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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Stem and progenitor cell compartments within adult mouse taste buds

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Abstract

Adult taste buds are maintained by the lifelong proliferation of epithelial stem and progenitor cells, the identities of which have remained elusive. It has been proposed that these cells reside either within the taste bud (intragemmal) or in the surrounding epithelium (perigemmal). Here, we apply three different *in vivo* approaches enabling single-cell resolution of proliferative history to identify putative stem and progenitor cells associated with adult mouse taste buds. Experiments were performed across the circadian peak in oral epithelial proliferation (04:00 h), a time period in which mitotic activity in taste buds has not yet been detailed. Using double label pulse-chase experiments, we show that defined intragemmal cells (taste and basal) and perigemmal cells undergo rapid, sequential cell divisions and thus represent potential progenitor cells. Strikingly, mitotic activity was observed in taste cells previously thought to be postmitotic (labelled cells occur in 30% of palatal taste buds after 1 h of BrdU exposure). Basal cells showed expression of the transcription factor p63, required for maintaining the self-renewal potential of various epithelial stem cell types. Candidate taste stem cells were identified almost exclusively as basal cells using the label-retaining cell approach to localize slow-cycling cells (0.06 ± 0.01 cells per taste bud; n = 436 taste buds). Together, these results indicate that both stem- and progenitor-like cells reside within the mammalian taste bud.

Introduction

Taste buds, the sensory endorgans for taste, are maintained by the lifelong addition and incorporation of new cells (reviewed by Miura *et al.*, 2006). Although taste buds are known to arise from local epithelium (Stone *et al.*, 1995; Okubo *et al.*, 2009; Thirumangalathu *et al.*, 2009), little is known about the locations and phenotypes of the stem and progenitor cell populations maintaining the continuous genesis of taste cells. Proliferation and lineage tracing experiments have led to two differing hypotheses, that the stem and progenitor cells maintaining adult taste buds are located either inside or outside the taste bud (Miura *et al.*, 2006; Okubo *et al.*, 2009).

Individual taste buds in mice consist of a tight cluster of ~30–150 intragemmal cells of four subtypes (types I–IV) (Murray, 1973; Ma *et al.*, 2007), and are surrounded by perigemmal cells (Sakai *et al.*, 1999). Taste cell types I–III are postmitotic (Hirota *et al.*, 2001) and function as supporting cells (type I), tastant receptors (type II) and presynaptic cells (type III) (Roper, 2007). In contrast, basal cells (type IV), which occur in contact with the basement membrane (Olivieri-Sangiacomo, 1972; Delay *et al.*, 1986), appear to exhibit mitotic activity. The extent of proliferation observed in basal cells has differed markedly between studies, with some authors describing high levels of proliferation (Delay *et al.*, 1986; Hendricks *et al.*, 2004) whereas others have found no evidence of mitotic activity (Beidler & Smallman, 1965; Asano-Miyoshi *et al.*, 2008). In these latter studies, mitotically active cells were observed immediately adjacent to the base of the taste bud.

Mitotic activity within the oral epithelia of rodents exhibits one of the strongest circadian rhythms in cell proliferation seen in any tissue (Potten *et al.*, 2002b; Luo *et al.*, 2009). The peak in proliferation (as measured by S phase) occurs around 04:00 h with the nadir at 16:00 h (Potten *et al.*, 2002b; Luo *et al.*, 2009; see also Supporting information, Fig. S1). Despite this marked circadian rhythm, few studies examining proliferation associated with taste buds have incorporated this feature into their experimental design (but see Hamamichi *et al.*, 2006). Furthermore, a systematic examination of proliferation across the peak in proliferative activity has not yet been undertaken.

As identification of taste bud stem and progenitor cell populations has proven elusive, here we used three different *in vivo* approaches to detect rapid and slow dividing cells associated with taste buds of the tongue (fungiform, circumvallate) and soft palate (palatal). All experiments were performed across the peak of the circadian rhythm in proliferation. To detect rapid re-entry into the cell cycle, we undertook two double labelling pulse-chase experiments using nucleotide analogues. The label-retaining cell approach (Kalabis *et al.*, 2008) was used to localize slow cycling candidate stem cells. The resolution afforded by these techniques provides the opportunity to examine the temporal history of proliferation at the single cell level.

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Materials and methods

Animals

Adult male and female CBA/CaH mice (6–8 weeks) were used in this study ($n = 35$). Animals were maintained on a 12/12-h light–dark cycle with lights off at 19:00 h (Potten *et al.*, 2002b). All procedures were approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee and were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) of the National Health and Medical Research Council of Australia. All mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/ml) and perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4 (PBS).

Proliferating cell types at the circadian peak

Mitotically active cells associated with taste buds were labelled using either 5-bromo-2′-deoxyuridine (BrdU) incorporation, Ki67 expression or expression of phospho-histone H3 [pSer^{10}]. BrdU is a thymidine analogue that is incorporated into dividing cells during S phase of the cell cycle and can be visualized immunohistochemically. In contrast, Ki67 is expressed in all active phases of the cell cycle (G_1, S, G_2 and M phases) (Scholzen & Gerdes, 2000). Both labels were applied across the peak (04:00 h) in the circadian rhythm in proliferation. In BrdU experiments, animals ($n = 5$) received a single intraperitoneal injection at 03:30 h of BrdU (380 μmol/kg body weight; Sigma, St Louis, MO, USA) dissolved in 0.007 M NaOH in 0.9% NaCl. The animals were then killed 1 h later. For Ki67 labelling, animals ($n = 5$) were killed at 04:00 h. Phosphorylation of histone H3 at Ser^{10} occurs from prophase to telophase (McManus & Hendzel, 2006), and can therefore be used to identify cells in M phase of the cell cycle. For phospho-histone H3 labelling, animals ($n = 5$) were killed during the period (12:30–13:30 h) in which most cells in the oral epithelia enter M phase (Potten *et al.*, 2002b).

Identification of rapid cycling progenitor cell types

To examine the temporal history of division in cells associated with taste buds, two double labelling pulse-chase experiments were performed to detect cells rapidly re-entering the cell cycle. In the first study, mice ($n = 4$) received two intraperitoneal injections (21:00 h, 03:00 h) of BrdU (380 μmol/kg body weight) to label a cohort of dividing cells across the circadian peak in proliferation. Animals were killed 1 or 2 days later at 04:00 h and immunolabelled for BrdU and Ki67 to localize cells that were mitotically active at both time points. The second study involved the sequential, equimolar administration of the thymidine analogues 5-chloro-2′-deoxyuridine (CldU) and 5-iodo-2′-deoxyuridine (IdU). Like BrdU, these halogenated deoxyuridines are incorporated into DNA during the S phase of mitosis and can be labelled immunohistochemically (Aten *et al.*, 1994). In the present study, animals ($n = 10$) initially received four intraperitoneal injections over 24 h (21:00, 03:00, 09:00 and 15:00 h) of CldU (380 μmol/kg body weight; MP Biomedicals, Irvine, CA, USA) dissolved in 0.007 M NaOH in 0.9% NaCl, to label the majority of the population of proliferating cells in the oral epithelia. These time points were chosen such that the early morning injection (03:00 h) was applied across the peak (04:00 h) in the circadian rhythm of proliferation; the bioavailability of CldU is estimated to be ~2 h (Bauer & Patterson, 2005; Taupin, 2007). The animals were then left for 1 or 2 days, thus ensuring that all cells that had incorporated CldU had left S phase, estimated to have a duration of 5.1–5.5 h in mouse oral epithelia (Burns *et al.*, 1975). Animals then received four intraperitoneal injections over 24 h (21:00, 03:00, 09:00 and 15:00 h) of IdU (380 μmol/kg body weight; MP Biomedicals) dissolved in 0.014 M NaOH in 0.9% NaCl. Animals were killed 6 h after the final injection and immunolabelled for CldU and IdU, to localize those cells that had rapidly re-entered the cell cycle after incorporation of CldU and subsequently also incorporated IdU.

p63 expression in proliferating cell types

Expression of p63 in mitotically active cells was examined by double labelling for p63 and BrdU or Ki67 across the circadian peak in proliferation. In BrdU experiments ($n = 2$), animals received a single intraperitoneal injection at 03:30 h of BrdU (380 μmol/kg body weight) and were then killed 1 h later. For Ki67 labelling ($n = 2$), animals were killed at 04:00 h.

Frequency and distribution of label-retaining cells

Nucleotide label retention has been shown to be a property of epithelial stem cells, and has been used previously to identify putative stem cell populations in a number of epithelial tissues (Bickenbach, 1981; Cotsarelis *et al.*, 1989, 1990; Taylor *et al.*, 2000; Braun *et al.*, 2003; Chan & Gargett, 2006; Kalabis *et al.*, 2008; Muffler *et al.*, 2008). Despite their reduced mitotic index, epithelial stem cells can be labelled by prolonged administration (pulse) of nucleotide analogues. Infrequently dividing stem cells can thereafter be identified as label-retaining cells as they retain the exogenous nucleotide analogues after long-term survival (chase) whereas these labels are lost through dilution in more rapidly dividing cells (Bickenbach, 1981; Cotsarelis *et al.*, 1990; Braun & Prowse, 2006; Blanpain & Fuchs, 2009). Because the basement membrane plays a critical role in governing the proliferative capacity of epithelial stem cells (Fuchs, 2008; Blanpain & Fuchs, 2009), only basal label-retaining cells were examined here.

To identify label-retaining cells associated with taste buds, mice ($n = 6$) received intraperitoneal injections of BrdU (380 μmol/kg body weight) every 6 h for a period of 48 h (21:00, 03:00, 09:00 and 15:00 h). A similar protocol has recently been used to detect label-retaining cells in the adult mouse brain (Golmohammadi *et al.*, 2008). Animals were killed 15 or 30 days after the last injection.

Tissue preparation

Following perfusion with 4% paraformaldehyde, the tongue and soft palate were removed and post-fixed overnight in the same fixative. Tissues were rinsed over 2 h in PBS and regions containing circumvallate or fungiform papillae were dissected from the tongue. Antigen retrieval was performed *en bloc* according to the method of Ino (2003). Tissues were initially immersed overnight in 10 mM sodium citrate buffer (pH 6.0) at 4°C and then heated to ~99°C for 20 min in citrate buffer in a food steamer. For Ki67 labelling, antigen retrieval was performed over 60 min in 0.1 M Tris-HCl buffer containing 5% urea, pH 9.5 (Hirota *et al.*, 2001). Specimens were then left to cool in the retrieval buffer for 1 h at room temperature. Subsequently, tissues were rinsed for 2 h in PBS, suspended in 4% Noble agar (Difco, Detroit, MI, USA) and sectioned at 35 μm on a vibratome. For Ki67 labelling, tissues were placed in 30% sucrose in PBS for 24 h at 4°C, frozen in OCT (Sakura Finetek, Tokyo, Japan) and sectioned at 10 μm on a cryostat.

Antibodies

The following primary antibodies were used: monoclonal mouse anti-BrdU (1 : 20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; G3G4), monoclonal mouse anti-BrdU (1 : 400; BD Bioscience, Franklin Lakes, NJ, USA; 347580), monoclonal rat anti-BrdU (1 : 250; AbD Serotec, Raleigh, NC, USA; MCA2060), monoclonal rat anti-cytokeratin 8 (1 : 40; Developmental Studies Hybridoma Bank; TROMA1), polyclonal rabbit anti-phospholipase C β2 (1 : 50; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-206), polyclonal rabbit anti-Ki67 (1 : 500; Leica Microsystems, Milton Keynes, UK; NCL-Ki67-p), monoclonal rabbit anti-Ki67 (1 : 50; Biocare Medical, Concord, CA, USA; CRM325), polyclonal rabbit anti-phospho-histone H3 [pSer^{10}] (1 : 200; Sigma; H0412), and monoclonal mouse anti-p63 (1 : 50; Santa Cruz Biotechnology; sc-8431). Fluorescent secondary antibodies used were: Alexa 488-conjugated goat anti-mouse IgG (1 : 200; Invitrogen, Carlsbad, CA, USA; A-11029), Alexa 594-conjugated goat anti-rabbit IgG (1 : 200; Invitrogen; A11012) and DyLight 649-conjugated goat anti-rat IgG (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA; 112-495-167).

Antibody characterization

Three antibodies directed against BrdU were used: a monoclonal rat antibody and two monoclonal mouse antibodies. Each antibody displayed an identical pattern of staining, reacting with the nuclei of cells that had incorporated BrdU during S phase of the cell cycle. Immunoreactivity was not observed in tissue sections from mice that had not been injected with BrdU. The first mouse anti-BrdU antibody (DSHB; lot no. 8/9/07) was used to identify proliferating cells associated with taste buds and in experiments involving double-labelling experiments with Ki67. This antibody is a monoclonal IgG raised against BrdU conjugated to bovine serum albumin.

For double nucleotide analogue labelling experiments, the rat anti-BrdU antibody and the second mouse anti-BrdU antibody (BD Biosciences) were used. The specificities of these two anti-BrdU antibodies have been extensively characterized for use in experiments involving double labelling for IdU and CldU (Aten *et al.*, 1992, 1994; Maslov *et al.*, 2004; Bauer & Patterson, 2005). The rat anti-BrdU antibody (clone BU1/75 ICR1; lot no. 240408) is a monoclonal IgG which reacts with BrdU and CldU, but exhibits no cross-reactivity to IdU (manufacturer's technical information; Aten *et al.*, 1992; Burns & Kuan, 2005). The mouse anti-BrdU antibody (clone B44; lot no. 07873), derived from hybridization of mouse Sp2/O-Ag14 myeloma cells with spleen cells from BALB/c mice immunized with iodouridine-conjugated ovalbumin, reacts with both BrdU and IdU (manufacturer's technical information; Gratzner, 1982; Aten *et al.*, 1992). This antibody also exhibits weak cross-reactivity to CldU, but antibodies bound to CldU-containing DNA can be removed selectively by washing in Tris buffer with a high salt concentration (Aten *et al.*, 1992; Maslov *et al.*, 2004; Bauer & Patterson, 2005). In the present study, specificity of the observed labelling was assessed using sections from animals injected with IdU that were labelled for CldU and from animals injected with CldU that were labelled for IdU. No labelling was detected in these control experiments.

The anti-cytokeratin 8 antibody (lot no. 9/10/07) is a rat monoclonal IgG raised against cytokeratin 8 purified from mouse trophoblastoma. This antibody reacts specifically with cytokeratin 8 in two-dimensional gels of HeLa cytoskeletons (Altmannsberger *et al.*, 1986). The anti-phospholipase C β2 antibody (lot no. G2009) is a rabbit polyclonal IgG raised against a synthetic peptide corresponding to residues 1170–1181 of human phospholipase C β2 (Yang *et al.*, 2007). This antibody detects a major band at 120 kDa in immunoblot analyses of RAW 264.7 whole-cell lysate (manufacturer's technical information). The polyclonal anti-Ki67 antibody (lot no. 301119) was raised against bacterially expressed residues 1159–1522 of human Ki67 (manufacturer's technical information). This antibody stained proliferating cells in the basal layer of the mouse tongue epithelium, comparable with those described previously (Hirota *et al.*, 2001) and detects a double band at 345 and 395 kDa in immunoblot analyses (IM-9 cells; Key *et al.*, 1993). The monoclonal anti-Ki67 antibody (clone SP6; lot no. 062708) is rabbit IgG raised against a synthetic peptide corresponding to the C-terminus of human Ki-67 (manufacturer's technical information). This antibody stained proliferating cells in the basal layer of the mouse tongue epithelium, comparable with those described previously (Hirota *et al.*, 2001) and detects a band at 359 kDa in immunoblots (SKBR3 cells, manufacturer's technical information) consistent with the molecular weight of the larger protein isoform (Scholzen & Gerdes, 2000). The anti-phospho-histone H3 [pSer^{10}] antibody (lot no. 092K4808) is a rabbit polyclonal IgG raised against a keyhole limpet haemocyanin-conjugated synthetic peptide (ARK[pS]TGGKAPRKQLC) corresponding to the N-terminus of human histone H3. This histone H3 sequence is identical in human and mouse. The antibody has been purified by absorption on the non-phosphorylated peptide, and staining in immunoblots (17-kDa band, Jurkat cells) is specifically inhibited with the immunizing peptide (manufacturer's technical information). The anti-p63 antibody (clone 4A4; lot no. l1508) is a mouse monoclonal IgG raised against amino acids 1–205 at the N-terminus of human p63. Immunoreactivity to this antibody is abolished in p63 null mice (Livera *et al.*, 2008).

Immunohistochemistry

Tissue sections were permeabilized in PBS containing 0.3% Triton X-100 (PBSTx) for 1 h. Sections to be labelled for BrdU incorporation were incubated in TrypLE Express (Invitrogen) for 3 h at room temperature and rinsed in PBSTx over 2 h. Tissues were then blocked for 1 h in 10% normal goat serum in PBSTx (NS-PBSTx). Primary antibodies were diluted in NS-PBSTx and applied overnight at 4°C. Sections were then rinsed for 4 h in several changes of PBSTx and incubated overnight at 4°C in the appropriate secondary antibodies diluted in PBSTx. Sections were counterstained by incubation overnight at 4°C in rhodamine-conjugated *Ulex europaeus* agglutinin I (1 : 100; Vector Laboratories, Burlingame, CA, USA), rhodamine-conjugated *Phaseolus vulgaris* leucoagglutinin (1 : 100; Vector Laboratories), or the nuclear stain Neurotrace 500/525 (1 : 50; Invitrogen). Subsequently, sections were rinsed for 4 h in PBSTx and mounted in Gelmount (Biømeda, Foster City, CA, USA).

Double labelling for CldU and IdU was achieved using two anti-BrdU antisera with differing affinities to the two thymidine analogues (Aten *et al.*, 1994). Sections were incubated overnight in the mouse monoclonal anti-BrdU antibody (cross-reactivity with IdU) at 4°C, washed in a high-stringency solution (40 mM Tris-HCl, 50 mM NaCl and Tween 1%, pH 8.0) for 1 h at room temperature to eliminate cross-reactivity with CldU, and incubated overnight with the rat monoclonal anti-BrdU antibody (cross-reactivity with CldU) at 4°C. Sections were then rinsed in PBSTx and incubated for 3 h at room temperature in the appropriate secondary antibodies.

Sections were viewed using a Leica TCS SP1 laser-scanning confocal microscope equipped with a Leica PL APO 40 × 1.25–0.75 NA oil-immersion objective lens. Images represent single optical sections (512 × 512 pixels) or *z*-projections of two to three adjacent optical sections, as indicated in the figure legends. Double-labelling of

individual nuclei was confirmed in the *x*, *y* and *z* axes, and all images were taken within the boundaries of the taste buds. Images were processed to adjust brightness and contrast using Adobe Photoshop 8.0 (Adobe Systems).

Statistics

Statistics are quoted as mean ± SEM. Significant differences in mean threshold values were determined using the D'Agostino & Pearson omnibus normality test, the Kruskal–Wallis test with Dunn's post test (Fig. 2; Fig. 5, analyses within taste buds; Table 1), the Mann–Whitney test (Fig. 5, analyses of perigemmal cells between taste buds), and the one-sample *t*-test (hypothetical value of 0; Fig. 5, analyses of taste cells between taste buds) (Prism, GraphPad). Exact *P*-values are reported for the Mann–Whitney test and one-sample *t*-test, while *P*-values are expressed as inequalities (at the 95 or 99.9% confidence intervals) for the Kruskal–Wallis test with Dunn's post test.

Results

As previous studies have identified functional and anatomical distinctions between taste bud types, here we examine mitotic activity in three different taste bud types of the tongue and soft palate. In the anterior tongue, taste buds are located within the fungiform papillae. Each papilla contains a single taste bud that resides within a depression located at the apex of the papilla (Fig. 1). Comparable depressions are also evident, although less pronounced, in the palate (see Fig. 2B). Only those cells located within these depressions were considered for analysis. At the rear of the rodent tongue, taste buds occur as rows within the walls of the single, large circumvallate papillae. As the perigemmal margins of individual taste buds in this papilla are less distinct, only those cells within or immediately adjacent to individual circumvallate taste buds were analysed.

The cell types associated with individual taste buds are shown in Fig. 1. Taste buds are comprised of two intragemmal cell types: taste (type I–III) and basal (type IV) cells. Many taste cells strongly express cytokeratin 8 (Knapp *et al.*, 1995; Asano-Miyoshi *et al.*, 2008) and send bipolar projections to the taste bud pore and the basement membrane. Cytokeratin 8 labelling was therefore used in the present study to delimit individual taste buds. As the intensity of cytokeratin 8 immunoreactivity in most taste buds is considerably greater in apical than basal regions, cytokeratin 8 labelling in several images (e.g. Fig. 1B) is shown at high intensity in order to show clearly the taste bud boundary. The subcellular localization of cytokeratin 8 within individual taste cells, however, can be seen in Fig. 1C. Labelling for the lectin *Ulex europaeus* agglutinin I was also used in some studies to establish taste bud boundaries, as this marker also labels subsets of taste cells (Wakisaka, 2005) (Figs. 2 and 3).

Within all three taste bud types, basal cells reside adjacent to the basement membrane amongst the proximal projections of taste cells. As a basal cell-specific marker has not yet been identified in mice, cells in the present study were designated as basal if they were located adjacent to the basement membrane and if their nuclei were bordered on both sides (100% overlap) by the proximal processes of taste cells (Fig. 1B–D). Outside the taste bud, perigemmal cells surround both basal and apical regions of the taste bud. In the present study, all cells whose nuclei did not overlap 100% with the proximal processes of the taste cells were designated as perigemmal.

TABLE 1. Number of slow cycling (label-retaining) cells per adult mouse taste bud

	Taste bud type		
Cell type	Fungiform (n = 51)	Palatal (n = 103)	Circumvallate (n = 282)
Basal cells (B)	0.12 ± 0.05	0.10 ± 0.03	0.04 ± 0.01
Perigemmal cells (P)	0.02 ± 0.02	0.01 ± 0.01	0.004 ± 0.004

Proliferating cell types at the circadian peak

In order to characterize the proliferating cell types associated with taste buds, animals were injected with BrdU across the peak in the circadian rhythm in proliferation (03:30–04:30 h) and killed after 1 h (Fig. 2). As the duration of S phase in mouse tongue epithelium is estimated to be 5.1–5.5 h (Burns *et al.*, 1975), killing the animals at 1 h after BrdU injection ensures that all labelled cells were still in the cell cycle at the time of fixation. A second group of animals was killed at 04:00 h and labelled for the proliferation marker Ki67 (Fig. 2), while a third was killed between 12:30 and 1:30 h for labelling with the M phase marker phospho-histone H3 (Fig. 3).

Mitotic activity was observed in both intragemmal cell types and in perigemmal cells for fungiform, palatal and circumvallate taste buds (Figs 2–4). Surprisingly, taste cells, previously thought to be postmitotic (Hirota *et al.*, 2001), demonstrated mitotic activity in all three taste bud types. These cells had large, elongated nuclei and exhibited labelling for three independent proliferation markers: BrdU, Ki67 and phospho-histone H3 (asterisks; Figs 2 and 3). Single optical sections taken at a series of planes through individual taste buds indicated that the labelled nuclei of these taste cells resided within the taste bud (Fig. 4). Labelled nuclei were not surrounded by cytoplasm exhibiting intense cytokeratin 8 immunoreactivity. Basal cells were also labelled by the three proliferation markers in fungiform, palatal and circumvallate taste buds (Figs 2A–D and 3B).

The occurrence of labelled cells was quantified by counting the mean number of each cell type per taste bud, as reconstructed from *z*-stack images (Fig. 2G–I). A comparison within the taste bud type showed that the mean number of labelled perigemmal cells was significantly greater than taste and basal cells in fungiform (n = 43 taste buds; $P < 0.001$), palatal (n = 58 taste buds; $P < 0.001$) and circumvallate taste buds (n = 71 taste buds; $P < 0.001$). A comparison between taste bud types revealed that the mean number of proliferating taste cells was similar between the three taste bud types (fungiform, 0.14 ± 0.06, n = 43 taste buds; palatal, 0.31 ± 0.07, n = 58 taste buds; circumvallate, 0.15 ± 0.04, n = 71 taste buds; all comparisons $P > 0.05$), while the mean number of proliferating basal cells was significantly greater in circumvallate taste buds (0.21 ± 0.05, n = 71 taste buds) than fungiform taste buds (0.02 ± 0.02, n = 43 taste buds; $P < 0.05$) but not palatal taste buds (0.16 ± 0.05, n = 58 taste buds). Direct statistical comparisons between circumvallate and other taste bud types for perigemmal cells were not made due to differences in quantification selection criteria (see above).

Identification of rapid cycling progenitor cell types

The temporal history of division in the mitotically active cell types was examined in two series of experiments using double-labelling approaches to detect cells rapidly re-entering the cell cycle (Fig. 5). In the first set of experiments, a cohort of dividing cells was labelled by systemic administration of BrdU across the circadian peak in proliferation. One or 2 days later, the animals were killed at the peak

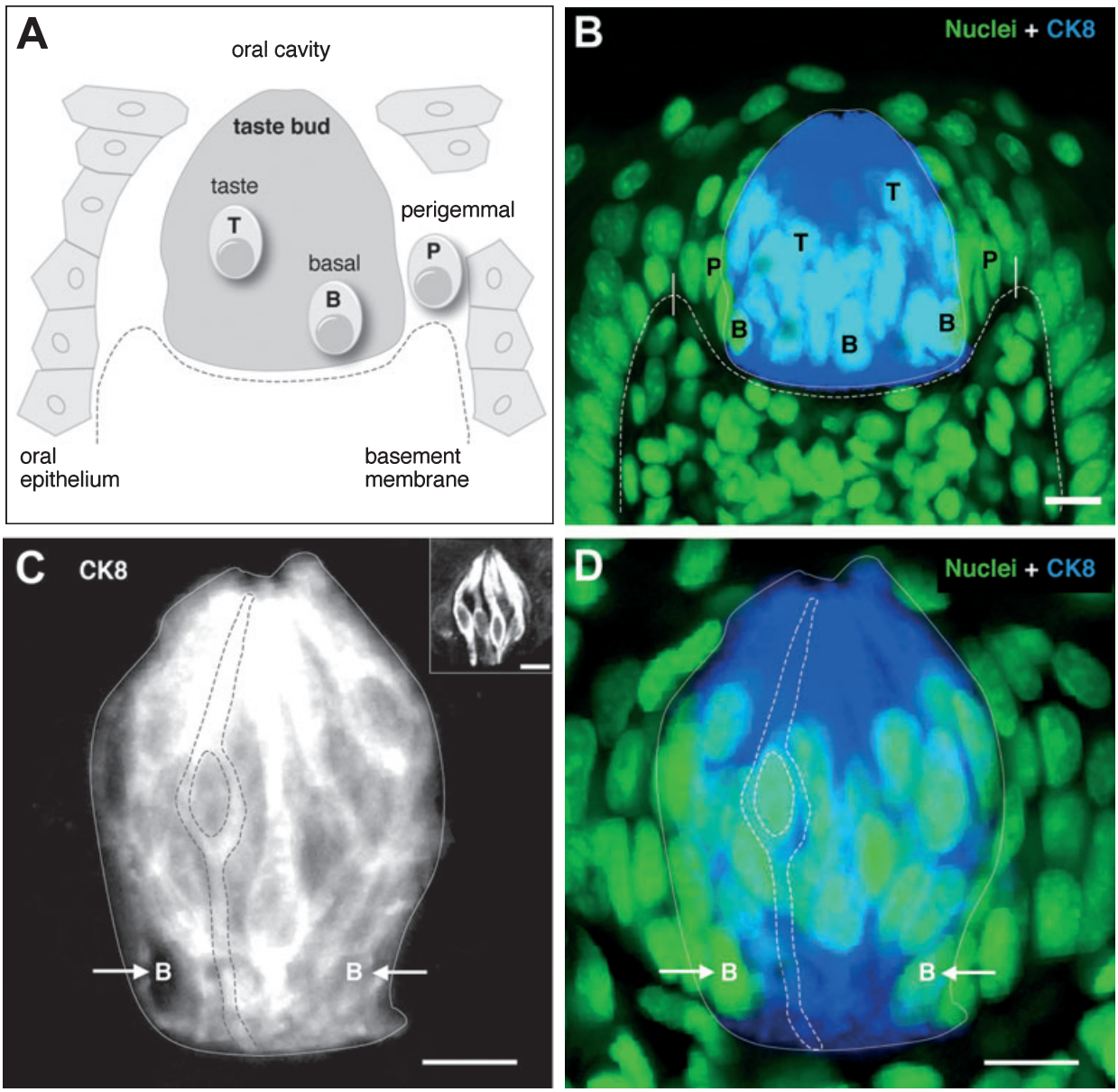

FIG. 1. Adult taste bud cell types. (A) Schematic illustrating the locations of intragemmal (taste cells, basal cells) and perigemmal cell types. (B) Confocal image of a fungiform taste bud showing examples of each cell type. The dashed line indicates the location of the basement membrane. The vertical lines indicate the region of study. Image is a *z*-projection of three adjacent optical sections. (C, D) Single optical sections through the same plane of a fungiform taste bud showing labelling for cytokeratin 8 (C) or cytokeratin 8 and a nuclear label (D). The arrows indicate the lateral margins of the nuclei of two basal cells. Basal cells were identified as cells located adjacent to the basement membrane whose nuclei are bordered on both sides (100% overlap) by the proximal processes of taste cells. Dashed lines show an individual taste cell with bipolar processes. Solid lines indicate taste bud boundaries. The inset in C shows the bipolar projections of individual taste cells immunolabelled for phospholipase C β2. Abbreviations: B, basal cell; P, perigemmal cell; T, taste cell; CK8, cytokeratin 8. The inset in C is a *z*-projection of ten adjacent optical sections. Voxel depths: C and D, 977 nm; inset in C, 366 nm. Scale bar = 10 μm.

in proliferation (04:00 h) and stained for both BrdU (to detect those cells in S phase during the initial period of BrdU administration) and Ki67 expression (to detect cells that were mitotically active at the time of fixation). Only cells undergoing rapid, sequential rounds of cell division (candidate progenitor cells) will be double labelled in these experiments.

Double labelling was observed in basal and perigemmal cells within all three taste bud types (Fig. 5A and C), indicating that these cells rapidly re-enter the cell cycle. Interestingly, double labelling of taste cells was also observed in palatal taste buds (Fig. 5B and D–F). Quantification of the number of double-labelled cells per taste bud was performed in fungiform (n = 56 sections) and palatal (n = 74 sections) taste buds, in which the boundaries of individual taste buds are clearly defined. A comparison within the taste bud types showed that the mean number of labelled perigemmal cells was significantly greater than taste and basal cells in both taste bud types (all comparisons $P < 0.001$; Fig. 5G and H). No differences in the distribution of double-labelled cells were observed at the two time points examined. A comparison between the taste bud types revealed that the mean numbers of taste cells ($P = 0.001$, d.f. = 73, $t = 3.4$) and perigemmal cells per palatal taste bud were significantly greater than the mean number per fungiform taste bud ($P = 0.011$).

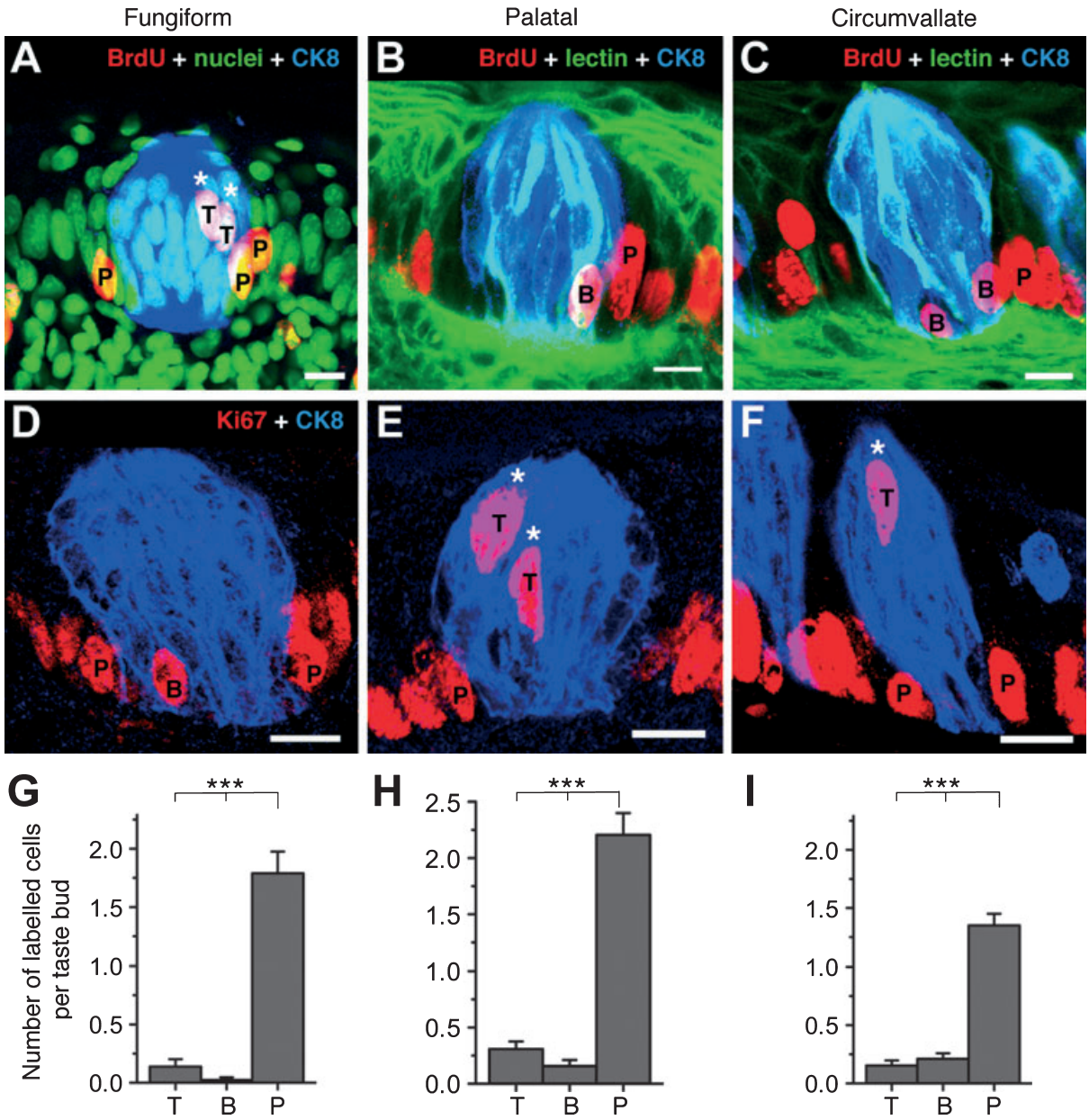

FIG. 2. All taste bud cell types are mitotically active at the peak in the circadian rhythm of proliferation. (A–C) Mitotically active BrdU-labelled cells following 1 h of exposure (03:30–04:30 h). (D–F) Mitotically active Ki67-labelled cells at 04:00 h. White asterisks indicate taste cells, previously thought to be postmitotic. (G–I) Histograms showing quantification of BrdU labelling (mean number of each cell type per taste bud ± SEM) in fungiform (G), palatal (H) and circumvallate (I) taste buds. Perigemmal cells were the predominant labelled cell type in all three taste bud types (***$P < 0.001$). Images are single optical sections (A, D, F) or *z*-projections of two (C) or three (B, E) adjacent optical sections. Voxel depths: A, 814 nm; B, 936 nm; C, 651 nm; E, 529 nm; F, 773 nm. Abbreviations as in Fig. 1. Scale bars = 10 μm.

These results were confirmed by a second series of double-labelling experiments, involving the sequential administration of CldU and IdU (Fig. 5I). By separating the administration of these two thymidine analogues at known intervals, it is possible to distinguish cells that re-enter the cell cycle and thus remain proliferative. Initially, animals received injections of CldU over 24 h to label the majority of the population of mitotically active cells. One or 2 days later, the animals subsequently received injections of IdU over 24 h. Consistent with our previous results, double-labelled basal and perigemmal cells were observed in all three taste bud types, as shown in Fig. 5I for a representative (circumvallate) taste bud.

p63 expression in proliferating cell types

To examine the phenotypes of the proliferating cell types, we investigated their expression of p63 (Fig. 6), a transcription factor required for maintaining the self-renewal potential of various epithelial stem cell types (Yang *et al.*, 1999; Truong *et al.*, 2006; Senoo *et al.*, 2007). This transcriptional regulator was also recently proposed to be expressed by taste progenitor cells (Okubo *et al.*, 2009). In agreement with studies by others, p63 expression was observed in perigemmal cells surrounding each taste bud type (Fig. 6). In contrast to these studies, however, expression of p63 was also observed in basal cells within the taste buds (Fig. 6A and C). Double labelling for p63 and

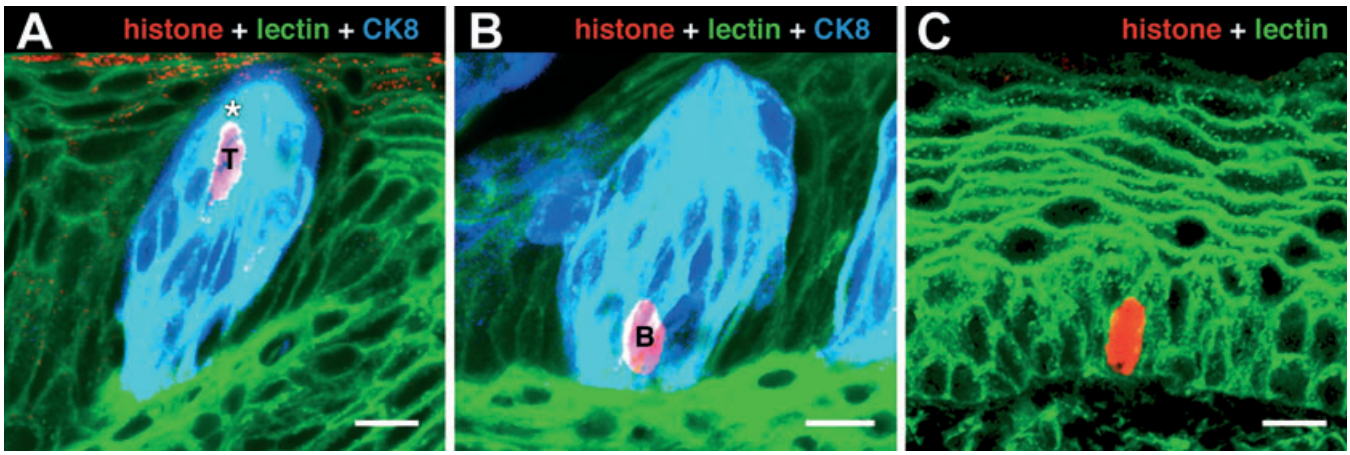

FIG. 3. Taste and basal cells enter M phase of the cell cycle. (A, B) Labelling of taste (A) and basal (B) cells in circumvallate taste buds (12:30–13:30 h) with the M phase marker phospho-histone H3. (C) Basal cell of the tongue epithelium labelled for phospho-histone H3 at the same time period. Images are *z*-projections of two adjacent optical sections. Abbreviations: B, basal cell; T, taste cell; CK8, cytokeratin 8; histone, phospho-histone H3 [pSer^{10}]. White asterisk denotes labelled taste cell. Voxel depths: A, 691 nm; B, 284 nm; C, 325 nm. Scale bars = 10 μm.

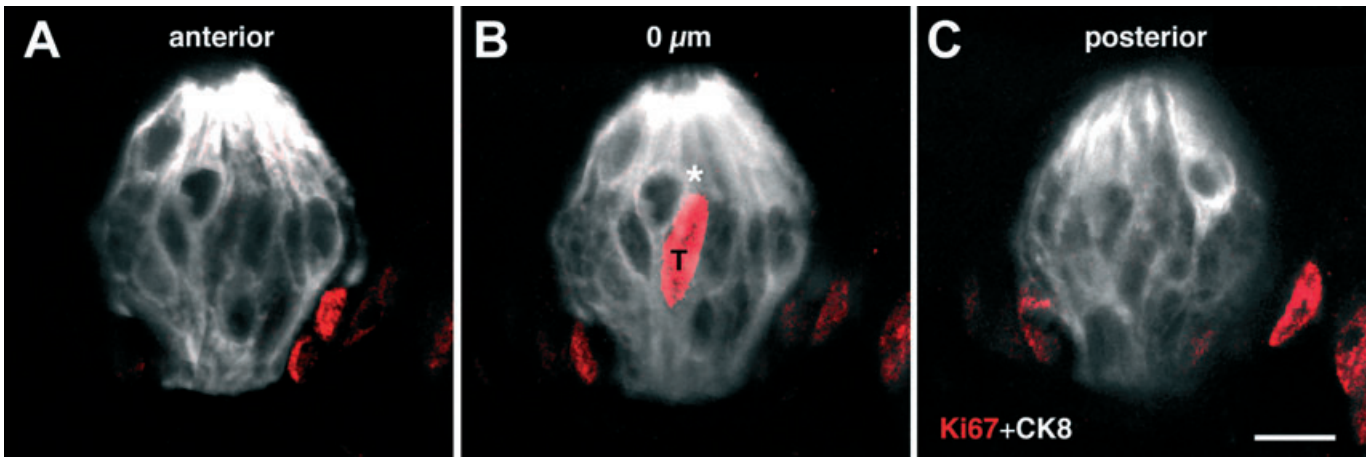

FIG. 4. Mitotically active taste cells reside in central regions of the taste bud. (A–C) Series of single optical sections through a palatal taste bud immunolabelled for Ki67 and cytokeratin 8 (CK8). The image in A represents an optical section anterior to a Ki67-labelled nucleus (seen in B) while the image in C is posterior to the labelled nucleus. White asterisk denotes labelled taste cell. Voxel depths: A–C, 244 nm. Scale bars = 10 μm.

BrdU (1 h exposure) or Ki67 demonstrated that all proliferating basal and perigemmal cells across taste bud types express this transcriptional regulator (Fig. 6). Proliferating taste cells do not express p63 (Fig. 6B).

Frequency and distribution of label-retaining cells

While the experiments described above detected rapid-cycling progenitor cells (e.g. transit amplifying cells), it is widely accepted that adult stem cells are characterized by their slow cycling (Lavker & Sun, 2000). As adult epithelial stem cells divide infrequently during normal tissue homeostasis, they can be visualized as nucleotide label-retaining cells (Bickenbach, 1981; Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000; Braun *et al.*, 2003; Chan & Gargett, 2006; Kalabis *et al.*, 2008; Muffler *et al.*, 2008).

In the present study, animals received injections of BrdU over 48 h to label a fraction of the slowly dividing cells in the tongue and soft palate. Animals were then killed after a chase of 30 days, a time point used previously to identify label-retaining cells in adult mouse oral epithelia (Bickenbach, 1981). In agreement with previous studies, strongly labelled cells were occasionally observed in the basal layer of the oral epithelia while no labelling was present in suprabasal cells (Fig. 7A). Strongly labelled basal cells were observed in fungiform, palatal and circumvallate taste buds (Fig. 7B–D), indicating that basal cells within these taste buds represent label-retaining cells. The mean number of labelled basal cells per taste bud was similar for fungiform (0.12 ± 0.05, $n = 51$), palatal (0.10 ± 0.03, $n = 103$) and circumvallate taste buds (0.04 ± 0.01, $n = 282$; all comparisons $P > 0.05$; Table 1). Sparse labelling of perigemmal cells was observed (one perigemmal cell per taste bud type). Taken together, these results suggest that slow cycling label-retaining cells (candidate stem cells) reside primarily within the basal cell populations of the taste bud.

Discussion

Adult taste buds consist of a heterogeneous population of taste cells differing in their anatomical and biochemical characteristics (Finger & Simon, 2000). Cell lineage analysis of the circumvallate taste buds of

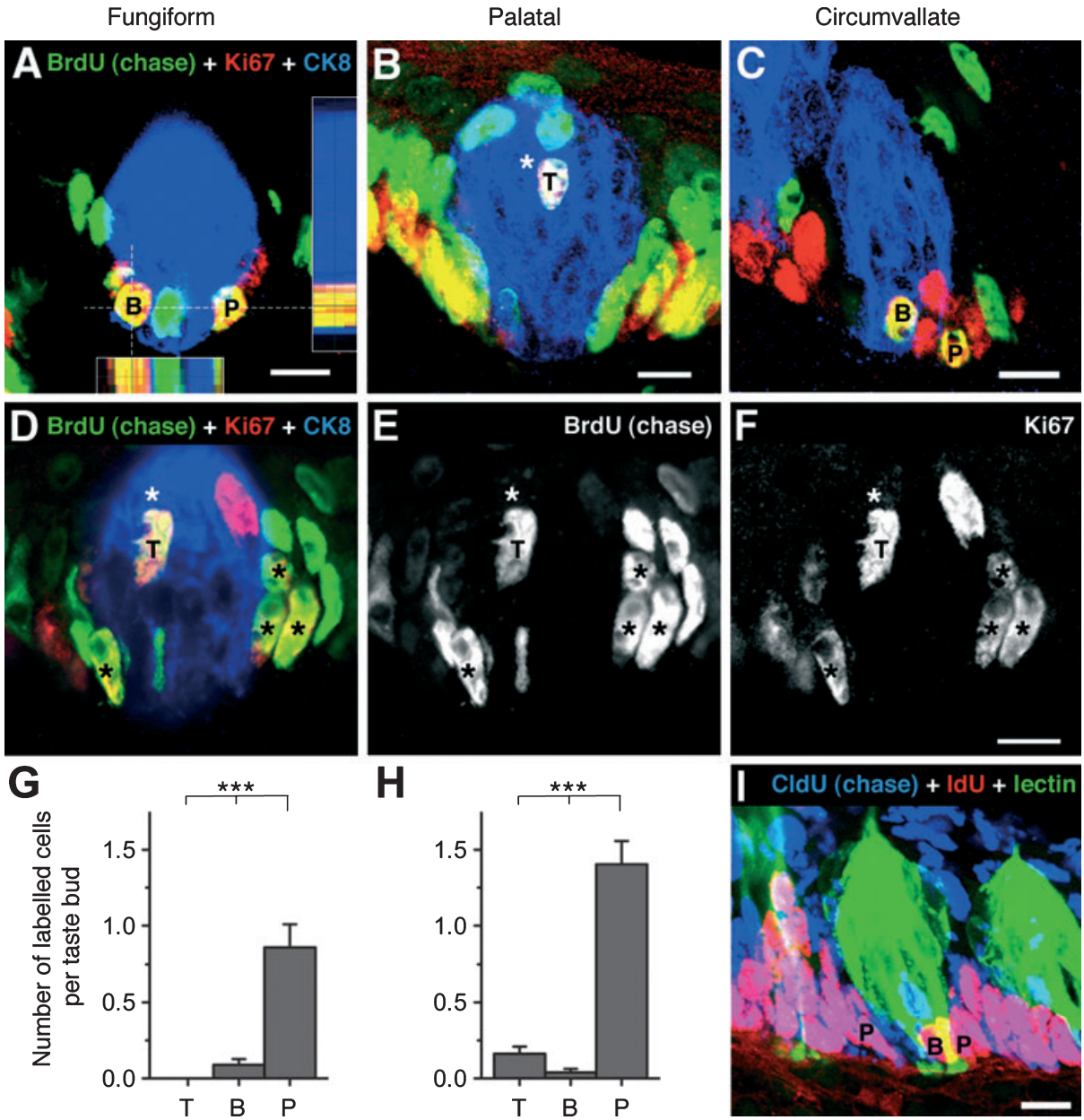

FIG. 5. Progenitor cell-like cells include taste, basal and perigemmal cells. (A–C) Taste bud cell types re-enter the cell cycle and undergo rapid sequential divisions, as shown by double proliferation marker labelling (BrdU chase + Ki67) after a 24-h (A, fungiform; B, palatal) or 48-h (C, circumvallate) chase. White asterisk indicates a double-labelled taste cell. (D–F) Single optical sections through a palatal taste bud. The merged image is shown in D and labelling for the two proliferation markers is shown again in separate images (E, BrdU; F, Ki67). (G, H) Histograms showing quantification of double labelling (BrdU chase + Ki67; mean number of each cell type per taste bud ± SEM) in fungiform (G) and palatal (H) taste buds after a 24-h chase. Perigemmal cells were the most prevalent cell type labelled in both taste bud types (***$P < 0.001$). Double-labelled taste cells were also observed in palatal taste buds. (I) Double nucleotide analogue labelling (CldU chase + IdU) of a basal cell in a circumvallate taste bud after a 48-h chase. Double-labelled taste cells were not observed using this technique. Images are single optical sections (C–F) or *z*-projections of three adjacent optical sections (A, B, I). Double-labelled nuclei are identified by white (taste cells) or black (perigemmal cells) asterisks. Voxel depths: A, 569 nm; B, 407 nm; I, 407 nm. Abbreviations as in Fig. 1. Scale bars = 10 μm.

adult mice has provided evidence that these taste buds arise from a population of at least eight progenitor cells, some of which give rise to restricted lineages (Stone *et al.*, 2002). In the present study, we used three *in vivo* approaches providing single cell resolution to identify rapid and slow cycling cells associated with taste buds in the tongue and soft palate. By applying proliferation markers at the peak in the circadian rhythm of proliferation, we demonstrate that each of the intragemmal and perigemmal cell types, including taste cells, re-enter the cell cycle and undergo rapid, sequential divisions. These cells thus represent potential taste progenitor cell types (Fig. 8A). In contrast, the label-retaining cell approach provided evidence of slowly dividing cells (candidate stem cells) primarily within the basal cell populations of the taste bud (Fig. 8B).

Studies of mammalian epidermis have suggested that homeostasis in adult tissues is maintained by stem cells residing in the basal cell layer adjacent to the basement membrane (Lavker & Sun, 2000; Fuchs & Horsley, 2008; Blanpain & Fuchs, 2009; Haegebarth & Clevers, 2009). These cells are defined by their infrequent division *in vivo* and

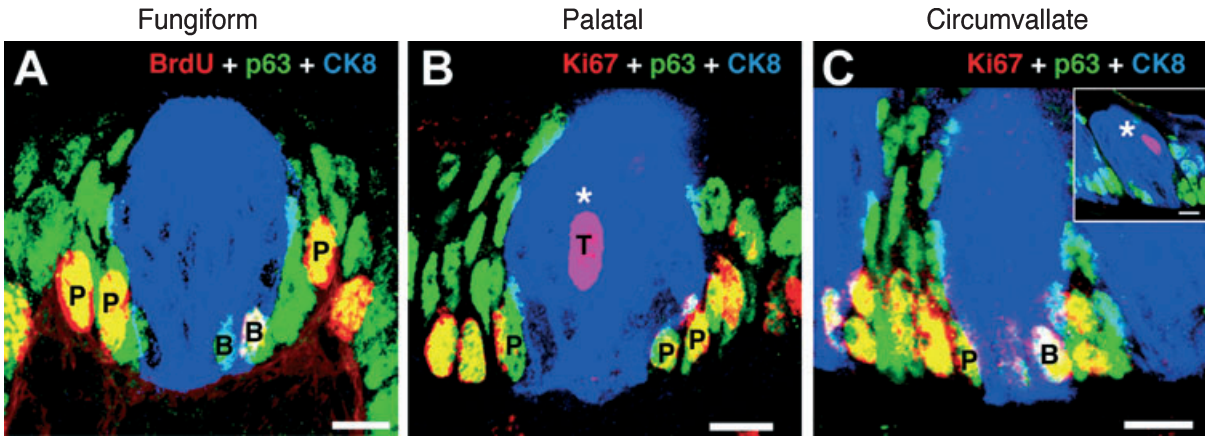

FIG. 6. Proliferating cells express the transcription factor p63. (A) Fungiform taste bud showing labelling for the p63 transcription factor and BrdU (1 h exposure). (B, C) Double labelling of palatal (B) and circumvallate (C) taste buds for p63 and Ki67. All labelling was performed at the circadian peak in proliferation. White asterisk indicates a labelled taste cell. Images are *z*-projections of two (B and C, inset in C) or three (A) adjacent optical sections. Abbreviations as in Fig. 1. Voxel depths: A, 895 nm; B, 569 nm; C, 325 nm; inset in C, 651 nm. Scale bars = 10 μm.

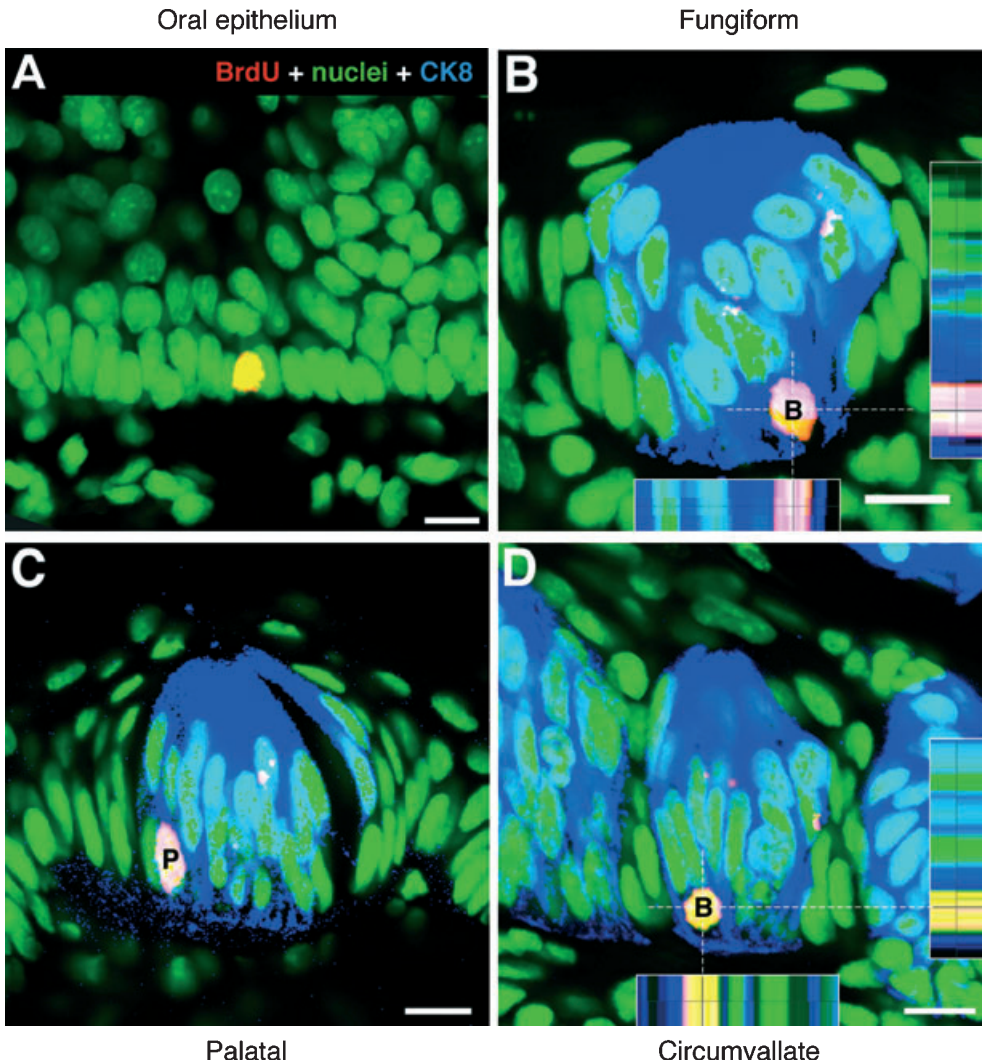

FIG. 7. Stem cell-like cells include basal cells only. (A) Label-retaining (slow cycling) cell in the basal layer of the oral epithelia (tongue). (B–D) Label-retaining cells in fungiform (B), palatal (C) and circumvallate (D) taste buds. Images are *z*-projections of two adjacent optical sections. Abbreviations as in Fig. 1. Voxel depths: A, 814 nm; B, 732 nm; C, 407 nm; D, 284 nm. Scale bars = 10 μm.

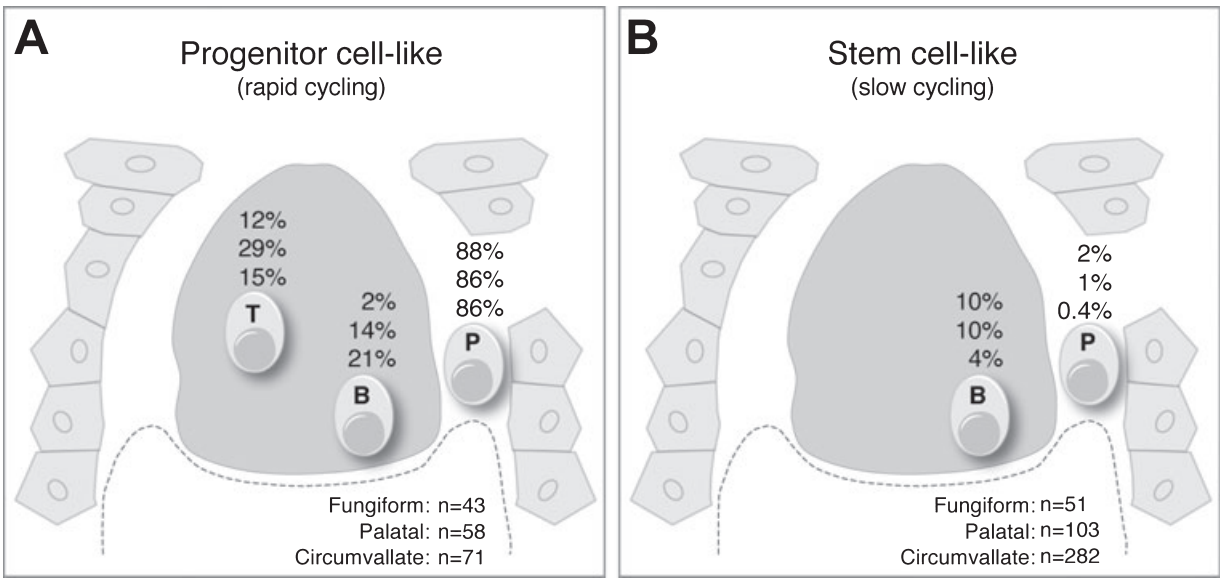

FIG. 8. Progenitor- and stem cell-like cells reside within the taste bud. (A, B) Schematics summarizing the taste bud cell types exhibiting features of progenitor and stem cells. The number of taste buds showing each cell type is indicated as a mean percentage. Taste buds were reconstructed from *z*-stack images. Abbreviations as in Fig. 1. *n* = number of taste buds examined.

by their characteristic ability to self-renew and produce multiple lineages of differentiated cells. It has been proposed that epithelial stem cells give rise to transit-amplifying progenitor cells which proliferate rapidly a finite number of times before undergoing terminal differentiation (Jones *et al.*, 1995; Lavker & Sun, 2000; Fuchs & Horsley, 2008; Blanpain & Fuchs, 2009). Comparable hierarchical stem/transit-amplifying cell models have also been proposed to maintain tissue homeostasis in oral epithelia (Hume & Potten, 1976; Potten *et al.*, 2002a) and taste buds (Stone *et al.*, 2002; Miura *et al.*, 2006).

Transit-amplifying progenitor cells undergo rapid cell division, and thus re-enter the cell cycle multiple times over short time periods. Consequently, these progenitor cell types can be distinguished from proliferating cells committed to terminal differentiation by using pulse-chase experiments with two proliferation markers (Potten *et al.*, 2002a; Vega & Peterson, 2005). The resolution afforded by these double-labelling techniques provides the opportunity to examine the temporal history of proliferation at the single cell level. To examine the proliferative history of taste bud cell types, we used two *in vivo* double-labelling approaches (BrdU chase + Ki67; CldU chase + IdU) to identify cells re-entering the cell cycle over a period of 1–4 days. The results obtained from the two approaches were consistent, indicating that cells within each intragemmal and perigemmal population undergo rapid, sequential cell divisions. These findings extend previous work using single proliferation markers (Beidler & Smallman, 1965; Conger & Wells, 1969; Murray & Murray, 1971; Farbman, 1980; Delay *et al.*, 1986; Hirota *et al.*, 2001; Hendricks *et al.*, 2004; Asano-Miyoshi *et al.*, 2008) by providing the first evidence that cells within each of the taste bud cell types exhibit characteristics of rapidly dividing (transit-amplifying) progenitors (Fig. 8A).

Although previous studies of adult rodent taste buds have concluded that taste cells (types I–III) are postmitotic (Hirota *et al.*, 2001), our study revealed mitotic activity in these cells during the circadian peak in proliferation. The labelling of taste cells with Ki67 and phospho-histone H3 as well as BrdU (Figs 2 and 3; see also Hirota *et al.*, 2001) suggests that these cells are actively dividing rather than incorporating BrdU during DNA repair or apoptosis. Our findings are supported by the identification of a taste cell in metaphase in an electron microscopic study of adult rabbit foliate taste buds (Toyoshima & Tandler, 1986). This cell exhibited ultrastructural features of a type II cell and possessed bipolar projections to the taste pore and basement membrane (Toyoshima & Tandler, 1986). Similarly, the labelled taste cells we observed in adult mouse taste buds possessed the large elongated nuclei characteristic of type II cells (Ma *et al.*, 2007). Preliminary experiments indicate, however, that the labelled taste cells do not express phospholipase C β2, a marker of type II cells (data not shown). As nuclear size and shape can change during mitosis (Webster *et al.*, 2009), further immunohistochemical and electron microscopic examinations will be necessary to fully elucidate the phenotypes of these proliferating cells.

Loss-of-function experiments in mammals have revealed a role for the transcription factor p63 in maintaining the proliferative potential of different epithelial stem and progenitor cells (Yang *et al.*, 1999; Truong *et al.*, 2006; Senoo *et al.*, 2007). On the basis of its localization within the oral epithelia, p63 expression has also been proposed to be characteristic of taste cell progenitors (Okubo *et al.*, 2009). Our findings are consistent with these studies, as double labelling for p63 and BrdU or Ki67 indicated that most proliferating cells associated with taste buds in the tongue and soft palate express this transcriptional regulator. Expression of p63 was not observed in mitotically active taste cells. As p63 plays a role in maintaining progenitor cells in a proliferative state, the absence of p63 expression in taste cells suggests they may have a more limited proliferative capacity than the other cell types.

A recent cell lineage mapping study provided evidence that many taste cells in adult mouse taste buds are the progeny of progenitor cells expressing cytokeratin 14 (Okubo *et al.*, 2009). As cytokeratin 14 immunoreactivity was not observed within taste buds in this study, the progenitor cells were concluded to be located outside the taste bud (Okubo *et al.*, 2009). In contrast, our studies indicate that subsets of both intragemmal and perigemmal cells in CBA/CaH mice exhibit cytokeratin 14 immunoreactivity (see supporting Fig. S2), as also observed previously in the rat (Asano-Miyoshi *et al.*, 2008). The expression of cytokeratin 14 both inside and outside the taste bud is

consistent with our identification of rapid-cycling progenitor cells in both regions and provides further evidence that taste progenitors cells reside within adult taste buds.

One of the most distinctive functional characteristics of adult stem cells is their infrequent division during normal tissue homeostasis (Cotsarelis *et al.*, 1990; Lavker & Sun, 2000; Blanpain & Fuchs, 2009). Nucleotide label retention, therefore, has proved to be a valuable tool for determining the anatomical localization of adult epithelial stem cells (Bickenbach, 1981; Cotsarelis *et al.*, 1990; Lavker & Sun, 2000; Braun & Prowse, 2006). Consistent with a previous study of label-retaining cells in mouse oral epithelia (Bickenbach, 1981), occasional strongly labelled basal cells were observed after 30 days in both the tongue and the palatal epithelia. Within the taste buds, label-retaining cells were detected amongst the basal cell populations of fungiform, palatal and circumvallate taste buds. In contrast, label-retaining cells were rarely observed amongst the perigemmal cell populations. Taken together, these results indicate that label-retaining cells occur predominantly within the taste buds and suggest that slow cycling stem cell-like cells reside within the basal cell populations (Fig. 8B).

The experimental approaches employed in this study provide evidence that basal cells within the taste bud represent heterogeneous populations, containing both rapid-cycling progenitor cells and slow-cycling stem cell-like cells (Fig. 8). Detailed characterization of the phenotypes of basal taste bud cells has been delayed by the lack of specific markers for these cells. Subsets of basal cells have been shown to express the Notch signalling pathway protein Hes6 and the intercellular signalling molecules bone morphogenetic protein 4 (BMP4) and sonic hedgehog (Shh) (Miura *et al.*, 2001, 2004; Yee & Finger, 2001; Seta *et al.*, 2006), providing additional evidence of the heterogeneity of these cells. Further detailed characterization of basal taste cell populations will be important, therefore, in determining the contributions of these cells to the lifelong maintenance of taste sensation.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. A marked circadian rhythm in proliferative activity is present in the oral epithelia.

Fig. S2. Expression of cytokeratin 14 in fungiform and circumvallate papillae and the soft palate.

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Abbreviations

BrdU, 5-bromo-2′-deoxyuridene; CldU, 5-chloro-2′-deoxyuridine; IdU, 5-iodo-2′-deoxyuridine.

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