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# Establishment of a Novel Lingual Organoid Culture System: Generation of Organoids Having Mature Keratinized Epithelium from Adult Epithelial Stem Cells

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Despite the strong need for the establishment of a lingual epithelial cell culture system, a simple and convenient culture method has not yet been established. Here, we report the establishment of a novel lingual epithelium organoid culture system using a three-dimensional matrix and growth factors. Histological analyses showed that the generated organoids had both a stratified squamous epithelial cell layer and a stratum corneum. Very recently, we showed via a multicolor lineage tracing method that Bmi1-positive stem cells exist at the base of the epithelial basal layer in the interpapillary pit. Using our new culture system, we found that organoids could be generated by single Bmi1-positive stem cells and that in the established organoids, multiple Bmi1-positive stem cells were generated at the outermost layer. Moreover, we observed that organoids harvested at an early point in culture could be engrafted and mature in the tongue of recipient mice and that the organoids generated from carcinogen-treated mice had an abnormal morphology. Thus, this culture system presents valuable settings for studying not only the regulatory mechanisms of lingual epithelium but also lingual regeneration and carcinogenesis.

Lingual dorsal epithelium contains 4 kinds of papillae: filiform, fungiform, foliate, and circumvallate papillae. Only 1 foliate papilla and approximately 10 circumvallate papillae have been observed in the posterior area of the tongue in mice. Recent analysis revealed that 200–400 filiform papillae and approximately 100 fungiform papillae reside in the anterior area of the mouse tongue<sup>1</sup>. Stratum corneum are seen in filiform papillae, but not in fungiform, foliate, and circumvallate papillae. In contrast, gustatory buds are seen in fungiform, foliate, and circumvallate papillae but not in filiform papillae. Although there have been many reports on culturing taste buds, the culture of lingual epithelium has not been well studied. Short-term (2–3 day) organ culture systems of embryonic (13–14 days of gestation) rat tongues have been established, and morphological development of papillae in such cultures have been reported<sup>2,3</sup>. With respect to adult lingual epithelial cell cultures, mouse lingual epithelial cells (LECs) can undergo expansion in the presence of an extracellular matrix (composed of collagen-Matrigel<sup>4</sup> or collagen-fibroblastic cell-matrix<sup>5</sup>) that offers a suitable environment for LECs. Ookura *et al.* cultured integrin  $\beta$ 1-positive LECs on a collagen-Matrigel-coated dish in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2), and then established a cell line (KT-1) with epithelial morphology<sup>4</sup>. However, the ability of KT-1 cells to generate a stratified keratinized epithelial layer was not examined in that report<sup>4</sup>. Luo *et al.* reported that keratin 5-positive cells obtained from the lingual epithelial layer could generate a multilayered squamous keratinized epithelium when these cells were cultured on a collagen-fibroblastic cell-matrix in the presence of EGF and FGF<sup>5</sup>. Thus, both systems need a specific cell separation procedure (namely integrin  $\beta$ 1-positive<sup>4</sup> or keratin 5-positive<sup>5</sup> cell separation) to induce sufficient epithelial cell growth, and the epithelial colony-forming ability of keratin 5-positive cells is very low (0.78%)<sup>5</sup>. It would be useful and convenient if multilayered keratinized epithelium could be generated at a higher efficiency *in vitro* from unseparated (whole) lingual epithelial cell populations containing lingual epithelial stem cells (LESCs).



LESCs are thought to be located in the basal layer of the lingual epithelium. Indeed, our recent study using *in situ* hybridization against *Bmi1* RNA showed that B cell-specific Moloney murine leukemia virus integration site 1 (*Bmi1*)-positive cells reside in the basal layer of lingual epithelium at a constant distance from each other (one *Bmi1*-positive cell per interpapillary pit)<sup>6</sup>. Moreover, multicolor lineage tracing methods using *Bmi1-CreER*; *Rosa26*-rainbow mice<sup>7,8</sup> clearly showed that *Bmi1*-positive stem cells are unipotent stem cells that maintain the epithelial cell layer but not the taste buds<sup>6</sup>. *Bmi1* belongs to the polycomb group family and is considered to play an important role in self-renewal and maintenance of adult stem cells<sup>9,10</sup>. Therefore, it is conceivable that the *Bmi1*-positive cells are one of LESCs and that the expression of *Bmi1* is a novel characteristic of LESCs.

In the present study, we attempt to establish a new lingual epithelial cell culture system using a three-dimensional matrix, EGF, the Wnt signaling-activator R-spondin1, and the TGF- $\beta$  signaling-inhibitor noggin. Three-dimensional culture is currently considered to be one of the best culture techniques for reproducing the physiological environment in which cells reside *in vivo*. This technique has been of great advantage for the culture of cells from various sources, including the oral mucosa<sup>11</sup>, the small intestine<sup>12</sup>, and the colon<sup>13</sup>. The 3 cytokines were selected for the following reasons: EGF is a cytokine commonly used in previously reported adult lingual epithelial cell cultures<sup>4,5</sup>. Noggin has been reported to increase the number of fungiform papillae in embryonic rat tongue organ culture<sup>3</sup>. R-spondin1 was used for the present culture because the Wnt/ $\beta$ -catenin signaling pathway has been shown to play an essential role in the adult mouse lingual epithelium<sup>14</sup>.

## Results

**Organoid formation from LECs.** LECs were collected from the C56BL/6 (B6) mouse tongue fragments by a two-step method; first, the epithelial cell layer was detached from the lamina propria mucosa using dispase<sup>5</sup>, and then LECs were released from the epithelial cell layer with a citrate-chelating buffer<sup>15</sup> that has a milder chelating activity than the standard chelating buffer, EDTA/PBS. LECs released into the buffer were collected as Fraction 1. The tongue fragments were then shaken in fresh buffer, and released cells were collected as Fraction 2. Further shaking did not release usable numbers of LECs. The cell number for Fraction 1 was  $1.68 \pm 0.43$  times higher than that of Fraction 2 (not significant), and  $(2.8 \pm 1.0) \times 10^4$  cells could be obtained from one tongue. Fig. 1a shows the representative morphological features of the cells in Fraction 1, with the cells displaying an epithelial cell appearance (being larger, with round-shaped nuclei and wide cytoplasm). There were no obvious morphological differences between the cell populations in Fractions 1 and 2.

The collected LECs were suspended in Matrigel and plated. Culture medium supplemented with EGF, noggin, and R-spondin1 was then added to the Matrigel. Organoid formation from the LECs was observed at a seeding efficiency of a single organoid from  $14.6 \pm 2.8$  LECs in Fraction 1. The seeding efficiency of LECs in Fraction 2 was slightly lower than that of LECs in Fraction 1 (not significant). Thus, Fraction 1 contained a higher number of LECs and showed a higher seeding efficiency than Fraction 2, and therefore Fraction 1 was used in each experiment in this study. Three different types of organoids were generated: round-shaped organoids with concentric cell arrangements (Fig. 1b), and rugged- and round-shaped organoids with a reticulated cell arrangement (Supplementary Fig. 1a).

**Morphological characterization of organoid cells.** Next, we attempted to morphologically characterize the organoid-constituting cells. After a 2-week culture, the organoids were harvested from the Matrigel using dispase. Using hematoxylin-eosin (HE) staining, we saw that round-shaped organoids with concentric cell arrangements

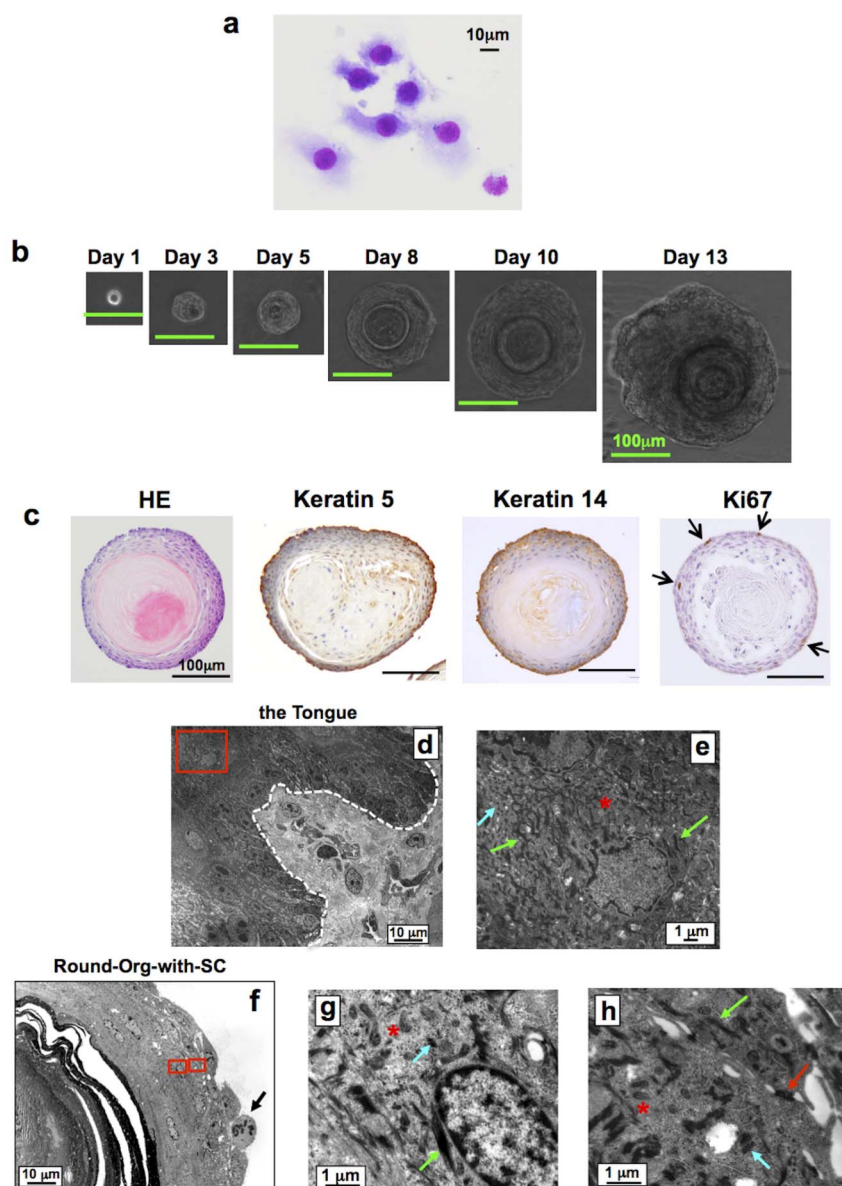
showed a stratum corneum (stained red with the eosin reagent; Fig. 1c); we called such organoids “round-Org-with-SCs.” Rugged-shape organoids (termed rugged-Org-with-CLs; Supplementary Fig. 1b) have a large central lumen containing dead cells with pycnosis, while round-shape organoids (termed round-Org-w/o-CLs; Supplementary Fig. 1b) did not have such a large lumen. Neither of the latter 2 kinds of organoids developed the stratum corneum.

As shown in Fig. 1c, the outer periphery of the round-Org-with-SCs was stained positively with anti-keratin 5 and keratin 14 antibodies, which are used to detect LESCs/progenitor cells<sup>5,16</sup>, indicating that LESCs/progenitor cells locate there and that differentiated cells migrate toward the center of organoids according to their maturation. Similar staining patterns with anti-keratin 5 and keratin 14 antibodies are seen in the rugged-Org-with-CLs and the round-Org-w/o-CLs (Supplementary Fig. 1b). As shown in Fig. 1c, several Ki67-positive cells (proliferating cells) were detected at a distance in the outer periphery of the organoids (indicated by arrows), and it is speculated that cell mitosis occurred mainly at these sites. No cells in any of the 3 different types of organoids were stained with either anti- $\alpha$ -amylase or anti-G $\alpha$ -gustducin (taste-cell-specific G protein) antibodies (data not shown), indicating that no salivary acinar cells or taste bud cells expanded in this culture system.

To further examine the organoid-constituting cells, transmission electron microscopic analysis was performed. Fig. 1d is a transmission electron micrograph of the tongue; basal and intermediate epithelium as well as lamina propria mucosa can be seen. Fig. 1e, which is a magnification of the areas surrounded by the squares in Fig. 1d, shows representative cells from the intermediate epithelium, in which many keratohyaline granules (blue arrow), keratin fibers (green arrow), and some lamellar granules (indicated by an asterisk) can be seen. The photograph of the round-Org-with-SC clearly shows the existence of a stratum corneum in the center and flattened cells surrounding the stratum corneum (Fig. 1f). On the outer side of the organoid, a cell with aggregated chromosomes (indicated by black arrow) was observed, suggesting that mitosis had been actively occurring at this site. Figures 1g, h are higher magnification photographs of the areas surrounded by the squares in Fig. 1f, and granules and fibers specific to cells in the intermediate epithelium were detected in both figures. In addition, the presence of desmosomes between the LECs was observed (red arrow; Fig. 1h). As shown in Supplementary Figs. 1c and d, such granules and fibers were also detected in the rugged-Org-with-CLs and the round-Org-w/o-CLs. The above results clearly show that the 3 types of organoids contain LECs belonging to the intermediate epithelium and indicate that these organoids are composed of LECs but not lingual salivary acinar cells.

The round-Org-with-SCs have a multilayer keratinized epithelium and a stratum corneum (Figs. 1c, f) and this morphology is characteristic of filiform papillae which are representative papillae in mouse tongues. Therefore, we focused the present study on the analysis of the round-Org-with-SCs.

**Cytokine requirement of organoid culture.** To investigate the cytokine dependency of organoid formation, LECs were cultured in the presence of different combinations of cytokines. As shown in Fig. 2a, only small organoids were seen on day 11 of culture in the cytokine combinations that did not contain EGF and in the culture without cytokines; however, thereafter these organoids began to enlarge (up to more than 100  $\mu$ m in diameter), and by day 23, a bias toward the generation of round-shape organoids was observed. In the EGF + noggin + R-spondin1 and EGF alone combinations, however, organoid growth began gradually to slow down after 11 days of culture, and active proliferation of stromal cells with fibroblastic cell morphology was frequently observed within the Matrigel and on the well surface (Fig. 2a). Similar observations were obtained for the EGF + noggin and EGF +



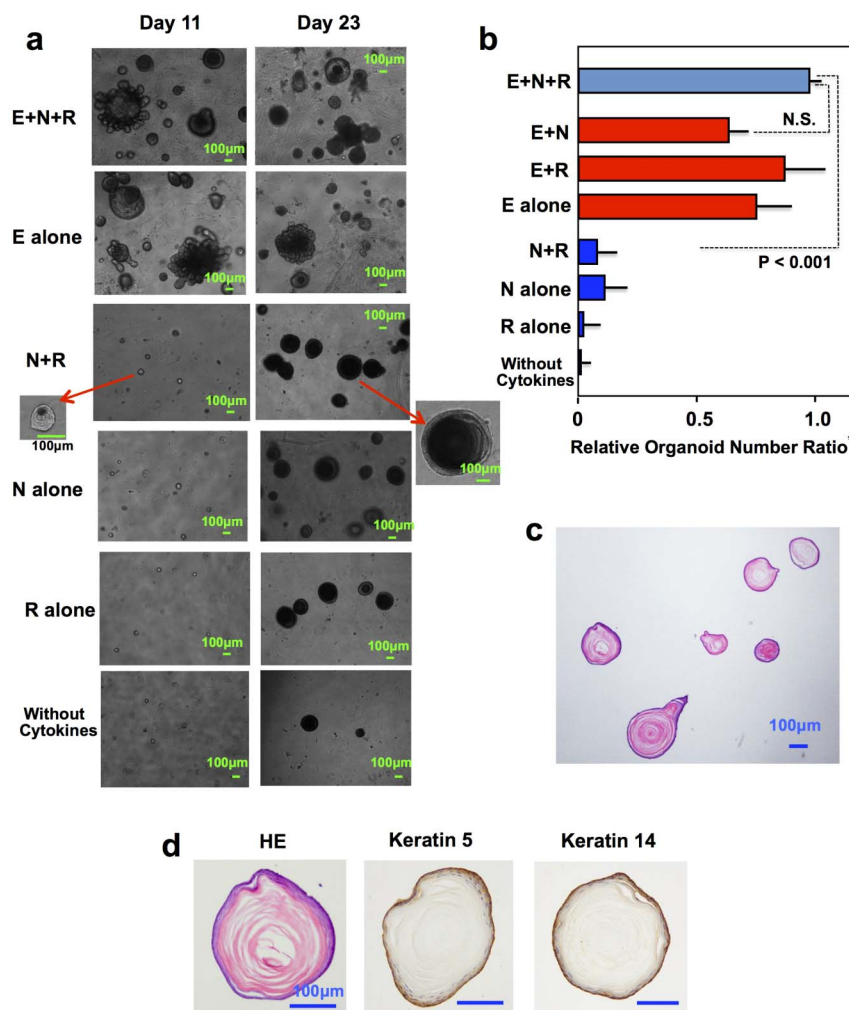
**Figure 1 | Morphological analysis of round-shape organoids with concentric cell arrangement.** (a) HE staining of separated LECs. (b) Time course of lingual organoid growth. LECs were cultured in the organoid culture system in the presence of EGF + noggin + R-spondin1 and round-shape organoids with concentric cell arrangement were generated. Phase-contrast image. (c) Histological analysis of organoids. Organoids were separated from Matrigel using dispase on day 14 of culture and their paraffin sections were stained with HE reagents. The organoid sections were also stained with anti-keratin 5 and keratin 14 antibodies. Positive cells were observed in the outer periphery of the organoids. Staining for Ki67 showed that some cells actively proliferated in the outer periphery (arrows). (d) Transmission electron micrograph of the normal tongue. The dotted line outlines the border between epithelium and lamina propria mucosa. Basal and intermediate epithelium (upper side of the dotted line) and lamina propria mucosa (lower side of the dotted line). (e) Higher magnification views of the boxed areas in (d). Many keratohyaline granules (blue arrow), keratin fibers (green arrow) and some lamellar granules (indicated by asterisk) were detected in the cytoplasm. (f) Transmission electron micrograph of organoids containing stratum corneum. Stratum corneum in the center and flattened cells surrounding the stratum corneum were observed. In the outer side of the organoid, a cell having aggregated chromosome (indicated by arrow) was detected. (g,h) Higher magnification views of the boxed areas in (f). Many keratohyaline granules (blue arrow), keratin fibers (green arrow) and lamellar granules (indicated by asterisk) were detected in the cytoplasm. Moreover, formation of desmosome between cells was observed (red arrow).

R-spondin1 combinations (data not shown). In contrast, stromal cell growth was infrequently detected in cytokine combinations that did not contain EGF and in the culture without cytokines (Fig. 2a). Fig. 2b shows the ratio of the number of organoids in each cytokine combination to the number of organoids in the standard EGF + noggin + R-spondin1 combination as determined on day 23. The organoid number was slightly lower in the EGF + noggin, EGF + R-spondin1, and EGF alone combinations than in the standard cytokine combination (not significant). On the other hand, a

significant reduction in organoid number was observed in those cytokine combinations that did not contain EGF as well as in the culture without cytokines.

In agreement with the above observations, HE staining of organoids collected from the noggin + R-spondin1 combination culture revealed that these culture conditions only produced organoids with a stratum corneum (a very thick stratum corneum and a very thin epithelial cell layer; Figs. 2c, d). Keratin 5 and keratin 14-positive cells were detected in the outer periphery of the organoids (Fig. 2d).





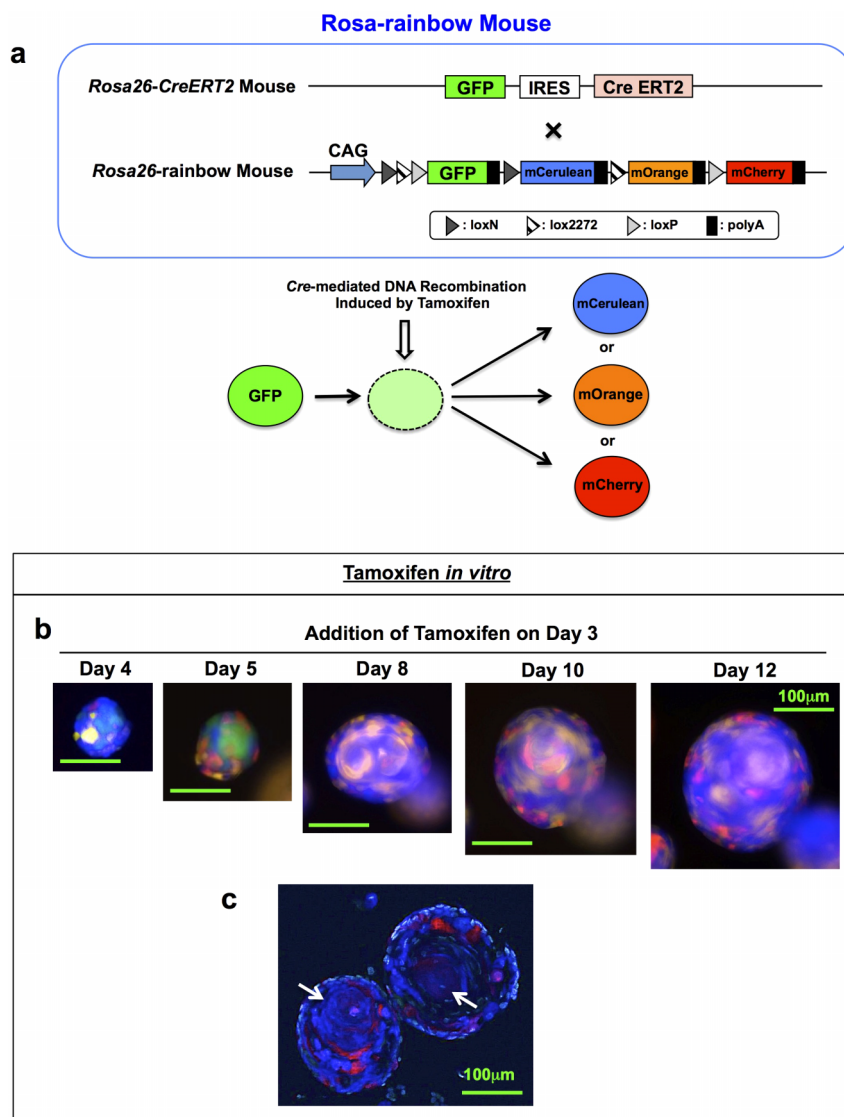
**Figure 2 | Characterization of organoids generated in different cytokine combinations.** (a) Skewed generation into the round-shape organoids in the cultures without EGF and in the culture without cytokine. LECs were cultured in the organoid culture system in the presence of various cytokine combinations or in the absence of cytokines. In the cultures with the cytokine combinations which did not contain EGF and in the culture without cytokines, organoids were very small (less than 50  $\mu\text{m}$ ) on day 11 of culture, but thereafter only round-shape organoids were generated. Phase-contrast image. (b) Comparison of organoid number in different cytokine combinations. The number of organoids was counted on day 23 of culture. (3 wells/sample). Mean  $\pm$  SD of 3 independent experiments. \*: Relative organoid number ratio = No. of organoids in culture of cytokine combination other than EGF + noggin + R-spondin1 or in culture without cytokines/No. of organoids in culture of the cytokine combination of EGF + noggin + R-spondin1. N.S.: not significant. E: EGF, N: noggin and R: R-spondin1. (c) HE staining of organoids obtained from the noggin + R-spondin1 combination. The formed organoids were separated from Matrigel on day 23 of culture and their frozen sections were stained with HE reagents. The organoids were composed of very thick stratum corneum and very thin epithelial layer. (d) Keratin 5 and keratin 14 staining of organoids obtained from the noggin + R-spondin1 combination. The cells in the outer periphery of the organoids were stained positively with anti-keratin 5 and 14 antibodies.

Similar results were obtained with organoids cultured with noggin alone, R-spondin1 alone, and in the absence of cytokines (data not shown).

Overall, these results indicate that EGF is important for generating the stratified epithelial layer and the stromal cell growth, but not essential for the generation of the stratum corneum.

**Multicolor tracing study using organoids derived from *Rosa26-CreERT2;Rosa26-rainbow* mice.** In recent years, lineage tracing methods based on Cre-mediated recombination have been developed. In *Rosa-CreERT2;Rosa26-rainbow* (*Rosa-rainbow*) mice, Cre-mediated excision of floxed cassettes by tamoxifen is induced in all the cells in the body (including tissue-specific stem cells) and thereby fluorescent colors of every cells change from green to different color (red-, orange- or blue-color) (Fig. 3a). As the progeny of the newly colored stem cells retain the changed color, it is possible to use this system to examine the fate and dynamics of individual stem

cells. Thus, even if specific markers for stem cells has not yet been found, we can examine the existence of stem cells and can trace the fate of individual stem cells using this system. We have recently used this system to trace the fate of individual LSCs *in vivo* and found that clonal expansion of single-color cells occurred in each interpapillary pit and each pit was finally occupied with LECs of a single color (red-, orange- or blue-color). This finding indicates that single LSCs in each interpapillary pit have an ability to constitute lingual epithelial layer in the pit<sup>6</sup>. In this study, we used this system to observe the organoid-forming process from individual labeled LSCs. LECs obtained from *Rosa-rainbow* mice were cultured in the organoid culture system (cytokine combination: EGF + noggin + R-spondin1) for 3 days. At this time point, lingual organoids composed of 50–100 cells had been generated, and then the active form of tamoxifen (4-hydroxytamoxifen) was added to the culture to induce Cre-mediated recombination in the organoid-constituting cells. As shown in Fig. 3b, individual cells



**Figure 3 | Observation of organoid-forming process using color-labeled cells.** (a) Schematic representations of gene constructs and Cre-mediated fluorescent color change in Rosa-rainbow mice. In Rosa-rainbow mice, fluorescent colors of individual cells change from green to different color (red-, orange- or blue-color) by Cre-mediated excision of floxed cassettes induced by tamoxifen and their descendant cells retain the changed color. (b) A representative growth pattern of organoid. LECs of Rosa-rainbow mice were cultured in the organoid culture system in the presence of EGF + noggin + R-spondin1 for 3 days and active form of tamoxifen was added to the culture. Individual cells were labeled by red-, orange- or blue-color after the Cre induction and the organoids showed mosaic patterns composed of the colored cells on day 5 of culture. Over time, the mosaic patterns disappeared and domains showing blue color expanded gradually and most parts of the organoids were occupied with blue color. Fluorescence image. (c) Frozen section of organoids collected on day 14 of culture. Most parts of the organoids were occupied with blue-colored cells. Stratum corneum is indicated by arrows. Nuclei were stained with Hoechst 33342. Fluorescence image.

begin to express different colors on day 4 of culture (1 day after the Cre induction), and organoids showing mosaic patterns were observed on day 5 of culture. Over time, the mosaic patterns disappeared and the blue-colored domains expanded gradually until most of the organoid cells were blue. This observation was confirmed by the analysis of frozen sections of the organoids harvested from the Matrigel on day 14 of culture (Fig. 3c), suggesting that a few LSCs selectively expanded in the organoids and that, as a result, clonal expansion occurred. The stratum corneum was observed in the center of the organoids (indicated by arrow), indicating that these organoids were round-Org-with-SCs.

**Multicolor tracing study of organoids derived from *Bmi1-CreER*; *Rosa26-rainbow* mice.** By lineage tracing methods using *Bmi1-CreER*; *Rosa26-rainbow* (*Bmi1-rainbow*) mice<sup>7,8</sup>, we recently showed that *Bmi1*-positive cells were able to generate lingual epithelium<sup>6</sup>.

Based on this finding, in this study we examined whether *Bmi1*-positive cells could generate organoids in our organoid culture system. Tamoxifen was injected into *Bmi1-rainbow* mice to induce fluorescent color change (from green to red, orange, or blue) in *Bmi1*-positive cells. LECs collected from the mice 2 days later were cultured in the organoid culture system. Fig. 4a shows the time course of formation of a representative blue-colored organoid. A phase-contrast image at day 10 shows that the organoid has a concentric cell arrangement and that this organoid is a round-Org-with-SC. On and after 17 days of culture, budding events were observed in the organoid, and its shape changed from round to rugged. As shown in Fig. 4b, all colored organoids were of a single color, and organoids containing more than one color were never detected. From this result, it is evident that each colored organoid was generated from a single *Bmi1*-positive cell. The frequency of occurrence of the color-changed round-Org-with-SC was less than

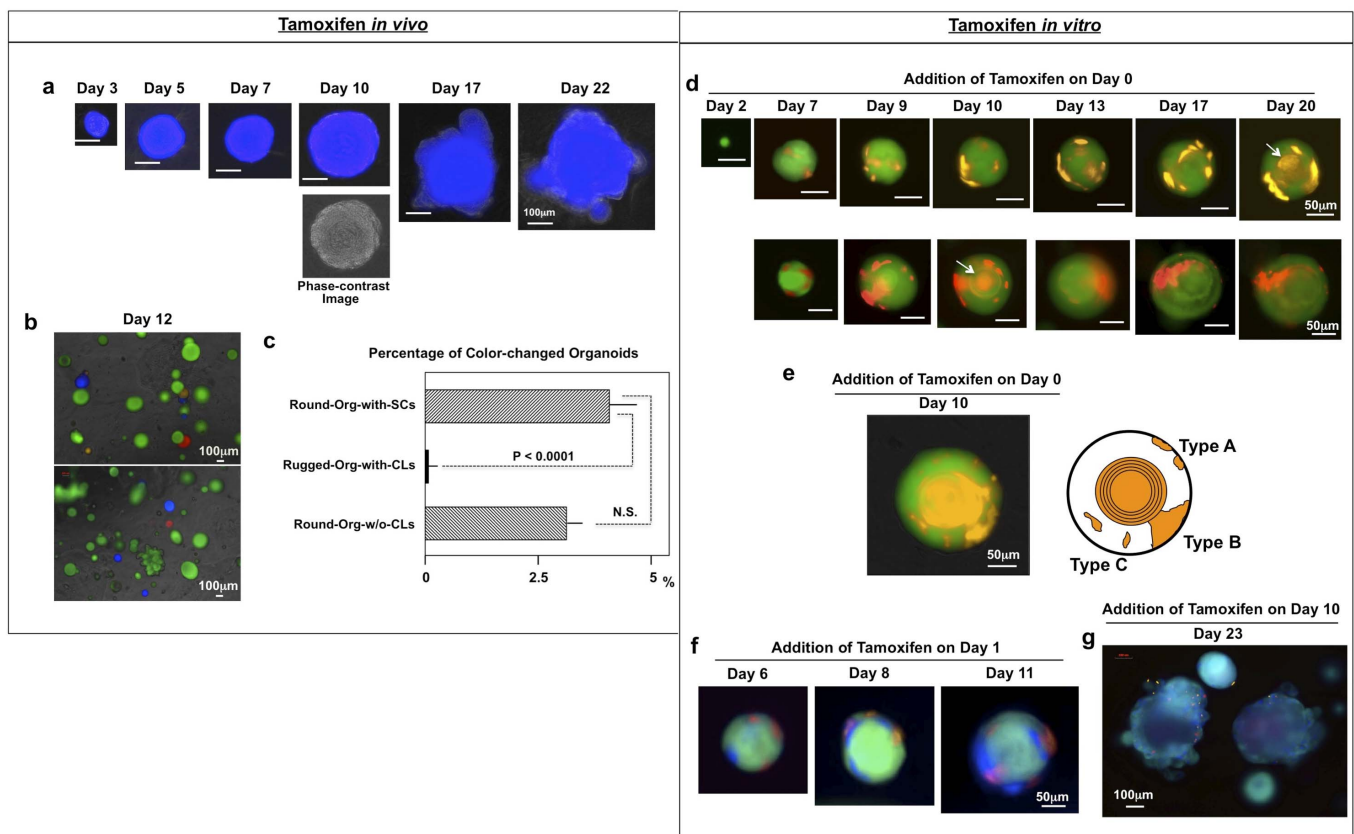


5% of all generated organoids (Fig. 4c). Interestingly, nearly all rugged-Org-with-CLs were green (Figs. 4b, c and Supplementary Fig. 2a), suggesting that those organoids were derived from Bmi1-negative cells. In contrast, color-changed round-Org-w/o-CLs were detected at a frequency of around 3% (Figs. 4b, c and Supplementary Fig. 2a). The above findings clearly show that Bmi1-positive cells have an ability to generate organoids. Accordingly, Bmi1-positive cells can be regarded as a type of LSCs.

Next, we added tamoxifen to the Bmi1-rainbow mouse organoid culture system at the beginning of culture to induce Cre-mediated recombination in the Bmi1-positive cells. Up to 6 days of culture, nearly all the generated organoids were green, and it was difficult to find organoids containing red, orange, or blue cells. However, on 7 days of culture, a few orange or red cells could be detected at the outer periphery of the green-colored organoids (Fig. 4d). Thereafter, the color-altered cells proliferated rapidly, and some of them spread toward the center of the organoids where they formed a stratum corneum (indicated by arrows), showing that these organoids are round-Org-with-SCs. It should be emphasized that no round-Org-with-SC had more than 1 color (other than green), suggesting that the number of Bmi1-positive cells was 1 per organoid at the time of

tamoxifen administration. The growth patterns of the Bmi1-positive cells in the round-Org-with-SCs could be classified roughly into 3 types (Fig. 4e): Bmi1-positive cells remaining on the outer periphery of the organoids (Type A); Bmi1-positive cells and/or their progeny spreading toward the center of the organoids and finally forming a stratum corneum (Type B); and Bmi1-positive cells and/or their progeny leaving the outer periphery of the organoids, migrating toward the center of the organoids, and remaining there (Type C).

Tamoxifen was administered *in vivo* at different time points: on the first day (Fig. 4f) or on day 10 of culture (Fig. 4g). In both these cases, round-Org-with-SCs with 2 or more different-colored cells were found. Such a phenomenon was also observed with round-Org-w/o-CLs; only blue cells were detected following the addition of tamoxifen at the beginning of culture, whereas both orange and blue cells were detected when it was added after the first day of culture (panels in the top and second rows of Supplementary Fig. 2b). In the rugged-Org-with-CLs, no colored cells were induced by tamoxifen on day 1, but many colored cells were induced following the addition of tamoxifen on day 10 (panels in the third and fourth of Supplementary Fig. 2b). Thus, the number of colored cells per each organoid became higher as the *in vitro* administration of tamoxifen



**Figure 4 | Capacity of Bmi1-positive cells to generate organoids.** (a–c) *in vivo* administration of tamoxifen. LECs obtained from Bmi1-rainbow mice that had been injected with tamoxifen 2 days before were cultured in the organoid culture system in the presence of EGF + noggin + R-spondin1. (a) Time course of organoid growth in a blue-colored round-Org-with-SC. Overlay of fluorescence image and phase-contrast image. (b) Representative photographs of organoids on day 12 of culture. Most organoids showed green color, but some round-shape organoids showed red-, orange- or blue-color. Overlay of fluorescence image and phase-contrast image. (c) Percentage of color-changed organoids in each type of organoids. The total number of generated organoids and the number of color-changed organoids were counted on day 12. N.S.: not significant. (d–g) *in vitro* administration of tamoxifen. Tamoxifen was added to the organoid culture medium of Bmi1-rainbow mice at the beginning of culture (d,e), on day 1 (f) or on day 10 of culture (g). Fluorescence image. (d) Time course of organoid growth in a round-Org-with-SC containing orange-colored or red-colored cells derived from Bmi1-positive cells. Fluorescence image indicates that these organoids had colored stratum corneum (indicated by arrows). (e) A representative round-Org-with-SC on day 10 of culture and a schematic representation of three different types of growth patterns of Bmi1-positive cells. (f) Time course of organoid growth in a round-Org-with-SC having two or more-colored cells. Addition of tamoxifen on day 1 of culture induced red-, orange- or blue-colored cells in a single organoid. (g) A representative photograph of round-Org-with-SC on day 23 of culture. Many colored cells were detected in each organoid, when tamoxifen was added on day 10.





was delayed. This observation indicates that the number of Bmi1-positive cells per each organoid gradually increased during culture period.

**Successful engraftment and maturation of organoids in the tongues of recipient mice.** Immature organoids from Bmi1-rainbow mice that had been cultured for only 3 days (Fig. 5a) were injected into the dorsal subepithelial area of the tongues of MHC-compatible B6 recipient mice (Fig. 5b). On the same day, tamoxifen was injected into the recipient mice. Within 1 week of grafting, the organoids had already formed stratified epithelial structures, and some organoids contain a stratum corneum (indicated by white arrows; Fig. 5c), implying that the engrafted organoids had been accepted, had maintained their proliferation potential in the environment of the recipient tongues, and had grown into mature organoids. In addition, some colored cells (Bmi1-positive cells) were detected in the organoids, and one organoid, seen in the lower right of the photograph, has a blue stratified epithelial cell layer (Fig. 5c). The formation of stratified epithelium by Bmi1-derived cells is more evident in the recipient tongues after 2 weeks, showing that red or blue cells migrated toward the center of organoids according to their maturation (Fig. 5d). As shown in Fig. 5e, the engrafted organoids that had developed the stratum corneum (indicated by arrows) had grown in the muscular layer but not in the epithelial layer, although we tried to inject the 3-day-cultured organoids into the subepithelial area, expecting that the expanded organoids would be incorporated into the epithelium. This is simply because it is technically difficult to inject the organoids into the subepithelial area (the thickness of the epithelium is less than 100  $\mu\text{m}$ ).

We also transplanted 1-week-cultured organoids from Bmi1-rainbow mice, and expansion and maturation of the organoids were observed in recipient tongues after 1 week (data not shown). However, the formation of stratified epithelium by Bmi1-derived cells was not clearly observed, suggesting that most Bmi1-positive cells had entered into a resting state during the 1-week culture period.

**Lingual organoid culture of carcinogen-treated mice.** The lingual organoid culture system will be very valuable if this culture system can be applied to the research on lingual carcinoma and can contribute to the understanding of the mechanism of carcinogenesis. It was reported that administration of 4-nitroquinoline 1-oxide (4-NQO) through the drinking water resulted in a high incidence of squamous cell carcinoma in mice<sup>17</sup>. The tongues of B6 mice treated with 4-NQO for 16 weeks (Fig. 6a) showed hyperplasia over the whole epithelium and carcinoma *in situ* in some parts of the epithelium (Fig. 6b). The number of LECs obtained from the tongues of the 4-NQO-treated mice was  $7.1 \pm 0.7$  times higher than that of LECs obtained from normal mice ( $p < 0.001$ , significant). These LECs made up a morphologically heterogeneous cell population (Fig. 6c). Neutrophils (indicated by arrows) were detected, suggesting that inflammation had occurred in the lingual epithelium. The seeding efficiency in organoid formation was one organoid from  $11.4 \pm 1.1$  LECs in the 4-NQO-treated mice but was not significantly different from that in normal mice. Abnormally shaped organoids (very large or slender organoids: indicated by arrows) were observed on day 18 of culture (Fig. 6d). Although the average diameter of the organoids in the NQO-treated mice did not differ significantly from that in normal mice (Fig. 6e), the range of diameters (126–704  $\mu\text{m}$ ) varied widely.

Organoids were harvested from Matrigel using dispase on day 18 of culture, and the frozen sections were stained with HE reagents. Organoids with abnormal round or slender shapes were detected (indicated by arrows; Fig. 6f). Ki67 staining of the frozen sections revealed that many proliferating cells were present in the middle epithelial layer (indicated by blue arrows) as well as in the outer periphery (indicated by black arrows) of the abnormally shaped organoid (Fig. 6g). In the organoids generated from normal mice

(Fig. 1c), Ki67-positive cells were detected only in the outer periphery of the organoids. Thus, structure disorder and accelerated proliferation were evident in the abnormal organoids.

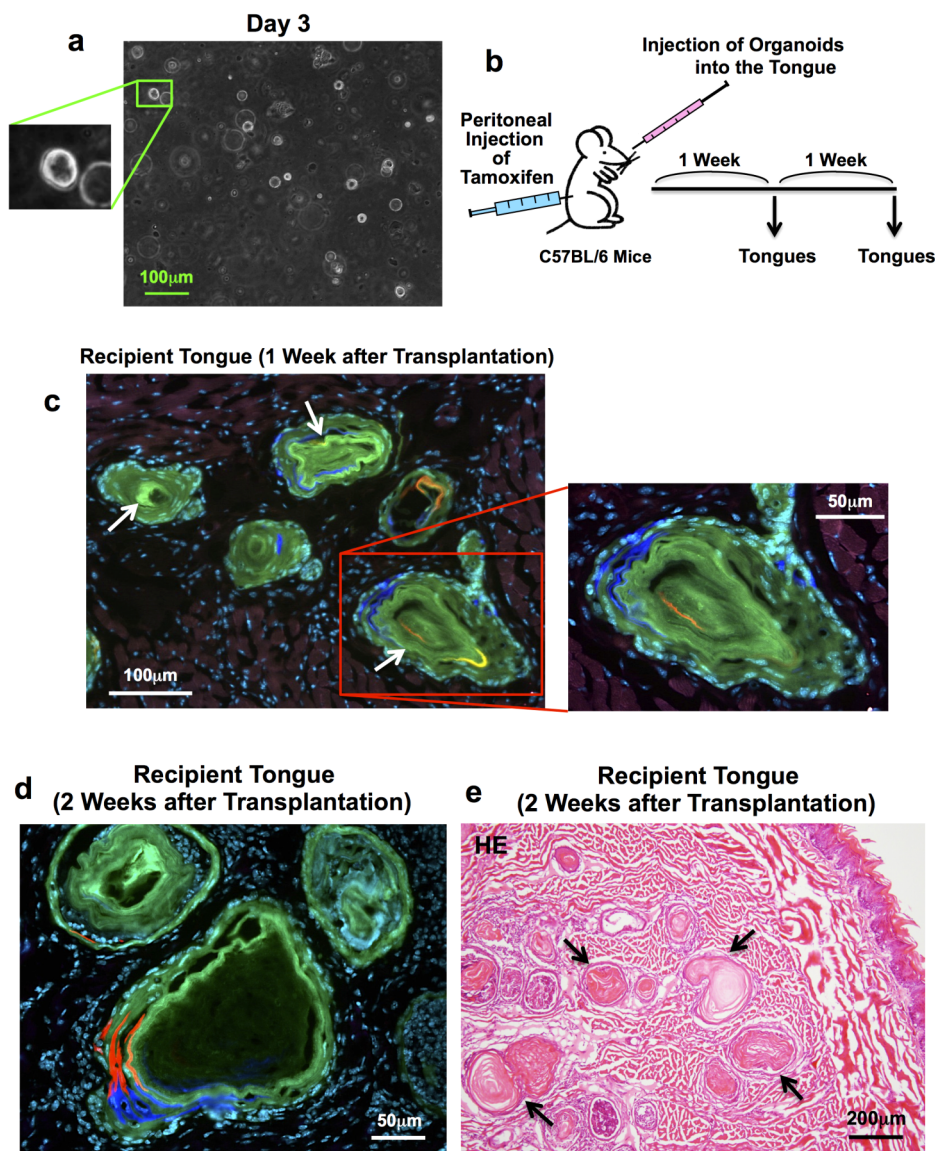
## Discussion

The main differences of this new culture system from previous lingual epithelial cell culture methods<sup>4,5</sup> are 1) the mild separation of LECs using dispase and citrate-chelating buffer, 2) the three-dimensional culture using Matrigel, and 3) the use of noggin and R-spondin1. In previous methods of lingual epithelial cell culture, 3 kinds of enzymes<sup>4</sup> (collagenase, esterase and dispase) or dispase plus EDTA/EGTA-chelating buffer<sup>5</sup> were used for the separation of LECs from mouse tongues. Although a high number of LECs ( $4.5 \times 10^5$  LECs per one tongue<sup>5</sup>) was obtained by these strong separation methods, additional separation process such as magnetic cell separation<sup>4</sup> or cell sorting<sup>5</sup> was also needed. As for the use of R-spondin1, which is an activator of Wnt signaling pathway, it is widely known that Wnt signals play an important role in the epithelial cell growth of the small intestine and the colon in mice and humans. Indeed, the addition of Wnt3a is necessary for the generation of mouse colonic organoids from single colonic stem cells<sup>13</sup>. In the organoid culture of mouse small intestinal crypts, Sato *et al.* showed that Wnt ligands were provided mainly from Paneth cells that were in contact with the ESCs<sup>12</sup>. It is currently not clear which kinds of cells produce Wnt ligands in our lingual organoid culture system. There is a possibility that LECs in the organoids themselves produce Wnt ligands via an autocrine mechanism. We have frequently observed the growth of stromal cells within the Matrigel and on the surface of the culture wells (Fig. 2a). Accordingly, the possibility that these cells provide Wnt ligands cannot be excluded.

In the present study, 3 different types of organoids were generated: round-Org-with-SCs (Fig. 1b), rugged-Org-with-CLs, and round-Org-w/o-CLs (Supplementary Fig. 1a). The latter 2 organoid types did not generate a stratum corneum, suggesting that these organoids had developed from LECs/progenitor cells from papillae other than filiform papillae. The relative proportion of organoids was skewed predominantly toward round-Org-with-SCs in the cytokine combinations not containing EGF and even in the culture without cytokines (Figs. 2a, c). This result shows that EGF plays an important role in epithelial cell growth, but is not necessarily essential for the generation of the stratum corneum.

In the organoid cultures from Bmi1-rainbow mice, the organoids following *in vivo* administration of tamoxifen (Figs. 4a, b) contained cells that were of a single color, but organoids from the *in vitro* administration (Fig. 4d) contained both green and red or orange cells. The organoid-forming process following *in vitro* administration might be explained as follows: Single Bmi1-positive cells divide asymmetrically and produce Bmi1-negative daughter cells before the induction of Cre-recombination by tamoxifen, even if tamoxifen is added at the beginning of culture. Thereafter, the induction of Cre-recombination occurs in Bmi1-positive cells but not in Bmi1-negative daughter cells. As a result, organoids having both green and color-changed cells are generated. A delayed addition (on day 10) of tamoxifen (Fig. 4g) induces the formation of organoids containing a higher number of colored cells in comparison with earlier addition (on day 1; Fig. 4f). This phenomenon may be explained by the following interpretation: Bmi1-positive cells are considered to be in a resting state based on a recent study from our group<sup>6</sup>. Organoids have a small number of Bmi1-positive cells at earlier culture times, because the organoids are growing rapidly. When the organoids reach a resting state later on in the culture, the rapid growth stops, and the number of Bmi1-positive cells increases. The addition of tamoxifen induces color change in each Bmi1-positive cell, resulting in the development of multicolored organoids.

Integrin  $\beta 1$ -positive<sup>4</sup> and keratin 5-positive<sup>5</sup> cells in the lingual basal layer have been proposed as candidates for LECs/progenitor



**Figure 5** | Expansion of engrafted organoids in the tongues of recipient mice. (a) Three-day-organoids generated from LECs of Bmi1-rainbow mice. Phase-contrast image shows that the organoids are 20–30  $\mu\text{m}$  in diameter and do not have stratum corneum showing that these organoids are immature. (b) Experimental protocol of organoid transplantation experiments. (c) A representative frozen section of the recipient tongue collected at 1 week after engrafting. Enlarged organoids were detected in the muscle layer. Stratum corneum is indicated by arrows. Nuclei were stained with Hoechst 33342. Fluorescence image. (d) A representative frozen section of the recipient tongue collected at 2 weeks after engrafting. Nuclei were stained with Hoechst 33342. Fluorescence image. (e) HE staining of paraffin-embedded sections of recipient tongues at 2 weeks after grafting.

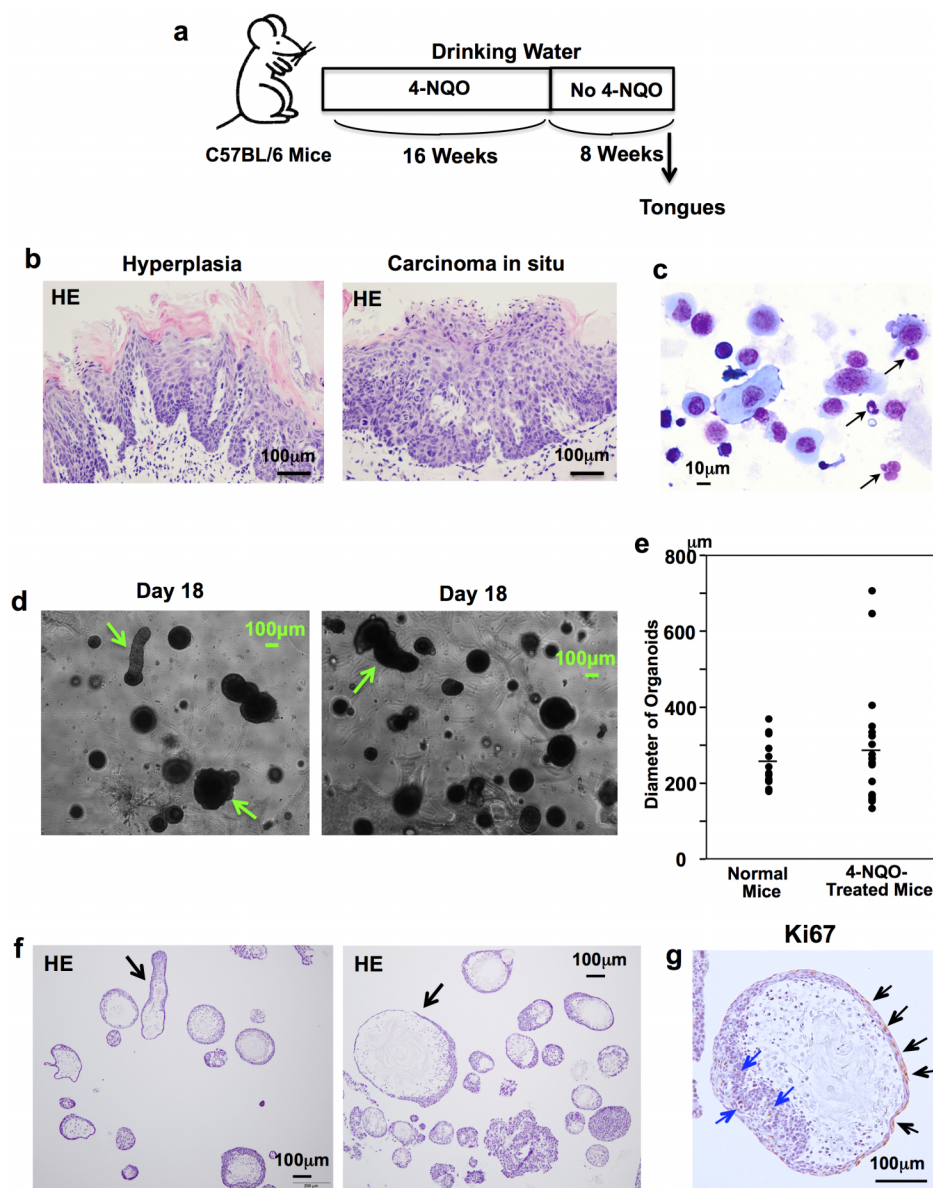
cells. In addition, Okubo *et al.* demonstrated that keratin 5-, keratin 14-, and Trp63-positive but Sox2-low positive basal cells are long-term progenitor cells that generate the keratinocyte lineage<sup>16</sup>. Our present study clearly shows that Bmi1-positive cells have the ability to generate organoids with a stratum corneum (Fig. 4a, b). This finding has confirmed our previous observations in a mouse tongue-based study<sup>6</sup>. Further investigation is required to identify actual LSCs, but our organoid culture system can contribute greatly to the identification of LSCs, because it is evident from this study that each organoid is generated from a single cell and that the differentiation of single LSCs into mature keratinized LECs is easily and efficiently induced in our culture system.

Surprisingly, the transplantation experiments with organoids (Fig. 5) showed that the grafted organoids expanded and matured in the muscular layer (but not in the epithelial layer) of the recipient tongues. Methodologies to inject organoids directly into the sub-epithelial area and induce proper rearrangement of the transplanted

organoids in the lingual epithelium need to be developed before considering the application of our culture system in lingual regenerative medicine to treat injury or following tumor excision. Recently, polyglycolic acid sheets and fibrin glue sprays have been used for covering open wounds after lingual tumor excision<sup>18</sup>. This technique represents an important advancement in the area of lingual regenerative medicine.

Ki67-positive cells were observed at constant distances in the outer periphery of organoids generated from normal mice (Fig. 1c), whereas many Ki67-positive cells were found in the middle epithelial layer of organoids generated from carcinogen-treated mice (Fig. 6g). In this way, the organoid culture system can elucidate the morphological and functional difference between normal and abnormal organoids. Moreover, there is a possibility that this culture system could contribute to screening of lingual anticancer drugs. Thus, our culture system provides a valuable strategy not only for the study of regulatory mechanisms of lingual epithelium but also for the study of





**Figure 6 | Lingual organoids generated from LECs of carcinogen-treated mice.** (a) Experimental protocol of 4-NQO administration. (b) HE staining of paraffin-embedded tongue sections obtained from carcinogen-treated mice. (c) HE staining of separated LECs. (d) Overview of generated organoids on day 18 of culture. Very large or slender organoids (indicated by arrows) were detected. Phase-contrast image. (e) Comparison of organoid diameters between normal mice and carcinogen-treated mice. There was no significant difference in organoid diameters between normal mice and carcinogen-treated mice. (f) HE staining of frozen sections of organoids. Organoids were separated from Matrigel using dispase on day 18 of culture and their frozen sections were stained with HE reagents. Abnormal shapes of organoids (indicated by arrow) were observed. (g) Ki67 staining of organoids. Frozen sections of the organoids were stained with anti-Ki67 antibody. Ki67-positive cells were detected in the middle epithelial layer (indicated by blue arrows) and in the outer side of the organoids (indicated by black arrows).

lingual reconstitution and lingual carcinogenesis in both mice and humans.

## Methods

**Mice.** Mice were bred and maintained at the Kansai Medical University Research Animal Facility in accordance with the Kansai Medical University guidelines. C57BL/6 (B6) mice (8–12-week-old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). *Bmi1*<sup>CreER/+</sup> (*Bmi1-CreER*), *Rosa26*<sup>CreERT2/+</sup> (*Rosa-CreERT2*), and *Rosa26*<sup>bw/+</sup> (*Rosa26-rainbow*) mice were purchased from the Jackson Laboratory (Sacramento, CA, USA) or generated as described previously<sup>4,5</sup>. Cre enzyme was induced to these F1 mice by intraperitoneal injection of tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil (Sigma) 2 days in advance; *Bmi1-CreER*;*Rosa26-rainbow* (*Bmi1-rainbow*) or *Rosa-CreERT2*;*Rosa26-rainbow* (*Rosa-rainbow*): 4 or 1 mg/10 g body weight, respectively. The experiments were approved in advance by the Kansai Medical University Welfare Committee.

**Separation of epithelial cells from mouse tongue.** The tongue taken from mice was cut into about 2 mm size fragments in cold PBS containing 0.5 mM dithiothreitol (DTT) and incubated in 50 units/ml dispase (BD Biosciences) for 60 min at 37°C. The tongue fragments were then twice washed with cold PBS and incubated in 10 ml of chelating buffer at 4°C with constant stirring for 10 min. The chelating buffer (pH 7.3) contained 27 mM trisodium citrate, 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 94 mM NaCl, 8 mM KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM KCl, 0.5 mM DTT, 55 mM D-sorbitol and 44 mM sucrose<sup>6</sup>. Cells released from the lingual fragments into the chelating buffer were collected as Fraction 1 after passing through a cell strainer (70 μm mesh size, #REF352350; BD Falcon). The tissue fragments were then transferred to 20 ml of fresh cold chelating buffer and vigorously shaken by hand (20 inversions). Cells released from the lingual fragments into the chelating buffer were collected as Fraction 2.

**Lingual organoid culture.** Collected lingual epithelial cells (LECs) were cultured in Matrigel (Becton Dickinson Biosciences, San Jose, CA), in the presence of cytokines.



Briefly, the LECs were suspended in Matrigel ( $0.5\text{--}1 \times 10^4$  cells/50  $\mu\text{l}$  of Matrigel) and plated 24-well plate (triplicate). The Matrigel was then added 0.75 ml of advanced DMED/F-12 medium supplemented with N-2, B-27, N-acetyl cysteine, Glutamax (Invitrogen) and cytokines (rmEGF: 50 ng/ml, rmnoggin: 100 ng/ml, rhR-spondin1-hFc: 1000 ng/ml). The EGF and noggin were purchased from Peprotech. The R-spondin1-hFc containing a C-terminal of human IgG was produced in our laboratory. The cDNA of rhR-spondin1 was kindly donated by Kyowa Hakko Kirin (Tokyo, Japan). Y-27632 (10  $\mu\text{M}$ , Sigma) was also added to the culture medium.

Every 2 or 4 days, all the culture medium in the wells was removed and fresh medium was added to the wells.

In some experiments, 4-hydroxytamoxifen (20 or 40 ng/ml) was added to the culture medium at the different time points to induce Cre enzyme. Sixteen hours later, the 4-hydroxytamoxifen in the culture medium was removed by replacement with fresh culture medium.

**Transmission electron microscopic examinations.** Organoids recovered from Matrigel using dispase (50 units/ml) were fixed with 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C for 2 hours, and then rinsed with 0.1 M phosphate buffer. The fixation of the organoids and the observation of the specimens under transmission electron microscope were processed in Applied Medical Research (Kobe, Japan).

**Histological examinations.** Organoids, recovered from Matrigel using dispase, were fixed with 4% paraformaldehyde overnight. Paraffin-embedded sections (4  $\mu\text{m}$ ) and frozen sections (7  $\mu\text{m}$ ) of the organoids were prepared and stained with Hematoxylin-eosin (HE) reagents or the following antibodies: rabbit anti-Ki67 monoclonal antibody (Dako), and rabbit anti-keratin 5, keratin 14 (Covance),  $\alpha$  amylase (Biomedica Corp. Foster City, CA) and  $G\alpha$ -gustducin (Santa Cruz) polyclonal antibodies. After being washed with PBS, they were incubated with peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience Inc., Tokyo, Japan) at room temperature for 30 min. They were visualized using metal-enhanced 3,3'-diaminobenzidine (DAB).

The frozen sections of organoids and recipient tongues were analyzed using an inverted fluorescent microscope (IX71, Olympus, Tokyo, Japan) after nuclei-staining by Hoechst 33342.

**Transplantation of organoids.** LECs obtained from Bmi1-rainbow mice were cultured in the organoid culture system in the presence of EGF + noggin + R-spondin1, and generated organoids were harvested from Matrigel using dispase on day 3 of culture. Recipient B6 mice were anesthetized with 2,2,2-tribromoethanol (Avertin, 0.4 mg/g body weight), and one thousand organoids in 20  $\mu\text{l}$  were injected into three different dorsal subepithelial area of the tongue using a Hamilton microsyringe. On the same day, tamoxifen (4 mg/10 g body weight) was injected intraperitoneally into the recipient mice. One or two weeks later, the tongues of the recipients were then removed and fixed. Frozen sections of the fixed tongues were examined histologically.

**Preparation of tongue carcinogenesis model mouse.** A carcinogen, 4-NQO (98% pure, #56-57-5, Wako Pure Chemical Ind., Osaka, Japan) was used to induce tongue tumors in this study. B6 mice were given drinking water containing 100  $\mu\text{g}/\text{ml}$  4-NQO for 16 weeks, and then given drinking water without 4-NQO for additional 8 weeks. The thus-treated mice were sacrificed at the 24th week after the start of the treatment. Their tongues were collected and LECs were cultured.

**Statistics.** Statistical differences were analyzed by the Student *t*-test. Reproducible results were obtained, and therefore representative sets of data are shown in the figures.

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## Author contributions

H.H. and H.U. contributed to the conception and design of the study, and to the analysis of data. H.H. performed the majority of the experiments. H.H. and H.U. wrote the manuscript. T.T. contributed to perform transplantation experiments of organoids. S.K., Y.T., Y.K., S.O., H.Y. and T.O. helped out with the experiments.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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