

## TECHNICAL REPORT

# Generation of Highly Expandable Intestinal Spheroids Composed of Stem Cells

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Many of early findings regarding intestinal stem cells (ISCs) and their niche in the human intestine have relied on colorectal cancer cell lines and labor-intensive and time-consuming mouse models. However, these models cannot accurately recapitulate the physiologically relevant aspects of human ISCs. In this study, we demonstrate a reliable and robust culture method for 3D expanding intestinal spheroids (InS<sup>exp</sup>) mainly comprising ISCs and progenitors, which can be derived from 3D human intestinal organoids (HIOs). We did functional characterization of InS<sup>exp</sup> derived from 3D HIOs, differentiated from human pluripotent stem cells, and optimization culture methods. Our results indicate that InS<sup>exp</sup> can be rapidly expanded and easily passaged, and show enhanced growth rates *via* WNT pathway activation. InS<sup>exp</sup> are capable of exponential cell expansion and cryopreservation. Furthermore, *in vitro*-matured HIO-derived InS<sup>exp</sup> proliferate faster than immature HIO-derived InS<sup>exp</sup> with preservation of the parental HIO characteristics. These findings may facilitate the development of scalable culture systems for the long-term maintenance of human ISCs and provide an alternative platform for studying ISC biology.

**Keywords:** Human intestinal organoid (HIO), Expandable Intestinal spheroid (InS<sup>exp</sup>), Intestinal stem cell, WNT pathway, Maturity memory

## Introduction

Human pluripotent stem cell (hPSC)-derived intestinal organoids (HIOs) recapitulate developmental processes, and can differentiate into the multiple cell types found within the human intestine *in vivo* (1, 2). HIOs are cultured three-dimensionally inside extracellular matrix

(ECM) domes such as a Matrigel dome, and showing the morphology of a miniaturized gut with a crypt-villus structure. Owing to the high structural and functional similarities between the *in vivo* human intestine and *in vitro* HIOs, HIOs can be used in various application studies such as disease modeling, drug screening, and development of regenerative therapies (3, 4). Although HIOs have high potential and applicability, there is a growing need for intestinal stem cell (ISC) model systems to study ISC biology. Most early findings related to human ISCs have involved expensive, labor-intensive, and time-consuming mouse models and colorectal cancer cell lines (5). Therefore, it is necessary to develop a robust and reliable culture method capable of exponential proliferation and long-term culture of ISCs.

To address these issues, we recently developed a culture method for 3D expanding intestinal spheroids (InS<sup>exp</sup>) derived from 3D HIOs differentiated from hPSCs. InS<sup>exp</sup> comprise mainly intestinal stem cells (ISCs) and progeni-

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tors. In this study, we investigated the exponential proliferation and long-term culture of ISCs by inducing rapid expansion of InS<sup>exp</sup> through WNT pathway activation.

## Materials and Methods

### Human pluripotent stem cells

The H9 human embryonic stem cells (hESCs) were purchased from WiCell Research Institute (Madison, WI, USA). Human induced pluripotent stem cells (hiPSCs) were generated as described previously (6).

### Differentiation of hPSCs into immature and mature 3D HIOs

hPSC-derived 3D HIOs were generated as reported previously (1, 2). The 3D HIOs were mechanically dissociated and re-embedded every 10~14 days. For *in vitro* maturation of HIOs, 1 ng/ml of IL-2 (R&D systems) was added to the culture medium when the spheroids were embedded. IL-2 was freshly added after at least two passages.

### Isolation and culture of 3D InS<sup>exp</sup> derived from HIOs

The 3D InS<sup>exp</sup> derived from HIOs were generated as previously reported (7). The growth medium was replaced every other day. To optimize the organoid growth, 1  $\mu$ M of CHIR99021 (Tocris) was included in the InS<sup>exp</sup> growth medium.

### Passaging of 3D InS<sup>exp</sup>

For passaging, InS<sup>exp</sup> were dissociated using trypsin-EDTA and a P1000 pipette was used to break up the Matrigel dome. The total mixture was transferred to a conical tube, incubated at 37°C in a water bath for 5 min, and mechanically dissociated using 10 rounds of pipetting. The dissociated cells were resuspended and centrifuged at 1,500 rpm, 4°C, for 5 min. After resuspending the cell pellet with Matrigel, the HIO-Matrigel mixture was plated onto a four-well culture plate and incubated at 37°C in a CO<sub>2</sub> incubator for more than 30 min to solidify the Matrigel, followed by the addition of InS<sup>exp</sup> growth medium supplemented with 10  $\mu$ l of Y-27632 (Tocris), and 1  $\mu$ M Jagged-1 (AnaSpec, Fremont, CA, USA) for the first 2 days. Subsequently, this medium was replaced with the InS<sup>exp</sup> culture medium, which was then replaced every other day.

### Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using the RNeasy kit (Qiagen), and cDNA was prepared using a Superscript

IV cDNA synthesis kit (Thermo Fisher Scientific Inc.). Quantitative polymerase chain reaction (qPCR) was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). Relative expression was calculated using the  $\Delta\Delta$ Ct method. The primers used in this study are listed in Table S1.

### Immunofluorescence analysis

Immunofluorescence staining was performed on 10% formalin-fixed InS<sup>exp</sup> as described previously (8). Cryosections were permeated with 0.1% Triton X-100, and then blocked for 1 hour with 4% bovine serum albumin. After blocking, the sections were incubated with primary antibodies at 4°C overnight and the secondary antibody at room temperature. Finally, 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml, Thermo Fisher Scientific Inc.) was used to counterstain the nuclei. Fluorescence images were acquired using a fluorescence microscope (IX51, Olympus, Tokyo, Japan) and LSM800 confocal microscope (Carl Zeiss, Gottingen, Germany). The list of primary antibodies used is provided in Table S2.

### Cell counting and cell size analysis

The InS<sup>exp</sup> were dissociated to single cells using 0.25% trypsin-EDTA for 10 min at 37°C. The dissociated cells were then diluted with advanced DMEM/F12 basal media and centrifuged for 5 min at 1,500 rpm. The single cells were resuspended in InS<sup>exp</sup> growth medium and stained with trypan blue. The cell counts and sizes were determined using the Countess 3 Automated Cell Counter (Thermo Fisher Scientific Inc.).

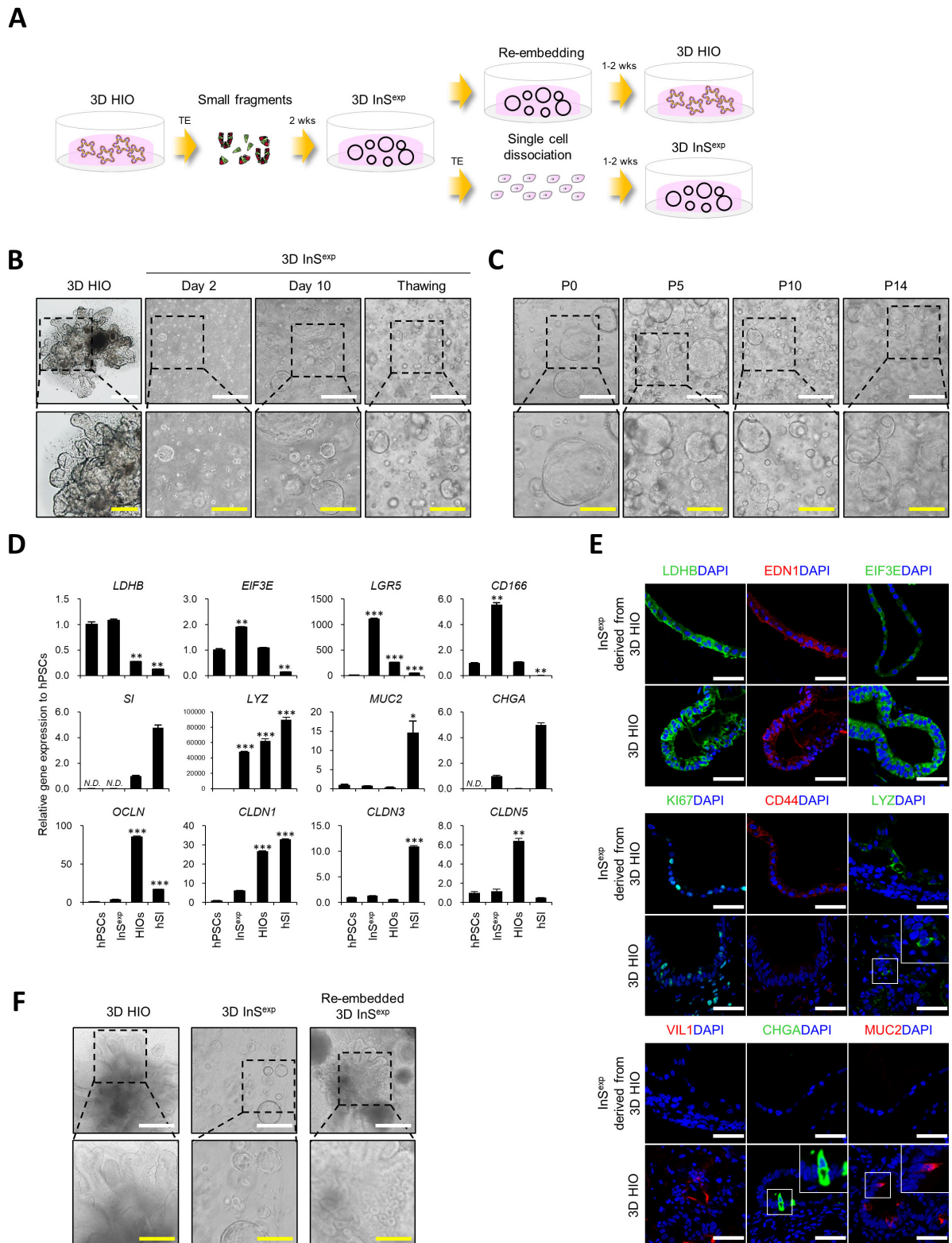
### Statistical analysis

All experiments were repeated at least three times and results are presented as means  $\pm$  standard error (SEM). Two-tailed student's t-test was performed to determine the significance of the data statistically.

## Results

### Establishment of the 3D InS<sup>exp</sup> culture system and characterization of marker expression

To develop a fast expandable 3D ISC culture method, we dissociated hPSC-derived HIOs into small fragments and embedded them in a Matrigel dome (Fig. 1A). Embedded cells formed morphologically distinct structures compared to HIOs and showed a hollow spherical shape (Fig. 1B). This new type of 3D intestinal organoids, designated as InS<sup>exp</sup>, is highly proliferative and easily passaged by single cell dissociation for more than 6 months without spontaneous



**Fig. 1.** Culture and characterization of InS<sup>exp</sup> derived from 3D HIOs. (A) Schematic diagram of the InS<sup>exp</sup> culture method. (B, C) Bright field images of InS<sup>exp</sup> by data, after thawing (B), and by passaging (C). White scale bars, 500  $\mu$ m. Yellow scale bars, 250  $\mu$ m. (D) Relative expression of intestinal epithelial cell marker genes in hPSCs, InS<sup>exp</sup>, and HIOs. (E) Immunofluorescence analysis of intestinal epithelial cell markers in InS<sup>exp</sup>. White scale bars, 50  $\mu$ m. (F) Bright field images of 3D HIOs, InS<sup>exp</sup>, and re-embedded InS<sup>exp</sup>. White scale bars, 500  $\mu$ m. Yellow scale bars, 250  $\mu$ m. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 using two-tailed  $t$ -test.

differentiation and cell death (Fig. 1C). The InS<sup>exp</sup> is mostly composed of ISCs and progenitors, and thus exhibits higher expression of stem cell/progenitor marker genes and proteins (Fig. 1D and 1E). Furthermore, the InS<sup>exp</sup> can be cryopreserved without any loss of morphology and function upon thawing (Fig. 1B). These data suggest that the newly developed InS<sup>exp</sup> culture method enables rapid expansion and cryopreservation through the enrichment of ISCs and progenitors. Interestingly, ISC and progenitor-enriched InS<sup>exp</sup> further differentiated into 3D HIOs by re-embedding in a new Matrigel without cell dissociation, and the generation of multiple budding structures in the re-embedded InS<sup>exp</sup> is similar to the structural characteristics of the original 3D HIOs (Fig. 1F).

### Optimization of 3D InS<sup>exp</sup> culture conditions through WNT pathway activation

As treatment with CHIR99021 has been reported to enhance the growth of mouse and human adult stem cell-derived intestinal organoids (9, 10), we supplemented a glycogen synthase kinase 3 (GSK-3) inhibitor CHIR99021 in the InS<sup>exp</sup> culture medium to optimize the culture medium. First, we performed a titration experiment with increasing concentrations of CHIR99021 to assess the optimal concentration range of WNT activator. The growth of InS<sup>exp</sup> was maximized by treatment with 1  $\mu$ M CHIR99021; however, InS<sup>exp</sup> growth was suppressed when treated with CHIR99021 at a concentration higher than 1  $\mu$ M (Fig. 2A and 2B). Interestingly, treatment with CHIR99021 at more than 6  $\mu$ M completely inhibited the growth of InS<sup>exp</sup> (Fig. 2A and B). As the growth of InS<sup>exp</sup> was maximized when treated with 1  $\mu$ M of CHIR99021, this was the concentration used in all subsequent experiments. We next examined the effect of WNT ligand depletion on the growth of InS<sup>exp</sup>. As expected, the growth rate of InS<sup>exp</sup> was significantly decreased by depletion of WNT ligands, WNT3A and R-spondin 1, from the growth medium, and was mostly recovered by supplementation with CHIR99021 (Fig. 2C and 2D). In line with this, the spheroid formation and growth of InS<sup>exp</sup> were completely diminished by treatment with WNT pathway inhibitors such as WNT C-59 and XAV939 (Fig. 2E). Therefore, these data indicate that the WNT pathway plays an essential role in controlling the growth of InS<sup>exp</sup>, and that the growth of InS<sup>exp</sup> can be enhanced by moderate activation of the WNT pathway using 1  $\mu$ M of CHIR99021. The WNT pathway was found to be essential for InS<sup>exp</sup> proliferation, as KI67<sup>+</sup> proliferating cells disappeared under WNT-depleted or WNT inhibitor-treated conditions; however, these did not induce

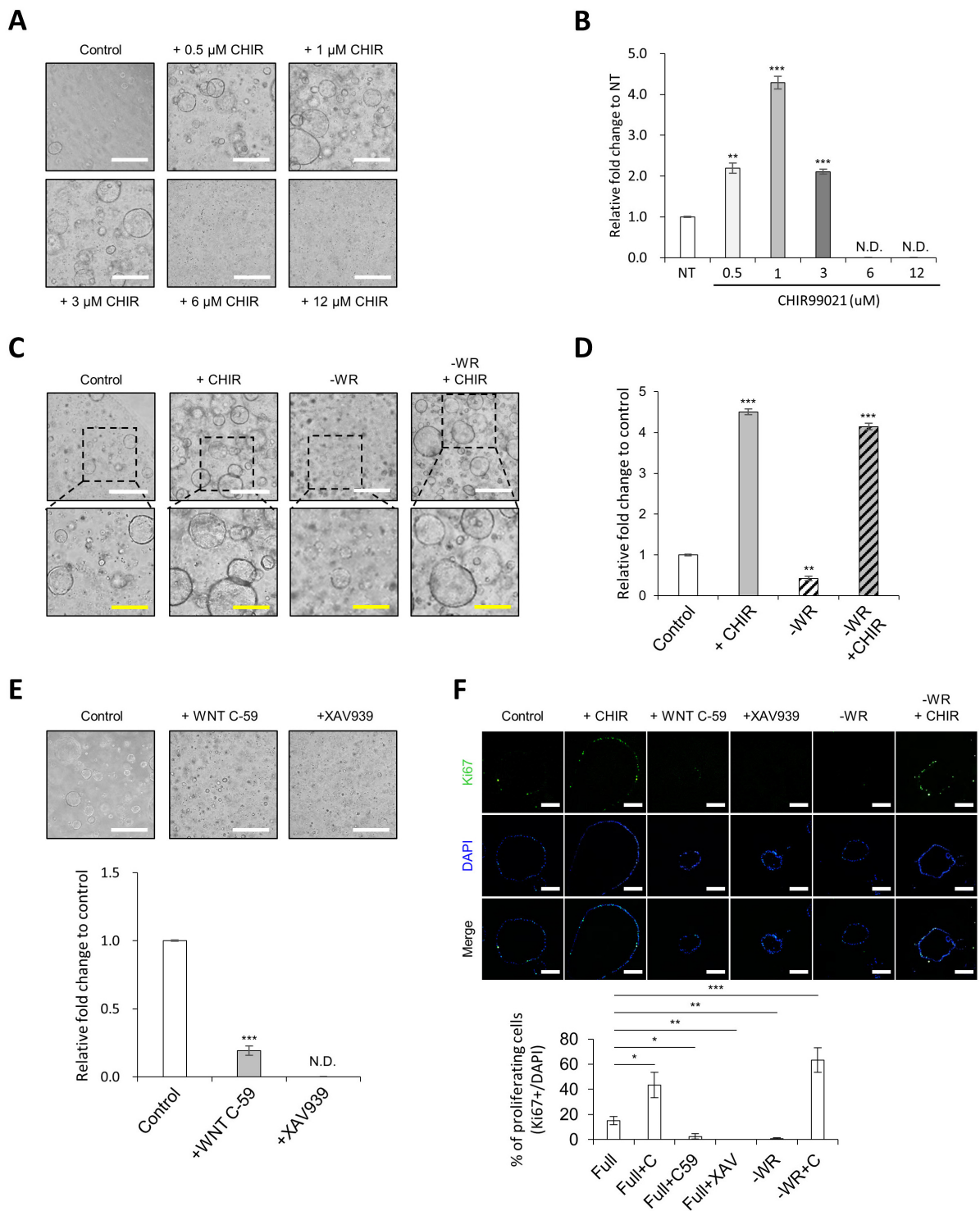
cell death (Fig. 2F). Taken together, we suggest that moderate activation of the WNT pathway augments the growth rate of InS<sup>exp</sup> by increasing cell proliferation, and that the growth medium was optimized by adding a low concentration of CHIR99021.

### Immature and mature HIO-derived 3D InS<sup>exp</sup> display distinct profiles of maturity gene expression

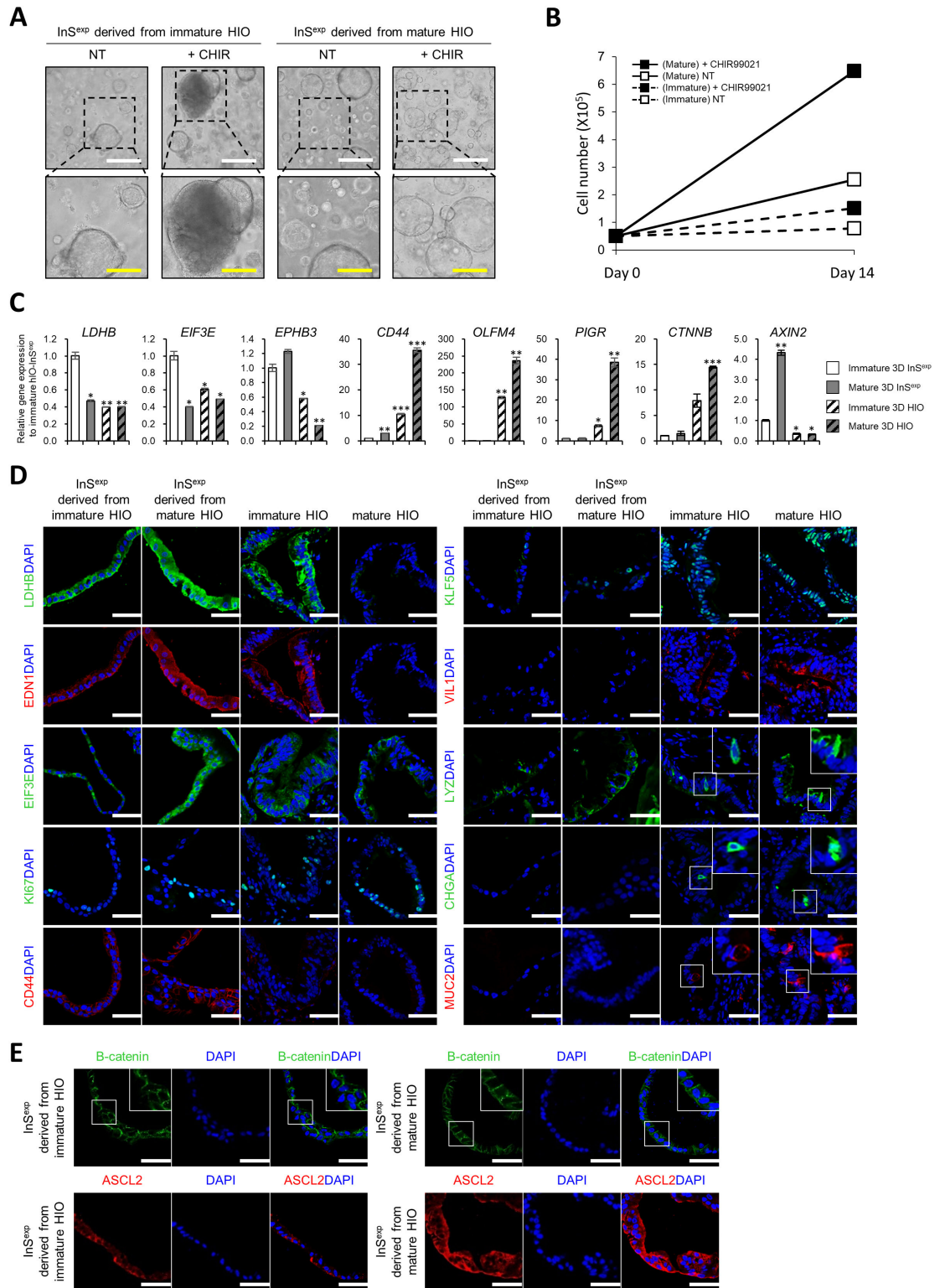
We previously demonstrated that hPSC-derived hIOs can be matured *in vitro* using interleukin-2 treatment, and these were more similar to the adult human small intestine (2). Therefore, we investigated whether 3D InS<sup>exp</sup> derived from immature or *in vitro*-matured HIOs exhibited distinct marker gene expression pattern and functional characteristics. First, when the same number of cells was embedded in a Matrigel dome, the spheroid formation efficiency of mature HIO-derived InS<sup>exp</sup> was significantly higher than that of immature HIO-derived InS<sup>exp</sup> (Fig. 3A). On day 14 of culture, the total cell number of mature HIO-derived InS<sup>exp</sup> was  $\sim 2.6$  times greater compared to that of immature HIO-derived InS<sup>exp</sup> ( $0.78 \pm 0.0265 (\times 10^5)$  in immature HIO-derived InS<sup>exp</sup> vs.  $2.55 \pm 0.046 (\times 10^5)$  in mature HIO-derived InS<sup>exp</sup>) (Fig. 3B). Interestingly, CHIR 99021 treatment showed that mature HIO-derived InS<sup>exp</sup> responded more sensitively to WNT pathway activation compared to immature HIO-derived InS<sup>exp</sup> (Fig. 3A and 3B). Furthermore, the WNT target genes were confirmed to be highly expressed in mature HIO-derived InS<sup>exp</sup> compared to that in immature HIO-derived InS<sup>exp</sup> (Fig. 3C~E). Activation of the canonical WNT pathway governs the expression of stemness- and proliferation-related genes (11, 12); the expression of these genes and proteins including CD44,  $\beta$ -catenin, ASCL2 and KI67 was also higher in mature HIO-derived InS<sup>exp</sup> than that in immature HIO-derived InS<sup>exp</sup> (Fig. 3C~E). Our findings indicate that immature and mature HIO-derived InS<sup>exp</sup> retain maturity-specific functional characteristics and gene expression profiles.

### Discussion

In intestinal biology and its applications, there is an increasingly evident need for the development of various intestinal epithelial models such as 3D hIO and 2D monolayer culture systems (7, 13). Here we present a novel culture method for the scalable expansion of human ISCs enriched from hPSC-derived HIOs. Unlike 3D HIOs, the InS<sup>exp</sup> have a spherical shape without a budding and villus structure, and mostly comprise ISCs and progenitors residing in the crypt region in HIOs (Fig. 1B and 1D).



**Fig. 2.** CHIR99021 treatment promotes growth rate of InS<sup>exp</sup>. (A, B) Effect of CHIR99021 on the InS<sup>exp</sup> growth rate at multiple concentrations. Bright field images of the InS<sup>exp</sup> (A) and Relative fold change in the InS<sup>exp</sup> cell number (B). White scale bars, 500  $\mu$ m. Data represent means $\pm$ SEM (n=4). (C, D) Recovery of InS<sup>exp</sup>-forming competency by addition of CHIR99021 in WNT3A- and R-spondin 1-depleted medium. Bright field images of InS<sup>exp</sup> (C) and Relative fold change in the InS<sup>exp</sup> cell number (D). White scale bars, 500  $\mu$ m. Yellow scale bars, 250  $\mu$ m. Data represent means $\pm$ SEM (n=4). (E) Inhibition of InS<sup>exp</sup> growth by treatment with WNT inhibitors. White scale bars, 500  $\mu$ m. Data represent means $\pm$ SEM (n=4). (F) Immunofluorescence analysis and quantification of proliferating cell markers in InS<sup>exp</sup>. Scale bars, 200  $\mu$ m. Data represent means $\pm$ SEM (n=5). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 using two-tailed t-test.



**Fig. 3.** Characterization of InS<sup>exp</sup> derived from immature and mature 3D HIOs. (A, B) Effect of CHIR99021 on InS<sup>exp</sup> derived from immature and mature HIOs. Bright field images of InS<sup>exp</sup> (A) and cell number increase of the InS<sup>exp</sup> after day 14 (B). White scale bars, 500  $\mu$ m. Yellow scale bars, 250  $\mu$ m. Data represent means  $\pm$  SEM (n=4). (C) Relative gene expression of intestinal epithelial cell markers and WNT target genes in InS<sup>exp</sup> derived from immature and mature HIOs. (D, E) Immunofluorescence analysis of the intestinal epithelial cell markers and WNT target genes in InS<sup>exp</sup> derived from immature and mature HIOs. White scale bars, 50  $\mu$ m. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 using two-tailed t-test.

Owing to the enrichment of ISCs and progenitors, InS<sup>exp</sup> can be cultured with rapid expansion kinetics and can be maintained long-term through multiple passaging and freeze-thaw cycles (Fig. 1B). The growth rate of InS<sup>exp</sup> was further increased by WNT pathway activation (Fig. 2A and 2B). Interestingly, the growth of InS<sup>exp</sup> was enhanced by treatment with a low concentration of CHIR99021, whereas it was inhibited by treatment with a high concentration of CHIR99021 (Fig. 2A and 2B). It is unclear how low and high concentrations of CHIR99021 have contrasting effects on InS<sup>exp</sup> growth; however, this phenomenon has been repeatedly observed in previous reports (10, 14). Although the reason for the distinct effects between low and high concentrations of CHIR99021 on InS<sup>exp</sup> growth remains unclear, we will elucidate the underlying molecular mechanism in future studies.

hPSC-derived HIOs are known to exhibit immature characteristics similar to the fetal intestine, and it can be matured similar to the adult intestine through *in vivo* transplantation or *in vitro* maturation (2, 15, 16). In this study, mature HIO-derived InS<sup>exp</sup> exhibited higher spheroid formation capability and growth rate compared to immature HIO-derived InS<sup>exp</sup> (Fig. 3A and 3B). The surface area of spheroids and total number of cells in the mature HIO-derived InS<sup>exp</sup> were significantly increased by treatment with CHIR99021, as they responded more sensitively to WNT pathway activation (Fig. 3A and 3B). These results suggest that the memory of the maturity of HIO is retained even in InS<sup>exp</sup>, as confirmed through the differences in the expression of the maturation marker genes between mature HIO-derived InS<sup>exp</sup> and immature HIO-derived InS<sup>exp</sup> (Fig. 3C and 3D). However, it is necessary to verify whether there is a difference in the epigenetic modifications including DNA methylation between mature HIO-derived InS<sup>exp</sup> and immature HIO-derived InS<sup>exp</sup> in further studies.

In conclusion, we established a culture method for InS<sup>exp</sup>, which can be used as a cell source for studying human ISC biology. Furthermore, as InS<sup>exp</sup> mostly comprise ISCs and progenitors and can be cryopreserved, they can be developed for use in regenerative medicine in the future, and provide an alternative platform for studying ISC biology.

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### Potential Conflict of Interest

The authors have no conflicting financial interests.

### Supplementary Materials

Supplementary data including two tables can be found with this article online at <https://doi.org/10.15283/ijsc21209>.

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