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Definitive endoderm differentiation is promoted in suspension cultured human iPS-derived spheroids more than in adherent cells

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ABSTRACT Human pluripotent stem cells (hPSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are very attractive cell sources for the treatment of diabetes mellitus, because numerous cells can be obtained using their infinite proliferation potential to overcome the paucity of donor islets. Advances in differentiation protocols make it possible to generate glucose responsive hPSC-beta cells, which can ameliorate hyperglycemia in diabetic mice. These protocols have mainly been based on an adherent culture system. However, in clinical applications, suspension culture methods are more suitable for large-scale culture. There are reports that suspension culture and spheroid formation promote differentiation in various cell types, including hPSCs, but, to our knowledge, there are no reports comparing gene expression patterns between suspension and adherent cultured human iPSCs (hiPSCs) during definitive endoderm (DE) differentiation. In this study, we chose several stage marker genes, not only for DE but also for posterior epiblast and primitive streak, and we examined their time course expression in suspension and adherent cultures by quantitative PT-PCR (qPCR), western blot, flow cytometry and immunocytochemistry. Our results demonstrate that expressions of these marker genes are faster and more strongly induced in suspension culture than in adherent culture during the DE differentiation process, indicating that suspension culture favors DE differentiation.

KEY WORDS: *definitive endoderm, pluripotent stem cell, induced pluripotent stem cell, adherent culture, suspension culture*

Introduction

Islet transplantation is a very effective therapy for Type1 diabetes, but many patients can't receive this treatment due to the shortage of donors. Human pluripotent stem cells (hPSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) may overcome this problem, because of their infinite proliferation potential and pluripotency, which allows them to produce all embryonic tissues. Over the last ten years, there has been great progress in methods to induce differentiation of hPSC-derived pancreatic beta cells (D'Amour *et al.*, 2006, Maehr *et al.*, 2009, Kunisada *et al.*, 2012, Rezania *et al.*, 2012). Moreover, these cells have been proven to secrete insulin in response to blood glucose level and normalize hyperglycemia in diabetic mice (Pagliuca *et al.*, 2014, Rezania *et al.*, 2014). Recently, we reported a 6-step differentiation protocol from hiPS cells into functional pancreatic beta cells (Yabe

Abbreviations used in this paper: BRA, brachyury; DE, definitive endoderm; EOMES, eomesodermin; FGF8, fibroblast growth factor 8; FOXA2, forkhead box protein A2; GSC, goosecoid; hiPSC, human induced pluripotent stem cell; HNF1B, hepatocyte nuclear factor 1 beta; HNF4A, hepatocyte nuclear factor 4 alpha; iPSC, induced pluripotent stem cell; MIXL1, mix paired-like homeobox; NANOG, nanog homeobox; NODAL, nodal growth differentiation factor; NGN3, neurogenin 3; OAZ1, ornithine decarboxylase antizyme; OCT3/4, PE, posterior epiblast; PDX1, pancreatic and duodenal homeobox 1; PGT, primitive gut tube; POU class 5 homeobox 1; PS, primitive streak; PSC, pluripotent stem cell; SOX2, SRY (sex determining region Y)-box 2; SOX17, SRY (sex determining region Y)-box 17.

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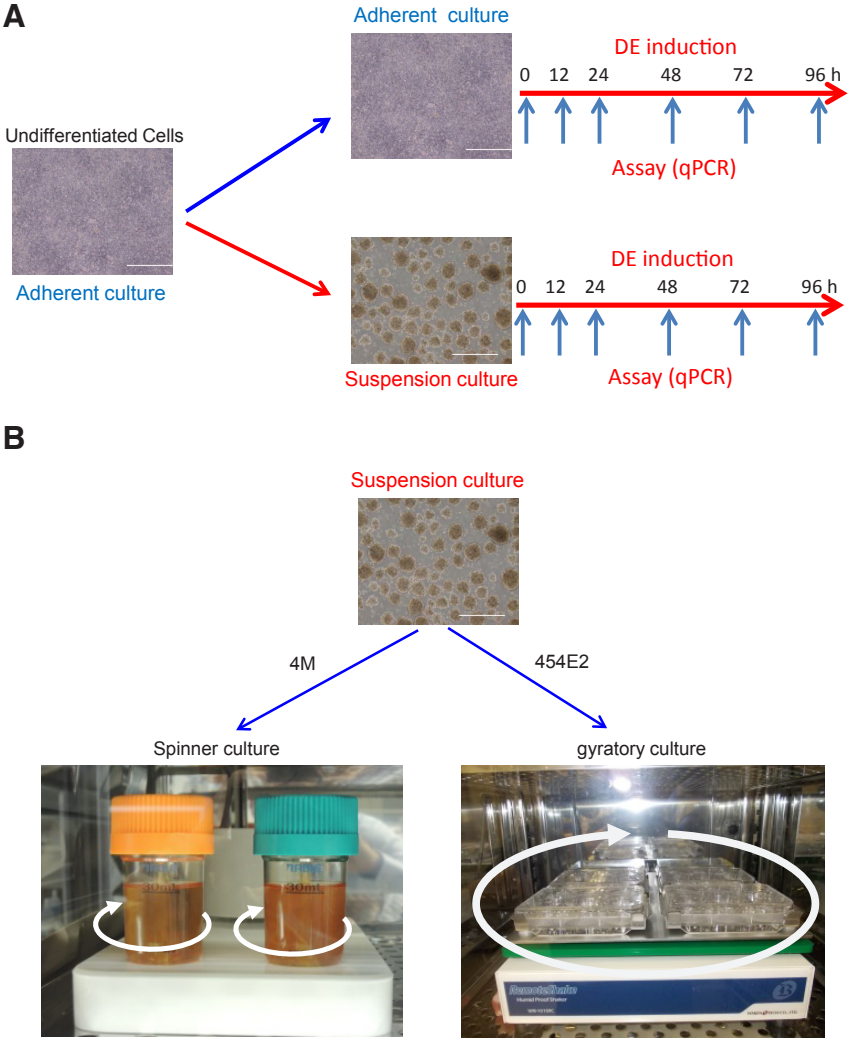

Fig. 1. Experimental design of this research. (A) *Scheme of assay by qPCR analysis.* **(B)** *Two types of culture systems: suspension or adherent culture.*

renewal have been developed by several groups (Rungarunlert *et al.*, 2009, Amit *et al.*, 2010, Olmer *et al.*, 2010,Singh *et al.*, 2010, Olmer *et al.*, 2012, Abbasalizadeh *et al.*, 2012), many differentiation protocols for beta cells have been based on adherent culture mimicking the developmental stages (D'Amour *et al.*, 2006, Maehr *et al.*, 2009, Kunisada *et al.*, 2012, Rezania *et al.*, 2012). Recently, however, new protocols based on suspension culture throughout the entire process have been reported (Schulz *et al.*, 2012, Pagliuca *et al.*, 2014, Russ *et al.*, 2015, Konagaya and Iwata, 2016, Mihara *et al.*, 2017, Yabe *et al.*, 2019). Viacyte, which is doing clinical trials on diabetes patients using encapsulated pancreatic progenitor cells (PEC-01) derived from human ESCs, reported a scalable suspension culture production system using gyration (Schulz *et al.*, 2012). Melton's group also reported a differentiation protocol for functional hES-derived beta cells in large scale suspension culture using a spinner system (Pagliuca *et al.*, 2014). We decided to use both suspension culture systems to characterize cells from adherent and suspension cultures and then compare them.

Definitive endoderm (DE), which is the innermost of the three principal germ layers and is generated from invaginating epiblast cells in the primitive streak during gastrulation, gives rise to the epithelial lining of the respiratory and digestive tracts, from which the thyroid, thymus, lungs, liver and pancreas arise along the anterior-posterior axis (Zorn and Wells, 2009). Because pancreatic beta-cells originate from DE, DE differentiation from hPSCs is the first critical process in differentiating hES/iPS-beta cells; therefore, much research has focused on this step (D'Amour *et al.*, 2005. Mclean *et al.*, 2007. Kunisada *et al.*, 2012. Loh *et al.*, 2014, Ninomiya *et al.*, 2015, Matsuno *et al.*, 2016. Yabe *et al.*, 2017). Although, we also quite recently reported the induction of islet-like cells from hiPS cells by suspension culture (Yabe *et al.*, 2019), detailed comparison of DE differentiation between adherent and suspension cultures has not been published as far as we know. We focused on this first and critical step for differentiation to pancreatic β cells.

In this study, we assumed two stages, posterior epiblast and primitive streak, before DE and examined the time course of gene expression of the putative markers of these stages. We demonstrated different patterns of expression during DE differentiation between suspension culture and adherent culture.

Results

Expression of undifferentiated marker mRNA

We first examined the proliferation of 4M and 454E2 in both adherent and suspension culture conditions. Adherent cultured cells increased about 1.5-fold between 0 and 96 h; suspension cultured cells increased about 2-fold between 0 and 96 h (Sup. Fig. 2).

To compare the gene expressions of adherent and suspension cultured hiPSCs during DE differentiation, we assumed two stages,

et al., 2017). We noted that the first step from the undifferentiated state toward definitive endoderm (DE) was the most important for efficient generation of functional beta cells; we achieved robust DE differentiation by adding FGF2, BMP4 and CHIR99021 (GSK-3βinhibitor) as well as Activin A to the medium. Almost all the cells were SOX17 (definitive endoderm marker) positive, and PDX1 (pancreatic progenitor marker) and NGN3 (endocrine progenitor marker) became positive for more than 90% of them. Although immunostaining revealed that insulin c-peptide was positive for about 30% of the cells, the glucose stimulated insulin secretion response of these cells was weak. We successfully overcame this problem by introducing 3D spheroid culture at the final step. About 30% of the cells were C-peptide positive in these spheroids, and the glucose responsive insulin secretion of these 3D cultured cells was enhanced twofold compared with adherent culture. Because of the importance of 3D structure for the function of pancreatic beta cells, we became interested in suspension culture.

For clinical applications of hES/iPS-beta cells, large amounts of cells are needed, and suspension culture is suitable for such large-scale production. Although suspension culture systems in self-

posterior epiblast and primitive streak, before DE. Then we chose putative markers for these stages and examined their time courses of gene expression (qRT-PCR) at several timepoints (0, 12, 24, 48, 72, 96 h after DE induction) by quantitative RT-PCR (Fig. 1A). We used two iPSCs lines: 4M and 454E2. For suspension culture, 4M was mainly cultured in a spinner system; 454E2 was cultured in a gyratory system (Fig. 1B). This differentiation protocol is summarized in Sup. Fig.1. Representative individual data are shown in Sup. Fig. S1-4 and the averages of fold induction data (n=3) in Fig. 2-5. We first investigated the gene expression of undifferentiated PSCs markers *OCT3/4*, *SOX2*, and *NANOG*, because down-regulation of these genes' expression is important for progression of DE differentiation. As shown in Fig. 2 and Sup.Fig. 3, although *OCT3/4* expression in the suspension culture was higher than in the adherent culture until 24 h, a reduction of *OCT3/4* expression started from 48 h in suspension culture, but it started at 72 h in adherent culture. The same pattern was noted in *NONAG* expression; the turning point for suspension culture was 48 h, but it was 72 h for adhesion culture. Unlike *OCT3/4* and *NANOG* expression, *SOX2* expression had already decreased at 12 h, and *SOX2* expression was lower in suspension culture than in adherent culture from 24 to 96 h. These results indicate that expressions of the undifferentiated PSCs markers *OCT3/4*, *SOX2* and *NONOG* were all downregulated in suspension culture earlier than in adherent culture.

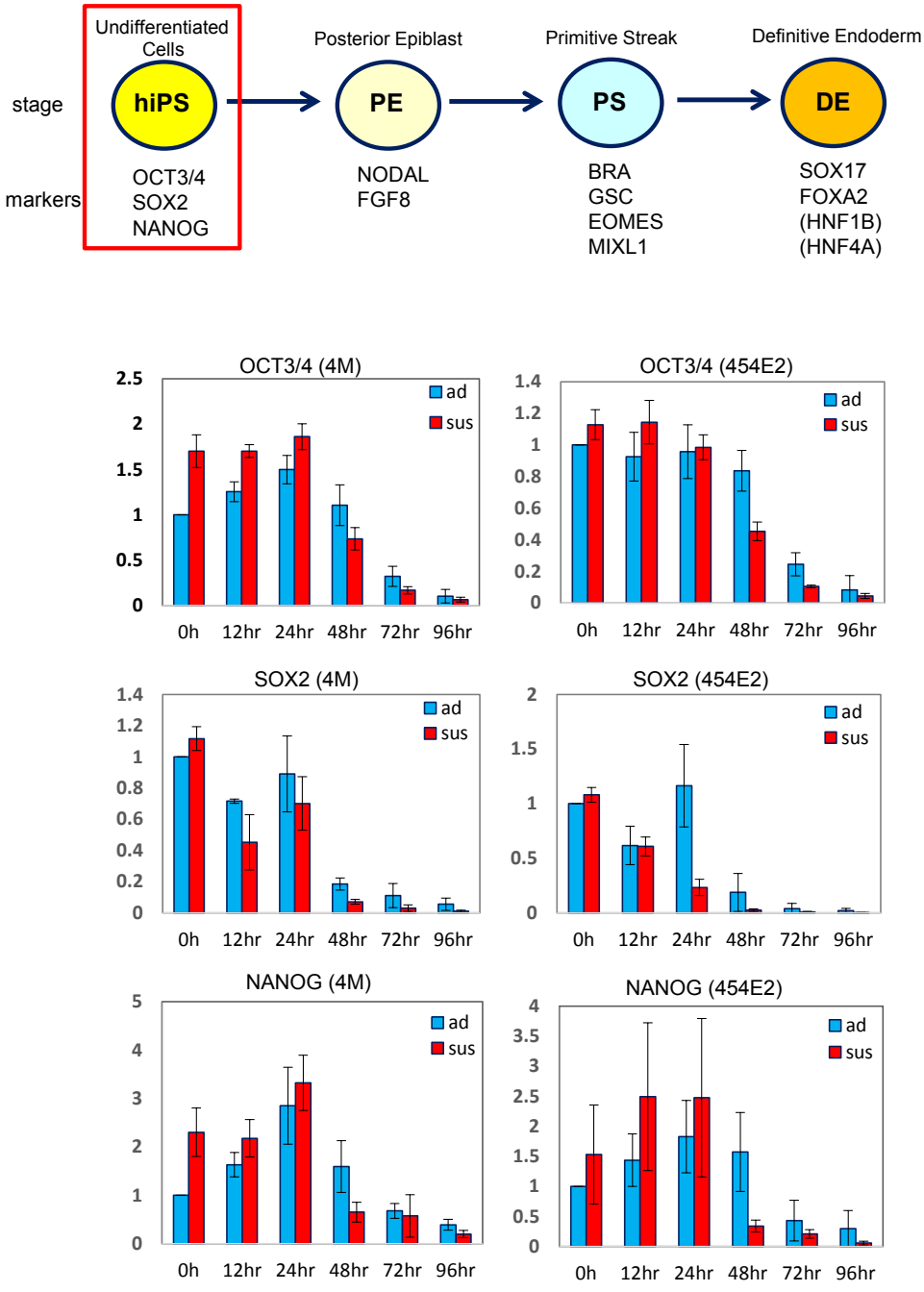

Fig. 2. Expression patterns of undifferentiated human induced pluripotent stem cell (hiPSC) markers. OCT3/4, SOX2 *and* NANOG *expression was examined by qPCR at 0, 12, 24, 48, 72, 96 h after definitive endoderm differentiation in suspension or adherent culture using two hiPSC lines (4M and 454E2). Y-axis indicates fold induction. Relative fold induction of each gene was calculated by selecting one sample at a certain timepoint and setting its expression level as 1. Selected samples for standardization are as follows: ad(0h) for* OCT3/4 *(4M), ad (0h) for* OCT3/4 *(454E2), ad (0h) for* SOX2 *(4M), ad (0h),* SOX2 *(454E2), ad (0h) for* NANOG *(4M), ad (0h) for* NANOG *(454E2,). ad, adherent culture sample; sus, suspension culture sample.*

Expression of posterior epiblast marker mRNA

Definitive endoderm is formed during gastrulation in the primitive streak, and the posterior epiblast is the area in which primitive streak emerges (Lu and Robertson 2001, Robb and Tam 2004). Because specification of the posterior epiblast is the initial step of this process, we next examined the expression profiles of *FGF8* and *NODAL*, regarded as the first indicators of posterior pattern formation in the epiblast (Fig.3, Sup. Fig.4). These two genes were rapidly up-regulated from 12 to 24 h, and stronger expression was observed in suspension culture than in adherent culture. Furthermore, these genes' expressions peaked at 24h in the suspension culture, but at 48h in adherent culture. These results suggest that pattern formation of the posterior epiblast occurred earlier in the suspension culture than in the adherent culture.

Expression of primitive streak marker mRNA

After patterning of the posterior epiblast, the primitive streak

forms, so we investigated four primitive streak markers: *Brachyury(BRA)*, *goosecoid(GSC)*, *Mix paired-like homeobox (MIXL1)* and *eomesodermin(EOMES)* (Fig. 4, Sup. Fig.5). These markers' expressions were more enhanced in suspension culture than in adherent culture from 12 h to 24 h. A remarkable difference was observed at 24 h, especially in 454E2. *BRA*, which is the downstream target of *WNT3a* (Yamaguchi *et al.*, 1999), is a good indicator of the progress of gastrulation toward DE, because *BRA* expression decreases and disappears in DE cells but persists in mesodermal cells after its peak. The peak of *BRA* expression was earlier in suspension culture (24 h) than adherent culture (48 h) and declined at 48 h in suspension culture. This shift of peak time may reflect the difference of progression speed of differentiation between suspension and adherent cultures.

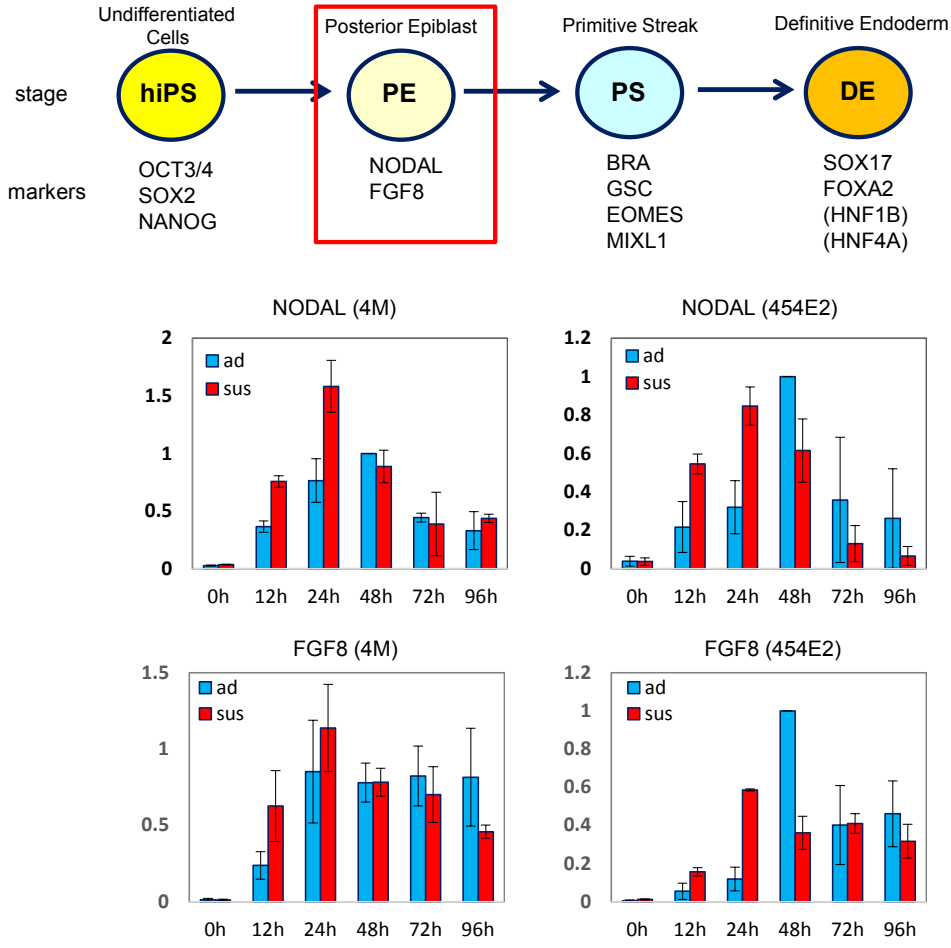

Fig. 3. Expression pattern of posterior epiblast markers. NODAL *and* FGF8 *expression was examined by qPCR at 0, 12, 24, 48, 72, 96 h in the same way as in Figure 2. Y-axis indicates fold induction. Relative fold induction of each gene was calculated as described in Figure 2. Selected samples for standardization are as follows: ad(48h) for* NODAL *(4M), ad (48h) for* NODAL *(454E2), ad (96h) for* FGF8 *(4M), ad (48h) for* FGF8 *(454E2). ad, adherent culture sample; sus, suspension culture sample.*

Expression of definitive endoderm marker mRNA

Definitive endoderm cells are generated during gastrulation, so we next examined the expression patterns of DE markers such as *SOX17* and *FOXA2* (Fig. 5, Sup.Fig. 6). Rapid up-regulation of *SOX17* expression was observed at 48 h with higher expression in suspension cultures than in adherent cultures. Although the expression level in suspension cultures of *SOX17* peaked at 48 h and remained almost the same between 48 and 72 h, that of *SOX17* in adherent cultures peaked later at 72 h and was lower at 48 h than in suspension cultures. *FOXA2* expression was up-regulated more and earlier in suspension cultures than in adherent cultures, and the higher expression level was sustained for 48-96 h.

Expression of primitive gut tube marker mRNA

Because the progression speed of differentiation was faster in suspension culture than in adherent culture, we surveyed the patterns of expression of primitive gut tube (PGT) markers such as *HNF1B* and *HNF4A*. As shown in Fig. 5 and Sup.Fig. 6, *HNF1B* expression was first activated at 48 h in suspension culture but only slightly in adherent culture, and it was remarkably enhanced at 72 h in suspension culture compared with adherent culture. This difference in expression level between suspension culture and adherent culture remained at 96 h. Higher activation of *HNF4A* expression was observed at 72 h in suspension culture than in adherent culture, and there was a significant difference in its expression level at 96 h. These results suggest that a transition towards PGT occurred in suspension cultures earlier than in adherent cultures.

Expression of representative genes at the protein level

We examined expression patterns at the protein level by western blot (Fig. 6). OCT3/4 protein was down-regulated at 48 h in both suspension and adherent cultures. In 4M, OCT3/4 protein disappeared in the suspension culture but remained in the adherent culture at 96 h. In 454E2, OCT3/4 protein was more reduced in suspension culture than in adherent culture at 48 h and lost in suspension culture but still present in adherent culture at 72 h. We observed stronger SOX17 bands in suspension culture at 48 h in both 4M and 454E2. FOXA2 protein was detected in 4M at 24 h in suspension culture, earlier than in adherent culture, but no obvious difference in FOXA2 protein level was observed in 454E2. Weak HNF4A protein was detected in suspension cultures at 72 h, and a denser HNF4A protein band was observed in suspension culture than in adherent culture at 96 h. The HNF1B protein level did not differ between suspension culture and adherent culture (data not shown).

Next, as cell-based analyses, we performed flow cytometry and immunocytochemistry (Fig. 7, Sup. Fig. 7). Flow cytometric analysis showed that there are no clear differences in positive rates of surface DE marker CXCR4 between adherent and suspension cultures, both of which was more than 97% (Fig. 7). On the other hand, the numbers of positive cells for SOX17, FOXA2, HNF1B and HNF4A were higher in suspension cultures than in adherent

cultures, and an especially remarkable difference was observed in 454E2 (Fig. 7) These results correspond to immunocytochemical ones (Sup. Fig. 7), in which positive cells were relatively uniformly distributed in aggregates. Taken together, these results suggest that the expressions of markers for protein levels also indicate earlier DE differentiation in suspension culture than in adherent culture.

Discussion

3D structure is critical for the function of pancreatic beta cells. In the present study, we compared the expressions of marker genes for developmental stages between suspension culture and adherent culture to uncover possible biological advantages of suspension culture in addition to the technical advantage of large scale culture. We considered the experimental design carefully, because it is critical for this study to maintain the same conditions between suspension culture and adherent culture. Cell interactions in 2D and 3D culture are basically different. In particular, PSCs are known to start differentiation after cell aggregation (embryoid body formation). Therefore, we did not use hanging drop methods to aggregate iPS cells immediately but rather adopted a gradual aggregation method from a single cell in suspension using spinner culture and gyratory culture, as described in Fig. 1B. We obtained relatively uniformly sized spheroids with both culture systems by adjusting the rotation speed. We were able to use the same medium and additives before and after the differentiation process in each set of experiments, although we used different basic media for 4M and 454E2. We regarded these spheroids and adherent cells as undifferentiated because we detected high expressions of *OCT3/4, SOX2* and *NANOG* at the beginning of differentiation (0h in Fig. 2). Proper circuit of undifferentiated marker gene expression is important for retaining undifferentiated PSCs and inhibiting differentiation of hPSCs (Boyer *et al.*, 2005, Pardo *et al.*, 2010, van den Berg *et al.*, 2010). The fact that PS or DE marker genes were barely detected before differentiation indicates that these cells did not start endoderm differentiation automatically.

The initial step of DE differentiation is patterning of the posterior epiblast, because the primitive streak initially emerges in that area (Lu and Robertson 2001, Robb and Tam 2004). We chose *FGF8* and *NODAL* as posterior epiblast marker genes and *BRA, GSC, MIXL1* and *EOMES* as primitive streak marker genes. We examined their time course expressions and found that they expressed earlier in suspension culture than in adherent culture. The rapid up-regulation of these genes suggested that the posterior epiblast was specifying more strongly in suspension culture than in adherent culture and subsequently initiating transition to primitive streak.

Interestingly, these gene expressions were detected as early as 12h after initiating differentiation and peaked

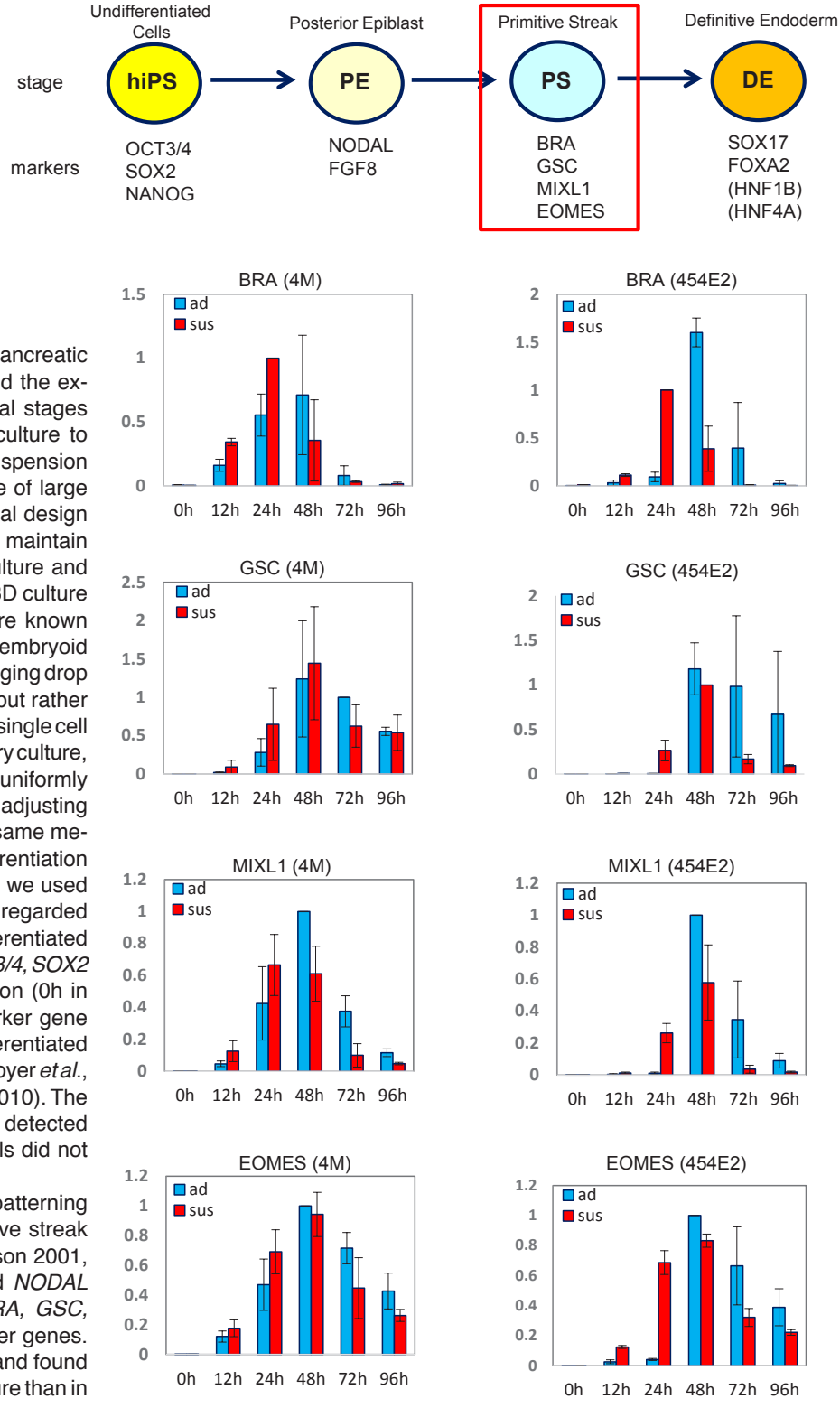

Fig. 4. Expression pattern of posterior primitive streak markers. BRA, GSC, MIXL1 *and* EOMES *expression was examined by qPCR at 0, 12, 24, 48, 72, 96 h in the same way as Figure 2. Selected samples for standardization are as follows: sus (24h) for* BRA *(4M), sus (24h) for* BRA *(454E2), ad (72h) for* GSC *(4M), sus (48h) for* GSC *(454E2), ad (48h) for* MIXL1 *(4M), ad (48h) for* MIXL1 *(454E2), ad (48h) for* EOMES *(4M), ad(48h) for* EOMES *(454E2). ad, adherent culture sample; sus, suspension culture sample.*

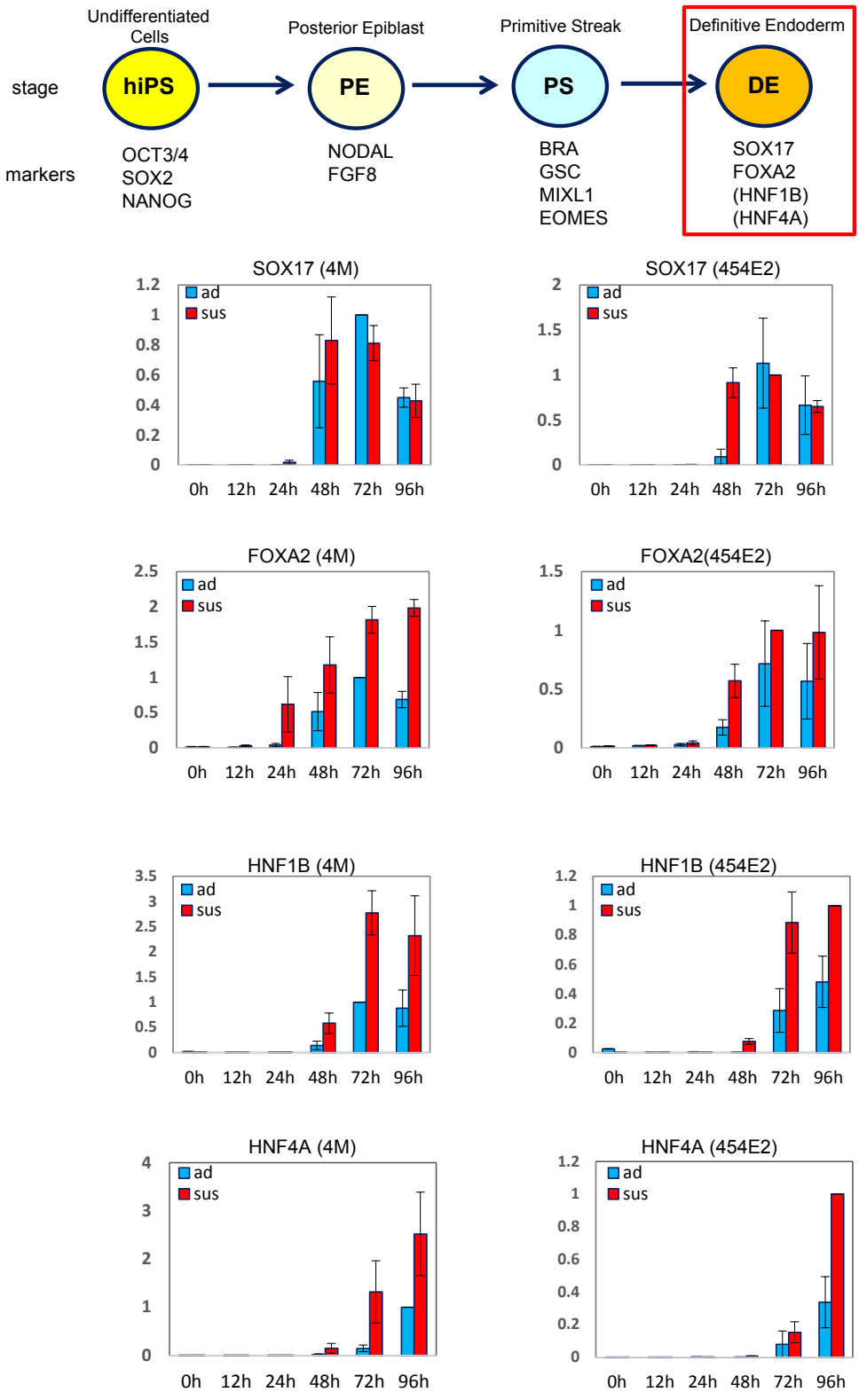

Fig. 5. Expression pattern of definitive endoderm and primitive gut tube markers. SOX17, FOXA2, HNF1B *and* HNF4A *expression was examined by qPCR at 0, 12, 24, 48, 72, 96 h in the same way as Figure 2. Selected samples for standardization are as follows: ad (72h) for* SOX17 *(4M), sus (72h) for* SOX17 *(454E2), ad (72h) for* FOXA2 *(4M), sus (72h) for* FOXA2 *(454E2), ad (72h) for* HNF1B *(4M), sus (96h) for* HNF1B *(454E2), ad (96h) for* HNF4A *(4M), sus (96h) for* HNF4A *(454E2). ad, adherent culture sample; sus, suspension culture sample.*

at 24h in suspension culture, even though undifferentiated hPSC marker gene expression was still high. It is generally thought that breakdown of this circuit due to down-regulation of undifferentiated hPSC marker gene expression triggers differentiation. Our results suggest that PE and PS differentiation processes start before undifferentiated hPSC marker gene expression shuts down. Of the three genes tested, *SOX2* down-regulated first. This might reflect a previous report that *OCT3/4* didn't block PS markers expression but inhibited DE markers expression; SOX2 repressed both PS and DE markers expression (Teo *et al.*, 2011).

During gastrulation, epithelial epiblast cells undergo an epithelial-mesenchymal transition (EMT) and give rise to mesoderm and endoderm (Kallri and Weinberg, 2009). As DE differentiation proceeds, expression of the epithelium marker E-cadherin decreases; in contrast, mesenchymal marker N-cadherin expression increases (D'Amour *et al.*, 2005). We also examined these genes' expression patterns and demonstrated the same behavior, in agreement with D'Amour *et al.*, 2005. No clear differences in expression levels were observed between suspension and adherent cultures (data not shown). However, the expressions of DE markers and a later stage of DE marker *SOX17* were initially stronger and peaked earlier at 48 h in suspension culture; they tended to decrease as differentiation proceeded. Stronger *FOXA2* expression was also observed in suspension culture and continued to increase until 96 h. These data parallel the report that SOX17 expression peaked faster than *FOXA2* expression and decreased after peak during DE differentiation (D'Amour *et al.*, 2005). In addition to the DE markers *HNF1B* and *HNF4A,* both PGT markers of the following stage of DE had a faster run-up and retained higher expression in suspension culture. The temporal dynamics of these PGT markers indicate that the transition from DE toward PGT proceeded faster in suspension culture. Of note, *HNF4A* mRNA is transcribed by two promoters, P1 or P2, which are used in different tissues and at different times during develop-

ment (Harries *et al*., 2008, Walesky and Apte 2015, Tuncer and Banerjee 2017). During liver development, although the P2 promoter is used initially, as differentiation proceeds, the promoter switches from P2 to P1, and only *HNF4A* mRNA transcribed with the P1 promoter is observed in adult liver (Harries *et al*., 2008, Walesky and Apte 2015). Although only the P2 promoter is used in adult pancreas, both P2 and P1 promoter-derived *HNF4A* mRNA are detected in fetal pancreas (Harries *et al*., 2008). Therefore, the expression profiles of P2 and P1 promoter-derived *HNF4A* mRNA are interesting as indicators of differentiation state in addition to that of PGT marker. Recently it was reported that P2-derived *HNF4A* mRNA significantly increased, but expression of P1-derived *HNF4A* mRNA did not change during DE differentiation in hiPSCs (Hanawa *et al*., 2017). We observed that both P2 and P1-derived *HNF4A* mRNA increased in both suspension and adherent cultures but that suspension cultures expressed these mRNAs at higher levels than adherent cultures during DE differentiation (data not shown). Although further research is needed to resolve this question, this difference in behavior of P1-derived *HNF4A* between this previous report and our results might be due to differentiation conditions. The higher expression of P2 and P1-derived *HNF4A* mRNA in suspension cultures might reflect differences in differentiation state. Moreover, the balance between the expression level of P2-derived *HNF4A* and that of P1-derived *HNF4A* mRNA might become a new indicator of DE cell subtype (Loh *et al*., 2014, Matsuno *et al*., 2016).

Although we examined only a limited number of marker genes and used only two hiPSCs lines, we found similar patterns of expression in mRNA and protein levels. Our data clearly show that expression of marker genes was faster and more strongly up-regulated in suspension culture than in adherent culture. To explain these results, we hypothesized an effect of ECM because interaction with proper ECM is thought to be important to maintaining undifferentiated states in hPSCs. Matrigel was actually used for adherent cultures in this study, because it is supposed to maintain the self-renewal ability strongly and stably (Xu *et al*., 2001). Therefore, undifferentiated states might be so tightly maintained in adherent culture that it takes more time to start differentiation. It is conceivable that downregulation of undifferentiated markers and transition from undifferentiated to differentiated states occur faster in suspension cultures due to the less contact with ECM. ECM has been suggested to play important roles not only in retaining undifferentiated states but also in DE differentiation. It was reported that fibrillar fibronectin was necessary for loss of pluripotency and that fibronectin-coated substrate improved DE differentiation in comparison with collagen and laminin substrate (Brafman *et al*., 2012, Taylor-Weiner *et al*., 2013). We confirmed that expression levels of fibronectin were higher in suspension cultures than in adherent cultures (data not shown). Interestingly, spheroid formation enhanced the expression and meshwork of fibronectin compared with monolayer in the case of human adipose-derived stem cells (Cheng *et al*., 2012). Another possibility is the effect of insulin/PI3K signaling. It is reported that Activin A specified DE only when insulin/PI3K signaling was suppressed (Maclean *et al*., 2007, Yu *et al*., 2015). We found that the expression level of IRS1, which is the effector of insulin signaling, was lower in suspension culture than adherent cultures (data not shown). Suppression of insulin/PI3K signaling might also contribute to the effective DE induction in our case. It is also reported that 3D culture of differentiated hPSCs such as hepatocyte or pancreatic endocrine progenitors promote function or maturation (Sengupta *et al*., 2014, Takeuchi *et al*., 2014, Yabe *et al*., 2017). Taken together, we can conclude that suspension culture favors DE differentiation. This argument is further supported by our quite recent data that iPS-derived islet-like cells generated by suspension culture ameliorate hyperglycemia in diabetic mice and that they produce human insulin c-peptide at a rate 30 times higher than our adherent based culture in mouse plasma (Yabe *et al*., 2019). Further study will be needed to elucidate the precise mechanism of differentiation in 3D culture.

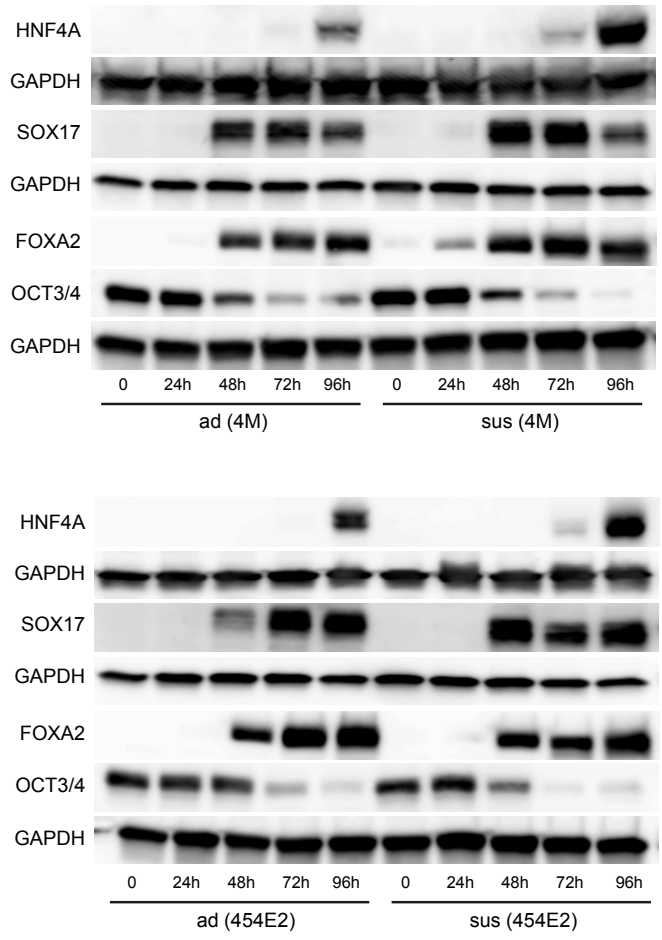

Fig. 6. Protein expression patterns of representative definitive endoderm (DE) and primitive gut tube (PGT) markers. *Protein expression was examined by Western blot at 0, 24, 48, 72, 96 h in suspension or adherent cultured using two hiPSCs lines (4M and 454E2). HNF4a was used as a PGT marker, and SOX17 and FOXA2 were used as DE markers. OCT3/4 was used as an undifferentiated marker. GAPDH was used as a control. The amount of sample protein loaded on each lane in the gel was as follows: HNF4A (44 μg), SOX17 (1.5 μg), FOXA2 and OCT3/4 (8.0 μg). ad, adherent culture sample; sus, suspension culture sample.*

Materials and Methods

Undifferentiated iPSC culture

The human iPSCs line TkDN4-M (4M), which was established from neonatal fibroblasts using retrovirus, was a kind gift from Dr. M. Ohtsu at The Institute of Medical Science, The University of Tokyo, and was cultured according to our previous protocol (Yabe *et al*., 2015). 4M was cultivated

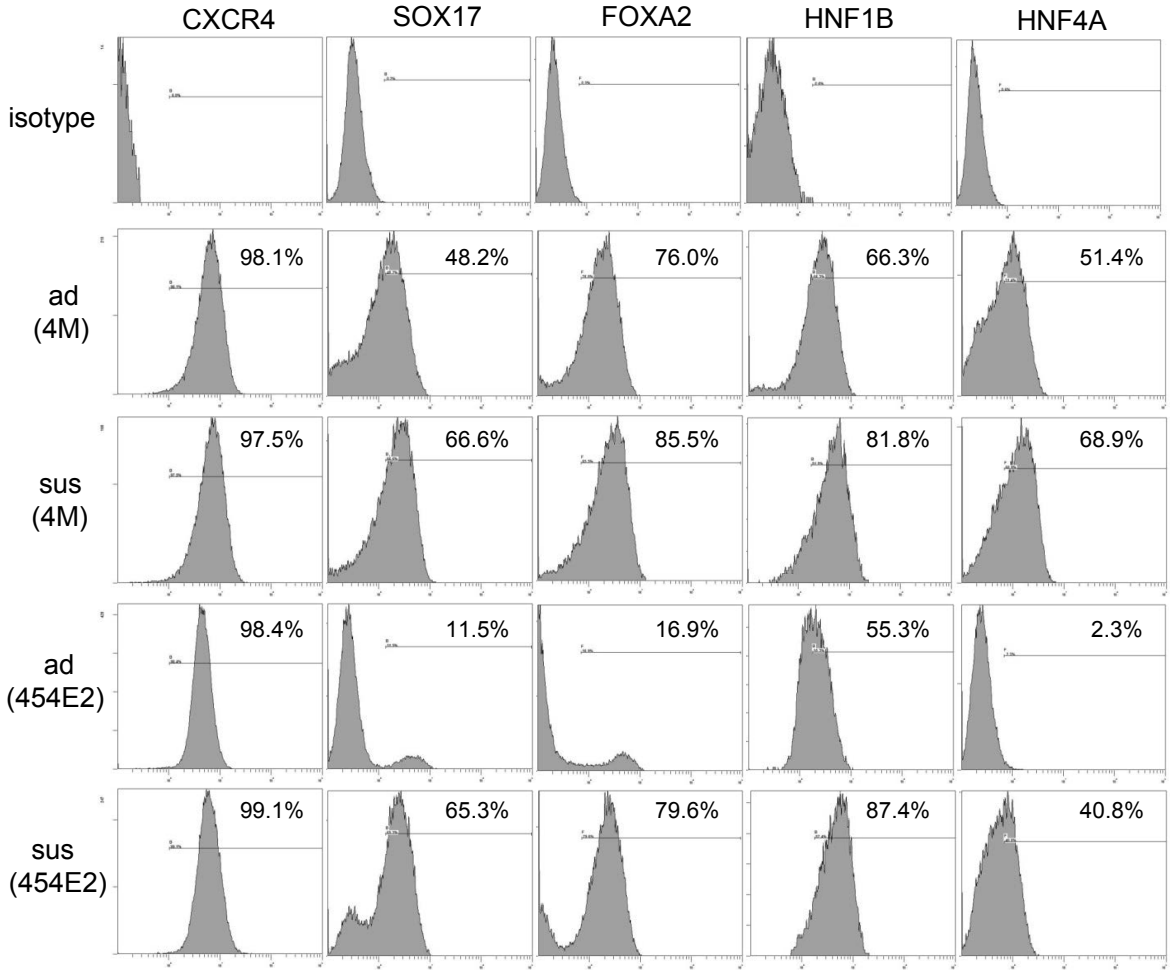

Fig. 7. Flow cytometric analysis of definitive endoderm (DE) and primitive gut tube (PGT) markers. *Flow cytometry was performed with cells harvested 96 h after DE differentiation in suspension or adherent culture using two hiPSCs lines (4M and 454E2). CXCR4, SOX17 and FOXA2 were used as DE markers and HNF1B and HNF4A were used as PGT markers. Isotype, isotype control; ad, adherent culture sample; sus, suspension culture sample.*

on mitomycin-C treated SNL feeder cells in hiPSs medium (DMEM/Ham's F12(Wako, Osaka, Japan) containing 20% Knockout Serum Replacement (KSR; GIBCO BRL, Palo Alto, CA, USA), 1xMEM non-essential amino acids (NEAA; Wako), 0.5x penicillin and streptomycin (PS; Wako), 0.055 mM 2-mercaptoehtanol (2-ME; GIBCO), and 7.5 ng/ml recombinant human fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ, USA). The human iPSCs line 454E2, which was established from adolescent dental pulp using episomal vectors, was a kind gift from CiRA, Kyoto University, and was maintained on vitronectin (GIBCO) in Essential8 medium (E8; GIBCO).

TABLE 1

PRIMERS USED FOR qRT-PCR

gene	forward	reverse
BRA	CGT CTC CTT CAG CAA AGT CAA G	CCC AAC TCT CAC TAT GTG GAT TC
EOMES	GTC GAG GTT CTT ACC AGA GGA AG	GGG TGT CTC TAT CCA AGA AGA GC
FGF8	ATC CGG ACC TAC CAA CTC TAC AG	GTC TCC ACG ATG AGC TTT GC
FOXA2	GAG ATC TAC CAG TGG ATC ATG GAC	CAC CTT CAG GAA ACA GTC GTT G
GSC	ACC GCA CCA TCT TCA CTG AC	TAA ACC AGA CCT CCA CTT TCT CC
HNF1b	GAG ATC CTC CGA CAA TTC AAC C	AAA CAG CAG CTG ATC CTG ACT G
HNF4a	AAG AGA TCC ATG GTG TTC AAG GAC	AGG TAG GCA TAC TCATTG TCA TCG
MIXL1	CCC TTG CCT CTA GCT TCT CAA C	CTC CTG ACT TCA GGT GAT CCA C
NANOG	CGA AGA ATA GCA ATG GTG TGA C	GTT GCT CCA GGT TGA ATT GTT C
NODAL	AGA GTC TGC TGA AAC GTT ACC AG	CCA CAT TCT TCC ACG ATC ATG
OAZ1	GTC AGA GGG ATC ACA ATC TTT CAG	GTC TTG TCG TTG GAC GTT AGT TC
OCT3/4	CGC TTC AAG AAC ATG TGT AAG CTG C	CTC TCA CTC GGT TCT CGA TAC TG
SOX2	ATA AGT ACT GGC GAA CCA TCT CTG	AAT TAC CAA CGG TGT CAA CCT G
SOX17	TAC ACA CTT CCT GGA GGA GCT AAG	CCA AAC TGT TCA AGT GGC AGA C

In vitro *differentiation*

Undifferentiated 4M was detached from feeder cells using CTK solution, rinsed with D-PBS several times, and then dissociated into single cells using Accumax (Innovative Cell Technologies, San Diego, USA). For adherent culture, dissociated 4M were plated on Matrigel-coated 6 well plates in mTeSR1 (VERITAS, Tokyo, Japan) containing 10μM Y-27632(Cayman Chemical, Ann Arbor, MI, USA), cultured for 1-2 days until they reached ~90% confluence, and further cultured in hiPSs medium for 1 day. The next day, DE induction was initiated. For suspension culture, dissociated 4M was seeded at a density of one million cells per ml in 30ml mTeSR1 with 10 μM Y-27632 in the spinner flask (ABLE, Tokyo, Japan) at a rotation rate of 45 rpm. The medium was replaced the next day, and, one day later, it was exchanged for hiPS medium. The next day, DE induction was started.

For adherent culture of 454E2, undifferentiated cells were dissociated using Accutase (Innovative Cell Technologies) and then seeded on Matrigel-coated 6-well plates in E8 with 10 μM Y-27632. The cells were cultivated for 2 days and then cultured in hiPS medium for 1 day. DE induction was started the next day. For suspension culture, dissociated 454E2 cells were

seeded into ultra-low attachment 6-well plates at a density of 10^6 cells/ml in 4ml E8 medium including 10 μM Y-27632 on orbital rotators set at 90 rpm. After overnight culture, the medium was exchanged. The next day, E8 was replaced with hiPS medium for 1 day; then DE induction was initiated. Human iPSCs were differentiated to DE according to following protocol: Day1-2: RPMI 1640 supplemented with 0.25% bovine serum albumin (BSA), 0.4x PS, 1 mM sodium pyruvate, 1x NEAA, 0.055 mM 2-ME, 80 ng/ml recombinant human activin A (Peprotech), 50 ng/ml FGF2, 20 ng/ml recombinant bone morphogenetic protein 4 (BMP4; Peprotech), 3 μM CHIR99021 (Biovision, Milpitas, CA, USA). Day3: RPMI 1640 supplemented with 0.25% BSA, 0.4x PS, 1 mM sodium pyruvate (SP; Wako), 1x NEAA, 0.055 mM, 2-ME, 80 ng/ml recombinant human activin A. Day4: RPMI 1640 supplemented with 0.5% KSR, 0.25% BSA, 0.4x PS, 1 mM SP, 1x NEAA, 0.055 mM 2-ME, 80 ng/ml recombinant human activin A. For 454E2, insulin-transferrin-selenium-ethanolamine (ITS-X, GIBCO) was further added to this medium (1:100,000). This differentiation protocol is summarized in Sup. Fig.1.

Quantitative RT-PCR

Total RNA was isolated and purified using Isogen. The cDNA was synthesized with PrimeScript II reverse transcriptase using random nonamers and oligos (dT18). Quantitative RT-PCR reactions were carried out on CFX96 Touch Deep Well (Bio-Rad, Hercules, CA, USA) using GoTaq qPCR master mix (Promega, Madison, WI, USA). Relative quantification was performed against a standard curve, and the expression levels of target genes were normalized against that of the housekeeping gene, ornithine decarboxylase antizyme (OAZ1). For the time course comparison of gene expression, one of the samples at a certain time point was selected, and relative fold induction was calculated for each gene. The primer sequence is listed in Table1.

Western blotting

Cells were collected in RIPA Buffer (Wako) with protease inhibitors (Roche, Basel, Switzerland), sonicated, and centrifuged. Whole cell lysate protein concentrations were determined by a BCA protein assay (TakaRa). Proteins were boiled in sample buffer (BIO-RAD) and loaded onto Mini-PROTEAN®TGX™Gels (BIO-RAD) separated by SDS-PAGE. Protein bands were transferred to Trans-Blot®Turbo™ membranes (BIO-RAD), which were then immersed in TBST containing the first antibody. Then the membranes were treated with Blocking One (Nacalai Tesque, Kyoto, Japan). After washing, the membranes were next immersed in the second antibody and reacted with Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA). The bands were observed by LAS 4000 mini (FujiFilm, Tokyo, Japan).

Western blotting was performed using anti-OCT3/4(1:1000, BD Bioscience), anti-FoxA2 (1:1000, Cell Signaling), anti-SOX17(1:5000, R&D Systems), anti-HNF1β (1:1000, Proteintech) anti-HNF4α (1:1000, Cell Signaling) and anti-GAPDH (1:5000, Thermo Fisher Scientific). Horseradish peroxidase–conjugated second antibody (1:2000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow cytometry

Differentiated cells were dissociated into single cells using accutase and washed with RPMI1640. Cells were fixed with 4% paraformaldehyde at RT for 30 min and washed with PBS several times. For surface marker CXCR4 staining, cells were incubated in blocking buffer (PBS+0.1% triton-X+3% FBS) at RT for 1 h and then incubated in blocking buffer with directly conjugated primary antibody (APC anti-human CD184 (CXCR4), 1:20, Biolegend, CA, USA) at 4 °C for 1 h. Stained cells were washed with blocking buffer and re-suspended in PBS+1% BSA and then analyzed on a Gallios flow cytometer (Beckman Coulter, CA, USA) using Gallios software. For intracellular staining (SOX17, FOXA2, HNF1B and HNF4A), fixed cells were incubated in methanol at 4°C for 10 min and washed with blocking buffer several times. Cells were incubated in blocking buffer at RT for 1 h and then incubated in blocking buffer with 1:100 diluted primary antibody (anti-FoxA2 (Cell Signaling), anti-SOX17(R&D Systems), anti-HNF1β (Proteintech) anti-HNF4α (Cell Signaling)) at 4°C overnight. Cells were washed with blocking buffer several times and incubated in blocking buffer with 1:400 diluted secondary antibody (donkey anti-rabbit Alexa 647 or donkey anti-goat Alexa 647, Thermo Fisher Scientific). Stained cells were washed with blocking buffer and re-suspended in PBS+ 1% BSA and then analyzed as mentioned above.

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