Short Report

Analysis of the Physiological Function of Myofibroblasts in Japanese Shorthorn Intestinal Spheroid Cultures

Ken Ito, Chisato Seya, Katsuyoshi Sato, Jun Watanabe, Masaki Yokoo

Department of Agribusiness, Faculty of Bioresource Sciences, Akita Prefectural University

Intestinal organoid/spheroid culture is a technique for the primary culture of intestinal epithelial cell 3D clusters. The L-WRN-conditioned medium has been used with this technique for various animals. We attempted to culture intestinal spheroids from the Japanese Shorthorn small intestine to better understand the high growth performance of this breed even under stressed conditions. However, after 11 days of culture, myofibroblasts (MyoFb) appeared around the intestinal organoids. After the MyoFb were removed, the intestinal spheroids were cultured alone, but died. Isolated MyoFb cells were cultured, and the cell structure was analyzed using RT-qPCR and immunocytochemistry. The isolated MyoFb cells were positive for MyoFb markers alpha-smooth muscle actin and vimentin, as well as Wnt2b, Rspo2, Rspo3, and Nog. These results suggested that MyoFb might provide support for intestinal spheroids by secreting proteins activating Wnt/β-catenin signaling.

Keywords: intestinal organoid, Japanese Shorthorn, myofibroblast, Wnt/β-catenin signaling

In Japan, four types of Wagyu occur, viz., Japanese Black, Japanese Brown Cattle, Japanese Polled, and Japanese Shorthorn (Gotoh et al., 2018). The Japanese Shorthorn breed was improved by crossbreeding with a native Japanese variety of Nanbu cattle (Morioka city) to increase beef production in the Meiji era. Nowadays, Japanese Shorthorn is mainly raised in the Tohoku (Northeast) region of Japan. Japanese Black comprises more than 80% of the Wagyu in Japan and is well researched from the aspect of genetics, breeding, feeding, and growth performance (Kahi & Hirooka, 2005; Nakajima et al., 2019; Setiaji & Oikawa, 2019; Ogata et al., 2019; Shibata et al., 2019a,b). In contrast, Japanese Shorthorn comprises approximately 1% of the cattles in Japan, despite this breed being resistant to extensive grazing and low temperatures and exhibiting higher levels of growth performance. In addition, it has a gentle character, high milk production, and is generally more successful at raising offsprings than the other breeds. However, these characteristics are based on little scientific evidence, and key questions remain unanswered, especially regarding the reason for the higher feed efficiency. Therefore, we revealed why Japanese Shorthorn exhibits high growth performance and analyzed the function of its intestinal epithelial cells.

The intestinal organoid culture method was developed by Sato et al. (2009) for culturing epithelial cells while retaining their 3D structure and functionality similar to their *in vivo* conditions. In addition, those cultured from an undeveloped intestinal tract such as during fetation are defined as “Spheroids,” and those cultured from an intestinal tract that has undergone differentiation to certain extent are defined as “Organoids” (Mustata et al., 2013). Initially, intestinal organoids were cultured from mouse and human intestines using Matrigel® Matrix, supplementing the culture medium with Wnt3a, R-spondins, and Noggin. To date, intestinal organoids have been cultured from a variety of animals, viz., cats, dogs, cows, mice, pigs, sheep, and chickens (Powell & Behnke, 2017). Powell and Behnke cultured various intestinal organoids using L-WRN-conditioned medium (L-WRN-CM) containing recombinant murine Wnt3a,

Correspondence to: Ken Ito, Department of Agribusiness, Faculty of Bioresource Sciences, Akita Prefectural University, 241-438 Kaidobata-Nishi, Shimoshinjo-Nakano, Akita, Akita 010-0195, Japan. E-mail: k-ito@akita-pu.ac.jp

R-Spondin3, and Noggin (Miyoshi & Stappenbeck, 2013). Using this conditioned medium made intestinal organoid and spheroid culture possible at a low cost. In addition, adding nicotinamide to the medium allowed for the long-term culture of intestinal organoids (Sato et al., 2011). Therefore, we tried to culture intestinal spheroid from the small intestine of stillborn Japanese Shorthorn using nicotinamide and L-WRN-CM to better understand Japanese Shorthorn's high growth performance.

In conclusion, long-term intestinal spheroid cultures could not be established as MyoFb was generated and died in the middle of the intestinal organoid culture. Moreover, even if MyoFb and the intestinal spheroids could be separated, the intestinal organoid would have been destroyed.

Therefore, in the present study, we searched for the sudden occurrence of MyoFb physiological functions and the cause of the death of intestinal organoids.

Materials and methods

Experiment 1

Intestinal spheroid culture using Japanese Short-horn

Preparation of L-WRN-CM

L-WRN cells were obtained from the ATCC (CRL-3276TM) and cultured following the ATCC protocol. Briefly, L-WRN cells were subcultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 0.5 mg/mL G418, and 0.5 mg/mL hygromycin until becaming overconfluent, then the first batch of conditioned medium was collected and centrifuged at 2,000 × g for 5 min at 4℃. The supernatant was the first batch of medium and was stored at 4℃. Every 24 h, 2–7 batches the conditioned media were collected and centrifuged. Batches 1–7 of the conditioned medium were pooled and filtered through a 0.20-µm filter. L-WRN-CM was stored at −80℃ until spheroid culturing. To prepare the spheroid culture medium, we used equal volumes of the conditioned medium and supplemented advanced DMEM/F12 (Thermo Fisher Scientific., MA., USA) medium.

Table 1. Medium composition for culturing intestinal spheroids

Stock solution	Final conc.
1 M HEPES	10 mM
100× GlutaMax™-1	1×
50× B27 supplement	1×
100× N_2 supplement	1×
500 µg/mL mEGF (dissolved in 0.1% BSA)	50 ng/mL
5 mg/mL gentamycin	50 µg/mL
125 µg/mL amphotericin B	2.5 µg/mL
500 µM A83-01 (dissolved in DMSO)	500 nM
L-WRN CM (1^{st}–7^{th})	Half of total volume
1 M nicotinamide (dissolved in DPBS)	10 mM
Advanced DMEM/F12	Up to final volume

L-WRN-conditioned medium was cultured according to the ATCC manual. The medium was replaced every 2–3 days.

Formulation details of culture media are shown in Table 1.

Animal care

The small intestines (jejunum) of stillborn Japanese Shorthorn cattle were obtained from the Agri-Innovation Education and Research Center (AIC) in the Akita Prefectural University. The protocols for animal experimentation were approved by the Animal Care and Use Committee of Akita Prefectural University (accession number: 19-05).

Intestinal spheroid culture from stillborn cattle

Jejunum samples were collected from stillborn cattle by laparotomy. The samples were immediately immersed in ice-cold Dulbecco's phosphate-buffered saline (DPBS). For culturing the organoids, the intestinal sample (~5 cm) was washed with ice-cold DPBS at least four times to wash away the debris. After the intestine was everted and ligated, > 2 mL of 25 mM EDTA in DPBS was injected into it. Next, the intestine was everted five more times to expand the sac as much as possible. The everted sac was incubated with 20 mL of 25 mM EDTA buffer for 40 min on ice. After the incubation, the everted sac was shaken vigorously for 30 s with 2% glucitol–3% sucrose in DPBS to discard villi and debris. To obtain the intestinal crypts, the supernatant was discarded and shaken vigorously for 1 min with glucitol–sucrose buffer. The crypt suspension was passed through a 100-µm cell strainer and centrifuged at 1,500 × g and 4℃ for 5 min.

The supernatant was discarded, and the spheroid culture medium was added to the suspension. An equal amount of Matrigel® Matrix (Corning Inc., NY, USA) was added and gently mixed with the crypt suspension. Next, 20 μL × 2/well of the suspension was seeded in a 4-well culture plate, and the Matrigel® Matrix was polymerized. After polymerization, the culture medium was added (500 μL/well) and incubated at 37℃ with 5% CO_2. The medium was replaced every 2 days.

Individual MyoFb and stillborn intestinal spheroid culture

On day 16 of the intestinal spheroids, we discarded the culture medium and added ice-cold DPBS to dissolve the polymerized Matrigel® Matrix. This was then centrifuged at 1,500 × g and 4℃ for 5 min, and the supernatant was discarded. New culture media was added and gently pipetted to transfer the spheroid and MyoFb cell mass to a new 4-well plate. Since the previous 4-well plate had MyoFb adhered to it, it was not discarded and used for the MyoFb isolation culture described later. The intestinal spheroid was detached from the aggregated MyoFb using a tapered glass capillary (Drummond Scientific Company Inc., PA, USA) under a stereomicroscope. The isolated intestinal spheroids were centrifuged at 1,500 × g and 4℃ for 5 min. The supernatant was discarded, and the spheroid culture medium was added to the suspension. An equal amount of Matrigel® Matrix was added and gently mixed. Next, 20 μL × 2/well of the suspension was seeded in a 4-well culture plate, and the Matrigel® Matrix was polymerized. After polymerization, the culture medium was added (500 μL/well) and the spheroid culture was started. The medium was replaced every 2 days.

To the detached MyoFb from the first 4-well plate, we added 0.05% trypsin-0.2 mM EDTA solution and incubated for 5 min at 37℃. The same volume of culture medium was added to deactivate the trypsin and centrifuged at 1,500 × g and 4℃ for 5 min, then the supernatant was discarded. Cell pellet and a remaining aggregated MyoFb were incubated with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin in DMEM (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) in the same T-25 cell culture flask, and culturing was maintained until the cells were semi-confluent. To passage the MyoFb, the culture medium was discarded and washed twice with DPBS. Then we added trypsin-EDTA solution and incubated for 5 min at 37℃. The same volume of culture medium was added to deactivate the trypsin, centrifuged at 1,500 × g and 4℃ for 5 min, and the supernatant was discarded. A new culture medium was added and gently pipetted to transfer into three new T-75 cell culture flasks.

Experiment 2

Physiological role of MyoFb generated during intestinal spheroid culture

Animal care

Small intestines (jejunum) of adult female Japanese Shorthorn cattle were obtained from a slaughterhouse in Akita city (Akita Meat Trading Center Inc., Akita, Japan). Testis samples were excised from two male Japanese Shorthorn cattle (5 and 6 months old) in AIC under anesthesia by a veterinarian. Some intestinal samples were used for PCR analysis (Experiment 3).

Preparation of MyoFb from the intestinal tract of adult Japanese Shorthorn as a control

The jejunum was collected from adult male Japanese Shorthorns in a slaughterhouse. The samples were washed with DPBS to remove debris and immersed in ice-cold DPBS. To conduct the PCR analysis, a part of the sample was immersed in RNAlater™ and stored at −80℃ following overnight incubation at 4℃. The samples were transported to the laboratory (~90-min journey) and washed three times with DPBS after cutting them to 3 × 3 cm pieces. Samples were minced into ≤ 5 mm and incubated at 37℃ for 60 min in 2% collagenase type II (Worthington Biochemical Corporation, NJ, USA) in DMEM. After being centrifuged (3,000 × g, 4℃, 5 min), cell pellets were washed twice with DMEM. Next, 0.01% collagenase

type II + 0.01% dispase II (FUJIFILM Wako Pure Chemical Corp.) in DMEM was added and incubated for 30 min at 37℃. After being centrifuged (3,000 × g, 4℃, 5 min), cell pellets were washed twice with DMEM. Then we added 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin in the DMEM and counted the number of cells after staining with trypan blue. The suspension contained 2.2×10^6 cells, which were seeded on a 100-mm^2 culture dish coated with pepsin-solubilized bovine dermal collagen type I, and cell culture began at 37℃ with 5% CO_2. Adult MyoFb were passaged after being cultured until reaching semi-confluency.

Gene expression of Wnt, Rspos, and Nog in MyoFb

Proliferated stillborn and adult MyoFb were seeded on 6-well culture plates coated with pepsin-solubilized bovine dermal collagen type I at a density of 3×10^5 cells/well. The medium was replaced every 2 days. After 6 days of culture, total RNA was isolated and purified with TRIzol® reagent (Thermo Fisher Scientific) and RNeasy Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The concentration of total RNA was measured with Nanodrop (Thermo Fisher Scientific) and the A260/280 ratio was determined to be > 2.0. To remove the genomic DNA, total RNA was treated with RNase-Free DNase Set (Qiagen). cDNA was synthesized with ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Tokyo, Japan) under the following cycling conditions: 37℃ for 30 min, 98℃ for 5 min, 50℃ for 5 min, and 12℃ for 5 min. the PCR was performed as follows: denaturing at 95℃ for 1 min; 35 cycles of 95℃ for 15 s, 62℃ for 30 s, and 72℃ for 30 s. The reaction solution was 20 μL and contained THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.), 50 ng of cDNA, and 0.3-0.3 pM of left and right primers each. The reactions were run on a LightCycler® 96 System (F. Hoffmann-La Roche, Ltd. Basel, Switzerland) using the intercalation method. After the Ct value was calculated, amplification was determined by the efficiency calibrated model. As housekeeping genes we used B2M (beta-2-microglobulin) and HPRT1 (hypoxanthine phosphoribosyltransferase 1). In this study, we selected HPRT1, which is the most stable gene. The target genes were referenced on GenBank (https://www.ncbi.nlm.nih.gov/) and Ensembl genome browser 99 (Hunt et al., 2018). The primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and contained exon-exon junctions. Table 2presents the primer sequences.

Table 2. RT-qPCR primers for MyoFb analysis

Target gene	GenBank ID	Sequences (5′→3′)	Product Size (bp)
Wnt2b	NM_001099363.2	Forward: GTAGACACGTCCTGGTGGTACAT Reverse: GCATGATGTCTGGGTAACGCTG	115
R-spondin2	NM_001206092.3	Forward: GGAATGTGTGGAAGGATGTGAGG Reverse: CAGGGTATCGTGTCTTTTGCTGG	144
R-spondin3	NM_001076034.1	Forward: GTCAGTATTGTGCACTGTGAGGC Reverse: GGTTACCCTTTGCTGATGGATGC	139
Noggin	XM_002695554.6	Forward: GGCCAGCACTATCTCCACATCC Reverse: AGCGTCTCGTTCAGATCCTTCTC	116
B2M	*ENSBTAT00000016359.3	Forward: TCGCTTCGTGGCCTTGGTCCTTCT Reverse: TGAACTCAGCGTGGGACAGCAGGT	263
HPRT1	NM_001034035.2	Forward: TGACACTGGGAAGACAATGCAGA Reverse: CCTTTTCATCAGCAAGCTCGCAA	91

Primers were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). *B2M used Ensemble gene ID (Ensemble genome browser 99: www.ensembl.org). These primers were checked with 3% agarose gel electrophoresis before use. B2M: Beta-2-microglobulin. HPRT1: Hypoxanthine phosphoribosyltransferase 1.

Immunocytochemistry (ICC) staining

Proliferated stillborn and adult MyoFb were seeded on 4-well chamber slides coated with pepsin-solubilized bovine dermal collagen type I at a density of 2×10^4 cells/well. On culture day 2, the medium was discarded, and the slides were washed three times for 5 min in DPBS. Next, the slides were incubated for 20 min in 4% paraformaldehyde. The slides were washed again three times for 5 min in DPBS and blocked with Blocking One Histo (Nacalai Tesque Inc., Kyoto, Japan) for 10 min.The slides were stained with a 1/200 dilution of the primary antibody for 1 h at 30℃ in a moist chamber. The slides were washed three times for 20 min in TBS-T and stained with a 1/200 dilution of the secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at 30℃ in a moist chamber. The slides were washed three times for 5 min in TBS-T and finally washed with TBS for 5 min. The samples were mounted with 90% of glycerol in TBS and observed with fluorescence microscopy.

The primary antibodies used were anti-vimentin mouse monoclonal antibody (14-9897-82, Thermo Fisher Scientific), anti-Wnt2b rabbit polyclonal antibody (ab50575, Abcam, Cambridge, UK), anti-Rspo2 rabbit polyclonal antibody (17781-1-AP, Proteintech Group, Inc., IL, USA), anti-Rspo3 rabbit polyclonal antibody (17193-1-AP, Proteintech), and anti-noggin rabbit polyclonal antibody (14772-1-AP, Proteintech).

The secondary antibodies used were donkey anti-mouse IgG (H+L) polyclonal antibody conjugated with Alexa Fluor® 594 (715-858-150, Jackson ImmunoResearch, Inc., PA, USA) and donkey anti-rabbit IgG (H+L) polyclonal antibody conjugated with Alexa Fluor® 488 (711-545-152, Jackson ImmunoResearch).

These antibodies were diluted with a diluent prepared with the Blocking One Histo (Nacalai Tesque Inc.) dilution buffer 20 times by TBS-T (TBS/0.05% tween 20).

Experiment 3

Gene expression of Wnt3/Wnt3a in the Japanese Shorthorn intestine

Animal care

The testis, as the positive control for Wnt3/Wnt3a, was excised from two male Japanese Shorthorn cattle (5 and 6 months old) in AIC under anesthesia by a veterinarian.

The intestine and testis samples were washed with ice-cold DPBS and sliced into ≤ 5 mm thick pieces. Th sliced samples were immersed in RNAlater™ and incubated at 4℃ overnight. The samples were stored at −80℃ until PCR analysis.

Wnt3a and Wnt3 PCR analysis

Total RNA was isolated with TRIzol® reagent from the intestine and testis samples. Total RNA was tre ated with DNase and purified using the RNeasy Min i Kit. The A260/280 ratio was determined to be > 2. 0.cDNA was synthesized with ReverTra Ace® qPCR RT Master Mix. The PCR reaction was performed as follows: denaturing at 95℃ for 1 min; 35 cycles of 95℃ for 15 s, 60℃ for 30 s, and 72℃ for 30 s. The reaction solution was 25 μL and contained GoTa q® Master Mix (Promega Corporation., WI, USA), 5 0 ng of cDNA, and 0.3-0.3 pM of left and right pri mers each.

The primer sequences used to amplify Wnt3a (Gen Bank ID: XM_019963736.1) were as follows: forwar d: 5′-TACCCGATCTGGTGGTCCCT-3′; reverse: 5′-G GGCATGATCTCCACGTAGTTCC-3′. The predicted product size was 135 bp. The primer sequences used to amplify Wnt3 (GenBank ID: NM_001206024.1) were as follows: forward: 5′-CCGTACGACCATCCT GGACC-3′; reverse: 5′-TACTTGTCCTTGAGGAAGT CGCC-3′. The predicted product size was 132 bp. T hen, 3% agarose gel electrophoresis was performed a t a constant voltage of 100 V for 45 min. XL-DNA ladder 50 plus (Apro Science Inc., Tokushima, Japa n) was used as the molecular marker.

Amino acid alignment of Wnt3a and Wnt3 in cattle and mice

The amino acid sequences of bovine and mouse Wnt3 and Wnt3a were aligned and their identity was calculated using the UniProt alignment tool (The UniProt Consortium, 2019) to confirm the activity of recombinant murine Wnt3a. The Wnt3a and Wnt3 amino acid sequences were obtained from the UniProt database. The following UniProt IDs were used in the alignment tool: Wnt3a: P27467(mouse), A0A4W2HLH5(cattle). Wnt3: P17553 (mouse), and A0A4W2CE35 (cattle).

Statistical analysis

The results are expressed as the means ± SEM. The generalized linear models were constructed in R (R Core Team., 2018). The object variable was specified as the gene expression level, and the explanatory variable was specified as the type of MyoFb. The significance was determined by Tukey's HSD test for multiple comparisons ($p < 0.001$).

Results

Experiment 1

Intestinal spheroid culture from Japanese Shorthorn

Isolated crypts were formed intestinal spheroid (Figure 1a and b). However, on culture day 10, MyoFb had appeared around a part of the intestinal shperoids (Figure 1c and d). On culture day 13, some intestinal spheroids formed a budding-like structure (Figure 1e). However, all intestinal spheroids were caught up in the MyoFb and eventually died. In contrast, MyoFb continued to proliferate. Next, we tried to isolate the intestinal spheroids from the MyoFb using a glass capillary. However, the isolated spheroids became atrophic and died on culture day 3 (Figure 1f).

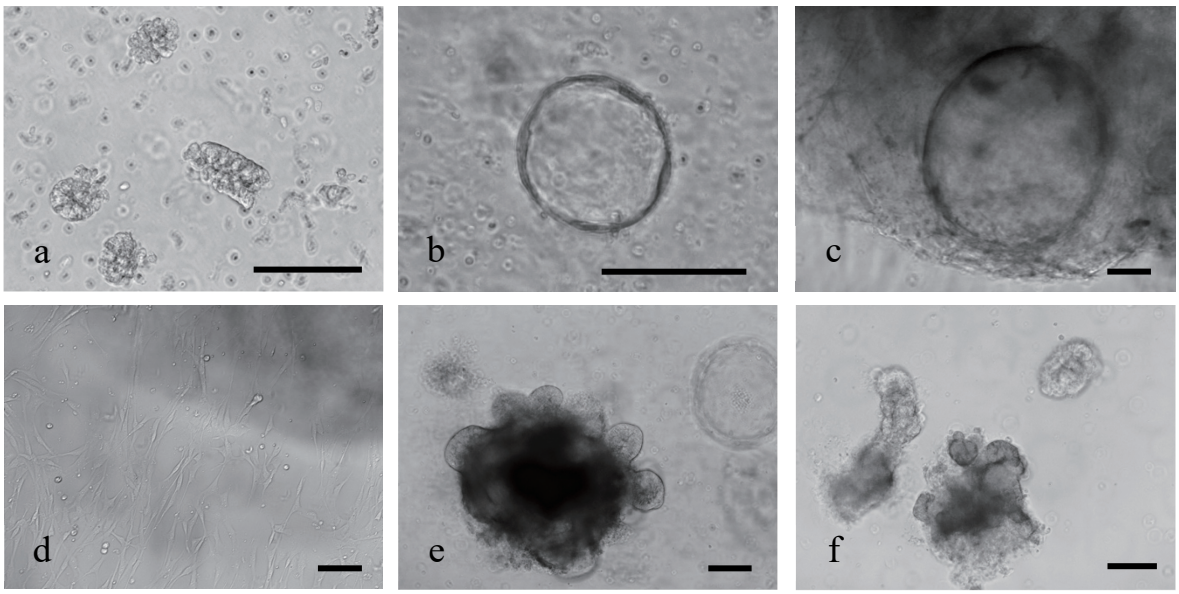

Figure 1. Culture of intestinal spheroids from the small intestine.
a) Isolated crypt from small intestine after passing a cell strainer. b) Intestinal spheroid on day 6. c) Intestinal spheroid on day 11. d) MyoFb on day 11. The shooting position is the same as c), but the focus is on MyoFb. e) Intestinal spheroid on day 14. f) Intestinal spheroid on day 3 after being isolated from myofibroblasts. Scale bar: 100 μm.

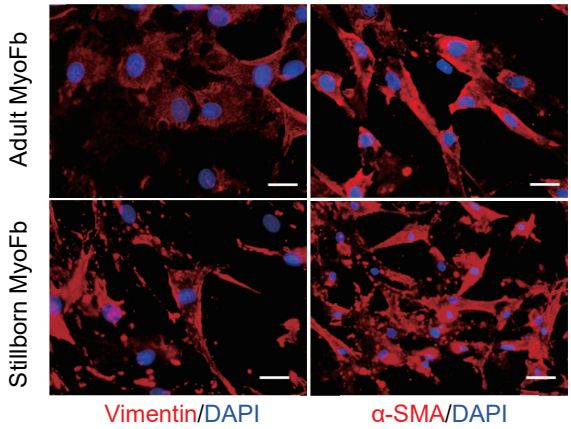

Figure 2. Identification of MyoFb stained with MyoFb markers.
α-SMA: alpha-smooth muscle actin. DAPI: 4',6-diamidi no-2-phenylindole. Scale bar: 50 μm.

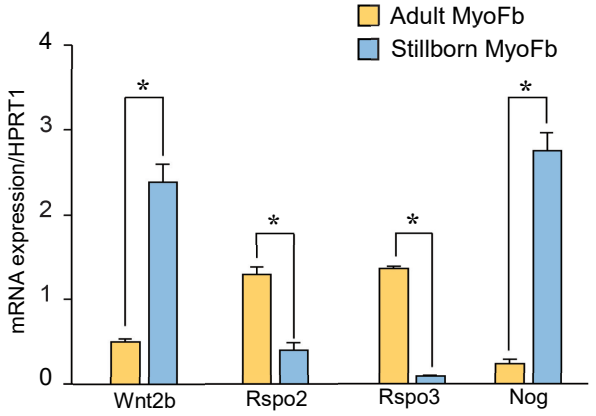

Figure 3. Wnt/β-catenin-related gene expression in MyoFb from adult and stillborn Japanese Shorthorn cattle.
* indicates statistically significant difference (P < 0.001) between adult MyoFb and stillborn MyoFb. Results were expressed as means ± SEM. n = 6.

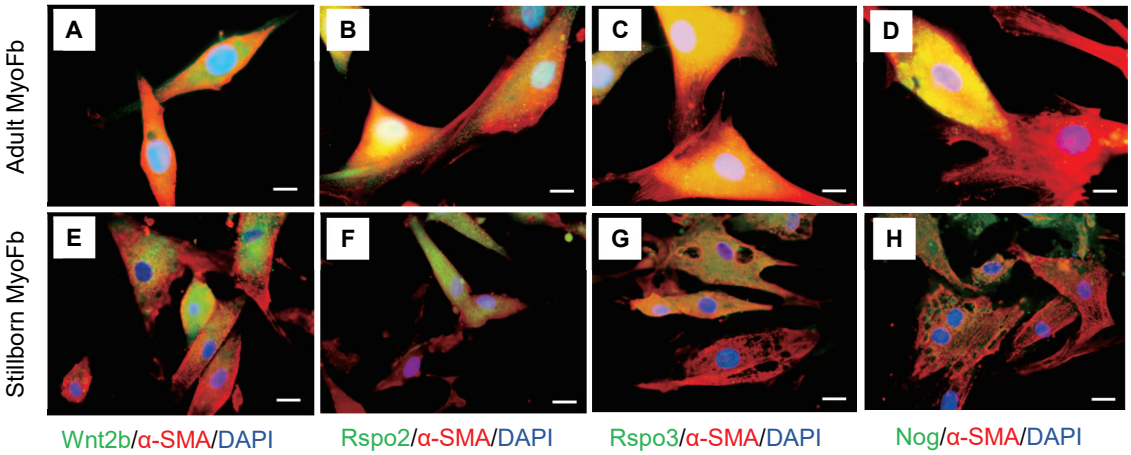

Figure 4. Secretion of Wnt/β-catenin signaling-related proteins MyoFb from adult and stillborn Japanese Shorthorn cattle.
A-D: Adult MyoFb stained with Wnt2b, Rspo2, Rspo3, Nog. *E-H*: Stillborn MyoFb stained with Wnt2b, Rspo2, Rs po3, and Nog. Rspo2: R-spondin2, Rspo3: R-spondin3, Nog: Noggin. α-SMA: alpha-smooth muscle actin. DAPI: 4',6-diamidino-2-phenylindole. Scale bar: 20 μm.

Experiment 2

Physiological function of MyoFb generated from spheroid culture

In this study, adult MyoFb was used as the control. The isolated and proliferated stillborn and adult MyoFb were positive for the MyoFb markers, vimentin and alpha-smooth muscle actin (α-SMA) (Figure 2). Next, we analyzed the gene expression of Wnt2b, Rspo2, Rspo3, and Nog, known to be secreted by stillborn and adult MyoFb. In stillborn MyoFb, the expression of Rspo2 and Rspo3 was lower than that in the adult MyoFb. In contrast, the Wnt2b and Nog expressions were significantly higher than adult MyoFb (Figure 3). Significant differences could be observed between stillborn and adult MyoFb, and gene expression could be detected. In addition, stillborn and adult MyoFb were stained with ICC. All cytokines were positive, showing that stillborn MyoFb secreted Wnt2b, Rspos, and Nog (Figure 4).

Experiment 3

Detection of Wnt3a and Wnt3 in the small intestine of the Japanese Shorthorn

Wnt3a was not expressed in the small intestine of the Japanese Shorthorn but in the testis as a positive control. Wnt3 was detected in both the small intestines and testes (Figure 5). The homology rates of the mouse Wnt3a amino acid sequence and the bovine Wnt3a and Wnt3 amino acid sequences were 95.18% and 83.15%, respectively. The amino acid sequence of the mouse Wnt3a binding site to the receptor was not different from that of the bovine Wnt3a and Wnt3 (Figure 6).

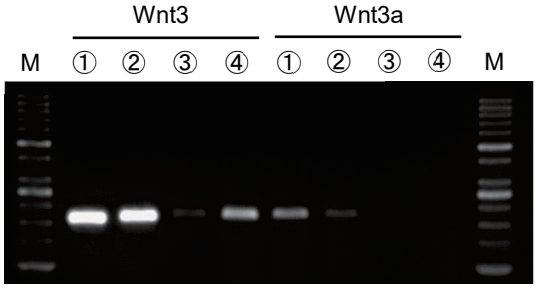

Figure 5. Detection of Wnt3a and Wnt3 by Endpoint PCR.
Testis was used as a positive control. The predicted product size of Wnt3a and Wnt3a was 135 bp and 132 bp, respectively. ①, ②: Testis. ③: female intestine. ④: male intestine. M: ladder marker.

Discussion

In this study, we aimed to culture intestinal spheroids from Japanese Shorthorn small intestine. However, culturing was difficult as MyoFb appeared and killed the intestinal spheroids. Therefore, intestinal spheroids were isolated from the MyoFb and cultured separately, but they also died eventually (Figure 1a–f). The outbreak of MyoFb killed the spheroid, presumably as the MyoFb proliferated to wrap around the spheroid, blocking the supply of culture components to the intestinal spheroid. However, since the spheroids died during MyoFb and spheroid isolation, MyoFb most probably supported spheroid growth. Intestinal MyoFb exists under the intestinal epithelial cells and supports the $Lgr5^{+}$ crypt base columnar cells by secreting Wnt2b and Rspos (Carulli et al., 2014; Kabiri et al., 2014; Koch, 2017).

First, we confirmed that the generated MyoFb (stillborn MyoFb) was in fact MyoFb using a-SMA and vimentin ICC staining. MyoFb and fibroblasts are morphologically similar but could be distinguished using cytoskeletal proteins, vimentin and α-SMA. MyoFb was positive for α-SMA and vimentin, whereas fibroblasts were only positive for vimentin (Roulis & Flavell, 2016). The ICC confirmed that stillborn MyoFb was MyoFb, as it was positive for both vimentin and α-SMA (Figure 2).

Next, we used ICC and RT-qPCR to test whether cytokines (e.g., Wnt2b) were secreted by stillborn MyoFb compared with adult MyoFb. Figures 3 and 4 show Wnt2b, Rspos and Nog expression in stillborn samples, also confirmed ICC. In particular, the Wnt2b gene expression level was high in stillborn MyoFb. Wnt2b enhances the Wnt/β-catenin signaling pathway and enables intestinal organoid culturing similar to Wnt3a and Wnt3 (Valenta et al., 2016).

Based on the above-described details, the synthesized Wnt3a amount in the L-WRN-CM used in this study could be potentially low, or the activity was low for cattles. Moreover, MyoFb could be potentially made up for the shortage of Wnt3a by secreting Wnt2b. However, it has been confirmed that organoid culture is possible

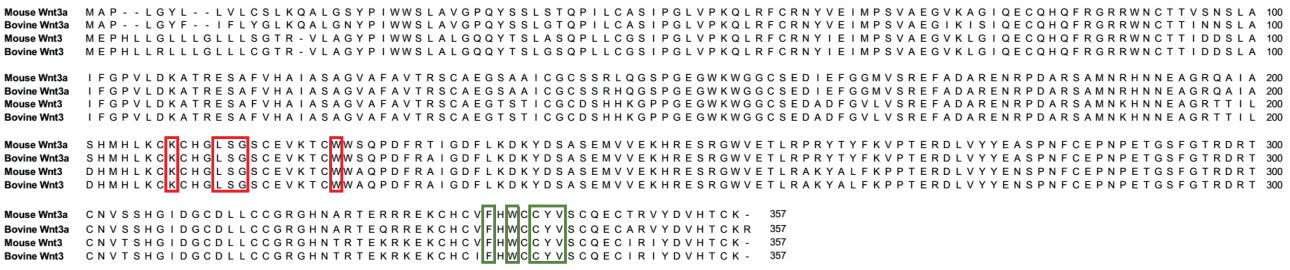

Figure 6. Amino acid sequence alignment results of Wnt3a and Wnt3 in mouse and bovine.

The red and green frames indicate the binding site with the receptor (Kumar et al., 2014).

using the L-WRN-CM medium used in this study using the intestinal tract of 5-week-old mice (data not shown). In addition, previous studies have enabled cattle intestinal organoid culture using L-WRN-CM. (Powell & Behnke, 2017). Therefore, considering the possibility that the expression pattern of the Wnt family is different in Japanese Shorthorn, Wnt3a and Wnt3 were investigated by PCR. Figure 5 shows that only Wnt3 gene expression, but not that of Wnt3a, was not detected in the cattle intestinal tract. Next, since the recombinant mouse Wnt3a expectedly displayed low activity against cattle, the amino acid sequences of mouse Wnt3a and bovine Wnt3 were compared. The homology between mouse and bovine Wnt3a and Wnt3 was as high as 95.18% and 83.15%, respectively, with no difference in the amino acid sequence for the Frizzled 8 binding site, which is the receptor for Wnt3a (Kumar et al., 2014) (Figure 6). Hamilton et al. (2018) used the IntestiCult™ Organoid Growth Medium, which is a serum-free medium that can be used for long-term maintenance of intestinal organoid cultures in cattles. In contrast, L-WRN-CM contained 10% FBS, which might adversely affect intestinal spheroid cultures because the FBS contained cytokines, such as bone morphogenetic proteins (BMPs), which restricts the stemness of intestinal $Lgr5^{+}$ stem cells (Qi et al., 2017). BMPs in FBS are derived from cattle and could be more active in cattle compared to other animal species. It is expected to be particularly effective for Japanese Shorthorn. In the future, it would be necessary to carry out spheroid culture considering the effects not only on Wnt but also on BMPs.

Acknowledgments

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References

Carulli, A. J., Samuelson, L. C., & Schnell, S. (2014). Unraveling intestinal stem cell behavior with models of crypt dynamics. *Integrative Biology*, 6, 243–257. https://doi.org/10.1039/c3ib40163d

Gotoh, T., Nishimura, T., Kuchida, K., & Mannen, H. (2018). The Japanese Wagyu beef industry: current situation and future prospects—A review. *Asian–Australasian Journal of Animal Sciences*, 31, 933–950. https://doi.org/10.5713/ajas.18.0333

Hunt, S. E., McLaren, W., Gil, L., Thormann, A., Schuilenburg, H., Sheppard, D., Parton, A., Armean, I. M., Trevanion, S. J., Flicek, P., & Cunningham, F. (2018). Ensembl variation resources. Database, 2018, bay119. https://doi.org/10.1093/database/bay119

Hamilton, C. A., Young, R., Jayaraman, S., Sehgal, A., Paxton, E., Thomson, S., Katzer, F., Hope, J., Innes, E., Morrison, L. J., &Mabbott, N. A. (2018). Development of in vitro enteroids derived from bovine small intestina crypts. *Veterinary Research*, 49, 54. https://doi.org/10.1186/s13567-018-0547-5

Kahi, K. A., & Hiooka, H. (2005). Genetic and economic evaluation of Japanese Black (Wagyu) cattle breeding schemes. *Animal Science Journal*, 83, 2021–2032. https://doi.org/10.2527/2005.8392021x

Kabiri, Z., Greicius, G., Madan, B., Biechele, S., Zhong, Z., Zaribafzadeh, H., Edison, Aliyev, J., Wu, Y., Bunte, R., Williams, B. O., Rossant, J., & Rossant, J.Virshup, D. M. (2014). Stroma provides and intestinal stem cell niche in the absence of epithelial Wnts. *Development*, 141, 2206–2215. https://doi.org/10.1242/dev.104976

Koch, S. (2017). Extrinsic control of Wnt signaling in the intestine. *Differentiation*, 97, 1–8. https://doi.org/10.1016/j.diff.2017.08.003

Kumar S, Žigman M, Patel TR, Trageser B, Gross JC, Rahm K, Boutros, M., Gradl, D., Steinbeisser, H., Holstein, T., Stetefeld, J., & Özbek S. (2014). Molecular dissection of Wnt3a-Frizzled8 interaction reveals essential and modulatory determinants of Wnt signaling activity. *BMC Biology*, 12, 44.

http://www.biomedcentral.com/1741-7007/12/44

Miyoshi, H., & Stappenbeck, S. T. (2013). In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. Nature Protocols, 8, 2471–2482. https://doi.org/10.1038/nprot.2013.153

Mustata, R. C., Vasile, G., Ferenandez-Vallone, V., Strollo, S., Lefort, A., Libert, F., Monteyne, D., Pérez-Morga, D., Vassart, G., & Garcia, M. I. (2013). Identification of Lgr5-independent sphroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Reports*, 5, 421-432. http://dx.doi.org/10.1016/j.celrep.2013.09.005

Nakajima, N., Doi, K., Tamiya, S., & Yayota, M. (2019). Effects of direct exposure to cold weather under grazing in winter on the physiological, immunological, and behavioral conditions of Japanese Black beef cattle in central Japan. *Animal Science Journal*, 90, 1033–1041. https://doi.org/10.1111/asj.13248

Ogata, T., Kim, Y. H., Masaki, T., Iwamoto, E., Ohtani, Y., Orihashi, T., … Sato, S. (2019). Effects of an increased concentrate diet on rumen pH and the bacterial community in Japanese Black beef cattle at different fattening stages. *Journal of Veterinary Medical Science*, 81, 968–974. https://doi.org/10.1292/jvms.19-0077

Powell, R. H., & Behnke, M. S. (2017). WRN conditioned media is sufficient for in vitro propagation of intestinal organoids from large farm and small companion animals. *Biology Open*, 15, 698–705. https://doi.org/10.1242/bio.021717

Qi, Z., Li, Y., Zhao, B., Xu, C., Liu, Y., Li, H., Zhang, B., Wang, X., Yang, X., Xie, W., Li, B., Han, J. J., & Chen, Y. G. (2017). BMP restricts stemness of intestinal Lgr5+ stem cells by directly suppressing their signature genes. *Nature Communi*cation, 8, 13824. https://doi.org/10.1038/ncomms13824

Roulis, M., & Flavell, R. A. (2016). Fibroblasts and myofibroblasts of the intestinal lamia propria in physiology and disease. *Differentiation*, 91, 116–131. https://doi.org/10.1016/j.diff.2016.05.002

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, 459, 262–265. https://doi.org/10.1038/nature07935

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G., Van Es, J. H., Van den Brink S, Van Houdt, W. J., Pronk, A., Van Gorp, J., Siersema, P. D., & Clevers H. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*, 141, 1762–1772. https://doi.org/10.1053/j.gastro.2011.07.050

Setiaji, A., & Oikawa, T. (2019). Genetics of heifer reproductive traits in Japanese Black cattle. *Asian-Australasian Journal of Animal Sciences*, 28, 197–202. https://doi.org/10.5713/ajas.19.0118

Shibata, M., Hikino, Y., Imanari, M., & Matsumoto, K. (2019a). Comprehensive evaluation of growth performance and meat characteristics of a fattening system combining grazing with feeding rice whole-crop silage in Japanese Black steers. *Animal Science Journal*, 90, 504–512. https://doi.org/10.1111/asj.13176

Shibata, M., Hikino, Y., & Matsumoto, K. (2019b). Influence of feeding a grass hay diet during the early stage of the fattening period on growth performance, carcass characteristics, and meat production in Japanese Black steers. *Animal Science Journal*, 90, 196–204. https://doi.org/10.1111/asj.13139

The UniProt Consortium. (2019). UniProt: a worldwide hub of protein knowledge. 47, D506–D515. https://doi.org/10.1093/nar/gky1049

Valenta, T., Degirmenci, B., Moor, A. E., Herr, P., Zimmerli, D., Moor, M. B., Hausmann, G., Cantù, C., Aguet, M., &Basler, K. (2016). Wnt ligands secreted by subepithelial mesenchymal cells are essential for the survival of intestinal stem cells and gut

homeostasis. *Cell Reports*, 15, 911–918.
https://doi.org/10.1016/j.celrep.2016.03.088

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