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Fluorescent labelling of intestinal epithelial cells reveals independent long-lived intestinal stem cells in a crypt

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ABSTRACT

Background and aims: The dynamics of intestinal stem cells are crucial for regulation of intestinal function and maintenance. Although crypt stem cells have been identified in the intestine by genetic marking methods, identification of plural crypt stem cells has not yet been achieved as they are visualised in the same colour.
Methods: Intestinal organoids were transferred into Matrigel® mixed with lentivirus encoding mCherry. The dynamics of mCherry-positive cells was analysed using time-lapse imaging, and the localisation of mCherry-positive cells was analysed using 3D immunofluorescence.
Results: We established an original method for the introduction of a transgene into an organoid generated from mouse small intestine that resulted in continuous fluorescence of the mCherry protein in a portion of organoid cells. Three-dimensional analysis using confocal microscopy showed a single mCherry-positive cell in an organoid crypt that had been cultured for >1 year, which suggested the presence of long-lived mCherry-positive and -negative stem cells in the same crypt. Moreover, a single mCherry-positive stem cell in a crypt gave rise to both crypt base columnar cells and transit amplifying cells. Each mCherry-positive and -negative cell contributed to the generation of organoids.
Conclusions: The use of our original lentiviral transgene system to mark individual organoid crypt stem cells showed that long-lived plural crypt stem cells might independently serve as intestinal epithelial cells, resulting in the formation of a completely functional villus.

1. Introduction

The intestinal environment varies according to the effects of different luminal substances, such as digested food and bacteria [1]. Therefore, to maintain its homeostasis, the intestine needs to adapt to the variable environment. Coordination of the various epithelial cells in each villus is critical for adapting to a variable environment. Intestinal stem cells at the base of crypts maintain individual villi by generating various types of epithelial cells over a lifetime [2]. It has been suggested that various cell signalling pathways [3] and transcription factors [4] destine epithelial cells to a specific cell lineage. However, the fate of intestinal stem cells themselves is not well understood. Previous reports have indicated that groups of stem cells are located in a crypt and act as reservoirs to continuously supply various types of cells to the villi over a lifetime [5]. Different subpopulations of intestinal stem cells, as defined by Lgr5 [6], Bmi1 [7], Lrig1 [8], mTert [9] and HopX [10] expression, have been demonstrated by genetic marking methods, a finding that has led to questions about which populations lie ancestrally upstream of others [11]. However, it has been reported that tamoxifen might ablate stem cells and induce regeneration of intestinal epithelial cells [11]. Further, a functional approach independent of stem cell markers has been reported. Continuous clonal labelling using the relative instability of dinucleotide repeat tracts during

Abbreviations: CBC, crypt base columnar; TA, transit amplifying; GFP, green fluorescence protein gene; Lgr5, leucine-rich-repeat-containing G-protein-coupled receptor 5; OLFM4, Olfactomedin 4.

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DNA replication has demonstrated both lower stem cell numbers per crypt and lower stem cell replacement rates [12]. These studies suggest that previous inferences of stem cell numbers and replacement rates derived from pulse-chase labelling may be overestimated. Thus, identification and tracking of individual stem cells in crypts would be helpful for understanding the dynamics of the fate of crypt stem cells. Most importantly, the Cre-loxP system is unable to detect individual stem cells in a crypt *in vivo* or *in vitro* because all previous stem cell markers are expressed in the plural cells at the base of a crypt [13].

In vitro organoid culture of intestinal epithelial cells while maintaining the crypt formation enables assessment of the dynamics of epithelial cells visually and sequentially [14,15]. However, it is difficult to directly introduce a transgene into the organoids for visualisation of a particular cell because of the matrix around the organoids. In this study, we applied an original approach independent of stem cell-specific markers to visualise stem cells in an organoid.

2. Materials and methods

2.1. Cell culture and chemicals

Human embryonic kidney-derived 293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin. Culture of the intestinal epithelium was performed as described previously [16]. Crypts of the proximal small intestine were obtained from adult heterozygous mice harbouring an Lgr5-EGFP-IRES-creERT2 knock-in allele and were purified. They were counted and embedded in Matrigel® (BD Biosciences, San Jose, CA) at 10,000 crypts/ml. For conventional culture, 30 μl of Matrigel® was seeded on 24-well plates. For live imaging experiments, 60 μl of Matrigel® was placed in 35-mm culture dishes. The medium was changed every 2 days. For cell passage, the medium was discarded and the Matrigel® was dissolved by the Cell Recovery Solution® (BD, Franklin Lakes, NJ, USA). The organoids were washed twice with phosphate buffer solution (PBS) and mechanically dissociated into crypt domains level by pipetting. Then transferred into fresh Matrigel® and organoid culture medium as above. The interval of cell passage was approximately once every week, with a 1:3 ratio for amplification. When necessary, Hoechst33342 (04915-81; Nacalai tesque) was added to the medium as indicated. Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2. Lentivirus infection to the organoids

Lentivirus production was performed according to the manufacturer's protocols. Lenti-virus supernatants were concentrated using Lenti-X™ (Clontech Laboratories, Inc.), leading to 100-folds increase in virus titer. Equal parts of the mixture of Matrigel® and virus solution and 293T cells were mixed together. After the mixture solidified, culture medium was overlaid. For the infection into the organoid, the medium was discarded and the Matrigel® was dissolved by the Cell Recovery Solution® (BD, Franklin Lakes, NJ, USA). The organoids were washed twice with PBS and mechanically dissociated into crypt domains level by pipetting. Then transferred into Matrigel® and mixed with equal parts of the lentivirus solution. The organoids in Matrigel® were divided into two wells in a 24-well plate. Organoid culture medium was overlaid after the Matrigel® solidified. The infection of the organoids was repeated three times during the passage of the organoids.

2.3. 3-Dimentional fluorescence analysis

Hoechst33342 was added to the medium for 10 min to detect the nuclei of organoids. After discarding the medium, 4% paraformaldehyde was added for 6 h to fix the organoids. Then the organoids in Matrigel® were put on a slide and mounted with VectaShield mounting medium (H-1000; Vector Laboratories, Burlingame, CA, USA). Whole organoids were visualised by confocal laser fluorescent microscopy FLUOVIEW FV10i (Olympus, Tokyo, Japan) to acquire high-resolution images of the specimens (optical section, 5 μm; *Z*-axis increment, 1 μm). Fluorescence from mCherry and Hoechst33342 was detected using filter sets for mCherry (excitation, 559 nm; emission, 610 nm) and Hoechst33342 (excitation, 405 nm; emission, 455 nm), respectively. A 3 dimensional (3D) picture was constructed from the sequential imaging of a whole organoid using FV10-ASW 3.1 software (Olympus).

2.4. Time-lapse live cell imaging

Live imaging was performed on the Delta Vision system (Applied Precision, Washington, USA) incorporating a fluorescent microscope IX-71 (Olympus) using a 20× 0.75NA UPlanSApo objective (Olympus). Fluorescence from mCherry was detected using filter sets for mCherry (excitation, 577/25; emission, 632/60). Time-lapse experiments were performed as previously described [16]. Differential interference contrast (DIC) and fluorescent images were acquired at 15-min intervals for 30 h or 45 h. The data were processed using softWoRx® (Applied Precision, Issaquah, WA) and, if necessary, image editing was performed using Adobe Photoshop CS5.1. Maximum intensity projections of the time series were exported into QuickTime format for presentation as Supplementary Movies.

3. Results

3.1. Lentivirus mixed into Matrigel enables direct infection of organoids

We first assessed the efficacy of the transgenic system that uses a lentivirus for gene transfer into an organoid. 293T cells were placed in a dish with or without Matrigel®, and lentiviruses coding for the green fluorescence protein (GFP) gene were added to the medium. The 293T cells in Matrigel® did not show green fluorescence, whereas 293T cells seeded on the dish showed fluorescence, which suggested that the lentiviruses were unable to pass through the Matrigel® (Fig. 1A). The lentiviruses were therefore mixed into the Matrigel® with 293T cells, which resulted in production of green fluorescence from the 293T cells in the Matrigel® (Fig. 1B). We used this method to introduce the mCherry fluorescence gene as a transgene into organoids generated from mouse small intestines. Although one-time introduction of the transgene did not lead to mCherry fluorescence even though mCherry gene expression was detected in the organoids (Supplementary Fig. 1), threefold repeated introduction of the transgene did lead to detection of mCherry fluorescence in the organoids (Fig. 1C). We isolated a single organoid with mCherry fluorescence by observation under a stereoscopic microscope (Fig. 1D); this single organoid was then mechanically divided into several pieces by using a needle to expand the organoids (Supplementary Fig. 2). After expansion, the divided organoids were removed from the Matrigel® and mechanically dissociated into the crypts by using a plastic pipette tip for continuous passage (Fig. 1E). Culture of these organoids for >1 year showed continuous mCherry fluorescence in every crypt of all organoids, which suggested that the mCherry gene had been transgenically introduced into the stem cells. Further analysis using confocal microscopy showed heterogeneous distribution of

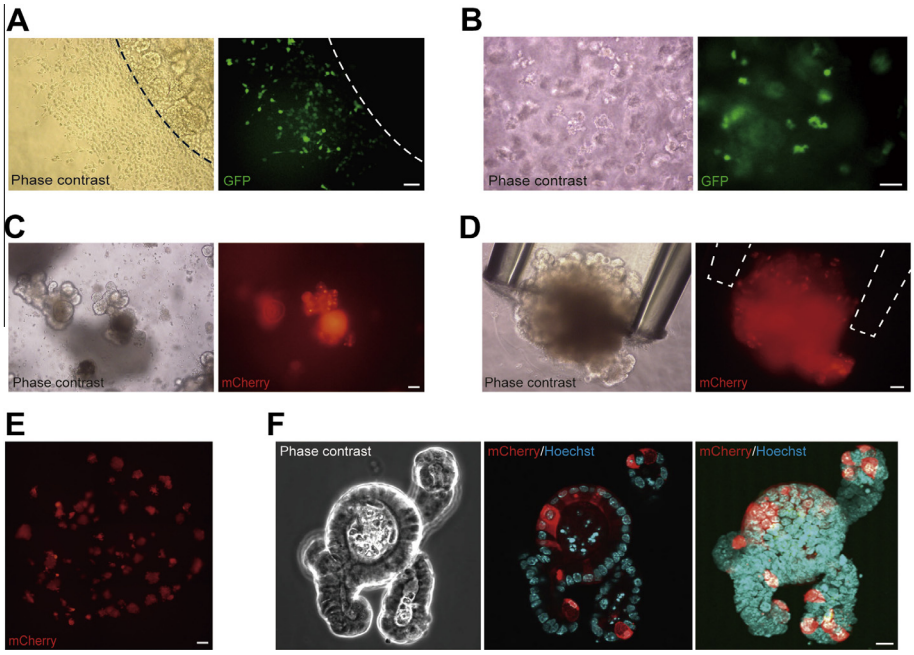

Fig. 1. Gene transduction into organoids resulted in the continuous fluorescence. (A) Lentiviruses coding for the green fluorescence protein gene were added to the medium. 293T cells mounted into the Matrigel® were not fluorescent (right side of each picture) whereas the 293T cells at the dish bottom were fluorescent (left side of each picture). (B) 293T cells mounted into the Matrigel® mixed with lentiviruses were fluorescent. (C) The organoids generated from small intestines were mounted into the Matrigel® mixed with lentivirus encoding the mCherry gene. The organoids were fluorescent after three times lentivirus infections. (D) A single organoid with mCherry fluorescence was collected into a plastic pipette tip. Scale bar: 50 μm. (E) mCherry fluorescent organoids expanded from a single organoids. Scale bar: 200 μm. (F) Heterogeneous expression of mCherry fluorescence in an organoid (mCherry-hetero organoid) over a year after the infection with lentiviruses. Left and centre panels are confocal imaging of an infected organoid. Right panel is 3-dimensional imaging of a whole organoid constructed by confocal microscopy. Scale bar: 10 μm.

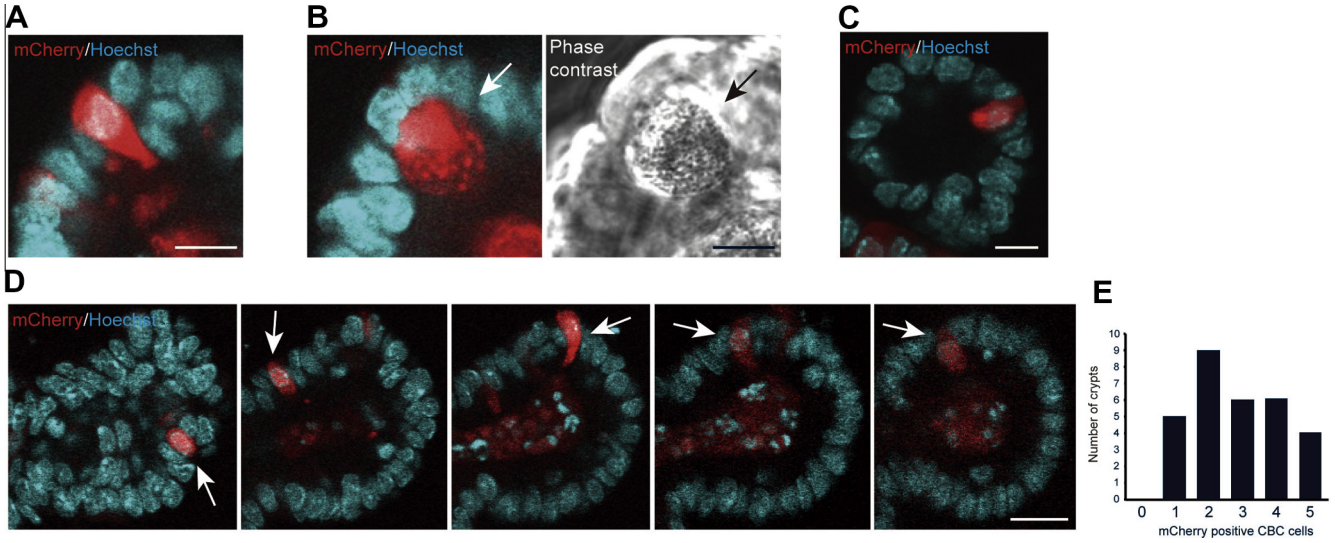

Fig. 2. Partial infection of the organoids enables the marking of individual stem cells in a crypt. (A) An mCherry positive crypt base columnar (CBC) cell in a crypt with a spindle shape. Green fluorescence protein fluorescence marking Lgr5 expression is lost after long-time culture. (B) An mCherry positive Paneth cell in a crypt with broad shape and granules. Scale bar: 5 μm. (C) Confocal imaging of a single mCherry-positive CBC cell in a crypt. 3D imaging of whole crypt is shown in Supplementary Movie 1. Scale bar: 10 μm. (D) Confocal imaging of five mCherry-positive CBC cells in a crypt. Sequential imaging of a whole crypt is shown in Supplementary Movie 2. Scale bar: 15 μm. (E) The number of mCherry-positive CBC cells in each 30 crypts of the mCherry-hetero organoids was counted. The maximum number of mCherry-positive CBC cells in a crypt was 5. No mCherry negative crypts were shown in all organoids.

the mCherry-positive cells in every crypt of all organoids (mCherry-hetero organoids) (Fig. 1F) (Supplementary Table 1).

3.2. Partial infection into the organoids allows the marking of individual stem cells

Because we could morphologically distinguish crypt base columnar (CBC) cell as a spindle shape cell from Paneth cell as a granular cell in the crypt (Fig. 2A and B), we counted the number of mCherry-positive CBC cells in the crypts that resulted in 1–5 mCherry-positive cells in each crypt (Fig. 2C–E, Supplementary Movies 1 and 2).

3.3. Fluorescent labelling of intestinal epithelial cells shows the different stem cells in a crypt

Our findings suggested that a single stem cell might be unable to occupy all intestinal cells in a crypt and villi. Staining of the

small intestinal stem cell specific marker, Olfactomedin 4 (OLFM4) [17] showed that OLFM4 was expressed in both mCherry-positive and -negative cells in the same crypt of mCherry-hetero organoids that have been cultured for >1 year (Fig. 3A). Moreover, HopX as a quiescent +4 stem cell marker and Musashi-1 as a CBC stem cell marker were also expressed in both mCherry-positive and -negative cells in the same crypt of mCherry-hetero organoids, suggesting that mCherry-positive and -negative cells might include stem cells (Fig. 3A). Paneth cells detected by lysozyme staining of a whole organoid were also derived from both mCherry-positive and -negative stem cells in the same crypt (Fig. 3B, Supplementary Movie 3). Time-lapse observation of the organoids in which only an mCherry-positive cell was located in a crypt revealed that a single mCherry-positive cell had divided into two cells. Finally, a single mCherry-positive cell supplied almost all CBC cells in a crypt in addition to transit amplifying (TA) cells (Fig. 3C, Supplementary Movies 4 and 5). However, the heterogeneity of mCherry expression has been maintained during continuous passages for >1 year,

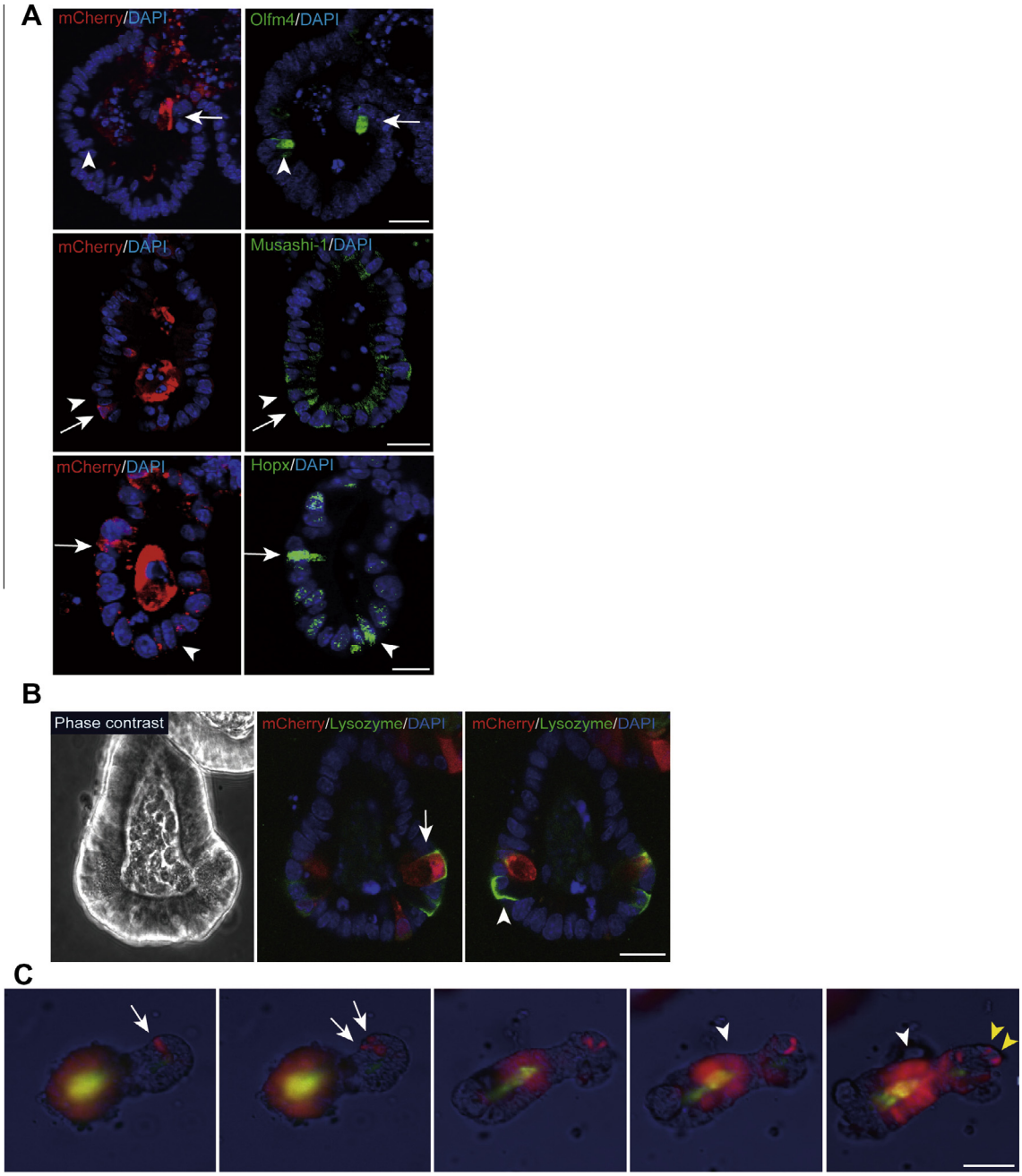

Fig. 3. Heterogeneity of intestinal stem cells in a crypt is maintained for over 1 year. (A) Immunofluorescent staining of mCherry and OLFM4, Musashi-1 and HopX (green) in serial sections of the mCherry-hetero organoid. OLFM4, Musashi-1 and HopX were expressed in both mCherry-positive (arrow) and -negative cells (arrow head) in the same crypt. Scale bar: 20 μm. (B) Immunofluorescent staining of mCherry and Lysozyme (green) in a whole mCherry-hetero organoid. Sequential confocal imaging shows both mCherry-positive (arrow) and -negative (arrow head) cells in a crypt. 3D imaging of a whole crypt is shown in Supplementary Movie 3. Scale bar: 20 μm. (C) Time lapse imaging of an organoid that has a single mCherry-positive cell in a crypt. 16 h 44 min later, a single mCherry-positive cell was divided into two cells (arrow). Following the first cell division, mCherry-positive cells supplied cells to the transit amplifying zone (white arrowhead) and crypt base (yellow arrowhead) for 45 h. Time lapse movies are shown in Supplementary Movie 4, 5. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which suggests that plural stem cells might fundamentally exist in a crypt to alternatively produce intestinal epithelial cells for the villi and crypt base.

3.4. Individual stem cells in a crypt can enable the generation of organoids

To characterise the mCherry-positive and -negative cells, we dissociated the mCherry-hetero organoids into individual cells (Fig. 4A). The organoids developed from a single cell showed that mCherry-positive and -negative organoids were individually generated (Fig. 4B). Each organoid was isolated under a stereoscopic microscope. Observation using confocal microscopy of whole organoids revealed that all cells expressed mCherry in the mCherry-positive organoids and that no cells expressed mCherry in the mCherry-negative organoids (Fig. 4C, Supplementary Movies 6–9). These findings indicate that both mCherry-positive and -negative cells in a crypt of the mCherry-hetero organoids have the capacity to generate organoids. Moreover, mCherry-negative organoids generated from mCherry-hetero organoids never became fluorescent again for >1 year, supporting the independency of mCherry-positive/negative stem cells in mCherry-hetero organoids. The

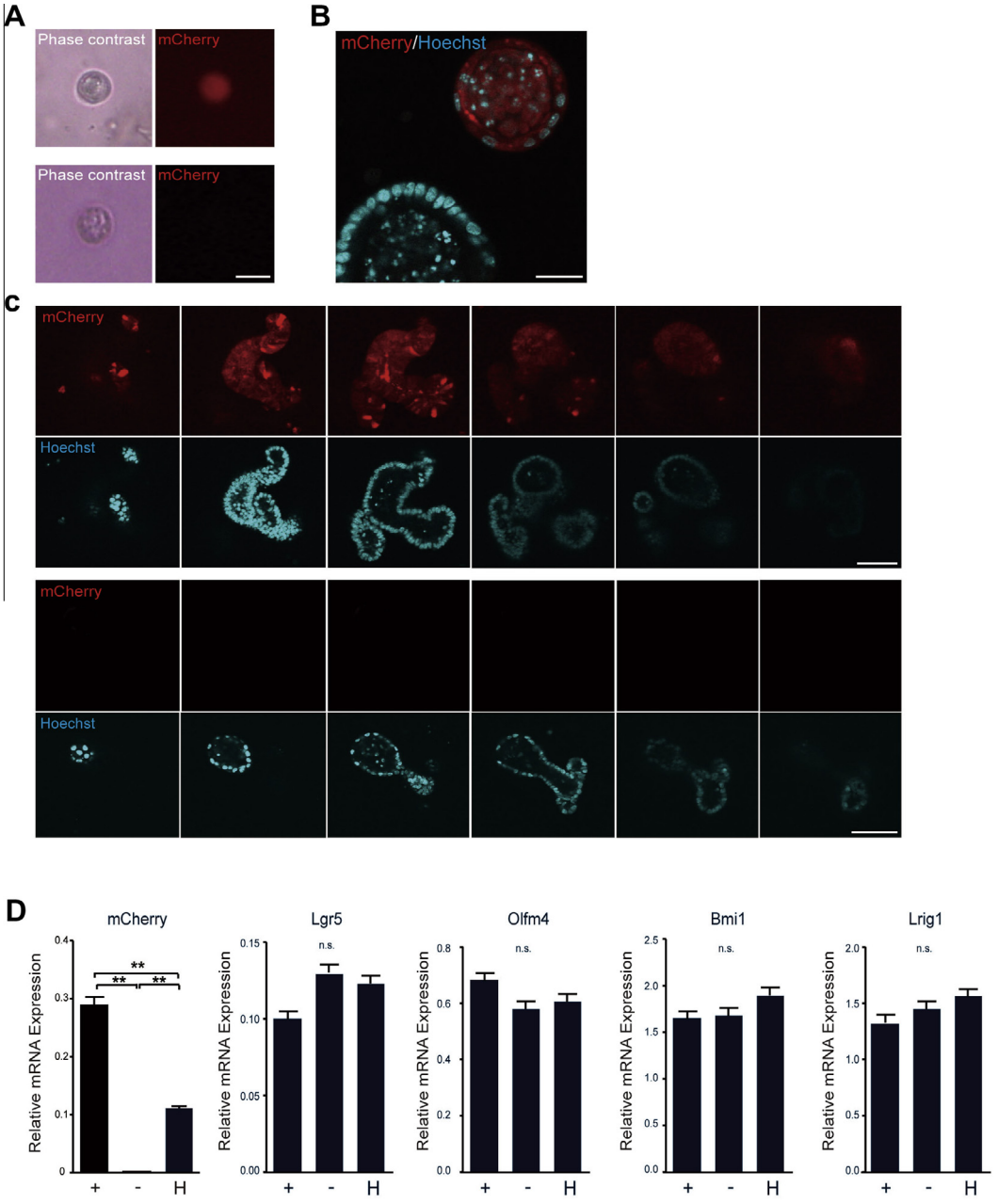

Fig. 4. Both mCherry-positive and -negative single cell reconstruct organoids. (A) mCherry-hetero organoids were dissociated as a single-cell level. Individual mCherry-positive and -negative cells were together mounted into the Matrigel® and cultured. Scale bar: 5 μm. (B) The organoids generated from a single mCherry-positive and -negative cell 7 days after the single-cell dissociation. Scale bar: 20 μm. (C) Sequential confocal imaging of a whole organoid. All cells were mCherry positive in an organoid generated from a single mCherry-positive cell (upper panel) whereas all cells were mCherry negative in an organoid generated from a single mCherry-negative cell (lower panel). Sequential imaging of a whole organoid is also shown in Supplementary Movies 6–9. Scale bar: 50 μm. (D) Quantitative real-time polymerase chain reaction (RT-PCR) for mCherry expression. +, completely mCherry-positive organoids generated from a single mCherry-positive cell in mCherry-hetero organoids. −, completely mCherry-negative organoids generated from a single mCherry-negative cell in mCherry-hetero organoids. H, mCherry-hetero organoid cultured for >1 year. Quantitative RT-PCR for intestinal stem cell markers. No significant differences were observed among the three organoids.

expression of the mCherry gene was greater in mCherry-positive organoids than in mCherry-hetero organoids, whereas mCherry-negative organoids did not express the mCherry gene (Fig. 4D). Finally, each organoid was characterised to confirm whether long-lived plural stem cells in the same crypt shared the functions of a villi and a crypt, resulting in the equal expression of a series of stem cell markers in each organoid (Fig. 4D).

4. Discussion

This study showed that plural stem cells in a crypt may be long-lived and produce different types of intestinal epithelial cells in villi. Although the use of lentivirus mixed with Matrigel® led to partial introduction of the transgene into organoids, random marking of the organoid cells showed that regulation of specific cell types, including stem cells, had occurred. Previous reports have indicated that individual cells dissociated from organoids could be inoculated with a transgene by retroviruses without Matrigel® [18]. Our method however enabled the introduction of a transgene into organoids in which the original morphology of the stem cells in a crypt *in vivo* might be kept. In particular, we could for the first time, visualise individual stem cells in a crypt which reflected the original position of stem cells *in vivo*. Sequential passages of the organoids generated from a single mCherry-hetero organoid for >1 year indicates that individual mCherry-positive cells in a crypt might be a long-lived stem cell.

Moreover, for the first time, we could divide the long-lived stem cells in a crypt into two subpopulations according to mCherry fluorescence. Previous reports have indicated that Cre-mediated recombination of stem cell marker promoters was useful for detection of intestinal stem cells [6]. However, all stem cells were marked at the same time, which made it impossible to distinguish each stem cell in a crypt. Recently, it has been reported that stem cells could be divided into four subpopulations according to colouring in a confetti mouse model [19]. Long-term clonal tracing of Lgr5-positive cell subpopulations in the confetti mouse resulted in the unification to a single colour in the villi, which suggested that a few long-lived stem cells in a crypt might serve all intestinal epithelial cells in the villi. However, it remains unknown whether individual stem cells in a crypt could supply all cells in a crypt and a villus. In this study, we found that a single mCherry-positive cell was located in a crypt, which suggested that mCherry-negative stem cells might supply almost all intestinal epithelial cells in a crypt and TA zone. Once an mCherry-positive stem cell shifts to the active phase, it supplies almost all CBC cells and TA cells. At the next passage of the organoids, the population of mCherry-positive cells in the crypt was reset, which suggests that individual long-lived stem cells alternately supply almost all CBC cells and TA cells. The observation of 1–5 mCherry-positive CBC cells in each mCherry-hetero organoid might have been caused by the difference in the time phase of mCherry-positive cell division.

Accordingly, these findings raised a question about whether individual long-lived stem cells in a crypt divide into the same cells. The organoids generated from individual long-lived stem cells distinguished by mCherry fluorescence showed no difference about the expression of stem cell markers, which suggests that these two subpopulations of stem cells have the equal capacity of stemness.

In the future, the comparison of gene expression between mCherry-positive and -negative cells in an organoid might be helpful to understand the difference among the subpopulations of the stem cells in a crypt. Although the general cell lineages of the intestine are regulated by a series of transcriptional factors [20], it remains unknown how the ratio of the respective cell lineages are regulated in each villus. In particular, the gene expression profiling is longitudinally different throughout the entire small intestine without Notch signal influence [21], although the differentiation-determined transcriptional factor Atoh1 was directly repressed by Hes1 via Notch signal [22]. This finding suggested that the comportment of stem cells with permanent lineage directivity in a crypt might be changed to adjust the proper cell distribution. In the future, an *in vivo* single-cell tracking method should be used to assess the lineage directivity of each intestinal stem cell in a crypt. Moreover, it remains unknown as to how many long-lived stem cells exist in a crypt because single colour markings of cells divided into only two subpopulations. Multi-colour markings of individual cells in a crypt might clarify the number of long-lived stem cells.

Another valuable feature of this system is that the dynamics of single stem cell in a crypt can be assessed. We could select the crypts in which single stem cell was marked by mCherry. Time-lapse analysis can be used to chronologically assess the regulation of a long-lived stem cell, such as cell quiescence, division, occupation of CBC cells and supply to differentiated cells. Time-lapse analysis would also be useful for determining the effects of various reagents on a stem cell to develop treatments that target intestinal stem cells. A system capable of measurement of single stem cell function should be established in the future.

In conclusion, the use of our novel lentivirus transgene system to mark individual stem cells in a crypt of an organoid demonstrated plural long-lived stem cells in a crypt.

Competing financial interests

The authors declare no competing financial interests.

Author contributions

K.T., M.W., T.N., R.O., Y.K., S.Y., T.M. and Y.N. conceived of and designed the experiments. N.H., R.H, K.F, and M.F. performed the experiments. S.H., K.T. and N.H. analysed the data. N.H. and K.T. wrote the manuscript.

Writing assistance

Crimson Interactive Pvt. Ltd.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.091.

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