Mash1-*expressing cells may be relevant to type III cells and a subset of PLCβ2-positive cell differentiation in adult mouse taste buds*

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REGULAR ARTICLE

Mash1-expressing cells may be relevant to type III cells and a subset of PLCβ2-positive cell differentiation in adult mouse taste buds

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Abstract

Mammalian taste bud cells have a limited lifespan and differentiate into type I, II, and III cells from basal cells (type IV cells) (postmitotic precursor cells). However, little is known regarding the cell lineage within taste buds. In this study, we investigated the cell fate of *Mash1*-positive precursor cells utilizing the Cre-loxP system to explore the differentiation of taste bud cells. We found that *Mash1*-expressing cells in Ascl1^{CreERT2}::CAG-floxed tdTomato mice differentiated into taste bud cells that expressed aromatic $_L$-amino acid decarboxylase (AADC) and carbonic anhydrase IV (CA4) (type III cell markers), but did not differentiate into most of gustducin (type II cell marker)-positive cells. Additionally, we found that *Mash1*-expressing cells could differentiate into phospholipase C β2 (PLCβ2)-positive cells, which have a shorter lifespan compared with AADC- and CA4-positive cells. These results suggest that *Mash1*-positive precursor cells could differentiate into type III cells, but not into most of type II cells, in the taste buds.

Keywords Mash1 · bHLH transcription factor · Taste bud · Fate maping · Cell lineage

Introduction

Mash1 (Ascl1) encodes a basic helix-loop-helix transcription factor that plays an important role in the differentiation of neurons in the nervous system, neuroendocrine cells, and sensory organs (Johnson et al. 1990, Lo et al. 1991, Guillemot et al. 1993). Previously, we reported that *Mash1* is expressed in basal cells and a subset of type III cells, but not in type II cells, within mature taste buds (Seta et al. 2006). Additionally, we revealed using *Mash1* knockout mice that *Mash1* plays an important role in the expression of aromatic $_L$-amino acid decarboxylase (AADC) and glutamate decarboxylase 67 (GAD67) in type III cells in the taste buds (Seta et al. 2011; Kito-Shingaki et al. 2014).

Mammalian taste buds are multicellular sensory organs comprising several distinct taste cells. Morphological and immunohistochemical studies revealed that taste buds possess four distinct cell types (Murray 1986; Finger and Simon 2000). In this classification of taste cells, types II and III cells serve as taste receptors, whereas type I cells are glia-like cells (Lindeman 1996; Yang et al. 2004). Type II cells express different immunohistochemical markers, including phospholipase C β2 (PLCβ2), gustducin, and protein gene product 9.5 (PGP9.5) (Yang et al. 2004; Yee et al. 2001; Kim et al. 2003; Clapp et al. 2004). Type III cells have also been distinguished using immunohistochemical markers. AADC, carbonic anhydrase IV (CA4), Synaptosome-Associated Protein 25 (SNAP25), and GAD67 are utilized as type III cell markers (Yee et al. 2001; Clapp et al. 2004; Takagi et al. 2018). These taste cells differentiate from basal cells (type IV cells) that arise from the local epithelium, and they are maintained through continuous cell renewal (Beidler and Smallman 1965; Farbman 1980; Delay et al. 1986; Stone et al. 1995). There are two hypotheses for the differentiation of taste cells: (1) basal cells (type IV cells) (postmitotic precursor cells) are restricted to individual cell types (Farbman 1965a, b; Fujimoto and Murray 1970; Pumplin et al. 1997), and (2) basal cells (type IV cells) that express Sonic hedgehog (Shh) give rise to all taste cell types (types I, II, and III) (Miura et al. 2006, 2014). However, little is known about the mechanism of basal cell development within taste buds.

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In this study, we utilized transgenic mouse strains (Ascl1^{CreERT2} and CAG-floxed tdTomato) for tracing the fate of *Mash1*-expressing cells in taste buds. The characteristics of Ascl1^{CreERT2}::CAG-floxed tdTomato mice (MT mice) are such that we could label *Mash1*-expressing cells temporarily by using the Cre-loxP system after tamoxifen induction. Prior to tamoxifen induction, CreERT2 was localized in the cytoplasm of *Mash1*-expressing cells. When tamoxifen is administered, CreERT2 is activated by transfer to the nucleus to induce the expression of tdTomato as a reporter red fluorescent protein. Perea-Martinez et al. (2013) reported that type II mature taste bud cells are differentiated from type IV basal cells after 2 days, and type III mature taste bud cells are differentiated after 3 days. To evaluate the relationship between *Mash1*-expressing cell fate and mature taste bud cells differentiation, we harvested MT mice at 2, 5, and 10 days later after 3 days of oral tamoxifen induction. The time point of 2 days was set because type II mature taste bud cells differentiated. We attempted to study the average lifespan of the total number of taste bud cells, which is 10 days, and 5 days is the intermediate time.

As mentioned above, this study investigated the cell fate of *Mash1*-positive precursor cells utilizing transgenic mouse strains (Ascl1^{CreERT2} and CAG-floxed tdTomato) to explore the differentiation of taste bud cells.

Materials and methods

Animals

All of the animals used in this study were maintained and handled according to the protocols approved by Kyushu Dental University Animal Care (Certification No: 17–010). Adult Ascl1^{CreERT2} knock-in (The Jackson Laboratory, Stock No: 012882) and CAG-floxed red fluorescent protein variant (tdTomato) mice (The Jackson Laboratory, Stock No: 007905) were used in the study. Ascl1^{CreERT2}::CAG-floxed tdTomato mice were generated by breeding Ascl1^{CreERT2} knock-in and CAG-floxed tdTomato mice and were referred to as MT mice. The genotyping of mice was performed using the following primers:

- Ascl1^{CreERT2} mutant sense, 5′-AACTTTCCTCCGGGGCTCGTTTC-3′
- Ascl1^{CreERT2} mutant antisense 5′-CGCCTGGCGATCCCTGAACATG-3′
- CAG-floxed tdTomato sense, 5′-AAGGGAGCTGCAGTGGAGTA-3′
- CAG-floxed tdTomato antisense, 5′-CCGAAAATCTGTGGGAAGTC-3′

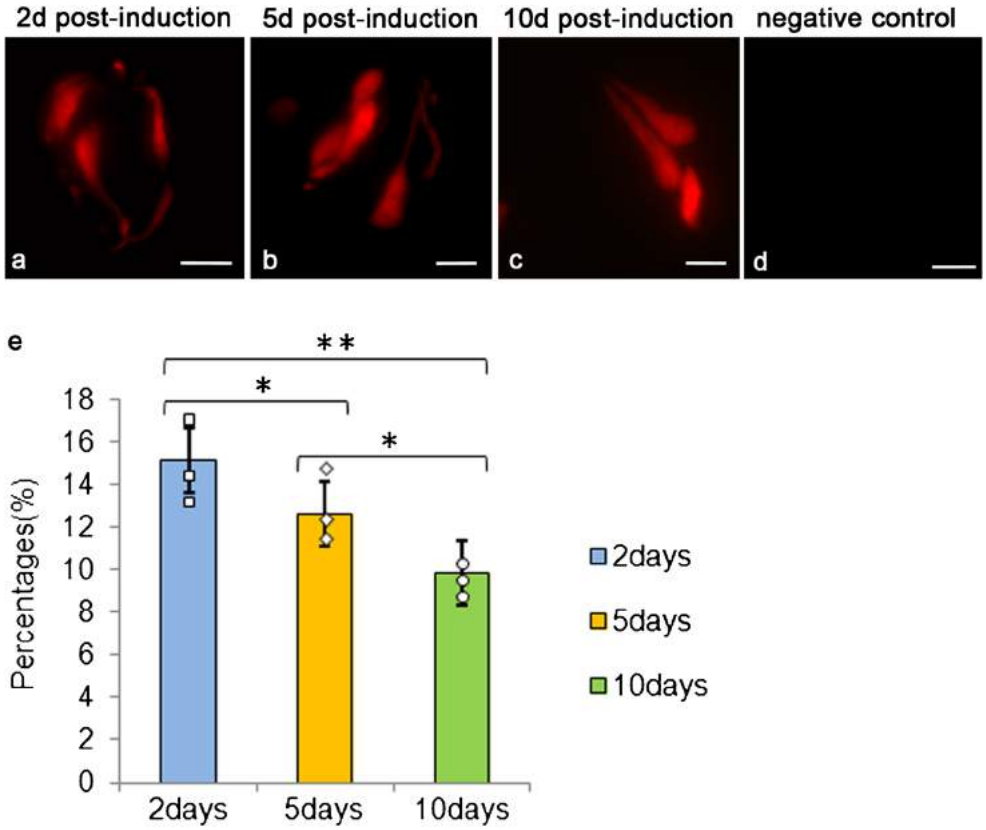

Fig. 1 tdTomato expression was identified after TAM induction in circumvallate papillae. tdTomato expression was exclusively identified in taste buds after TAM induction in Ascl1^{CreERT2}::CAG-floxed tdTomato mice (**a**–**c**). tdTomato expression was not identified in taste buds after TAM induction in CAG-floxed tdTomato mice (**d**). Scale bars: 10 μm. **e** The number of tdTomato-expressing cells gradually declined over time post-administration of tamoxifen. It reveals significantly decreased tendency during three continuous harvest time. Error bars indicate as mean ± SE ($n = 3$ for each harvest time). The individual results for each animal indicate as dots. Results were analyzed by using one-way ANOVA and Tukey-Kramer post hoc test. * $P < 0.05$, ** $P < 0.001$

Tissue preparation

MT mice were administered tamoxifen (100 mg/kg; Sigma, T5648) in sunflower seed oil (Sigma, S5007), which induces the translocation of Cre-ERT2 to the nucleus, in which it excises the reporter gene, via oral gavage. A 22-gauge feeding needle was used for oral gavage.

At the age of 4–11 months, MT and CAG-floxed tdTomato (as the control) mice of both sexes were given tamoxifen once every day for 3 days. CAG-floxed tdTomato (as the control) mice were also given tamoxifen. After 2, 5, and 10 days of tamoxifen administration, mice were anesthetized via intraperitoneal pentobarbital (50 mg/kg) and perfused with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The posterior portion of the tongue containing the circumvallate papillae were removed and fixed overnight in the same fixative. The tissues were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Sections (6–8 μm) were cut using a cryostat, thaw-mounted onto MAS-coated Superfrost slides (Matsunami, Tokyo, Japan), and stored at − 80 °C.

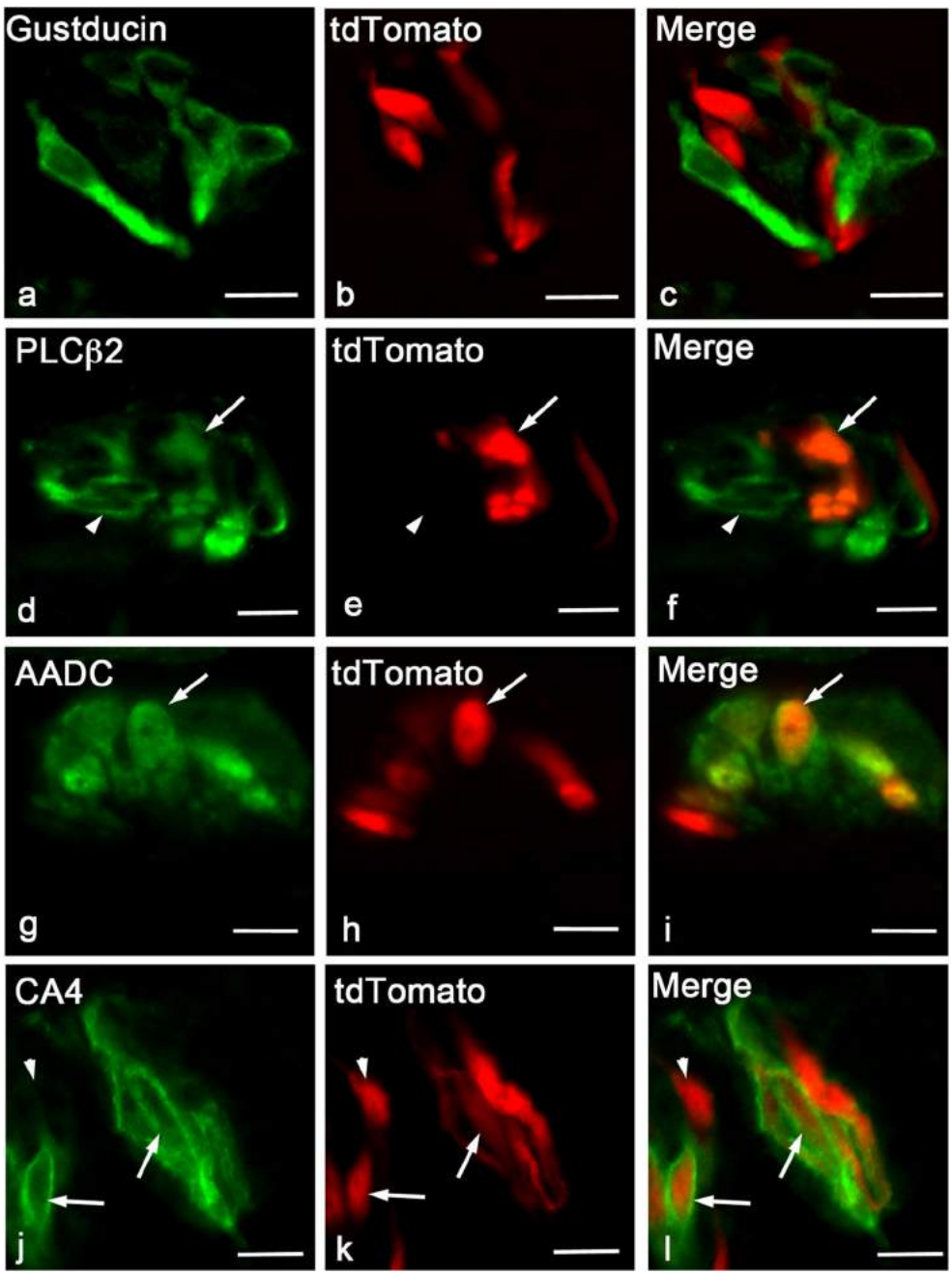

Fig. 2 tdTomato-positive cells with taste cell markers in taste buds at 2 days post-induction. The upper six panels were type II cell markers (gustducin, **a**–**c**; PLCβ2, **d**–**f**) results. The lower six panels were type III cell markers (AADC, **g**–**i**; CA4, **j**–**l**) results. Coexpression cells were indicated by arrow. Non-coexpression cells were indicated by arrowhead. Scale bars: 10 μm

Immunohistochemistry

The sections were rehydrated using phosphate-buffered saline (PBS), and followed by incubation with rabbit anti-AADC (1:200; Gene Tex), goat anti-CA4 (1:200; R&D Systems), rabbit anti-gustducin (1:1000; Santa Cruz), and rabbit anti-PLCβ2 (1:1000; Santa Cruz) as primary antibodies overnight at 4 °C in a humidified chamber. After washing with PBS, the sections were incubated with CF488A conjugated donkey anti-rabbit IgG (1:1000; Biotium) or CF488A conjugated donkey anti-goat IgG (1:1000; Biotium) as secondary antibodies for 1 h at room temperature. Slides were washed with PBS and coverslipped with Vectashield (Vector Laboratories, USA). Negative controls were created by substitution of PBS for the primary antibodies. Antibodies in taste buds have been previously validated (anti-AADC, DeFazio et al. 2006; anti-CA4, Chandrashekar et al. 2009; anti-gustducin, Boughter et al. 1997; anti-PLCβ2, Rössler et al. 1998). All immunostained sections were viewed using a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). Immunostained images were contrasted and color-adjusted and plates were created using Adobe Photoshop CS5 for Macintosh.

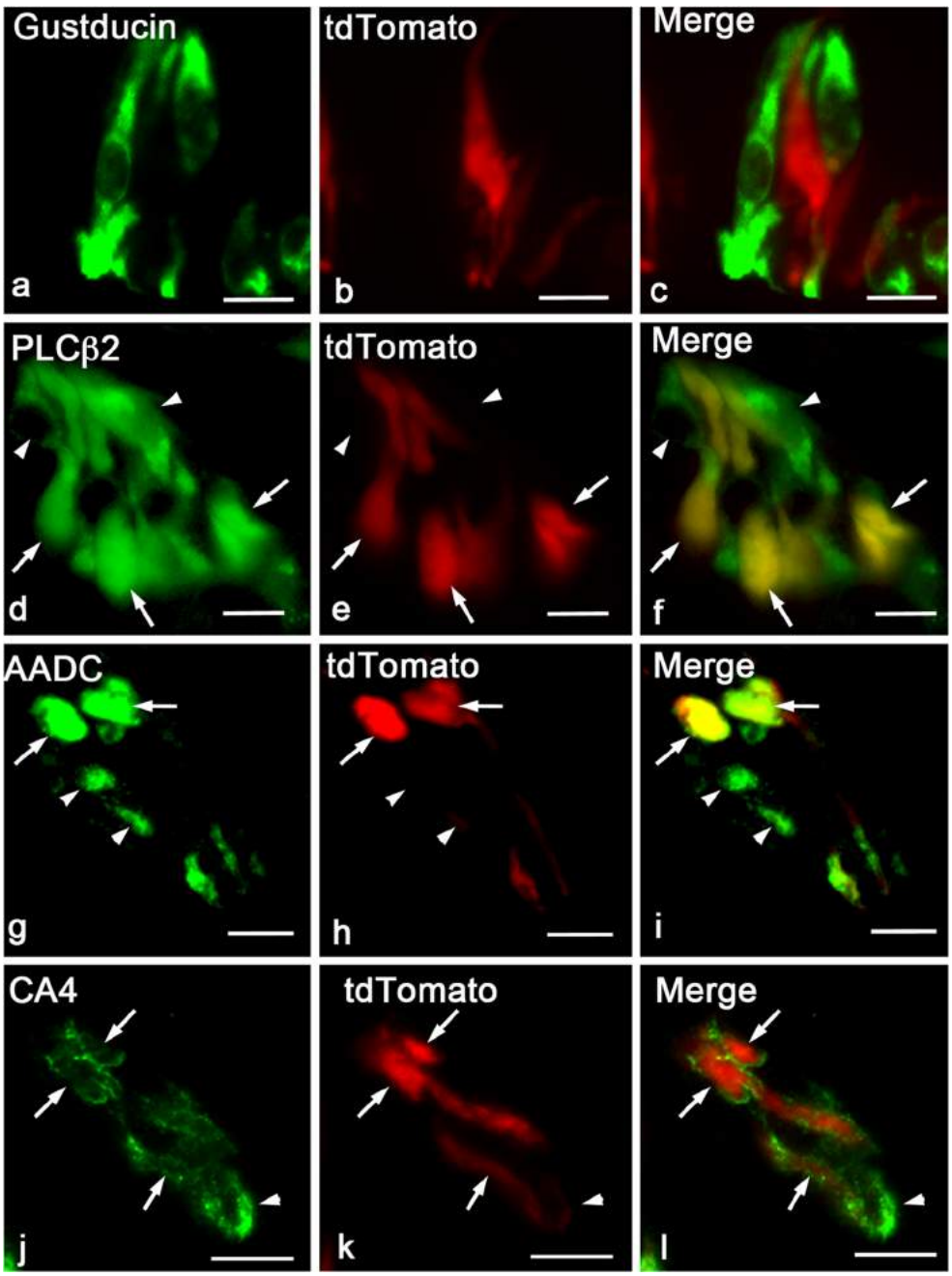

Fig. 3 tdTomato-positive cells with taste cell markers in taste buds at 5 days post-induction. The upper six panels were type II cell markers (gustducin, **a**–**c**; PLCβ2, **d**–**f**) results. The lower six panels were type III cell markers (AADC, **g**–**i**; CA4, **j**–**l**) results. Coexpression cells were indicated by arrow. Non-coexpression cells were indicated by arrowhead. Scale bars: 10 μm

Statistical analyses

All statistical analyses were performed using Bell Curve for Excel (version 2.13). One-way ANOVA was used for evaluating population normality. After analysis of variance, the Tukey-Kramer method was used to evaluate the difference of tdTomato-expressing cell percentages per taste bud between the groups at each harvest time. The same procedure was also performed to evaluate the difference of coexpression cell percentages per taste bud between the groups at each harvest time. The significance level was set at 0.05 for each test.

Result

Mash1-expressing cells were only observed within the taste buds of adult mice (Seta et al. 2006). To trace the fate of *Mash1*-expressing cells into the mature taste cells, we used the Cre-flox genetic fate mapping strategy. In Ascl1^{CreERT2}::CAG-floxed tdTomato (MT) mice, *Mash1* lineage cells were labeled with tdTomato following tamoxifen treatment. A small subset of tdTomato-expressing cells within taste buds was observed in circumvallate papillae (Fig. 1a–c). As expected, tdTomato-expressing cells were not observed in the other papillary elements, such as the papilla groove

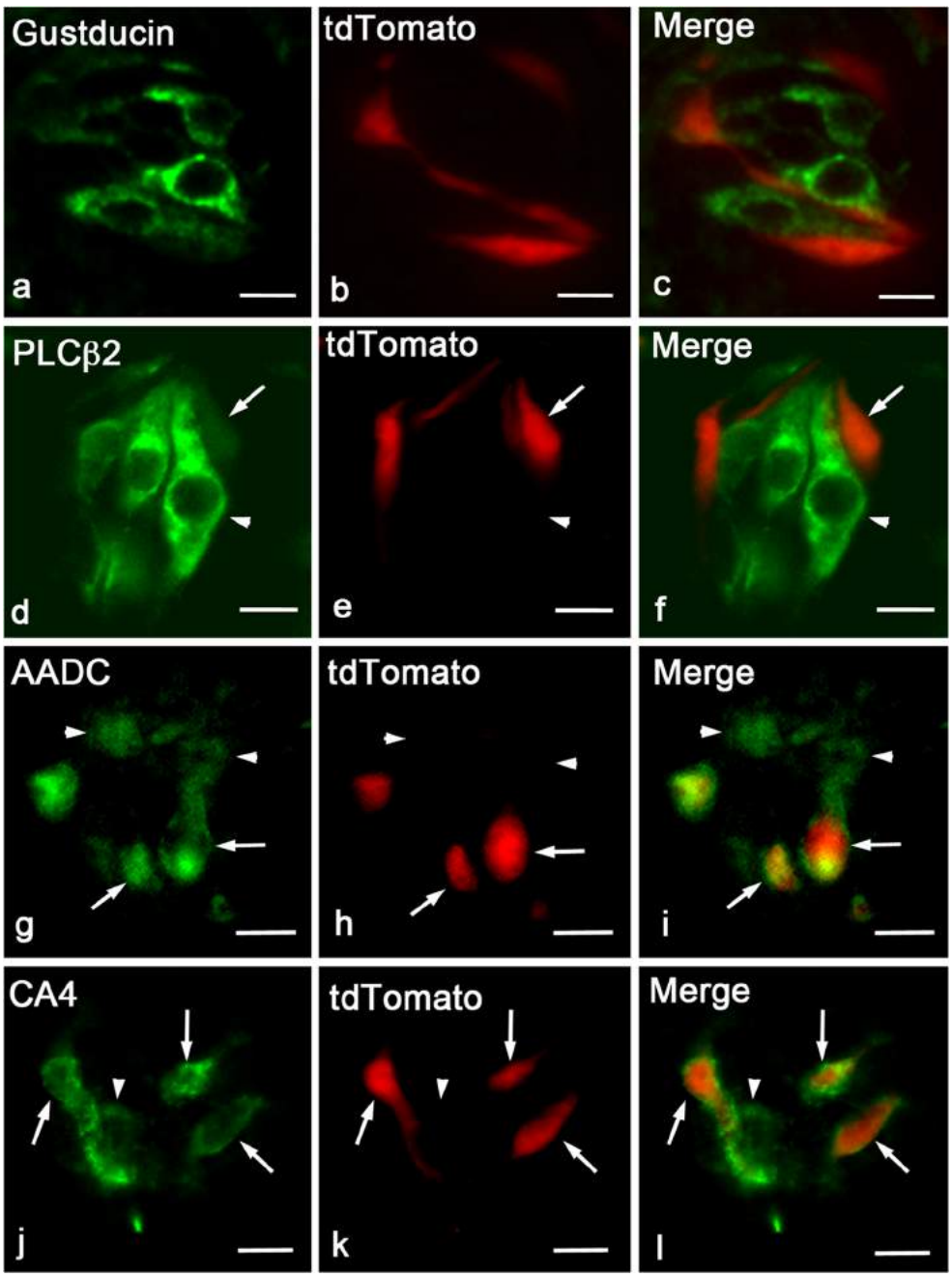

Fig. 4 tdTomato-positive cells with taste cell markers in taste buds at 10 days post-induction. The upper six panels were type II cell markers (gustducin, **a**–**c**; PLCβ2, **d**–**f**) results. The lower six panels were type III cell markers (AADC, **g**–**i**; CA4, **j**–**l**) results. Coexpression cells were indicated by arrow. Non-coexpression cells were indicated by arrowhead. Scale bars: 10 μm

epithelium. The number of tdTomato-expressing cells gradually declined over time after the administration of tamoxifen (Fig. 1e). There were no tdTomato-expressing cells within taste buds in CAG-floxed tdTomato (T) mice after tamoxifen treatment as the negative control (Fig. 1d).

To investigate the fate of *Mash1*-expressing precursor cells, we performed immunohistochemistry using taste cell markers on sections of circumvallate papillae from MT mice administered tamoxifen and then harvested 2, 5, or 10 days later. Gustducin is a marker of a subset of

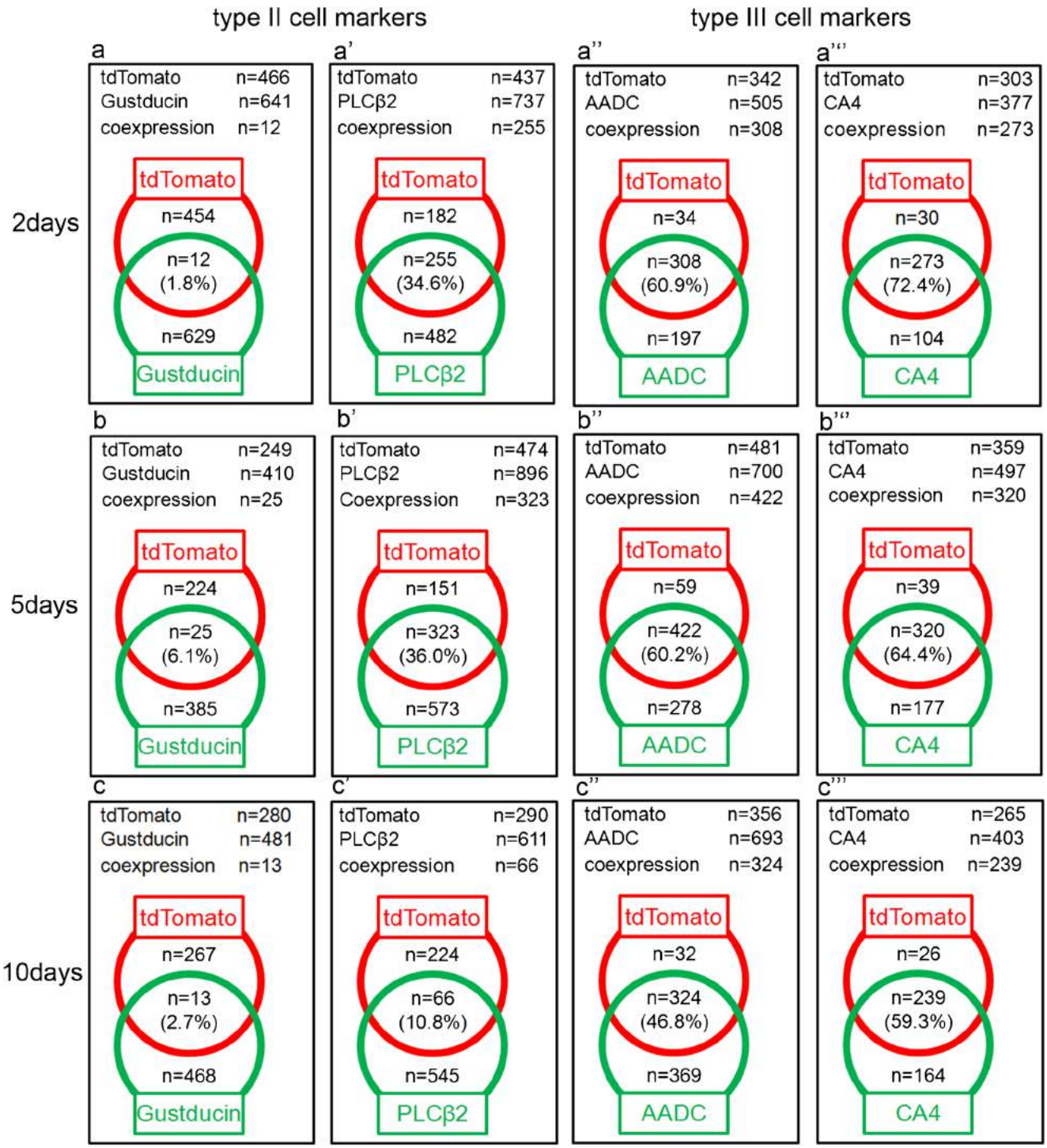

Fig. 5 The left six panels were type II cell markers (gustducin, **a**–**c**; PLCβ2, **a'**–**c'**) results. The right six panels were type III cell markers (AADC, **a''**–**c''**; CA4, **a'''**–**c'''**) results. The number of each cell type marker–positive cells, tdTomato-positive cells, and coexpression cells were shown in each Venn diagram. The coexpression rate of each type cell markers was also shown in the overlapped region of Venn diagram at 2, 5, and 10 days post-induction

type II taste cells (Clapp et al. 2004; Yee et al. 2001). Immunoreactivity for gustducin was observed in a large population of taste cells. Within circumvallate taste buds, a small number of gustducin-positive taste cells were labeled with tdTomato (Fig. 5; 2 days, 1.8%; 5 days, 6.1%; 10 days, 2.7%). PLCβ2 is a marker of type II taste cells (Clapp et al. 2004). Immunoreactivity for PLCβ2 was also present in a large subset of mouse circumvallate taste buds. Some PLCβ2-positive taste cells were labeled with tdTomato (Fig. 5; 2 days, 34.6%; 5 days, 36.0%; 10 days, 10.8%). The number of tdTomato-labeled PLCβ2-positive taste cells was significantly reduced by tamoxifen administration depending on the duration of treatment (Fig. 6).

AADC and CA4 are type III cell markers (Yee et al. 2003; Seta et al. 2011). Taste cells with AADC and CA4 immunopositivity were found within taste buds (Figs. 2, 3, and 4g–l). Some type III cell marker–positive taste cells were labeled with tdTomato (Fig. 5; AADC: 2 days, 60.9%; 5 days, 60.2%; 10 days, 46.8%; CA4: 2 days, 72.4%; 5 days, 64.4%; 10 days, 59.3%). The number of tdTomato-labeled AADC- and CA4-positive taste cells was significantly reduced by tamoxifen administration depending on the duration of treatment (Fig. 6).

Discussion

This present study clarified the fate of *Mash1*-expressing taste cells within mature taste buds. Using Ascl1^{CreERT2}::CAG-floxed tdTomato mice, we found that *Mash1*-expressing cells also expressed AADC and CA4 (type III cell markers). We additionally observed that *Mash1*-expressing cells could

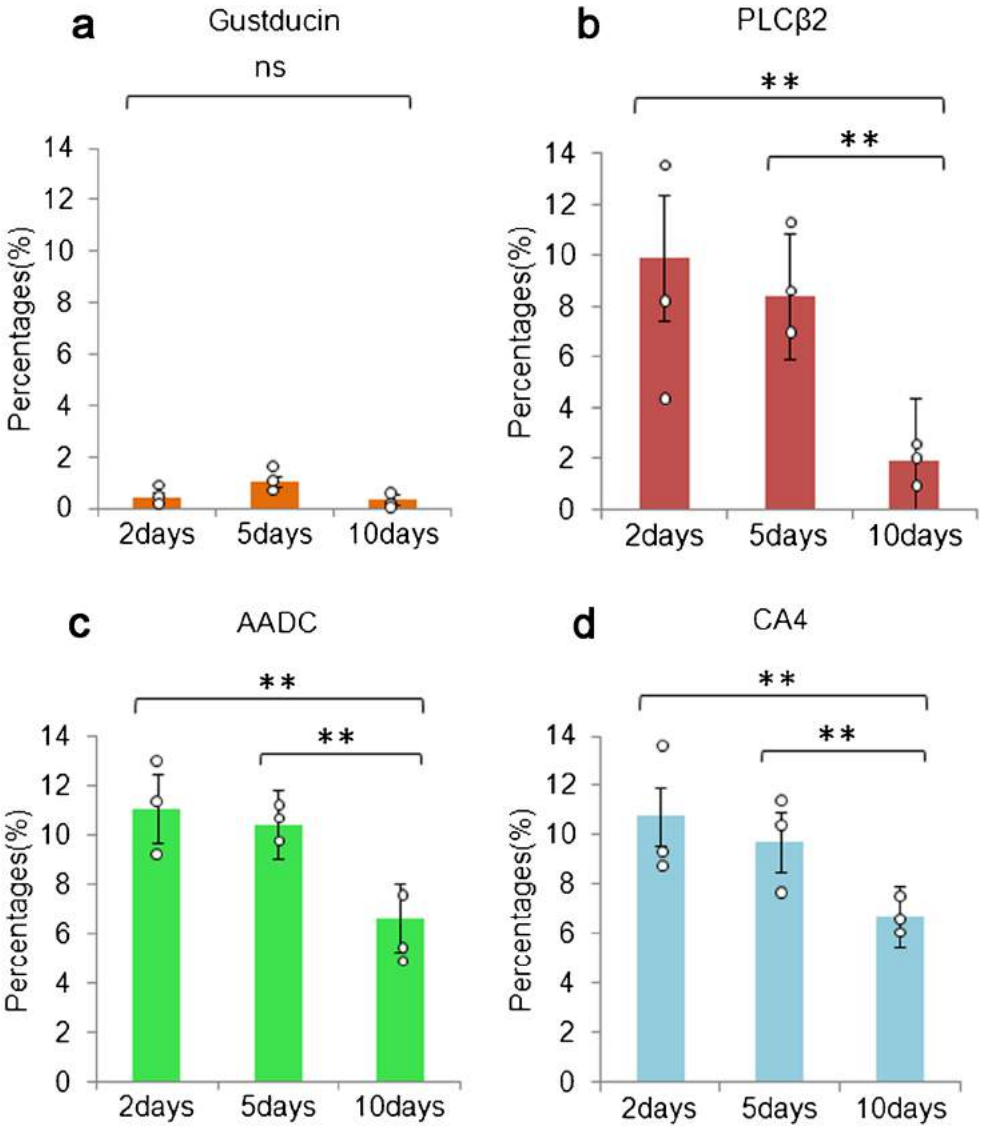

Fig. 6 For gustducin, there is no significant difference among the harvest times (**a**). For PLCβ2 (**b**), AADC (**c**), and CA4 (**d**), the percentage of coexpressing cells per taste bud decreased significantly between 2 and 10 days, and 5 and 10 days. The percentage of coexpressing cells in AADC and CA4 were approximately 6% at 10 days. In PLCβ2, the percentage of coexpression cells was only 1.8% at 10 days. Error bars indicate mean ± SE ($n = 3$ for each harvest time). The individual results for each animal are indicated as dots. Results were analyzed by using one-way ANOVA and Tukey-Kramer test. *ns* no significance * $P < 0.05$, ** $P < 0.001$

differentiate into PLCβ2-positive cells, and these cells had shorter lifespans than AADC- and CA4-positive cells.

Mash1 has been demonstrated to play an important role in differentiation in neural and other tissues (reviewed by Bertrand et al. 2002). *Mash1* is expressed in basal cells and some elongated taste cells within mature taste buds (Seta et al. 2006), and AADC- and GAD67-expressing type III cells were not detected in *Mash1* mutant mice (Seta et al. 2011; Kito-Shingaki et al. 2014). Furthermore, we illustrated that the number of cells expressing type III cell markers was significantly reduced in the circumvallate taste buds of *Mash1* mice using the Cre-loxP system (Takagi et al. 2018). In the present study, *Mash1*-expressing cells marked by CreERT2 in Ascl1^{CreERT2}::CAG-floxed tdTomato mice expressed type III cell markers within taste buds. These results coincide with the previous findings and indicate that *Mash1*-expressing precursor cells differentiate into type III cells in taste buds.

Mammalian taste buds comprise four distinct cell types according to ultrastructural and immunohistochemical studies (for a review, see Finger and Simon 2000). Within these cell types, basal cells are assumed to be precursor cells and other elongated cells are assumed to be mature taste cells (types I–III cells). Previously, we found that *Mash1* was expressed in basal cells and the majority of differentiated type III taste cells (Seta et al. 2006). These *Mash1*-expressing elongated cells coexpressed type III cell markers (NCAM and serotonin), but not type II cell markers (gustducin and PLCβ2). In this study, we found that *Mash1*-expressing precursor cells could differentiate into type III cell marker–expressing cells and PLCβ2-expressing cells. We have revealed that some AADC-expressing taste cells were positive for PLCβ2 (Seta et al. 2007). Taken together, *Mash1*-expressing precursor cells differentiate into PLCβ2-expressing cells that may express AADC. Miura et al. (2006) mentioned that *Mash1*-expressing cells represent an immature cell state of both types II and type III cells. Our present results indicate that *Mash1*-expressing precursor cells could not differentiate into most gustducin-positive type II cells, suggesting that most gustducin-positive type II cells differentiate from basal cells that do not express *Mash1*. However, only a few gustducin-positive cells were expressed as tdTomato. These results suggest that a small population of type II cells (gustducin and PLCβ2-positive cells) could have possibly differentiated from *Mash1*-positve precursor cells. On an average, taste bud cells have a lifespan of 10–14 days (Beidler and Smallman 1965; Farbman 1980; Delay et al. 1986; Stone et al. 2002). Perea-Martinez et al. (2013) have shown that types II and III cells have different lifespans. Specifically, type II cells were eliminated with a half-life of 8 days, whereas type III cells survived much longer, with a half-life of 22 days. Our results indicated that type III cell marker–expressing taste cells that differentiated from *Mash1*-expressing precursor cells have a longer lifespan than PLCβ2-positive cells that differentiated from *Mash1*-expressing precursor cells. Another possibility could be that PLCβ2-expressing type III cells may be immature. The expression of PLCβ2 in type III cells may represent an error in the differentiation process, and these cells have a shorter lifespan than normal type III cells caused by some mechanism identifying differentiation errors and removing such cells from the taste buds.

In conclusion, the present study demonstrated that *Mash1*-expressing precursor cells differentiated into type III cells within mature taste buds, but not into most of type II cells. Among taste cells that differentiated from *Mash1*-expressing precursor cells, type III cell marker–expressing cells survive much longer than PLCβ2-positive cells. It remains to be revealed whether PLCβ2-positive cells arising from *Mash1*-expressing precursor cells are type II or type III cells remains to be revealed. Further studies using *Mash1*-expressing precursor cells will provide new insights into taste cell differentiation.

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Compliance with ethical standards

Conflict of interest The authors state that there is no conflict of interest.

Ethical approval All of the animals used in this study were maintained and handled according to the protocols approved by Kyushu Dental University Animal Care (Certification No: 17–010).

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