Video Article

The Organoid Reconstitution Assay (ORA) for the Functional Analysis of Intestinal Stem and Niche Cells

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Abstract

The intestinal epithelium is characterized by an extremely rapid turnover rate. In mammals, the entire epithelial lining is renewed within 4 - 5 days. Adult intestinal stem cells reside at the bottom of the crypts of Lieberkühn, are earmarked by expression of the *Lgr5* gene, and preserve homeostasis through their characteristic high proliferative rate[1]. Throughout the small intestine, *Lgr5*$^+$ stem cells are intermingled with specialized secretory cells called Paneth cells. Paneth cells secrete antibacterial compounds (*i.e.*, lysozyme and cryptdins/defensins) and exert a controlling role on the intestinal flora. More recently, a novel function has been discovered for Paneth cells, namely their capacity to provide niche support to *Lgr5*$^+$ stem cells through several key ligands as Wnt3, EGF, and Dll1[2].

When isolated *ex vivo* and cultured in the presence of specific growth factors and extracellular matrix components, whole intestinal crypts give rise to long-lived and self-renewing 3D structures called organoids that highly resemble the crypt-villus epithelial architecture of the adult small intestine[3]. Organoid cultures, when established from whole crypts, allow the study of self-renewal and differentiation of the intestinal stem cell niche, though without addressing the contribution of its individual components, namely the *Lgr5*$^+$ and Paneth cells.

Here, we describe a novel approach to the organoid assay that takes advantage of the ability of Paneth and *Lgr5*$^+$ cells to associate and form organoids when co-cultured. This approach, here referred to as "organoid reconstitution assay" (ORA), allows the genetic and biochemical modification of Paneth or *Lgr5*$^+$ stem cells, followed by reconstitution into organoids. As such, it allows the functional analysis of the two main components of the intestinal stem cell niche.

Video Link

The video component of this article can be found at https://www.jove.com/video/56329/

Introduction

The intestinal epithelium is the most rapidly self-renewing tissue in the mammalian body and, as such, has been the object of a plethora of studies aimed at the identification and functional characterization of the adult stem cells residing at the bottom of the crypt of Lieberkühn, earmarked by expression of the *Lgr5* gene and dependent on canonical Wnt signals[1]. Notably, *Lgr5*$^+$ stem cells are flanked and supported by specialized niche cells, *i.e.* Paneth cells, which also depend on Wnt signaling for their maturation[2]. Together, these two cell types underlie self-renewal of the intestinal epithelial lining and preserve the daily homeostatic equilibrium: *Lgr5*$^+$ stem cells rapidly divide and give rise to progenitor and more specialized intestinal epithelial cells; Paneth cells provide essential niche factors (*e.g.*, Dll1, Wnt3, EGF) to *Lgr5*$^+$ stem cells[2]. The capacity of intestinal crypts when plated *ex vivo* to form organized and self-renewing structures called organoids, or "mini-guts", has been exploited as an experimental tool to provide insight into processes such as self-renewal and differentiation in normal and pathological conditions including cancer[4]. Organoid cultures have been established from several tissues, including the intestine, pancreas, liver, and kidney, from both mouse and human samples[4]. The extraction method and the growth factors employed to develop these organoid cultures are tissue-specific and designed to drive multi-lineage differentiation and mimic as closely as possible the original stem cell niche *in vivo*. Organoids may have potential applications including the treatment of genetic diseases, the assessment of therapeutic efficacy in cancer, the analysis of drug toxicity, or the study of organogenesis *in vitro*[5].

Overall, the main limitation of organoid cultures when established from tissue samples is a lack of cell-specificity. For example, intestinal organoids established from whole intestinal crypts do not allow the analysis of individual cellular components encompassed within the tissue source (*e.g.*, whole crypts contain *Lgr5*$^+$, Paneth, and progenitor cells).

Here, we describe a novel method, referred to as ORA, that combines the advantages of intestinal organoid cultures with the functional analysis of its most fundamental components, namely stem (*Lgr5*$^+$) and niche (Paneth) cells. This is achieved through the unique ability of Paneth and *Lgr5*$^+$ cells to physically associate with each other when co-incubated and give rise to organoids[2,6,10]. We took advantage of this feature and pre-treated the two cell types individually before allowing them to reconstitute organoids. When doing so, each cell component can be exposed to

any given drug, growth factor, biochemical inhibitor, genetic modification, or chemical treatment prior to reconstitution and organoid formation. Therefore, using the ORA assay will allow the determination of whether a specific drug treatment or genetic modification has a specific effect on the stem cells or their niche counterpart.

Protocol

All procedures were done according to local animal welfare laws and guidelines.

1. Preparation of Instruments, Culture Media, and Dishes

1. Autoclave 1 set of intestinal scissors, normal scissors, and forceps in a sterile container.
2. Place a 96-well (flat bottom) dish in an incubator at 37 °C.
3. Prepare 10 mL of complete culture medium with the reagents listed in table of materials.
4. Incubate the complete medium at 37 °C in a water bath.
5. Thaw reconstituted basement membrane by placing it in an ice bucket. The reconstituted basement membrane will become liquid at 4 °C .
6. Fill 4 Petri dishes with cold phosphate-buffered saline or abbreviated PBS (4 °C).

2. Isolation of Small Intestinal Crypts

1. Sacrifice by CO_2 inhalation a *Lgr5*-eGFP-IRES-CreER^{t2} mouse on a C57BL6/J background. Dissect the peritoneum longitudinally with a pair of scissors.
2. Hold the stomach with the forceps and cut it transversally in half.
3. Using the intestinal scissors from now on, pull out the intestine and place it in a Petri dish containing PBS.
   NOTE: Intestinal scissors have a sharp and a blunt tip. The blunt tip of these scissors is meant to not damage the crypt-villus architecture while opening the intestine.
4. Start by placing the blunt tip into the stomach and gently push it through the pylorus. Proceed by cutting with the scissors and pulling with the forceps.
5. Once the whole small intestine is opened longitudinally, wash it in cold PBS by holding it with the forceps and gently rinse it in the PBS solution with U-shaped movements.
6. Once all the stool remnants are cleared, proceed to flatten the intestine on a cutting board, luminal side up. The luminal side is easily recognizable by the absence of blood vessels and by its pale appearance when compared with the outer part.
7. With a glass slide, gently remove the villi by scraping the flattened intestine. Perform this step twice along the whole length of the tissue.
8. Cut the small intestine with a sterile surgical blade into 2 - 5 mm pieces.
9. Place the small intestinal fragments in a 50 mL tube containing 10 mL of ice cold PBS.
10. Clean the tissue fragments, removing any remaining impurities by pipetting them up and down in the PBS. Discard supernatant and repeat this step until the PBS is completely clear.
11. Add 15 mL cold PBS, to bring the total final volume of PBS to 25 mL. Add 2 mM ethylenediaminetetraacetic acid (EDTA) and incubate for 45 min on a roller at 4 °C.
12. Discard the PBS/EDTA.
13. Add 10 mL of PBS and detach the crypts by harshly pipetting the tissue fragments up and down (at least three times). Collect the supernatant.
14. Repeat step 2.13 four times. Add culture medium to reach a final volume of 50 mL.
15. Pellet the cells by centrifugation (300 x g for 5 min).
16. Resuspend the pellet in 10 mL of culture medium and pellet the crypts by centrifugation (80 x g for 3 min).

3. Single Cell Preparation

1. Discard the supernatant of the pelleted crypts and resuspend them in 1 mL trypsin like-enzymes together with 50 µL DNAse (50 µg/mL).
2. Incubate the cells in a water bath at 32 °C for 2 min.
3. Add 10 mL of culture medium. Dissociate the crypts into single cells by harshly pipetting up and down at least 5 times.
4. Filter the solution through a 40 µm strainer (to eliminate clumps and other impurities) and pellet the single cells at 300 x g for 5 min.

4. Flow Cytometry and Plating

1. Prepare 50 mL of 1x Hank's buffered salt solution or HBSS/2% fetal cow serum (FCS) solution and resuspend the pellet in 1 mL for every 10^6 cells
2. Add to the cell suspension BV421 Lin$^-$ (CD31, CD45, TER119) at a concentration of 1:100, and CD24-APC and CD117-PE both at a concentration of 1:250 antibodies for 30 min.
3. Sort *Lgr5*$^+$ stem cells and Paneth cells into separate low bind tubes. For details on sorting protocols for *Lgr5*$^+$ and Paneth cells, please see Roth *et al.*[7] and Schewe *et al.*[6]
   1. Centrifuge the sorted cells at 300 x g for 5 min. Resuspend the cells in 100 µL medium with the components described as in 1.3 (see the **Table of Materials**).
4. Treat (*e.g.*, by chemical or genetic modification) the Paneth cells (n = 2000) or the *Lgr5*$^+$ stem cells (n = 2000).
   1. To treat cells, use 5 µL of liposome-mediated transfection reagent in a total volume of 100 µL culture medium and small interfering RNA (siRNA) at a concentration of 100 nM. Incubate for 30 min at 37 °C for siRNA or for the time necessary for the chosen modification.

5. Wash the cells twice in 500 µL culture medium.
6. Centrifuge at 300 x g for 5 min and resuspend the cells in 10 µL culture medium.
7. Pool the two cellular components (Paneth or *Lgr5*$^{+}$ cells) in a 20 µl total volume and centrifuge at 300 x g for 5 min at RT.
8. Co-incubate the Paneth and *Lgr5*$^{+}$ cells for 10 min at RT.
9. Remove 10 µL of the supernatant and leave a meniscus of liquid to be sure to not aspirate the cell pellet.
10. Add 30 µL of reconstituted basement membrane to the cells for a total volume of 40 µL, resuspend the cells and plate in a pre-warmed 96 well plate. After 10 min, add 200 µL of complete medium (see the **Table of Materials**). Change medium every 48 h.
11. Count organoid multiplicity at day 5.

Representative Results

The organoid reconstitution assay allows the separate functional analysis of the essential niche and stem cell components of the intestinal epithelium, here demonstrated by small interfering RNA (siRNA) of the *Apc* gene.

To achieve this aim, we first utilized fluorescent activated cell sorting (FACS) to separate 8000 *Lgr5*$^{+}$ cells and 6000 Paneth cells from inbred C57BL6/J mice. In **Figure 1a**, a flowchart of the ORA assay is depicted. Organoid reconstitution capacity was assessed by incubating *Lgr5*$^{+}$ cells with siRNA oligonucleotides directed against the mRNA of *Apc* or encompassing a scrambled sequence (SCR) as a control. After treatment, the same siRNA-treated *Lgr5*$^{+}$ cells were either directly plated in organoid medium/reconstituted basement membrane, or incubated with an equal number of untreated Paneth cells and subsequently plated out. As a control, untreated *Lgr5*$^{+}$ cells were plated alone (*i.e.*, without Paneth cells) to determine the number of organoids derived from untreated *Lgr5*$^{+}$ cells. Furthermore, as an additional control, Paneth cells alone were plated to check for the background of organoid formation derived from the contamination of Paneth-Lgr5 cell doublets sorted in the Paneth cell gate. As expected, siRNA mediated knockdown of the murine *Apc* gene in *Lgr5*$^{+}$ stem cells (and the resulting activation of the canonical Wnt/β-catenin signaling pathway) positively affected organoid multiplicity when compared to untreated counterparts or the scrambled control (**Figure 1B**). The constitutive Wnt activation also rescued the requirement for Paneth cells, as previously reported[1]. Furthermore, these organoids appeared as hollow spheres (spheroids), a well described phenotype for *Apc*-mutant or Wnt-stimulated organoids (**Figure 1C**, panel 1), when compared with the morphology of those obtained from Paneth-Lgr5 cell doublets (**Figure 1C**, panel 2)[8,9]. Collectively, these results demonstrate that reconstituting Paneth cells and *Lgr5*$^{+}$ cells can give insight on cell-specific mechanisms within these two cell types; morphological analysis of the organoids by microscopy can be valuable in this respect since the morphology of the organoids reflects their cell composition (*e.g.*, spheroids are constituted by *Lgr5*$^{+}$ cells only). Another important parameter to be taken into account is the complexity of the organoid (*e.g.*, the number of crypt budding events).

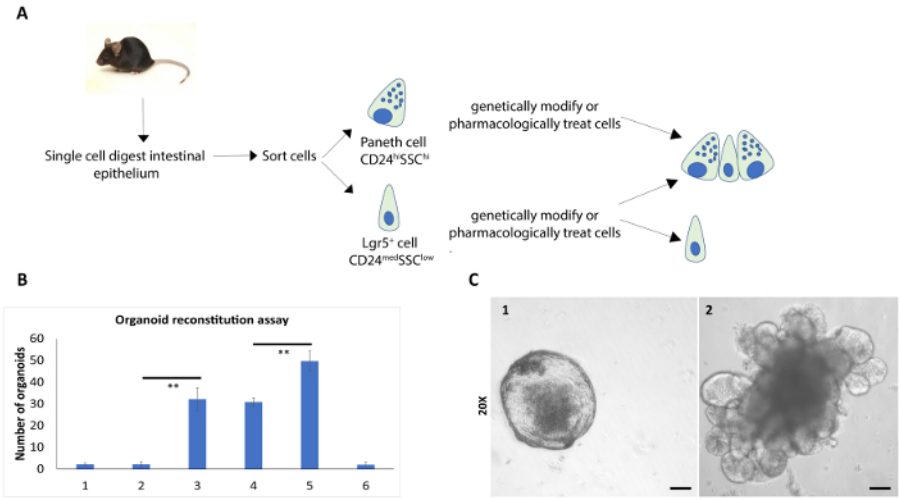

Figure 1. Effect of siRNA mediated knockdown of *Apc* in Lgr5^{+} cells. (A) Flowchart of the experimental procedure. *Lgr5*^{EGFP} reporter mice (*Lgr5*$^{EGFP\text{-}IRES\text{-}creERT2}$)[1] were employed as a source of *Lgr5*$^{+}$ sorted stem cells. Paneth cells were sorted as indicated and previously described[6,7]. **(B)** Organoid multiplicity observed upon reconstitution of *Lgr5*$^{+}$ stem cells and Paneth cells. When indicated cells were pretreated with siRNA oligonucleotides directed against the *Apc* (siRNA *Apc*) or scrambled (SCR) control sequence. The asterisks indicate statistically significant differences (n = 3, *p <0.05 **p <0.001). Error bars refer to SD. (1) *Lgr5*$^{+}$ cells, (2) *Lgr5*$^{+}$ cells SCR, (3) *Lgr5*$^{+}$ cells siRNA *Apc*, (4) *Lgr5*$^{+}$ cells + Paneth cells, (5) *Lgr5*$^{+}$ cells siRNA *Apc* + Paneth cells, (6) Paneth cells. **(C)** Representative images of organoids derived from (1) *Lgr5*$^{+}$ cells siRNA *Apc*, (2) *Lgr5*$^{+}$-Paneth cells. Scale bar = 10 µm. Please click here to view a larger version of this figure.

Discussion

The ORA allows the refined functional analysis of the two essential components of the intestinal stem cell niche, namely *Lgr5*$^{+}$ and Paneth cells. This approach has been previously employed by us and others with slight modifications[6,10,11]. Here, we present the ORA procedure as a reproducible and standardized laboratory protocol. Also, we report on the effects of *Apc* downregulation in *Lgr5*$^{+}$ stem cells, as an example of the possibility of implementing genetic (or biochemical[6]) modifications to the sorted cellular components. Collectively, the data show that siRNA mediated knockdown of *Apc* enhances the stem cell function of *Lgr5*$^{+}$ cells, as indicated by the increased organoid multiplicity (**Figure 1B**).

Several advantages of using intestinal 3D organoid cultures have already been listed in reviews[4], including the striking resemblance of their organization with that of the crypt-villus architecture *in vivo*, especially when compared to the architecture from standard 2D culture methods with

immortalized cell lines. However, one of the major limitations of this method is that in most cases, organoid cultures are established from whole intestinal crypts, a process which does not allow the functional analysis of its individual components and, in particular, of the stem and niche cells, here represented by *Lgr5*$^{+}$ and Paneth cells, respectively.

The ORA overcomes these limitations. Stem and niche cells can be separately sorted from the animal and reconstituted to generate organoids. As such, this approach allows the functional analysis of these two cellular components by either employing mouse models carrying specific genetic modifications or exposed to specific stress factors (*e.g.*, DSS and/or specific diets); or by directly modifying the sorted cells genetically (siRNA, CRISPR-Cas9) or biochemically, before reconstituting them to generate organoids. The multiplicity, morphology, self-renewal capacity, and extent of differentiation (and eventually the extent of metaplastic changes) of the resulting organoids can be employed as functional read-outs. In the case of genetic manipulation, such as siRNA, when a morphological effect is not evident, cells should be analyzed one hour after transfection to validate the knockdown of the mRNA of interest. Paneth and *Lgr5*$^{+}$ can also be sorted from mice genetically engineered with different mutations to study whether different mutations in stem and niche cells lead to altered interactions between these two cell types and a different organoid formation efficiency and/or morphology.

Critical steps within the protocol are the removal of the villi (step 2.7) and the resuspension of the cells in HBSS/2%FCS (step 4.1). Removal of the villi is critical to enrich Paneth and *Lgr5*$^{+}$ cells located in the lower third of the crypts and to improve the efficiency of sorting. Although FACS protocols commonly suggest sorting cells in PBS/FCS, the use of HBSS instead of PBS increased and kept cell viability stable throughout the sorting time. Other important steps are steps 4.7 and 4.8, where physical association of Paneth and *Lgr5*$^{+}$ *cells* allows these lineages to form doublets that will eventually give rise to organoids.

Disclosures

The authors have no competing financial interest or other conflicts of interest

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