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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