Fine-tuning of epithelial taste bud organoid to promote functional recapitulation of taste reactivity

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Abstract
Taste stem/progenitor cells from posterior mouse tongues have been used to generate taste bud organoids. However, the inaccessible location of taste receptor cells is observed in conventional organoids. In this study, we established a suspension-culture method to fine-tune taste bud organoids by apicobasal polarity alteration to form the accessible localization of taste receptor cells. Compared to conventional Matrigel-embedded organoids, suspension-cultured organoids showed comparable differentiation and renewal rates to those of taste buds in vivo and exhibited functional taste receptor cells and cycling progenitor cells. Accessible taste receptor cells enabled the direct application of calcium imaging to evaluate the taste response. Moreover, suspension-cultured organoids can be genetically altered. Suspension-cultured taste bud organoids harmoniously integrated with the recipient lingual epithelium, maintaining the taste receptor cells and gustatory innervation capacity. We propose that suspension-cultured organoids may provide an efficient model for taste research, including taste bud development, regeneration, and transplantation.

Keywords Taste bud organoids · Suspension-culture · Taste receptor cells · Single-cell RNA sequencing · Apicobasal polarity · Calcium imaging · Genetic alteration · Transplantation

Introduction

Taste buds in mice contain 50–100 differentiated taste receptor cells, which are located in the lingual and palatal epithelium [1, 2]. These differentiated taste receptor cells can be categorized as Type I (glial-like cells), Type II (receptor cells), and Type III (presynaptic cells) cells defined by their various molecular receptors and responses to tastants [3–5]. On the other hand, immature cells in the basal compartment of taste buds that give rise to taste receptor cells are categorized as Type IV cells (precursor cells) [6, 7]. However, the crucial factors that regulate cell fate have not been fully established.

Lgr5 (leucine-rich repeat-containing G protein-coupled receptor 5) marks bipotential stem/progenitor cells in the posterior tongue, which give rise to taste and non-taste epithelial cells [8, 9]. These Lgr5-positive cells can form taste bud organoids in ex vivo cultures, similar to those formed by Lgr5-positive cells from the intestine, colon, and liver [10–13]. As an ex vivo model for taste research, these organoids provide a platform for assessing the regulation of the cell cycle and cell motility through lineage tracing.
and live-cell imaging [14]. Furthermore, signaling pathways such as Wnt, Shh, BMPs, and Notch have been characterized in taste bud organoids via transcriptome analysis and gain-/loss-of-function, which suggests a similarity between taste bud organoids and taste buds at the molecular level [15]. Thus, taste bud organoids can contribute to new discoveries regarding taste cell biology, including the processes from cycling taste stem/progenitor cells to differentiated taste receptor cells. However, the accessibility of taste receptor cells to these organoids remain problematic, since the taste receptor cells are enclosed inside the organoid and, therefore, are not accessible for functional studies.

In enteroids, organoids derived from gastrointestinal stem cells, the removal of the extracellular matrix scaffold induces reversal of apicobasal polarity by suspension-culture. This enables the apical surface to evert and face outward (apical out organoids), thereby increasing access to the apical surfaces of the enteroids [16–19]. Apical out organoids derived from human liver and porcine intestine have also been reported to possess more in vivo-like characteristics than conventional Matrigel-embedded organoids [20, 21]. Thus, we hypothesized that removal of the extracellular matrix could reverse the apicobasal polarity by the suspension-culture method, to generate functional taste bud organoids with accessible taste receptor cells and taste bud-like morphology. Suspension-cultured organoids showed higher similarity in differentiation and renewal abilities to taste buds than Matrigel-embedded organoids. Suspension-cultured organoids also contained stem cells and could be serially passaged for five passages (10 weeks).

Another purpose of a 2D- or 3D-culture of taste receptor cells is to investigate the interaction between the latter and tastants [22]. The inaccessibility of taste receptor cells to tastants in conventional organoids is one of the greatest barriers for assessing the function of taste receptor cells ex vivo [3, 23–25]. In conventional taste bud organoids, a significant taste-evoked response has been observed through calcium imaging [22]. However, this calcium imaging analysis was performed on 2D-cultured cells that migrated from organoids, not organoids themselves. Thus, it was assumed that this technical difficulty might be due to the localization of taste receptor cells in conventional organoids, which are distant from the organoid surface [22, 26]. Here, we confirmed that suspension-cultured taste bud organoids could exhibit the reversal of apicobasal polarity and thus produce taste receptor cells localized on the apical surface in contact with the external environment. Apically localized taste receptor cells became accessible for taste-evoked calcium imaging analysis to study tantant responses.

In regenerative medicine, adult stem cell-derived organoids are an ideal source of sustainable genetic stability and tissue identity [27–30]. Organoids serve as a model for studying organogenesis, as a platform for studying genetic defects, and as a source for regenerative medicine [31]. The screening of target genes and their corresponding receptors can be performed simply and efficiently upon taste bud organoids using genetic alterations, compared to generating transgenic mice [32]. Suspension-cultured organoids can be genetically altered, similar to Matrigel-embedded organoids, which are used, in regenerative therapy.

Previous transplantation experiments using lingual organoids have demonstrated that organoids can be successfully engrafted and matured in the tongues of recipient mice [33]. However, the transplanted organoids that were developed in the muscle layer rather than the epithelial layer formed a keratinized layer without taste receptor cells. The suspension-cultured taste bud organoids were integrated into the epithelial layer of the recipient tongue after transplantation. The integration of transplanted organoids with the surrounding lingual epithelium and the maintenance of taste receptor cells and gustatory innervation were also enriched after suspension-culture.

In summary, suspension-cultured organoids induced the apical localization of taste receptor cells. The taste receptor cell differentiation rate from stem/progenitor was found to be associated with the taste buds. Calcium imaging analysis was possible on the whole organoid due to the apical localization of taste receptor cells. Suspension-cultured organoids were also capable of genetic alterations and transplantation to mirror their corresponding organs.

Results
Suspension-culture altered the apicobasal polarity of taste bud organoids
To examine whether a suspension-culture could alter morphology, especially the apicobasal polarity of taste bud organoids, Matrigel-embedded organoids cultured for 4 days were transferred to suspension-culture in the same culture medium using low-attachment plates (Fig. 1A). The size of suspension-cultured organoids increased until 12 h of culture (Fig. 1B). After 14 days of culture, the size of the Matrigel-embedded organoid increased dramatically and a keratinizing inner core was observed (Fig. 1C, D). On the other hand, the size of the suspension-cultured organoid was smaller with the absence of the keratinizing inner core (Fig. 1C’, D’). To confirm whether this morphology was sustainable, organoids were cultured for 30 days. Similar to previous observations, the keratinizing inner core was conserved in Matrigel-embedded organoids cultured for 30 days (Fig. S1A). Suspension-cultured organoids remained smaller and had no inner core (Fig. S1A’). The inner core in Matrigel-embedded organoids was further confirmed by the localization of filaggrin (stratum corneum) and keratin 10.
Fig. 1 Suspension-cultured organoids exhibit reversal of apicobasal polarity and contain Shh-expressing/-responsive cells. A Schematic of suspension-culture method for taste bud organoids. Single cell-derived taste bud organoids are formed in Matrigel and later transferrered to low-attachment plates for suspension-culture with the same culture medium. B Time-lapse imaging of suspension-cultured organoids under a bright-field microscope. The size of the organoid increased gradually to roughly 50 μm after 24 h. C, E' Bright-field images show that Matrigel-embedded organoids are larger than suspension-cultured organoids after a 14-day culture. D, D' An eosin-stained keratinizing inner core is observed in the Matrigel-group but not in the suspension-group. E The basal cell marker p63 is detected in the outer region of Matrigel-embedded organoids. E' After suspension-culture, p63-positive cells are scattered and found in the inner region. F–I' 14-day cultured organoids are immunostained using anti-EAAT1, TRPM5, SNAP25 and Keratin 8 antibodies. F Type I taste receptor cells marked by EAAT1 (green) are observed inside organoids (arrowhead). G' EAAT1 positive cells are found in the outer region of organoids (arrowhead). G Type II taste receptor cells marked by TRPM5 (red) are found in the inner region of organoids (arrowhead). G' TRPM5 positive cells are detected in the outer region contacting the medium (arrowhead). H Type III taste receptor cells marked by SNAP25 (green) are randomly distributed in the inner region of organoids (arrowhead). H' SNAP25 positive cells are detected in the outer region of suspension-cultured organoids. I K8 positive cells, a pan taste receptor cells marker, are observed in the inner region of Matrigel-embedded organoids (arrowhead). I' K8 positive cells are found in the outer region of organoids (arrowhead). J Most of K8 positive cells are localized internally in the Matrigel-embedded group but are localized in the outer region in the suspension-cultured group. Weak expressions of Shh (K), Gli1 (L) and Gli3 (M) in Matrigel-embedded organoids are partially detected by whole-mount in situ hybridization. K' Shh is detected in several cells inside the suspension-cultured organoids. L' Gli1 is broadly expressed in the inner region. M' Gli3 positive cells are detected inside the suspension-cultured taste bud organoids. Scale bars in (B–C', E, F–M) = 50 μm; (D, D', E', K'–M') = 25 μm. Arrowheads indicate localization of ZO-1, β-catenin and p63. Red squares indicate CVP and FOP (CVP: circumvallate papilla, FOP: foliate papillae). All panels represent data from at least five independent specimens (n > 5) (p < 0.05)
(granular and spinous layers), which suggested that keratin is a major component of the matrix (Fig. S1B, C). The tight junction protein, ZO-1, was detected between the keratin matrix and the cells of the organoids, whereas the basolateral protein β-catenin was detected on the basolateral surface of Matrigel-embedded organoids (Fig. S1D, E). Conversely, in suspension-cultured organoids, ZO-1 was localized on the outer surface in contact with the culture medium, while β-catenin was observed in the inner region of the organoid (Fig. S1D′, E′). p63-positive basal cells were observed in the outer regions of Matrigel-embedded organoids but were localized in the inner regions after suspension-culture (Fig. 1E, E′). In summary, these results demonstrated that the removal of the extracellular matrix scaffold induces a reversal in the polarity of taste bud organoids after suspension-culture. To investigate whether apicobasal polarity reversed (apical out) organoids formed directly in the suspension-culture without the intermediate Matrigel-culturing step, single cells from the tissue were directly cultured in a low-attachment dish. No organoid formation was observed after 7 days of culture (data not shown, n > 10). This indicates that stem cells require an extracellular matrix scaffold for the initiation of taste bud organoid formation.
Fig. 2 Suspension-cultured organoids show higher similarity to taste buds. **A** Taste receptor cell markers are significantly higher in suspension-cultured organoids after 14 days. CVP and FOP are used as technical and topological control, respectively. **B** The number of single cell-derived organoid forming units is larger in the suspension group than in the Matrigel and CVP epithelium groups after 7 days of culture. **C** Representative Ki67 staining (red) shows proliferating cells in the outer region of Matrigel-embedded organoids (arrowhead). **D** Double staining of BrdU (green) and K8 (red) shows that proliferative cells are partially differentiated into taste receptor cells (arrowhead) 5 days after BrdU incorporation. **E** Ki67 positive proliferating cells are broadly located in suspension-cultured organoids (arrowhead). **F** A higher number of BrdU/K8-positive cells are found in suspension-cultured organoids 5 days after incorporation (arrowhead). **G** Ki67 positive cells are found in the perigemmal region (arrowhead). **H** BrdU-incorporated cells (green) are found in the intragemmal and perigemmal regions 5 days after BrdU incorporation. **I** BrdU label retaining cells are categorized as progenitor, precursor, apical flat, and taste cell populations based on double staining with anti-K14/SHH/K8 and anti-BrdU antibodies. Pie charts show the cell populations at 5 and 14 days after BrdU incorporation. Numbers indicate the percentage of each cell population among BrdU label retaining cells. The proportion of each cell population is similar among the suspension group and adult CVP epithelium. **J** Uniform manifold approximation projection (UMAP) of single cell transcriptome data obtained from Matrigel-embedded and suspension-cultured organoids. The colors attribute to the different cell populations identified. **K** Suspension-cultured organoids show a higher cell number of lingual stem, precursor, and taste receptor cells. **L** UMAP plot comparing the subtypes of taste receptor cells from Matrigel-embedded and suspension-cultured organoids. **M** Suspension-cultured organoid showing a higher number of all types of taste receptor cells compared to a Matrigel-embedded organoid. Scale bars = 50 µm. Arrowheads indicate Ki67-positive cells or BrdU labeled cells. At least five independent batches of organoids were analyzed for RT-qPCR and organoid formation assays (n > 5). Matrigel/suspension organoids and adult mice were labeled with BrdU and processed for cell categorization experiments (n > 5). Data represented are mean ± SD; RT-qPCR data were compared by Tukey’s multiple comparison test. ***, **** indicate p < 0.01, p < 0.001 and p < 0.0001, respectively, ns—not significant

**Differentiated taste receptor cells and Shh-expressing/-responsive cells were found in suspension-cultured organoids**

To assess the differentiation of taste receptor cell localization in Matrigel-embedded and suspension-cultured organoids, immunostaining against EAAT1 for Type I taste receptor cells, TRPM5 for Type II taste receptor cells, SNAP25 for Type III taste receptor cells, and Keratin 8 (K8) as a pan taste receptor cell marker were performed. After 14 days of culture, EAAT1-, TRPM5-, SNAP25-, and Keratin 8-positive taste receptor cells were observed at random locations in the inner region of Matrigel-embedded organoids (Fig. 1F–I). Differentiated taste receptor cells expressing EAAT1, TRPM5, SNAP25, and K8 were also detected in suspension-cultured organoids, but most of the taste receptor cells were found in the outer region (Fig. 1F–I). Statistically, differences in the localization of K8-positive cells were confirmed between the two culture methods. 84.8% of the K8-positive cells were located in the inner region of the Matrigel-embedded organoids, while 87.6% of K8-positive cells were found in the outer region of the suspension-cultured organoids (p < 0.05) (Fig. 1J). To validate the specificity of taste receptor cell labelling, adult mice circumvallate papillae (CVP) were immunostained with differentiated taste receptor cell markers as a reference control. EAAT1, TRPM5, SNAP25, and K8 were all detected in the taste buds of the CVP (Fig. S1F–I). These data indicate that suspension-culture induced the outer localization of taste receptor cells efficiently within taste bud organoids.

It is well known that the Shh signaling pathway is essential for taste cell maintenance and differentiation in the mice taste epithelium [7, 34–36]. To determine whether the Shh signaling pathway is activated in taste bud organoids, in situ hybridization for Shh, Gli1, and Gli3 was performed on Matrigel-embedded and suspension-cultured organoids. Shh expression was undetectable in Matrigel-embedded organoids (Fig. 1K). In contrast, Shh-expressing cells were found in both the outer and inner regions of suspension-cultured organoids (Fig. 1K′). A weak expression of Gli1 or Gli3 was detected in Matrigel-embedded organoids (Fig. 1L, M). Gli1 expression was broadly detected (Fig. 1L′), while Gli3-positive cells were partially observed in the inner region of suspension-cultured organoids (Fig. M′). In situ hybridization for keratin 5 was used as a positive control for Matrigel-embedded organoids [33]. Keratin 5 was broadly detected in the outermost region of the Matrigel-embedded organoids (Fig. S2E). The expression levels of Shh, Gli1, and Gli3 in both types of organoids were further confirmed by reverse transcription-PCR. No expression of Shh and weak expressions of Gli1 and Gli3 were detected in Matrigel-embedded organoids (Fig. S2A), whereas strong expressions of Shh, Gli1, and Gli3 were found in suspension-cultured organoids (Fig. S2A′). cDNA from the epithelium of CVP and the surrounding non-taste epithelium were used as controls. Shh expression was observed in the CVP epithelium, while Gli1 and Gli3 were observed in the non-epithelium (Fig. S2B–D). These results suggest that the Shh signaling pathway was induced in suspension-cultured organoids.

To investigate whether the presence of the Shh signaling pathway in the suspension-cultured organoids was responsible for polarity reversal, suspension-cultured organoids were grown in culture media supplemented with a Shh inhibitor, 1 µM cyclopamine. Media supplemented with 1% ethanol served as a control. Zo-1 and K8-positive cells were localized on the apical side of the organoid in both control and cyclopamine-treated organoids (Fig. S2F–G′). These results indicate that apicobasal polarity alteration by suspension-culture was independent of Shh signaling.
Similarity on differentiation rate of suspension-cultured organoids to taste buds

The expression levels of taste receptor cell marker genes among CVP, Matrigel-embedded, and suspension-culture taste bud organoids were analyzed using RT–qPCR. Foliate papillae (FOPs) were used as topological controls. After 14 days, Type I (nucleotide triphosphate diphosphohydrolase-2, NTPdase II), Type II (Taste 1 receptor member 1, Tas1r1), and Type III (carbonic anhydrase 4, Car4) taste receptor cell markers were significantly higher in the suspension-cultured organoid than in the Matrigel-embedded organoid (Fig. 2A). Moreover, the expression level of the calcium-sensing receptor, which is broadly expressed in the posterior tongue papillae [37], was higher in suspension-cultured organoids than in Matrigel-embedded organoids (Fig. 2A). Thus, suspension-cultured organoids showed an improved taste receptor cell differentiation capacity than Matrigel-embedded organoids. The expressions of the subtypes of Type II taste receptor cells detected among suspension-cultured organoids included (Taste 1 Receptor Member 2, 3) Tas1r2, Tas1r3, and stem/progenitor cell markers, Lgr5 (leucine rich repeat containing G protein-coupled receptor 5), and Sox9 (SRY-box transcription factor 9) (Fig. S3A). Thus, both stem cell and taste receptor cell markers were maintained after suspension-culture.

To evaluate the expansion potential of suspension-cultured organoids for further passaging, the organoid formation efficiency was analyzed. 10 days cultured Matrigel-embedded and suspension-cultured organoids could form organoids after passaging. Cells dissociated from suspension-cultured organoids formed organoids more efficiently than those from Matrigel-embedded organoids and adult CVP epithelium (Fig. 2B). Furthermore, suspension-cultured organoids were serially passaged to evaluate the self-renewal capacity of stem/progenitor cells in the organoid. Serial passaging of the cells from the dissociated suspension-cultured organoids revealed that these organoids could be passaged for up to five generations (10 weeks), beyond which the organoid morphology and differentiation were affected (Fig. S3B, B’). This result indicates that suspension-cultured organoids contain functional stem/progenitor cells capable of self-renewal, albeit with limited regenerative potential.

Proliferating progenitor cells marked by Ki67 are essential for the renewal of taste receptor cells [9]. To assess the presence of proliferative cells, Ki67 staining was performed. A small number of Ki67-positive proliferative cells were found in the outer region of the Matrigel-embedded organoids (Fig. 2C, arrowhead). Comparatively, a large number of Ki67-positive proliferating cells were found in suspension-cultured organoids (Fig. 2E, arrowhead). Ki67-positive cells were found in the perigemmal region of the CVP epithelium (Fig. 2G, arrowhead). The fate of the proliferative cells was confirmed by analyzing BrdU incorporation. BrdU incorporation in mice and taste bud organoids was analyzed by double immunostaining for BrdU and K8. Several K8/BrdU double-positive cells were observed in suspension-cultured organoids, but few were observed in Matrigel-embedded organoids and none in adult CVP epithelium (Fig. 2D, F, H). At 3, 5, 7, 10, and 14 days after incorporation, BrdU-labeled cells in organoids or adult CVPs were analyzed and then categorized into four groups based on double staining with various combinations of anti-K8/K14/SHH antibodies (K8: taste receptor cells, K14: progenitor cells, SHH: precursor cells) (Fig. 2I). The proportion of apical flat cells and progenitor cells was significantly higher at 5 days in adult mouse CVPs, as well as in suspension-cultured organoids. At 14 days, the majority of BrdU-labeled cells in adult mice CVPs and suspension-cultured organoids were taste receptor cells (Fig. 2I). However, the proportion of each cell population was different in Matrigel-embedded organoids at 5 and 14 days when compared to the remaining groups (Fig. 2I). The fate of progenitor cells in suspension-cultured organoids showed a higher similarity to adult CVP epithelium compared to Matrigel-embedded organoids.

Single-cell RNA sequencing (scRNA-seq) of the Matrigel-embedded and suspension-cultured taste bud organoids was performed to confirm the differences between them. Using the marker gene analysis software, Seurat 3, 6 clusters based on five cell types were identified (Figs. 2J and S3C). The number of taste stem cells, taste precursor cells, taste receptor cells, and lingual epithelial cells were higher in suspension-cultured organoids than in Matrigel-embedded organoids. However, the number of progenitor cells increased in Matrigel-embedded organoids (Fig. 2K). Furthermore, the taste receptor cell subtypes (Type I, Type II, and Type III) were identified in the taste receptor cell cluster (Fig. 2L). Taste receptor cell subtypes, Type I, Type II, and Type III, were increased in suspension-cultured taste bud organoids (Fig. 2M). The genes used for analysis are shown in Fig. S3C, D. Collectively; these data suggest that the suspension-cultured organoid has improved differentiation and stem cell potential than Matrigel-embedded organoids.

Suspension-cultured organoids can be used for calcium imaging and genetic alteration

Calcium imaging for tastants stimuli have been performed on 2D-cultured cells, derived from the taste bud organoid, since taste receptor cells are localized inside of Matrigel-cultured taste bud organoid [22]. To examine the response of taste receptor cells in suspension-cultured organoid to variable tastants, localized outside of organoid, calcium imaging was performed on intact 3D-suspension-cultured organoids using Fluo-4 AM calcium indicator dye. An
increase in cytosolic calcium was observed in response to
tastant sensing clearly (Fig. 3A). Similarly, these changes
in fluorescence intensity were measured in the cells, in
response to sucrose (30 mM), denatonium (20 mM), citric
acid (50 mM), and NaCl (200 mM) solutions (Fig. 3B, Sup-
plementary movies). These taste-evoked calcium activities
corresponded to the localization of taste receptor cells spe-
cifically. However, a low or no fluorescence intensity change
was observed in response to the MSG (300 mM) solution
(data not shown), possibly because the umami receptors have
not yet been well characterized [38]. Overall, these results
indicate that calcium imaging could be directly performed
on suspension-cultured taste bud organoids to evaluate the
response to tastants.

Recently, suspension-culture technique has been intro-
duced to organoid studies [18, 19]. To confirm that the
genetic screening and functional studies could be applic-
able to suspension-cultured organoid, similar to conven-
tional Matrigel-cultured organoid, genetic alterations
were performed during passaging and compared between
Matrigel-cultured and suspension-cultured taste bud orga-
noids. Pou2f3, a transcription factor required for the gen-
eration of Type II taste receptor cells [39], was chosen as
the target. Taste bud organoids with Pou2f3 gene knock-
out (KO) were generated by electroporation following the
methods used in a previous study [40]. Matrigel-embedded organoids were used as positive controls. The plasmids encoding Cas9, GFP, gRNA targeting Pou2f3, and a control plasmid expressing tdTomato were electroporated together. Cells in the experimental group (Cas9 plasmid + tdTomato control plasmid) were positive for both tdTomato and GFP, but only tdTomato fluorescence was observed in the control group (tdTomato control plasmid only) (Fig. 3C, C' and G, G'). To select transgenic organoids, a plasmid expressing gRNA targeting Tp53 (pCas9-gRNATp53-GFP) was electroporated together with pCas9-gRNAPou2f3-GFP, to allow the selection of transfected organoids by Nutlin-3 [41]. No notable morphological differences between the control and the Pou2f3 KO organoids were observed (Fig. 3D, D' and H, H'). Using Sanger sequencing, site mutations in the alleles of Pou2f3 in KO organoids were reported, which led to changes in the amino acid coding (Fig. S4). No difference in K8 localization was observed between the control and Pou2f3 KO organoids (Fig. 3E, E' and I, I'). However, the number of TRPM5-positive Type II taste receptor cells was dramatically reduced in Pou2f3 KO organoids compared to the control (Fig. 3F, F' and J, J'). Thus, genetic alterations were successfully explored in suspension-cultured taste bud organoids.

Transplanted suspension-cultured organoids integrate with host epithelium

To evaluate the engraftment capacity, taste bud organoids obtained from Rosa26-tdTomato-flox/+ were cultured for 7 days (diameter ~ 40 μm) and transplanted underneath the lingual epithelium of recipient mice (Fig. S6A–C). The recipient mouse tongues were harvested and analyzed at 8 weeks after transplantation. 8 weeks after transplantation, 8–15 engrafted organoids could be detected in the lingual epithelium of the recipient mice. Matrigel-embedded organoids were integrated into the lingual epithelium. Both tdTomato and K8-positive taste cells were detected; however, few PGP9.5-positive neuronal components were found surrounding the transplanted organoids (Fig. 4A–D). Suspension-cultured organoids also integrated with lingual epithelium formed a structure similar to that of fungiform papillae.
papillae (FFP), with a narrow opening stromal core structure (Fig. 4A′, E). tdTomato-positive cells were found surrounding the K8-positive taste receptor cell cluster (Fig. 4B′, C′). PGP9.5-positive neural components were detected in the mesenchyme and surrounding transplanted suspension-cultured organoids (Fig. 4D′). In the FFP, K8-positive cells in taste buds without tdTomato were observed (Fig. 4F, G). PGP9.5-positive neuronal cells were detected through the stromal core to the taste bud (Fig. 4H). Serial sections showed morphological similarities (taste bud-like structure) and differences (lack of stromal core structure) between the FFP and transplanted organoids (Fig. S5A–A″, B–B″, C–C″). The stromal core was not opened to the mesenchyme beneath the taste bud in the Matrigel-embedded taste bud organoid (arrowhead, Fig. S5A–A″). However, a narrow stromal core opening was observed after suspension-culture when compared to FFP (arrows, Fig. S5B–C″). Reduced innervation density was observed in transplanted Matrigel-embedded organoids compared to suspension-cultured organoids and FFP. This might be due to the expression levels of Bdnf and Nt-3 (Fig. S5D, E), which are known to support taste bud and non-taste epithelium innervation, respectively [42–44]. Transplanted tdTomato-positive suspension organoids were also harvested and analyzed at 4 weeks after transplantation. Integration with lingual epithelium and the existence of PGP9.5-positive neuronal components were observed in transplanted Matrigel-embedded organoids compared to suspension-cultured organoids (Fig. S6D–G). These results suggest that suspension-cultured organoids have the potential for engraftment and innervation following lingual transplantation.

Discussion

This study aimed to establish an enhanced taste bud organoid culture system with reversed apicobasal polarity that would result in a model that mimics the taste bud, including the differentiation of taste receptor cells and taste sensing capacity. After suspension-culture, the apicobasal polarity of the taste bud was altered. Compared to Matrigel-embedded organoids, suspension-cultured organoids showed advantages, such as taste receptor cell localization on the apical surface (outer region of taste bud organoid) and an increased number of stem/precursor and taste receptor cells (Fig. 2). The reversed apicobasal polarity by suspension-culture led to the absence of a keratinizing inner core. The morphology of suspension-cultured organoids improved the accessibility to the tastants from the outer environment compared to that of Matrigel-embedded organoids, due to apical localization of taste receptor cells, which further allowed for direct calcium imaging of taste bud organoids. The suspension-cultured organoids could also be genetically altered and transplanted for regenerative medicine studies (Figs. 3 and 4).

A recent study suggested that the removal of extracellular matrix components, such as Matrigel or basement membrane extract, lead to a polarity reversal in enteroids (organoids derived from primary gastrointestinal tissue). The enteroids with apical surfaces facing the medium (apical out) exhibited a functional absorption of nutrients and a barrier function toward pathogens, which were not applicable to enteroids whose apical surfaces faced inward (basal out) [18, 19]. Recent studies have generated apical-out lung organoids that help in the identification of novel cell populations for SARS-CoV-2 [45]. By applying a similar technique, we found a correlation between the reversal of apicobasal polarity and apical localization of taste receptor cells in suspension-cultured organoids, which strongly affected the functional potential of taste bud organoids. The reversal of apicobasal polarity may broaden the practical applications of taste bud organoids, similar to that in enteroids and lung organoids.

Achieving the correct proportion of various cell populations is essential for taste epithelium homeostasis [15]. The proportions of taste cells in suspension-cultured organoids had an improved similarity to those of taste buds than Matrigel-embedded taste cells. Moreover, the number of proliferating cells was higher in suspension-cultured organoids than in the Matrigel group. This difference might be due to the putative mitogenic effect of Shh-expressing cells [7, 46] and its interaction with Shh-responsive progenitor cells. However, these data represent a correlation between cell populations, rather than a functional consequence, such that the role of Shh in taste cell differentiation requires further study.

Taste bud cells are motile inside the taste bud organoid, which suggests the importance of cell motility during taste bud development [47]. The motility of developing taste bud cells has also been investigated in zebrafish larvae [14, 48], which further suggests that the displacement of progenitor and mature taste cells is essential for taste bud assembly, but the underlying mechanisms remain unknown [47, 48]. Slow-cycling progenitor cells migrate toward the budding region and differentiate into fast-cycling progenitors in taste bud organoids [14]. The localization of p63-positive basal cells and Ki67-positive progenitor cells changed after suspension-culture, which might alter cell motility in suspension-cultured organoids (Figs. 1E, E′, 2C, and E). The association between taste cell motility and taste cell differentiation has been investigated in zebrafish larvae; however, evidence linking these phenomena to mammals has not yet been reported [47]. It is possible that taste receptor cells in Matrigel-embedded organoids have limited cell motility owing to the internal accumulation of the keratin matrix. Suspension-cultured taste bud organoids may thus eliminate this barrier and resemble the motility of epithelial cells.
in vivo. Whether and how epithelial cell motility is altered in suspension-cultured organoids, requires further analysis.

Overall, suspension-cultured taste bud organoids could be an accessible model for taste bud studies. This induced organoid showed the advancement of practical uses, including calcium imaging analysis, genetic screening, regenerative medicine, and the recapitulation of taste epithelium homeostasis. Previous studies on calcium imaging for tastant detection have been performed on 2D-cultured cells derived from the taste bud organoid [22]. The taste receptor cells were inaccessible in the conventional organoids due to the inward localization of the taste receptor cells within the organoid, which did not contact the medium. Hence, calcium imaging of the entire organoid was not possible. Specific taste receptor cells responded to a particular tastant in the organoid, except for sour tastants (Fig. 3B). Similar observations were made in previous studies, which showed that sour taste is achieved by the non-calcium pathway [22]. Hence, most of the cells showed a change in fluorescence intensity upon the addition of a sour tastant. The suspension-cultured taste bud organoids with reversed apicobasal polarity allowed for monitoring of taste-evoked Ca\textsuperscript{2+} responses in the whole organoid.

Suspension-cultured organoids are susceptible to genetic alterations equivalent to those of Matrigel-embedded organoids. By targeting the Pou2f3 domain, we demonstrated that Type II taste receptor cells could be specifically knocked down (Fig. 3). Genetic alterations in organoids may make it possible to identify the cause of cancer initiation and progression by culturing cancerous cells [33]. The potential for genetic alteration as a treatment for lingual carcinoma and understanding the mechanism of its carcinogenesis could be further explored using suspension organoids.

Previous lingual organoid transplantation experiments showed an integration of the organoid within the recipient tongue; however, the organoid developed in the muscle layer and did not express taste receptor cells [33]. The transplantation of suspension-cultured organoids demonstrated the integration of the organoid with the lingual epithelial layer while maintaining taste receptor cells and nerve innervation. The clinical use of suspension-cultured organoids for the treatment of patience with taste loss due to cancer or injury requires further exploration.

Materials and methods

**Mice**

Adult mice were housed in a temperature-controlled room (22 °C) under artificial illumination on a regular 12-h day/night cycle and 55% relative humidity with access to food and water ad libitum. Mice used in the study were adult (6–8 weeks ± 5 days) male and female animals.

**Organoid matrigel culture**

Tongues from sacrificed adult mice were dissected and injected with ~0.5 mL of Dispase II (2.2 unit/mL, Roche) in PBS for 25 min at 37 °C. Tongue epithelium was detached off gently from the underlying tongue mesenchyme. The epithelium of circumvallate and foliate papillae were dissected and incubated with TrypLE Express for 30 min at 37 °C and centrifuged at 800 rpm for 20 min. The cell pellet was resuspended in Matrigel and seeded onto 24-well culture plates (50 μL Matrigel). Matrigel was allowed to polymerize for at least 10 min at 37 °C. Taste culture medium based on DMEM/F12 supplemented with, N2 (1%), B27 [2% (vol/vol)], R-spondin-1 (200 ng/mL,), Noggin (100 ng/mL), Jagged-1 (1 μM), Y27632 (10 μM), N-acetylcysteine (1 mM), epidermal growth factor (50 ng/mL) was added to the plate. Growth media was changed every 3 days. For passage culture, organoids were transferred into TrypLE solution and dissociated into small pieces mechanically by fine glass pipette. The solution was then passed through cell strainer to obtain single cells. Cell were pelleted by centrifuging at 600×g for 5 min. The single cells were re-embedded into fresh Matrigel and plated in 24-well plates.

**Organoid suspension culture**

Single lingual epithelial cells were embedded and cultured in Matrigel for 4 days with growth media as mentioned above. Matrigel-embedded organoids were washed with ice-cold basal medium for breaking down the Matrigel by inverting and subsequently incubating on ice for 20 min. Organoids were gently centrifuged at 900 rpm for 5 min at 4 °C, the supernatant was removed carefully by Pasteur pipette without disturbing the pelleted organoids. Fresh ice-cold basal medium was added for washing. This process was repeated 3 times. The organoids were re-suspended in growth media and transferred to ultra-low attachment 96-well culture plates (Corning Costar). Suspended organoids were cultured at 37 °C with 5% CO\textsubscript{2}.

**In situ hybridization**

Whole-mount in situ hybridization was performed as previously described [49]. In brief, Organoids were washed and fixed in 4% paraformaldehyde (PFA), dehydrated in methanol and stored at –20 °C. On the day of the experiment, organoids were rehydrated and treated with proteinase K for permeabilization. Organoid were fixed in 0.25% glutaraldehyde in 4% PFA, and prehybridized in hybridization solution at 68 °C for 2 h. Hybridization was carried out by
incubating with Digoxigenin-labeled RNA probes overnight at 68–70 °C. Mouse complementary Shh/Gli1/Gli3/K5-inserted plasmids were used for synthesizing the RNA probes.

**Histology and immunofluorescence**

Organoids were fixed in 4% paraformaldehyde and processed until OCT using standard procedures. Sections (7 μm) were prepared for hematoxylin/eosin staining, and immunostaining. For immunostaining, the slides were boiled in citrate buffer (pH6.0). Blocking was done by 1% goat serum or 5% bovine serum albumin in PBS. The slides were incubated with antibodies against β-catenin (1:100), ZO-1 (1:100), EAAT1 (1:100), TRPM5 (1:200), SNAP25 (1:200), PGP9.5 (1:200), Filaggrin (1:100), Keratin 10 (1:100), Keratin 8 (1:100), RFP (1:200), BrdU (1:200), Ki67 (1:100), p63 (1:200) at 4 °C overnight. The following day slides were washed and sequentially incubated with a secondary antibody (1:200, Invitrogen, United States) and counterstained with DAPI to visualize nuclei. The sections were examined using confocal laser microscope (Leica DMi8).

**Organoid-forming efficiency assays and serial passaging**

Matrigel-embedded and suspension-cultured organoids were generated as described above. For organoid forming efficiency assay, organoids were dissociated into single cells and single cells were re-plated at a density of 25,000 cells in 50 μL of Matrigel droplet. One week after seeding organoids forming efficiency was determined by quantification of organoid number and size under the bright field microscope. Serial photos with different focusing level of whole Matrigel drops were analyzed using Fiji [50], size 25-infinite, circularity 0.5–1.0

**BrdU incorporation analysis**

Adult mice Circumvallate Papilla (CVP) injected with BrdU (Sigma) or organoids treated with BrdU in media were collected and processed for wax section. Anti-BrdU antibody and anti-K14/SHH/K8 antibody were used to perform double immunostaining for counting the progenitor cells, precursor cells and taste cells. Apical flat cells were categorized by their significant different morphology.

**Reverse transcription–polymerase chain reaction (RT–PCR) and real time–quantitative polymerase chain reaction (RT–qPCR)**

Total RNA was extracted from Matrigel-embedded organoids/suspension-cultured organoids/ CVP epithelium/ non-taste lingual epithelium using TRIzol (Thermo Fisher Scientific). cDNA was made from the mRNA of each type of tissue using Maxime RT PreMix according to manufacturer’s instructions (iNtRON Biotechnology). RT–PCR was run using specific primers which are intro-spanning. The amplicons were confirmed by electrophoresis and subsequent sequencing. RT–qPCR was performed using a StepOnePlus Real-Time PCR System (Applied BioSystems). The expression levels of each gene are expressed as normalized ratios against the B2m housekeeping gene.

**Generation of transgenic organoids**

Sequence of Pou2f3 gRNA targeting the exon of mouse Pou2f3 gene were designed using the online tool [http://zlab.bio/guide-design-resources](http://zlab.bio/guide-design-resources) (Pou2f3 #1 to #4). Pou2f3-targeting gRNAs were cloned into the pSpCas9-2A-GFP vector (Addgene plasmid #48138), as described in original article [51]. For the selection by TP53, the pSpCas9-Tp53-2A-GFP plasmid was used. Plasmid DNA was prepared using the Maxiprep plasmid extraction kit (QIAGEN) and diluted in RNAse-free water at 2–4 μg/μL. Plasmid DNA was mixed with 0.05% FastGreen dye for visualization during application. pSpCas9-Pou2f3-2A-GFP and pSpCas9-Tp53-2A-GFP were co-applied. Organoids were briefly dissociated into small pieces and washed with Opti-MEM and basal medium sequentially. A BTX ECM630 electroporation system (Fisher Scientific) and a pair of L-shaped electrodes with gold tips (Fisher Scientific) were used for electroporation. Plasmids–dye mixture was added into the mixture of BTXpress electroporation buffer and dissociated organoids (50 μL). After electroporation (Poring Pulse: 50 V, 10 ms ON, 50 ms OFF, Pulse Number = 4, Transfer Pulse: Poring Pulse: 20 V, 50 ms ON, 50 ms OFF, Pulse Number = 5), extra 450 μL electroporation buffer was added into the mixture and incubated on ice for 30 min. The dissociated organoids were pelleted down and embedded into Matrigel for culturing. The culture medium was supplemented with Nutlin-3, 10 mM (Cayman Chemicals) for the selection of mutated cells (only Tp53 mutant cells can grow). Organoids were derived from the mutated cells were expanded in culture. sgRNA transfection and subsequent selection were performed at least in three different batches and using four different sgRNA.
Genotyping of organoids

For genotyping the mutant lines, organoids were lysed and its genomic DNA was isolated with DNAeasy Blood & Tissue kits (Qiagen) according to the manufacturer’s instructions. Primers for Tp53 and Pou2f3 were used for PCR amplification of the targeted region. The amplified PCR products were cloned into pGEM-T Vector (Promega). Plasmid DNA was extracted from the growing of 20 colonies for the sequencing of both genes.

Calcium imaging

Calcium imaging experiments were conducted with methods previously described [22] in the confocal dish (SPL Life Sciences). Cultured organoids (12–14 days) were transferred to the confocal dish and allowed to attach for up to 2 days in the same culture media. Before calcium imaging, the organoids were washed with HBSS buffer containing Ca²⁺ and Mg²⁺ ions (Gibco). The organoids were then loaded with 5 µM Fluo-4, AM (Invitrogen) in HBSS buffer containing 0.01% pluronic F-127 (Invitrogen) and incubated at 37 °C for 30 min. Organoids were washed with HBSS buffer containing 2.5 mM probenecid (Invitrogen) and further incubated for 30 min at 37 °C. Images were recorded using Leica DMi8 confocal microscope. The imaging data were analyzed using the Leica LAS X software. Values of intensity change are presented (n > 10).

In vivo transplantation assay and tissue processing

Suspension-cultured organoids derived from Rosa26-tdTomato^flox/+ mice were collected for transplantation 4 days after culture. Organoids were washed by ice-cold basal medium and 50 μL of medium containing 25–50 organoids were injected underneath the lingual epithelium (beneath the epithelium of lateral border) of the recipient mice. Engrafts were detected 4 weeks and 8 weeks after transplantation. Animals were sacrificed and tongues were collected, fixed and embedded in paraffin blocks for sectioning. 7 μm-thick sections were processed for HE staining and immunofluorescence using anti-tdToamto, anti-K8 and anti-PGP9.5 antibodies. Images of immunofluorescence were acquired using Leica DMi8.

Adenovirus infection of culture organoids

CVP and FOP epithelium of Rosa26-tdTomato^flox/+ mice, were dissociated into single cells and were mixed with adenovirus that shuttled pAAV.CMV.HI.eGFP-Cre.WPRE. SV40 (Addgene #105545-AAV2) in 100 μL organoid culture medium [52]. With gentle agitation every 10 min, the mixture was incubated in a 37 °C 5% CO₂ incubator for 60 min. The infected cells were centrifuged at 800 rpm for 30 min at 4 °C, mixed with 70 μL ice-chilled Matrigel and plated onto 24-well plates. The infection efficiency was confirmed by green and red fluorescence by CQ1 fluorescent microscopy (YOKOGAWA).

Single-cell RNA-seq library preparation and sequencing

The 10× Genomics Chromium platform was used to capture and barcode the cells to generate single-cell Gel Beads-in-Emulsion (GEMs) by following the manufacturer’s protocol. Briefly, along with the reverse transcription master mix, cell suspensions were loaded onto 10× Genomics Single Cell 30 Chips. During this step, cells were partitioned into the GEMs along with gel beads coated with oligonucleotides. These oligonucleotides enable mRNA capture inside the droplets by 30 bp oligo-dT after cell lysis and provide barcodes to index cells (16 bp) as well as transcripts (12 bp UMI). Following reverse transcription, cDNAs with both barcodes were amplified, and a library was constructed using the Single Cell 3’ Reagent Kit (v3.1 chemistry) for each sample. The resulting libraries were sequenced on an Illumina NovaSeq 6000 System in a 2× 150 bp paired-end mode.

Sample demultiplexing, barcode processing and UMI counting

Sample demultiplexing; barcode processing and UMI counting were performed using the official 10× Genomics pipeline Cell Ranger (v3.1.0) (https://support.10xgenomics.com). Briefly, raw base call files generated by Illumina sequencers were demultiplexed into reads in FASTQ format using the bc12fastq developed by Illumina (https://github.com/brownjc/bc12fastq). The raw reads were trimmed from the 3’ end to get the recommended number of cycles for read pairs (Read1: 28 bp; Read2: 90 bp). The reads of each library were then processed separately using the “cellranger count” pipeline to generate a gene-barcode matrix for each library. During this step, the reads were aligned to a mouse reference genome. Cell barcodes and UMIs associated with the aligned reads were subjected to correction and filtering. The statistical analysis and visualization of scRNA-seq data was performed by NGeneS Inc. (Ansan-si, Republic of Korea) using Seurat v3 (Cluster and UMAP) in R and R Studio environment.

Statistical analysis

The graphic results were expressed as the mean ± standard deviation (SD). A GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyze the data. Comparison of multiple groups was performed by one-way
ANOVA followed by Tukey’s multiple comparisons test. A p value < 0.05 was considered significant.

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Author contributions AAA and SZ designed and performed the experiments. HYK, KWK, and SJM provided expertise and feedback. JML and HSJ conceived experiments and coordinated the entire project. All authors read and approved the manuscript.

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Data availability All mouse organoids scRNA-seq data have been deposited at the Gene Expression Omnibus (GEO) under the accession number GSE191169.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval All experiments were performed according to the guidelines of the Intramural Animal Use and Care Committee of the College of Dentistry, Yonsei University (2019-0312).

Consent to participate No human subjects were used in this study.

Consent to publish No human subjects were used in this study.

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