

*Full Paper***Isolation and Characterization of Bovine Intestinal Subepithelial Myofibroblasts**Koichi Iwanaga<sup>1</sup>, Takahisa Murata<sup>1,\*</sup>, Masatoshi Hori<sup>1</sup>, and Hiroshi Ozaki<sup>1</sup><sup>1</sup>Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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**Abstract.** Intestinal subepithelial myofibroblasts (ISMFs) are mesenchymal cells that exist under the epithelium of intestines. Primarily isolated ISMFs from rodents have been applied to experiments. However, due to the size of their intestines, the available cell number is limited. Thus, we attempted to isolate ISMFs from bovine colon as an alternative material. After detachment of smooth muscle and epithelial layers, colonic mucosa was explanted. After 2-week incubation,  $\alpha$ -SMA (+) / vimentin (+) / desmin (–) ISMFs were harvested and applied for experiments. First we examined the effect of cell passage on morphology and proliferation activity of bovine ISMFs. Although 3rd and 7th passage bovine ISMFs did not exhibit any changes, 11th passage ISMFs showed rounded enlarged shape and lost proliferation potential. On the contrary, rat ISMFs displayed the above senescent changes at earlier passage (passage 4). In intracellular  $\text{Ca}^{2+}$  concentration measurement, bioactive substances (0.3 – 1  $\mu\text{M}$  ATP, 0.1 – 1  $\mu\text{M}$  serotonin, 10 – 100 nM endothelin-1, and 1 – 10 nM bradykinin) dose-dependently induced an increase in intracellular  $\text{Ca}^{2+}$  concentration in bovine ISMFs (passage 3 and 7). However, at passage 11, impairment in intracellular  $\text{Ca}^{2+}$  responses was observed. Thus, bovine ISMFs might be a novel useful tool with long life span and good cellular responses to investigate physiological/pathophysiological roles of ISMFs.

**Keywords:** intestinal, myofibroblast, bovine, isolation, characterization

**Introduction**

Intestinal subepithelial myofibroblasts (ISMFs) are mesenchymal cells that exist under the basement membrane of the gastrointestinal tract (1, 2). ISMFs exhibit the ultrastructural features of both smooth muscles cell and fibroblasts, and thus ISMFs are supposed to be intermediate cells of these two cells (1, 3, 4). However the physiological roles of ISMFs are not fully understood.

To analyze the functions of ISMFs, primary ISMFs isolated from experimental animals including rats and mice are often used for experiments (5 – 7). However, because the tissue size of the gastrointestinal tract of these animals is small, they can provide only a relatively small number of cells. Furthermore, most somatic cells are known to have a finite life span, termed Hayflick limit

or cellular senescence (8, 9). These limitations in the experimental tool restrict further investigations of ISMFs. Thus alternative primary isolated cells are required.

Because bovine colon is very large, a sufficient number of ISMFs can be easily obtained at one time. Furthermore, bovine tissue is easily available from the slaughterhouse. Thus we assumed that bovine intestinal tissue is a novel convenient source for ISMFs isolation.

In this paper, we attempted to isolate ISMFs from bovine colon using an explant procedure and characterized their physiological and pharmacological features for the future evaluation of these cells.

**Materials and Methods***Materials*

The following reagents were used: endothelin-1 (Peptide Institute, Inc., Osaka); ethylenediaminetetraacetic acid, norepinephrine, Triton X-100 (Wako Pure Chemical, Osaka); TRIzol, trypsin-ethylenediaminetetraacetic

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acid, antimycotic/antibiotic, ethidium bromide (Invitrogen, Carlsbad, CA, USA); fura2-AM, HEPES (Dojindo, Kyoto); AMV Reverse Transcriptase XL (Life Sciences, Inc., North St. Petersburg, FL, USA); fetal bovine serum (Lot No.6C0282; JRH Biosciences, Lenexa, KS, USA); AmpliTaq Gold (Life Technologies Japan, Tokyo); cremophor EL (Nacalai Tesque, Kyoto); serotonin, bradykinin, angiotensin II, ionomycin (calcium salt), Dulbecco's modified Eagle's medium (DMEM), ATP, 4',6-diamidino-2-phenylindole (DAPI),  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Hanks' balanced salt solution ( $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HBSS) (Sigma, St. Louis, MO, USA). The following antibodies were used: goat anti-vimentin (Sigma); mouse anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Dako, Glostrup, Denmark); rabbit anti-mouse and rabbit anti-goat IgG Alexa 568 dye conjugates (Invitrogen).

### *Animals*

Sprague-Dawley (SD) rats were obtained from Charles River Laboratories (Yokohama). Animal care and treatments were conducted in accordance with the institutional guidelines outlined within the Guide to Animal Use and Care from the University of Tokyo. Experimental protocol was approved by the institutional Animal Care and Use committee at the University of Tokyo.

### *Cell culture and ISMFs isolation*

Bovine colon was obtained from a nearby slaughterhouse and rat colon was harvested from 4–5-week-old male SD rats. Bovine and rat ISMFs were isolated by the method described in our report (10). Briefly, colon was dissected and the smooth muscle layers were detached from the mucosal layer by using scissors and forceps. The mucosal samples are treated three times with 1 mM ethylenediaminetetraacetic acid in sterilized  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HBSS at 37°C for 30 min to remove epithelial cells. Epithelial cell-free mucosa was separated into several pieces (1 cm  $\times$  1 cm). Five pieces of them were cultured in a 100-mm round dish using sterilized DMEM with 10% fetal bovine serum. Medium was changed every two days. After about 1-week incubation, ISMFs started to proliferate and spread around epithelial-free mucosa. After 2-weeks incubation, bovine and rat ISMFs reached confluence. Both the number of isolated bovine and rat ISMFs are approximately  $1.5 \times 10^6$  cells. Then, the epithelial-free mucosa was removed and cells were subcultured (1:4 dilution for bovine ISMFs and 1:3 dilution for rat ISMFs). The observations of these cells were conducted with an inverted microscope.

### *Reverse-transcription polymerase chain reaction (RT-PCR)*

Total RNA was extracted from bovine ISMFs or

whole colon tissue by the acid guanidinium thiocyanate-phenol-chloroform method employing the TRIzol Reagent. RT-PCR was performed as follows: The first strand of cDNA was synthesized using a random 9-mer RT-primer and AMV Reverse Transcriptase XL at 30°C for 10 min, 55°C for 1.5 h, 99°C for 5 min, and 4°C for 5 min. PCR amplification was performed using Taq DNA polymerase (AmpliTaq Gold) and synthetic gene-specific primers for  $\alpha$ -SMA, desmin, vimentin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The oligonucleotide primers were designed as follows:  $\alpha$ -SMA expected product size = 240 base pairs, sense: ACTGGGACGACATGGAAAAG, antisense: CATCTC CAGAGTCCAGCACA; desmin expected product size = 374 base pairs, sense: GGGACATCCGTGCTCAGTAT, antisense: GTCCAAGGCCATCTTGACAT; vimentin expected product size = 220 base pairs, sense: CGCT CAAAGGGACTAACGAG, antisense: TCCAGCAGCT TCCTGTAGGT; GAPDH expected product size = 220 base pairs, sense: CAGGGCTGCTTTTAATTCTG, antisense: AGCACCAGCATCACCCCACT. Denaturation was done at 95°C for 10 min, followed by amplification using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedicals, Otsu).

PCR was performed using 30 cycles. Each cycle consisted of 0.6 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C. PCR products were separated by electrophoresis on a 2% agarose gel containing 0.2  $\mu\text{g/ml}$  ethidium bromide. The resultant fluorescent bands were visualized using an ultraviolet transilluminator (Toyobo Life Science, Osaka).

### *Immunostaining of cultured ISMFs*

Bovine ISMFs grown on glass cover slips were fixed with 4% paraformaldehyde at 37°C for 5 min. Cover slips were washed three times with phosphate-buffered saline and treated with 0.05% Triton X-100 for 5 min. Then permeabilized cells were incubated with mouse monoclonal anti- $\alpha$ -SMA (1:100 dilution) or goat anti-vimentin (1:50 dilution) in 10% rabbit serum as a first antibody overnight at 4°C. After two washes with phosphate-buffered saline, cells were incubated with a 1:200 dilution of rabbit anti-mouse IgG Alexa 568 dye conjugate or rabbit anti-goat IgG Alexa 568 dye conjugate as a second antibody at 37°C for 1 h. Then, nuclei were labeled with DAPI (1  $\mu\text{g/ml}$ ) at room temperature for 5 min and cells were washed three times with phosphate-buffered saline. The images were captured using Eclipse E800 fluorescence microscope (Nikon, Tokyo).

### *Measurement of ISMFs proliferation activity*

Bovine ISMFs or rat ISMFs ( $1.0 \times 10^5$  cells) were plated onto each well of a 6-well culture plate and

incubated in DMEM containing 10% fetal bovine serum. After 24-, 48-, and 72-h incubation, cells were treated with 0.1% trypsin-EDTA at 37°C for 3 min to detach cells from the plate. Cell numbers were counted using a hemocytometer.

#### Measurement of $[Ca^{2+}]_i$

ISMFs cultured on glass cover slips were washed with HEPES-buffered solution (125.4 mM NaCl, 11.5 mM glucose, 5.9 mM KCl, 1.2 mM  $MgCl_2$ , 1.5 mM  $CaCl_2$ , 10 mM HEPES, pH 7.4). Cells were loaded with fura2-AM by exposure to HEPES-buffered solution containing 3  $\mu$ M fura2-AM with 0.01% cremophor EL for 40 min in a dark room at 37°C. Every 3 s, the fluorescence of an image at 340 nm was divided by the fluorescence of an image at 380 nm to provide a resultant ratio (F340/F380) by means of a fluorescence imaging system (Hamamatsu Photonics, Hamamatsu).  $Ca^{2+}$ -free HEPES-buffered solution was made by adding 0.5 mM EGTA instead of 1.5 mM  $CaCl_2$ .  $[Ca^{2+}]_i$  was calculated by the method described in the previous report (11). The area under the  $\Delta[Ca^{2+}]_i$  per time curve (AUC) for 1 min was calculated to assess the response. This experiment was conducted after serum starvation (without fetal bovine serum for 24 h).

#### Statistical analyses

The results of the experiments are expressed as the

mean  $\pm$  S.E.M. Statistical evaluation of the data was performed by the unpaired Student's *t*-test for comparisons between two groups and by one-way ANOVA, followed by the Bonferroni post test for comparison between more than two groups. A value of  $P < 0.05$  was regarded as significant.

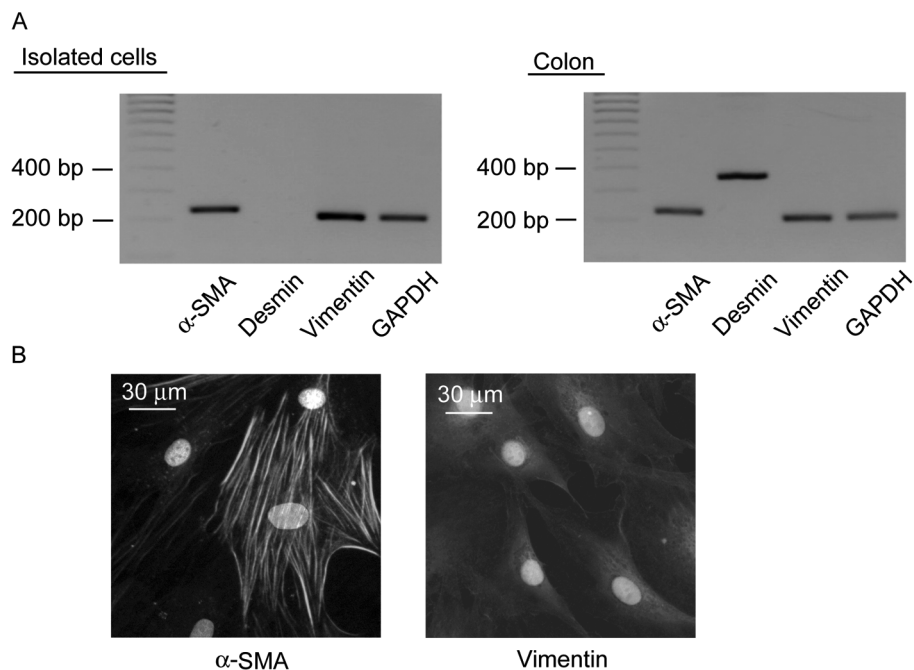
## Results

#### Expression of ISMFs markers in isolated cells

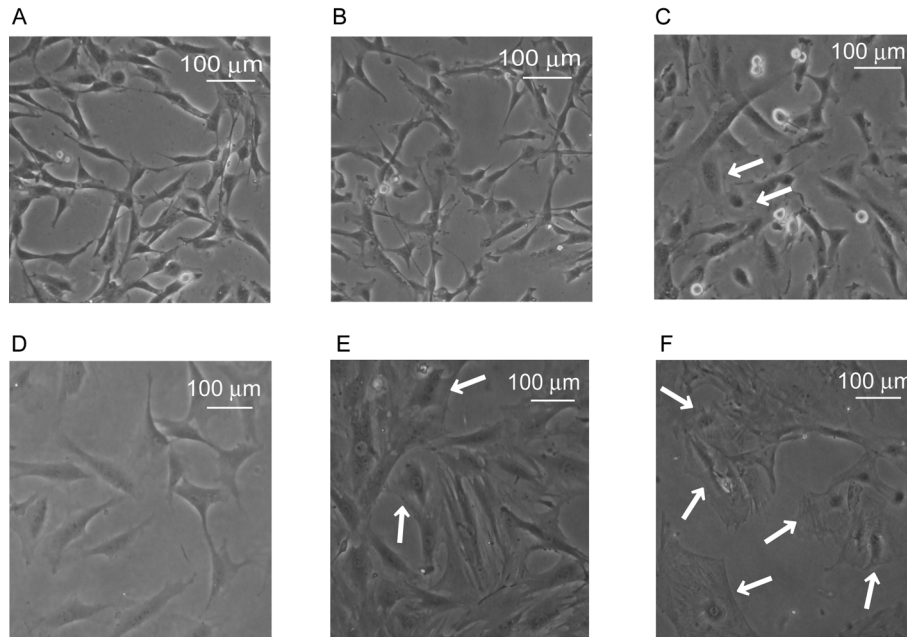
ISMFs are known to express  $\alpha$ -SMA and vimentin but not desmin (1, 2, 10). We initially examined the mRNA expressions of these markers in isolated cells. Figure 1A shows representative pictures of gel electrophoresis results from RT-PCR (30 cycles,  $n = 4$ ). As shown in the left panel of this figure, isolated cells expressed  $\alpha$ -SMA and vimentin but did not express desmin. The right panel represents the positive controls for these markers in the total extracted RNA from bovine colon. We also confirmed that more than 99% of isolated cells (passage 3 – 11) expressed  $\alpha$ -SMA and vimentin by immunostaining (Fig. 1B,  $n = 4$ ).

#### Morphological features of bovine and rat ISMFs

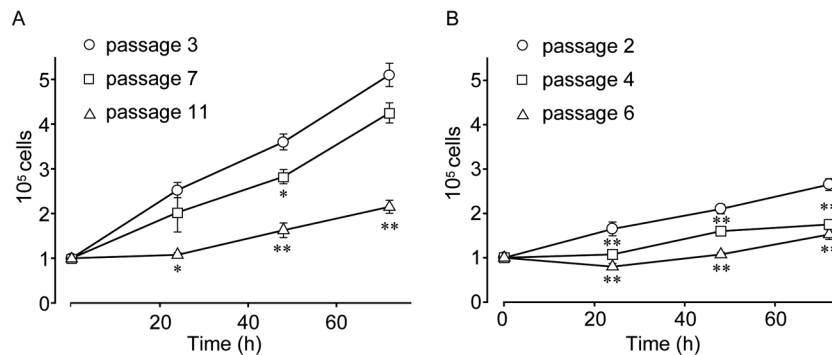
As shown in Fig. 2A – C ( $n = 4$ ), bovine ISMFs were spindle-shaped and had fibroblastoid morphology (Fig. 2A, passage 3; 2B, passage 7). However, at passage 11, bovine ISMFs became round in shape with wider



**Fig. 1.** The expression of ISMFs markers in cells isolated from bovine colon. A) mRNA expression of  $\alpha$ -SMA, desmin, and vimentin in cells isolated from bovine colon (left panel) and colon tissue (right panel). Total RNA was extracted from isolated cells or whole colon tissue and then subjected to RT-PCR. Figures represent gel electrophoresis of the PCR products. B) Isolated cells were immunostained for  $\alpha$ -SMA (left panel) and vimentin (right panel). Nuclei were labeled with DAPI.



**Fig. 2.** Morphological features of bovine and rat ISMFs. A – C) Typical pictures of bovine ISMFs (A, passage 3; B, passage 7; C, passage 11). D – F) Typical pictures of rat ISMF (D, passage 2; E, passage 4; F, passage 6). White arrows indicate the ISMFs that become round in shape with wider cytoplasm. Pictures of cells were photographed by using an inverted microscope.



**Fig. 3.** Growth curves of bovine and rat ISMFs. A) The number of bovine ISMFs at passage 3, 7, and 11 after 24-, 48-, and 72-h incubation. B) The number of rat ISMFs at passage 2, 4, and 6 after 24-, 48-, and 72-h incubation. Cells are detached from culture plate at each time and then counted by using a hemocytometer. Data are presented as the mean  $\pm$  S.E.M. of 4 separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. passage 3 of bovine ISMFs (A) or passage 2 of rat ISMFs (B).

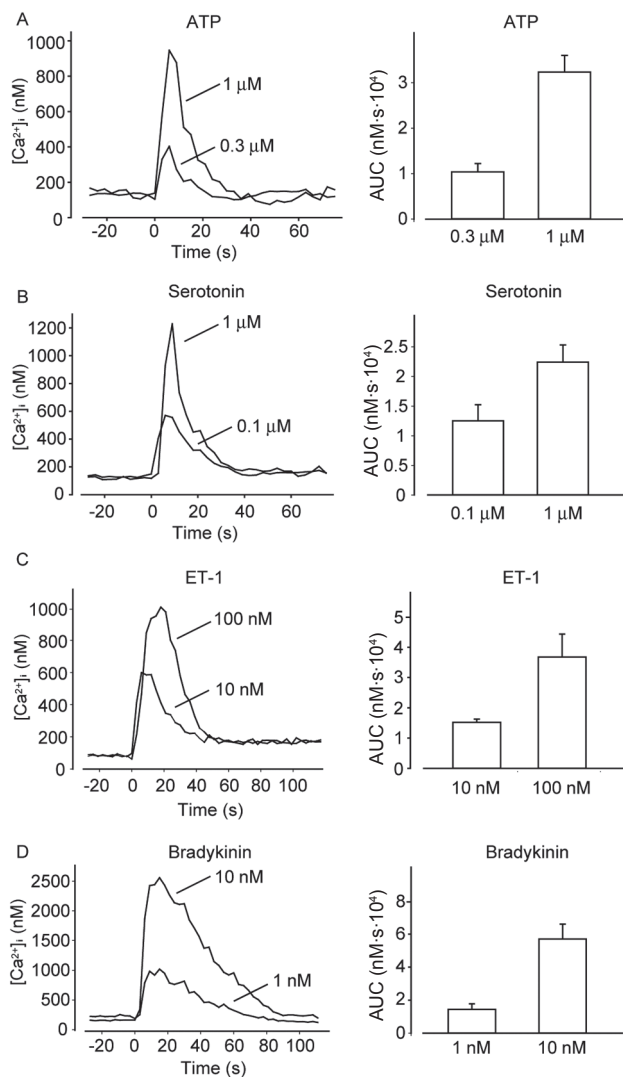
cytoplasm and lost their typical ISMFs morphology (Fig. 2C, white arrows). As for the rat ISMFs, cells were also spindle-shaped and had fibroblastoid morphology at passage 2 (Fig. 2D,  $n = 4$ ). However, at earlier passages of 4 and 6, cells became round in shape and lost typical morphological features of ISMFs (Fig. 2: E and F, white arrows,  $n = 4$ ).

#### *Proliferation activity of bovine and rat ISMFs*

To assess the proliferation activity, we counted the number of cells after 24-, 48-, and 72-h incubation of  $1.0 \times 10^5$  bovine ISMF cells (Fig. 3A,  $n = 4$ ) or rat ISMFs

(Fig. 3B,  $n = 4$ ). As shown in Fig. 3A, bovine ISMFs at passage 3 and 7 constantly proliferated; however, the proliferation activity of bovine ISMFs at passage 11 was significantly suppressed (After 72-h incubation: passage 3,  $5.10 \pm 0.26 \times 10^5$  cells; passage 7,  $4.25 \pm 0.23 \times 10^5$  cells; passage 11:  $2.15 \pm 0.14 \times 10^5$  cells). On the other hand, rat ISMFs represented lower proliferation activity even at earlier passages compared with bovine ISMFs and more quickly lost it in a passage-dependent manner (After 72-h incubation: passage 2,  $2.65 \pm 0.13 \times 10^5$  cells; passage 4,  $1.75 \pm 0.09 \times 10^5$  cells; passage 6,  $1.53 \pm 0.11 \times 10^5$  cells).





**Fig. 4.** Effects of bioactive substances on  $[Ca^{2+}]_i$  in bovine ISMFs. Typical traces of  $[Ca^{2+}]_i$  mobilization are shown in left panels and summarized data are shown in right panels expressed as the AUC for 1 min (A, 0.3 and 1  $\mu$ M ATP; B, 0.1 and 1  $\mu$ M serotonin; C, 10 and 100 nM endothelin-1 (ET-1); D, 1 and 10 nM bradykinin). Each agent was added at time 0 s and acted on the cells for 2 min. Analytical data are presented as the mean  $\pm$  S.E.M. of 4 separate experiments.

#### $[Ca^{2+}]_i$ responses to ATP, serotonin, endothelin-1, and bradykinin

Next we examined the effect of ATP, serotonin, endothelin-1, and bradykinin on  $[Ca^{2+}]_i$  mobilization in bovine ISMFs (passage 3–7) loaded with fluorescent  $Ca^{2+}$  indicator, fura-2. Typical traces of  $[Ca^{2+}]_i$  elevation out of 4 independent experiments are shown in Fig. 4 (A, ATP; B, serotonin; C, endothelin-1; D, bradykinin). ATP (0.3 and 1  $\mu$ M) and serotonin (0.1 and 1  $\mu$ M) rapidly elevated  $[Ca^{2+}]_i$ , which then settled to the basal level within 40 s. Endothelin-1 (10 and 100 nM) and bradykinin (1 and 10 nM) induced a transient increase

in  $[Ca^{2+}]_i$ , which then gradually decreased to a level above resting level. As shown also in Fig. 4, bovine ISMFs responded to ATP, serotonin, endothelin-1, and bradykinin in a dose-dependent manner (0.3  $\mu$ M ATP,  $1.04 \pm 0.18$  nM·s·10<sup>4</sup>; 1  $\mu$ M ATP,  $3.23 \pm 0.37$  nM·s·10<sup>4</sup>) (0.1  $\mu$ M serotonin,  $1.25 \pm 0.27$  nM·s·10<sup>4</sup>; 1  $\mu$ M serotonin,  $2.24 \pm 0.30$  nM·s·10<sup>4</sup>) (10 nM endothelin-1,  $1.52 \pm 0.11$  nM·s·10<sup>4</sup>; 100 nM endothelin-1,  $3.68 \pm 0.75$  nM·s·10<sup>4</sup>) (1 nM bradykinin,  $1.44 \pm 0.34$  nM·s·10<sup>4</sup>; 10 nM bradykinin,  $5.70 \pm 0.91$  nM·s·10<sup>4</sup>). We demonstrated that bovine ISMFs neither respond to norepinephrine (1  $\mu$ M) nor angiotensin II (100 nM) (n = 3 each, data not shown).

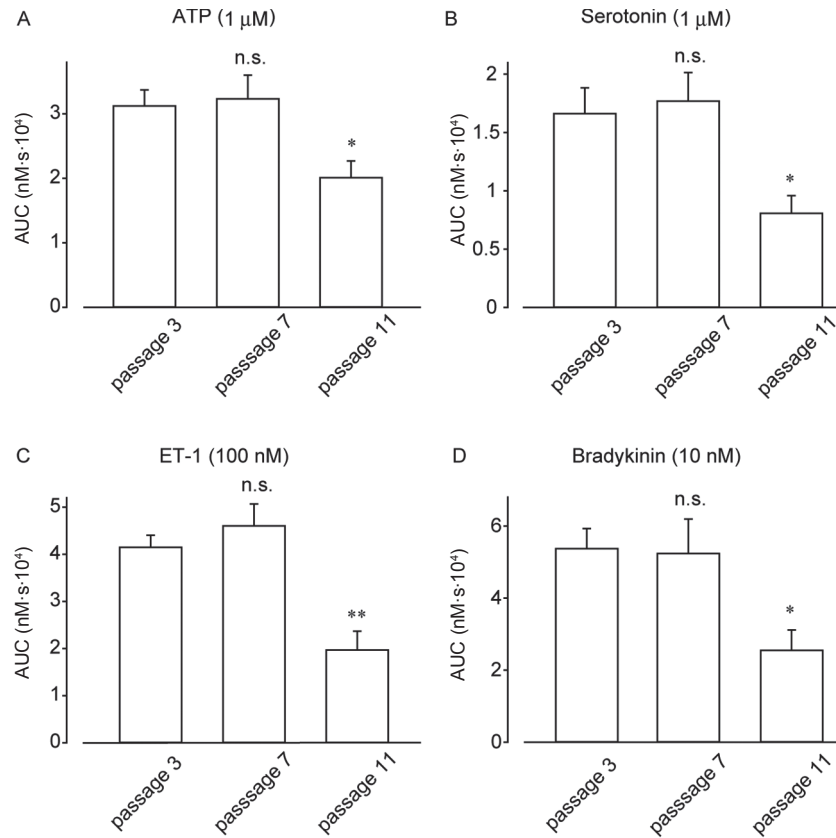
#### $[Ca^{2+}]_i$ responses of bovine ISMFs at passage 3, 7, and 11

We examined the effect of ATP (1  $\mu$ M), serotonin (1  $\mu$ M), endothelin-1 (100 nM), and bradykinin (10 nM) on  $[Ca^{2+}]_i$  mobilization in bovine ISMFs at passage 3, 7, and 11. As shown in Fig. 5,  $[Ca^{2+}]_i$  responses of bovine ISMFs were well-maintained through the 7th passage, but significant decrease was observed at passage 11 (n = 4–8): 1  $\mu$ M ATP (passage 3,  $3.12 \pm 0.25$  nM·s·10<sup>4</sup>; passage 7,  $3.23 \pm 0.37$  nM·s·10<sup>4</sup>; passage 11,  $2.00 \pm 0.26$  nM·s·10<sup>4</sup>); 1  $\mu$ M serotonin (passage 3,  $1.66 \pm 0.22$  nM·s·10<sup>4</sup>; passage 7,  $1.77 \pm 0.25$  nM·s·10<sup>4</sup>; passage 11,  $0.81 \pm 0.15$  nM·s·10<sup>4</sup>); 100 nM ET-1 (passage 3,  $4.15 \pm 0.26$  nM·s·10<sup>4</sup>; passage 7,  $4.60 \pm 0.46$  nM·s·10<sup>4</sup>; passage 11,  $1.97 \pm 0.40$  nM·s·10<sup>4</sup>); 10 nM bradykinin (passage 3,  $5.37 \pm 0.56$  nM·s·10<sup>4</sup>; passage 7,  $5.23 \pm 0.96$  nM·s·10<sup>4</sup>; passage 11,  $2.55 \pm 0.57$  nM·s·10<sup>4</sup>).

#### Discussion

Since ISMFs form a lining beneath the epithelium of small and large intestines (1, 2), investigators have isolated rodent ISMFs from epithelium-denuded mucosa by an explant procedure. In this study, we applied this procedure to isolate bovine ISMFs. ISMFs are characterized as cells that express  $\alpha$ -SMA and vimentin, but not desmin. Expression pattern of these markers in bovine ISMFs is clearly distinguished from  $\alpha$ -SMA (+) / desmin (+) smooth muscle cells or  $\alpha$ -SMA (–) / vimentin (+) fibroblasts (1, 2, 12, 13). We confirmed that the isolated cells express these ISMFs markers by RT-PCR (Fig. 1A). We also confirmed that more than 99% of the isolated cells express  $\alpha$ -SMA and vimentin by immunostaining (Fig. 1B).

Most somatic cells have a finite life span and undergo cellular senescence during successive subcultures (8, 9). The mechanism of cellular senescence is as follows. As cell divides, telomeres that protect the ends of chromosomes shorten and the attrition of telomeres leads to instability of chromosomes (14). Due to the changes



**Fig. 5.** Effects of bioactive substances on  $[Ca^{2+}]_i$  mobilization in bovine ISMFs at passage 3, 7, and 11 (A, 1  $\mu$ M ATP; B, 1  $\mu$ M serotonin; C, 100 nM endothelin-1 (ET-1); D, 10 nM bradykinin). Data are expressed as the AUC for 1 min. Analytical data are presented as the mean  $\pm$  S.E.M. of 4–8 separate experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. passage 3 of bovine ISMFs.

in gene expression, cellular morphology is abnormally changed (rounded and giant configurations) and the rate of cell proliferation is decreased. (15–17). Our results showed that bovine ISMFs maintained their normal morphology and proliferation activity even after 7 passages with 1:4 dilution (Fig. 2: A, B and 3A), while rat ISMFs quickly lost their cellular properties after 4 passages with 1:3 dilution. Since bovine ISMFs possess a longer life span and higher proliferation activity, these cells can be characterized as more easily-handled experimental tools than rat ISMFs. Furthermore, the large bovine tissue allows us to harvest a large number of ISMF at one time. Although we did not clarify the mechanism underlying this difference in cellular life span between bovine and rat ISMFs, our data strengthen the advantages of bovine ISMFs for experimental application.

ISMFs are known to express various receptors to neurotransmitters, vasoactive substances, and neuropeptides. Indeed previous reports showed that ATP, serotonin, endothelin-1, and bradykinin, but not norepinephrine or angiotensin II, elevated  $[Ca^{2+}]_i$  in ISMFs isolated from rat duodenum (7, 18). To characterize cellular reactivity, we measured  $[Ca^{2+}]_i$  in bovine ISMFs at passage 3–7.

Of interest, bovine ISMFs exhibited the same cellular reactivity to bioactive substances as rat ISMFs described in previous reports: that is, ATP, serotonin, endothelin-1, and bradykinin (Fig. 4: A–D), but not norepinephrine or angiotensin II (data not shown), dose-dependently increased  $[Ca^{2+}]_i$  in bovine ISMFs. These  $[Ca^{2+}]_i$  responses were maintained between passage 3 and 7, but not passage 11 (Fig. 5: A–D), consistently with the morphological changes and proliferation activities (Figs. 2 and 3).

In summary, we succeeded in isolating ISMFs from bovine colon and characterized their pharmacological features by analyzing  $Ca^{2+}$  signals. We demonstrated here that bovine ISMFs keep high proliferation activity and physiological reactivity until passage 7. Thus bovine ISMFs might be a powerful and beneficial tool to broaden the knowledge of the physiological and/or pathophysiological roles of ISMFs.

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