Efficient establishment and long-term maintenance of 3-dimensional mouse intestinal organoids using a novel defined and serum-free medium

Ryan K. Conder[1], Michael J. Riedel[1], Terry E. Thomas[1], Allen C. Eaves[1,2], and Sharon A. Louis[1]
[1] STEMCELL Technologies Inc., Vancouver, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver, B.C., Canada

Introduction

The small intestinal epithelium is a rapidly renewing monolayer that consists of well-characterized cell types that reside in two distinct domains: the crypt and villus. At the base of the crypt domain, undifferentiated stem cells produce four types of mature cells responsible for all functions of the adult intestine. These are the enterocytes, goblet, enteroendocrine and Paneth cells. The recent demonstration that intestinal stem cells can be maintained *in vitro* via novel 3D culture techniques has the potential to greatly impact the field of epithelial stem cell biology[1]. These 'organoids' can be derived from isolated intestinal crypts or single Lgr5-expressing stem cells and maintained using both a specialized medium and a complex spatial arrangement that mimics the stem cell niche. Furthermore, established organoids can be maintained long-term through routine passaging, indicating maintenance of the stem cell pool[2]. We have developed a serum-free and defined medium that supports the establishment and long-term maintenance of mouse intestinal organoids.

Materials and Methods

Small Intestinal Crypt Culture

Upper intestines of C57BL6/J mice were dissected and washed several times in PBS. The intestinal fragments were then incubated for 20 minutes with Gentle Cell Dissociation Reagent (GCDR) at room temperature (RT) to separate the crypts and villi from the intestinal basal surface. The crypts were then isolated from the villi through centrifugation, counted and re-suspended in a 50:50 mixture of Matrigel® and IntestiCult™ Organoid Growth Medium (OGM) at 6,000 crypts/mL. A 50 μL droplet of the suspension was gently placed into the center of pre-warmed 12-well culture plate wells, creating a dome containing ~300 crypts/well. The domes were solidified at 37°C for 5 min and the wells were then flooded with 750 μL of IntestiCult™ OGM. Crypts were cultured at 37°C for 4 - 7 days with 3 times weekly medium changes. After 7 days, organoids were passaged by treating cultures with GCDR for 15 min at RT followed by mechanical disruption into smaller aggregates. The resultant suspension was mixed with IntestiCult™ OGM at a 1:6 ratio and then re-plated as above to establish secondary cultures. This protocol was repeated to generate long-term cultures.

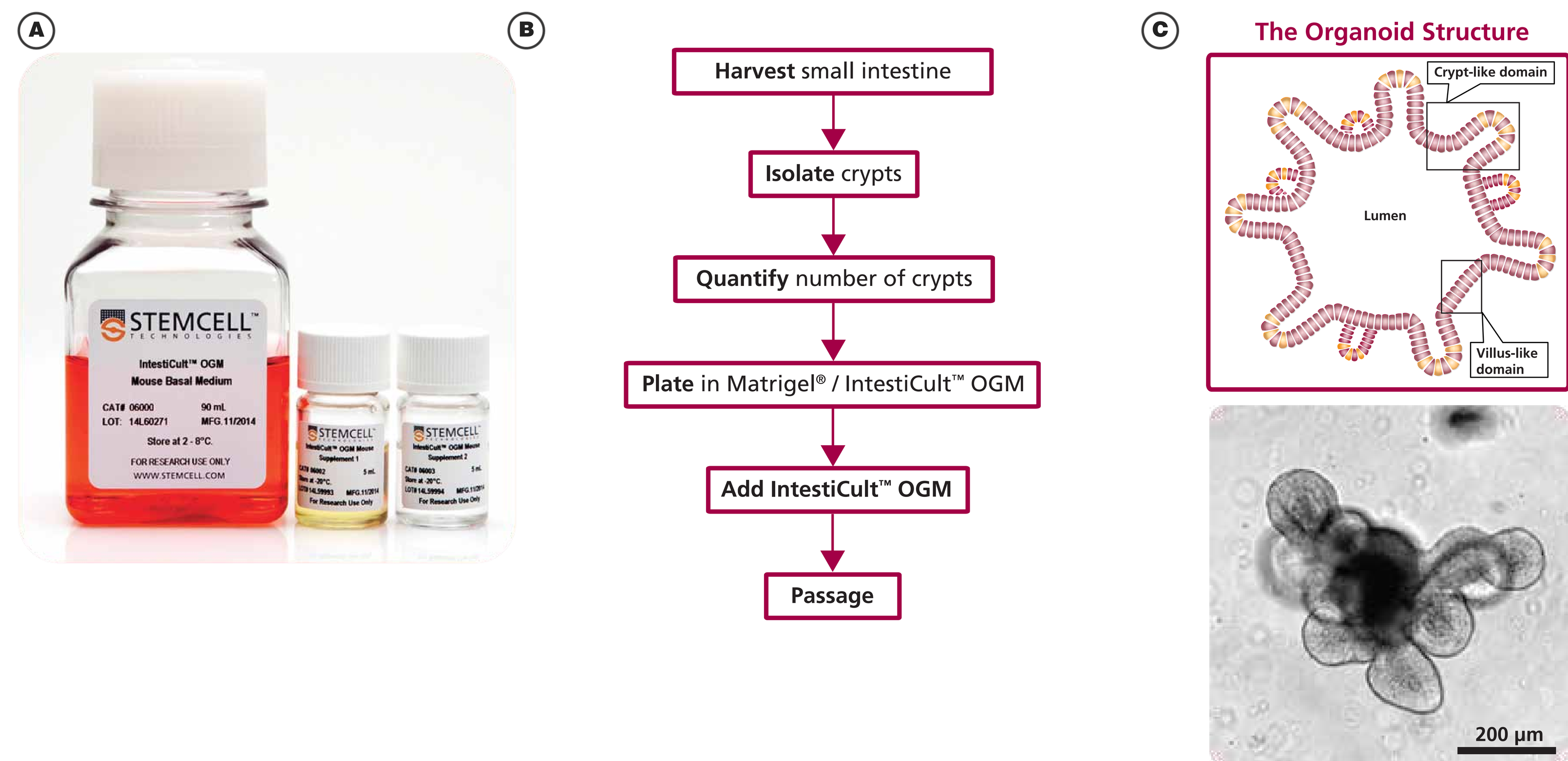

Figure 1. A) IntestiCult™ OGM consists of a basal medium and two supplements. **B)** Workflow of organoid establishment from primary mouse intestinal crypt using IntestiCult™ OGM. **C)** Schematic of a mature intestinal organoid (above) and brightfield image of a mature (day 5) mouse intestinal organoid (below). The central lumen is surrounded by an epithelial monolayer with budding crypt-like domains.

Colon Crypt Culture

The same procedure as the small intestinal crypt culture was followed for the collection of large intestinal crypts. Tissue samples were taken from the region of large intestine directly posterior to the cecum and anterior to the rectum.

Single Cell Dissociation

Small intestinal crypts were dissociated and then incubated with GCDR for an additional 45 min. at RT. During this time, the crypts were agitated by pipetting approximately every 10 minutes. The dissociated tissue was then passed through a cell strainer with a pore size of 20 μm. Single cells were plated in a 96 well plate in a 50:50 mixture of 10 μl of Matrigel® and IntestiCult™ OGM and then flooded with 100 μl IntestiCult™ OGM or IntestiCult™ OGM + Y-27632 (10 μM).

Growth Percentage

For each culture, a growth percentage of crypts was determined by calculating the number of organoids growing on day 3 as a fraction of the total number of crypts that had begun to form an epithelium approximately 4 hours after plating. The growth percentage as a representative metric (**Figure 2**) for the efficiency of organoid establishment was tracked for each culture.

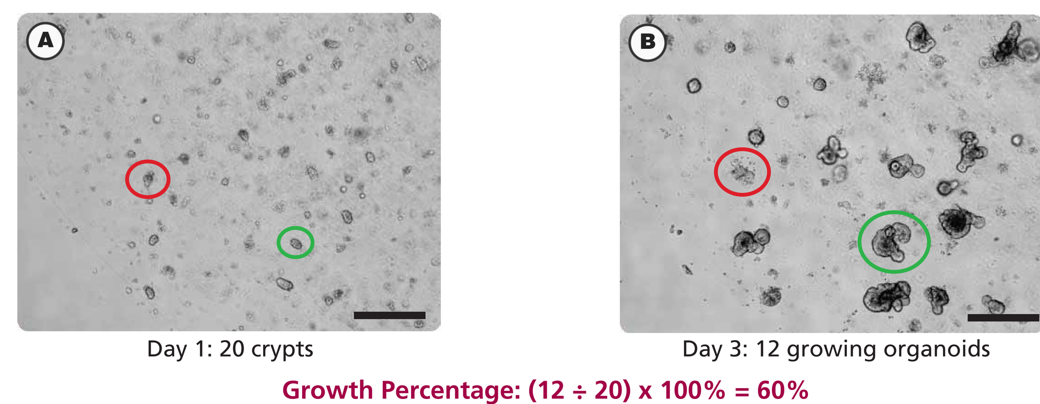

Figure 2. Example of growth percentage calculation. A) Cultured mouse intestinal crypts 1 day after plating. This image shows 20 crypts with potential to form organoids. **B)** Image shows the same culture on day 3, with 12 of the 20 crypts growing into mature organoid structures. The green circle highlights a crypt that has successfully grown. The red circle highlights a crypt that has differentiated prematurely. Equation demonstrates the growth percentage calculation for this field of view. Scale bar = 200 μm.

Results

Timecourse

The growth of individual organoids was tracked from the time of plating until a multi-crypt domain-containing structure was ready for passaging. At approximately 3 - 4 hours post-isolation, individual crypts undergo a morphogenesis from their elongated state into a sphere comprising a single-layered epithelium. At 2 - 3 days (day 2 shown), initial 'buds' form at high-Wnt, stem cell-rich domains of the sphere mimicking intestinal crypt formation. For the rest of the growth phase, the organoids grow in size, extruding cells into the central lumen structure and continue to develop multiple buds extending from the initial structure (day 5 shown).

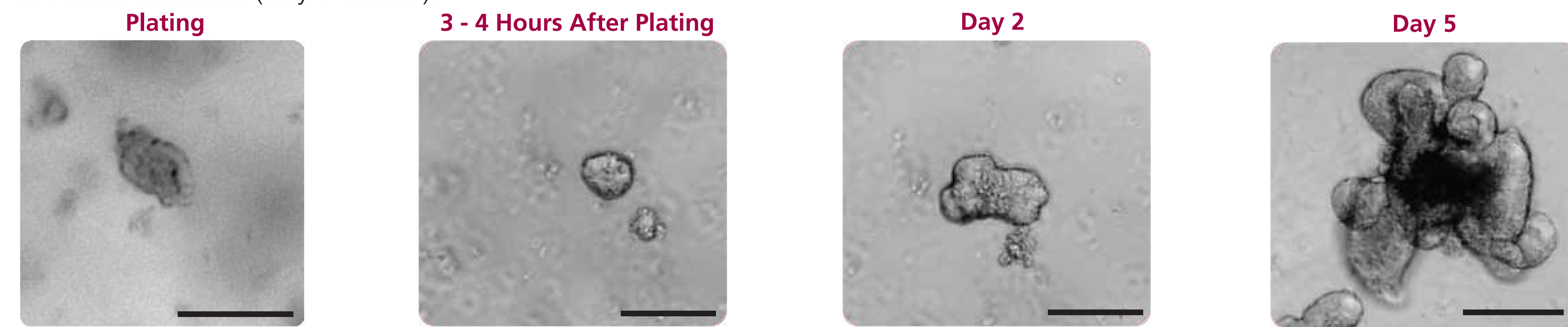

Figure 3. Isolated mouse intestinal crypt displaying morphological changes from initial plating to mature organoid. Scale bar = 200 μm.

Established Organoids: Establishment and Long-Term Maintenance

We assessed the growth efficiency of organoid cultures in IntestiCult™ OGM (see Materials and Methods). The intestinal organoids maintained a functional stem cell niche and could be passaged over long durations. Upon passaging, efficiency of organoid formation increased to >90%. Organoids were assessed for their functional properties over 10 passages and they maintained similar structures to the early passaged organoids.

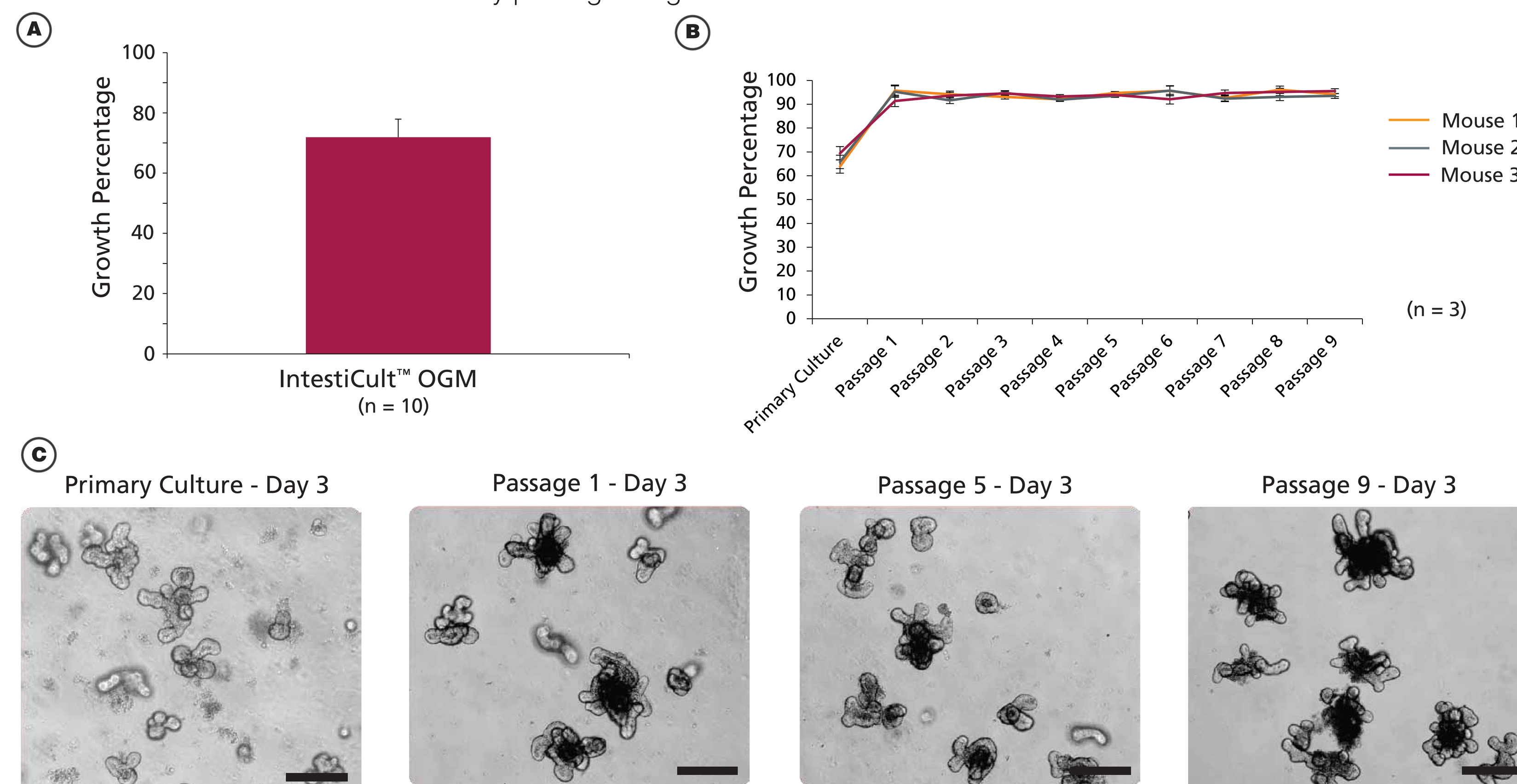

Figure 4. A) Formation of organoids from primary small intestinal derived crypts was assayed on day 4 at 71.9 ± 6.1% (mean ± SD; n = 10). **B)** The efficiency of organoid formation increased after passaging and remained consistently high over at least 10 passages. Three independent experiments demonstrate growth percentages between 92.4 ± 0.7% and 94.8 ± 1.5% (average mean ± SD; n = 3) **C)** Representative images of organoids from initial small intestinal crypts primary culture – day 3, passage 1 – day 3, passage 5 – day 3 and passage 9 – day 3 demonstrating a maintenance of the complex budding phenotype. Scale bar = 200 μm.

Colonic Organoids: Establishment and Long-Term Maintenance

Organoids were established from primary mouse colonic crypts in IntestiCult™ OGM (see Materials and Methods). Upon passaging, colonic organoids grew with increased efficiency approaching that of small intestinal organoids. This higher efficiency was maintained in subsequent passages (**Figure 4B**).

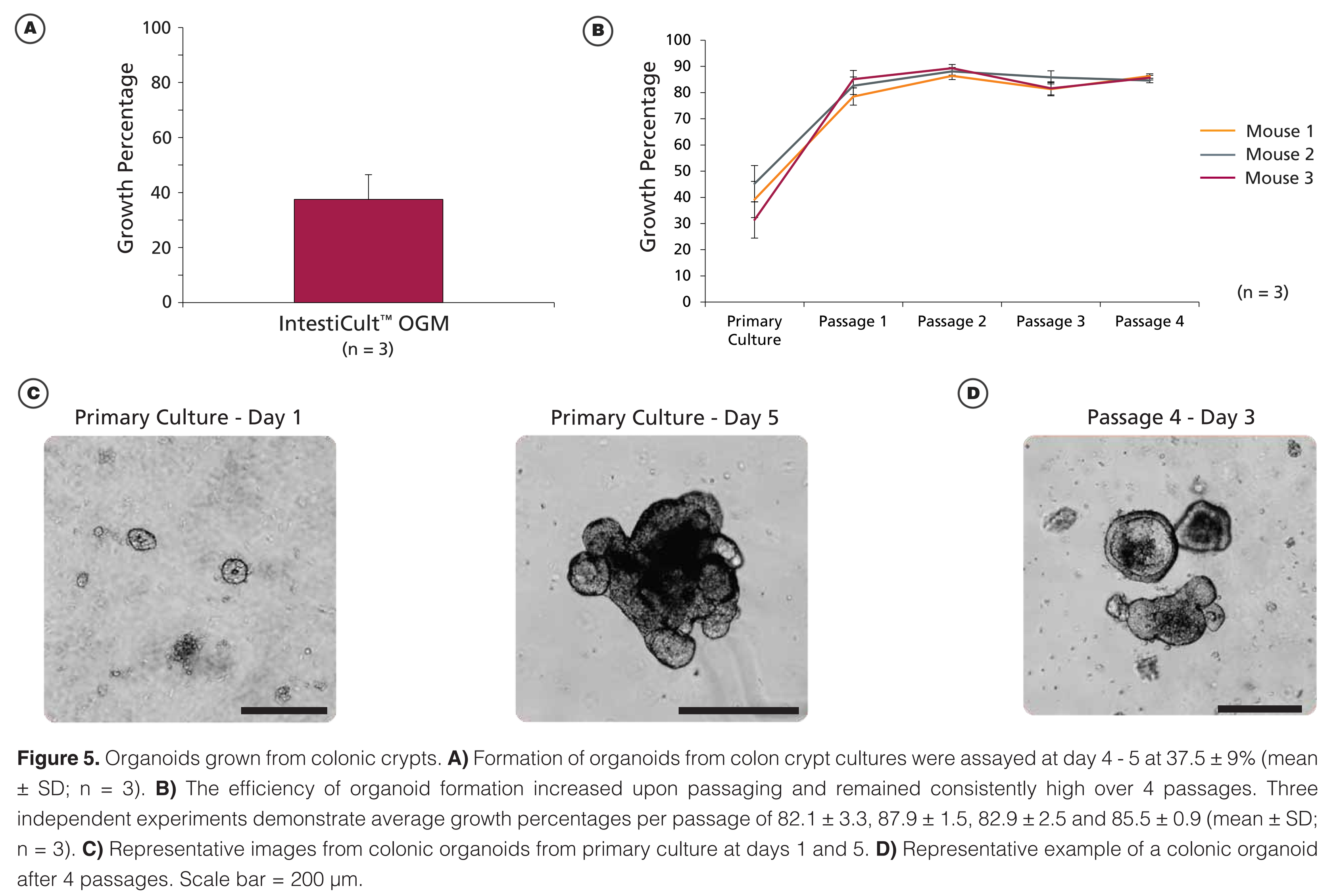

Figure 5. Organoids grown from colonic crypts. **A)** Formation of organoids from colon crypt cultures were assayed at day 4 - 5 at 37.5 ± 9% (mean ± SD; n = 3). **B)** The efficiency of organoid formation increased upon passaging and remained consistently high over 4 passages. Three independent experiments demonstrate average growth percentages per passage of 82.1 ± 3.3, 87.9 ± 1.5, 82.9 ± 2.5 and 85.5 ± 0.9 (mean ± SD; n = 3). **C)** Representative images from colonic organoids from primary culture at days 1 and 5. **D)** Representative example of a colonic organoid after 4 passages. Scale bar = 200 μm.

Single Small Intestinal Cells: Establishment and Maintenance

As many experiments using organoids require the dissociation of the crypts to single cells, we assayed the growth efficiency of organoid culture from single small intestinal cells. Plated single cells grew into organoids that were indistinguishable from small intestinal crypt cultures both with and without the addition of ROCK inhibitor. Once established, the organoid cultures were able to be expanded and maintained over multiple passages.

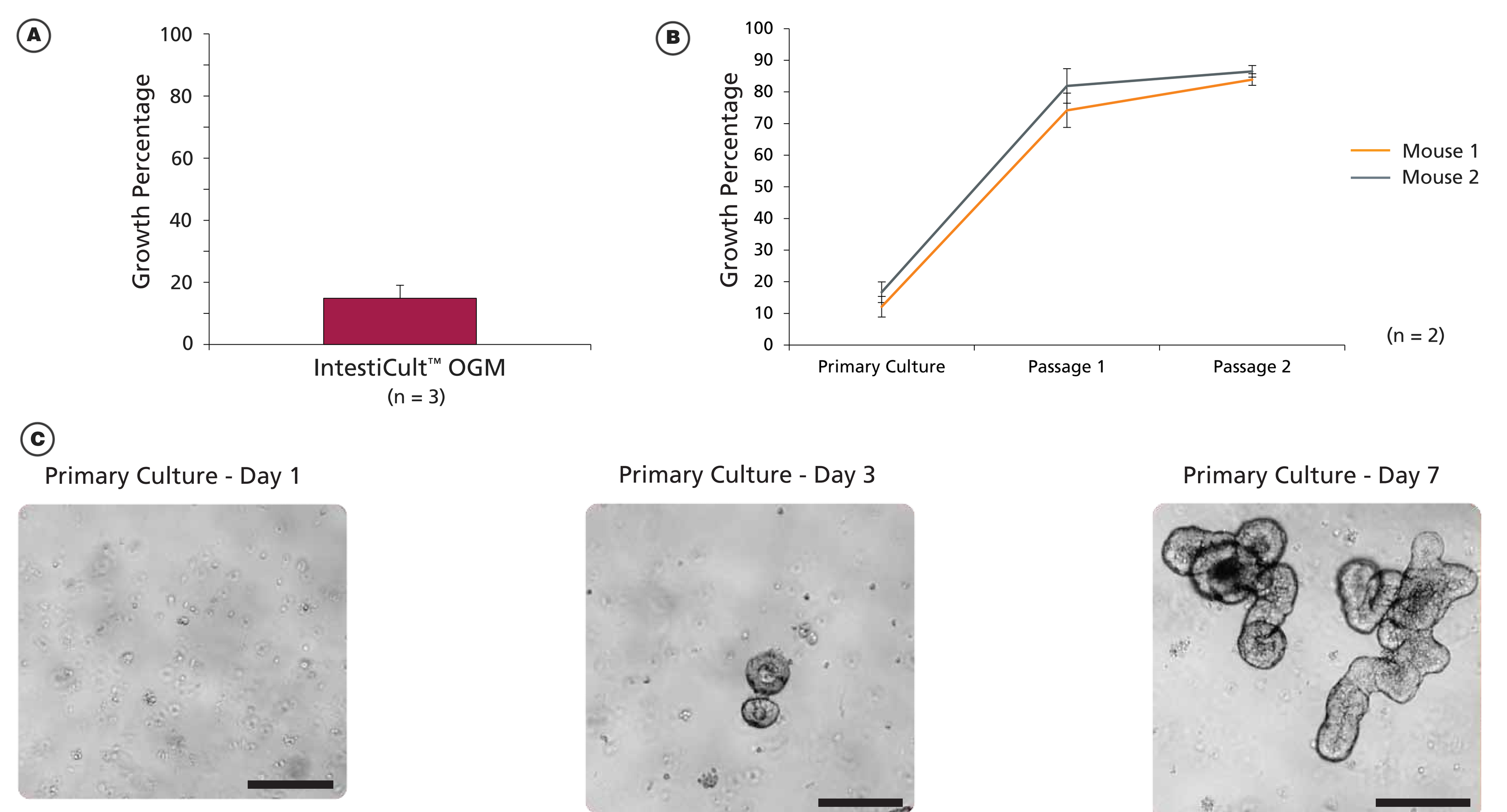

Figure 6. Organoids grown from single small intestinal epithelial cells. **A)** Formation of organoids from small intestinal crypts dissociated into single cells were assayed at day 4 - 5 at 14.9 ± 4.2% (mean ± SD; n = 3). **B)** The efficiency of organoid formation increased upon passaging and remained consistently high over 2 passages. Two independent experiments demonstrate average growth percentages per passage of 78.1 ± 5.4, 85.2 ± 1.8 (mean ± SD; n = 2). **C)** Timecourse images of small intestinal organoids from primary culture as single cells at day 1, forming cystic organoids at day 3 and establishing complex budding at day 7. Scale bar = 200 μm.

Intestinal Cell Types

The adult intestine consists of a self-renewing stem cell population and four differentiated cell types. Each has been characterized with markers that are visible through immunohistochemistry. The stem cell population at the crypt base expresses the Wnt target, Lgr5. The absorptive enterocytes (EC), which line the intestine, express the Villin (Vil) protein in a polarized fashion on the apical side of the epithelium. The secretory goblet cells (GC) and enteroendocrine cells can be visualized by their products, mucus (Muc2) and intestinal hormone Chromogranin A (ChgA), respectively. The Paneth cells, intercalated between stem cells at the base of the crypt, secrete antibacterial enzymes such as Lysozyme (Lys).

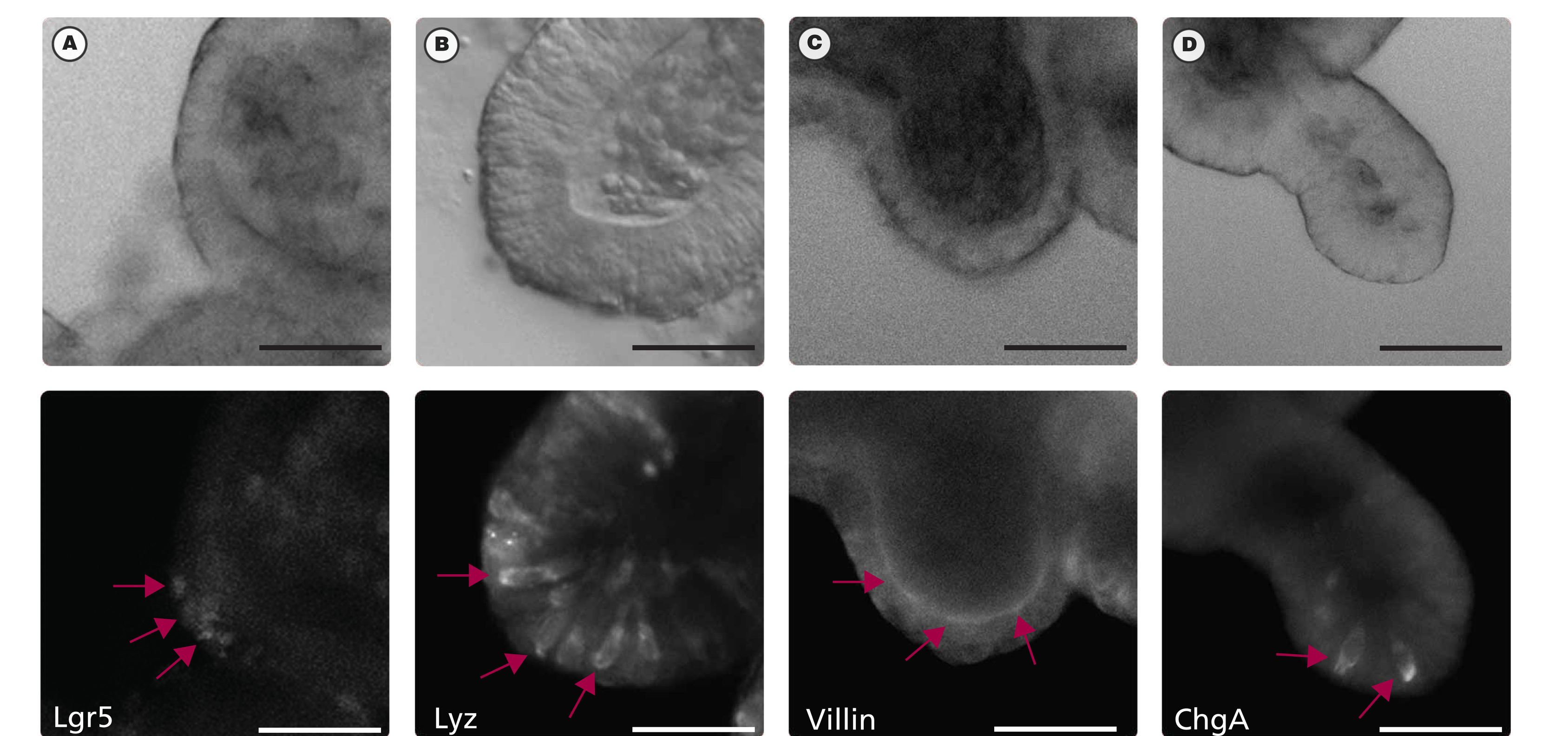

Figure 7. Day 5 intestinal organoids grown from primary crypt cultures maintain markers of the mammalian intestine. Brightfield (top) and corresponding immunofluorescence images (bottom) demonstrating the localization (arrows) of **(A)** intestinal stem cells (Lgr5), **(B)** Paneth cells (Lyz), **(C)** polarized enterocytes (Villin) and **(D)** enteroendocrine cells (ChgA). Scale bar = 200 μm.

Conclusions

IntestiCult™ Organoid Growth Medium:

- Complete, serum-free and defined cell culture medium for mouse intestinal organoids
- Supports maintenance of Lgr5+ stem cells within organoids for multiple passages
- Maintains growth of all native intestinal cell types and produces organoids containing all mature intestinal cell types
- Supports organoid growth from isolated crypts or single cells from the small intestine as well as colonic crypts

[1] Barker, N. and Clevers, H. Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138(5): 1681-96, 2010.
[2] Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(14): 262-66, 2009.