

Mash1-expressing cells could differentiate to type III cells in adult mouse taste buds

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Short Running Title: Mash1 needs to type III cell

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## **Abstract**

The gustatory cells in taste buds have been identified as paraneuronal; they possess characteristics of both neuronal and epithelial cells. Like neurons, they form synapses, store and release transmitters, and are capable of generating an action potential. Like epithelial cells, taste cells have a limited life span and are regularly replaced throughout life. However, little is known about the molecular mechanisms that regulate taste cell genesis and differentiation. In the present study, to begin to understand these mechanisms, we investigated the role of Mash1-positive cells in regulating adult taste bud cell differentiation through the loss of Mash1-positive cells using the Cre-loxP system. We found that the cells expressing type III cell markers—aromatic L-amino acid decarboxylase (AADC), carbonic anhydrase 4 (CA4), glutamate decarboxylase 67 (GAD67), neural cell adhesion molecule (NCAM), and synaptosomal-associated protein 25 (SNAP25)—were significantly reduced in the circumvallate taste buds after administration of tamoxifen. However gustducin and Phospholipase C beta2 (PLC beta2)—markers of type II taste bud cells—were not significantly changed

in the circumvallate taste buds after administration of tamoxifen. These results suggest that Mash1-positive cells could be differentiated to the type III cells, not to the type II cells in the taste bud.

### **Mini-abstract**

This study using Cre-loxP system suggests that Mash1 could play an important role of differentiation of the type III cells in the taste bud.

### Keywords

Mash1; Paraneuron; Sensory organ; Taste bud; Type III cell

## Introduction

Mammalian taste buds are mainly observed in the gustatory papillae (fungiform, foliate, and circumvallate papillae) of the tongue (Finger and Siomon, 2000). Taste buds are multicellular end organs that comprise several distinct cell types. Taste bud cells are differentiated from surrounding epithelial cells, and undergo continuous renewal and are regularly replaced throughout the lifespan (Beidler and Smallman 1965, Farbman 1980; Delay et al., 1986; Stone et al., 2002).

Mash1 (*Ascl1*) is a mammalian achaete-scute homolog of the proneural gene, which encodes basic helix-loop-helix (bHLH) transcription factors (Johnson et al., 1990, Guillemot and Joyner 1993). Mash1 is expressed in precursors of the both central and peripheral neurons (Lo et al., 1991; Guillemot et al., 1993). A null mutation in the Mash1 gene eliminates most olfactory neurons, sympathetic and parasympathetic neurons, and enteric neurons of the foregut, suggesting that Mash1 plays a role in determining the cell fate of specific neural lineages (Guillemot et al., 1993; Sommer et al., 1996; Blaugrund et al., 1996).

Previous studies indicated that Mash1 is also expressed in the taste papillae of mouse embryos and in a subset of adult taste bud cells (Seta et al., 1999; Kusakabe et al., 2002; Miura et al., 2003, 2005; Nakayama et al., 2008).

Recently, we observed that Mash1 was expressed in some basal cells and in the majority of differentiated type III taste cells but never in type II taste cells of adult taste buds (Seta et al., 2006). Furthermore, we demonstrated that Mash1 is required for the expression of aromatic L-amino acid decarboxylase (AADC) and glutamate decarboxylase 67 (GAD67) in type III cells in the taste buds using Mash1 knockout (KO) mice (Seta et al., 2011, Kito-Shingaki et al., 2014).

However, as Mash1 KO mice die prior to taste bud formation in the taste papillae, it is not possible to determine how Mash1 affects the differentiation of adult taste bud cells. **A conditional gene recombination system can be used to explore the Mash1 function in adult taste buds. In  $Ascl1^{CreERT2}$  mice, the entire coding region of the endogenous achaete-scute complex homolog 1 (Mash1) gene is replaced by a CreERT2 fusion protein. The Cre activity is induced after administering tamoxifen. CAG-floxed neo-DTA mice contain the CAG promoter that is followed**

by the neomycin resistance cassette that is flanked by the loxP sequence and the diphtheria toxin A (DTA) gene.  $Ascl1^{CreERT2::CAG}$ -floxed neo-DTA mice are generated by breeding  $Ascl1^{CreERT2}$  knock-in mice with CAG-floxed neo-DT mice. Tamoxifen-inducible Cre-mediated recombination results in the removal of the loxP-flanked DNA sequence, and the DTA gene is driven by the CAG promoter in Mash1-expressing cells.

In the present study, we investigated the role of Mash1-positive cells in the differentiation of adult taste bud cells utilizing several transgenic mouse strains ( $Ascl1^{CreERT2}$ , CAG-floxed neo-DTA, and GAD67-GFP) to explore the mechanisms that regulate taste bud cell differentiation.

## 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. Adult animals used in this study were *Ascl1*<sup>CreERT2</sup> knock-in mice (The Jackson Laboratory, Stock No: 012882), CAG-floxed neo-diphtheria toxin A (DTA) mice (Matsumura et al., 2004), and glutamate decarboxylase 67 (GAD67)-GFP knock-in mice (Tamamaki et al., 2003). *Ascl1*<sup>CreERT2::CAG-floxed neo-DTA::GAD67-GFP</sup>(MGD) mice were generated by breeding *Ascl1*<sup>CreERT2</sup> knock-in mice, CAG-floxed neo-DT mice with GAD67-GFP knock-in mice, and were referred to as MGD mice. The genotyping of mice used the following primers:

*Ascl1*<sup>CreERT2</sup> mutant sense, 5'-AACTTTCCTCCGGGGCTCGTTTC-3';

*Ascl1*<sup>CreERT2</sup> mutant antisense, 5'-CGCCTGGCGATCCCTGAACATG-3';

GAD67-GFP sense, 5'-GGCACAGCTCTCCCTTCTGTTTGC-3';

GAD67-GFP mutant antisense, 5'-CTGCTTGTCGGCCATGATATAGACG-3';

CAG-floxed neo-DTA sense,

5'-GCCTTCTATCGCCTTCTTGACGAGTTCTTC-3';

CAG-floxed neo-DTA antisense, 5'-CTACATAACCAGGTTTAGTCCCG-3'.

### **Tissue preparation**

MGD mice were injected intraperitoneally with tamoxifen (100mg/kg; Sigma,

T5648) in sunflower seed oil (Sigma, S5007), which has the effect of

translocating Cre-ERT2 to the nucleus, where it excises the reporter gene.

At the age of 6–8 weeks, MGD and *Ascl1*<sup>CreERT2::GAD67-GFP</sup> (MG) (as the control) mice were administered tamoxifen once every 24 h for 3 days, 5 days, and 10 days, according to Kim et al. (2011). *Ascl1*<sup>CreERT2::GAD67-GFP</sup> (MG) (as the control) mice were administered tamoxifen once every 24 h for 10 days. Mice were anesthetized by intraperitoneally administering pentobarbital (50 mg/kg), and perfused through the left ventricle with 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4. The tongues of perfused mice were fixed overnight in



the same fixative and embedded in OCT compound (Sakura, Torrance, CA, USA).

Cryostat sections (6–8  $\mu\text{m}$ ) were mounted on MAS-coated Superfrost slides

(Matsunami, Japan) and stored in airtight boxes at  $-80^{\circ}\text{C}$ .

### **Immunohistochemistry**

For immunohistochemical staining, the sections were blocked for 2 h in 5% goat

serum in phosphate buffered saline (PBS) and incubated with rabbit

anti-aromatic L-amino acid decarboxylase (AADC) (1:200; Gene Tex), goat

anti-carbonic anhydrase IV (CA4) (1:200; R&D system), rabbit anti-gustducin

(1:1,000; Santa Cruz), rabbit anti-phospholipase C beta2 (PLC beta2) (1:1,000;

Santa Cruz), rabbit anti-neural cell adhesion molecule (NCAM), and rabbit

anti-synaptosomal-associated protein 25 (SNAP25) as primary antibodies

overnight at  $4^{\circ}\text{C}$  in a humidified chamber. After rinsing with PBS, sections were

incubated with Alexa Fluor 488 conjugated donkey anti-rabbit IgG (1:1,000) or

Alexa Fluor 546 conjugated donkey anti-goat IgG (1:1,000) as secondary

antibodies overnight at  $4^{\circ}\text{C}$ . Slides were rinsed with PBS and coverslipped with

Vectashield (Vector Laboratories, USA). Negative controls were performed by the omission of each of the first primary antibodies.

All images were obtained using a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). Digital images were contrasted and color adjusted, and plates were created using Adobe Photoshop CS5 for Macintosh.

#### Statistical analyses

Statistical significance was assessed using one-way ANOVA and analysis of variance followed by the Tukey–Kramer test for each group. Significance was defined as  $p < 0.05$ .

## Results

After administering tamoxifen to MGD mice for 3 days, 5 days, and 10 days, there was no significant difference in the size and shape of the circumvallate papilla. Moreover, there was no significant difference in the distribution or number of taste buds in the circumvallate papillae between the tamoxifen-treated MGD mice and the control mice (Table1).

To investigate the influence of the loss of Mash1-positive cells in the differentiation of taste bud cells, we performed immunohistochemistry using taste cell markers. PLC beta2 is a marker of type II and a subset of type III taste cells (Clapp et al. 2001). Immunoreactivity of PLC beta2 was present in a large subset of mouse circumvallate taste buds (Fig.1a–h). Gustducin is a marker of a subset of type II taste cells (Clapp et al., 2001; Yee et al., 2001).

Immunoreactivity of gustducin was also present in a large subset of mouse circumvallate taste buds (Fig.1i–p). The expression of these type II cell markers did not significantly change compared with the control (Fig.2). Next we investigated the influence of the loss of Mash1 on the expression of type III cell

markers in taste buds. GAD67 is a subset of type III cell markers (Kito-Shingaki et al., 2014). The expression of GAD67 in taste bud cells was missing in Mash1 mutant mice. In MGD mice after administration of tamoxifen for 10 days, GAD67-expressing taste cells were significantly reduced compared with those of control mice (Fig.3a–h). AADC, CA4, NCAM and SNAP25 are also type III cell markers (Yee et al., 2003, Seta et al., 2011); these type III cell markers were found at a rate of about five to seven immunoreactive cells per taste bud in the control mice (Fig.3e, m, u, Fig. 4e, m). The observed reduction in the number of immunopositive cells for type III cell markers depended on the length of administration of tamoxifen (Fig.5). AADC and CA4 levels were significantly reduced to approximately 75% of those in the control mice after administration of tamoxifen for 5 days and 10 days, respectively (Fig.3i–x). On the other hand, NCAM and SNAP25 were significantly reduced compared to those of the control after administration of tamoxifen for 10 days (Fig.4a–p; Table 2).

## Discussion

This study shows the effect of the loss of Mash1-positive cells in adult taste bud cells. We found that no significant difference in the number and size of taste buds in the adult circumvallate papilla. After administering tamoxifen for 10 days, the expression of type III cell markers was significantly reduced compared with that of the control mice. However, the expression of type II cell markers did not significantly change compared with that of the control.

Recent studies have demonstrated that Mash1 is not only expressed in the precursors of the nervous system but also in neuroendocrine cells and sensory organs. Studies evaluating the loss-of-function of Mash1 revealed that Mash1 appears to act during a late stage of neuronal differentiation, neuroendocrine cells, and olfactory neurons (Guillemot et al., 1993). Furthermore, gain-of-function of Mash1 experiments showed that Mash1 may play a role in regulating subtype-specific aspect of neural differentiation (Lo et al., 1998).

Recently, we demonstrated that AADC-IR- and GAD67-expressing type III cells disappeared, although no significant differences in gene expression in the

developing papillae or taste bud formation in the soft palate of Mash1 mutant mice were observed (Seta et al., 2011, Kito-Shingaki et al., 2014). In this study, we observed that the tamoxifen-induced elimination of Mash1-expressing cells did not affect taste bud morphology and formation in adult mice. These results indicate that Mash1 does not play a role in taste bud cell differentiation from the surrounding epithelial cells.

On the basis of the ultrastructural features of taste bud cells, mammalian taste buds possess four distinct morphologically identifiable types of cells (Murray 1986). Among these taste cell types, only type III cells make a synaptic contact with nerve terminals; these cells are considered as taste receptor cells for sour taste. Mash1 was expressed in the subset of basal cells and also in that of mature taste cells (Seta et al., 1999). In this study, we observed that type II cell markers do not change compared with those of the control mice. This result suggests that the tamoxifen-induced elimination of Mash1-expressing basal cells do not affect type II cells differentiation, and there are two types of basal cells: one is Mash1-expressing basal cell and the other is Mash1-negative basal cell.

We previously demonstrated that AADC- and GAD67-expressing cells were missing in Mash1 KO mice. The type III cell markers NCAM and SNAP25 were, however, expressed in the soft palate epithelial tissue of Mash1 KO mice using RT-PCR, and NCAM-immunopositive cells were observed in the soft palate taste buds of Mash1 KO mice (Seta et al., 2011). In this study, the expression of all type III cell markers was significantly reduced compared with that of the control at 10 days after the administration of tamoxifen. There is, however, a difference in the reduction rate among type III cell markers. The expression of AADC, CA4 and, GAD67 was reduced to a greater extent than was that of NACM and SNAP25. Our results suggest that there are two subsets of type III cells in taste buds: 1) Mash1 appears to be necessary in the differentiation of a subset of type III cells—AADC- and GAD67-positive cells in the taste buds and 2) Mash1 does not appear to be necessary in the differentiation of a subset of type III cells—NACM and SNAP25-positive cells. **Altogether our investigations revealed that Mash1 may have essential roles in the differentiation of a subset of type III cells (AADC, CA4, and GAD67-expressing cells).** Unfortunately, MGD mice do

not completely lose Mash1-expressing cells by the administration of tamoxifen.

Thus, the precise function of Mash1 in taste bud cell differentiation remains to be determined.

In conclusion, the present study indicates that Mash1 is required to regulate the expression of type III cell markers in adult taste buds. Further studies that target transcription factors will provide a better understanding of taste bud cell differentiation.

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### **Conflict of interest**



The authors declare that they have no conflict of interest.

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### Figure legends

Figure 1. Expression of type II cell markers (PLCbeta2 in a-h, gustducin in i-p) after administration of tamoxifen in mouse circumvallate papilla. Dashed line indicates outline of taste bud. The expression of these type II cell markers did not significantly change compared with that of the control. Scale bars = 10  $\mu$ m.

Figure 2. Variation in the number of type II cell markers for immunoreactive cells per taste bud. White bar indicates the control group. Dot, stripe and black bars indicate 3 days, 5 days, and 10 days, respectively, for the experimental group. \*P < 0.05

Figure 3. Expression of type III cell markers (GAD67 in a-h, AADC in i-p, and

CA4 in q-x) after administration of tamoxifen in mouse circumvallate papilla.

Dashed line indicates outline of taste bud. A reduction was observed in the number of immunopositive cells for type III cell markers depending on the length of administration of tamoxifen. Scale bars = 10  $\mu$ m.

Figure 4. Expression of type III cell markers (NCAM in a-h, and SNAP25 in i-p) after administration of tamoxifen in mouse circumvallate papilla. Dashed line indicates outline of taste bud. A reduction was also observed in the number of immunopositive cells for type III cell markers depending on the length of administration of tamoxifen. Scale bars = 10  $\mu$ m.

Figure 5. Variation in the number of type III cell markers for immunoreactive cells per taste bud. White bar indicates the control group. Dot, stripe and black bars indicate the experimental group at 3 days, 5 days and 10 days. \*P < 0.05



Table 1

Comparison of the size and number of taste buds between the control group and experimental group.

Table 2

Comparison of the number of immunoreactive cells per taste bud between the control group and experimental group.