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Redefining definitive endoderm subtypes by robust induction of human induced pluripotent stem cells

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ABSTRACT

Many reports have described methods that induce definitive endoderm (DE) cells from human pluripotent stem cells (hPSCs). However, it is unclear whether the differentiation propensity of these DE cells is uniform. This uncertainty is due to the different developmental stages that give rise to anterior and posterior DE from anterior primitive streak (APS). Therefore, these DE cell populations might be generated from the different stages of APS cells, which affect the DE cell differentiation potential. Here, we succeeded in selectively differentiating early and late APS cells from human induced pluripotent stem cells (hiPSCs) using different concentrations of CHIR99021, a small molecule Wnt/β-catenin pathway activator. We also established novel differentiation systems from hiPSCs into three types of DE cells: anterior and posterior domains of anterior DE cells through early APS cells and posterior DE cells through late APS cells. These different DE cell populations could differentiate into distinct endodermal lineages in vitro, such as lung, liver or small intestine progenitors. These results indicate that different APS cells can produce distinct types of DE cells that have proper developmental potency and suggest a method to evaluate the quality of endodermal cell induction from hPSCs.

1. Introduction

In mouse embryogenesis, the pluripotent epiblast at embryonic day (*E*) 5.5 differentiates into the anterior primitive streak (APS) at E6.5, which subsequently generates definitive endoderm (DE) at E7.0 – E7.5. Then, DE is patterned along the anterior–posterior axis into distinct gut tube (GT) regions, such as foregut, midgut and hindgut, at E8.5, and endoderm organ buds are generated from specific anteroposterior domains of late gut tube (LGT) regions at E9.5 (Kimura et al., 2006).

It has recently been reported that human pluripotent stem cells (hPSCs), such as human embryonic stem cells or induced pluripotent stem cells (hiPSCs), can differentiate into the cells constituting endoderm derivative organs, such as pancreas, liver, lung and small intestine (Toyoda et al., 2015; Rezania et al., 2014; Pagliuca et al., 2014; Takayama et al., 2014; Kajiwara et al., 2012; Ghaedi et al., 2015; Gotoh et al., 2014; Watson et al., 2014; McCracken et al., 2011). In these reports, hPSCs are induced through primitive streak (PS) into DE cells, which are then induced to endoderm derivative cells. The DE cell induction from hPSCs has been demonstrated by showing the expression of two well-known DE marker genes, SOX17 and FOXA2, but it is still unknown whether different endodermal derivatives can differentiate from the same progenitors which express these markers. Furthermore, DE cells can be classified into two distinct populations, anterior and posterior DE cells, in vertebrate development. These cells are further patterned along the anterior-posterior axis of the embryo, and the derivatives of each are distinct (Zorn and Wells, 2009). Therefore, it is assumed that hPSC-derived DE cells might have heterogeneity.

It has been reported that DE cells are generated from PS cells (Arnold et al., 2009; Stern et al., 2006; Tam and Loebel, 2007). The formation of PS is an indispensable step for DE differentiation, as otherwise defects in DE formation occur (Conlon et al., 1994; Waldrip et al., 1998). Fate-mapping experiments using mouse embryos have demonstrated that DE progenitors are specified in APS cells that express a T-box transcriptional factor, *Eomesodermin* (*Eomes*) (Lawson et al., 1991; Tam et al., 1997; Costello et al., 2011). Moreover, anterior and posterior DE cells have been reported to originate from different developmental stages of PS (Matsushita, 1999). Accordingly, it is expected that anterior and posterior DE cells are the descendants of different developmental stages of

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Table 1
Published induction methods of definitive endoderm (DE).

Inducer	Treatment	Reference
Activin A (+ FBS)	100 ng/ml activin A+0.2% FBS	D'Amour et al., Nat Biotechnol, 2005
	100 ng/ml activin A+0.5% FBS	Agarwal et al., Stem Cells, 2008
	100 ng/ml activin A+20% FBS	Shim et al., Diabetologia, 2007
Activin A+Wnt (+ FBS)	100 ng/ml activin A+25 ng/ml WNT3A+0.2% FBS	D'Amour et al., Nat Biotechnol, 2006 Kelly et al., Nat Biotechnol, 2011 Sui et al., Cell Transplant, 2012
	100 ng/ml activin A+25 ng/ml WNT3A or 3 μM CHIR99021+2% FBS	Kunisada et al., Stem Cell Res, 2011
	100 ng/ml activin A+20 ng/ml WNT3A+0.2–0.5% FBS	Rezania et al., Diabetes 2012
	100 ng/ml activin A+50 ng/ml WNT3A	Hay et al., PNAS, 2008
Activin A+BMP (+FGF)	50 ng/ml activin A+50 ng/ml BMP4	Phillips et al., Stem Cells Dev, 2007 Teo et al., Stem Cells, 2012
	100 ng/ml activin A+10 ng/ml BMP4+20 ng/ml FGF2	Vallier et al., PLoS One, 2009
	100 ng/ml activin A+50 ng/ml BMP4+100 ng/ml FGF2	Xu et al., Mech Dev, 2011
	100 ng/ml activin A+0.25 ng/ml BMP4+2.5 ng/ml FGF2	Nostro et al., Development, 2011
Activin A+BMP + FGF + PI3K inhibitor	100 ng/ml activin A+10 ng/ml BMP4+20 ng/ml FGF2+10 μM LY294002	Touboul et al., Hepatology, 2010
Activin A+BMP inhibitor	100 ng/ml activin A+250 nM DM3189	Loh et al., Cell Stem Cell 2014

FBS: fetal bovine serum, BMP: bone morphogenetic protein, FGF: fibroblast growth factor, PI3K inhibitor: phosphoinositide 3-kinase inhibitor.

Eomes(+) APS cells. Although previous reports have generated DE cells from hPSCs using different methods (Table 1), it is unclear whether those reports addressed the heterogeneity of DE cells.

In this study, we differentiate early and late developmental stages of APS cells from hiPSCs. Then, we establish selective differentiation methods toward three different types of DE cell populations: anterior domain of anterior DE (AADE), posterior domain of anterior DE (PADE) and posterior DE (PDE). These hiPSC-derived DE cell populations can give rise to their own derivatives in vitro. Moreover, our results indicate that the differentiation potency of endodermal cells is restricted by the DE stage.

2. Materials and methods

2.1. Cell culture

For feeder-free cultures, hiPSCs (585A1 cells) (Okita et al., 2011; Kajiwara et al., 2012) were maintained with Essential 8 medium (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. For routine passaging, hiPSC colonies were dissociated by an enzymatic method with 0.5 mM EDTA (Wako, Osaka, Japan) and split at a ratio of 1:100 upon adding 10 μM Y-27632 (Wako).

2.2. Differentiation into anterior primitive streak (APS)

hiPSC colonies grown with 80% confluency were dissociated into single cells by an enzymatic method with 0.5 mM EDTA. The cells were re-suspended in RPMI 1640 medium (NACALAI TESQUE, Kyoto, Japan) containing 2% (vol/vol) growth factor-reduced B27 supplement (GFR-B27, Thermo Fisher Scientific), 50 U/ml penicillin/streptomycin (P/S, Thermo Fisher Scientific) and 10 μM Y-27632 supplemented with 100 ng/ml recombinant human/mouse/rat activin A (R&D Systems, Minneapolis, MN) and 3 μM CHIR99021 (Axon Medchem, Groningen, Netherlands) for early APS or with 100 ng/ml activin A and 8 μM CHIR99021 for late APS, seeded on Matrigel (Becton Dickinson, Franklin Lakes, NJ)-coated plates at a density of 9×10^4 cells/cm^2 and cultured for one day.

2.3. Differentiation into definitive endoderm (DE)

Early APS was further patterned into anterior domain of anterior DE (AADE) or posterior domain of anterior DE (PADE) by 2 days of differentiation culture in RPMI 1640 medium with 2% GFR-B27, 50 U/ml P/S and 100 ng/ml activin A for AADE or 100 ng/ml activin A and 500 nM LDN193189 (Axon Medchem) for PADE. Late APS was differentiated in RPMI 1640 medium with 2% GFR-B27, 50 U/ml P/S and 100 ng/ml activin A to generate posterior DE (PDE).

2.4. Differentiation into gut tube (GT) and late gut tube (LGT)

For GT cell induction, three types of DE cells (AADE, PADE and PDE) were cultured in Improved MEM Zinc Option (iMEM) medium (Thermo Fisher Scientific) for 3 days. Then, GT cells were differentiated into LGT cells using the same media for 3 days.

2.5. Differentiation into liver and lung lineage cells

The LGT cells were further differentiated into hepatocyte-like cells and alveolar epithelial progenitor cells according to previously reported differentiation protocols (Kajiwara et al., 2012; Gotoh et al., 2014). In brief, the hiPSC-derived LGT cells were cultured in hepatocyte culture medium (Lonza, Tokyo, Japan) containing 20 ng/ml recombinant human hepatocyte growth factor (HGF; PeproTech, Rocky Hill, NJ) and 20 ng/ml recombinant human oncostatin M (OsM; PeproTech) for 6 days to induce differentiation into hepatocyte-like cells. For the differentiation of alveolar epithelial progenitor cells, the LGT cells were cultured in DMEM/F12 plus Glutamax medium (Thermo Fisher Scientific) containing 1x B27 and N2 supplements (Thermo Fisher Scientific), 50 U/ml P/S, 0.05 mg/ml of L-ascorbic acid (Sigma-Aldrich, Tokyo, Japan), 0.4 mM of monothioglycerol (Wako), 100 ng/ml of recombinant human bone morphogenetic protein (BMP)4 (R&D Systems), 0.5 μM of all-trans retinoic acid (ATRA; Sigma-Aldrich) and 3.5 μM of CHIR99021 for 4 days.

2.6. Immunostaining

The cells were fixed with 4% paraformaldehyde (PFA)/PBS for 20 min at 4 °C. After washing with PBS, the cells were blocked with 5% normal donkey serum (Funakoshi, Tokyo, Japan)/PBST (PBS/0.1% Triton X-100) for 1 h at room temperature. The primary antibodies were diluted in blocking solution and incubated with the samples overnight at 4 °C. After washing with PBS three times, the cells were incubated with secondary antibodies for 1 h at room temperature. The secondary antibodies used in this study included Alexa Fluor 488-, 546-or 647-conjugated donkey antibodies against mouse, rabbit or goat IgG, and were used at 1:200 dilution. For nuclear staining, Hoechst 33342 trihydrochloride trihydrate (Thermo Fisher Scientific) was used at 1:200 dilution. The primary antibodies used are detailed in Table 2.