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Research article

Development of an evaluation system using intestinal organoids for drug efflux transport analysis by an imaging approach

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Abstract

There are several *in vitro* systems that enable evaluation of the absorption direction, but there are few quantitative systems that enable easy evaluation of the excretion direction. Enteroids, organoids derived from intestine, have been frozen and passaged for various research. But it is not clear how the freezing and passaging affect the expression and function of transporters. We investigated the effects of passage and cryopreservation of enteroids. We focused on P-gp (P-glycoprotein) and compared the transfer rates of rhodamine 123 (Rh123) into the lumen of enteroids with and without a P-gp inhibitor. mRNA expression levels did not change significantly before and after passage and cryopreservation. Accumulation of Rh123 in the lumen of enteroids was observed. With some P-gp inhibitors, excretion of Rh123 into the lumen of enteroids was inhibited and the nonexcreted Rh123 accumulated in enteroids epithelial cells. The transfer rate of Rh123 into the lumen of enteroids with a P-gp inhibitor was significantly decreased compared to that of without a P-gp inhibitor. Before and after passage and cryopreservation, the transfer rate was almost the same as that of primary cultured enteroids. We succeeded in easily evaluating whether a component is a substrate of P-gp using enteroids.

Keywords: Enteroids; efflux transporter; P-glycoprotein, cryopreservation; passage.

Introduction

ATP-binding cassette (ABC) transporters including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein (MRP) have been shown to play important roles in excretion of xenobiotics and biological defense. [1,2] These transporters are expressed wherever needed in tissues. [3-5] Luminal drugs or food ingredients may cause some drug-drug, drug-food and food-food interactions. These interactions would increase the risk of adverse events and should be avoided. Therefore, it is important to investigate the absorption and metabolism mechanisms. Methods that have been devised to clarify the mechanism of gastrointestinal absorption of drugs include methods that enable *in vitro* evaluation with maintenance of the physiological state in the gastrointestinal tract in which cells and tissues other than absorbing epithelial cells are mixed. [6,7] However, there has been few experimental systems that enable easy evaluation of the functions of efflux transporters.

Enteroids made from the small intestine are organoids in which stem cells contained in the crypts differentiate and proliferate into various intestinal epithelial cells, and these cells adhere to each other to self-assemble. [8] Due to the characteristics of the three-dimensional structure, enteroids maintain a physiological state in which tissues and endocrine, goblet and Paneth cells other than absorbing epithelial cells coexist, so it is considered that gastrointestinal absorption mimicking the actual small intestine is performed. [8-10] Enteroids have a closed structure with a gastrointestinal lumen inside. It has been a useful tool for mass transport analysis as an *in vitro* system close to the *in vivo* system. [11-14] We focused on the above-mentioned characteristics of enteroids and came up with the idea of constructing a simple evaluation system for the involvement of efflux transporters.

In addition, although an experimental system using enteroids would have many advantages, it requires a lot of time and effort to acquire a procedure because it requires many operations that rely on experience in the isolation process of the small intestinal crypt. Cultured cells have also been used as a tool for transport analysis in pharmacokinetics. Some cultured cells are given a recommended number of passages because the longer the cells are in culture, the more they change their characteristics. There are reports of significant increases in P-gp expression in Caco-2 cells at different numbers of passages. [15] Warrier also reported that the expression revels of P-gp and MRP2 differ in Caco-2 cells depending on the number of passages, culture days, and culture conditions. [16] It has been known that enteroids can be frozen and passaged. [10,17,18] These reports led us to wonder whether the expression revels of transporters may also change in organoids depending on passages and cryopreservation. However, little has been investigated about how the expression of transporters that affect pharmacokinetics changes in organoids after passage or cryopreservation.

The aim of this study was 1) to investigate the expression level of efflux transporters in freezing and passaging of enteroids and 2) to establish a novel mass transport experimental system using enteroids and apply it to evaluation of the functions of efflux transporters. To evaluate the drug efflux system, we focused on one of the major efflux transporters, P-gp, that functions by utilizing energy from ATP hydrolysis. We have developed a simple evaluation system that can quantify the influence of P-gp even if the shape and size of each enteroid are different.

Materials and methods

Chemicals

Rhodamine 123 (Rh123), verapamil hydrochloride and Y-27632 dihydrochloride were purchased from Sigma-Aldrich (MI, USA). Cyclosporin A was purchased from Toronto Research Chemicals Inc. (ON, Canada). Reagents were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan) unless otherwise noted. All reagents were of the highest grade available and were used without further purification.

Animals

Male C57BL/6J mice, aged 6 weeks (20-25 g in weight), were obtained from Jla (Tokyo, Japan). All mice were housed in plastic cages (360 mm × 120 mm × 160 mm, Natsume Seisakusyo Co., Ltd., Tokyo, Japan). There were 3-4 mice in each cage. The rearing facilities were specific pathogen-free and were maintained at 23 ± 3ºC (room temperature) and 50 ± 10% relative humidity with a 12-h light/dark cycle. For acclimation, mice were allowed free access to food (Picolab® Rodent Diet 20 (Lab Supply Inc., Texas, USA)) and water for at least 1 week as described previously. [19] The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals (18-0044)".

Crypt isolation and preparation of organoids

Crypts were isolated by a procedure described in a previous report with some modification. [20] The proximal jejunum of 5 cm in length was removed from mice euthanized by cervical dislocation. The small intestine

was flushed with ice-cold PBS (phosphate buffer saline) and cut open lengthwise on ice. The villi were scraped off using a scalpel blade (Feather Safety Razor Co. Ltd., Osaka, Japan), and remaining tissue was washed with ice-cold PBS (phosphate buffer saline) (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2PO_4, 1.47 mM KH_2PO_4) and incubated in 30 mM EDTA (ethylenediaminetetraacetic acid) with Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) and rotated 5 rpm for 10 min at room temperature. The tissue was shaken vigorously 20 times in fresh ice-cold HBSS to exfoliate crypts followed by transfer to ice-cold fresh HBSS (fraction #1) and the tissue was shaken 50 times in the same way in fresh ice-cold HBSS (fraction #2) and the shaking step was repeated (fraction #3). The tissue was shaken 100 times in the same way and fraction #4 was obtained. Each fraction was centrifuged at 400 g for 4 min at 4ºC and isolated crypts were resuspended in HBSS supplemented with 10 μM Y-27632. Y-27632 is a potent ROCK (Rho-associated coiled-coil forming kinase: Rho-binding kinase) inhibitor that acts in a specific and ATP antagonistic manner. Ham et al. reported that Y-27632 increased the survival rate of enteroids in preparation of enteroids and culture, cryopreservation. [18] According to the report, we added Y-27632 in our medium. After counting the crypts of each fraction, the fraction with >80% purity of crypts was centrifuged at 400 g for 4 min at 4ºC. It is important to ensure high purity of the crypts. The supernatant was removed and a total of 125 crypts were gently mixed with 12.5 μL of IntestiCult™ OGM (IntestiCult™ Organoid Growth Medium (mouse) (STEMCELL Technologies Inc., Vancouver, Canada). IntestiCult™ OGM was composed of IntestiCult™ OGM basal medium/IntestiCult™ OGM Mouse Supplement 1/IntestiCult™ OGM Mouse Supplement 2=90/5/5 (v/v/v) and 100 units/mL of penicillin/streptomycin. Twelve point five μL of Matrigel (Corning Inc., NY, USA) was then added to this suspension and plated in 48-well plates (Thermo Fisher Scientific, MA, USA) at 37ºC. After polymerization of Matrigel with incubation for 10 min at 37ºC, 250 μL of IntestiCult™ OGM was added and 500

μL of PBS at 37ºC was added. Matrigel-embedded crypts were cultured in an atmosphere of 5% CO_2/95% air at 37ºC. The culture medium was changed every day or every 2 days. Bright-field microscopic observations of morphological changes in enteroids from seeding to 7 days later are shown as Supplemental Figure 1. The U-shaped portion of the crypts begin to close about 2 hours after seeding (Supplemental Fig. 1-A), while the villi fragments do not grow. 1-2 days after seeding, the crypts become spherical (Supplemental Figs. 1-B, C), crypt-like domains appear from day 3 (Supplemental Fig. 1-D), and crypt-like domains proliferate and elongate from day 4 onward (Supplemental Figs. 1-E, F).

Cell culture and cryopreservation

For passage, IntestiCult™ OGM was removed from the enteroids after 7 days of culture and Gentle Cell Dissociation Reagent (STEMCELL Technologies Inc.) was added and mixed to crush Matrigel. The enteroids were centrifuged at 440 × g for 5 min at 4ºC and the supernatant was removed. Ten milliliters of DMEM/F12 with 15 mM HEPES (STEMCELL Technologies Inc.) was added and mixed. The centrifugation and removal of the supernatant were then repeated. The enteroids were seeded at the density of 150 crypts/well into a new 48-well plate as described above. For cryopreservation, IntestiCult™ OGM was removed from the enteroids after 7 days of culture and ice-cold PBS was added and mixed to crush Matrigel. After centrifugation twice as described above, CryoStor CS10 (STEMCELL Technologies Inc.) with 10 μM Y-27632 was added to the pellet and transferred to a cryotube (200 crypts/1 mL of CryoStor CS10). The enteroids was preserved in liquid nitrogen after freezing at -80ºC for 24 h.

154

155 *RT-PCR and quantitative real-time PCR analysis*

156

157 RT-PCR (reverse transcription polymerase chain reaction) was performed as described in a previous

158 report with some modification. [21] Total RNA was prepared from mouse enteroids using RNAiso Plus (Takara Bio

159 Inc., Shiga, Japan). Single-strand cDNA was made from total RNA by reverse transcription using ReverTra Ace®

160 qPCR RT Master Mix with gDNA remover (TOYOBO Co. Ltd., Osaka, Japan). PCR was performed with 10 μg

161 of genetic template DNA using Sapphire Amp Fast PCR Master Mix (Takara Bio) through 30 cycles of 98ºC for

162 5 sec, 55ºC for 5 sec and 72ºC for 5 sec followed incubation for 1 min at 94ºC. The sequences of the specific

163 primers are shown in Table 1. The PCR products were subjected to electrophoresis on a 1% agarose gel and then

164 visualized by ethidium bromide staining with UV light.

165 Real-time qPCR was performed with 10 μg of genetic template DNA using TB Green™ Premix Ex

166 Taq™ II (Tli RNaseH Plus) (Takara Bio) through 2-step PCR with 40 cycles of 95ºC for 5 sec and 60ºC for 30 sec

167 after preincubation for 30 sec at 95ºC and dissociation reaction were performed after PCR for 5 sec at 95ºC and

168 for 30 sec at 60ºC. The PCR products were normalized to amplified Gapdh (glyceraldehyde 3-phosphate

169 dehydrogenase), which was the internal reference gene (housekeeping gene). A standard curve was established

170 between the threshold cycles (Ct) and the log10 (copy numbers) by using Applied Biosystems sequence detection

171 system software, version 110.61. The software calculates the relative amount of the target gene to the housekeeping

172 gene based on the Ct value.

173

Uptake study in enteroids

The enteroids were seeded at the density of 150 crypts/well into a new 48-well plate (flat bottom, non-pyrogenic, polystylene). Enteroids of 5-7 days after seeding were used for an uptake assay. At this time, enteroids with a size of about 50-100 μm and a proliferated and elongated crypt-like domain were used. The medium was aspirated from the wells to be used for the experiment. Rh123 (final conc. of 1 μM) in Advanced DMEM/F12 (Thermo Fisher Scientific) with or without P-gp inhibitors (cyclosporine A, verapamil and quinidine) (each final conc. of 20 μM) [22] or Rh123 (final conc. of 1 μM) in transport buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM HEPES, no glucose, pH 7.5 adjusted with Tris) with or without mitochondrial ATP production (final conc. of 10 mM) were prepared. These solutions were pre-warmed to 37°C and added at 250 μL/well. They were incubated up to 60 min in an atmosphere of 5% CO_2/95% air at 37ºC or 4ºC. After the designated time, the enteroids were analyzed by using a fluorescence microscope (BZ-9000, KEYENCE Corp., Osaka, Japan).

Analytical procedures

In a photographic image of enteroids 60 min after uptake taken by the microscope, a two-dimensional profile plot of pixel intensities along a straight line in the fluorescence image was made using Image J (NIH). For analysis of the profile of enteroids and calculation of the substrate transfer rate of enteroids to the lumen, an image taken in a bright field was subjected to negative/positive reversal processing and then colored by pseudo color

processing, and the fluorescent blur was removed by haze reduction. The concentration gradient on an arbitrary straight line in the image obtained by these treatments was graphed by ImageJ, and the range from A to B and C to D was defined as the epithelial cell, and the range from B to C was defined as the lumen. A, B, C and D on an arbitrary straight line were selected as a contoured signal in the whole enteroid (Supplemental Figure 2). An arbitrary straight line is drawn so that it passes near the center of the enteroid. It is drawn from any points to include the epithelial cell and the lumen area. The straight line used when the contour of the enteroid was determined was fitted to the fluorescence image of the uptake experiment, and the transfer rate of the substrate to the lumen of the enteroid was calculated on the basis of defined A, B, C and D. Specifically, the concentration in the lumen was calculated by subtracting the background value from the average value of the fluorescence intensity in the range of B to C. The concentration in epithelial cells was calculated by subtracting the background value from the average value of the fluorescence intensities in the ranges of A to B and of C to D. The background value was the average value of the fluorescence intensity in the area where there was no enteroid. From these calculations, the ratio of the concentration in the lumen to the concentration in epithelial cells was defined as the transfer rate of the substrate to the lumen of the enteroid. The formula for calculating the transfer rate was as follows:

$$\frac{\text{Lumen concentration}}{\text{Concentration in epithelial cells}} = \frac{(Average\ of\ fluorescence\ intensity\ from\ B\ to\ C) - (background)}{(Average\ of\ fluorescence\ intensity\ from\ A\ to\ B\ and\ C\ to\ D) - (background)}$$

Statistical analysis

Student's t-test was used to determine the significance of differences between two group means.

Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test or the Tukey-Kramer test. Statistical significance was defined as $p<0.05$.

Results

mRNA expression levels of P-gp, Bcrp and Mrp2 in enteroids

In the first part of this study, the mRNA expressions levels of ABC transporters were confirmed. We had already confirmed the mRNA expression of Mdr1, Mrp2, Bcrp, Gapdh and Villin in our prepared mouse enteroids. There was no significant difference in the mRNA expression levels of any of the transporters (Mdr1, Mrp2 and Bcrp) depending on the number of passages and number of culture days (Fig. 1). The expression of Villin, an intestinal epithelium marker, in primary cultured enteroids was almost the same regardless of the number of culture days.

We then evaluated the expression of these transporters before and after cryopreservation of enteroids. At day 3 of cryopreservation after thawing, the mRNA expression levels of Mdr1 and Mrp2 were significantly increased compared with the levels before cryopreservation (Fig. 2). The expression of Bcrp tended to increase at the same time point. There was no significant difference in the relative expression levels of these transporters when corrected with Villin (data not shown).

Accumulation of Rh123 and effects of P-gp inhibitors and inhibitors of mitochondrial ATP production on Rh123 accumulation in enteroids

The fluorescence of Rh123, a substrate of P-gp, accumulated in the lumen of enteroids over time when exposed to enteroids (Fig. 3A). We confirmed that there was no accumulation of Rh123 fluorescence in the enteroid lumen up to 60 min in accordance with our expectation that transporters would not work at 4ºC (data not shown). On the other hand, when cyclosporin A as a P-gp inhibitor was exposed in combination with Rh123, it was observed that the enteroids accumulated not in the lumen but in the outer peripheral part, that is, in epithelial cells. (Fig. 3B). We then investigated whether similar results could be obtained with inhibitors other than cyclosporine A. Fig. 4 shows bright and dark field images and a profile plot of fluorescence intensity of Rh123 along the yellow line in the dark field image. It was confirmed that Rh123 accumulated in epithelial cells of the enteroid in the presence of P-gp inhibitors (verapamil and quinidine), whereas it accumulated in the lumen of the enteroid in the absence of a P-gp inhibitor (Figs. 4C, 4D). In addition, we investigated the accumulation of Rh123 using inhibitors of ATP production and transport buffer (no glucose) in order to confirm that these results were based on the inhibition of P-gp activity by the inhibition of ATP production. Accumulation of Rh123 in enteroids was similar to that of P-gp inhibitors even when ATP was depleted (Fig. 4E). These results showed that an ATP production inhibitor inhibited the activity of P-gp and inhibited the extracellular efflux of Rh123 that accumulated in epithelial cells.

Quantitative analysis of Rh123 accumulation in enteroids

We next evaluated the effects not only qualitatively but also quantitatively. We analyzed fluorescence

254 images by the methods described in analytical procedures in Materials and Methods. The average ratios of the
255 concentration in the lumen to the concentration in epithelial cells were 0.68, 0.77 and 0.81 for cyclosporine A,
256 verapamil and quinidine, respectively, in the P-gp inhibitor group and 1.4 in the ATP production inhibitor group,
257 which were significantly reduced compared to the ratio in the control group (3.0) (Fig. 5A). On the other hand, the
258 transfer rate of antipyrine, a negative control, [23] was 3.0. There was no significant difference between the control
259 and antipyrine groups.

260 We focused on the effects of passage and cryopreservation on the accumulation of Rh123 in enteroids.
261 The transfer rates in the control group and the negative control group were about 3.0 even when the enteroids were
262 used after passage and after cryopreservation (Figs. 5B, 5C). In addition, the values in the P-gp inhibitor groups
263 were significantly smaller than the value in the control group. Regardless of whether enteroids were used after
264 passage or cryopreservation, the transfer ratio from the epithelium to the lumen was approximately 1.3 the P-gp
265 inhibitor combination group (verapamil and quinidine). However, it was shown that the inhibitory effect was
266 slightly smaller than that of the enteroid in the case of passage after primary culture in conditions (data not shown).

267

268 **Discussion**

269 The enteroid prepared from the small intestine is an organoid in which the stem cells contained in the
270 crypts grow and differentiate into mainly four types of cells (absorptive, goblet, endocrine and Paneth cells) and
271 these cells adhere to each other to self-assemble. [8] Enteroids maintain a physiological state in which cells and
272 tissues other than absorbing epithelial cells coexist, and substance transport mimicking the actual small intestine
273 has been performed. [8,9] In recent years, there have been increasing reports of studies of ES cell- and iPS cell-

derived organoids and human intestinal organoids. [24,25] Several experimental models using enteroids have been used as a tool for analysis of various functions of the small intestine as a useful as an *in vitro* system that is close to the *in vivo* system. [20, 26-28] There are some systems that enable evaluation of the absorption direction (apical to basolateral). [12] Injections into the lumen of the enteroids are necessary to evaluate the direction of absorption. This injection a solution into an enteroid of about 100 nm would require skillful technique. On the other hand, there are few systems that enable easy and proper evaluation of the excretion direction (basolateral to apical). Due to the characteristics of three-dimensional structures and a structure in which the inside of enteroid is closed on the side of the gastrointestinal lumen, we tried to construct an evaluation system of the influence of efflux transporters. Although there have been several reports using enteroids to study the influence of efflux transporters, [13,14,29,30] reproducibility, that is, the influence of subculture and cryopreservation of enteroids, has not been investigated in detail. There are some reports that some cultured cells increase or decrease in transporter expression depending on the number of passage and days in culture. This suggested the need to confirm the same in enteroids. In this study, we investigated that expression levels of efflux transporters and the influence of passages and cryopreservation in murine enteroids.

Firstly, the mRNA expressions levels of ABC transporters were confirmed by RT-qPCR. There was no significant difference in the mRNA expression level of any of the transporters (Mdr1, Mrp2 and Bcrp) with different numbers of culture days (Fig. 1). The expression of Villin, an intestinal epithelium marker, in primary cultured enteroids tended to decrease daily. There were no significant differences in the mRNA expression level of Villin in the number of culture days after passage. A comparison before and after passage showed that the expression of each transporter tended to increase. Liu et al reported that the expression levels of cystic fibrosis

transmembrane conductance regulator (cftr) and other acid-base transporters were almost the same as the levels before passage. [10] Our results are almost consistent with their results. We then investigated the expression levels of these transporters before and after cryopreservation of enteroids. At day 3 of cryopreservation, the mRNA expression levels of Mdr1 and Mrp2 were significantly increased (Fig. 2). The expression of Bcrp tended to increase at the same point. However, after correcting these expression levels with Villin, there was no significant difference for each transporter (data not shown). Fuller et al. reported that crypt expansion and viability of enteroids after cryopreservation were almost the same as those before cryopreservation. [17] It is thought that transporter expression was increased due to an increase in the percentage of epithelial cells in enteroids. In the present study, we found significant differences in the expression of Mdr and Mrp2 at the mRNA level, but the ratio was less than 2, which is not considered a physiologically meaningful difference.

We focused on P-gp, the major efflux transporter, and analyzed the function in detail. Rh123 accumulated in the lumen of enteroids up to 60 min (Fig. 3). These results are consistent with the results of a previous study. [11] For additional analysis, we obtained a profile plot of pixel intensities at 60 min after uptake of Rh123 with or without each P-gp inhibitor. Excretion of Rh123 into the lumen of enteroids was inhibited by several P-gp inhibitors and the Rh123 that was not excreted accumulated in enteroid epithelial cells (Figs. 4A-4D). As well as using P-gp inhibitors, almost the same result was obtained even when an inhibitor of ATP production was used (Fig. 4E). We considered that we could eliminate the effect of glucose-derived ATP production if when using both substances as an inhibitor and transport buffer without glucose. These are based on that sodium azide inhibits mitochondrial complex IV, and sodium fluoride inhibits ATP production by inhibiting glycolytic enolase. [31] In Fig. 4-E, the effect of ATP-depletion on the accumulation of Rh123 is less than those of other P-gp inhibitors. In this

study, we used 10 mM ATP-depleting agents and a transport buffer with no glucose, but glucose was included (4.5 g/L, equivalent to 25 mM) as the constitutive medium (DMEM) of the Matrigel in which the enteroids were embedded. It is possible that glucose was supplied by this Matrigel and did not create the condition of ATP depletion that we intended.

We next evaluated the effects of P-gp inhibitors and an inhibitor of ATP production on the excretion of Rh123 to lumen of enteroids using a profile plot of pixel intensities (Supplemental Fig. 2). Zhang et al. reported the functions of Bcrp and Mrp2 using enteroids. [13,14] They measured the fluorescent substrates of these transporters in whole enteroids containing the lumen and epithelial cells. We then thought that it would be easier to evaluate by dividing the amount excreted into the lumen of enteroid by the amount accumulated in epithelial cells. We focused on the effects of passage and cryopreservation on the accumulation of Rh123 in enteroids. When two parameters of fluorescence, exposure time and excitation light control, were changed at the same time and fluorescence images were taken, the ratio of the calculated intraluminal concentration to the epithelial cell concentration was almost the same (data not shown). We therefore considered that the data calculated from a single fluorescence image could be regarded as almost the same even under different measurement conditions. It is possible to evaluate the function of an efflux transporter after eliminating the differences in the solidity of the enteroid and the measurement conditions. We then analyzed the translocation rate of Rh123 into the enteroid lumen by defining the ratio of luminal Rh123 concentration to epithelial cell concentration. The ratios of the concentration in the lumen to the concentration in epithelial cells was 0.50, 0.77 and 0.77 were for cyclosporine A, verapamil and quinidine, respectively, in the P-gp inhibitor group and 1.53 in the ATP production inhibitor group, which were significantly reduced compared to the ratio in the control group (Fig. 5A). The transfer rates in the control group

and the negative control group were about 3 even when the enteroids were used after passage and after cryopreservation (Figs. 5B, 5C). In addition, the values in the P-gp inhibitors group were significantly smaller than the value on the control group. However, it was shown that the inhibitory effect was slightly smaller than that of the enteroid in the case of primary culture in both conditions. Although the control and negative control values were not different before and after passage and cryopreservation, the inhibitory rate of Rh123 lumen transfer by the inhibitor was slightly lower than that in the primary culture in enteroids after freezing or passage. It is necessary to further elucidate the reason why the function is slightly decreased despite the fact that the expression level does not change. These values obtained at this stage should be considered as reference values since the activity of P-gp may have changed. Since this experimental system showed the same tendency at the time of freezing and passage as that at the time of primary culture, there is a possibility that it can be used as a simple screening tool. Recently, Okada et al., reported the establishment of the preparation of human intestinal organoids using cryopreserved human small intestinal epithelial cells and reported that their properties were similar to those of biopsy-derived human intestinal organoids. In this study, we have reported the results in mice. [25] Further studies are needed to investigate the various influences of long-term cryopreservation.

The effects of passage and cryopreservation on the expression and function of efflux transporters of enteroids were investigated in this study. There are some surfactants that could be substrates for P-gp among those contained in emulsions such as Cremophor EL. [32] We previously reported that emulsions and solid dispersions could significantly improve the absorption of lutein and coenzyme Q10, which are lipophilic nutrients. [33-35] In the case of a formulation using some new surfactants, [35] it would be necessary to evaluate the effect of the formulation itself on the small intestine. The technology of injection into the lumen of enteroids may also allow easier

evaluation of the effects of these formulations such as those administered orally.

For this study, it is important to use a good fluorescent substrate of the target efflux transporter. Drug-drug interactions and food-drug interactions would increase the risk of adverse events and should be avoided. Our results should lead a reduction in the number of experimental animals and enable easy evaluation of the possibility of drug-drug or drug-food interactions. Further studies to apply this system to evaluation of the intestinal absorption mechanism are in progress.

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Conflict of interest

The authors report no conflicts of interest in this work.

Declaration of generative AI and AI-assister technologies in the writing process

The authors did not use any AI and AI-assisted technologies in the writing of the manuscript.

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Figure legends

Fig. 1 mRNA expression levels of efflux transporters before and after passage of murine enteroids.

Primary cultured enteroids at days 3, 5 and 7 and cultured enteroids at days 3, 5 and 7 after passage were collected. mRNA expression levels of Villin and efflux transporters (Mdr1, Mrp2 and Bcrp) were measured by real-time qPCR. The mRNA level was calculated by the relative calibration curve method with the expression level on day 3 of the primary cultured enteroid as the standard. Each column represents the mean with S.D. of 6 measurements.

Fig. 2 mRNA expression levels of efflux transporters before and after cryopreservation of murine enteroids.

Primary cultured enteroids at days 3, 5 and 7 and enteroids cultured at days 3, 5 and 7 after thawing following cryopreservation were collected. mRNA expression levels of Villin and efflux transporters (Mdr1, Mrp2 and Bcrp) were measured by real-time qPCR. The mRNA level was calculated by the relative calibration curve method with the expression level on day 3 of the primary cultured enteroid as the standard. Each column represents the mean with S.D. of 8 measurements. *; Significantly different by one-way ANOVA and by the Tukey-Kramer test.

Fig. 3 Accumulation of Rh123 in enteroids up to 60 min.

The cultured enteroids were treated in 1 μM Rh123 (A) and 1 μM Rh123 + 20 μM cyclosporin A (B) for up to 60 min. Images of the enteroids were taken by a fluorescence microscope at the designated times. Scale bar shows 100 μm. The data shown are typical results from 3 independent experiments.

Fig. 4 Effects of P-gp inhibitors and an inhibitor of ATP production on the accumulation of Rh123 in

enteroids.

Cultured enteroids were treated in 1 μM Rh123 (A) and 1 μM Rh123 + 20 μM cyclosporin A (B), + 20 μM verapamil (C), + 20 μM quinidine (D) and 1 μM Rh123 + 10 mM inhibitor ATP depletion (sodium azide and sodium fluoride) with buffer without glucose (only (E)) for up to 60 min. Images of the enteroids were taken by a fluorescence microscope at 60 min after addition of reagents. A bright field image and a dark field image and a profile plot of pixel intensities obtained by the method described in analytical procedures in Materials and Methods in each condition are shown. Scale bar shows 100 μm. The data shown are typical results from 3 independent experiments.

Fig. 5 Comparison of transfer rates of Rh123 into the lumen of enteroids in the presence of P-gp inhibitors and an inhibitor of ATP production (A) and effects of passage (B) and cryopreservation (C) on the transfer rate of Rh123 into the lumen of enteroids.

(A) Cultured enteroids were treated in 1 μM Rh123 only and 1 μM Rh123 + 20 μM cyclosporin A, verapamil, quinidine (P-gp inhibitors), antipyrine (negative control of P-gp inhibitor) and 1 μM Rh123 + 10 mM inhibitor ATP depletion (sodium azide and sodium fluoride) with buffer without glucose for up to 60 min. Images of the enteroids were taken by a fluorescence microscope at 60 min after addition of reagents. The transfer rate of Rh123 to the lumen of the enteroid was calculated by the image. Each column represents the mean ± S.D. with 3-6 measurements.

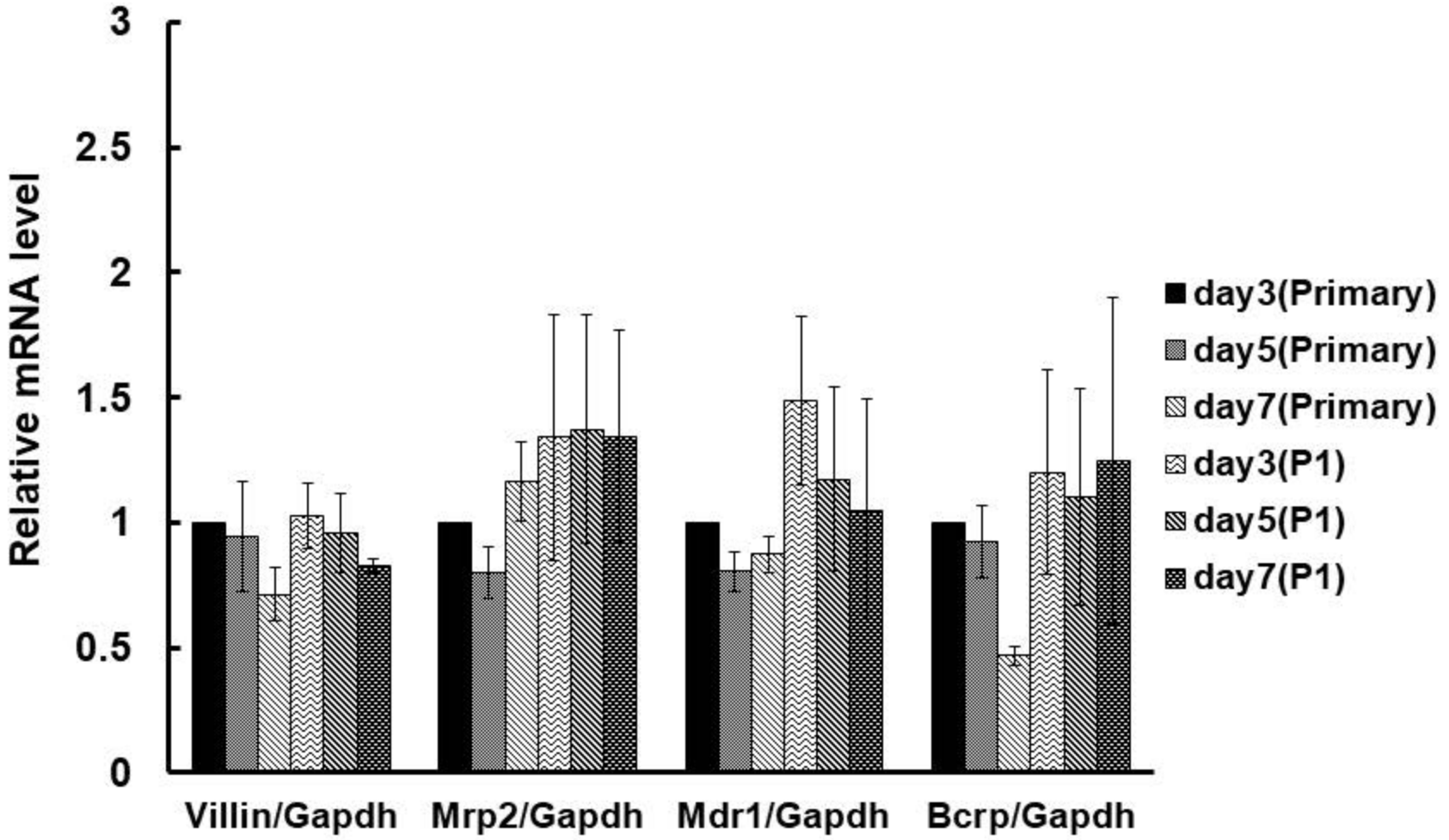
(B, C) Cultured enteroids were treated in 1 μM Rh123 only and 1 μM Rh123 + 20 μM verapamil, quinidine (P-gp inhibitors), and antipyrine (negative control of P-gp inhibitor) for up to 60 min. Images of the enteroids were taken

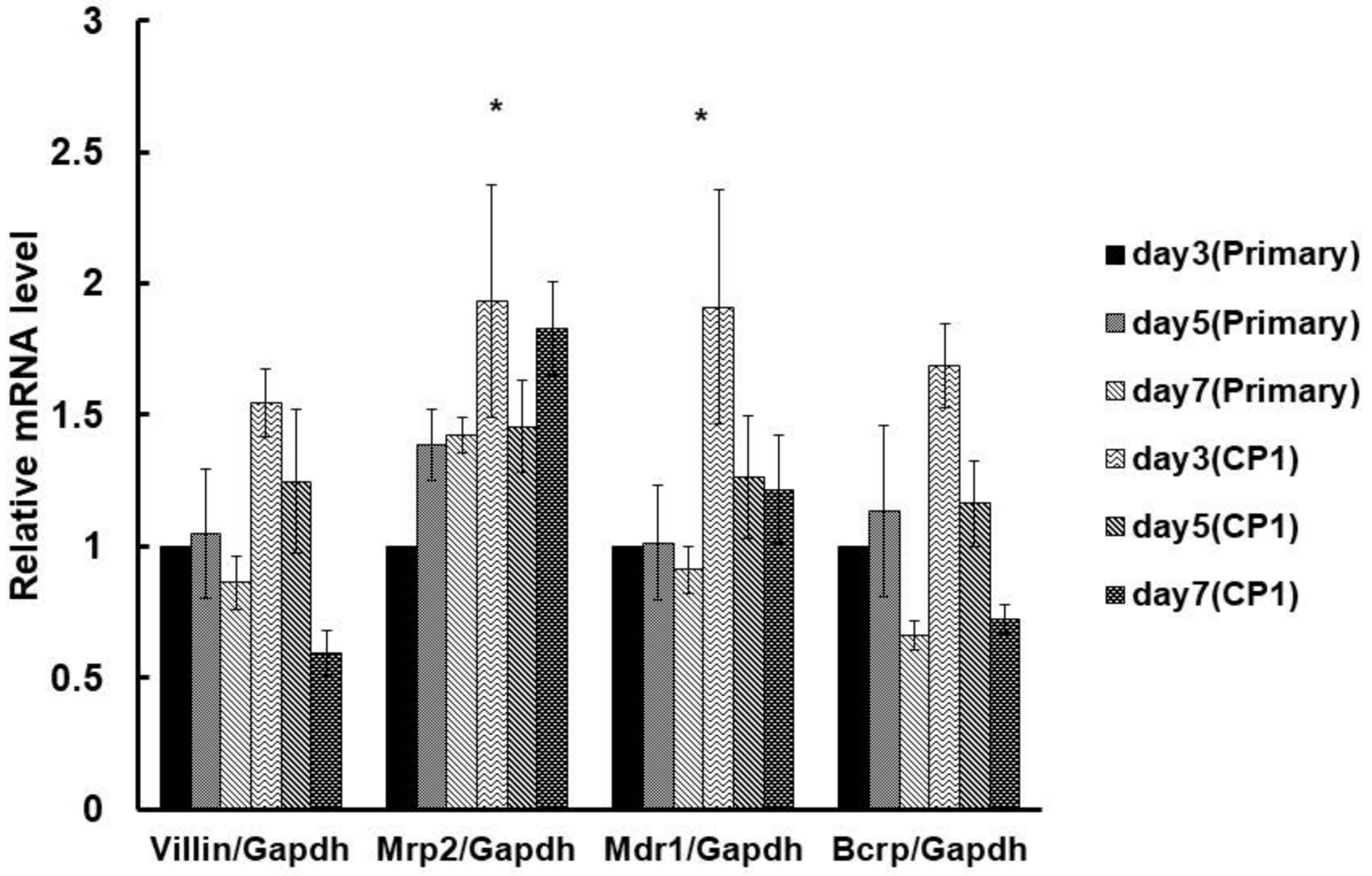
509 by a fluorescence microscope at 60 min after addition of reagents. The transfer rate of Rh123 to the lumen of the

510 enteroid was calculated by the image. Each column represents the mean ± S.D. with 3-5 measurements.

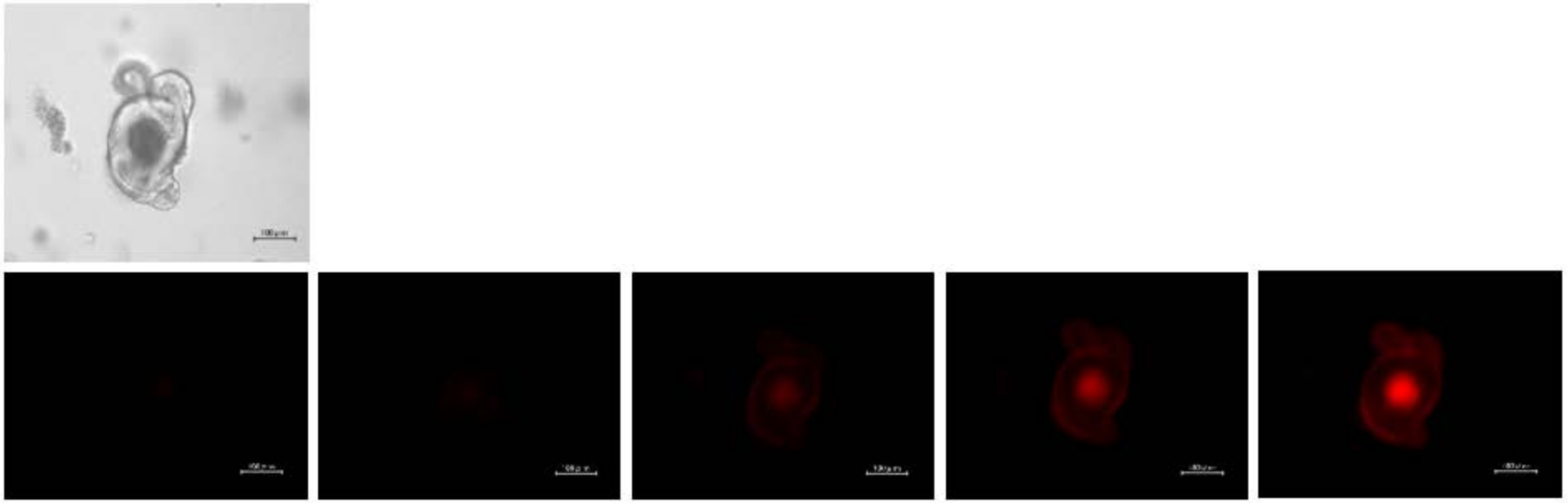
511 *; significantly different from control $p<0.05$ by Dunnett's test

512


Relative mRNA level
3
2.5
2
1.5
1
0.5
0
Villin/Gapdh
Mrp2/Gapdh
Mdr1/Gapdh
Bcrp/Gapdh
day3(Primary)
day5(Primary)
day7(Primary)
day3(P1)
day5(P1)
day7(P1)

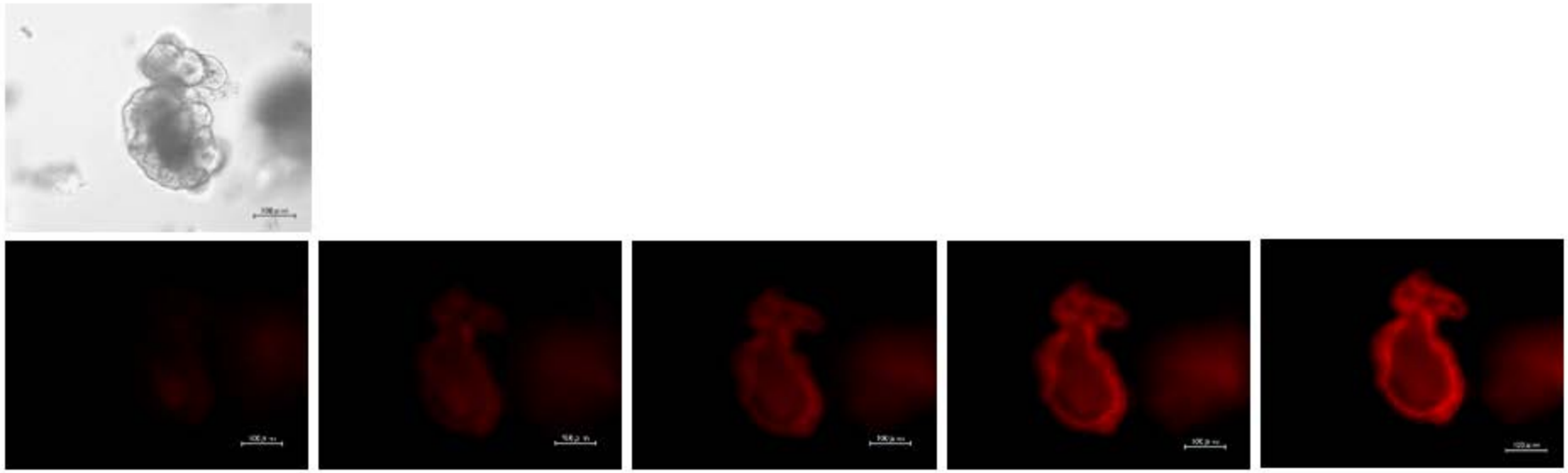

Relative mRNA level
3
2.5
2
1.5
1
0.5
0
*
*
Villin/Gapdh
Mrp2/Gapdh
Mdr1/Gapdh
Bcrp/Gapdh
day3(Primary)
day5(Primary)
day7(Primary)
day3(CP1)
day5(CP1)
day7(CP1)

A) Rh123



4 min 15 min 30 min 45 min 60 min

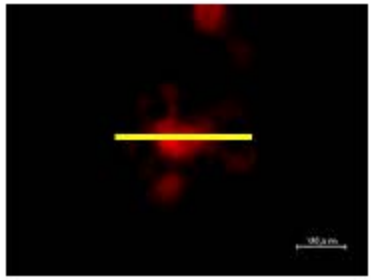
B) Rh123 + cyclosporin A

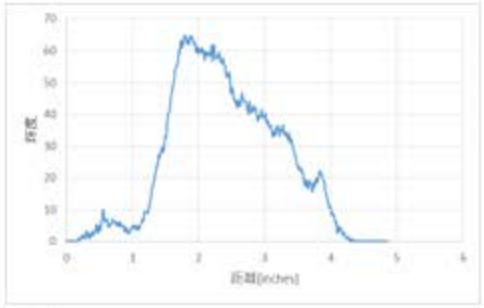


4 min 15 min 30 min 45 min 60 min

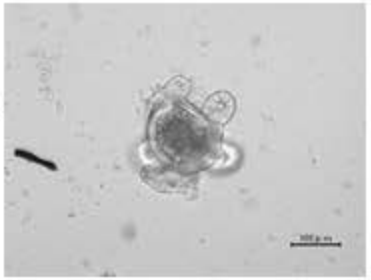
A) Rh123

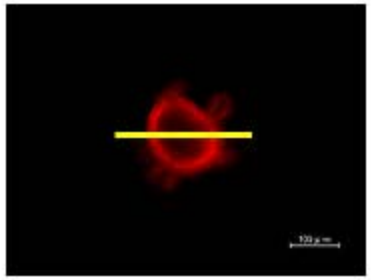


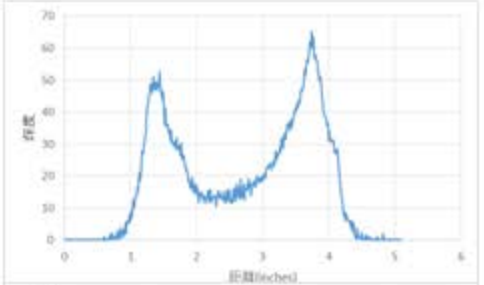




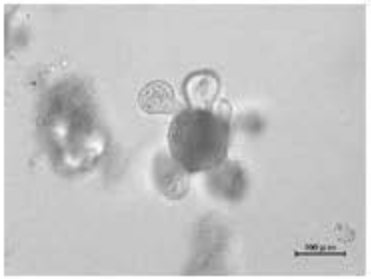
B) Rh123 + cyclosporin A

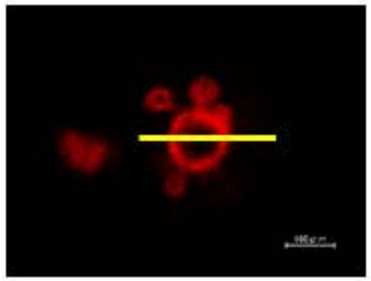


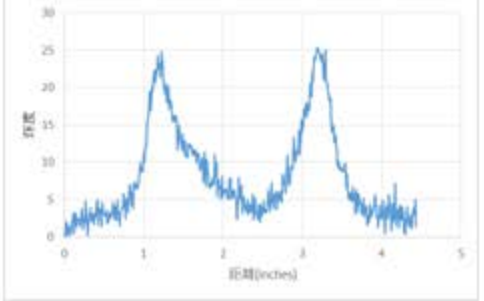




C) Rh123 + verapamil

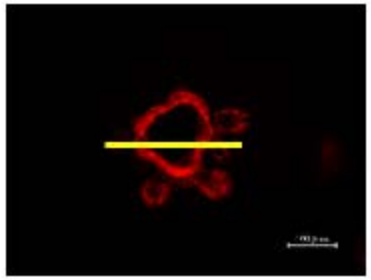


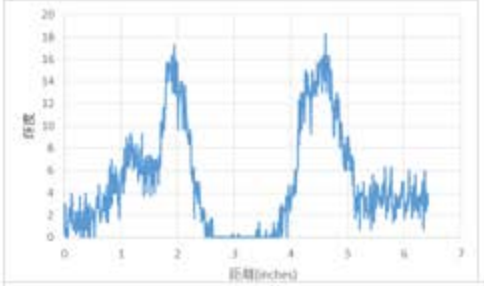




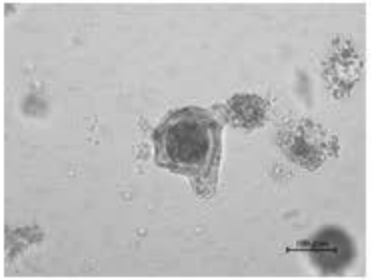
D) Rh123 + quinidine

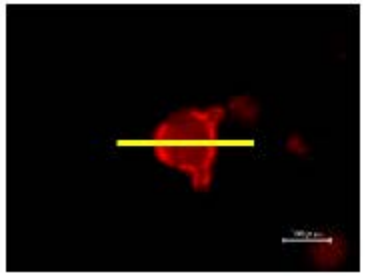


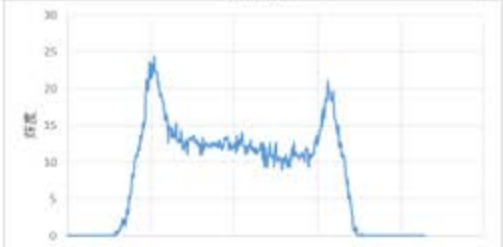


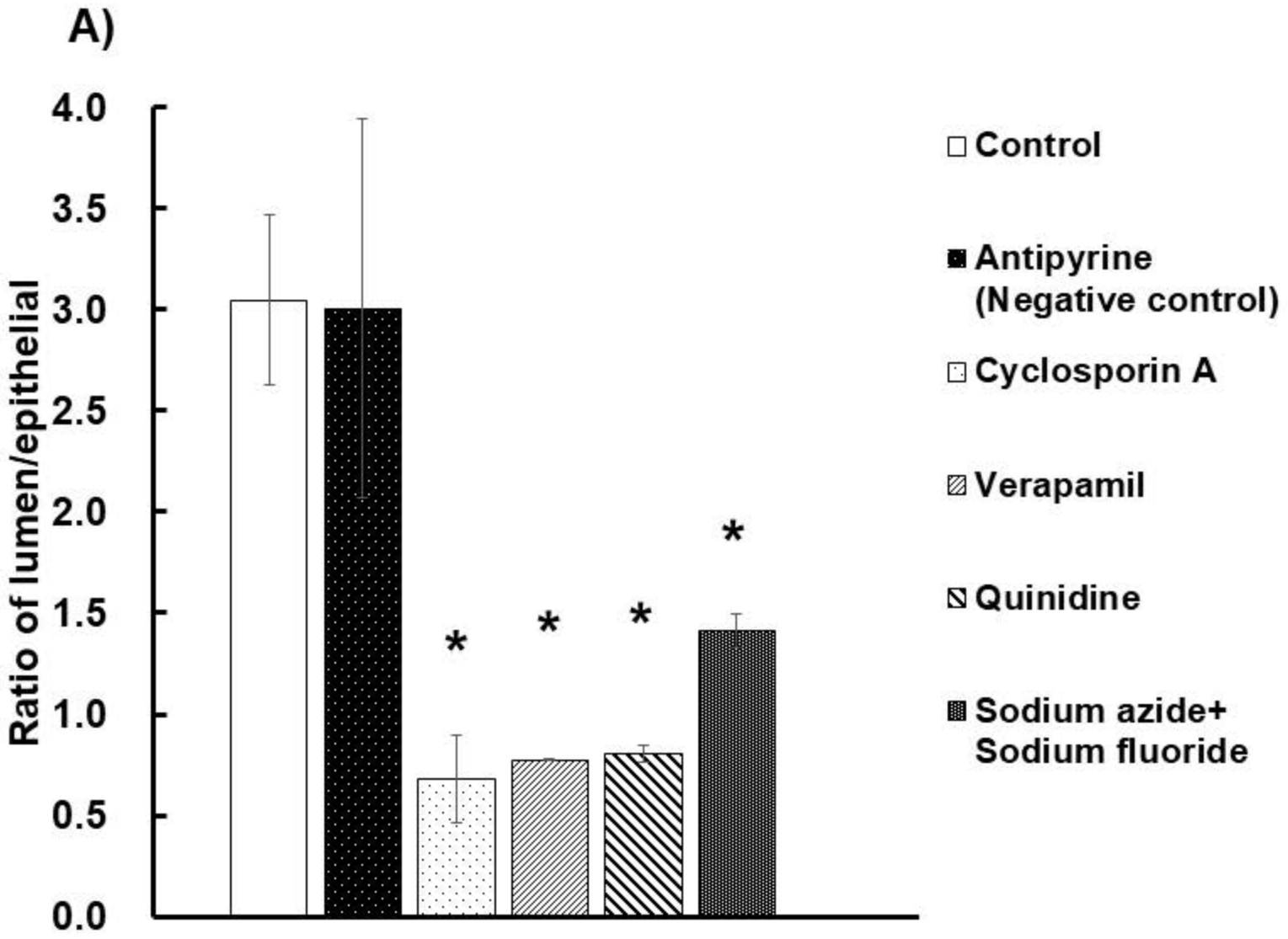


E) Rh123 + inhibitor of ATP depletion








A)
Ratio of lumen/epithelial
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
Antipyrine
(Negative control)
Cyclosporin A
Verapamil
Quinidine
Sodium azide+
Sodium fluoride

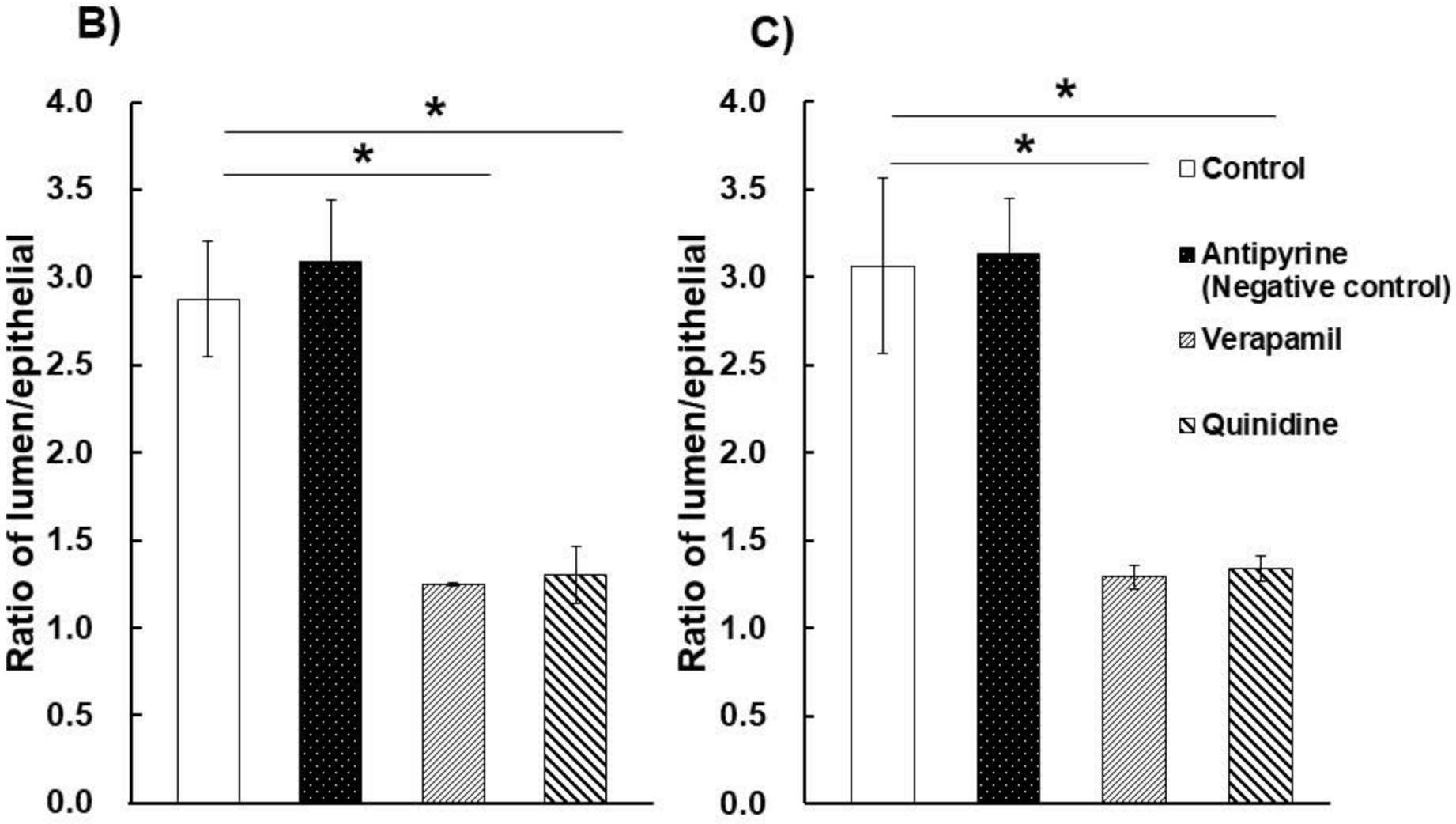

B)
C)
Ratio of lumen/epithelial
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
Antipyrine
(Negative control)
Verapamil
Quinidine

Table 1 Primer sequences used for qPCR analysis

Primer name	Primer sequence	Product size
mGapdh_Fwd	5’-ACAGTCCATGCCATCACTGCC-3’	266 bp
mGapdh_Rev	5’-GCCTGCTTCACCACCTTCTTG-3’	
mVillin_fwd	5'-CTTCTTCGATGGTGACTGCTAT-3'	203 bp
mVillin_rev	5'-AAGTCTCGCTCTCGTTGCCT-3'	
mMrp2_fwd	5’-CGACCATCCGGAACGAGTT-3’	57 bp
mMrp2_rev	5’-GCAGCCTGTGTGCGATAGTG-3’	
mMdr1_fwd	5’-GGGCACAAACCAGACAACAT-3’	117 bp
mMdr1_rev	5’-TCCGCTCTTCACCTTCAGAT-3’	
mBcrp_fwd	5’-AAATGCTGTTCAGGTTATGTGGT-3’	185 bp
mBcrp_rev	5’-TCCGACCTTAGAATCTGCTACTT-3’	