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Rapid establishment of murine gastrointestinal organoids using mechanical isolation method

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ABSTRACT

Gastrointestinal (GI) diseases, including pathological dysplasia, inflammation, neoplasia and injury, suffer millions of patients globally per year. Organoids, three-dimensional cell mass structures supported by biomaterials in dishes, are currently used as a research model for diseases of the small intestine. However, the traditional enzymatic-digestion method for establishing small-intestinal organoids (EnzyOs) is time consuming and often loses the bulk of crypts, a more efficient and reliable method needs to be developed. In this study, using mouse GI organoids as a model, we formulated a rapid mechanical isolation method that could efficiently isolate and culture villi-crypts into small intestinal organoids (MechOs). Primary duodenum organoids generated by MechOs displayed three types of morphology: spheroid, semi-budding and budding, while EnzyOs produced much less budding. Moreover, primary duodenum organoids from MechOs could be subcultured and presented similar gene expression profiles of small intestine specific markers as that from EnzyOs. Importantly, the MechOs method could also be used to generate other types of organoids derived from the stomach, jejunum –ileum, sigmoid–rectum and bile cysts. Taken together, the results show that MechOs could efficiently and economically generate digestive system organoids, providing a potential basis of epithelial organoids for the clinical treatment of gastroenterological diseases.

1. Introduction

The alimentary canal is a long muscular tube (including the oral cavity, pharynx, esophagus, stomach, small intestine, colon, rectum and anus) that responsible for food delivery, nutrient absorption, water–electrolyte balance and waste excretion. High-risk pathological factors (*e.g.*, abnormal temperature, microbial infection, etc) can easily disrupt digestive system homeostasis, causing inflammation, neoplasia or even cancer. These complicated and overwhelming stresses result in millions of people suffering from conditions such as gastric and duodenal ulcers, adenocarcinoma, and inflammatory bowel disease every year [1–3]. To combat these digestive problems, many disease models have been developed *in vivo* and *in vitro* over the last few decades, including patient-derived cancer (PDC) cell lines and patient-derived xenografts (PDXs). PDC cell lines permit the culturing of indefinite cancer cell lines from patients for high-throughput drug screening and disease modeling [4]. PDXs allow the simulation of tumor development, invasion process and pathological outcomes via allogeneic transplantation [5–10]. However, the limitation of these methods is caused by cell line variations, long periodical cycles and high cost [7,11], which impede their general application in gastroenterological disease research.

An organoid is a three-dimensional cell mass supported by biomaterials in a dish that is able to highly recapitulate and

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maintain structural features, genetic stability and physical functions of primary tissues in site. Recent studies have shown that organoids derived from pluripotent or adult stem cells can be cultured to model gastrointestinal epithelium with long-term expansion of primary epithelial cells *in vitro*, which have been widely used for studying gastrointestinal physiology and pathophysiology [12–17]. To do so, Clevers's group has successfully established a traditional method to isolate and cultivate murine small intestinal villi-crypts into organoids *ex vivo* [18], which indicates that the strong expansion and proliferation abilities of small intestinal organoids are largely dependent on *Lgr5*$^{+}$ (Leucine-rich repeat containing G protein coupled receptor 5)-adult stem cells existing below paneth cells at the bases of villi-crypts. Namely, isolating villi-crypts is a key step in establishing these organoids. However, increasing evidence has shown that when ethylenediamine-tetraacetic-acid (EDTA) incubation or collagenase digestion is used to loosen epithelium and release villi-crypts in this traditional method, many villi-crypts are lost and very few left for organoids culture and formation [18,19].

In this study, to better isolate small intestine villi-crypts from epithelium niches and to augment the efficiency of organoids establishment *in vitro*, we developed a rapid mechanical isolation method (MechOs), along with the traditional enzyme releasing with EDTA/collagenase digestion (EnzyOs), to isolate and culture mouse duodenum villi-crypts into organoids. We compared the difference of organoids generated by these two methods in morphology, yield and specific gene expression patterns. In addition, we also explored the potential application of MechOs in establishing other digestive organ epithelial organoids including duodenum development. Taken together, our results shed new light on the rapid establishment of *in vitro* digestive system organoids and provide potential strategies for disease modeling and clinic therapeutic treatment.

2. Materials and methods

2.1. Ethical statement

All procedures performed in the studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Fudan University (Approval No. 20190221–026).

2.2. Animals

C57BL/6 mice (SPF, female) aged from 0 to 33 weeks were purchased from Shanghai Lingchang Biotech LTD. Raise mouse in SPF condition and shift light switch each 12 h. Agreement and permission of experimental animals were obtained from the local Institute of Animal Ethnical Committee.

2.3. Culture medium

Advanced DMEM (Advanced DMEM/F12 + 20% FBS), basal medium (Advanced DMEM/F12 + 1% HEPES+ 1% L-glutamine), digestion medium, completion DMEM (Advanced DMEM/F12 + 1% FBS), and WRN conditioned medium was made as described previously [18].

2.4. Preparation of organoids from mechanical isolation method (MechOs) and enzyme digestion method (EnzyOs)

Briefly, for MechOs, incubate pieces with 1 × cold Chelating Buffer containing 2 mM EDTA (Sangon Biotech) at 4 °C and 20–30 rpm rotate for 30 min. Then, aspirate the supernatant and transfer the tissue pieces into a new 10 cm dish, press it with a sterile cover glass plate, squeeze the pieces gently for 5 times to release villi-crypts from niches. To collect villi-crypts, rinse glass plate with 10 ml cold Basal Medium and transfer the villi-crypts suspension to a new 15 ml conical tube on ice with filtering through 70 μm strainer (fisherbrand) for purity. For EnzyOs, incubate pieces with 10 ml Digestion Buffer and rotate (50–60 rpm) for 2 h at room temperature. Then, aspirate the supernatant and wash the small pieces with Completion DMEM for 5 times (300×*g*, 4 °C, 5 min) to release villi-crypts; re-suspend the villi-crypts pellet with Sorbitol DMEM and then filter through 70 μm strainer to avoid tissue contamination.

Before seeding the droplets, count the concentration of the villi-crypts suspension and centrifuge at 300×*g*, 4 °C for 5 min. To get a 3-dimensional structure for organoids growth, re-suspend the villi-crypts pellet with 1 × Matrigel® Matrix (Phenol-Red Free, CORNING) and seed in pre-warmed droplets, reverse the plate and incubate it in 37 °C, 5% CO_2, humidified atmosphere for 20 min to promote arch-dome droplets solidifying. Lastly, immerse Matrigel-villi-crypts complex dome droplets with 50% WRN conditioned medium and back the plate to the incubator for cultivation.

Immunofluorescent staining, RNA extraction and quantitative PCR were performed as described previously were performed as described previously [12].

2.5. Generation of primary organoids from corpus stomach, antrum stomach, jejunum-ileum, sigmoid-rectum, and bile cyst using MechOs in vitro

Establishment of jejunum-ileum organoids are same as described in duodenum MechOs. For sigmoid-rectum organoids, cut approximately 5 cm sigmoid-rectum off from the intestine tube. The others are same as duodenum MechOs. For bile cyst organoids, utilize sterile utensils to take the whole bile cyst off and cut it open gently to drop off juice under the microscope. Wash the bile cyst with cold chelating buffer for 3 times. Transfer the tissue into a new dry sterile 10 cm dish, then cover the cyst with a sterile glass cover plate in a dish on ice, and squeeze the tissue vigorously for 3 times. Suspend the bile cyst debris with Basal Medium and mix it gently in 10 cm dish. Pick out big bile cyst pieces and transfer the suspension into a new 15 ml conical tube. Centrifuge it directly at 300×*g*, 4 °C for 5 min without filtering. For corpus and antrum stomach organoids, isolate mouse corpus stomach and antrum stomach; remove fatty and blood vessels under microscope. Steps for glands isolation, embedding and seeding are same as duodenum MechOs. Finally, add growth medium with human recombinant accordingly.

2.6. Sub-culture and cryopreserve organoids

Mouse duodenum organoids could be sub-cultured by mechanical disrupting regardless of its origin. Firstly, change medium with Basal Medium, then pipet up and down vigorously for 15 times to disrupt the organoids into debris. Aspirate the organoids debris suspension and transfer to a 15 ml conical tube on ice for 5 min to melt Matrigel. Pipet vigorously for 5 times again and centrifuge at 400×*g*, 4 °C for 5 min. For sub-culture, the next steps are same as the primary organoids. Generally, passage organoids at a ratio of 1: 2 every 3 days. For cryopreservation, remove the supernatant as much as possible; re-suspend the organoids pellet with Cryopreservation Medium. Store it at - 80 °C, transfer it to liquid nitrogen cabinet for long term storage.

2.7. Statistical analysis

Data analysis is performed by GraphPad Prism 8, Las AF Lite, and

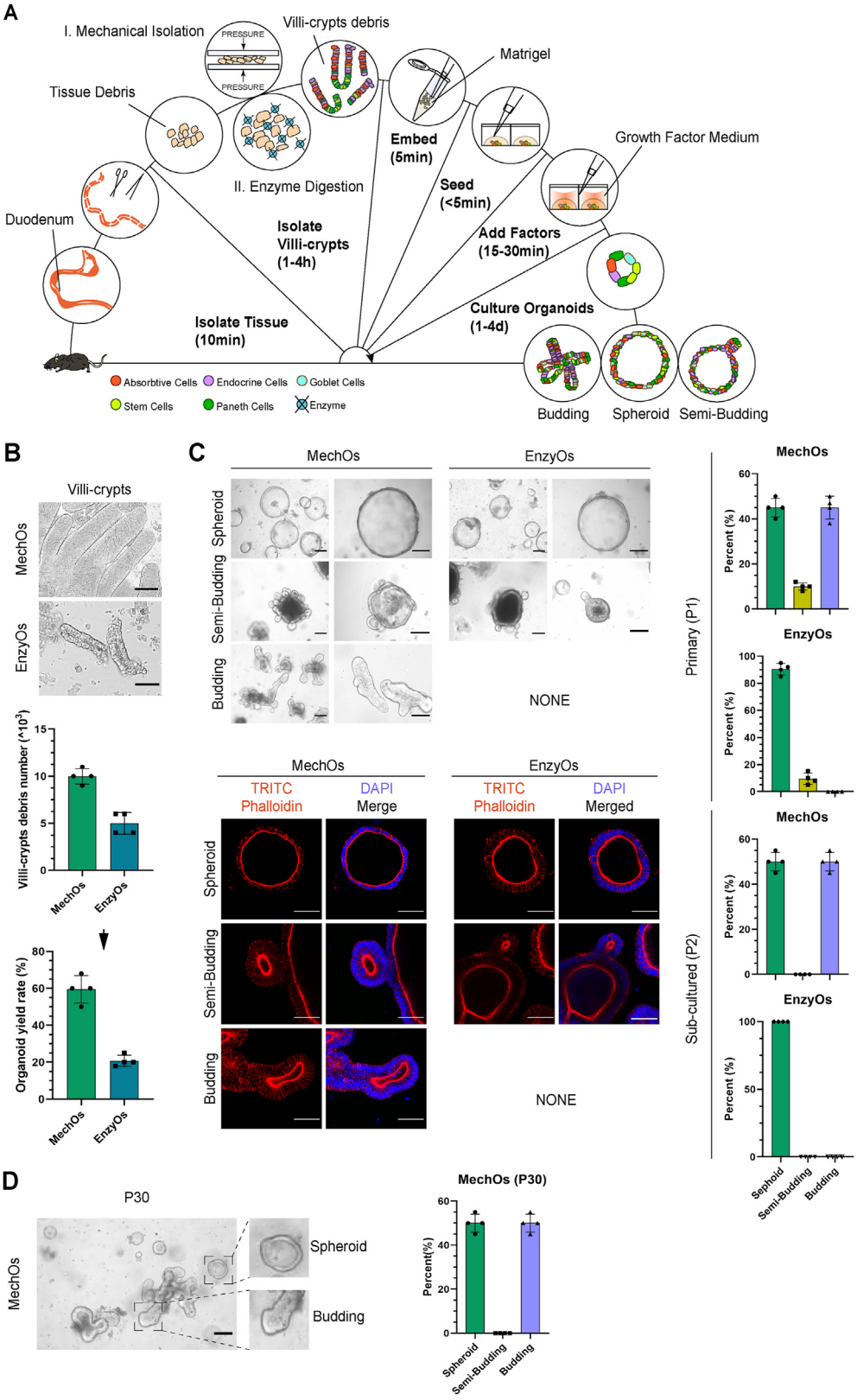

Fig. 1. Generation of mouse duodenum organoids by MechOs and EnzyOs.
(**A**) Schematic for construction of mouse duodenal organoids by MechOs and EnzyOs. Key steps included mouse sacrifice, disassociation of the duodenum, villi-crypts isolation, embedding with Matrigel, seeding villi-crypts with Matrigel droplets and adding conditioned medium. (**B**) Quantification and morphology of villi-crypts debris and organoids yield generated by MechOs or EnzyOs. *Middle panel,* average number of villi-crypts debris was calculated from four repetitions. *Bottom panel,* quantification of organoid yield from 100 villi-crypts debris generated by MechOs or EnzyOs; Scale bar, 100 μm. (**C**) Representative organoid morphology from MechOs and EnzyOs. *Right panel:* Relative percentages of spheroid, semi-budding and budding organoids in primary (P1) culture and second-passage (P2) subculture were quantified. *Bottom panels:* Representative organoid with nucleus and cytoskeleton stained by DAPI and phalloidin, respectively. (**D**) Representative morphology of mouse subcultured (P30) MechOs. *Right panel:* Relative percentages of organoids in thirtieth passage (P30) were quantified. Scale bar, 50 μm.

Bio-Rad CFX Maestro programs. p value < 0.05 is considered significant.

3. Results

3.1. MechOs but not EnzyOs yields budding morphology of mouse duodenum organoids

To better generate mouse duodenal organoids, we used MechOs along with the traditional enzymatic-digestion method (EnzyOs) to release villi-crypts from murine duodenal epithelial villi-crypts niches (Fig. 1A). The results showed that in the MechOs group, almost all integrated villi-crypts appeared as U-shapes after they were squeezed out of the epithelial niches, while a great variety of villi-crypts were cracked and broken in the EnzyOs group (Fig. 1B). Importantly, because villi-crypts could be further disrupted after suspension and filtering (particularly the incomplete and fragile debris found in the EnzyOs group), we quantified villi-crypts debris in both groups. The results showed that MechOs produced approximately twice as many villi-crypts debris as EnzyOs (Fig. 1B, middle panel), and about 59.5% of villi-crypts grew into organoids in the MechOs group, while only 20.75% did so in the EnzyOs group (Fig. 1B, bottom panel).

Interestingly, primary organoids in the MechOs group displayed three types of morphological patterns: spheroid, semi-budding and budding. Meanwhile, those in the EnzyOs group presented spheroid and semi-budding forms only, with no budding (Fig. 1C). Buds from either semi-budding or budding organoids are assumed as U-shape in duodenum epithelial villi-crypts *in vivo* (Fig. 1C, top panel; an organoid with nuclear and cytoskeletal staining is shown in the bottom panels).

To further analyze the differences between MechOs and EnzyOs, we also subcultured both types of organoids via vigorous pipetting to create an amplification culture. As shown in Fig. 1C (right panels), in the MechOs group, we observed that semi-budding organoids accounted for only 10% of primary organoids, while almost no semi-budding grew up after subculture. In contrast, both spheroid and budding organoids remained the majority of organoids (from 45% *vs.* 45%–50% *vs.* 50%, respectively) in the primary and subcultured organoids. In the primary EnzyOs group, semi-budding organoids accounted for nearly 10% of all organoids; the other 90% were spheroid. When passaged, all duodenal organoids were spheroid, none of them semi-budding, and we observed no budding organoids in either the primary or subcultured group.

3.2. MechOs and EnzyOs had similar small intestinal gene expression profiles

To learn whether MechOs could functionally and genetically mimic the duodenum in site, and whether they differed from EnzyOs, we attempted to detect their specific murine-duodenum gene expression patterns, including gene markers for goblet cells (*mMUC2* and *mTFF3*) [20], intestinal cells (*mCDX1*, *mCDX2* and *mLyz*) [21,22], Paneth cells (*mReg3γ* and *mMMP7*) [20,23], stem cells (*mSOX9* and *mLgr5*) [24,25], and cell proliferation (*mKi-67*) [26]. As shown in Supplementary Fig. S1, although expression of most of these duodenum-specific genes was relatively lower in organoids than in the duodenum in site (except for *mKi-67*, which was higher in EnzyOs), no significant difference between MechOs and EnzyOs wer observed in comparison with the duodenum in site.

To further evaluate the genomic stability of duodenal organoids created by MechOs, we passaged primary organoids *in vitro* for expansion. Unexpectedly, we found that semi-budding organoids were almost completely lost after MechOs long-term passage (30 times, 3 months), with only spheroid and budding forms remaining, in contrast with primary organoids (Fig. 1D). To deeply explore whether the *in vitro* passaged MechOs remained genetic stable, we extracted total ribonucleic acid (RNA) from both primary organoids and the thirtieth passage of MechOs to analyze small-intestinal gene expression via qPCR. The results showed that there was no significant difference in the expression of most duodenum specific genes between primary and thirty times passaged duodenum organoids, although the expression of *mMuc2*, *mLyz* and *mPdx1* was relatively lower in the sixth-passage group (Supplementary Fig. S2). This indicated that mouse duodenum organoids cultured and expanded over long term *in vitro* using MechOs can stably maintain genetic profiles in dishes.

3.3. Development of mouse duodenum impaired production and morphology of organoids generated by MechOs

To analyze the effect of mouse duodenal development on the production and morphology of organoids, we individually isolated infant (0 w), adult (9 w) and elderly (33 w) mouse villi-crypts and cultured them into organoids via MechOs. The results showed that all villi-crypts from mice at all three stages of life retained their "U-clip" structure, and the number of villi-crypts debris was approximately 10×10^3 on average regardless of mouse age (Fig. 2A, right panel). However, the yield of duodenal organoids from the infant group was much higher than that from the adult and elderly groups (71.25% *vs.* 57.5% and 51.5%, respectively; Fig. 2A, right panel). Unexpectedly, when we cultured and subcultured these primary organoids, they presented different features. The ratio of spheroid to budding infant duodenal organoids created by MechOs remained at nearly 9:1 in both the primary and subcultured groups, while semi-budding organoids were rarely observed at all (Fig. 2B, top panels). For adult duodenal MechOs, the spheroid: semi-budding: budding ratio varied from nearly 4.5: 1: 4.5 to 5: 0: 5 in the primary and subcultured groups (Fig. 2B, middle panels). Elderly duodenal MechOs displayed a pattern similar to that of their adult counterparts, with spheroid: semi-budding: budding ratios of 5: 1: 4 and 4.5: 0: 5.5 in the primary and subcultured groups, respectively (Fig. 2B, bottom panels).

To address whether gene expression profiles in MechOs differed depending on its origin (infant, adult or elderly mouse duodenum), we also detected the expression of mouse intestine specific genes simultaneously. Interestingly, we found that both goblet cell markers and Paneth cell marker were dramatically downregulated as age increased (Fig. 2C, top panels), while intestinal markers, aneth cell marker and stem cell marker were downregulated in the elderly group only. In contrast, expressions of intestinal marker, pancreas–duodenum marker, stem cell marker, proliferation marker were greatly upregulated in both adult and elderly duodenal organoids, even though upregulation in the adult group was much higher than in the elderly group. In addition, no significant difference in intestinal stem cell marker was observed among all three MechOs groups. These findings suggested that organoids cultivated and expanded using MechOs can highly display the mouse duodenum physical active functions of different ages *in vitro*.

3.4. Duodenum MechOs differed from jejunum-ileum MechOs in infant mice

Considering that the high yield rate of mouse infant duodenal organoids indicated strong self-renewal and proliferative abilities, to identify whether the MechOs process impaired the physical activities of mouse small intestinal organoids from different parts, we separately isolated villi-crypts from infant duodenum and jejunum-ileum and cultured them into organoids. We isolated

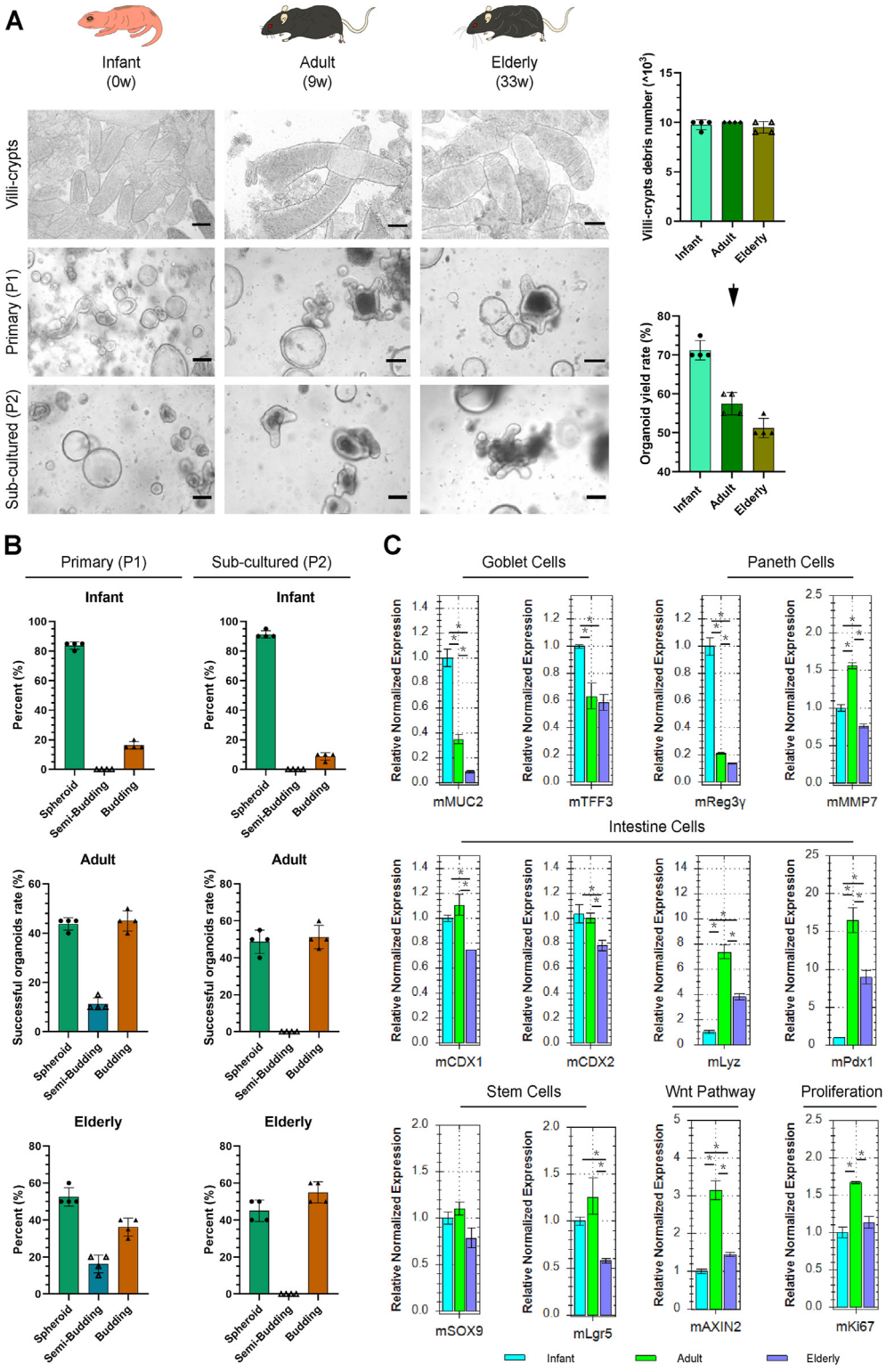

Fig. 2. Development of mouse duodenum impaired production and morphology of organoids by MechOs.
(A) Representative morphology of duodenal villi-crypts derived from mice ranging in age from infant to elderly, and their organoids (P1, P2) generated by MechOs. *Right panel:* Relative percentage of villi-crypts debris and organoid yield were quantified; n = 4 mice. Scale bar, 50 μm. (**B**) Relative percentages of spheroid, semi-budding and budding P1 and P2 organoids in primary culture were quantified. (**C**) Profiles of duodenum-specific gene expression in primary organoids from infant, adult and elderly mice. *$p < 0.05$.

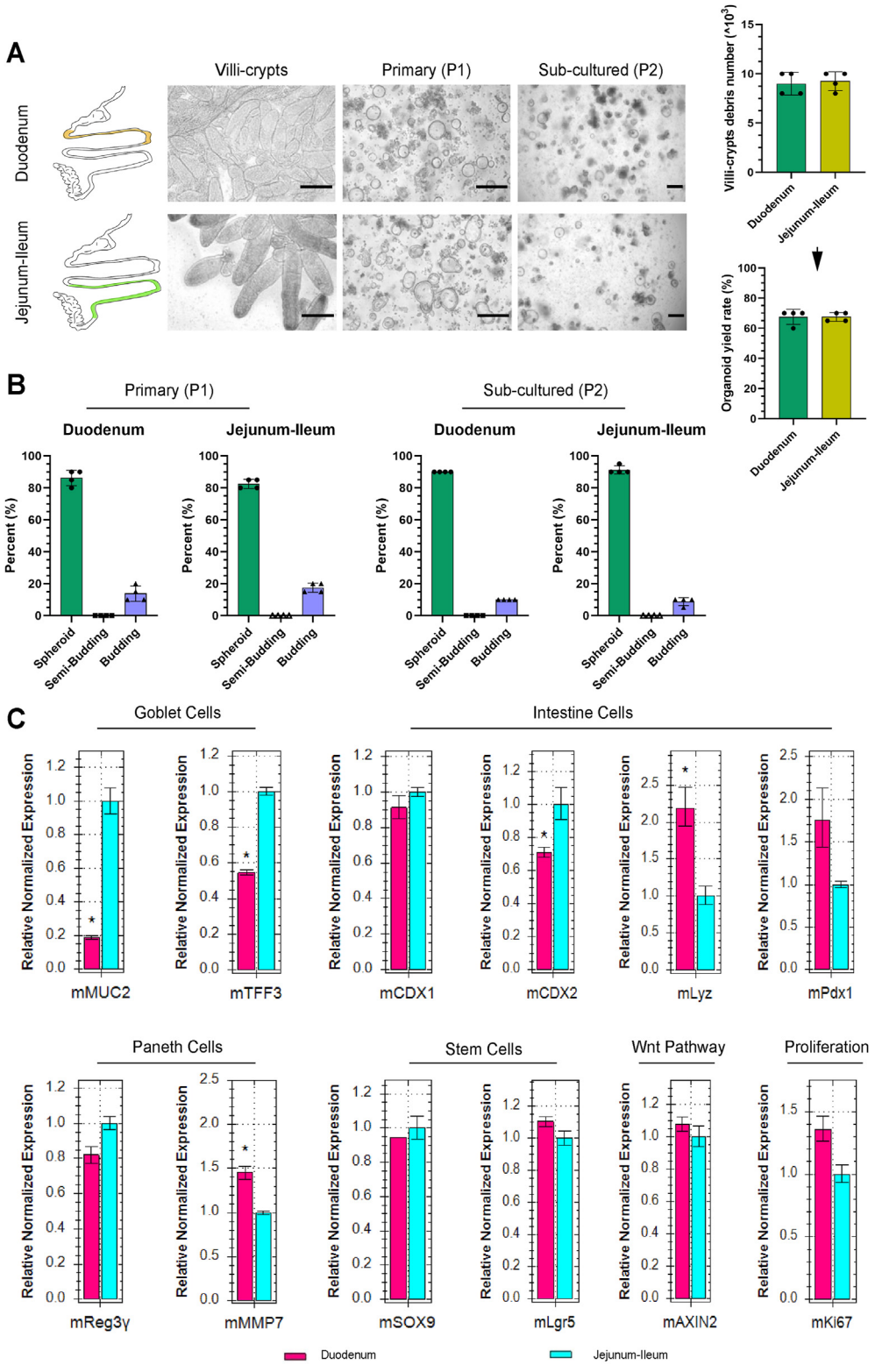

Fig. 3. Duodenum MechOs differed from jejunum–ileum MechOs in infant mice.
(A) Schematic representation of villi-crypts and of primary and subcultured MechOs from infant mouse duodenum and jejunum–ileum. *Right panels:* Relative number of villi-crypts debris and organoid yield from infant mouse duodenum and jejunum–ileum. Scale bar, 100 μm. (**B**) Relative percentages of spheroid, semi-budding and budding P1 and P2 organoids were quantified. (**C**) Expression profiles of specific genes in duodenum and jejunum–ileum MechOs. *$P < 0.05$.

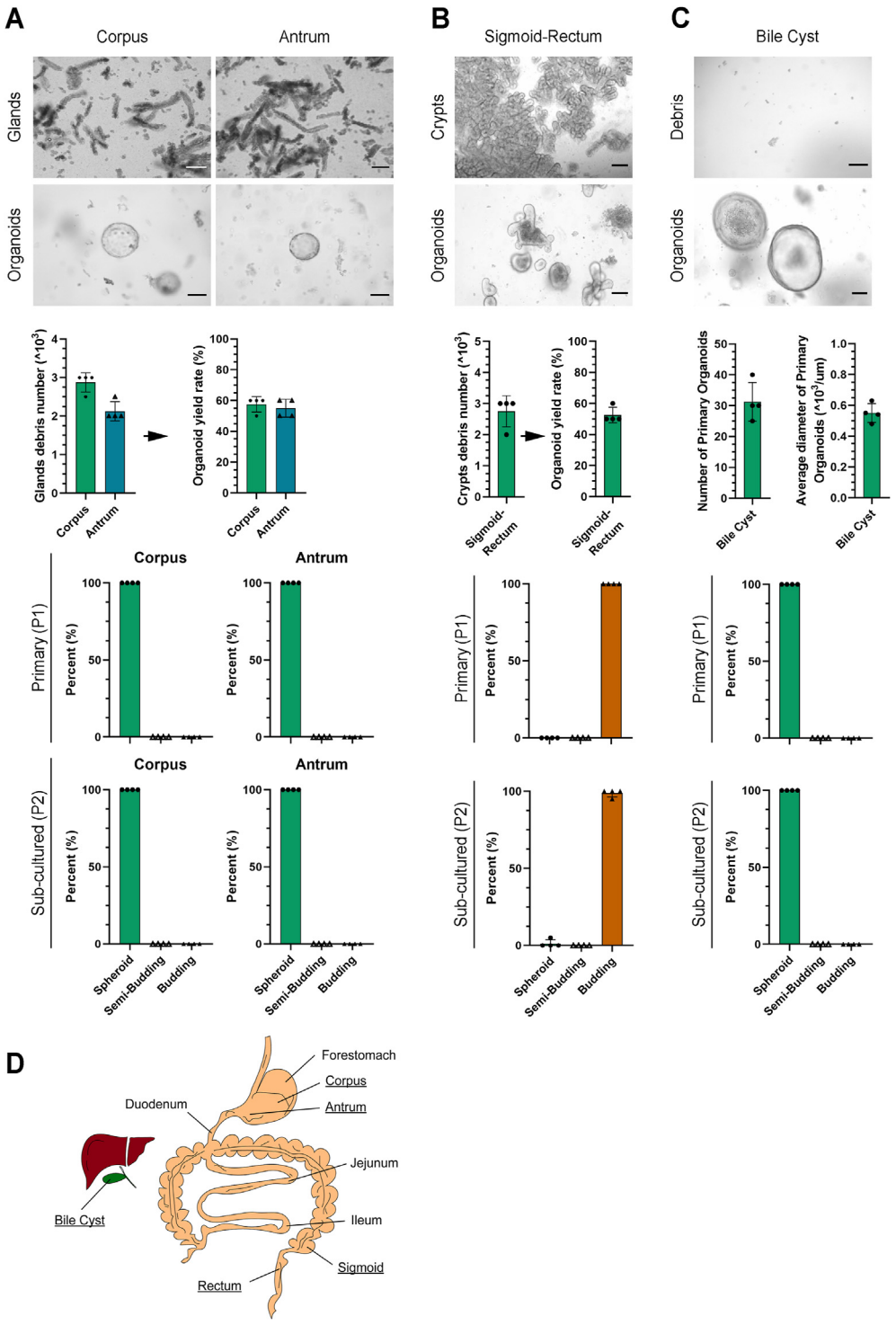

Fig. 4. Establishment of other mouse digestive system organoids using MechOs.
(**A**) Features of gastric-corpus and gastric-antrum glands and organoids produced via MechOs. Scale bar, 100 μm. (**B**) Features of crypts and organoids from sigmoid-rectum tissue produced via MechOs. Scale bar, 100 μm. (**C**) Features of epithelial cells and organoids from bile cyst tissue produced via MechOs. Scale bar, 100 μm. (**D**) Schematic representation of how MechOs is suitable for generation of organoids from mouse digestive system.

9.25×10^3 jejunum-ileum villi-crypts debris, nearly as many as in our duodenum experiment, and 67.5% of them could be successfully cultured into organoids (Fig. 3A, right panels). The spheroid: semi-spheroid: budding ratio in the morphology of infant jejunum-ileum primary organoids did not significantly change (82.5%, 0% and 17.5% *vs.* 91.25%, 0% and 8.75%, respectively) during subculture (Fig. 3B).

Since we did not observe any apparent difference in morphology between infant duodenal and jejunum-ileum organoids produced by MechOs, we detected the expression of duodenum-specific biomarker genes. Notably, goblet cell markers and intestinal marker were upregulated, while intestinal marker was down-regulated, in jejunum-ileum MechOs compared with duodenum

MechOs (Fig. 3C). This suggested that infant mouse duodenum did not differ genetically from infant mouse jejunum-ileum, especially in terms of genes that controlled goblet cells.

3.5. Establishment of other digestive system organoids using MechOs

To test whether MechOs was suitable and effective for establishing different gastroenterological organoids, we attempted to individually isolate adult stem cells (ASC) from epithelium *in site* by mechanical squeezing. Interestingly, in the upper gastrointestinal tract, we were able to release long integrated stomach epithelial glands and collect approximately 3×10^3 corpus glands debris and 2×10^3 antrum glands debris via MechOs (Fig. 4A, top panels); 57.5% of glands from the gastric corpus glands and 55% from the gastric antrum glands could be successfully cultivated into organoids (Fig. 4A, middle panels). Surprisingly, almost all organoids from both types of gastric glands were spheroid in morphology, no matter they were primary or subcultured (Fig. 4A, bottom panels). In contrast with these glands and with those from the small intestine, the 2.75×10^3 crypts debris isolated from the sigmoid-rectum by mechanical squeezing were relatively short and small. Only about 52% of them could successfully grow into organoids, and all organoids derived from the sigmoid-rectum via MechOs were budding, whether primary or subcultured (Fig. 4B).

In addition, only approximately 30 primary organoids could be derived from an entire bile cyst, and the diameters of these organoids averaged up to 550 μm 5 days post-seeding (Fig. 4C). Bile cyst organoids presented as spheroid whether they were primary or subcultured, which was reminiscent of bile cysts wrapped by the liver in the abdominal cavity.

4. Discussion

Although the traditional EnzyOs method has enabled most researchers to establish and expand organoids over the long term *in vitro*, yield is highly relied on villi-crypts releasing factors, including temperature, time and enzyme type [27,28]. To improve the villi-crypts releasing rate, in this study, we established a practical MechOs method and compared it with the traditional EnzyOs method for preparing adult mouse duodenal organoids. Interestingly, we found that MechOs allowed almost all integrated duodenal villi-crypts to be squeezed out of the epithelial niches, and approximately 60% of these villi-crypts debris could successfully grow into organoids. By contrast, EnzyOs failed to release the bulk of villi-crypts, and only 20% could be grown into organoids. It is worth mentioning that no significant difference between MechOs and EnzyOs in duodenum specific gene expression profiles were observed, while MechOs save time (~10 min *vs* > 2 h for villi-crypts releasing) and materials, produce a high yield, and maintain the stability of most of their genes after subculture and expansion *in vitro*.

In summary, we successfully established a practical MechOs method to rapidly generate several types of digestive organoids, including stomach, small intestine, colon, rectum and bile cyst *in vitro*, which provides a potential tool for other adult epithelial organoids cultivation and disease modeling in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.03.151.

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