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Research Article

A serum-free medium developed for in vitro expansion of murine intestinal stem cells

Mahmoud S. Mohamed[1,*], *Yun Chen*[2,3,*] *and Chao-Ling Yao* [2]

[1] Graduate School of Biotechnology and Bioengineering, Yuan Ze University, Chung-Li, Taoyuan, Taiwan
[2] Department of Chemical Engineering and Materials Science, Yuan Ze University, Chung-Li, Taoyuan, Taiwan
[3] Department of Surgery, Far Eastern Memorial Hospital, Pan-Chiao, New Taipei, Taiwan

Intestinal stem cells (ISCs) are located at the base of the intestinal crypts and have the ability to self-renew as well as to differentiate into mature epithelial cells. Recently, ISCs have received much attention for the treatment of many intestinal diseases. However, many challenges face those studying ISCs because insufficient ISCs are available. Therefore, the development of a culture medium for ISC expansion is an important necessity for basic research and clinical application. In this study, we described the technique used to develop a serum-free medium for expanding ISCs in vitro. Furthermore, five serum substitutes were selected and optimized in order to maintain the long-term proliferation and enteroid-forming ability of ISCs: (i) ethanolamine; (ii) ascorbic acid phosphate; (iii) transferrin; (iv) glutathione; and (v) sodium selenite. Analysis of gene expression of Lgr5, Bmi1, Msi1 and PTEN demonstrated that our serum-free medium sustained the expression of genes involved in ISC-related functions in the expanded ISCs. Additionally, the expression intensity of surface markers, including Lgr5, CD24 and CD44, on serum-free expanded cells in crypts was greatly increased. Taken together, our results demonstrate that the number of ISCs can be expanded and their functionality maintained in our serum-free medium, indicating the suitability of this serum-free expansion medium for increasing the numbers of ICSs available for basic research and clinical applications in the future.

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1 Introduction

Stem cells that have the abilities of self-renewal and differentiation are located in many adult mammalian organs. One of these organs is the intestinal tract. Anatomical studies of the intestine have shown that it is composed of two main parts, the small intestine and the colon [1, 2]. Additionally, histological studies have shown that the intestine is lined with epithelium [1]. The intestinal epithelium is the most rapidly renewed tissue in the body [3, 4]. Within the crypt-villus axis, four main lineages of cells have been distinguished: (i) enteroendocrine cells; (ii) absorptive enterocytes; (iii) mucin-secreting goblet cells; and (iv) Paneth cells [1]. The intestinal stem cells (ISCs) of the small intestine are located at the base of the crypts [1, 3–5], and have the ability to self-renew and differentiate into another active lineage called transit-amplifying progenitors, which in turn give rise to types of different mature epithelial cells [4, 6].

Correspondence: Dr. Chao-Ling Yao, Department of Chemical Engineering and Materials Science, Yuan Ze University, No. 135 Yuan-Tung road, Chung-Li city 32003, Taiwan
E-mail: d897601@alumni.nthu.edu.tw

Abbreviations: Adv medium, advanced DMEM/F12 medium supplemented with ISC growth supplement cocktail and 2% serum; **Adv-SF medium**, Adv medium without serum addition; **FBS**, fetal bovine serum; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **ISCs**, intestinal stem cells; **Lgr5**, leucine-rich repeat-containing G-protein-coupled receptor 5; **Opt medium**, Optimum medium; **Opt-SF medium**, Serum-free optimum medium; **PBS**, phosphate-buffered saline; **PTEN**, phosphatase and tensin homolog; **qRT-PCR**, Quantitative real-time polymerase chain reaction; **SBS**, short bowel syndrome

* These authors contributed equally to this work.

Recent studies have identified some marker genes for ISCs, including Msi1, Bmi1, CD24, CD44, and Lgr5 (leucine-rich repeat-containing G-protein-coupled receptor 5). Lgr5 is a Wnt target gene and is a seven-transmembrane receptor that is expressed in the crypt base columnar cells at the base of the crypts [7, 8]. Bmi1 encodes a member of the PRC1 group and is a polycomb-ring finger oncogene that is necessary for the self-renewal of ISCs [9]. Msi1 is a regulator of Notch signaling and its expression has been observed in the murine intestine [10]. Furthermore, PTEN (phosphatase and tensin homolog) was shown to be an ISC marker that is involved in bone morphogenetic protein signaling [6]. Recently, CD24 and CD44 were used to isolate ISCs from human intestinal epithelial cell populations [11].

Many patients suffer from various intestinal diseases, including short bowel syndrome (SBS). SBS results from malabsorption due to the surgical removal of up to 70% of the small intestine [12]. This disease is considered one of the major causes of mortality in humans every year. Although there are various treatments for SBS, such as sequential intestinal lengthening, total parenteral nutrition and intestinal transplantation, each treatment has its limitations. Therefore, an ISC-based therapy for this disorder has received much attention [13–16]. Based on previous trials, we hypothesized that ex vivo expansion of ISCs is the first important step in obtaining sufficient cell numbers for stem cell therapy of intestinal diseases.

A serum-free expansion medium is important because its use avoids the problems of viral contaminants and the undefined components of serum [17–20]. Thus, the aim of this study was to improve the proliferation abilities of ISCs in vitro and to develop a serum-free culture medium for their expansion using a technique that was based on a combination of a fractional factorial design and a steepest ascent approach. Our results showed that we succeeded in enhancing the growth of ISCs and developed a serum-free system. Analysis of gene expression, including Lgr5, Bmi1, PTEN, Msi1 and CD24, confirmed that our serum-free medium formulation maintains the ISC-related gene expression of the cultured ISCs. Additionally, flow cytometric analysis of Lgr5, CD24, and CD44 protein levels confirmed those results. Moreover, our serum-free medium can maintain ISC proliferation for at least six weeks in vitro. Finally, we believe that our serum-free medium is a promising tool for use in basic research and clinical applications.

2 Materials and methods

2.1 Animals

All protocols and procedures for the animal experiments performed in this study were approved by the committee on laboratory animal research of the Far Eastern Memorial Hospital, Taiwan. Three-week-old C57BL/6JNarl mice weighing 50–60 g were used for the experiments. The mice were provided food and water, and were housed with a 12:12 h day-night cycle at approximately 20°C.

2.2 Harvesting of the intestinal crypts

After sacrificing the mice, the small intestines were removed and flushed with cold phosphate-buffered saline (PBS). ISCs were harvested from the whole small intestine from ligment of Treitz to ileocecal junction. The intestines were cut into 5 mm sections, opened longitudinally, transferred into 3 mM EDTA in PBS (Gibco, Carlsbad, CA) containing 2% gentamicin (Gibco) and rocked on an orbital shaker for 30 min at 4°C. After shaking, the tissue pieces were placed in cold PBS and shaken on an orbital shaker for 5 min. The tissue was mixied by vortexing to release the crypts into the solution and was then filtered through 70 μm cell strainer. The filtrate was then centrifuged at 200 × g for 10 min at 4°C.

2.3 In vitro culture of intestinal crypts

Initially 5,000 crypts were plated in cold laminin-rich matrigel (BD Biosciences, San Jose, CA) in 12-well plates and were overlaid with advanced DMEM/F12 (GIBCO) that was supplemented with 2% fetal bovine serum (FBS, GIBCO) and ISC growth supplement cocktail (50 ng/mL epidermal growth factor (EGF, R&D systems, Minneapolis, MN), 500 ng/mL R-spondin (R&D systems), 100 ng/mL of Noggin (Pepro Tech EC Ltd, London, UK), 1 μM Jag-1 peptide (Anaspec, San Jose, CA), 2.5 ng/mL Wnt3a (Sigma), 10 μM Y-27632 (Sigma), 2 mM L-glutamine (Sigma, St. Louis, MO), 100 units/mL of penicillin and 100 μg/mL of streptomycin (GIBCO)). The crypts were maintained in culture and the medium was changed every 3 days.

2.4 Experimental design

The basal medium, advanced DMEM/F12 supplemented with a group of essential growth factors, is a standard formulation used in most of the previous studies involving the culture of ISCs[18]. However, our preliminary data show that when the other basal medium, general DMEM/F12, is supplemented with the same group of growth factors as used to culture crypts, a 43% reduction in the proliferation of ISCs occurred (Fig. 1). After screening both types of basal media, we found nine different components in the advanced DMEM/F12 that are not pressent in general DMEM/F12. The nine components are: (i) 8.63×10^{-3} mM ascorbic acid phosphate (Sigma); (ii) 1.38 mM Albumax (Sigma); (iii) 9.74×10^{-5} mM human transferrin (Sigma); (iv) 1.72×10^{-3} mM insulin (Sigma); (v) 3.26×10^{-3} mM glutathione (Sigma); (vi) 2.6×10^{-6} mM ammonium metavanadate (Sigma); (vii) 3×10^{-7} mM

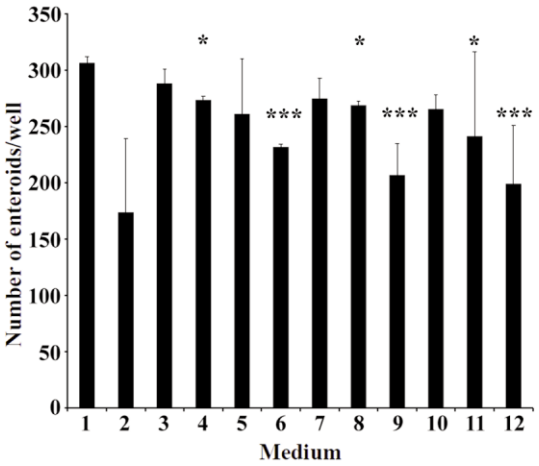

Figure 1. Effects of different growth factors on the proliferation of ISCs in vitro. A 12-well plate was seeded with 5000 fresh crypts, and the enteroids in each well were counted at day 7. The concentration of nine components added in media 3–12 are: 8.63×10^{-3} mM ascorbic acid phosphate; 1.38 mM Albumax, 9.74×10^{-5} mM human transferrin; 1.72×10^{-3} mM insulin; 3.26×10^{-3} mM glutathione; 2.6×10^{-6} mM ammonium metavanadate, 3×10^{-7} mM manganous chloride, 2.89×10^{-5} mM sodium selenite and 1.95×10^{-2} mM ethanolamine. Each experiment was replicated three times. *$p < 0.05$ and ***$p < 0.001$ compared with the medium 1 and medium 3.

manganous chloride (Sigma); (viii) 2.89×10^{-5} mM sodium selenite (Sigma); and (ix) 1.95×10^{-2} mM ethanolamine (Sigma). Therefore, we decided to use the general DMEM/F12 medium and these nine components in our research to develop serum-free medium formulation. We utilized four sequential approaches to develop the new serum-free medium formulation, as described in Sections 2.4.1–2.4.4.

2.4.1 Growth media

We designed various growth media (Table 1) to determine the effect of the absence of each factor on the proliferation of the in vitro cultured intestinal crypts. Following plating, the crypts change from normal crypt structures to spherical structures. According to the recommendations of the ISC Consortium (ISCC; http://iscc.coh.org), these structures were termed enterospheres. During culture, the enterospheres undergo extensive budding to give rise to more complex structures, termed enteroids (Fig. 2). At day 7, the number of enteroids in each well was counted.

2.4.2 Factor screening

The matrix of the $2^{(6-2)}$ fractional factorial design was used to determine which factors were significant (Supporting information, Table S1). The experimental data collected when the fractional factorial design plan was implemented was processed using SPSS (Statistical Package for the Social Science) software to obtain a first-order regression model. This model utilized the following equation:

Table 1. Composition of various culture conditions in Figure 1.

Medium	Description
1	Advanced DMEM/F12 with 2% FBS
2	General DMEM/F12 with 2% FBS
3	General DMEM/F12 with 2% FBS and 9 components[a)] (equivalent in composition to Medium 1)
4	Medium 3 without ascorbic acid
5	Medium 3 without Albumax
6	Medium 3 without transferrin
7	Medium 3 without insulin
8	Medium 3 without glutathione
9	Medium 3 without ammonium metavanadate
10	Medium 3 without manganous chloride
11	Medium 3 without sodium selenite
12	Medium 3 without ethanolamine

a) The nine components are 8.63×10^{-3} mM ascorbic acid phosphate, 1.38 mM Albumax, 9.74×10^{-5} mM human transferrin, 1.72×10^{-3} mM insulin, 3.26×10^{-3} mM glutathione, 2.6×10^{-6} mM ammonium metavanadate, 3×10^{-7} mM manganous chloride, 2.89×10^{-5} mM sodium selenite, and 1.95×10^{-2} mM ethanolamine.

$$\text{ISCs (enteroids/well)} = a_0 + \Sigma\, a_i x_i \quad (1)$$

in which the a_i values are the fitted coefficients and the x_i values are coded variables of the medium components that were investigated. This model can be used to identify the effective component, and the fitted coefficients of components in the model provide the ratio to develop the steepest ascent path to obtain the optimal concentration cocktail for ISC expansion. The larger the fitted coefficient was, the more significant was the effect on ISC expansion by the associated component, where a negative coefficient implied an inhibitory effect on ISC expansion. The direction toward the maximum enteroid number was determined according to the gradient of the subsequent experiments.

2.4.3 Optimization of effective concentration

For the design of steepest ascent path to determine the optimal concentrations of each medium component see Supporting information, Table S2.

2.4.4 Comparison of optimum medium with other commercially available media

Optimum medium (Opt) is general DMEM/F12 supplemented with our serum-free formula (see step 5 in Supporting information, Table 2), ISC growth supplement cocktail and 2% FBS; Serum-free optimum medium (Opt-SF) is the Optimum medium without serum addition. Adv medium is advanced DMEM/F12 medium supplemented with ISC growth supplement cocktail and 2% FBS. Adv-SF is adv medium without serum addition. General medium is general DMEM/F12 medium supplemented with ISC growth supplement cocktail and 2% FBS. General-SF is General medium without serum addition.

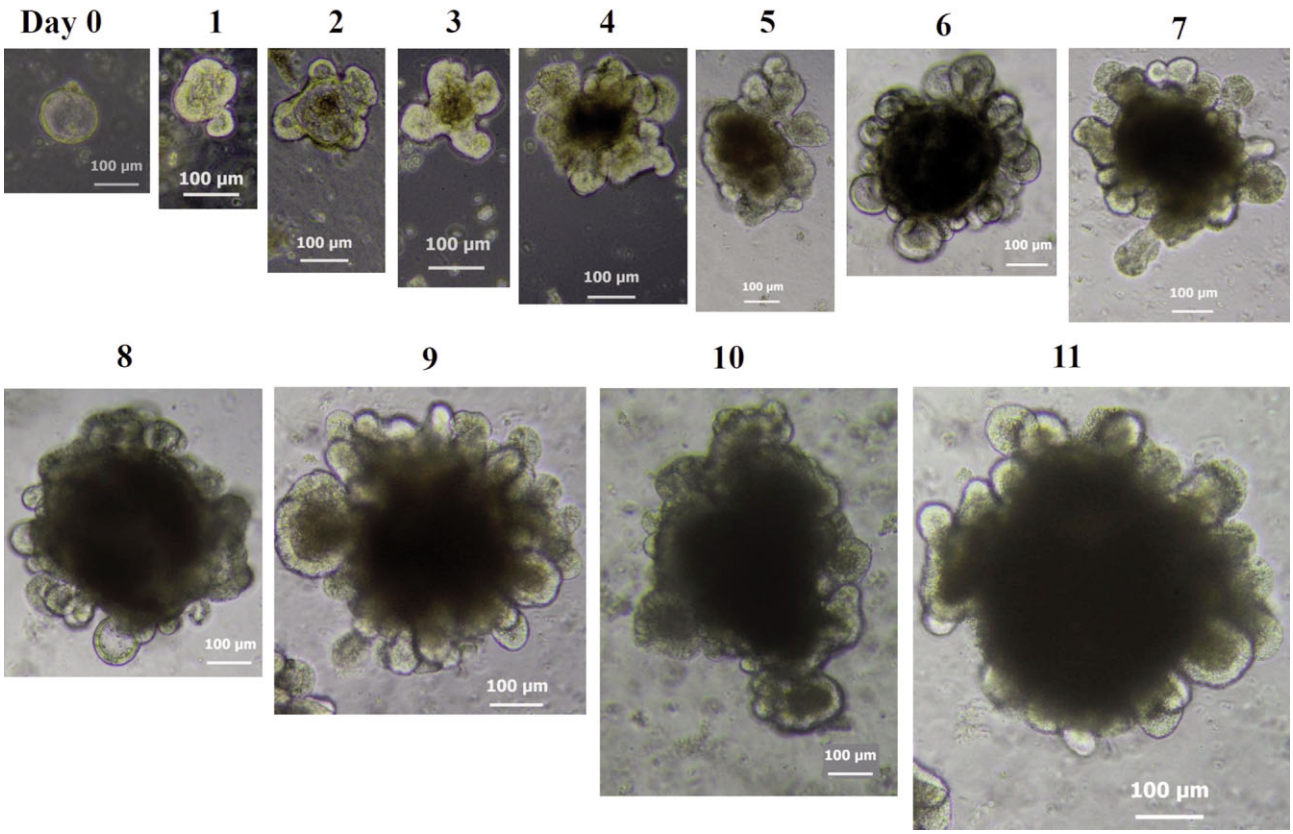

Figure 2. Time-course study of crypt morphologies cultured in vitro. The representative images show that the successfully grown crypts formed enteroids. These crypts were cultured in the control medium, advanced DMEM/F12 containing 2% serum. Each experiment was replicated three times. Scale bar, 100 μm.

2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

A sample of 1×10^6 cells from each treatment was collected and total RNA isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRA, Japan) by the reverse transcriptase polymerase chain reaction. The primers used are listed in Supporting information, Table S3 [21, 22]. qRT-PCR was performed using SYBR Green (Thermo Fisher Scientific, San Diego, CA) and StepOne™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Each target mRNA level was normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

2.6 Flow cytometric analysis of surface antigen expression

At day 7, the enteroids in each well were collected and mechanically dissociated into single cells. Samples of 1×10^6 cells were washed using PBS and then stained with FITC-conjugated anti-mouse CD24 (Biolegend, San Diego, CA), PE-conjugated anti-mouse CD44 (Biolegend) or primary Lgr5 antibody (Abgent, San Diego, CA) followed with FITC-conjugated goat-anti-rabbit secondary antibody (BD Biosciences) and analyzed by flow cytometry (BD Biosciences). A replicate sample was stained with specific IgG1 as an isotype control.

2.7 Long-term culture

To determine whether ISCs grown in our optimum and serum-free conditions can continue to self-renew, we re-cultured the intestinal enteroids. After 1 week of culturing, enteroids were collected and mechanically dissociated by repeated pipetting to break the large enteroid structures into small pieces. Then, a density of 100 pieces was re-cultured per well (in a 24-well plate). The same procedure was repeated every 7 days and the enteroid numbers counted.

2.8 Statistical analysis

Each experiment was replicated three times and expressed as mean ± standard deviation. The Student's *t*–test was used to evaluate the differences between the various groups and the control. Statistical significance was set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3 Results

3.1 ISC growth and enteroid structure in vitro

Before screening the effective factors to replace the serum, the growth ability and enteroid formation of ISCs in the control condition (advanced DMEM/F12 containing 2% serum) was checked. Our time-course study showed that the cultured crypts formed spherical structures filled with apoptotic cells, the so-called enteroids. These enteroids have villus domains and crypt domains that continue budding in vitro, and the size of enteroid grew up to 500 μm after a 10-day culture (Fig. 2). In the present study, we could maintain the growth of ISCs for long periods until the enteroid structures formed.

3.2 Screening of growth factors

In the first screening of the 9 different components that are included in the advanced DMEM/F12 basal medium, the results for enteroid formation after one week in culture showed that transferrin, ammonium metavanadate, and ethanolamine are the most significant factors ($p < 0.001$); as in their absence, the number of enteroids formed dramatically decreased to 232 ± 2.6, 207 ± 27.8 and 199 ± 51.5 respectively, compared with that in the control medium (medium 1 or medium 3 in Fig. 1) which contained 306 ± 5.7 enteroids/well. Additionally, ascorbic acid, glutathione, and sodium selenite had a minor effect on enteroid formation ($p < 0.05$). The other three components slightly affected ICS proliferation ($p > 0.05$) (Fig. 1).

3.3 Fractional factorial design screening

To determine the most effective ingredients among the six factors that were selected in the first screening, we performed a $2^{(6-2)}$ fractional factorial design. The matrix design (Supporting information, Table S1) included 16 different runs. The results of the linear first-order models were regressed according to the data shown in Supporting information, Table S1, using the following equation:

$$(\text{enteroids/well}) = 3.83 + 3.63\,x_1 - 3.38\,x_2 + 4.87\,x_3 + 1.5\,x_4 + 3.88\,x_5 + 4.25\,x_6 \quad (2)$$

where x_1, x_2, x_3, x_4, x_5 and x_6 are the coded variables for transferrin, ammonium metavanadate, ethanolamine, ascorbic acid phosphate, glutathione, and sodium selenite, respectively. Equation 2 showed that transferrin, ethanolamine, ascorbic acid phosphate, glutathione, and sodium selenite enhanced the growth of the ISCs because these components had positive coefficients in Equation 2. It was found that ammonium metavanadate inhibited ISC growth, as shown by its negative coefficient. The most significant component was ethanolamine, which had the highest positive coefficient.

3.4 Medium optimization

The steepest ascent approach that was combined with the fractional factorial was designed to optimize the concentration of the ingredient cocktail in the serum-free system [19, 20]. The concentrations of these factors that were selected are shown in Supporting information, Table S2. The results for the different media compositions obtained using the steepest ascent approach showed that the maximal number (146 ± 50.2) of enteroids/well was found at step 5. The optimal concentrations for transferrin, ethanolamine, ascorbic acid, glutathione, and sodium selenite in general DMEM/F12 basal medium were determined to be 5.4×10^{-3} mM, 1.5 mM, 0.185 mM, 0.194 mM and 1.95×10^{-3} mM, respectively.

3.5 Culture and morphology of ISCs in serum-free conditions

After optimizing the concentrations of the growth factors, we designed six different media to compare the effect of serum-free conditions on the proliferation of ISCs (Fig. 3A). To evaluate the formulations that were developed, the number of enteroids/well was counted in the optimal (Opt) medium and serum-free medium (Opt-SF, optimal medium without 2% FBS) that were developed in this study as well as in the advanced DMEM/F12 + 2% FBS (Adv) medium. As shown in Fig. 3A, a higher number of enteroids/well was attained in our optimal medium (Opt) than in the control (Adv) medium, at 235 ± 17.6 and 194 ± 31.7, respectively. Furthermore, the growth pattern observed in our optimal medium without serum (Opt-SF, 183 ± 28.7) was significantly increased in comparison to the general DMEM/F12 medium with/without 2% serum (143 ± 43.6/82 ± 50.5), and was slightly higher than in the Adv medium without serum (Adv-SF, 175 ± 59.7).

As previously mentioned, ISCs can grow in vitro and form spherical structures known as enteroids [18]. These enteroids have both crypt and villus domains (Fig. 2), which demonstrate the morphology of the enteroids after one week of culture in each medium formulation. Similarly, the micrographs show that the enteroid structures attained the same morphology in all of the media after one week of culture (Fig. 3B). Thus, the enteroids that formed in our serum-free medium were determined to show normal morphology.

3.6 Expression of ISC marker genes

To investigate whether our Opt and Opt-SF media could sustain the ISC-related gene expression in the ISCs throughout the culture period, we evaluated the levels of mRNA expression of ISC marker genes (Fig. 4, A–E). The results revealed that the expression level of Lgr5 was increased after culture for one week in all these media compared with that of the freshly isolated intestinal

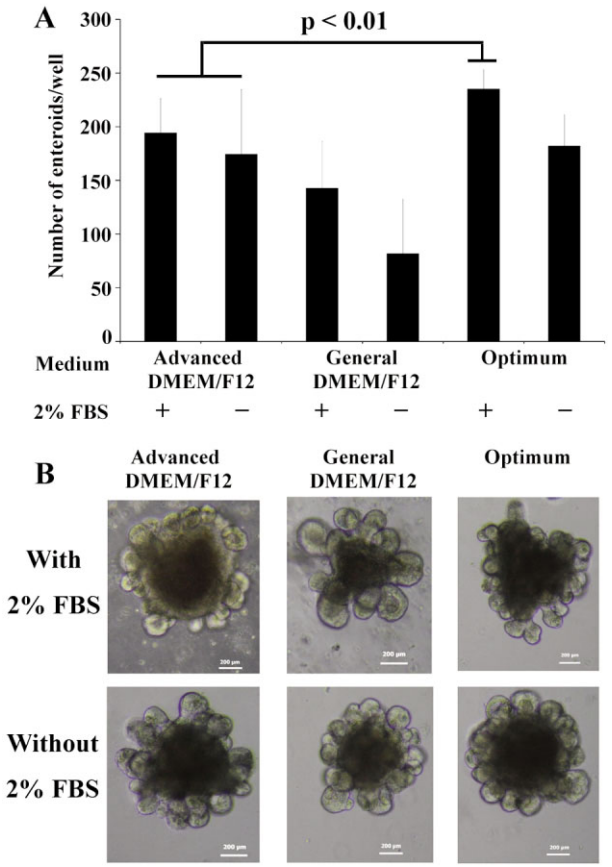

Figure 3. Various culture conditions for the ISCs growth. **(A)** The number of enteroids / well was counted at day 7 in the advanced DMEM/F12 medium, general DMEM/F12, and optimal medium developed in this study with or without 2% FBS. The number of enteroids / well was the average from three separate experiments in which the initial density was 1000 crypts / 24-well plate. Each experiment was replicated three times and the error bars were the standard deviations of the three replicated experiments. **(B)** The representative images show the morphology of enteroids at day 7 in the advanced DMEM/F12 medium, general DMEM/F12, and optimal medium developed in this study, with or without 2% FBS. Each experiment was replicated three times.

crypts. The level of expression in Opt medium was higher than that in the control medium (Adv). The Lgr5 expression in Opt-SF medium was also higher than that in Adv-SF medium. Similarly, the expression levels of Bmi1, PTEN, and CD24 in Opt and Opt-SF were up-regulated compared with those in Adv or Adv-SF media. However, the expression of Msi1 in Adv medium was slightly increased compared with that in Opt medium, and Msi1 expression in Adv-SF and Opt-SF was similar. Taken together, these results indicate that our optimal and serum-free media (Opt-SF) sustained ISC-related gene expression in vitro.

3.7 Flow cytometric analysis of surface antigen expression

Lgr5 is a seven-transmembrane receptor. To detect the expression level of this protein, we labeled cells that were isolated from the intestinal enteroids with Lgr5, CD24, and CD44 antibodies. The flow cytometric histograms shown (Fig. 4, F–J) demonstrate that Lgr5 protein levels were highly increased after one week of culture in Opt or Opt-SF media compared with that of the freshly isolated crypts. Similarly, CD24 levels were significantly increased after one week of culture in Opt or Opt-SF medium compared with that of the freshly isolated crypts. However, CD44 levels were slightly increased in the crypts that were cultured in Opt-SF and control Adv-SF. Thus, these results confirmed that our Opt and Opt-SF media sustained the expression of ISC-related surface antigens, Lgr5, CD24, and CD44 in the cultured ISCs.

3.8 Long-term culture of ISCs

To confirm that our optimal and serum-free media can maintain ISC growth for long periods of time, the cultured enteroids were subcultured. After the second round of culturing of the collected enteroid pieces, we found that 100 pieces grew into 107, 91 and 87 enteroids at day 14 in case of Opt, Adv and Opt-SF, respectively. Whereas, the number of enteroids formed at day 21 from the same density was 114, 101 and 90 enteroids in case of Opt, Adv and Opt-SF, respectively, and the same trend was maintained for 6 weeks (Fig. 5). These results suggested that our optimal and serum-free media are able to maintain the growth of ISCs for long periods of time.

4 Discussion

The main functions of the intestinal lumen in the intestinal tract are to digest and absorb nutrients and form a barrier against pathogens. The intestinal lumen consists of an epithelial layer and the intestinal epithelium is the most rapidly self-renewing tissue in mammals. ISCs, like other adult stem cells, have the ability to self-renew and differentiate. The ISCs are located at the base of the crypts in the small intestine [23]. ISC isolation and culture is a difficult process due to the lack of definitive markers and absence of suitable long-term culture methods. Previous studies employed DNA-label retention to label ISCs and identify their location. This method is based on the slow cell cycling and the quiescent properties of stem cells. The label-retaining cells can retain a DNA-synthesis label following cellular injuries [24]. Using this method, Potten identified ISCs in the intestinal crypts at positions 4–9 [25]. However, the mechanisms underlying this method are still unclear, and its current application for ISC identification is limited. Recently, putative markers to

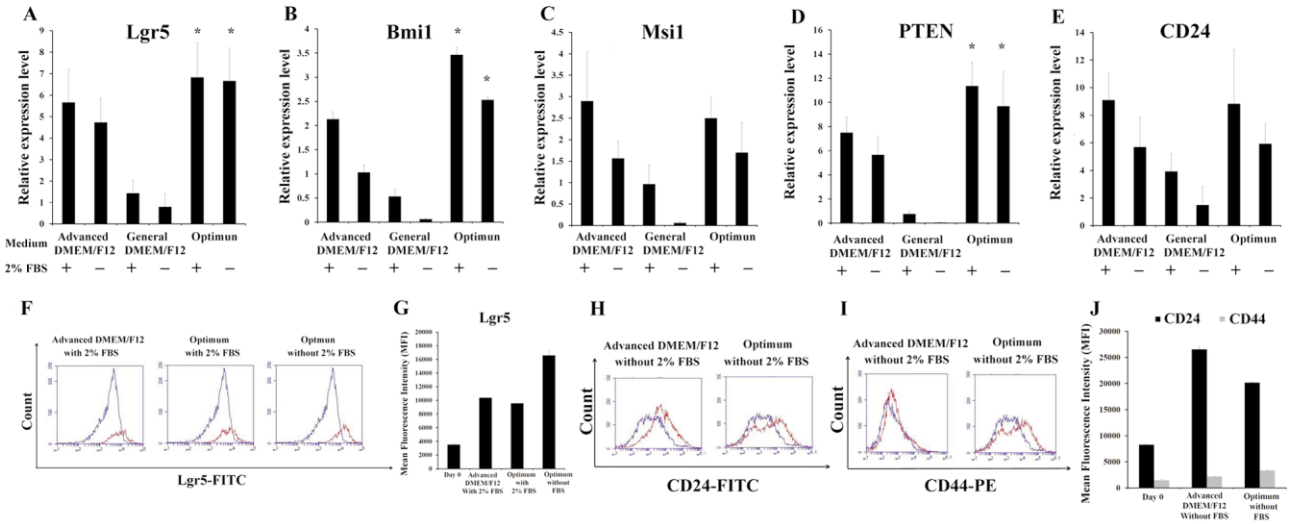

Figure 4. qRT-PCR results for the relative expression of ISC marker genes and flow cytometric analysis of surface antigen labeling. The cells for the gene expression analysis were collected from each medium after one week in culture. (**A**) Expression levels of Lgr5. (**B**) Expression levels of Bmi1. (**C**) Expression levels of Msi1. (**D**) Expression levels of PTEN. (**E**) Expression levels of CD24 in cells at day 7 in the advanced DMEM/F12 medium, general DMEM/F12, and optimal medium developed in this study, with or without 2% FBS. The expression levels represent the fold changes compared with the levels in the freshly isolated crypts that were used as the control. The fold-induced values were calculated using the ($2^{-\Delta\Delta Ct}$) method and the expression was normalized to that of GAPDH. *$p < 0.05$ compared with the advanced DMEM/F12 with 2% serum medium. The representative histograms of (**F**) Lgr-5. (**H**) CD 24. and (**I**) CD44, and mean fluorescence intensity (MFI) of (**G**) CD24. and (**J**) CD44. labeling for ISCs at day 7 in the indicated medium. Cultured cells are grey bar or red peak on each panel. Freshly isolated crypts are black bar or blue peak on each panel. Each experiment was replicated three times and the error bars are the standard deviations of the three experiments.

identify ICSs, such as Bmi1, Msi1, Ascl2, and Lgr5, have been described. Barker and his colleagues determined that Lgr5 is a putative marker gene for ISCs and is restricted to the base of the crypts [26].

Recently, ISCs have received much attention due to the need for stem cell therapies for the treatment of different intestinal diseases. Many studies have focused on regenerating the damaged parts of the intestine via stem-cell therapy, intestinal transplantation, and tissue engineering. The different choices of stem cell sources include mesenchymal stem cells and intestinal organoid units [27]. Thus, the possibility of using ISCs for tissue engineering has inspired researchers to improve their isolation methods and to optimize the culture systems for ISCs to obtain sufficient populations for clinical use [28].

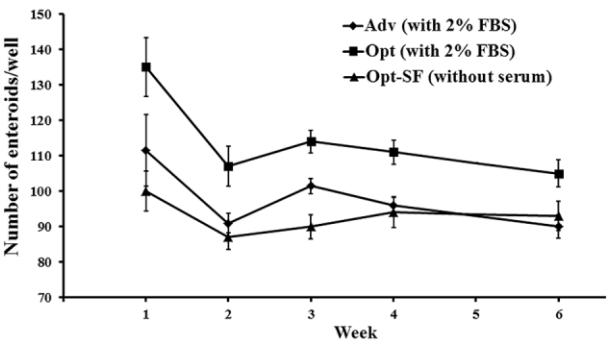

Figure 5. Long-term culture of ISCs. Long-term culture of ISCs in the advanced DMEM/F12 with 2% FBS (black diamonds), Optimum medium with 2% FBS (black squares), and Optimum medium without serum (black triangle). The initial density was 1000 crypts/24-well plate at day 0. Enteroids were subcultured every week and a density of 100 enteroid pieces/well was used. ***$p < 0.001$ compared with the advanced DMEM/F12 medium with 2% FBS. Each experiment was replicated three times and the error bars were the standard deviations of the three replicated experiments.

The goal of this study was to optimize the culture conditions, and to develop a serum-free medium, for ISCs. The development of serum-free medium is important to avoid the shortcomings of viral contaminants, physiological variability and batch-to-batch variation arising from serum components [28, 29]. In the present study, we chose nine components as serum substitutes. These nine components are included in the advanced DMEM/F12 basal medium. However, the general DMEM/F12 basal medium lacks these components. As a result, the latter cannot support ISC proliferation because these components appear to be necessary for ISC growth. Therefore, we decided to use these nine components to develop a novel and optimal serum-free medium. According to the first screening, we determined that transferrin, ethanolamine, and ammonium metavanadate significantly affected the proliferation of ISCs. In addition, ascorbic acid, glutathione and sodium selenite were determined to have a less marked effect. Furthermore, Albumax, insulin, and manganous chloride had a very weak effect (Fig. 1). Thus, we selected the first six factors for further screening. The fractional factorial design and the steepest

ascent approach were combined to optimize the serum-free medium for ISC expansion [30]. We identified five significant factors: (i) transferrin; (ii) ethanolamine; (iii) ascorbic acid; (iv) glutathione; and (v) sodium selenite. Concentration optimization via the steepest ascent approach showed that step 5 in Supporting information, Table 2 was the optimal condition for the proliferation of the ISCs. The concentration of FBS that was used in all of the media formulations was 2%. Thus, in the final screening step, we eliminated the serum and examined the effect of serum-free medium on ISC proliferation. As shown in Fig. 3, our Opt and Opt-SF media improved enteroid formation by the ISCs compared with the control media, Adv and Adv-SF. Although transferrin, ethanolamine, ascorbic acid, glutathione, and sodium selenite are common in the general cell culture media, this is the first report to indicate these five factors are required for ISC growth and can replace the role of serum in ISC culture. In the future, this serum-free medium can be used as the basal medium to identify other effective factors and obtain improved culture results. Additionally this medium enables the possibility of investigating the individual roles and synergistic effects of the various factors on the mechanisms involved in ISC metabolism.

As previously mentioned, Lgr5 is a Wnt target gene that is highly expressed at the base positions 4–9 of the crypt, which is known as the sites of the stem cells of the small intestine. Not only Lgr5, but also Bmi1, Msi1 and PTEN were used to label the ISCs, and the expression of these genes are considered to be reliable biomarkers [6]. The qRT-PCR-based gene expression analysis that we performed showed that the expression levels of Lgr5, Bmi1 and PTEN were highly up-regulated after one week culture of the ISCs in both our Opt and Opt-SF media (Fig. 4, A-E) compared with their expression levels in the control media, Adv and Adv-SF. Additionally, Msi1 expression was very similar in the control media (Adv and Adv-SF) and our media (Opt and Opt-SF). Potten and his colleagues have shown the Msi1 gene expression patterns in the murine intestine [31]. Previous studies showed that CD24 could be used a marker for isolating and identifying ISCs [11, 32, 33]. Thus, we used CD24 as an ISC marker to confirm our gene expression results. The CD24 expression level was greatly increased in our media (Opt and Opt-SF) compared to that in the control media (Adv and Adv-SF). Therefore, our results suggested that Opt and Opt-SF maintain the expression of ISC-related genes and surface antigens of ISCs in vitro. For further confirmation of these results, Lgr5, CD24, and CD44 antibodies were used to label ISCs for cytometric flow analysis. The content of $Lgr5^+$ cells increased in the crypts that were grown in Opt and Opt-SF compared with that in the control (Adv) medium and in the freshly isolated crypts (Fig. 4, F–J). Additionally, the number of $CD24^+$ cells was greatly increased by culturing in Opt-SF, and came close to the levels obtained using Adv-SF. Similarly, the number of $CD44^+$ cells in crypts that were grown in Opt-SF was slightly increased compared to in Adv-SF. Taken together, these results suggested that our optimal and serum-free media enhanced the proliferative abilities of ISCs and maintain the expression of ISC-related genes and surface antigens during long-term culture.

In conclusion, we have systematically developed a serum-free medium based on the general DMEM/F12 as the basal medium for mouse ISC expansion in vitro. Freshly isolated ISCs from intestinal crypts could grow and maintain the spherical enteroid structures in our optimum and serum-free media for at least 6 weeks of culture. We believe that this study provides a promising step towards improving the expansion of ISCs in vitro. In addition, our serum-free medium can be used for clinical applications as well as in the tissue engineering field.

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5 References

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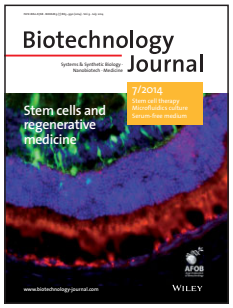

Special Issue: Stem cells and regenerative issue. This is the second special issue of Biotechnology Journal in collaboration with the Asian Federation of Biotechnology and is edited by Prof. Byung-Soo Kim and Prof. Jiandong Ding. The cover shows a fluorescence image of a rat eye tissue section. Transplanted neural stem cells expressing GFP (green) have integrated into the host retina. Light-detecting photoreceptors (rods and cones) of the host eye are stained red and all cell nuclei are stained blue. The image is from the article by Jisun Oh et al. http://dx.doi.org/10.1002/biot.201400019.

Biotechnology Journal – list of articles published in the July 2014 issue.

Editorial: Scientific and engineering progress in stem cell and regenerative medicine research
Byung-Soo Kim and Jiandong Ding
http://dx.doi.org/10.1002/biot.201400226

Review
Induced pluripotent stem cells for modeling of pediatric neurological disorders
Jiho Jang, Zhejiu Quan, Yunjin J. Yum, Hyo Sook Song, Seonyeol Paek and Hoon-Chul Kang
http://dx.doi.org/10.1002/biot.201400010

Review
Stem cell therapy and cellular engineering for treatment of neuronal dysfunction in Huntington's disease
Kyung-Ah Choi, Insik Hwang, Hang-soo Park, Seung-Ick Oh, Seongman Kang and Sunghoi Hong
http://dx.doi.org/10.1002/biot.201300560

Review
Preserving human cells for regenerative, reproductive, and transfusion medicine
Waseem Asghar, Rami El Assal, Hadi Shafiee, Raymond M. Anchan and Utkan Demirci
http://dx.doi.org/10.1002/biot.201300074

Review
Cell sheet engineering for regenerative medicine: Current challenges and strategies
Toshiyuki Owaki, Tatsuya Shimizu, Masayuki Yamato and Teruo Okano
http://dx.doi.org/10.1002/biot.201300432

Mini-Review
Antibody approaches to prepare clinically transplantable cells from human embryonic stem cells: Identification of human embryonic stem cell surface markers by monoclonal antibodies
Hong Seo Choi, Won-Tae Kim and Chun Jeih Ryu
http://dx.doi.org/10.1002/biot.201300495

Research Article
Multipotent adult hippocampal progenitor cells maintained as neurospheres favor differentiation toward glial lineages
Jisun Oh, Gabrielle J. Daniels, Lawrence S. Chiou, Eun-Ah Ye, Yong-Seob Jeong and Donald S. Sakaguchi
http://dx.doi.org/10.1002/biot.201400019

Research Article
Biologically synthesized silver nanoparticles induce neuronal differentiation of SH-SY5Y cells via modulation of reactive oxygen species, phosphatases, and kinase signaling pathways
Ahmed Abdal Dayem, BongWoo Kim, Sangiliyandi Gurunathan, Hye Yeon Choi, Gwangmo Yang, Subbroto Kumar Saha, Dawoon Han, Jihae Han, Kyeongseok Kim, Jin-Hoi Kim, and Ssang-Goo Cho
http://dx.doi.org/10.1002/biot.201300555

Research Article
Angiogenic/osteogenic response of BMMSCs on bone-derived scaffold: Effect of hypoxia and role of PI3K/Akt-mediated VEGF-VEGFR pathway
Yi Zhou, Xiaoxu Guan, Mengfei Yu, Xinhua Wang, Wenyuan Zhu, Chaowei Wang, Mengliu Yu and Huiming Wang
http://dx.doi.org/10.1002/biot.201300310

Research Article
S-Fms signalobody enhances myeloid cell growth and migration
Masahiro Kawahara, Azusa Hitomi and Teruyuki Nagamune
http://dx.doi.org/10.1002/biot.201300346

Research Article
A serum-free medium developed for in vitro expansion of murine intestinal stem cells
Mahmoud S. Mohamed, Yun Chen and Chao-Ling Yao
http://dx.doi.org/10.1002/biot.201400016

Research article
Detachably assembled microfluidic device for perfusion culture and post-culture analysis of a spheroid array
Yusuke Sakai, Koji Hattori, Fumiki Yanagawa, Shinji Sugiura, Toshiyuki Kanamori and Kohji Nakazawa
http://dx.doi.org/10.1002/biot.201300559

Research Article
The hollow fiber bioreactor as a stroma-supported, serum-free ex vivo expansion platform for human umbilical cord blood cells
Xue Cao, Kenneth Y. C. Kwek, Jerry K. Y. Chan, Qingfeng Chen and Mayasari Lim
http://dx.doi.org/10.1002/biot.201300320