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Short Communication

Effect of Wnt signaling pathway activation on the efficient generation of bovine intestinal organoids

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ABSTRACT Recent progress has been made to establish intestinal organoids for an *in vitro* model as a potential alternative to an *in vivo* system in animals. We previously reported a reliable method for the isolation of intestinal crypts from the small intestine and robust three-dimensional (3D) expansion of intestinal organoids (basal-out) in adult bovines. The present study aimed to establish next-generation intestinal organoids for practical applications in disease modeling-based host-pathogen interactions and feed efficiency measurements. In this study, we developed a rapid and convenient method for the efficient generation of intestinal organoids through the modulation of the Wnt signaling pathway and continuous apical-out intestinal organoids. Remarkably, the intestinal epithelium only takes 3-4 days to undergo CHIR (1 μM) treatment as a Wnt activator, which is much shorter than that required for spontaneous differentiation (7 days). Subsequently, we successfully established an apical-out bovine intestinal organoid culture system through suspension culture without Matrigel matrix, indicating an apical-out membrane on the surface. Collectively, these results demonstrate the efficient generation and next-generation of bovine intestinal organoids and will facilitate their potential use for various purposes, such as disease modeling, in the field of animal biotechnology.

Keywords: apical-out, bovine, intestinal organoid, Wnt signaling pathway activation

INTRODUCTION

The gastrointestinal (GI) tract plays an important role in increasing productivity and maintaining homeostasis in animals (Haq et al., 2021). Of them, the small intestine consists of the duodenum, jejunum, and ileum. Specifically, the epithelium of the small intestine tract is composed (Haq et al., 2021) of a variety of intestinal cell types (e.g., Paneth cells, enteroendocrine cells, goblet cells and enterocytes). Each cell performs a variety of functions, such as nutrient absorption, electrolyte uptake, hormone secretion and host-pathogen interactions (Olayanju et al., 2019; Lee et al., 2021). However, *in vitro* two-dimensional (2D) culture systems have difficulty expressing cellular diversity in the intestinal epithelium (Hamilton et al., 2018).

Recently, a scaffold-based three-dimensional (3D) culture system has provided a reliable alternative platform for the establishment of intestinal organoids *in vitro*.

Recent progress has been made to establish intestinal organoids in livestock, including bovine (Lee et al., 2021), porcine (Powell et al., 2017), chicken (Pierzchalska et al., 2017) and equine (Stewart et al., 2018). These findings may provide a valuable tool for potential alternatives to *in vivo* systems in the field of animal biotechnology for various purposes. In particular, we previously reported a reliable method for the isolation of intestinal crypts from the small intestine and robust 3D expansion of intestinal organoids (basal-out) in adult bovines (Rallabandi et al., 2020; Lee et al., 2021). However, there are practical limitations associated with the use of intestinal organoids, such as host-pathogen interactions and nutrient absorption, because intestinal organoids cultivated in Matrigel all have basal-out structures. Thus, development of apical-out organoid culture system (polarity reversal) is required.

The Wnt signaling pathway plays a critical role in the self-renewal of intestinal stem cells, and activation of the Wnt signaling pathway is required for the maintenance of function and homeostasis in intestinal organoids (Krausova et al., 2014; Li et al., 2018). The canonical Wnt signaling pathway is the most studied, and these events lead to the inhibition of Axin-mediated β-catenin phosphorylation and the thereby stabilization of β-catenin, which accumulates and translocates to the nucleus to form complexes with TCF/LEF and activates Wnt target gene expression (MacDonald et al., 2009). Generally, secretion of Wnt from Paneth cells in intestinal epithelial cells is enough to support the self-renewal of intestinal stem cells (Sato et al., 2011). In addition, for the establishment of human small intestinal organoids, the addition of Wnt must be required (Sato et al., 2011).

In this study, we first report a rapid, convenient method that enabled us to use for the efficient generation of bovine intestinal organoids by modulating the WNT signaling pathway. Furthermore, we evaluated epithelial barrier function using a FITC-dextran 4 kDa permeability assay and gene expression levels using several specific markers involved in intestinal stem cells and epithelium characteristics. We also established an apical-out bovine intestinal organoid culture system through suspension culture without Matrigel matrix after CHIR treatment.

MATERIALS AND METHODS

Maintenance and cultivation of bovine intestinal organoids

Adult bovine (> 24 months)-derived intestinal organoids were recently established (Lee et al., 2021) and used in this study. Intestinal organoids were subjected to passage approximately once a week upon maturation in 1 mL of intestinal human organoid medium (Stem Cell Technologies). Briefly, the medium was gently aspirated and rinsed with ice-cold PBS without disturbing the organoid dome. To harvest the organoids, a 10× volume of enzyme-free cell disassociation buffer (1 mL) was added to a Matrigel dome (100 μL) in each well and incubated for 10 min in an incubator. Organoids were dislodged by gentle pipetting and collected by centrifugation at 200 × g for 5 min. The pellet was resuspended in the desired amount of medium and Matrigel in a 1:1 ratio, and each well (140-150 organoids) was distributed into three parts in subsequent passages and seeded in 24-well plates. The medium was replaced every 3 days and subcultivated once a week.

Morphological classification of bovine intestinal organoids and CHIR treatment

Bovine intestinal organoids were mainly classified into various morphologies, such as spheroidal (round shaped), stomatocyte, budding (spheroids with extension) and mature villi and crypt-like structures, at each passage (Rozman et al., 2020). The number of organoids according to each morphology was counted at 3-day intervals before passaging. Furthermore, CHIR990221 (Stem Cell Technologies) was used as a Wnt activator and added to the medium in each well of the 24-well plates at various concentrations of 0 μM, 0.1 μM, 1 μM and more. The morphological changes of bovine intestinal organoids were monitored daily under a microscope and counted at 1-day intervals before passaging.

RNA isolation

Total RNA for prepared samples, including intestinal organoids, was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) as described previously (Lee et al., 2007; Lee et al., 2020). RNA quality was assessed by an Agilent 2100 bioanalyzer using an RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using an ND 2000

Spectrophotometer (Thermo Inc., DE, USA).

Quantitative RT-PCR

Quantitative RT-PCR was performed to assess the expression of several markers of intestinal stem cells and epithelium in both CHIR-treated and untreated bovine intestinal organoids (BIO). Each total RNA sample was prepared using TRIzol reagent (Invitrogen, USA). Total RNA (1 μg) was reverse transcribed using the Superscript III First-Strand Synthesis System (Invitrogen). The PCR mixture was prepared by adding 2 μL PCR buffer, 1.6 μL 2.5 mM dNTP, 10 pmol each forward and reverse primer, 1 μL 20 × Eva green, 0.2 μL Taq DNA polymerase, and 2 μL cDNA to a final volume of 20 μL. PCR was performed by means of an initial incubation at 94℃ for 3 min, followed by 40 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s, using a melting curve program (in-increasing temperature from 55 to 95℃ at a rate of 0.5℃ per 10 s) and continuous fluorescence measurement. Sequence-specific products were identified by generating a melting curve. The Ct value represents the cycle number at which a fluorescent signal increases to a level significantly higher than the background, and gene expression was quantified by the 2-ΔΔCt method (Livak et al., 2001). qPCR primers for each target gene and 18S ribosomal RNA (rRNA) in a previous study were used (Lee et al., 2021). Gene expression was normalized to that of bovine 18S rRNA. qPCR analysis of mRNAs was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Generation of apical-out intestinal organoids

Adult bovine-derived intestinal organoids were cultivated in a Matrigel dome. To harvest the organoids, a 10 × volume of enzyme-free cell disassociation buffer (1 mL) was added to a Matrigel dome (100 μL) in each well and incubated for 10 min in an incubator. To generate apical-out intestinal organoids, the organoids were then collected by centrifugation at 200 × g for 5 min. The pellet was seeded in ultralow-attachment 24-well plates using intestinal human organoid medium (Stem Cell Technologies) through suspension culture without Matrigel matrix. The morphology of apical-out intestinal organoids was monitored daily under a microscope to check reversal polarity (Li et al., 2020).

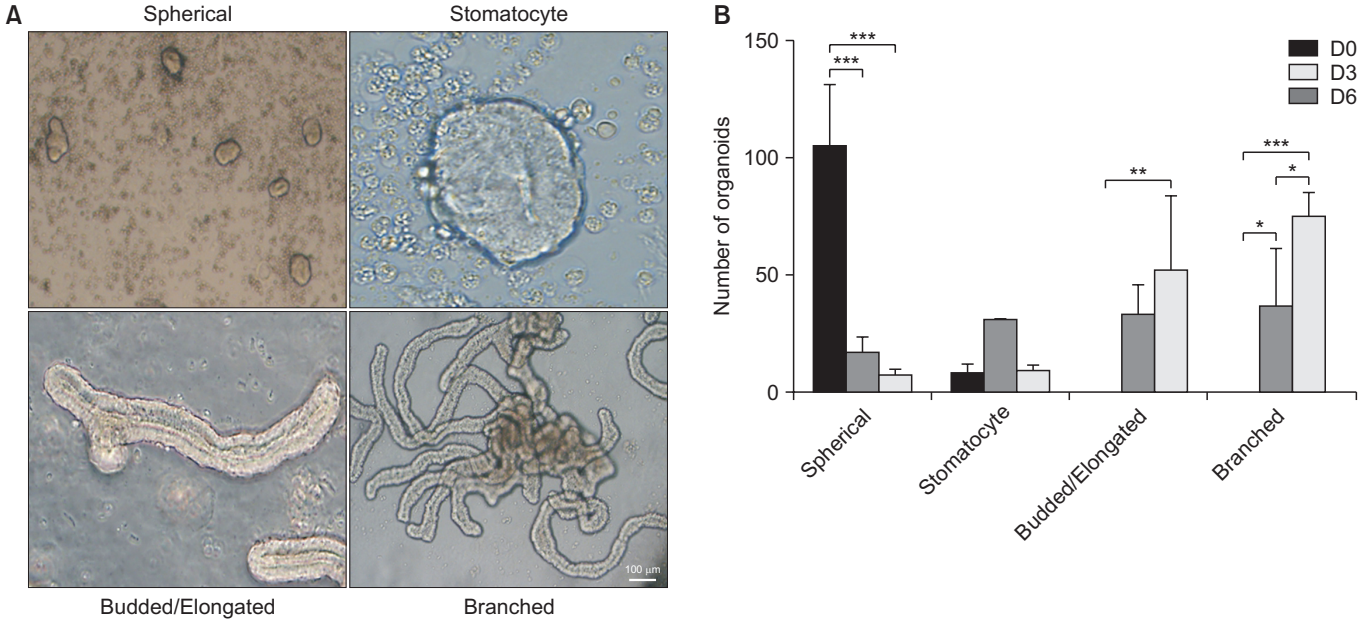

Fig. 1. Morphological classification of bovine intestinal organoids. (A) Bovine intestinal organoids were classified into various morphologies, such as spheroidal (round shaped), stomatocyte, budding (spheroids with extension) and mature villi and crypt-like structures, at each passage. Spherical: 200 μm, Stomatocyte: 50 μm, Budded/Elongated: 100 μm, Branched: 100 μm. (B) The number of intestinal organoids at spontaneous differentiation according to each morphology was counted at 3-day intervals before passaging. Significant differences between groups were analyzed by two-way ANOVA of variance. A p value less than or equal to 0.05 indicated statistical significance (*p value ≤ 0.05, **p value ≤ 0.01, ***p value ≤ 0.001).

Epithelial barrier permeability assay using FITC-dextran

Epithelial barrier function was tested by diluting powdered fluorescein isothiocyanate (FITC)-dextran (4 kDa) (Sigma-Aldrich) in nuclease-free water, which resulted in a 1 mg/mL working solution. Bovine intestinal organoids in both the CHIR treatment and non-treatment groups were placed in 24-well plates and allowed to grow until fully developed into crypt and villi structures. Then, 25 ng/mL FITC-dextran was added to each well, and the plate was incubated under normal growth conditions. The permeability was observed using luminal absorption and recorded for up to 3 hr at 30-min intervals under a Leica CTR6000 fluorescence microscope (Leica, Wentzler, Germany). The fluorescence intensity was calculated using ImageJ software.

Statistical analysis

The significance between groups was analyzed by two-way ANOVA of variance or Student's t test using GraphPad Prism V 6.0 software (San Diego, CA, USA). The results are expressed as the mean ± standard error (n ≥ 3, where n is the number of replicates). The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Morphological classification of bovine intestinal organoids

The recapitulating capacity of the organoids was previ-

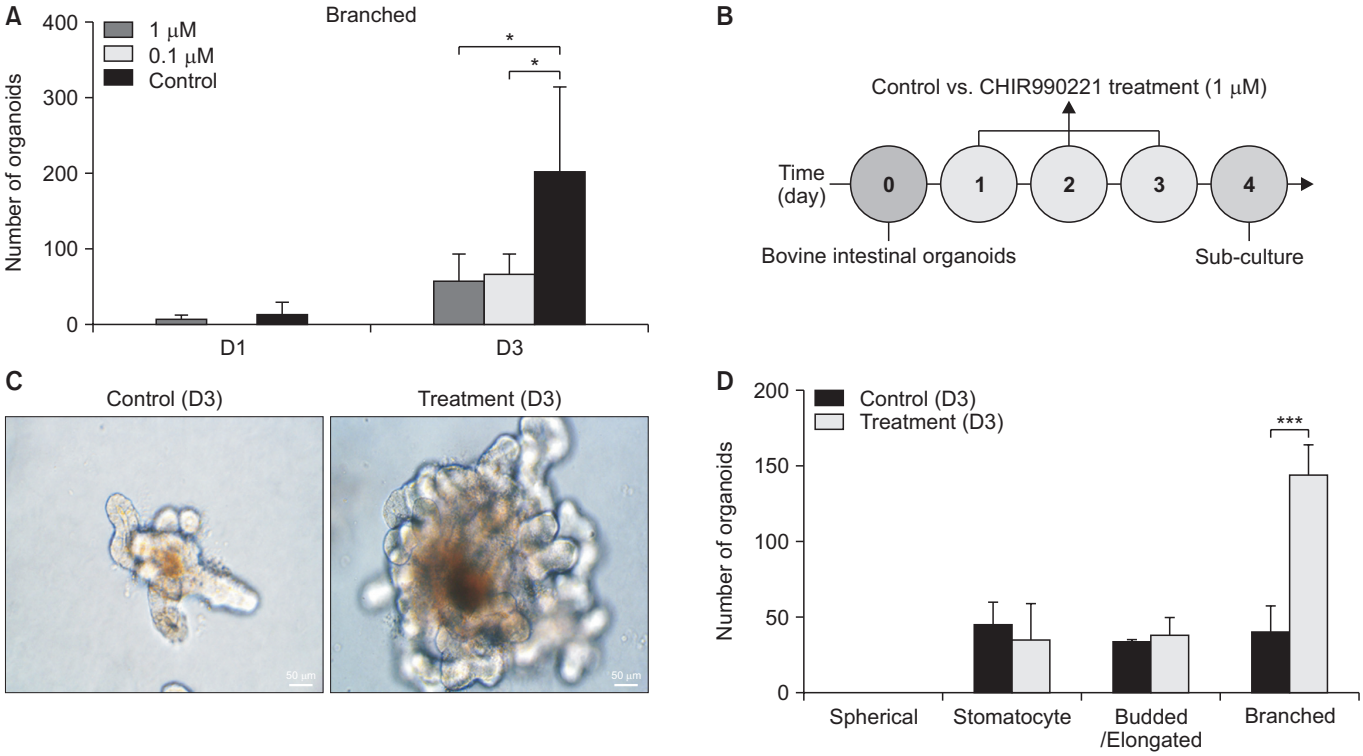

Fig. 2. Effect on WNT activator treatment in bovine intestinal organoids. (A) Search for the optimal concentration of CHIR treatment in bovine intestinal organoids (BIO) that induced morphological changes from spheroidal to branched structures. Treatment with 1 μM CHIR in BIO at Day 3 caused significant morphological changes compared to treatment with 0 μM and 0.1 μM CHIR. Significant differences between groups were analyzed by Student's t test. A p value less than or equal to 0.05 indicated statistical significance (*p value ≤ 0.05). (B) Schematic illustration of the experimental procedures for CHIR treatment and the subculture of bovine intestinal organoids. After treatment with 1 μM CHIR on Day 4, bovine intestinal organoids were subcultured into subsequent passages upon maturation. (C) Representative image of bovine intestinal organoids after treatment with 1 μM CHIR on Day 3. The organoids clearly have mature villi and crypt-like structures (branched structures). Scale bar: 50 μm. (D) The number of bovine intestinal organoids (BIO) after treatment with 1 μM CHIR according to each morphology was counted at 1-day intervals before passaging. Surprisingly, BIO treatment with 1 μM CHIR on Day 3 caused significant morphological changes from spheroidal to branched structures. Significant differences between groups were analyzed by Student's t test. A p value less than or equal to 0.05 indicated statistical significance (*p value ≤ 0.05).

ously demonstrated by the stable growth for more than 10 passages (P10) and the long-term maintenance (Lee et al., 2021), and these were classified into detailed structures such as spheroidal, stomatocyte, budded/elongated and branched structures at each passage from Day 0 to the fully grown structure on Day 7, as shown in Fig. 1A. Furthermore, we monitored and counted bovine intestinal organoids with several characteristic morphologies at 3-day intervals before passaging. Fig. 1B represents the number of organoids according to each morphology, indicating that intestinal organoids were mainly spheroidal structures at Day 0 and branched structures at Day 7, while they had stomatocyte, budded/elongated structures at Day 3 at each passage. Collectively, these results show that adult bovine-derived intestinal organoids were substantially grown by experiencing spheroidal, stomatocyte, budded/elongated and branched structures at each passage.

Effect on Wnt activator treatment in bovine intestinal organoids

In this study, we set out to search for the optimal concentration of CHIR treatment in bovine intestinal organoids (BIO) that induced morphological changes from spheroidal to branched structures. First, we added daily CHIR as a Wnt activator to the medium in each well of the 24-well plates at various concentrations of 0 μM, 0.1 μM, 1 μM and more and monitored the cells under a microscope at 1-day intervals. Surprisingly, we found that treatment with 1 μM CHIR in BIO at Day 3 caused significant morphological changes in spheroidal into branched structures compared to treatment with 0 μM and 0.1 μM CHIR (Fig. 2A). In addition, treatment with more than 1 μM CHIR in BIO had severe toxicity and the resultant lethality. To examine whether treatment with 1 μM CHIR in BIO is expandable, we sub-cultured cells into subsequent passages upon maturation (Fig. 2B), indicating that treatment with 1 μM CHIR in BIO was successfully expandable. Furthermore, we counted at 1-day intervals before passaging after treatment with 1 μM CHIR in BIO. As shown in Fig. 2C and 2D, treatment with 1 μM CHIR in BIO at Day 3 efficiently induced the branched structure. Consistent with the spontaneous differentiation of BIO, intestinal organoids treated with 1 μM CHIR were classified

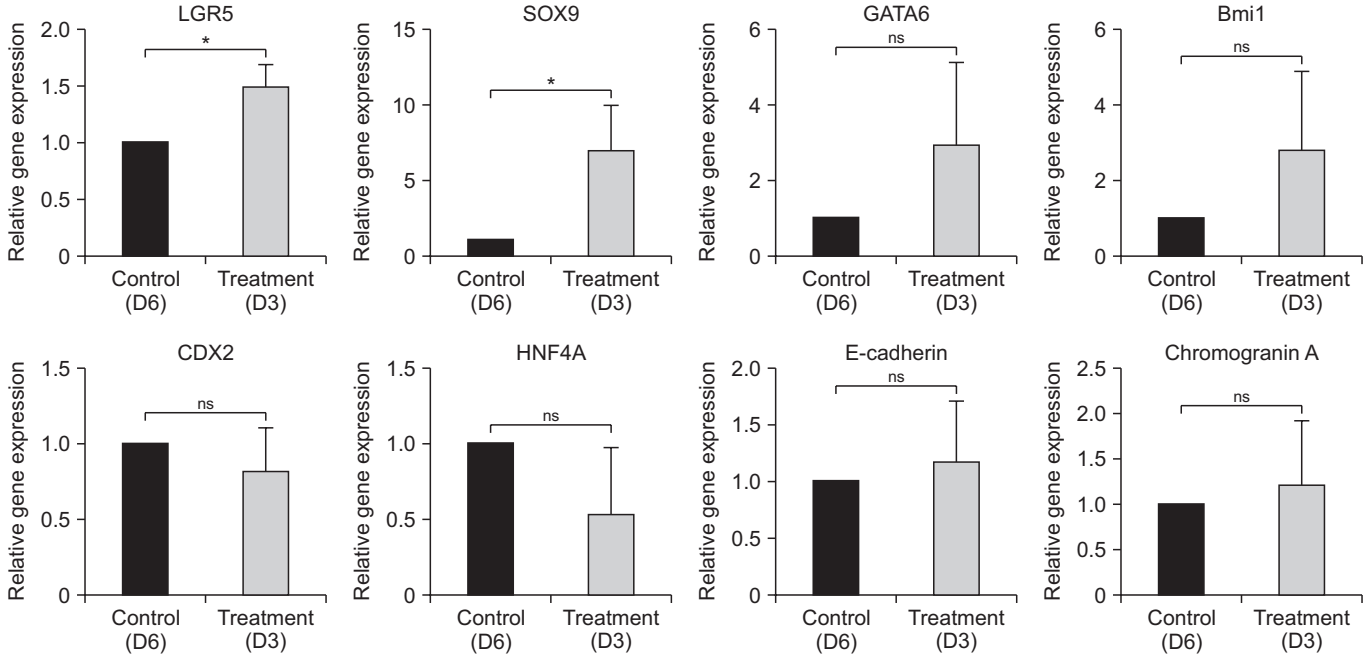

Fig. 3. Gene expression profiling of bovine intestinal organoids after treatment with 1 μM CHIR. Gene expression profiling of bovine intestinal organoids after treatment with 1 μM CHIR using quantitative RT-PCR. Quantitative RT-PCR was performed to evaluate the gene expression of bovine intestinal organoids (BIO) using several markers of intestinal stem cell (LGR5, GATA6, HNF4A, Bmi1, CDX2 and SOX9) and epithelium (Chromogranin A and E-cadherin) characteristics with those of spontaneous differentiation at Day 6 as a control. Gene expression was normalized to that of 18S rRNA and analyzed by the 2-ΔΔCt method. Significant differences between groups were analyzed by Student's t test. A *p* value less than or equal to 0.05 indicated statistical significance (*p value ≤ 0.05).

into detailed structures, such as spheroidal, stomatocyte, budded/elongated and branched structures, at each passage from Day 0 to the fully grown structure on Day 3, as shown in Fig. S1. Taken together, our results clearly demonstrate that treatment with 1 μM CHIR in BIO induced the differentiation and morphological changes of spheroids into branched structures at a significant level.

Gene expression profiling and generation of apical-out intestinal organoids

Next, to characterize the genetic potential of bovine intestinal organoids derived from the small intestines of adults after treatment with 1 μM CHIR, we investigated the spatial expression of several specific markers involved in intestinal stem cell and epithelium characteristics. As shown in Fig. 3, the treatment of 1 μM CHIR in BIO at Day 3 regarding intestinal stem cell-related genes such as LGR5 ($p < 0.05$) and SOX9 ($p < 0.05$) was significantly

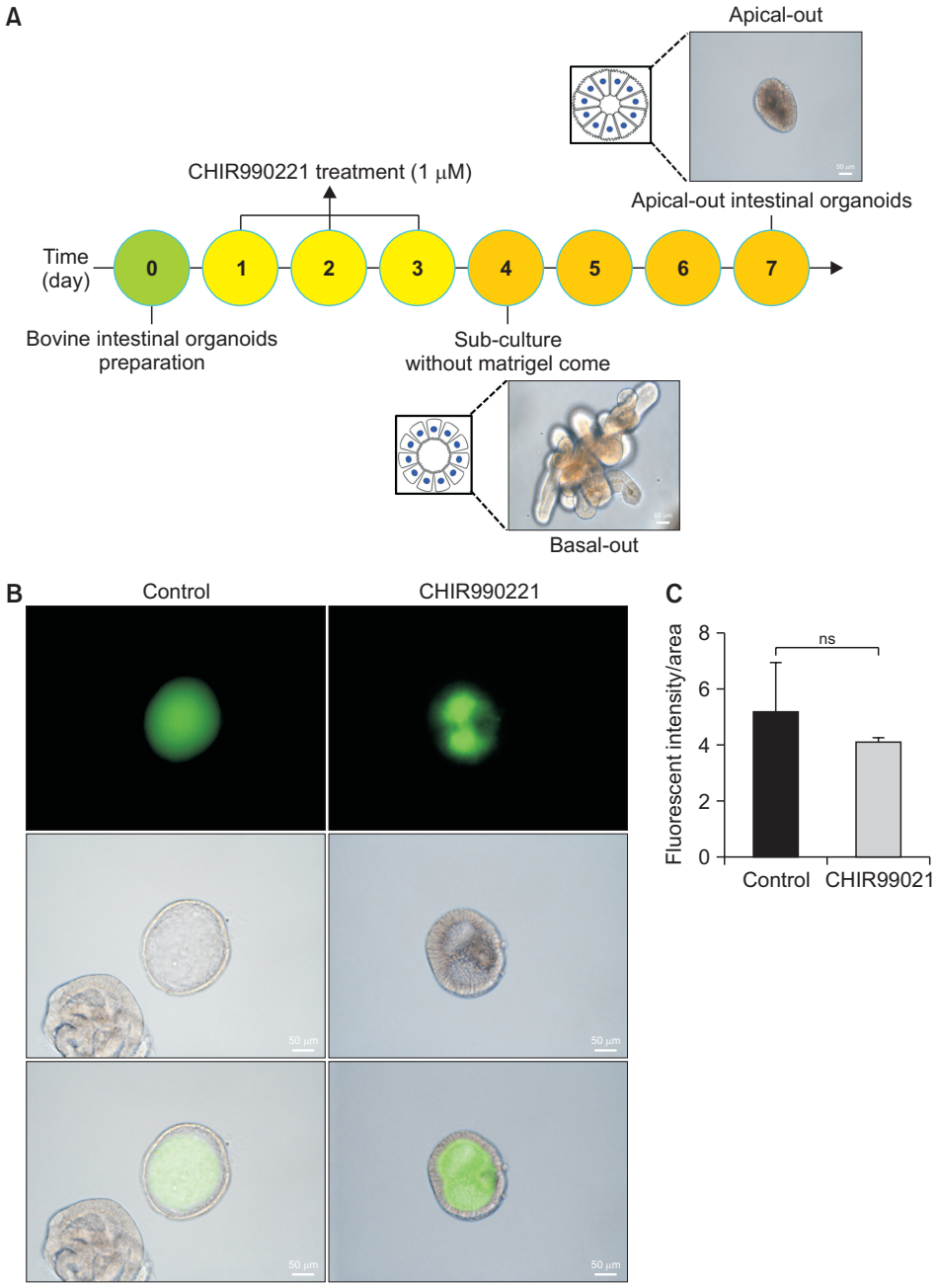

Fig. 4. Generation of apical-out intestinal organoids after treatment with 1 μM CHIR. (A) Schematic illustration of the experimental procedures regarding the generation of apical-out intestinal organoids after treatment with 1 μM CHIR. Apical-out intestinal organoids through suspension culture without Matrigel matrix after treatment with 1 μM CHIR began to emerge at Day 7. Scale bar: 50 μm. (B) The paracellular permeability characteristics of the epithelial layer in apical-out intestinal organoids for comparison of treatment with 1 μM CHIR at Day 3 and spontaneous differentiation at Day 6. (C) Comparison of fluorescent intensity regarding apical-out intestinal organoids generated after treatment of 1 μM CHIR at Day 3 and spontaneous differentiation at Day 6. In addition, we generated apical-out intestinal organoids after spontaneous differentiation at Day 6 as a control (Lee et al., 2021). The fluorescence intensity was calculated using ImageJ software. There was no significant difference in the fluorescence intensity levels. Significant differences between groups were analyzed by Student's t test. A p value less than or equal to 0.05 indicated statistical significance (*p value ≤ 0.05).

expressed compared to that of spontaneous differentiation in BIO at Day 6 as a control, while intestinal stem cell-related genes such as GATA6, Bmi1, CDX2 and HNF4 and intestinal epithelium such as Chromogranin A and E-cadherin were not significantly different, indicating that the genetic properties of bovine intestinal organoids after treatment with 1 μM CHIR at Day 3 were highly similar to those of spontaneous differentiation at Day 6. Generally, intestinal pathogens penetrate the apical membrane of intestinal epithelial cells (Li et al., 2020). However, intestinal organoids cultivated in Matrigel are all basal-out. Thus, there are practical limitations associated with the use of intestinal organoids, such as host-pathogen interactions and nutrient absorption. In this study, we generated apical-out intestinal organoids after treatment of 1 μM CHIR (Fig. 4A). Furthermore, we tested the paracellular permeability character of the epithelial layer using fluorescent tracers. We previously reported that FITC-dextran 4 kDa did not reach the apical surface due to the basal-out structure of bovine intestinal organoids (Lee et al., 2021). However, basal-out bovine intestinal organoids can be used for co-culture with microbes to study host-bacterial interactions (Zhang et al., 2014; Puschhof et al., 2021). Moreover, to further characterize the cellular potentials of apical-out bovine intestinal organoids, we compared apical-out intestinal organoids generated after treatment of 1 μM CHIR at Day 3 with previously described method (spontaneous differentiation at Day 6 as a control). As shown in Fig. 4B and 4C, there was no significant difference in the fluorescence intensity levels. Together, these results show that the cellular and genetic properties of bovine intestinal organoids after treatment with 1 μM CHIR at Day 3 were highly similar to those of spontaneous differentiation at Day 6.

CONCLUSION

In this study, we first established a rapid, convenient method for the efficient generation of bovine intestinal organoids by modulating the WNT signaling pathway and continuous apical-out intestinal organoids to overcome the current limitation of basal-out intestinal organoids cultivated in Matrigel matrices. Finally, these next-generation bovine intestinal organoids will facilitate their potential use for various purposes, such as disease modeling and feed efficiency measurement, in the field of animal biotechnology.

Author Contributions: Conceptualization, B.R.L.; methodology, data curation and formal analysis, B.R.L., K.W.P., H.Y., H.W., S.A.O., P.L., I.S.H.; writing-original draft preparation, B.R.L., K.W.P.; supervision, P.L.; funding acquisition and project administration, B.R.L. All authors have read and agreed to the published version of the manuscript.

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Ethical Approval: The experimental use of Hanwoo cattle was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Science (NIAS-2019-366), Korea.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: The datasets during and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

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Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

SUPPLEMENTARY MATERIALS

Supplementary material can be found via https://doi.12750/JARB.37.2.136.

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