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Stem Cell: Advanced Research and Therapy

Nakamura S, et al. Stem Cell Adv Res Ther 2017: SCRT-111.

Research Article

DOI: 10.29011/SCRT-111. 000011

# Simple Isolation of Pancreatic Progenitor Cells from Human Induced Pluripotent Stem Cells Using the ALDEFLUOR

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**Citation:** Nakamura S, Takizawa S, Yokoyama T, Tomotsune D, Fenming Y, Sasaki K (2017) Simple Isolation of Pancreatic Progenitor Cells from Human Induced Pluripotent Stem Cells Using the ALDEFLUOR.Stem Cell Adv Res Ther 2017: SCRT-111. DOI: 10.29011/SCRT-111. 000011

**Received Date:** 20 December, 2016; **Accepted Date:** 04 January, 2017; **Published Date:** 11 January, 2017

## Abstract

Acquiring progenitor cells from pluripotent stem cells is important for regenerative medicine because of their proliferative capability and restricted differentiation toward target cells. However, simple and effective methods to isolate pancreatic progenitor cells from human induced pluripotent stem cells (hiPSCs) have not been established yet. Centroacinar and intercalated duct cells near acini are positive for aldehyde dehydrogenase (ALDH) 1 and can be simply isolated by cell sorting using a fluorescent non-toxicsubstratesystem, ALDEFLUOR. In this study, we sorted hiPSC-derived pancreatic progenitor cells using ALDEFLUOR and determined whether they could differentiate into endocrine progenitor cells, which would benefit clinical applications using hiPSCs. A modified stepwise method was used to differentiate hiPSCs into pancreatic progenitor cells. Their gene and protein expression of ALDH and pancreatic duodenal homeobox 1 (Pdx1) was investigated by reverse transcription polymerase chain reaction and immunohistochemistry, respectively. ALDEFLUOR-positive cells comprised about28% of total cells and were isolated by a fluorescence-activated cell sorter.They were differentiated toward pancreatic endocrine progenitor cells by treatment with Noggin, fibroblast growth factor 7 (FGF7), and epidermal growth factor (EGF), and immunostained for neurogenin 3 (Ngn3), a marker of pancreatic endocrine progenitor cells. Undifferentiated hiPSCs did not express ALDH1 and were not isolated by ALDEFLUOR sorting. These results showed that ALDEFLUOR provides a simple and efficient method to sort hiPSC-derived pancreatic progenitor cells for potential use in regenerative medicine.

## Introduction

Because the proliferation of adult pancreatic cells is poor, cell transplantation of pancreatic endocrine cells, especially insulin-producing cells, is required to cure diabetes mellitus permanently without drugs or insulin. Pluripotent stem cells including embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) are a valuable source to supply target cells.

There are two kinds of methods for pancreatic cell differentiation: stepwise direct differentiation from undifferentiated cells through the progenitor cell stage to target cells, and differentiation of isolated progenitor cells to target cells. The former has prevailed in this field, but it is difficult to remove a mixture of unexpected cells [1,8]. There are fewer reports of the latter method because of the lack of adequate surface markers. Cai et al. reported that cells sorted by CXCR4 include more than 70% pancreatic duodenal homeobox 1 (Pdx1)-expressing cells [9]. In addition, pancreatic endoderm progenitors can be sorted with an anti-CD142 antibody, and endocrine cells can be sorted with anti-CD200 and -CD318 antibodies [10]. However, sorting based on immunoreactions requires relatively more processes and time to perform. Moreover, the above markers are not always specific for target cells.

Rovira et al. showed that aldehyde dehydrogenase (ALDH) protein is restricted to the tips of branching pancreatic ductules in mouse embryonic pancreas at embryonic day 12.5 and in adult mouse pancreas [11]. It is also exclusively expressed in a Pdx1-

dependent manner [12] and marks the source of endocrine precursors in the developing human pancreas [13].

ALDHs are a group of NAD (P+)-dependent detoxification enzymes that are critical to protect organisms against various aldehydes [14, 15] and their activity can be detected rapidly within 1 h using a fluorescent dye in the ALDEFLUOR reagent system [16]. Cells expressing high levels of ALDH1 become brightly fluorescent and can be identified and sorted using a fluorescence-activated cell sorted for further purification and characterization.

Therefore, in this study, we established a new simple method to sort human pancreatic stem/progenitor cells from hiPSCs using the ALDEFLUOR fluorescent dye to pancreatic progenitor cells, and confirmed that the sorted cells expressed Pdx1 and differentiated into β progenitor cells.

## Materials and Methods

### Cell lines and culture

iPSC lines 201B7 and 253G1, which were established by Takahashi et al. [17] were purchased from Kyoto University.

These pluripotent stem cells maintain an undifferentiated state on mitomycin C (Sigma, St. Louis, MO)-inactivated mouse embryonic fibroblasts (Kitayama Labes, Ina, Japan) in Dulbecco's modified Eagle medium/nutrient mixture F-12(DMEM/F-12; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 20% KnockOut serum replacement (Invitrogen, Carlsbad, CA), 100 µM nonessential amino acids (Wako), 2 mM L-glutamine (Wako), 100 µM 2-mercaptoethanol (Sigma), and 4 ng/ml basic fibroblast growth factor (bFGF; Wako). The medium was exchanged every day. Passaging was performed every 4 days.

### Pdx-1-positive cell differentiation

Previously, a stepwise differentiation method was developed to acquire exocrine pancreatic cells from mouse ESCs [5], which was modified to apply to human ESC differentiation [18]. In this study, to obtain Pdx1-positive cells more effectively, the protocol was modified by incorporation of some of the method to induce endocrine cells developed by Toyoda et al. [8] (Fig. 1).

This protocol consisted of three steps. Step 1 was 4 days of culture in RPMI 1640 medium (Gibco BRL, Rockville, MD) with 100 ng/ml activin A (Sigma), 1 µM CHIR99021(StemRD, Burlingame, CA), 2% (vol/vol) B27 (Gibco BRL), 50 U/ml penicillin, and 50 µg/ml streptomycin to induce definitive endoderm (DE) differentiation from undifferentiated hiPSCs. Step 2 was 3 days of culture to produce primitive gut tubes from DE. The cells were cultured in RPMI 1640 medium containing 50 ng/ml FGF7 (R&D systems, Minneapolis, MN) instead of activin A and CHIR99021, and the concentration of B27 was reduced to 1%. Finally, step 3 was also 3 days culture in medium containing 0.5 µM 3-keto-N-aminoethyl-aminocaproyl-dihydrocinnamoyl cyclopamine (KAAD-cyclopamine; CALBIOCHEM, Merck, Darmstadt, Germany), 100 ng/ml Noggin (Repro-tech, Rocky Hill, NJ),1 µM all-trans retinoic acid (RA) (Sigma), and 1% B27 (Gibco BRL) to differentiate primitive gut tubes into posterior foregut including Pdx1-positive cells.

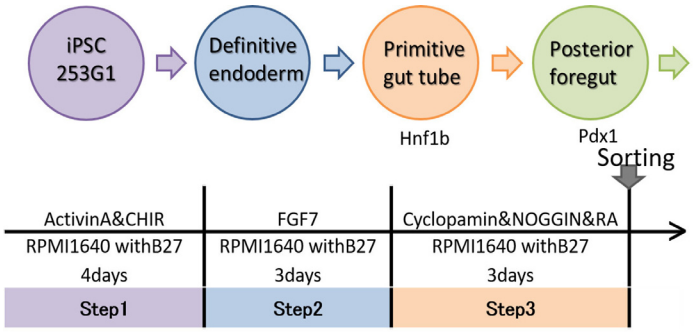



**Fig. 1:** Protocol to produce pancreatic progenitor cells (posterior foregut). The protocol includes three steps from iPSCs through definitive endoderm and primitive gut tube to posterior foregut using activin A and CHIR99021 in step 1, FGF7 in step 2, andcyclopamine, Noggin, and RA in step 3.

### ALDEFLUOR assay and sorting of ALDEFLUOR-positive cells

The differentiated cells produced by the above stepwise method were prepared for the ALDEFLUOR reaction (intracellular enzymatic reaction) using an ALDEFLUOR Kit (Stem Cell technologies,Vancouver, Canada) according to the manufacturer's instructions to identify and isolate ALDEFLUOR-positive cells. The cells were dissociated into single cells with versene (Gibco BRL) before staining. The brightly fluorescent ALDH-expressing cells were detected in the green fluorescent channel (520–540 nm) of a fluorescence-activated cell sorter, FACSAria II (Becton Dickinson, Franklin Lakes, NJ), and sorted as ALDEFLUOR-positive cells. Some of the samples served as the negative control using an

ALDH enzyme inhibitor, diethylaminobenzaldehyde (DEAB).

### Differentiation of pancreatic endocrine progenitor cells from ALDHFLUOR-positive cells

ALDEFLUOR-positive cells were sorted and cultured for 4 days in DMEM/F-12 containing 100 ng/ml Noggin, 100 ng/ml FGF7, and 50 ng/ml EGF (R&D systems). Then, the cells were prepared for immunohistochemical analysis to confirm differentiation of endocrine progenitor cells.

## Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from the total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Yokkaichi, Japan). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio) in a Thermal Cycler Dice® Real Time System (Takara Bio). The cDNA was amplified by 30 s of initial denaturation at 95°C, followed by 40 cycles of heating at 95°C (5 s) and then at 60°C (60 s). The expression of each mRNA was determined using specific primers for ALDH1, Pdx1, Hnf1b, Oct4, Nanog, and Sox2. Gene expression values were normalized to that of the housekeeping gene β-actin. For the negative control, distilled water was substituted for the cDNA template. The primer sequences were as follows: ALDH1, forward, 5′- TCCTGGTTATGGGCCTACAG -3′, reverse, 5′- CTGGCCCTGGTGGTAGAATA -3′; Pdx1, forward, 5′- TGGATGAAGTCTACCAAAGC -3′, reverse, 5′- GGTCAAGTTCAACATGACAG -3′; Hnf1b, forward, 5′- TCACAGATACCAGCAGCATCAGT -3′, reverse, 5′- GGGCATCACCAGGCTTGTA -3′; Oct4, forward, 5′- TCTATTTGGGAAGGTATTCAGC -3′, reverse, 5′- ATTGTTGTCAGCTTCCTCCA -3′; Nanog, forward, 5′- AGCTACAAACAGGTGAAGAC -3′, reverse, 5′- GGTGGTAGGAAGAGTAAAGG -3′; Sox2, forward, 5′- GGGGGAATGGACCTTGTATAG -3′, reverse, 5′- GCAAAGCTCCTACCGTACCA -3′; β-actin, forward, 5′- TGGCACCCAGCACAATGAA -3′, reverse, 5′- CTAAGTCATAGTCCGCCTAGAAGCA -3′.

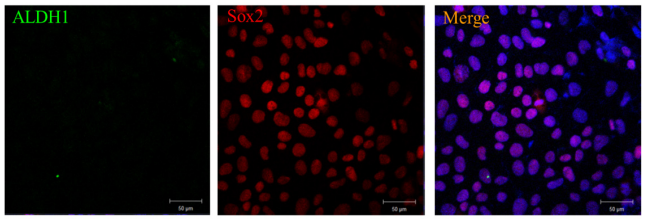
### Immunofluorescence

Immunostaining procedures have been described previously [5]. Briefly, cultured cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde/PBS (pH 7.4) for 15 min, permeabilized with 0.1% Triton X-100 in PBS, and then treated with 1.5% normal donkey or goat serum to block nonspecific staining. After 30 min, the following primary antibodies were applied to the cells at 4°C overnight: rabbit anti-Sox2(an undifferentiated marker, 1:400; Cell Signaling Technology,Danvers, MA), mouse anti-ALDH1(1:100; Santa Cruz Biotechnology, Dallas, TX), goat anti-PDX-1(1:200; R&D Systems), and rabbit anti-neurogenin 3 (Ngn3; a marker for pancreatic endocrine progenitor cells, 1:200; Affinity Bioreagents, Golden, CO). After three washes with PBS, the samples were incubated with diluted secondary antibodies conjugated with either Alexa Fluor 488 or 568 and then mounted with ProLong® Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Waltham, MA). The specimens were observed using a ZEISS Axio Observer Z1 or ZEISS LSM5EXCITER confocal scanning microscope (Carl Zeiss, Oberkochen, Germany).

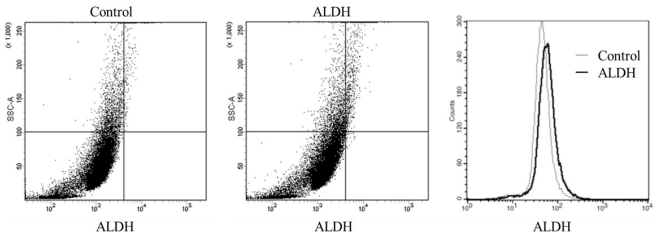
## Results and Discussion

### Undifferentiated hiPSCs do not express ALDH1

The ALDEFLUOR assay to detect ALDH1 has been applied to identify progenitor cells and cancer stem cells [16]. However, not all progenitor cells or stem cells express ALDH1. Undifferentiated hiPSCs were positive for Sox2 and not immunohistochemically positive for ALDH1 (Fig. 2A). This observation was supported by flow cytometry showing no difference between ALDEFLUOR-treated cells and the control (Fig. 2B). iPSCs had no ALDH1 and were not positive in the ALDEFLUOR assay. This feature is essential to apply the assay to sort pancreatic progenitor cells from undifferentiated iPSCs.




2A




2B

**Fig. 2A:** Immunostaining of undifferentiated hiPSCs. DAPI-positive cells show Sox2 expression but do not express ALDH. Bars = 50μm

**2B:** Sorting of bright ALDEFLUOR-positive cells (ALDH positive) from undifferentiated hiPSCs by florescence-activated cell sorting. Both control and ALDH regions almost overlap.

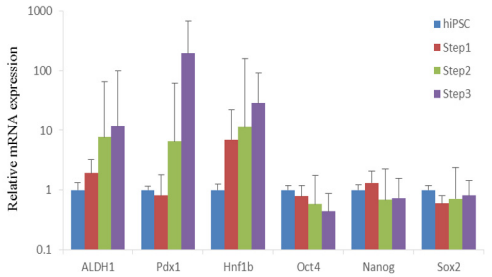
### ALDH1-positive cells appear during pancreatic differentiation

During embryogenesis, differentiated pancreatic progenitor cells begin to express ALDH1 [12]. Real-time PCR analysis demonstrated that mRNA expression of ALDH1 was up-regulated as differentiation proceeded from step 1to 3, which was similar to

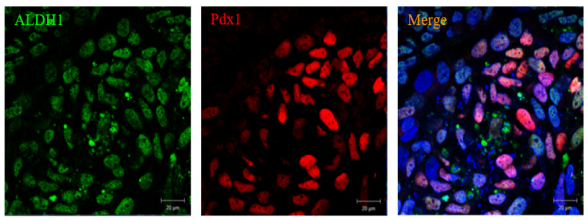
the increase in expression of Pdx1 and Hnf1b (pancreatic progenitor marker) (Fig. 3A). ALDH1 mRNA expression at step 3 was increased by 10-fold compared with the undifferentiated state. The increase of Pdx1 began from step 2 and reach a 100-fold increase in step 3. Hnf1b showed a similar increase as ALDH1. Expression changes of differentiation or progenitor markers were closely associated with that of ALDH1.

The expression of undifferentiated markers Oct4, Nanog, and Sox2 were down-regulated, although they were expressed continuously throughout differentiation stages.

Next, immunocytochemistry confirmed that ALDH1-positive cells were also positive for Pdx1 (Fig. 3B). In step 3, the hiPSC-derived posterior foregut stage, a large number of ALDH1-positive cells expressed Pdx1, indicating the appearance of pancreatic progenitor cells.




3A




3B

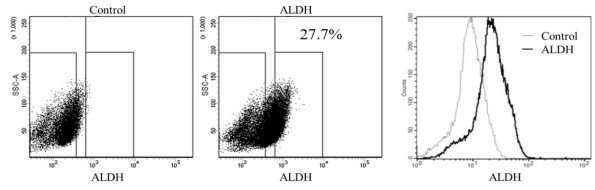
**Fig. 3A:** Effects of the stepwise method on differentiation. Real-time PCR analysis of ALDH1, Pdx1, Hnf1b, Oct4, Nanog, and Sox2. Compared with undifferentiated hiPSCs (blue column), ALDH, Pdx1, and Hnf1b expression increases according to each step, while undifferentiated markers decrease.

**3B:** Immunostaining of differentiated cells in step 3. Dual immunostaining showed that ALDH1-positive cells also express Pdx1. Bars = 20μm

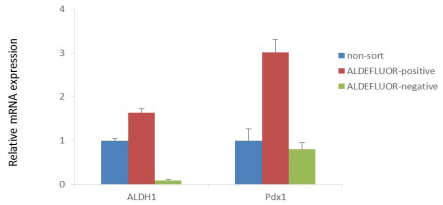
## Sorted ALDH1-positive cells differentiate into pancreatic endocrine progenitor cells

ALDH1-positive cells immunocytochemically found in step 3 were confirmed by the ALDEFLUOR assay (Fig. 4A). When the gate was set according to the DEAB control, ALDEFLUOR-positive cells were about 28% of differentiated cells (Fig. 4B). Real-time PCR analysis demonstrated that mRNA expression levels of Pdx1 and ALDH1 were higher in ALDEFLUOR-positive cells compared with ALDEFLUOR-negative cells and non-sorted differentiated hiPSCs. Pdx1 was expressed moderately in ALDEFLUOR-negative cells despite almost no ALDH1 expression. Based on these results, the ALDEFLUOR assay is an efficient method with low cell damage to specifically sort pancreatic progenitor cells from undifferentiated hiPSCs.

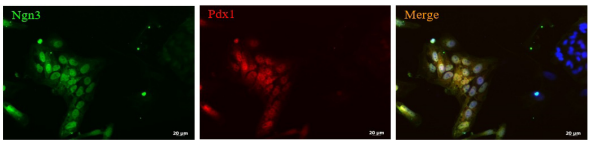
Sorted ALDEFLUOR-positive cells were cultured for 4 days under differentiation conditions toward endocrine progenitor cells and prepared for immunocytochemistry to confirm expression of a biomarker for pancreatic endocrine progenitor cells, Ngn3 (Fig. 4C). ALDH-positive cells were also positive for Ngn3. Several studies have shown that endocrine progenitor cells originate from both ALDH1 and Pdx1-positive cells [19–22]. Our study showed that, after further induction by Noggin, FGF7 and EGF, ALDEFLUOR-positive cells had differentiated into Ngn3-positive cells. Ngn3, a pro-endocrine transcription factor, is necessary for commitment to the endocrine fate [23]. Furthermore, it has been shown that Ngn3-positive cells represent pancreatic endocrine cells in humans using ESCs [7]. Therefore, sorted hiPSC-derived cells by the ALDEFLUOR assay can be a source of pancreatic endocrine cells. It was also demonstrated that Ngn3-negative cells appeared to lose Pdx1 expression by immunocytochemistry(Fig. 4C). According to Rovira et al., these cells may be differentiated acinar cells [11].




4A




4B




4C

**Fig. 4A:** Sorting of differentiated cells in step 3 using fluorescence-activated cell sorting after ALDEFLUOR staining. The gate was set according to the DEAB control. ALDEFLUOR-positive cells were about 28% of the total cell population.

**4B:** Real Time-PCR analysis of sorted positive and negative cells and non-sorted cells. Sorted positive cells express more ALDH1 and Pdx1

than other cells.

**4C:** Immunostaining of β progenitor cells differentiated from sorted positive cells. Ngn3-positive cells also express Pdx1. DAPI-positive cells in the right side do not express Ngn3 or Pdx1. Bars = 20μm

## Acknowledgments

We thank Dr. K. Kametani, Ms. K. Suzuki, and Ms. A. Yoshitome for technical support and Ms. J. Maruyama for secretarial assistance.

## Funding

This work was partially supported by JSPS KAKENHI Grant Numbers JP24240076 and JP15K01280.

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