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Establishment of intestinal organoids from small intestine of growing cattle (12 months old)

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Abstract

Recently, we reported the robust *in vitro* three-dimensional (3D) expansion of intestinal organoids derived from adult bovine (> 24 months) samples. The present study aimed to establish an *in vitro* 3D system for the cultivation of intestinal organoids derived from growing cattle (12 months old) for practical use as a potential alternative to *in vivo* systems for various purposes. However, very few studies on the functional characterization and 3D expansion of adult stem cells from livestock species compared to those from other species are available. In this study, intestinal crypts, including intestinal stem cells, from the small intestines (ileum and jejunum) of growing cattle were isolated and long-term 3D cultures were successfully established using a scaffold-based method. Furthermore, we generated an apical-out intestinal organoid derived from growing cattle. Interestingly, intestinal organoids derived from the ileum, but not the jejunum, could be expanded without losing the ability to recapitulate crypts, and these organoids specifically expressed several specific markers of intestinal stem cells and the intestinal epithelium. Furthermore, these organoids exhibited key functionality with regard to high permeability for compounds up to 4 kDa in size (e.g., fluorescein isothiocyanate [FITC]-dextran), indicating that apical-out intestinal organoids are better than other models. Collectively, these results indicate the establishment of growing cattle-derived intestinal organoids and subsequent generation of apical-out intestinal organoids. These organoids may be valuable tools and potential alternatives to *in vivo* systems for examining host-pathogen interactions involving epithelial cells, such as enteric virus infection and nutrient absorption, and may be used for various purposes.

Keywords: Growing cattle, Three-dimensional (3D) cultivation, Intestinal organoids, Apical-out structure, Characterization

INTRODUCTION

The small intestine consists of the duodenum, jejunum, and ileum and performs a variety of functions,

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Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Lee BR.
Data curation: Park KW, Yang H, Lee MG, Ock SA, Wi H, Lee P, Hwang IS, Yoo JG, Park CK, Lee BR.
Formal analysis: Park KW, Yang H, Lee MG, Ock SA, Wi H, Lee P, Hwang IS, Yoo JG, Park CK, Lee BR.
Methodology: Park KW, Yang H, Lee MG, Ock SA, Wi H, Lee P, Hwang IS, Yoo JG, Park CK, Lee BR.
Validation: Lee BR.
Investigation: Park KW, Yang H, Lee MG, Ock SA, Wi H, Lee P, Hwang IS, Yoo JG, Park CK, Lee BR.
Writing - original draft: Park KW, Lee BR.
Writing - review & editing: Park KW, Yang H, Lee MG, Ock SA, Wi H, Lee P, Hwang IS, Yoo JG, Park CK, Lee BR.

Ethics approval and consent to participate
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.

such as nutrient absorption, electrolyte uptake, hormone secretion and host-pathogen interactions in intestinal epithelium that is composed of a variety of intestinal cell types such as Paneth cells, enteroendocrine cells, goblet cells and enterocytes [1,2]. Recently, a scaffold-based three-dimensional (3D) culture system has provided a reliable alternative platform for the establishment of pluripotent and adult stem cells derived intestinal organoids *in vitro*. *In vitro* 3D organoid systems are valuable tools and potential alternatives to *in vivo* systems [3], and these *in vitro* organoid systems have been used for novel practical applications in various fields, such as animal science, animal biotechnology, and biomedicine [1].

Recent progress has been made to establish intestinal organoids derived from livestock, including bovine [2], porcine [4], chicken [5] and equine [6]. In particular, we previously reported a reliable method for the isolation of intestinal crypts from small intestines and the robust 3D expansion of intestinal organoids (basal-out) derived from adult bovine [2,7]. Genearally, basal-out intestinal oragnoids have the intestianl basal membrane on the surface. However, there are practical limitations associated with the potential use of intestinal organoids, such as host-pathogen interactions and nutrient absorption, because intestinal organoids cultivated in Matrigel have all basal-out structures [8–10]. Thus, development of apical-out organoid culture system (polarity reversal) is required. Moreover, very few studies on the functional characterization and 3D expansion of adult stem cells isolated from livestock species compared to those from other species are available [11].

In this study, we successfully established growing cattle-derived intestinal organoids and apical-out intestinal organoids and characterized their cellular potentials using gene expression profiling and immunocytochemistry. Furthermore, we investigated the permeability of the epithelial barriers of these apical-out and basal-out organoids using fluorescein isothiocyanate (FITC)-dextran.

MATERIALS AND METHODS

Experimental designs and animals

The aim of present study was the identification of adult intestinal stem cells and the cultivation of intestinal organoids derived from growing cattle (12 months old). Hanwoo growing cattle (*Bos Taurus coreanae*) were used for these experiments, and the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Science (NIAS-2019-366), Korea.

Isolation of intestinal crypts from the small intestines of growing cattle and establishment of intestinal organoids

The isolation of intestinal crypts from growing cattle and establishment of intestinal organoids were conducted as previously described [2]. Briefly, ileum and jejunum fragments were obtained from the small intestines of growing cattle (12 months old). After washing ice-cold phosphate-buffered saline (PBS) and 1% penicillin/streptomycin (Sigma-Aldrich, New York, NY, USA), The fragments were dissected vertically and washed thoroughly with washing buffer to remove debris. To remove the mucosal and submucosal layers were used to scrape a glass slide. The remaining muscle layer was cut into 3- to 5-mm pieces and repeatedly washed by shaking vigorously until the supernatant was clear. After centrifuged at 300×g, the collected pellet was resuspended in 25 mL of Cell Disassociation Solution (Stem Cell Technologies, Vancouver, Canada) and incubated at room temperature for 40 min on a rocker to release the crypts. Intestinal crypts were added in 1 mL of intestinal human organoid medium (Stem Cell Technologies) and the number of intestinal crypts was counted using a hemocytometer under an inverted microscope. A total seeding mixture with

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100–150 crypts and Matrigel in a 1:1 ratio was prepared and carefully placed in the middle of the wells of a 24-well plate. After 20 min, the matrigel dome was completely polymerized and gently added 1ml of organoid growth medium to each well.

Passage and cryopreservation of growing cattle-derived intestinal organoids

The growing cattle-derived intestinal organoids were passaged approximately once a week upon maturation as previously described [2]. Briefly, the medium was completely removed and the organoid dome was rinsed with ice-cold PBS. The organoids were collected by centrifugation at 300×g for 5 min after removing the Matrigel dome using enzyme-free cell disassociation buffer. The pellets were seeded in the amount of medium and Matrigel in a 1:1 ratio, and each well (140–150 organoids) was passaged into three parts in 24-well plates. The medium was replaced every 3 days, and the organoids were subcultured once a week. The number of organoids was counted in triplicate every week. For cryopreservation, the organoids were resuspended in preserving solution composed of 90% medium and 10% dimethyl sulfoxide (Sigma-Aldrich), and transferred to a liquid nitrogen tank after stored at −80℃ for 24 hr.

Histology and immunohistochemistry

After being thoroughly washed with ice-cold PBS, ileum and jejunum fragments from the small intestine were fixed in 10% neutral-buffered formalin (Sigma-Aldrich). The fragments were subsequently embedded in a paraffin block, and the fragments were vertically and horizontally sectioned at a thickness of 3–5 μm. The sections were then deparaffinized in xylene, rehydrated with water using a graded series of alcohol solutions, and processed prior to hematoxylin and eosin (Merck, Darmstadt, Germany) staining. For immunohistochemical analysis, the sections were permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with 0.1% normal goat serum for 1 h to block nonspecific binding after antigen retrieval by boiling in a sodium citrate buffer solution. The samples were incubated overnight at 4℃ with the appropriate primary antibodies. The antibodies used in the present study are shown in Table 1. After washing with PBS, the samples were incubated with anti-mouse and anti-rabbit secondary antibodies coupled to Alexa Fluor-488 and Alexa Fluor-594 (Molecular Probes, Eugene, OR, USA), for 1 h at room temperature, respectively. Diamidino-2-phenylindole (DAPI) was used to counterstained these fluorescent samples. Images were captured using an Olympus X100 confocal microscope (Olympus, Tokyo, Japan).

Immunocytochemistry

The organoids were maintained in 24-well plates until maturation. The medium was removed from

Table 1. Antibodies used for characterization of growing cattle-derived intestinal organoids

Antibody	Host species	Company (Catalog No.)
LGR5	Mouse	Origene Technologies (TA503316)
Bmi1	Rabbit	abcam (ab38295)
Mucin2	Mouse	Santan Cruz Biotechnology (SC-515032)
E-cadherin	Mouse	BD Biosciences (61081)
F-actin	Rabbit	abcam (ab83746)
Chromogranin A	Rabbit	abcam (ab85554)
Cytokeratin 19	Rabbit	abcam (ab84632)
Ki67	Rabbit	Cell Signaling Technology (9123)

the wells, and the organoids were gently washed by cold PBS. Briefly, the organoids were incubated in neutrally buffered 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature. After permeabilization with 0.5% Triton X-100 (v/v) (Sigma-Aldrich) in PBS for 30 min at room temperature, the blocking step was performed using 3% bovine serum albumin in PBS for 1 h at room temperature. Then, the organoids were thoroughly washed with PBS and incubated overnight at 4℃ with the appropriate primary antibodies, as shown in Table 1, at their appropriate dilutions. The protein expression against specific antibody was measured by incubating the samples with corresponding secondary antibodies coupled to Alexa Fluor-488 and Alexa Fluor-594 (Molecular Probes) for 1 h at room temperature. These fluorescent samples were counterstained with DAPI and mounted on glass slides using ProLong Gold antifade (Thermo Fisher Scientific, Waltham, MA, USA) mounting medium. Fluorescent images were analyzed under an Olympus X100 confocal microscope (Olympus).

Generation of apical-out intestinal organoids

Growing cattle-derived intestinal organoids were cultivated in a Matrigel dome as previously described [12]. 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 organoids were re-seeded and cultivated in ultralow-attachment 24-well plates with intestinal human organoid medium (Stem Cell Technologies) through suspension culture without Matrigel matrix. The morphology of the apical-out intestinal organoids was monitored daily under a microscope to evaluate polarity reversal [8].

Epithelial barrier permeability assay using fluorescein isothiocyanate-dextran

FITC-dextran (4 kDa) (Sigma-Aldrich) was diluted in nuclease-free water and resulted in a 1 mg/mL working solution to evaluate epithelial barrier function. Growing cattle-derived basal-out and apical-out intestinal organoids were placed in 24-well plates. Then, 25 ng/mL FITC-dextran was added to each well, and the plate was incubated under normal growth conditions. The permeability was monitored using luminal absorption and recorded at 30-min intervals for up to 3 h under a Leica CTR6000 fluorescence microscope (Leica, Wentzler, Germany). The fluorescence intensity was calculated using ImageJ software.

RNA Isolation

Total RNA was isolated from prepared samples, including intestinal organoids, using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [13,14]. The RNA quality and quantification was assessed by an Agilent 2100 bioanalyzer using an RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands).

Quantitative real-time polymerase chain reaction

Quantitative RT-PCR was performed to assess the expression of several markers regarding intestinal stem cells and epithelium in ileum and jejunum tissues and growing cattle-derived intestinal organoids. Total RNA (1 μg) was reverse transcribed using the Superscript III First-Strand Synthesis System (Invitrogen, Waltham, MA, USA). The PCR mixture was prepared by adding 2 μL 10 pmol of each forward and reverse primer, 7 μL nuclease-free water, 10 μL SYBR Green qPCR Mater Mix, and 1 μL cDNA to a final volume of 20 μL. PCR was performed as follows: initial incubation at 94℃ for 3 min, followed by 40 cycles of 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. Sequence-specific products were identified by generating a melting curve. The

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Ct value represents the cycle number at which a fluorescent signal increased to a level significantly higher than the background, and relative gene expression was determined by the $2^{-\Delta\Delta}$Ct method [15]. The qPCR primers for each target gene and 18S ribosomal RNA (rRNA) that were used as previously described (Table 2). Gene expression levels were normalized to that of bovine 18S rRNA and performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

All data are expressed as the mean ± standard error from three independent experiments. The significance between groups was analyzed by two-way ANOVA of variance or Student's *t*-test using GraphPad Prism V 6.0 software (GraphPad, San Diego, CA, USA). The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of intestinal stem cells from small intestine of growing cattle

First, we selected ileum and jejunum fragments from the small intestine to identify suitable sites from which to more effectively isolate intestinal crypts of growing cattle (12 months old). Intestinal ileum and jejunum tissues were histologically analyzed using hematoxylin and eosin staining to identify distinct crypt and villus structures, including intestinal stem cells. Detailed views of vertical and horizontal sections from ileum and jejunum tissues showed the integral structures of the intestinal epithelium gland, such as crypts at the bottom and finger-shaped villi on the apical side, indicating the possibility of growth potentials for derivation of intestinal organoids from both the jejunum and ileum in growing cattle (Fig. 1A). In addition, to investigate the genetic properties of ileum and jejunum tissues, quantitative RT-PCR was performed. As shown in Fig 1B, intestinal stem cell-related genes, such as *LGR5* ($p < 0.01$), *Bmi1* ($p < 0.001$), *CDX2* ($p < 0.001$) [16], *HNF4A* ($p < 0.01$) [17], *FOXA3* ($p < 0.001$) [2], and *SOX9* ($p < 0.001$) [18], and intestinal epithelium-related genes, such as *MUC2* ($p < 0.001$), *Chromogranin A* ($p < 0.001$), and E-cadherin ($p < 0.001$), were expressed at significantly higher levels in the ileum than in the jejunum. Furthermore, to identify intestinal stem cells of the ileum *in vivo*, immunohistochemical analysis of several markers related to intestinal stem cells and epithelial cells was conducted. As shown in Fig. 1C, the ileum of the small intestine exhibited distinct expression pattern, which included leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), which was found to be expressed with F-actin

Table 2. Primers used for the gene expression analysis of growing cattle-derived intestinal organoids

Gene name	Forward	Reverse
18S rRNA	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
LGR5	GTGTTCAGAGCCGCAGTGTA	GATTCCGAAGCAAAAATGCA
Bmi1	TTCATTGATGCCACAACCAT	CAGCATCAGCTGAAGGATGA
CDX2	CATCACCTCACCACCATCC	GTTTTCACTTGGCTGCCCAG
SOX9	TTCATGAAGATGACCGACGA	GTCCAGTCGTAGCCCTTGAG
FOXA3	GCAAGATGCTTACCCTGAGC	AGGTAGCAGCCGTTCTCAAA
HNF4A	CGAGCAGATCCAGTTCATCA	GAAGGCTGTGGAGTCTCAGG
MUC2	TTCGACGGGAGGAAGTACAC	TTCACCGTCTGCRTCATTCAG
E-cadherin	CCAGGTGACCACACTTGATG	ATACACATTGTCCCGGGTGT
Chromogranin A	TATCAATCCTGCGACATCAG	CTGTCTCCGTCCGAGTCTTC

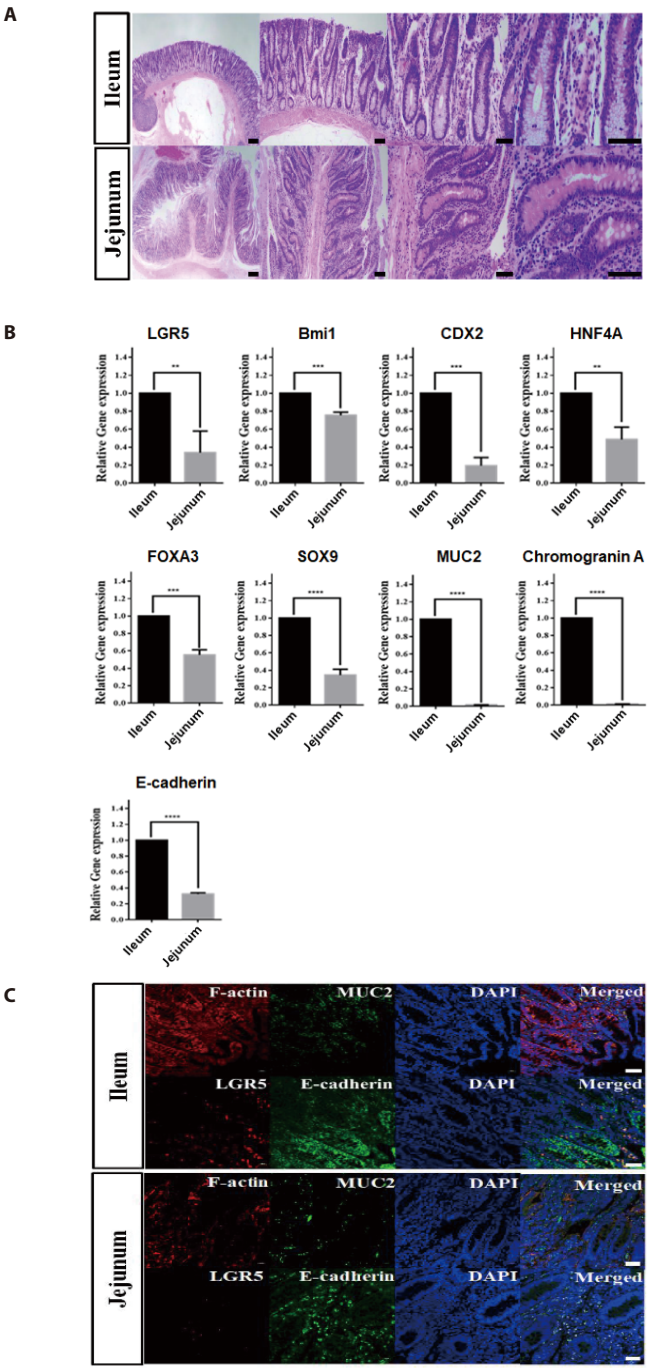

Fig. 1. Identification of intestinal stem cells in small intestine of growing cattle. (A) Hematoxylin and eosin staining of distinct crypt and villus structures, including intestinal stem cells, in the jejunum and ileum of the small intestine of growing cattle. Scale bar: #1: 40 μm, #2: 100 μm, #3: 200 μm, #4: 400 μm. (B) Gene expression profiling of jejunum and ileum tissues from the small intestine of growing cattle using quantitative RT-PCR. Quantitative RT-PCR was performed to evaluate the gene expression of jejunum and ileum tissues using several markers of intestinal stem cells (*LGR5*, *BMI1*, *CDX2*, *SOX9*, *HNF4A* and *FOXA3*) and epithelium (*MUC2*, *Chromogranin A* and *E-cadherin*). Gene expression was normalized to that of 18S rRNA and analyzed by the $2^{-\Delta\Delta}$Ct 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 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (C) Immunohistochemical staining of LGR5, MUC2, F-actin and E-cadherin in the jejunum and ileum of the small intestine in growing cattle. The fluorescently stained crypts were counterstained with diamidino-2-phenylindole (DAPI). Scale bar: 200 μm. RT-PCR, real-time polymerase chain reaction.

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in the intestinal epithelial cytoskeleton. Moreover, the fluorescently stained villi and crypt structures showed epithelium-specific expression of MUC2 in goblet cells and E-cadherin in adherent junctions, indicating that the authenticity of intestinal stem cells and epithelium in the ileum of the small intestine in growth cattle. However, the jejunum had a relatively low expression compared to the ileum of the small intestine. Collectively, these results strongly suggested that the ileum of the small intestine had the most growth potentials for the derivation of intestinal organoids from growth cattle.

Long-term cultivation and characterization of growth cattle-derived intestinal organoids

Intestinal crypts were isolated from the small intestine (ileum and jejunum) of healthy growth cattle (12 months old), and cultivated in IntestiCult medium at Matrigel dome. Fig. 2A illustrates the experimental procedure for the isolation of growth cattle-derived intestinal crypts, including the induction and cultivation of intestinal organoids. In this study, we attempted to produce intestinal organoids by isolating crypts from the jejunum and ileum of the small intestine to generate organoids specific to different regions of the small intestine. Interestingly, although there was no particular difference between the jejunum and ileum in growing cattle in terms of crypt and villus structure, intestinal organoids were confirmed to be formed by ileum (Fig. 2B). However, organoid formation did not occur jejunum. According to our previous results, intestinal organoids were described to exhibit detailed structures, such as spheroidal, stomatocyte, budded/elongated and branched structures, at each passage from Day 0 to the fully mature structure observed on Day 7 [2,19]. As shown in Fig. 2B, these organoids showed various morphologies, such as spheroidal

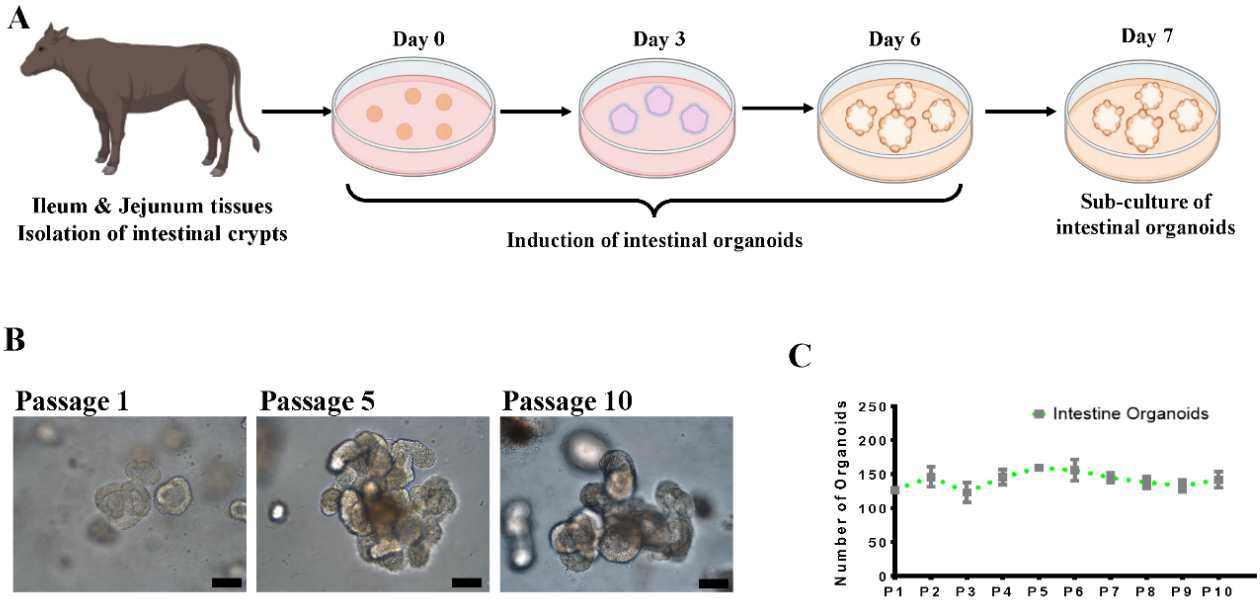

Fig. 2. Isolation of intestinal crypts and three-dimensional (3D) cultivation of intestinal crypts, including intestinal stem cells, from growing cattle. (A) Experimental procedures for the isolation of intestinal crypts from the small intestine (ileum and jejunum) and the 3D cultivation of intestinal crypts, including intestinal stem cells, from growing cattle. This figure was created with BioRender (Toronto, ON, Canada). (B) 3D expansion of intestinal crypts, including intestinal stem cells, from growing cattle. These organoids showed various morphologies, such as spheroidal (round shaped), mature villi and crypt-like structures, from the P1 to P10 generations at early passages. Scale bar: 50 μm. (C) Growth rate of growing cattle-derived intestinal organoids showing the number of organoids/well (mean n = 3 wells) cultured in a 100 μL Matrigel dome in each well. Growing cattle-derived intestinal organoids were maintained for up to 10 generations without losing the ability to recapitulate the crypts or continuous proliferation observed *in vivo*.

(round shaped) and mature villi and crypt-like structures, from the P1 to P10 generations at early passages. Furthermore, they showed stable growth for more than 10 passages with an average of 130–150 organoids present in the Matrigel dome at each generation, indicating the continuous proliferation and recapitulating capacity of the ileum-derived intestinal organoids (Fig. 2C). These results were consistent with establishment of the jejunum-derived intestinal organoids from adult bovines [2]. To more characterize the cellular potential of the ileum-derived intestinal organoids (P5) in growing cattle, we investigated the spatial expression of several specific markers of in intestinal stem cells and the epithelium. Intestinal organoids do not consist of only one cell but consist of various cells, such as stem cells, Paneth cells, and intestinal endocrine cells [10,20]. Consistent with previous results [2], the ileum-derived intestinal organoids had distinct patterns of gene expression, such as LGR5 and Bmi1 expression. Moreover, the fluorescently stained organoids showed epithelium-specific expression of Mucin2 in goblet cells, which contributes to epithelial barrier integrity, E-cadherin in adherent junctions, F-actin in the intestinal epithelial cytoskeleton, Chromogranin A in enteroendocrine cells, and Cytokeratin 19 in enterocytes, suggesting that the organoids closely mimicked the *in vivo* organ physiology (Fig. 3A). In addition, we investigated the genetic expression patterns of the ileum-derived intestinal organoids through expression profiling. As shown in Fig. 3B, the expression of intestinal stem cell-related genes, such as *LGR5* ($p < 0.001$), was significantly higher in the ileum-derived intestinal organoids on Day 0 than on Day 3 and Day 6, while the expression of intestinal stem cell-related genes, such as *Bmi1* ($p < 0.05$), *CDX2* ($p < 0.05$), *HNF4A* ($p < 0.05$) and *SOX9* ($p < 0.05$), was significantly higher in the ileum-derived intestinal organoids on Day 3 than on Day 0 and Day 6. In addition, the expression of intestinal

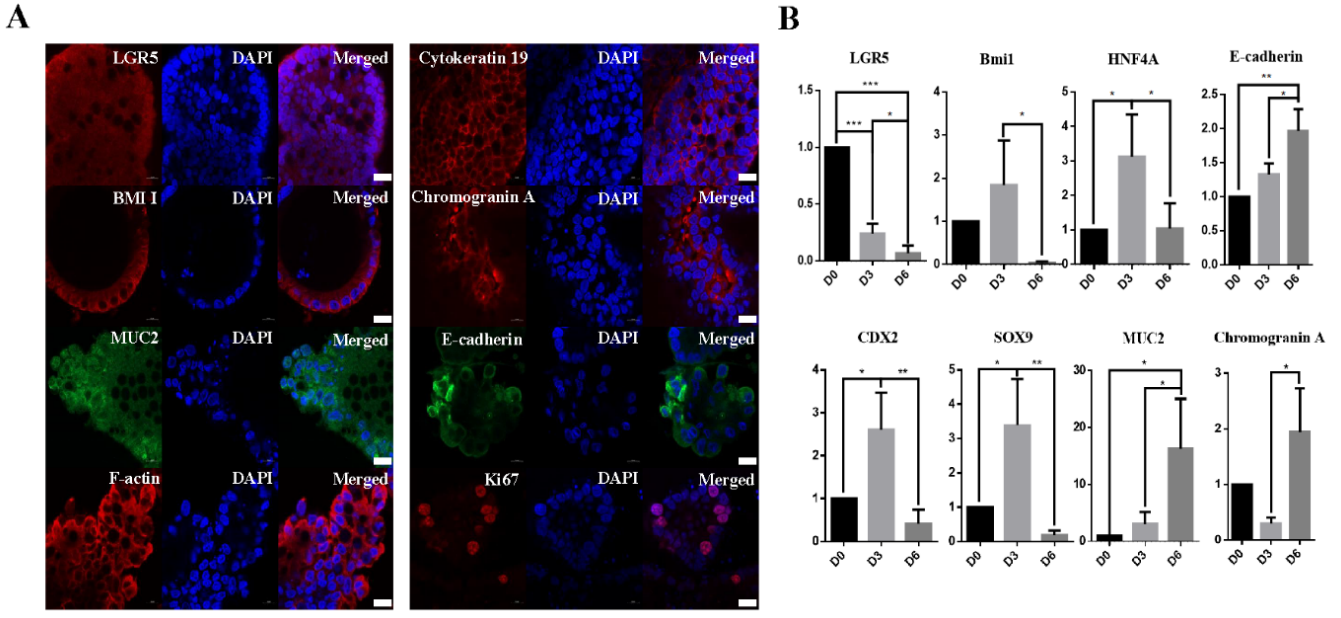

Fig. 3. Characterization and gene expression profiling of growing cattle-derived intestinal organoids. (A) Immunostaining of several intestinal organoid markers, such as intestinal stem cell markers (LGR5, BMI1 and Ki67) and epithelial markers (MUC2, Chromogranin A, E-cadherin, F-actin and Cytokeratin 19), in growing cattle-derived intestinal organoids on Day 6. The organoids were counterstained with DAPI. Scale bar: 10 μm. (B) The expression of intestinal stem cell-related genes, such as LGR5 ($p < 0.001$), was significantly higher in growing cattle-derived intestinal organoids on Day 0 than on Day 3 and Day 6, while the expression of intestinal stem cell-related genes, such as *Bmi1* ($p < 0.05$), *CDX2* ($p < 0.05$), *HNF4A* ($p < 0.05$) and *SOX9* ($p < 0.05$), was significantly higher in growing cattle-derived intestinal organoids on Day 3 than on Day 0 and Day 6. In addition, the expression of intestinal epithelial markers, such as *MUC2* ($p < 0.001$), *Chromogranin A* ($p < 0.05$) and *E-cadherin* ($p < 0.01$) was significantly higher in growing cattle-derived intestinal organoids on Day 6 than on Day 0 and Day 3. Gene expression was normalized to that of 18S rRNA and analyzed by the $2^{-\Delta\Delta}$Ct method. One-way ANOVA and Student's *t*-test were used to analyze significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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epithelial markers, such as *MUC2* ($p < 0.001$), *Chromogranin A* ($p < 0.05$) and *E-cadherin* ($p < 0.01$) was significantly higher in the ileum-derived intestinal organoids on Day 6 than on Day 0 or Day 3. Moreover, the expression of intestinal epithelial markers, such as *Chromogranin A* ($p < 0.001$) and *E-cadherin* ($p < 0.001$) was specifically expressed in the ileum-derived intestinal organoids on Day 6 than bovine embryonic fibroblast (BEF) (Fig. 4). This confirmed the development and differentiation of intestinal stem cells into intestinal organoids over time. Taken together, these results demonstrated that the genetic expression patterns and cellular potentials of the ileum-derived intestinal organoids were highly similar to those observed *in vivo*.

Generation of apical-out intestinal organoids and assessment of their epithelial barrier permeability

Recently, intestinal organoids have been considered potential alternatives to *in vivo* systems and have become the focus of research about livestock, including cattle, pig and chicken [1,11,21,22]. In particular, intestinal organoids have been described as the ideal model of mucosal permeability since they that can model the absorption of bacteria, viruses, and nutrients [7,9,23–26]. However, there are practical limitations associated with the potential use of intestinal organoids, such as host-pathogen interactions and nutrient absorption, because intestinal organoids cultivated in Matrigel have all basal-out structures [8–10]. To overcome the limitations of current growing cattle-derived basal-out intestinal organoid models cultivated in Matrigel matrices, we generated apical-out intestinal organoids through suspension culture without Matrigel matrix, showing that these organoids exhibited an apical-out membrane on their surfaces (Fig. 5A). From our results, on day 3 of culture in suspension without the Matrigel matrix, the apical-out form began to emerge. In addition, we investigated the spatial expression of a specific marker, F-actin, associated with epithelial characteristics in apical-out-derived intestinal organoids derived from growing cattle. Based on our results, it was confirmed that F-actin was strongly expressed on the surface, showing that intestinal epithelial cells were inverted and formed differently compared with those observed in basal-out organoids (Fig. 5A). The intestinal epithelium plays an important role in the absorption of nutrients through the membrane and diffusion of small molecules across the intestinal barrier

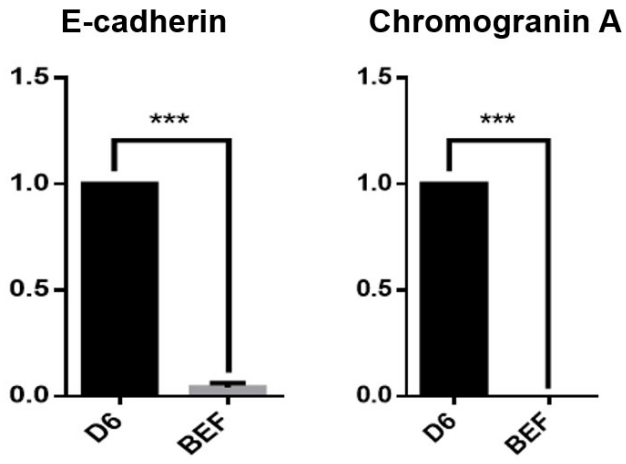

Fig. 4. Investigation on comparison of the expression of intestinal epithelial markers. The expression of intestinal epithelial markers, such as *Chromogranin A* ($p < 0.001$) and *E-cadherin* ($p < 0.001$) was specifically expressed in the ileum-derived intestinal organoids on Day 6 than bovine embryonic fibroblast (BEF). Gene expression was normalized to that of 18S rRNA and analyzed by the $2^{-\Delta\Delta}$Ct method. One-way ANOVA and Student's *t*-test were used to analyze significant differences. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

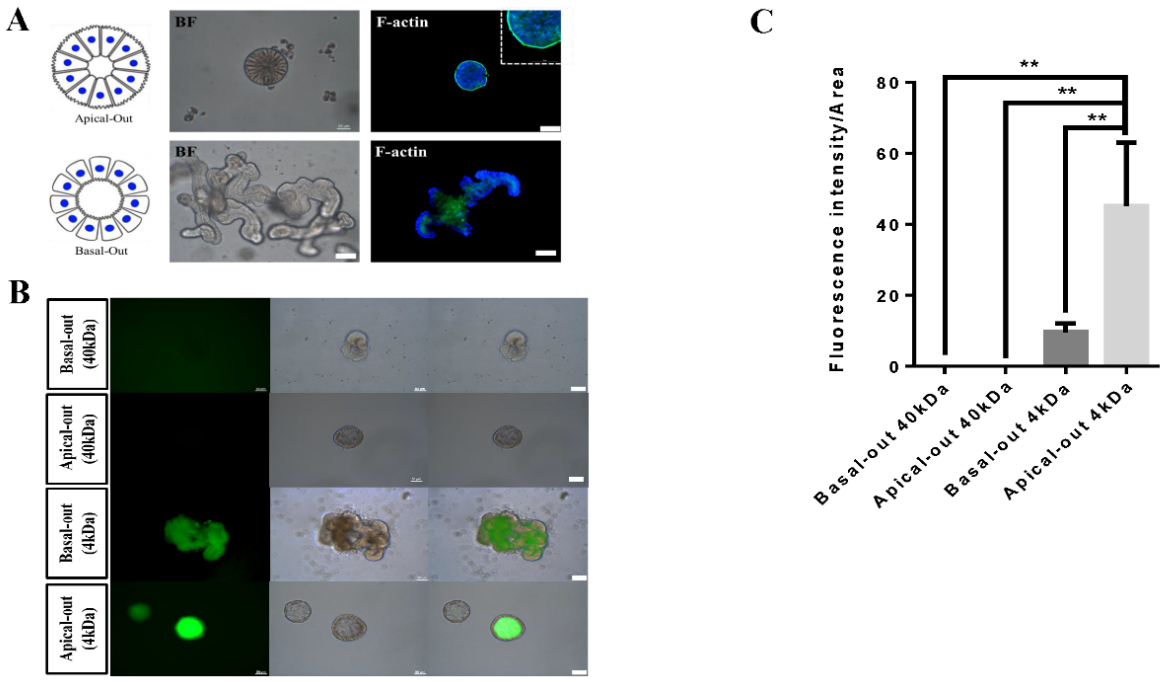

Fig. 5. Generation of apical-out intestinal organoids and assessment of paracellular permeability of growing cattle-derived intestinal organoids. (A) Apical-out organoids were formed without Matrigel after 3 days of suspension culture. Scale bar: 25 μm. Images of stained organoids; nuclei and F-actin were stained with DAPI and an antibody, respectively. Immunostaining showed that the epithelium of apical-out organoids was inverted compared to basal-out organoids. Scale bar: 50 μm. (B) Assessment of paracellular permeability of the epithelial layers of basal-out and apical-out organoids using fluorescent staining. Intestinal organoids were stained with FITC-dextran to evaluate barrier function. The organoids exhibited better permeability for FITC-dextran 4 kDa than FITC-dextran 40 kDa. (C) ImageJ software was used to analyze the fluorescence intensity of each organoid. One-way ANOVA and Student's *t*-test were analyzed by fluorescent intensity (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

[27,28]. Furthermore, we tested the paracellular permeability of the epithelial layer using fluorescent tracers. We previously reported that FITC-dextran 4 kDa did not reach the apical surface of bovine intestinal organoids due to their basal-out structures [2]. Generally, intestinal pathogens penetrate the apical membrane of intestinal epithelial cells [8]. However, apical-out bovine intestinal organoids overcame this limitation. Markedly, they showed the key functionality with regard to a high permeability for compounds up to FITC-dextran 4 kDa in size, while FITC-dextran 40 kDa failed to enter the organoid lumen because of intestinal epithelial barrier integrity. In addition, when the fluorescence of the apical-out and the basal-out structure were compared, it was confirmed that the transmittance of the apical-out structure was significantly higher and were better than other models (Figs. 5B and 5C), indicating apical-out intestinal organoids is the best model to study for various purposes. Together, these functional testing results suggested that apical-out intestinal organoids were physiologically relevant and exhibited properties similar to those of the gut *in vivo*. Finally, these novel organoids may be useful for evaluating nutrient absorption capacity and in experiments on intestinal epithelial cells.

CONCLUSION

In this study, we successfully established the ileum-derived intestinal organoids from growing cattle and generated a more reliable research model for intestinal organoids by producing organoids with apical-out structures and characterized their cellular potentials. Growing cattle-derived intestinal organoids have potential for use in various purposes in the field of animal biotechnology, such as

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disease modeling and feed efficiency measurement.

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