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Temporal changes in NCAM immunoreactivity during taste cell differentiation and cell lineage relationships in taste buds

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Abstract

Neural cell adhesion molecule (NCAM) is a type III cell marker in the taste buds. In order to clarify the cell type of *Mash1*-expressing cells in taste buds, expression of NCAM was examined in *Mash1*-expressing taste cells of adult mice in comparison with *gustducin*- and *T1r3*-expressing cells, using a combination of NCAM immunohistochemistry and *in situ* hybridization. About 98% of *Mash1*-expressing cells were NCAM immunopositive (IP), suggesting that *Mash1*-expressing cells should be categorized as type III cells. Unexpectedly, small subsets of *gustducin*- and *T1r3*-expressing cells were also found to be NCAM-IP, contradicting previous immunohistochemical studies in rats, in which gustducin-IP cells were observed specifically in type II cells, which do not have NCAM immunoreactivity. Examinations of developing taste buds showed temporal changes in the ratio of NCAM-IP cells in *gustducin*- and *T1r3*-expressing cells; the ratio of NCAM-IP cells in these gene-expressing cells were ~90% at 0.5 days after birth and decreased markedly during development. In contrast, the majority of *Mash1*-expressing cells showed constant NCAM immunoreactivity throughout development. In addition, BrdU-labeling experiments showed that the differentiation of *Mash1*-expressing cells precedes those of *gustducin*- and *T1r3*-expressing cells in taste buds of adult mice. These results suggest that *T1r3*- and *gustducin*-expressing cells are NCAM-IP at the beginning of cell differentiation, and that NCAM immunoreactivity in *gustducin*- and *T1r3*-expressing cells might remain from the previous developmental stage expressing *Mash1*.

Key words: cell lineage, *Mash1*, neural cell adhesion molecule (NCAM), taste bud, *T1r3*

Introduction

Taste buds are the sensory organs for taste and are maintained under continuous cell renewal, even in adults. Taste cells are constantly differentiated from local epithelia (Stone *et al.*, 1995), and their average life span is estimated to be ~10 days (Beidler and Smallman, 1965; Farbman, 1980). Therefore, taste buds consist of cells at various maturation stages of taste cell differentiation. Recently, expression of bHLH transcription factors, *Mash1* and *NeuroD*, was reported in mouse taste buds (Kusakabe *et al.*, 2002; Suzuki *et al.*, 2002). The sequential expression of *Mash1* followed by *NeuroD* is reported in the differentiation of olfactory neurons (Cau *et al.*, 1997, 2002). These transcription factors might be related to a regulatory gene cascade and the differentiation of taste cells. Based on ultrastructural characteristics, taste bud cells have been classified into several cell types: basal cells, type I (dark) cells, type II (light) cells and type III (intermediate) cells (Kinnamon *et al.*, 1985; Delay *et al.*, 1986; Yee *et al.*, 2001; Clapp *et al.*, 2004). Immunohistochemical studies in rats have shown histochemical markers associated with cell types. The H blood group antigen is predominant in type I cells (Pumplin *et al.*, 1999). *Gustducin* is reported to be expressed in a subset of type II cells (Boughter *et al.*, 1997; Yang *et al.*, 2000), but a marker gene expressed in all type II cells has not been reported. Although there has been debate regarding the criteria of type III (intermediate) cells in rodents, a cell type that is well defined in rabbits, recent immunohistochemical studies in rats distinguished type III cells in combination with ultrastructural analysis, and showed the relationship among immunohistochemical markers for type III cells (Yee *et al.*, 2001). The majority of type III cells predominantly expresses neural cell adhesion molecule (NCAM), while serotonin-positive cells were observed in a subset of type III cells, and ubiquitin carboxyl terminal hydrolase (PGP 9.5) was expressed in a subset of both type II and type III cells. It is generally accepted that type I, II and III cells are derived from basal cells; however,

the cell lineage relationships among the cell types in taste buds remain unclear. Conflicting views have been published: (i) taste buds are composed of one cell lineage, and morphological changes occur transiting from basal to dark (type I) to intermediate (type III) to light (type II) cells during maturation (Delay *et al.*, 1986); (ii) taste buds are composed of at least two cell lineages, which belong to different cell types, an idea based on the difference in life span and morphology between dark cells and light cells (Farbman, 1980; Pumplin *et al.*, 1997). As for regulatory gene expression, a previous report showed that *NeuroD*-expressing cells express *gustducin* but not NCAM, suggesting that *NeuroD* is expressed in type II cells (Suzuki *et al.*, 2002). On the other hand, we have reported the segregated expression pattern of *Mash1* and *gustducin* (Kusakabe *et al.*, 2002), suggesting that *Mash1*-expressing cells may be of another cell type than type II cells. However, the cell type of *Mash1*-expressing cells remains unclear. It is expected that clarification of the relationship between the transcription factors and cell types in taste buds will provide a clue to cell lineage relationships in taste buds.

In the present report, we first examined NCAM expression in *Mash1*-expressing cells in adult mice to clarify the cell type, using a combination of NCAM immunohistochemistry and *in situ* hybridization. Almost all *Mash1*-expressing cells were NCAM-IP, suggesting that *Mash1*-expressing cells should be categorized as type III cells. Unexpectedly, NCAM immunoreactivity was also observed in small subsets of *gustducin*- and *T1r3*-expressing cells, contradicting the previous reports in rats (Yang *et al.*, 2000). NCAM immunoreactivity was then examined in *Mash1*-, *gustducin*- and *T1r3*-expressing cells during development. In addition, the BrdU-labeling experiments were performed to examine the timing of the differentiation of *Mash1*-, *gustducin*- and *T1r3*-expressing cells in adult mice.

Materials and methods

Experimental animals

The animals used in this study as adults were 10- to 12-week-old C57BL/6N mice. These animals were initially purchased from Charles River Japan (Yokohama, Japan), and were bred at the National Food Research Institute. We followed the guidelines of our institute for the care and use of experimental animals.

NCAM immunohistochemistry after *in situ* hybridization

Mouse tongues were dissected, placed in embedding compound (OCT; Sakura Finetech., USA, Los Angeles, CA), and rapidly frozen in a liquid nitrogen bath. Tissues were sectioned at 5 μm, and *in situ* hybridization was performed as previously described (Miura *et al.*, 2004). Antisense RNA probes were transcribed *in vitro* with digoxigenin-UTP using an RNA transcription kit (Roche Diagnostics GmbH, Mannheim, Germany) from a linearized plasmid containing one of the following cDNA inserts: *Mash1* (10012: Genbank U68534-760: Genbank M65603), *gustducin* (Kim *et al.*, 2003), *T1r3* (Kitagawa *et al.*, 2001) and *Shh* (Kitamura *et al.*, 1997). Following hybridization, sections were incubated with an alkaline phosphatase anti-digoxigenin goat antibody (Roche Diagnostics GmbH) and anti-NCAM rabbit polyclonal antibody (2 μg/ml; Chemicon, Temecula, CA) in TBSB [Tris-buffred saline, TBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 1% blocking reagent (Roche Diagnostics GmbH)] at 4°C overnight. After they were washed in TBST (TBS containing 0.05% Tween 20), the signal for *in situ* hybridization was detected using 2-hydroxy-3-naphthoic acid-2′-phenylanilidephosphate (HNPP)/Fast Red alkaline phosphatase substrate (Roche Diagnostics GmbH). Sections were rinsed in TBS, incubated with a biotinylated anti-rabbit IgG (10 μg/ml; Vector, Burlingame, CA) in TBSB for 2 h at room temperature and then washed in TBST. To detect the signal for NCAM, sections were incubated with streptavidin–Alexa 488 (Molecular Probes, Eugene, OR) for 30 min and washed in TBST. Double color fluorescent images were merged using Photoshop (Adobe Systems, San Jose, CA). Signals in every fourth section were analyzed in order to avoid any double counting of cells. NCAM immunoreactivity adjacent to the nucleus of taste bud cells was counted as the signal indicating its expression in order to avoid counting the signals for nerve fibers as taste cell signals. Sections from one or two circumvallate papillae were analyzed to count the number of NCAM-positive cells in *Mash1*-, *gustducin*- and *T1r3*-expressing cells. In the cases of *gustducin*- and *T1r3*-expressing cells at 0.5 days after birth, the data were collected from six circumvallate papillae because the number of positive cells for these genes were very small: 37 *gustducin*- and 22 *T1r3*-expressing cells were found in counts of six whole circumvallate papillae.

BrdU labeling and the detection after *in situ* hybridization

Adult mice were injected intraperitoneally with bromodeoxyuridine (BrdU, 50 mg/kg; Roche Diagnostics GmbH). Mouse tongues were dissected and treated in the same manner as that used for *in situ* hybridization at 3 and 12 h and 1, 2, 3 and 4 days after BrdU injection. After hybridization with the digoxigenin-labelled cRNA probe, signals for *in situ* hybridization were obtained by incubation with HNPP/Fast Red alkaline phosphatase substrate (Roche Diagnostics GmbH). The sections were washed in TBS, incubated in TBSB for 15 min and washed again in PBS. The sections were then incubated with anti-BrdU monoclonal antibody (Roche Diagnostics GmbH) for 30 min at 37°C according to the instructions for use of the BrdU Labeling and Detection Kit II (Roche Diagnostics GmbH). After washing in PBS, the sections were incubated with an Alexa 488–anti mouse IgG (Molecular Probes) for 2 h at room temperature and washed in TBS. Double-color fluorescent images were merged using Photoshop (Adobe Systems). Signals in every

fourth section were analyzed in order to avoid any double counting of the gene-expressing cells.

BrdU detection after NCAM immunohistochemistry

Sections were incubated with anti-NCAM rabbit polyclonal antibody (2 μg/ml; Chemicon, Temecula, CA) overnight, washed and then incubated with biotinylated anti-rabbit IgG (10 μg/ml; Vector, Burlingame, CA). After washing, they were incubated with streptavidin–Alexa 568 (Molecular Probes, Eugene, Oregon) for 30 min and washed. The sections were incubated at 65°C for 90 min, and BrdU signals were then detected with anti-BrdU monoclonal antibody (Roche Diagnostics GmbH) and Alexa 488–anti mouse IgG (Molecular Probes). Double-color fluorescent images were merged using Photoshop (Adobe Systems). Signals in every fourth section were analyzed. The number of BrdU NCAM double-positive cells per circumvallate trench wall was counted in each section at 3 and 12 h and 1, 2, 3 and 4 days after BrdU injection.

Results

NCAM immunoreactivity in *Mash1*-, *gustducin*- and *T1r3*-expressing cells

The cell type of *Mash1*-expressing cells in taste buds remains unclear, although we have previously reported that *Mash1* has an expression pattern distinct from *gustducin* and *T1r2*, suggesting that *Mash1*-expressing cell is not a type II cell (Kusakabe *et al.*, 2002). To clarify whether *Mash1*-expressing cells can be categorized as type III cells, *Mash1*-expressing cells were examined for immunoreactivity for NCAM, a type III cell marker in taste buds, using a combination of *in situ* hybridization and NCAM immunohistochemistry. The same procedures were used to test *gustducin*- and *T1r3*-expressing cells for NCAM. Almost all of the *Mash1*-expressing cells (98.2%, 372/379) in the taste buds were NCAM-immunopositive (IP), suggesting that *Mash1*-expressing cells should indeed be categorized as type III cells (Figure 1, Table 1). The majority of NCAM-IP cells in the taste buds expressed *Mash1*. Expression of *gustducin* and *T1r3* was observed primarily in NCAM-immunonegative (IN) cells; however, small subsets of *gustducin*- and *T1r3*-expressing cells were unexpectedly found to be NCAM-IP: 12.9% (42/325) of *gustducin*- and 7.73% (30/388) of *T1r3*-expressing cells (Figure 1, Table 1). This result contradicted previous results in rats (Yang *et al.*, 2000). If *gustducin*- and *T1r3*-expressing cells are derived from *Mash1*-expressing cells, as speculated previously (Kusakabe *et al.*, 2002), it is expected that NCAM immunoreactivity in *gustducin*- and *T1r3*-expressing cells is a remnant from the *Mash1*-expressing stage, and is down-regulated in the maturation process of the taste cells. We next examined the ratio of NCAM-IP cells in *Mash1*-, *gustducin*- and *T1r3*-expssing cells during development. Double-color *in situ* analysis in circumvallate papillae was performed in the circumvallate papillae at 0.5, 2.5 and 10.5 days after birth. At 0.5 days after birth, taste bud structure was hardly observed, and the signals for *gustducin* and *T1r3* were very rare but clearly detected. Only 37 *gustducin*- and 22 *T1r3*-expressing cells were observed through the analysis of six circumvallate papillae, suggesting that the expression of these genes may have just begun to turn on, while *Mash1*-expression was detected in almost every slice. The NCAM-IP cells were mainly observed in the dorsal epithelium of circumvallate papillae at 0.5 days, consistent with previous reports (Takeda *et al.*, 1992). In contrast to the results in adulthood, at 0.5 days, the vast majority (~90%) of *gustducin*- and *T1r3*-expressing cells were NCAM-IP (Figure 2, Table 1). The ratios of NCAM-IP cells for each type of gene-expressing cells in the circumvallate papillae are summarized in Table 1 and Figure 3. The ratios of NCAM-IP cells in *gustducin*- and *T1r3*-expressing cells decreased markedly during taste bud development. In contrast, the vast majority of *Mash1*-expressing cells were constantly NCAM-IP during development. However, the proportion of NCAM-IP cells in younger stage (88.9% at 0.5 days after birth) was lower than that in elder stage (98.2% at adulthood), raising the possibility that *Mash1*-expressing cells may be NCAM-IN at the onset of *Mash1* expression.

The difference in the timing of differentiation of cells expressing *Mash1*, *gustducin* and *T1r3* in adult mouse

We have previously reported that the expression of *Mash1* precedes those of taste reception-related genes such as *gustducin* and *T1r2* during development, suggesting the differential timing of cell differentiation (Kusakabe *et al.*, 2002). To clarify whether the differentiation of *Mash1*-expressing cells precedes those of taste reception-related gene-expressing cells in adult mice, BrdU-labeling experiments were performed. The ratios of BrdU-positive cells in *Mash1*-, *gustducin*- and *T1r3*-expressing cells at various intervals after BrdU injection are shown in Figure 4. The BrdU signals in *gustducin*- and *T1r3*-expressing cells began to be obvious at 2 days after BrdU injection (Figure 5, Table 2), consistent with previous immunohistochemical results, in which the BrdU signal began to be detected at 2.5 days after BrdU injection (Cho *et al.*, 1998). The BrdU signals gradually increased in these cells in the subsequent days. In *Mash1*-expressing cells, the BrdU signal increased more rapidly and was clearly observed at 1 day after BrdU injection (Figure 5, Table 2); a few signals, in <1% of *Mash1*-expressing cells, were observed at 3 and 12 h. At 2 days, BrdU signals in *Mash1*-expressing cells reached a peak level, and 11% (37/335) of *Mash1*-expressing cells were BrdU-positive. The ratio was kept at an almost constant level or slightly decreased in subsequent days. The results in *Shh*-expressing cells (originally published in Miura *et al.*, 2004) are also shown in Figure 4 and Table 2 for the sake of comparison with *Mash1*-expressing cells. In

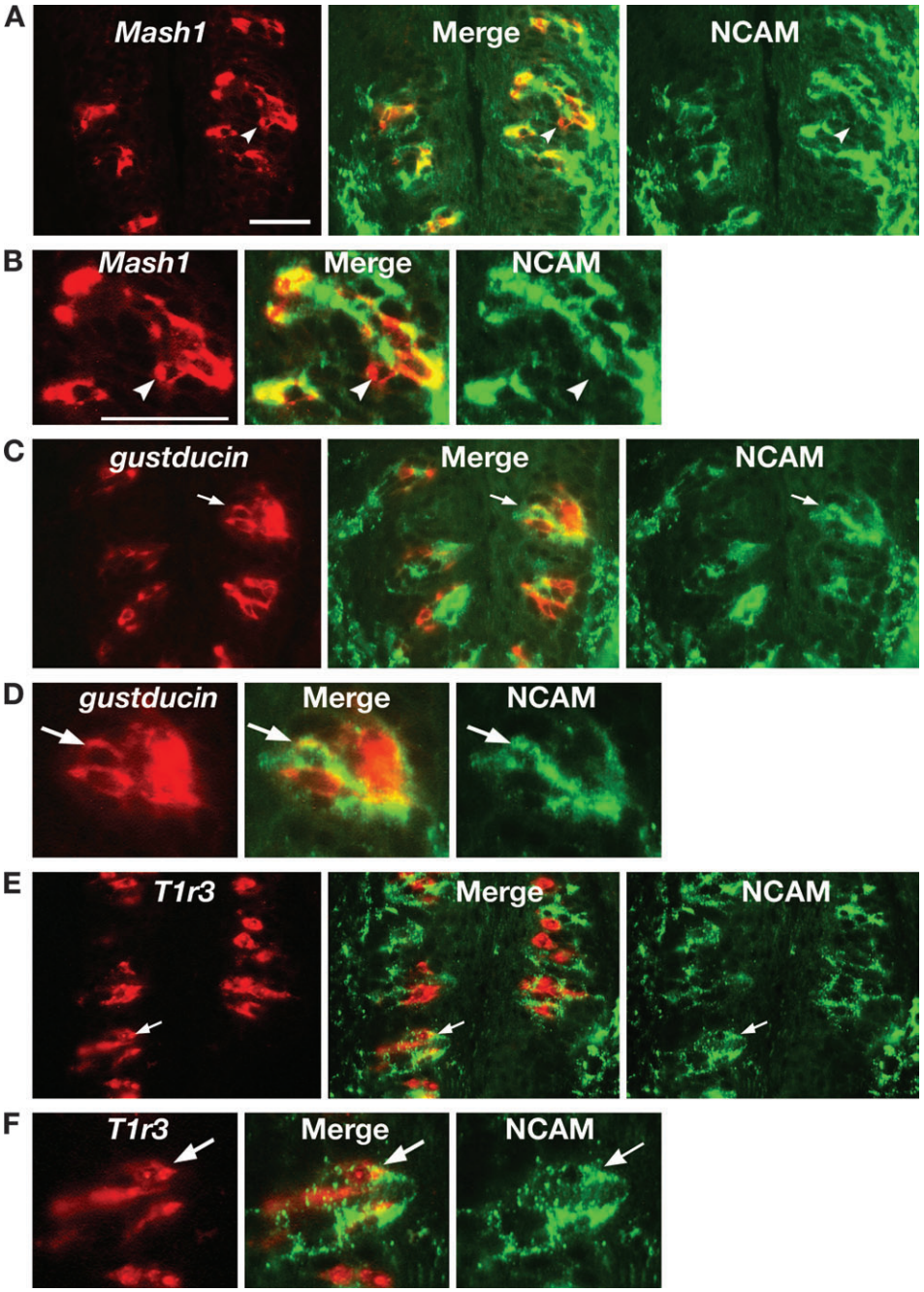

Figure 1 The immunoreactivity of NCAM in *Mash1*-, *gustducin*- or *T1r3*-expressing cells in the taste buds of adult mouse circumvallate papillae. **(A)** Almost all *Mash1*-expressing cells were NCAM-IP. NCAM-IN cells were very rare in *Mash1*-expressing cells (see arrowhead). **(B)** A high magnification view. **(C, E)** *Gustducin* and *T1r3* were mainly observed in NCAM-IN cells. Small subsets of these cells showed NCAM immunoreactivity (arrows in **C, E**). **(D, F)** High magnification views. The scale bars indicate 50 μm in A for A, C, E and in B for B, D, F.

Shh-expressing cells, BrdU signals were observed earlier than those in *Mash*-expressing cells, and the signal began to decrease 2 days after BrdU injection (Figure 5, Table 2).

The timing of differentiation of NCAM-IP cells

To compare the timing of differentiation of NCAM-IP cells to those of *Mash1*-, *gustducin*- and *T1r3*-expressing cells, BrdU-positive cells with NCAM immunoreactivity were examined at 3 and 12 h, and 1, 2, 3 and 4 days after BrdU-injection (Figure 6). In NCAM-IP cells, BrdU signals were clearly observed at 1 day after BrdU injection and reached peak level at 2 days, like that in *Mash1*-expressing cells (Figures 6 and 7). This result suggests that the differentiation timing of NCAM-IP cells is close to that of *Mash1*-expressing cells and earlier than those of *T1r3*- and *gustducin*-expressing cells

(Figure 4). The change of the rate of BrdU-positive cells in NCAM-IP cells during 4 days after BrdU injection was similar to that in *Mash1*-expressing cells. The number of NCAM-IP cells was hard to be estimated because of the NCAM signals for nerve fibers in taste bus. However, the BrdU NCAM double-positive cells could be identified clearly. Then the number of BrdU NCAM double-positive cell per trench in each section was analyzed instead of the ratio of the number of BrdU-positive cells in NCAM-IP cells to the total number of NCAM-IP cells.

Discussion

In the present report, we revealed a gradual decrease in the ratio of NCAM-IP cells in *gustducin*- and *T1r3*-expressing cells during development, while the majority of *gustducin*- and *T1r3*-expressing cells were NCAM-IP at 0.5 days after birth. These observations indicate that the *gustducin*- and *T1r3*-expressing cells may be NCAM-IP at the beginning of cell differentiation and that NCAM immunoreactivity may be reduced during their maturation process. In this case, NCAM immunoreactivity appears to represent younger populations in *gustducin*- and *T1r3*-expressing cells in adult mouse taste buds. In addition, *Mash1*-expressing cells in taste buds were found to be NCAM-IP. These findings appear to support the idea that *gustducin*- and *T1r3*-expressing cells are derived from *Mash1*-expressing cells; in taste cell differentiation, NCAM immunoreactivity in *gustducin*- and *T1r3*-expressing cells may be a remnant from the *Mash1*-expressing cell stage, and might be downregulated during maturation process. This idea of taste cell differentiation has been originally proposed based on the segregated expression patterns between *Mash1* and taste reception-related genes, and differential expression timing of these genes during development in our previous report (Kusakabe *et al.*, 2002). In the present study, BrdU-labeling experiments provided additional evidence that, in adult mice as in developing mice, the differentiation of *Mash1*-expessing cells precedes those of *gustducin*- and *T1r3*-expressing cells in taste buds. BrdU signals in *Mash1*-expressing cells were rapidly elevated before those in *gustducin*- and *T1r3*-expressing cells. In addition, previous studies showed that the expression of *NeuroD* in taste buds overlapped *gustducin* but not NCAM (Suzuki *et al.*, 2002). Taken together, our findings suggest that *Mash1* expression precedes *NeuroD*, and may not overlap *NeuroD* in taste buds. *Mash1* and *NeuroD* are bHLH transcription factors critical for neuronal differentiation, and the *NeuroD* expression following *Mash1* is known to be essential for the differentiation of olfactory neurons (Lee *et al.*, 1995; Cau *et al.*, 1997, 2002). Sequential expression of *Mash1* and

Table 1 NCAM-IP cells in each type of gene-expressing cells in circumvallate papillae of taste buds

Genes	Adult	10.5 days after birth	2.5 days after birth	0.5 days after birth
Mash1	98.2% (372/379)	97.8% (268/274)	93.5% (172/184)	88.9% (48/54)
gustducin	12.9% (42/325)	39.9% (109/273)	54.3% (51/94)	89.2% (33/37)
T1r3	7.73% (30/388)	20.8% (61/293)	49.3% (36/73)	90.9% (20/22)

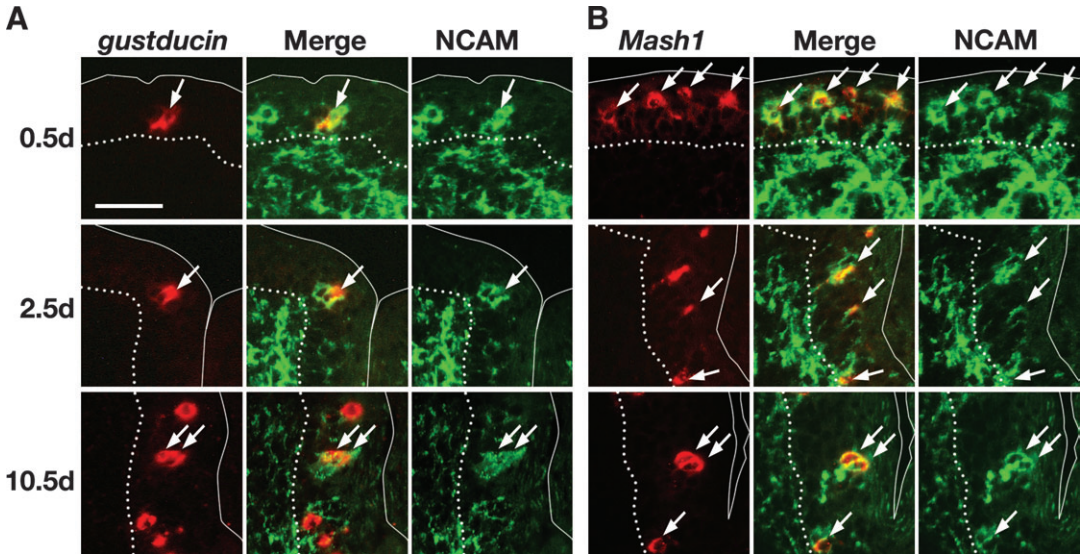

Figure 2 The immunoreactivity of NCAM in **(A)** *gustducin*- or **(B)** *Mash1*-expressing cells in the circumvallate at 0.5, 2.5 and 10.5 days after birth. At 0.5 days after birth, taste bud structure was hardly observed, but signals for *gustducin* and *Mash1* were detected. At 10.5 days, taste bud structures were observed, and the signals for *gustducin* had increased. Arrows indicate the NCAM signals overlapping with *gustducin* or *Mash1*. Dotted lines indicate the position of epithelial basement membrane, and white lines indicate the outline of epithelia. The upper side of the figure corresponds to the dorsal side of the tongue. The scale bar indicates 50 μm.

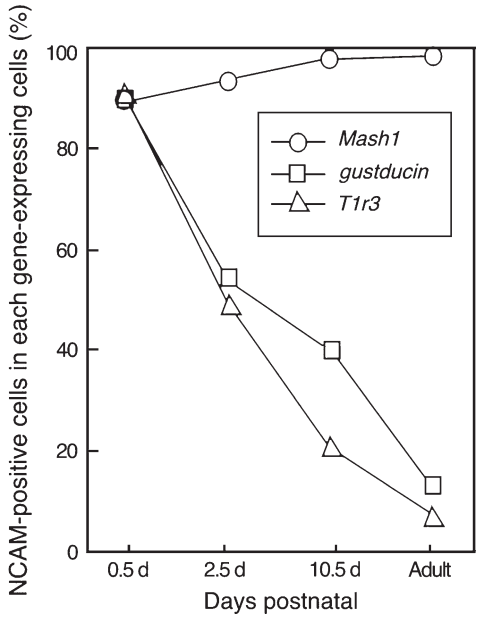

Figure 3 Temporal changes of the ratio of NCAM-IP cells in cells expressing *Mash1, gustducin* and *T1r3*. At 0.5 days after birth, the majority of *Mash1*-, *gustducin*- and *T1r3*-expressing cells were NCAM-IP. *Mash1*-expressing cells constantly showed NCAM immunoreactivity in large populations, although the ratio of NCAM-IP cells at the younger stage of these cells was slightly lower than that at the advanced stage. In contrast to *Mash1*-expressing cells, the ratio of NCAM-IP cells in *gustducin*- or *T1r3*-expressing cells markedly decreased during development.

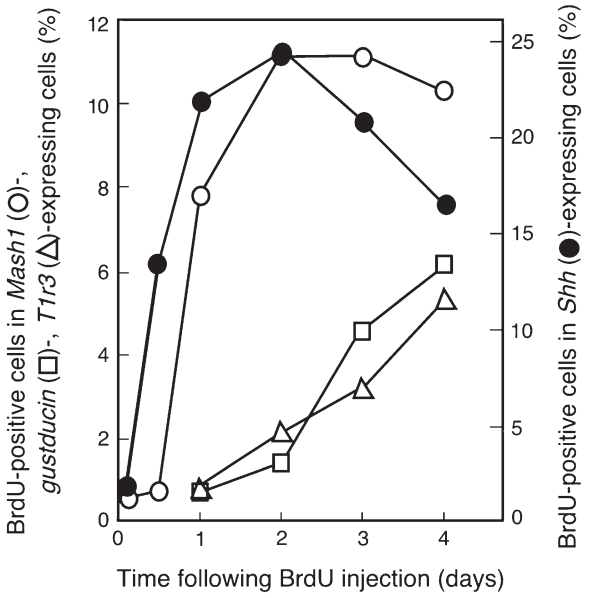

Figure 4 Temporal changes of the ratios of BrdU-positive cells in *Mash1*-, *gustducin*-, *T1r3*- or *Shh*-expressing cells after BrdU injection. In *Mash1*-expressing cells, BrdU signals were obvious at 1 day after injection. The ratio of BrdU signals in *Mash1*-expressing cells reached a peak level at 2 days and remained at that level for the next 2 days. In *Shh*-expressing cells, BrdU was clearly observed at 12 h after injection, slightly earlier than that in *Mash1*-expressing cells. After reaching peak level at 2 days, the levels declined, unlike the levels in *Mash1*-expressing cells. In *gustducin*- and *T1r3*-expressing cells, BrdU signals were obvious at 2 days after injection and increased over the next 2 days examined.

NeuroD might be involved in the differentiation of the taste receptor cells expressing *gustducin* and *T1r3*. On the other hand, we did not observe an apparent decrease of the ratio of BrdU-positive cells in *Mash1*-expressing cells inversely related to the increase of BrdU-signal in *gustducin*- and *T1r3*-expressing cells. We would expect to observe this if all *Mash1*-expressing cells become *gustducin*- or *T1r3*-expressing cells. This suggests there are cell populations expressing *Mash1* independently from the differentiation of *gustducin*- and *T1r3*-expressing cells. *Mash1* might continue to be expressed in particular populations of *Mash1*-expressing cells maybe throughout cell life. Further studies, especially with *in vitro* culture systems or gene transfer systems to induce taste cell differentiation, are needed to elucidate the function of *Mash1* and *NeuroD* in taste cell differentiation, and the cell lineages from *Mash1*-expressing cells to cells expressing taste reception–related genes. On the other hand, BrdU signals in *Shh*-expressing cells were observed earlier than that in *Mash1*-expressing cells. It is possible that *Mash1*-expressing cells are derived from *Shh*-expressing cells; overlap expression of *Shh* and *Mash1* was observed in the basal region of taste buds to some extent (unpublished data), supporting this possibility. Further analysis of the basal cells in taste buds will provide better understanding of the progenitor cells for taste buds.

NCAM immunoreactivity in taste buds has been considered as a marker for type III cells, which is distinguished from type II cells including gustducin-IP cells (Takeda *et al.*, 1992; Nelson and Finger, 1993; Yang *et al.*, 2000; Yee *et al.*, 2001). In the present report, we showed that almost all *Mash1* expression in adult mouse taste buds occurred in NCAM-IP cells, suggesting that *Mash1*-expressing cells could be categorized as type III cells. However, NCAM immunoreactivity was observed also in *gustducin*- and *T1r3*-expressing cells, contradicting previous studies in which NCAM- and gustducin-immunoreactivities were linked to type III cells and type II cells, respectively, and did not overlap. Our results in mice seem to indicate that NCAM immunoreactivity is not so strictly linked to type III cells and may be observed also in type II cells, or that *gustducin* and *T1r3* are expressed also in type III cells. In this study, to examine the co-expression pattern, we used the combination of immunohistochemistry for NCAM and *in situ* hybridization for *Mash1*, *gustducin* and *T1r3*, unlike previous reports, in which the immunohistochemical method was used for *gustducin* and NCAM. Because the appearance of mRNAs is expected to precede the protein products of these genes, it is possible that the overlap, which could not be observed immunohistochemically, was detected by our method in the present report. Alternatively, the difference in species may be more important. Immunoelectron microscopic analysis of the cell type of *gustducin*-expressing cells has been performed only in rats

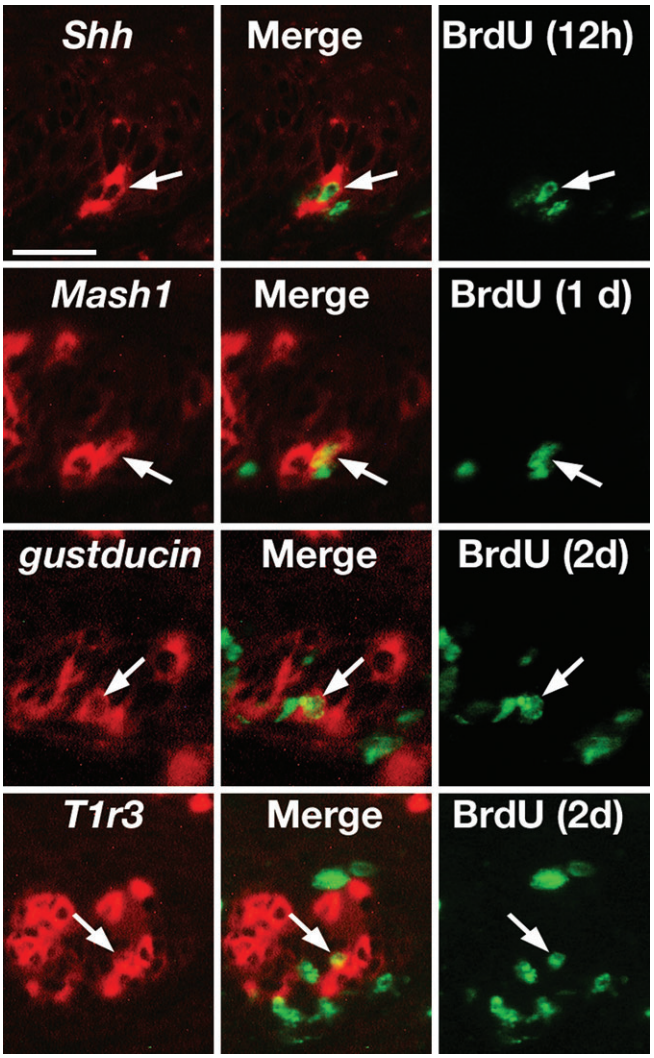

Figure 5 BrdU signals in *Shh*-, *Mash1*-, *gustducin*- or *T1r3*-expressing cells at the early stage when an obvious signal was first observed. Arrows indicate the positions of the BrdU signals as they overlap expression of each gene. The time periods after BrdU injection are given in parentheses. The scale bar indicates 25 μm.

Table 2 BrdU-positive cells in each type of gene-expressing cells in circumvallate papillae of taste buds

Gene	Time					
	3 h	12 h	1 day	2 days	3 days	4 days
Shh	2.09%	13.4%	21.7%	24.4%	20.6%	16.4%
	(5/239)	(28/209)	(76/350)	(38/156)	(49/238)	(37/226)
Mash1	0.546%	0.710%	7.73%	11.0%	11.1%	10.2%
	(2/366)	(4/563)	(14/181)	(37/335)	(41/371)	(74/722)
gustducin			0.648%	1.36%	4.51%	6.10%
			(3/463)	(5/368)	(18/399)	(33/541)
T1r3			0.794%	2.10%	3.16%	5.25%
			(5/630)	(7/334)	(5/158)	(33/628)

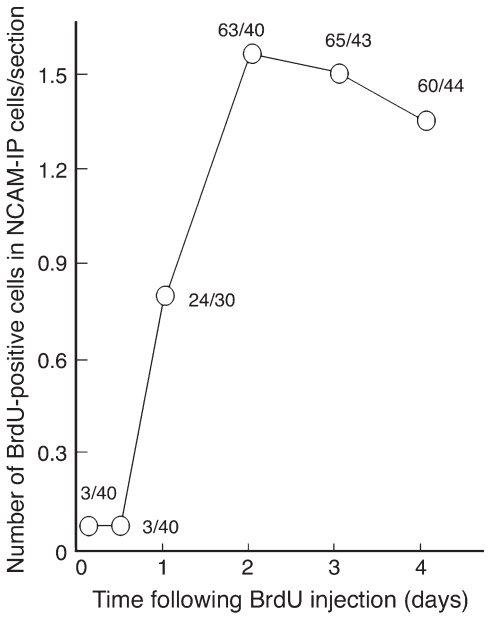

Figure 6 Temporal change of the number of BrdU NCAM double-positive cells per circumvallate trench wall in each section after BrdU injection. BrdU signals were obvious at 1 day after injection. The numbers near each point denote the number of BrdU NCAM double-positive cells/the number of circumvallate trench sections analyzed.

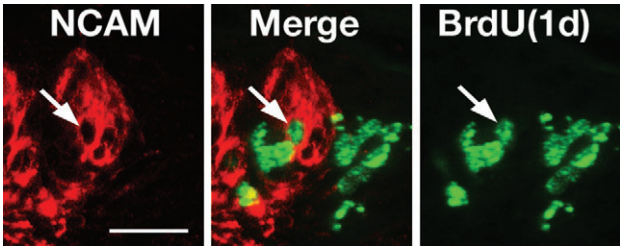

Figure 7 BrdU signals in NCAM-IP cells at 1 day after BrdU injection. Arrow indicates the position of the BrdU signal in NCAM-IP cell. BrdU signals were clearly observed at 1 day after BrdU injection. The scale bar indicates 25 μm.

(Yang *et al.*, 2000), and the case in mice remains unclear. Furthermore, a morphological difference between mice and rats has been reported in the cell types of taste buds regarding synaptic connection and dense-cored vesicles (Kinnamon *et al.*, 1985; Delay *et al.*, 1986; Pumplin *et al.*, 1997; Clapp *et al.*, 2004). In either case, based on our hypothetical model for taste cell differentiation, type II cells expressing *gustducin* or *T1r3* are likely to be derived from NCAM-IP type III cells expressing *Mash1*. This is partially consistent with the previous idea that taste cell maturation progresses from type I (dark) → type III (intermediate) → type II (light), originally proposed by Delay (1986). However, our model is different from this scheme in the following two points:

1. Type III cells expressing *Mash1* are assumed to comprise two distinct populations: one becomes type II cells

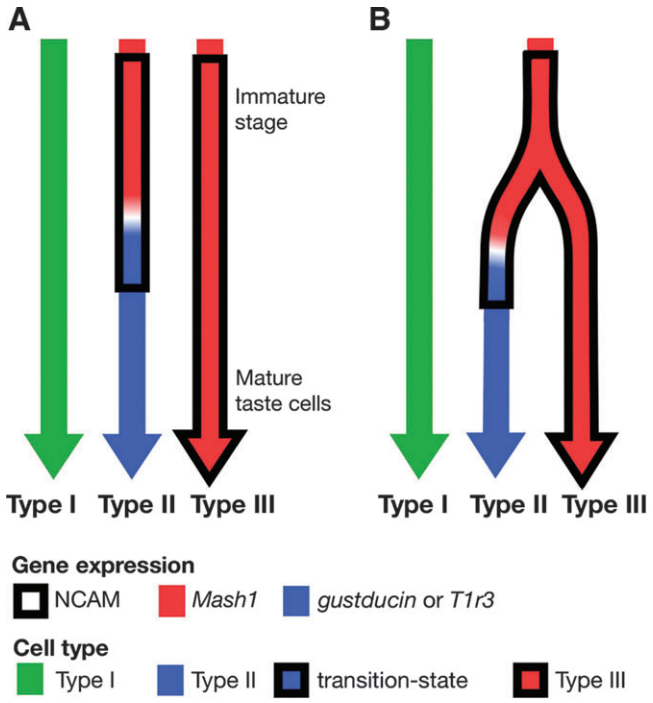

Figure 8 A schematic model of possible cell lineage relationships between type II and type III cells in taste buds. *Gustducin*- and *T1r3*-expressing cells might be derived from *Mash1*-expressing cells, while some portion of *Mash1*-expressing cells seems to stay in the type III cell state throughout cell life. Progenitors of these cells are separated (A) or shared (B). *Mash1*-expressing cells might be NCAM-IN just after the onset of *Mash1* expression. Type I cells may be in separate cell lineages from type II and III cells.

expressing *gustducin* or *T1r3* and the other remains in the type III cell state expressing *Mash1*. It is unclear whether these two populations share progenitor cells, while a serotonin-IP subpopulation in type III cells was reported to tend to be related to a separate lineage based on the results of mosaic mouse analyses (Stone *et al.*, 2002).

2. Type I cells are not assumed to be progenitors of type III cells expressing *Mash1*, because of the rapid differentiation of *Mash1*-expressing cells: BrdU signals were observed in *Mash1*-expressing cells at 3 and 12 h after BrdU injection, although those were very rare. There is no delay in differentiation timing of *Mash1*-expressing cells in comparison with the case of dark cells (type I) (Delay *et al.*, 1986). Furthermore, the sequential expression of *Mash1* and *NeuroD* suggests molecular similarities in cell differentiation between taste cells and neurons, while the gene expression related to Delta-Notch signaling was recently reported in taste buds (Seta *et al.*, 2003). Delta-Notch signaling is known to be involved in neuron/glia cell fate determination at an early phase of the development (Artavanis-Tsakonas *et al.*, 1999; Wakamatsu *et al.*, 2000; Grandbarbe *et al.*, 2003), and type I cells are believed to be glia-like cells (Lindeman, 1996; Lawton *et al.*, 2000). It is possible that Delta-Notch signaling might be involved in cell fate determination between type I cell and type III cells in the early phase of taste cell differentiation.

A schematic model of possible cell lineage relationships in taste buds is presented in Figure 8. In this scheme, the cells expressing *gustducin* or *T1r3* in the mature stage are assumed to be type II cells, and NCAM-IP cells expressing *Mash1* to be type III cells. However, the cell type of NCAM-IP cells expressing *gustducin* or *T1r3* is ambiguous; these cells may be in a transition state. Further immunoelectron microscopic analysis at the onset of the differentiation of *gustducin*- and *T1r3*-expressing cells can provide important information regarding the classification of this cell type on the basis of morphological characteristics.

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