in a bubble of water, and then cells were adhered onto glass slides by evaporating the water on a hot-plate (55°C). The slides were stored at -80°C before use.

**Immunofluorescence.** Tissue sections or dissociated taste bud cells were refixed in 4% paraformaldehyde in 0.1 M PBS for 30 minutes on ice and washed with TBST (50 mM TBS containing 0.05% Tween 20). After blocking in TBST containing 1% blocking reagent (Roche), slides were incubated overnight with primary antibodies at 4°C, washed in TBST and treated with secondary antibodies for 2-3 hours at room temperature. Primary antisera and dilutions used include: guinea pig anti-β-galactosidase 1:500 (Gift from Dr. T.E. Finger, University of Colorado School of Medicine), mouse anti-GFP 1:1000 (Wako, 012-20461), rabbit anti-PLCβ2 1:200 (Santa Cruz, sc-206), rabbit anti-TrpM5 1:1000 gift from Dr. E. Liman, University of South California), mouse anti-IP3R3 1:100 (BD Bioscience, 610313), rabbit anti-Snap25 1:100 (Calbiochem, 567343), rabbit anti-NTPdase2 1:1000 (purchased from L.G. Lavoie and J. Sévigny, Université Laval, Canada). Secondary antibodies (dilution): Goat antibody (1:1000, Invitrogen) for rabbit IgG-Alexa568 or -Alexa546, guinea pig IgG-Alexa488, and mouse IgG-Alexa488. The mouse anti-EGFP antibody was used only for dispersed cell samples in combination with anti-mouse
IgG-Alexa488, since the intensity of EGFP fluorescence was markedly reduced in dispersed cell samples after fixation. Nuclei were counterstained with To-Pro-3 (Invitrogen).

**Image acquisition and analysis.** For immunofluorescence images, projected Z series of 0.75 µm optical sections of tissue sections and single optical sections of dispersed cells were obtained with Olympus Fluoview or Leica TCS SP5 II confocal microscope. Nomarski images of X-gal stained sections were obtained with a Zeiss Axioplan2 microscope and Axiocam cooled CCD camera. For tissue sections, every sixth section was tallied to avoid any double counting of marker-immunoreactive and/or LacZ/EGFP-positive cells. Images were contrast adjusted and sized in Adobe Photoshop.

**Statistical analysis.** For data in Figure 1, a one-way ANOVA test was performed, followed by Fisher’s LSD post hoc test, while for data in Figure 2, a Student’s t-test was employed.

**ACKNOWLEDGEMENTS**

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**Conflict of interest:** The authors declare no conflicts.

**Role of Authors:** HM conceived and carried out genetic experiments, imaging, data analysis and wrote the paper. JKS performed imaging and data analysis, and edited the manuscript. SH contributed to the writing of the manuscript. LAB conceived the experimental approaches, and contributed to imaging, analysis, writing and editing of the manuscript.
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FIGURE LEGENDS

Figure 1. Lineage labeling of Shh-expressing taste bud cells in ShhcreER^T2;R26RLacZ mice results in reporter gene expression in taste buds in all taste fields. A. A round, Xgal-positive cell (blue) is evident in the basal compartment of a CV taste bud of a bigenic mouse given 2 daily tamoxifen injections (i.p.) after a 2 day chase. Broken versus dotted white lines indicate a taste bud and the basement membrane, respectively. B. After 4 injections and a 7 day chase, a β-galactosidase-immunoreactive fusiform cell (green) is present in a CV taste bud of a ShhcreER^T2;R26RLacZ mouse (ToPro nuclear counterstain: purple). * in B left panel indicates nucleus of the Shh-descendent cell (green) in the middle and right panels. Scale bar in A,B: 20µm. C. The mean number of reporter+ cells per labeled taste bud profile does not vary with respect to tamoxifen treatment protocol; 4 daily i.p. injections + 1 week chase (gray bars) or 8 daily gavage doses of tamoxifen with a 1 or 10 day chase (open bars). D. The proportion of taste buds with labeled cells is significantly higher following the same total dose of tamoxifen given over 8 days (open bars) versus given in 4 days (gray bars). SP: soft palate, FF: fungiform papillae, CV: circumvallate papillae. One-way ANOVA, and Fisher’s LSD post hoc test. Mean ± SEM; n = 3-5 mice per treatment group.

Figure 2. The number of labeled cells per taste bud (A), and the proportion of taste buds with labeled cells (B) are significantly increased in ShhcreER^T2;CAG-CAT-EGFP mice treated with tamoxifen, either with a short term labeling paradigm (4 daily injections + 1 week chase; gray bars) or long term method (3 week access to tamoxifen in the drinking water; black bars). Pairwise comparisons assessed with Student’s t-test. Mean ± SEM; n = 3-5 mice per treatment group.

Figure 3. Shh-expressing basal cells give rise to both Type II and Type III taste cells in the SP, FF and CV taste fields. A,B. Beta-galactosidase-ir cells (green; arrows) descendental from Shh-expressing cells become Trpm5-ir Type II cells (red in A) or Snap25-ir Type III cells (red in B) in ShhcreER^T2;R26RLacZ mice, shown here after 4 daily injections of tamoxifen and a 7-day chase. C,D. Similarly, EGFP-expressing cells (green; arrows) become PLCβ2-ir Type II cells (red in C) or
Snap25-ir Type III cells (red in D) in ShhcreER\textsuperscript{T2}:CAG-CAT-EGFP mice, shown here following tamoxifen for 21 days via drinking water. Scale bars 20 µm. TO-PRO3 nuclear counterstain (blue). Images are stacks of 2 or 3 confocal sections taken at 0.75 µm intervals. Dorsal is up in all panels.

**Figure 4.** Type I taste cells descendent from Shh-expressing basal cells cannot be identified unequivocally in tissue sections. **A.** In ShhcreER\textsuperscript{T2}:CAG-CAT-EGFP taste buds (4 daily injections, 7 day chase), EGFP-labeled taste cells (green) are surrounded by NTPDase2-ir processes of Type I cells (red), but double labeled cells cannot be identified unambiguously (see text). Images are single confocal sections (0.75 µm). **B.** A lineage-labeled EGFP-expressing cell (green) has a Type I cell morphology, including an apical fan-like process (green arrowhead), a slender nucleus (red asterisk), a process that tightly wraps a neighboring PLCβ2-ir Type II cell body (red: note a slim rim of PLCβ2-ir cytoplasm surrounds the round Type II cell nucleus (white arrowhead), and an additional process that wraps the apical process of another PLCβ2-ir cell (white arrow). Images represent a stack of 3 confocal sections (0.75 µm/optical section) of an SP taste bud from a ShhcreER\textsuperscript{T2}:CAG-CAT-EGFP mouse given tamoxifen for 21 days via the drinking water. For all panels, nuclei are counterstained with TO-PRO3 (blue, but white in rightmost panel in B), scale bars are 20 µm, and dorsal is up.

**Figure 5.** Shh-expressing basal cells give rise to Type I cells. In dispersed taste bud samples from each taste field, numerous EGFP-expressing taste cells (green) are also NTPDase2-ir, indicative of Type I cells (red). Taste buds were taken from ShhcreER\textsuperscript{T2}:CAG-CAT-EGFP mice after 4 tamoxifen injections and a 7-day chase. Scale bars: 50 µm. TO-PRO3 nuclear counterstain (blue). Fluorescent images are single confocal sections.
Table 1. Percentage of Type II (PLCβ2-ir) and Type III (Snap25-ir) cells among reporter-expressing cells.

<table>
<thead>
<tr>
<th>Reporter (vehicle)</th>
<th>treatment + chase days (route)</th>
<th>Soft palate</th>
<th>Fungiform</th>
<th>Circumvallate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Type II</td>
<td>Type III</td>
<td>Type II</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R26RLacZ (mineral oil)</td>
<td>8d + 10d (gavage)</td>
<td>9.4 ± 5.4%</td>
<td>N.D.</td>
<td>14.8 ± 7.0%</td>
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<td>3.0 ± 3.0%</td>
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<tr>
<td></td>
<td>R26RLacZ (mineral oil)</td>
<td>8.8 ± 5.4%</td>
<td>1.0 ± 1.0%</td>
<td>24.9 ± 12.6%</td>
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<tr>
<td></td>
<td>16eod§+ 10d (gavage)</td>
<td>(48/104)</td>
<td>(56/99)</td>
<td>(93/193)</td>
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<tr>
<td></td>
<td></td>
<td>(74/105)</td>
<td>(58/166)</td>
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<td>CAG-CAT-EGFP (corn oil)</td>
<td>4d + 1 wk (i.p. injection)</td>
<td>36.6 ± 8.2%</td>
<td>2.7 ± 1.1%</td>
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<td>1.5 ± 0.8%</td>
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<td>(250/99)</td>
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<td>(260/318)</td>
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<td></td>
<td>21d + 0d (drinking water)</td>
<td>30.2 ± 1.4%</td>
<td>1.8 ± 0.6%</td>
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<td></td>
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<td>(477/99)</td>
<td>(455/97)</td>
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<td></td>
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<td>(268/323)</td>
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Mean ± SEM. N = 3 mice.

(Total number of reporter expressing cells/Total number of taste buds examined).

N.D. Not done.

*Type II cells identified by IP3R35-ir. §eod: every other day.
A

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114x110mm (300 x 300 DPI)
Figure 5
114x112mm (300 x 300 DPI)