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Original Article

Efficient reprogramming of human fibroblasts using RNA reprogramming with DAPT and iDOT1L under normoxia conditions

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ABSTRACT

Introduction: Human induced pluripotent stem cells (hiPSCs) are generated through the reprogramming of somatic cells expressing a defined set of transcription factors. The advent of autologous iPSCs has enabled the generation of patient-specific iPSC lines and is expected to contribute to the exploration of cures and causes of diseases, drug screening, and tailor-made regenerative medicines. Efficient control of hiPSC derivation is beneficial for industrial applications. However, the mechanisms underlying somatic cell reprogramming remain unknown, while reprogramming efficiency remains extremely low, especially in human cells.

Methods and results: We previously reported that chemical inhibition of the NOTCH signaling pathway and DOT1L promoted the generation of hiPSCs from keratinocytes, but the mechanisms and effect of this double inhibition on other types of cells remain to be investigated. Here, we found that the NOTCH/DOT1L inhibition markedly increased iPSC colony generation from human fibroblast cells via mRNA reprogramming, and mesenchymal to epithelial transition (MET)-related genes are significantly expressed in the early phase of the reprogramming. We successfully derived hiPSC lines using a single-cell sorting system under efficient reprogramming conditions.

Conclusions: This user-friendly reprogramming approach paves the way for the development of hiPSC derivations in industrial applications of disease modeling and drug screening.

1. Introduction

Somatic cells can be reprogrammed to induce pluripotent stem cells (iPSCs) through the forced expression of a defined set of transcription factors [1,2]. iPSCs provide a valuable source of patient-specific cells for research and drug development and can potentially be used in medical applications such as regenerative medicine. However, the mechanisms underlying somatic cell reprogramming remain to be fully understood, and the reprogramming efficiency remains extremely low, especially in human

Abbreviations: ACTB, actin beta; ALP, alkaline phosphatase; ALPL, alkaline phosphatase, liver/bone/kidney; CDH1, cadherin 1; CDH2, cadherin 2; CDKN1A, cyclin dependent kinase inhibitor 1A; DSC2, desmocollin 2; EGFR, epidermal growth factor receptor; ERBB3, erb-B2 receptor tyrosine kinase 3; FOXH1, forkhead box H1; KLF4, Krüppel-like factor 4; KRT14, keratin 14; LIN28A, Lin-28 homolog A; MMP2, matrix metallopeptidase 2; NANOG, nanog homeobox; NODAL, nodal growth differentiation factor; PDGFRA, platelet derived growth factor receptor alpha; POU5F1, POU class 5 homeobox 1; SOX2, sex determining region Y-box transcription factor 2; TMEFF1, transmembrane protein with epidermal growth factor like and two follistatin like domains 1; TP53, tumor protein p53; TWIST1, twist family basic helix-loop-helix transcription factor 1; VIM, vimentin.

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cells [3,4]. Designing strategies to improve the efficiency of iPSC generation is warranted for practical applications such as drug screening and patient-specific regenerative medicine. Recent studies have shown that somatic cell reprogramming is accompanied by remodeling of donor cell transcription and chromatin status, which is characterized by the rapid induction of cell proliferation and downregulation of the expression of somatic genes, followed by a mesenchymal–epithelial transition (MET) [5]. Furthermore, recent advancements in reprogramming technologies have enabled the selection of improved reprogramming methods, including cell types, under a variety of conditions.

Reprogramming efficiency can be improved using epigenetic modulators such as the histone H3 methyltransferase disruptor of telomeric silencing 1-like inhibitor (iDOT1L) [6,7]. However, little is known about the mechanism underlying chemical reprogramming processes, such as mRNA reprogramming, in human fibroblast cells through genome integration-free reprogramming. In our previous study, pharmacological inhibition of the NOTCH signaling pathway (via N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester [DAPT] or dibenzazepine [DBZ]) boosted the reprogramming of iPSCs from human keratinocytes without c-MYC and Krüppel-like factor 4 (KLF4) factors [8]. Considering this evidence, a combination of small-molecule compounds along with mRNA reprogramming was expected to promote reprogramming efficiency in human fibroblasts.

Taking advantage of iPSCs technology at the industrial level requires automated iPSC generation and cultivation. However, iPS cell reprogramming and maintenance are expensive and require constant supervision and cell quality assessment by highly trained personnel [9,10]. Techniques and culture products for human iPSCs continue to advance; however, generating human iPS cells remains a challenge at the industrial level. The traditional generation of human iPS cells relies mostly on the expertise of the operator's variabilities, including the isolation of individual iPS cell colonies, which is a laborious and time-consuming process. Therefore, to establish a stable, efficient, and robust system for generating human iPSCs, development of iPSCs technology with broad practical applications is warranted.

Here, we demonstrated that two inhibitors, DAPT and iDOT1L, significantly improved the efficiency of iPSC generation from human fibroblast cells, without altering p53 activity. During the reprogramming process, we showed that MET-related gene expressions were significantly modulated compared to those during conventional, non-additional chemical reprogramming conditions. This underscores that chemical combination reprogramming enables us to generate iPSCs using mRNA factors even under non-hypoxic conditions. Our study unraveled some of the mechanisms involved in the reprogramming process and suggested the possibility of cost and time reduction for the clinical application of tailor-made regenerative medicine.

2. Materials and methods

2.1. Ethics statement

Human fibroblast cells (D32) were collected from healthy donors after obtaining signed informed consent. The experiments were approved by the Institutional Review Board of the National Center for Child Health and Development (NCCHD) of Japan (permit number: #385). All the experiments using human cells and tissues were performed in accordance with the tenets of the Declaration of Helsinki.

The animal use protocol was approved by the Institutional Animal Care and Use Committee of NCCHD (Permit Number: A2003-002). All animal experiments were based on the three Rs (refined, reduced, and replaced), and all efforts were made to minimize animal discomfort and reduce the number of animals used.

2.2. Cell culture

BJ human foreskin fibroblast cells (BJ fibroblasts; Stemgent, Cambridge, MA, USA) were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 2 mM GlutaMAX, and 1% penicillin/streptomycin (all reagents from Thermo Fisher Scientific, Waltham, MA, USA). Human iPSCs were generated in our laboratory and routinely maintained in StemFlex Medium (Thermo Fisher Scientific) on vitronectin (Thermo Fisher Scientific) [11]. The medium was changed every other day except on the day after passage and cells were passaged approximately once per week using an enzyme (TrypLE Select Enzyme; Thermo Fisher Scientific).

2.3. iPSC reprogramming

BJ fibroblasts or human adult fibroblasts (D32 fibroblasts) were plated on laminin-511 at 7.5×10^4 cells per well in a six-well plate (Sumitomo Bakelite, Tokyo, Japan) 1 day prior to reprogramming in BJ fibroblast medium. On the day of reprogramming, the medium was replaced with NutriStem hPSC XF culture medium (Reprocell, Yokohama, Kanagawa, Japan). BJ fibroblasts at passage 15–17 were used in all experiments. As a conventional method of RNA reprogramming, cells were reprogrammed for 4 days using the Stemgent StemRNA 3rd Gen Reprogramming Kit with non-modified RNA of OCT4, NANOG, SOX2, KLF4, c-MYC, and LIN28 reprogramming factors (RNA-reprogramming kit; Reprocell) according to the manufacturer's instructions. Cells were maintained in a humidified atmosphere of 37 °C, 5% O_2 and 5% CO_2 culture conditions (hypoxia condition). From day 5 onward, the complete medium was replaced daily with NutriStem medium until the first cell passage, and then the medium was replaced with StemFlex medium.

In a combination of conventional RNA reprogramming with two small molecules, 5 μM DAPT (R&D Systems, Minneapolis, MN, USA) and 3 μM iDOT1L (Abcam, Cambridge, UK) were added to the RNA reprogramming kit from day 1. Cells in RNA reprogramming with DAPT and iDOT1L were maintained under the following incubator conditions: in a humidified atmosphere at 37 °C, 5% CO_2 in air (normoxia condition).

Live-cell staining with TRA1-60 was performed using the TRA1-60 Alexa Fluor™ 488 conjugate kit for live-cell imaging (Thermo Fisher Scientific) according to the manufacturer's instructions on day 10 following the first four transfections. The reprogramming efficiency was calculated by measuring the area of TRA1-60-positive iPS cell colonies using a BZ-X700 digital microscope and hybrid cell count software (Keyence, Osaka, Japan).

RNA reprogramming with DAPT and iDOT1L was used for single-cell iPSC derivation with a cell sorting protocol. All cells per well were collected on day 10 of reprogramming, and single-cell sorting was conducted using CELL SORTER SH800 (Sony, Tokyo, Japan). Each sorted single cell was distributed to each well of a 96-well plate (smilon) precoated with Laminin-511 (Nippi, Tokyo, Japan) in 100 μl of NutriStem medium, followed by the iPSC reprogramming protocol with 5 μM DAPT and 3 μM iDOT1L.

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from the cell pellet using a QuickGene RNA Cultured Cell Kit (KURABO, Osaka, Japan), and DNA was removed using DNase (Thermo Fisher Scientific). First-strand

complementary DNA (cDNA) was synthesized using SuperScript IV VILO (Thermo Fisher Scientific). Gene expression was analyzed using Qiagen RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany), which were commercially produced for simultaneous and reliable assessment of gene expression. Total RNA (100 ng) was used with a PCR array kit, and PCR was performed using the TaqMan method (TaqMan Gene Expression Master Mix, Thermo Fisher Scientific) in a QuantStudio 12 K Flex Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer's instructions. Threshold cycle values were normalized to those of the housekeeping genes actin-beta (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase 1 (HPRT1), and ribosomal protein large P0 (RPLP0), and translated to relative values. ACTB was used as the internal control. The fold changes were calculated using the values obtained by means of the $2^{-\Delta\Delta Ct}$ method at each time point.

Representative mRNA levels of EMT-related genes in mRNA-induced reprogrammed cells were assessed using the RT^2 Profiler PCR Array Human Epithelial to Mesenchymal Transition (#PAHS-0909ZA: Qiagen).

The following primers were used: ALPL (Hs00559643_m1), ATCB (Hs99999903_m1), CDKN1A (Hs00355782_m1), LIN28A (Hs04189307_g1), NANOG (Hs04260366_g1), PDGFRA (Hs00998018_m1), POU5F1 (Hs03005111_g1), and TP53 (Hs01034249_m1).

2.5. Flow cytometry analysis

One million cells were suspended in 100 μl Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific) with 10% FBS containing each specific antibody. Antibody concentrations were determined according to the manufacturer's instructions. To determine the surface marker concentrations, Brilliant Violet 421 (BV421)-conjugated antibodies against Tra-1-60 (Becton Dickinson, Mountain View, CA, USA) were used. As an isotype control, BV421 conjugated non-specific mouse IgM (Becton Dickinson) was used as the antibody. The cells were then incubated for 30 min at 4 °C, the cells were washed with DPBS containing 10% FBS, and then suspended in 500 μl of DPBS containing 10% FBS for further analysis. Cell fluorescence was determined using a flow cytometer (Attune Nxt; Thermo Fisher Scientific).

2.6. Embryoid body formation for the in vitro differentiation assay

Human iPSC colonies were washed with DPBS, and cells were collected using TrypLE Select enzyme (Thermo Fisher Scientific). Dissociated cells were seeded at 1×10^4 cells per well in 96-well round (U) bottom ultra-low attachment plates (Nunclon Sphera, Thermo Fisher Scientific) to form embryoid bodies (EBs) in DMEM/F12 based medium with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (all reagents from Thermo Fisher Scientific). The resulting EB cultures were maintained in 96-well plates for 7 days and then replated onto tissue culture dishes coated with 0.1% gelatin (Sigma) for further 7 days.

2.7. Immunofluorescence staining

Cells were cultured in a glass-bottom dish (AGC Techno Glass, Shizuoka, Japan) and fixed with 4% paraformaldehyde for 20 min at 4 °C before being permeabilization with 0.1% Triton X-100 (Sigma) for 10 min at 25 °C. After blocking with 5% normal goat serum in DPBS for 30 min at RT, samples were incubated with primary antibodies at 4 °C overnight. After washing with DPBS, the samples were incubated with secondary antibodies conjugated to Alexa 488 or 546 (Thermo Fisher Scientific) for 30 min at 25 °C. After washing with DPBS, mounting medium containing DAPI was used. The following primary antibodies were used: anti-βIII tubulin (TUJ1; G712A, Promega, Madison, Wisconsin, USA), α-smooth muscle cell actin (α-SMA; A2547, Sigma), SOX17 (MAB1924; R&D Systems), OCT3/4 (SC5729, Santa Cruz Biotechnology, Santa Cruz, California, USA), NANOG (RCAB004P–F, ReproCELL, Kanagawa, Japan), SSEA4 (MAB4304, Merck Millipore, Billerica, Massachusetts, USA), and TRA-1-60 (MAB4360, Merck Millipore). Images were acquired with a BZ-X700 microscope (Keyence). All antibodies, except for the anti-TUJ1 antibody (1:300), were used at a 1:150 dilution in 5% normal goat serum.

2.8. Western blotting

Cells were washed with 4 °C DPBS and lysed with lysis buffer containing protease inhibitors. After addition of lysis buffer, lysates were sonicated, and supernatants were heated to 95 °C for 5 min. Then, 10 μg of proteins were resolved using SDS-PAGE on Mini-PROTEAN TGX Gels 4%–15% (#4561083, Bio-Rad Laboratories, Hercules, CA, USA), transferred to Immobilon-P Transfer Membrane 0.45 μm (IPVH00010, Merck Millipore). Following blocking with 1% skimmed milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 30 min at room temperature, membranes were incubated with specific primary antibodies diluted in TBS-T with 1% skimmed milk at 4 °C overnight. Membranes were washed three times with TBS-T containing 1% skimmed milk and incubated for 1 h at room temperature with the corresponding horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2,000, ab205718, Abcam). Finally, the antibody–antigen complex was visualized using the ECL Select western blotting Detection Reagent (RPN2235, Cytiva, Tokyo, Japan). Rabbit polyclonal antibody (pAb) against p21 and p53 (1:1,000, 10355-1-AP, 21891-1-AP, Proteintech, Chicago, IL, USA), rabbit pAb against histone H3 di methyl K79 (1:4,000, ab3594, Abcam), rabbit monoclonal antibody (mAb) against cleaved Notch1 (1:1,000, 4147, Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal antibody against beta actin (1:10,000, 66009-1-Ig, Proteintech) were used.

2.9. Alkaline phosphatase (ALP) staining

Well-grown human iPS cells on culture plates were fixed with 4% paraformaldehyde for 20 min at 4 °C. Fixed cells were stained with the BCIP/NBT substrate system (Sigma) according to the manufacturer's instructions. Images were acquired using a BZ-X700 microscope (Keyence).

2.10. Statistical analysis

The values shown in the graphs are expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical analysis for comparison between groups was performed using paired Student's *t*-test. Differences were considered statistically significant at a value of $P < 0.05$.

3. Results

3.1. DAPT and iDOT1L promote the generation of TRA1-60-positive colonies through reprogramming of human fibroblast cells

Our previous study on iPSC reprogramming demonstrated that additional supplementation with the Notch signaling (DAPT) and the histone methyltransferase inhibitors (iDOT1L) promoted the reprogramming of human keratinocytes using retrovirus vectors with only OCT4 and SOX2, rendering c-MYC and KLF4 dispensable [8]. However, the efficiency of iPSC reprogramming varies between

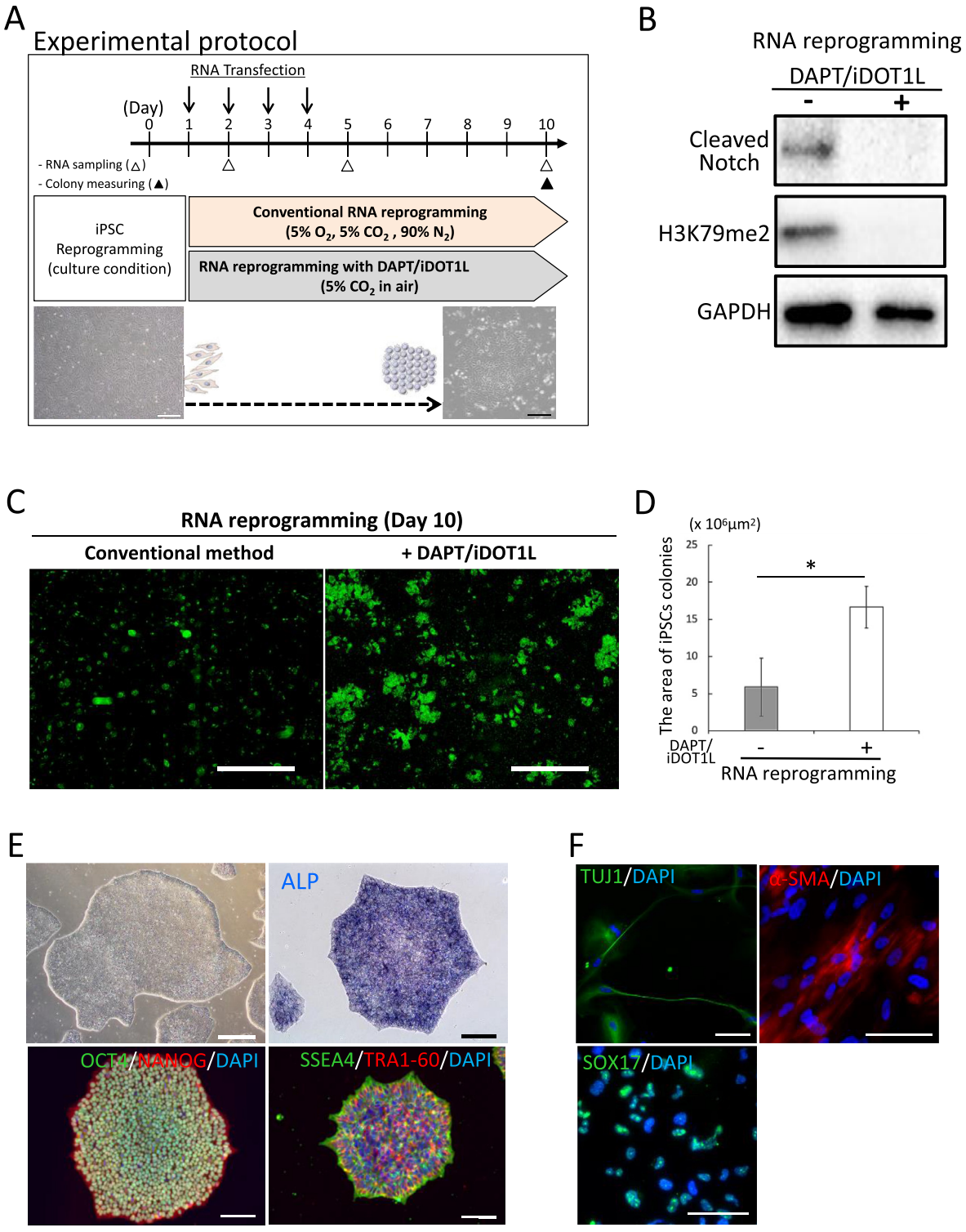

Fig. 1. Characterization of human iPS cells using RNA reprogramming with NOTCH and DOT1L inhibitors. (A) Experimental design of the study. Human fibroblasts were reprogrammed over 4 days using the Stemgent StemRNA 3rd Gen Reprogramming Kit with non-modified RNAs of OCT4, NANOG, SOX2, KLF4, c-MYC, and LIN28 reprogramming factors. Conventional RNA reprogramming was performed under hypoxic conditions. RNA reprogramming with NOTCH and DOT1L inhibitors involved treatment with DAPT and iTOT1L from day 1 to first cell passage under normoxia conditions. White scale bar = 500 μm. Black scale bar = 100 μm. (B) Western blotting analysis. Cells subjected to RNA reprogramming with DAPT and iDOT1L showed low expression of cleaved-Notch and H3K79 me2. (C) TRA1-60 positive areas (green) were measured automatically under the microscope at Day 10 via TRA1-60 live staining. Scale bar = 5 mm. (D) The DAPT/iDOT1L treated cells (white bar) showed significantly increased TRA1-60 positive area compared with cells subjected to conventional RNA reprogramming (gray) as a control. Bars represent mean ± standard error, n = 3 independent experiments. Statistical significance was identified using Student's *t*-test (*$P < 0.05$). (E) Representative images of iPS cells derived through RNA reprogramming with two inhibitors. Upper left; Cells are stably maintained under feeder-free conditions. Scale bar = 500 μm. Upper right; Alkaline phosphatase (ALP) staining of iPS cells by the two inhibitors. Scale bar = 100 μm. Bottom; iPS cells were positively stained for OCT4, NANOG, and SSEA4. Scale bar = 100 μm. (F) Differentiation of iPSCs was performed *in vitro* via EBs into the three germ layers *in vitro* via EBs expressed markers of the three germ layers. Immunohistochemical analyses of markers of the ectoderm (TUJ1, green), mesoderm (αSMA, red), and endoderm (SOX17, green) layers are shown. Scale bar = 100 μm.

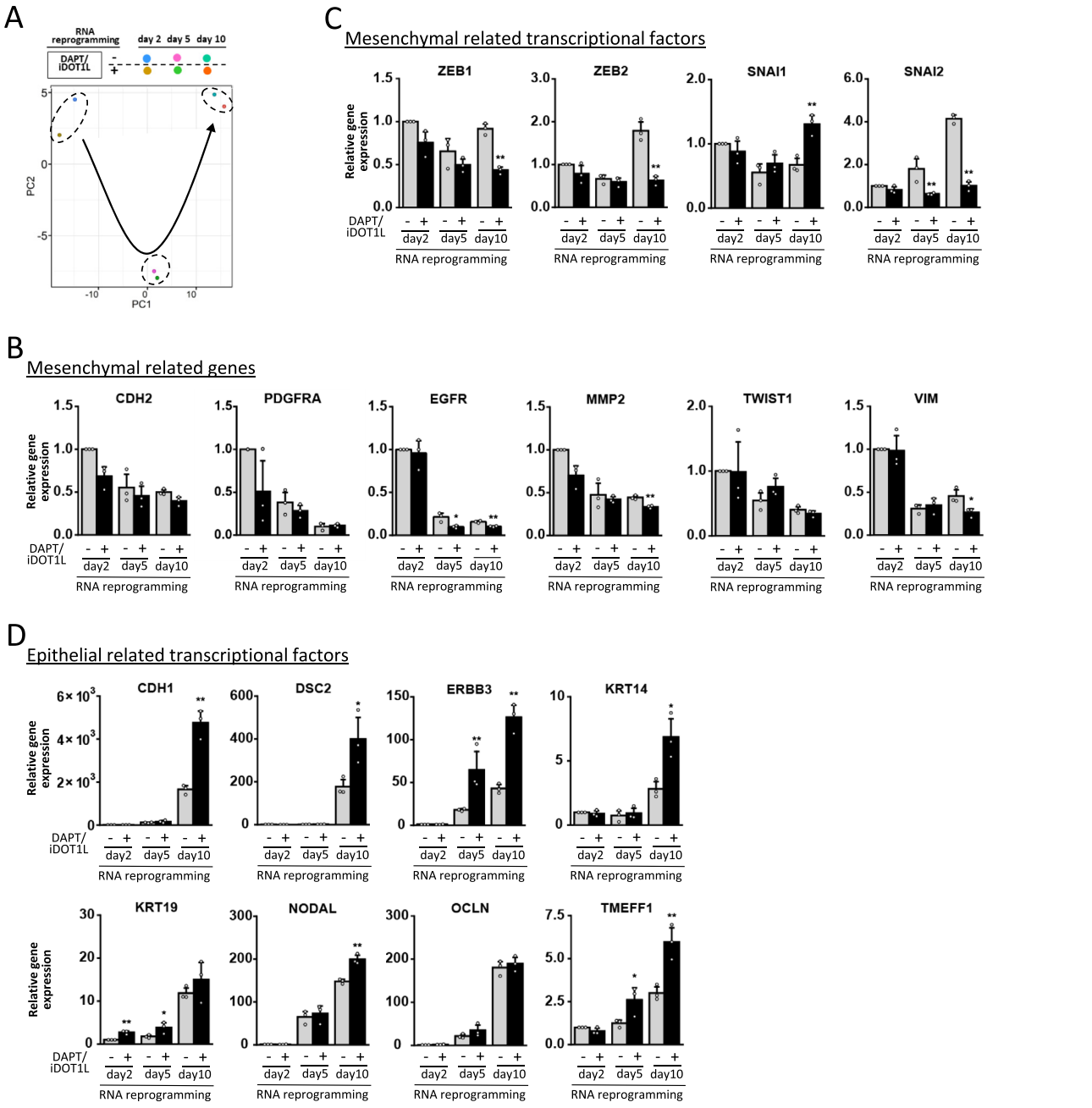

Fig. 2. Gene expression levels of the mesenchymal-epithelial transition (MET) in RNA reprogramming cells with or without DAPT and iDOT1L. (A) The sequential changes in mRNA expression of MET-related genes during the DAPT/iDOT1L-RNA reprogramming using qRT-PCR for 84 MET-related genes. Principal component analysis (PCA) showed the correlation of RNA reprogramming with BJ fibroblasts along with their reverting to pluripotency. (B–D) The expression levels of representative mesenchymal and epithelial markers were compared. The average expression levels and standard deviations of each gene in the conventional RNA reprogramming cells (gray columns) and the DAPT/iDOT1L-RNA reprogramming cells (black columns) are indicated. The data are reported as the mean ± SE. Statistically significant differences were identified between conventional RNA reprogramming cells and DAPT/iDOT1L-RNA reprogramming cells between each time point using Student's t-test (n = 3). *P < 0.05, **P < 0.01. GAPDH was used as an internal control. Relative gene expression to conventional RNA reprogramming cells at day 2.

cells and depends on cellular characteristics such as epigenetic state and virus transduction efficiency. RNA reprogramming is an attractive approach characterized by non-integration of the genome and is vector-free but requires hypoxic culture conditions and multiple RNA reprogramming inductions, resulting in reduced robustness. We evaluated the reprogramming efficiency of human BJ fibroblasts using RNA reprogramming with two inhibitors under physiological oxygen conditions. We used an mRNA transfection system containing OCT4, SOX2, KLF4, c-MYC, NANOG, and LIN28 as the reprogramming factors (Fig. 1A for an overview of the reprogramming schedule). First, we confirmed that the two inhibitors DAPT and iDOT1L functioned well in the protocol. Western blotting analysis showed that reprogrammed cells treated with the two chemicals expressed dramatically low levels of cleaved-Notch and H3K79 me2, which are downstream products of γ-secretase and DOT1L, respectively (Fig. 1B, Supplementary Fig. 1a). To measure reprogramming efficiency, a major pluripotent marker, TRA1-60, was measured automatically under a microscope. The DAPT/iDOT1L-treated cells showed a significantly increased TRA1-60 positive area compared to conventional RNA reprogramming trials as a control (Fig. 1C and D). Picked TRA1-60 positive cells upon RNA reprogramming with DAPT and iDOT1L confirmed stable

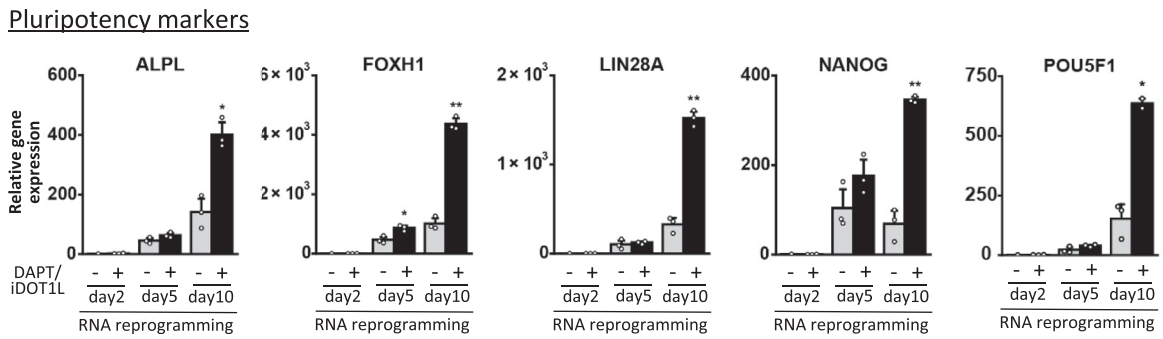

Fig. 3. Gene expression levels of pluripotency markers in RNA reprogramming cells with or without DAPT and iDOT1L. The sequential changes in mRNA expression of pluripotency markers during DAPT/iDOT1L-RNA reprogramming. The average expression levels and standard deviations of each gene in the conventional RNA reprogramming cells (gray columns) and the DAPT/iDOT1L-RNA reprogramming cells (black columns) are indicated. The data are reported as the mean ± SE. Statistically significant differences were identified between conventional RNA reprogramming cells and DAPT/iDOT1L-RNA reprogramming cells between each time point using Student's t-test (n = 3). *P < 0.05, **P < 0.01. GAPDH was used as an internal control. Relative gene expression to conventional RNA reprogramming cells at day 2.

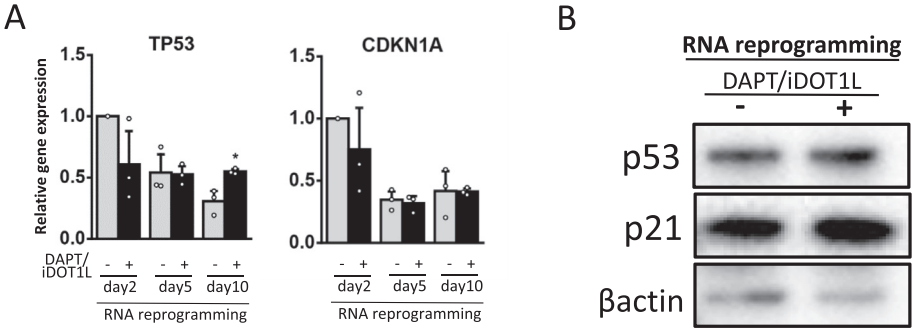

Fig. 4. Expression of p53 and p21 during the reprogramming process examined using qRT-PCR and western blotting analysis for BJ fibroblasts. (A) In the DAPT/iDOT1L method, the mRNA expression of TP53 (p53) and CDKN1A (p21) were examined in the early reprogramming. The data are reported as the mean ± SE. Statistically significant differences were identified between conventional RNA reprogramming cells and DAPT/iDOT1L-RNA reprogramming cells between each time point using Student's t-test (n = 3). *P < 0.05, **P < 0.01. GAPDH was used as an internal control. Relative gene expression to conventional RNA reprogramming cells at day 2. (B) Western blotting analysis showed p53 and p21 in protein levels in the conventional method and DAPT/iDOT1L RNA reprogramming cells.

pluripotent stem cell lines. A representative BJ iPSCs line showed typical human pluripotent stem cell characteristics (Fig. 1E and F). Embryoid body (EB) differentiation assay revealed that three germline markers (TUJ1: ectoderm, α-SMA: mesoderm, SOX17: endoderm) were expressed (Fig. 1F).

3.2. DAPT and iDOT1L promote mesenchymal-epithelial transition and accelerate the acquirement of pluripotency by BJ fibroblasts

Mesenchymal-epithelial transition (MET) is a critical, early event during somatic cell reprogramming [3,12,13]. To elucidate the reprogramming mechanism at the molecular level, we examined the sequential changes in the mRNA expressions of MET-related genes during DAPT/iDOT1L-RNA reprogramming using a PCR array system. We performed principal component analysis (PCA) of qRT-PCR for 84 MET-related genes. PCA showed a correlation between RNA reprogramming with BJ fibroblasts and reverting to pluripotency (Fig. 2A). Heat map was also generated to identify MET-related genes analyzed throughout the reprogramming (Supplementary Fig. 2). Expressions of mesenchymal-related genes such as *CDH2*, *EGFR*, *MMP2*, *TWIST1*, and *VIM* were gradually downregulated in both RNA reprogramming protocols (Fig. 2B). The expressions of some of the core MET-associated transcriptional regulators, such as *ZEB1*, *ZEB2*, *SNAI1*, and *SNAI2* were not fully downregulated and were expressed at significantly lower levels during DAPT/iDOT1L-RNA reprogramming, except for *SNAI1* (Fig. 2C). This expression pattern of *SNAI1* and *SNAI2* in hiPSCs is similar to that reported in previous studies; SNAI1 is highly expressed in hiPSCs, but SNAI2 expression is very low in hiPSCs [14]. ZEB1 and ZEB2, known as E-cadherin repressors, were expressed at significantly lower levels during DAPT/iDOT1L-RNA reprogramming at day 10 than during conventional RNA reprogramming. The array PCR data also showed that expressions of important epithelial-related genes such as *CDH1*, *DSC2*, *ERBB3*, *KRT14*, *NODAL*, and *TMEFF1* were significantly higher during DAPT/iDOT1L-RNA reprogramming cells on day 10 than during conventional RNA reprogramming (Fig. 2D). Moreover, the expressions of pluripotency marker genes, *ALPL*, *FOXH1*, *LIN28*, *NANOG*, and *POU5F1* were significantly increased during DAPT/iDOT1L-RNA reprogramming cells at day 10 (Fig. 3A). These sequential data suggest that the DAPT/iDOT1L method promotes mesenchymal–epithelial transition following the MET trajectory and accelerates the acquirement of pluripotency by BJ fibroblasts.

3.3. DAPT/iDOT1L did not downregulate the expression of p53 and p21 mRNAs and proteins

Several studies have reported that forcibly expressing pluripotent factors induces the overexpression of p53, a tumor suppressor gene, and p53 induces cell apoptosis or expression of p21, a cell cycle-related gene. This process inhibits iPSC reprogramming [15]. Although the inhibition of p53 dramatically increases iPSCs reprogramming efficiency, p53 inhibition causes accumulation of DNA damages and results in DNA mutations [16]. To investigate whether DAPT/iDOT1L inhibition caused the expression of p53 and p21, thereby affecting the reprogramming process, we conducted

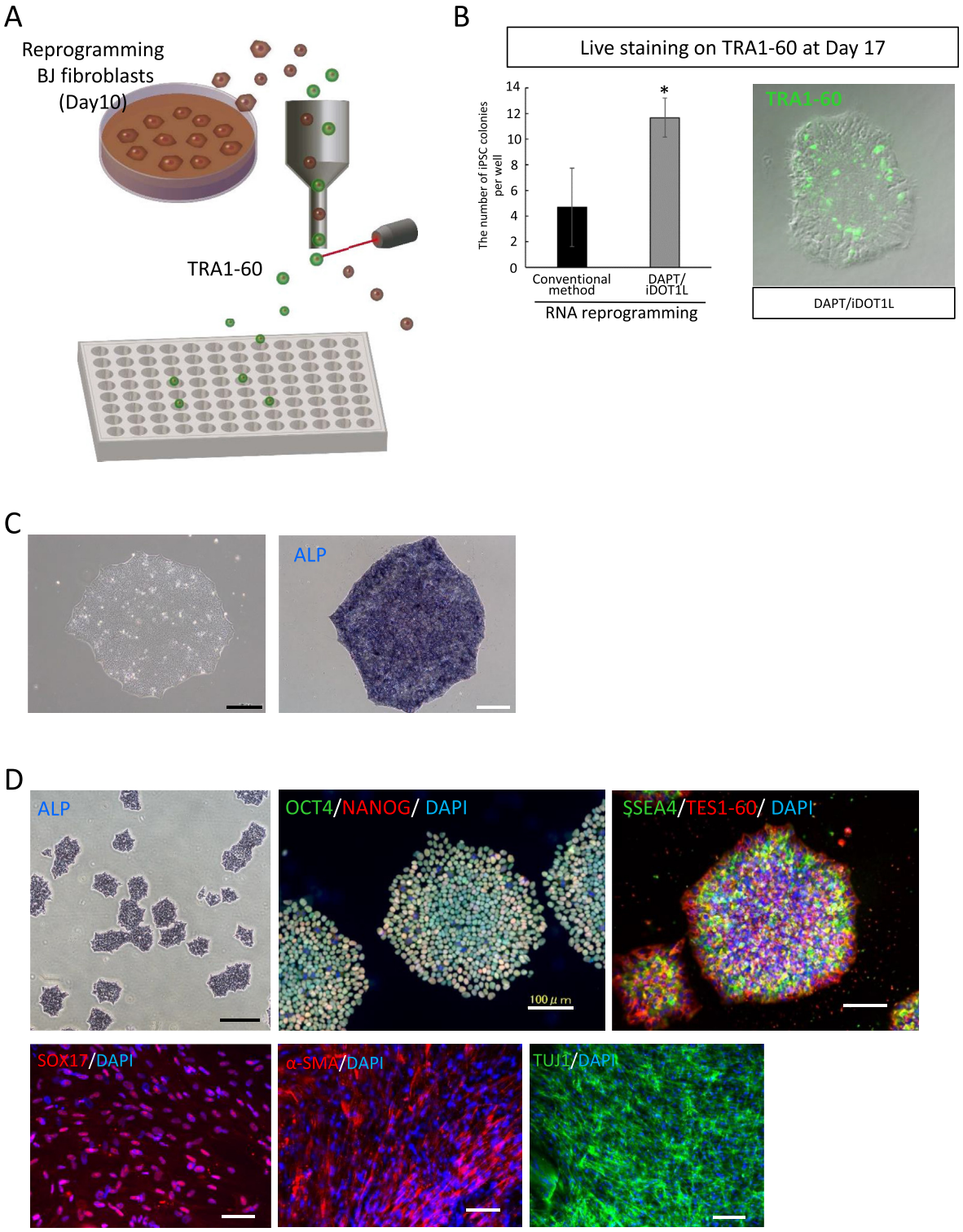

Fig. 5. Single-cell sorting for RNA reprogramming of human somatic cells using DAPT/iDOT1L. (A) Experimental design of single cell sorting of BJ fibroblasts for RNA reprogramming with DAPT/iDOT1L. BJ fibroblasts were collected at day 10 of RNA reprogramming under feeder-free condition and then sorted using TRA1-60 into each well of three 96 well plates. Following further 7 days of culture, we counted the number of TRA1-60 positive iPSC colonies. (B) The number of iPS colonies was significantly increased in DAPT/iDOT1L treated cells. Left: Bars represent mean ± standard error, n = 3 independent experiments. Statistical significance was identified using Student's *t*-test (*$P < 0.05$). Right: Representative reprogrammed colony positively expressing TRA1-60. (C) We randomly selected TRA1-60 positive cells followed by single cell sorting and stably expanded iPS cells from BJ fibroblasts. (D) iPS cells were newly generated from human primary adult fibroblasts following the DAPT/iDOT1L RNA reprogramming protocol in a single cell sorting system. Upper: The sorted cells showed that pluripotency markers such as ALP, OCT4, NANOG, SSEA4, and TRA1-60 were present. Black scale bar = 500 μm. White scale bar = 100 μm. Lower: Differential markers as SOX17 (endoderm), α-SMA (mesoderm), and TUJ1 (ectoderm) in EB differentiation assay were observed. In RNA reprogramming with DAPT/iDOT1L, single cell sorting without feeder cells enabled us to obtain stable human iPS cell lines. Scale bar = 100 μm.

qRT-PCR and western blotting analyses for BJ fibroblasts along with RNA reprogramming. In the DAPT/iDOT1L method, the mRNA expression levels of *TP53* (p53) and *CDKN1A* (p21) did not decrease during early reprogramming (Fig. 4A). Western blotting analysis showed that p53 and p21 protein levels did not change compared to those during the conventional method (Fig. 4B, Supplementary Fig. 1b). Therefore, DAPT/iDOT1L promoted reprogramming efficiency in BJ fibroblasts but not via p53 or p21 suppression. In summary, these results show that double inhibition of NOTCH/DOT1L promotes MET independent of the p53 or p21 pathway.

3.4. Single cell sorting could be used for RNA reprogramming of human somatic cells using DAPT/iDOT1L

A reliable and robust procedure for human iPS cell generation includes a fundamental platform for high-throughput application of hiPSCs in disease modeling, drug screening, and personalized medicine. We verified the DAPT/iDOT1L RNA reprogramming protocol using a single-cell sorting system (Fig. 5A). To verify this approach to stably generate iPS cells, we used BJ fibroblasts as a donor resource for RNA reprogramming. BJ fibroblasts were collected at day 10 of RNA reprogramming under feeder-free conditions, and a single cell was sorted using TRA1-60 into each well of three 96-well plates. After 7 days of culture, we counted the number of TRA1-60 positive iPSC colonies (Fig. 5B). The number of iPS colonies was significantly increased in DAPT/iDOT1L-treated cells (Fig. 5B). We randomly selected TRA1-60-positive cells followed by single-cell sorting and stable expansion of ALP-positive cells (Fig. 5C). To verify that this procedure is independent of a certain cell line, we used human primary adult fibroblasts derived from our laboratory with IRB permission. Adult human fibroblasts were reprogrammed using the DAPT/iDOT1L RNA reprogramming protocol in a single-cell sorting system. We successfully derived iPS cells stably, and a representative cell line was examined for pluripotency markers and differentiation ability. Newly derived cells showed positive staining for pluripotential markers such as ALP, OCT4, NANOG, SSEA4, and TRA1-60 (Fig. 5D). Differential markers such as SOX17 (endoderm), α-SMA (mesoderm), and TUJ1 (ectoderm) were confirmed through the EB differentiation assay (Fig. 5D). For RNA reprogramming with DAPT/iDOT1L, single-cell sorting without feeder cells enabled us to obtain a stable human iPS cell line.

In summary, RNA reprogramming with DAPT/iDOT1L can accelerate reprogramming of human fibroblast cells without decreasing p53 expression, and we showed that this protocol is an efficient and robust system for human iPS cell derivation. In addition, RNA reprogramming with DAPT/iDOT1L can be efficiently used to generate iPS cell lines through a single-cell sorting system under feeder-free conditions. This efficient and robust condition for generating human iPS cells could be a beneficial technical platform for the broad practical applications of hiPSCs.

4. Discussion

For the practical application of human iPS cells, it is crucial to have robust and efficient derivation conditions that do not employ viral or DNA-based reprogramming delivery vectors, even under feeder-free systems. In this study, we used RNA reprogramming technology to enable safer delivery of reprogramming factors into cells compared with other protocols such as episomal DNA and Sendai virus vectors [17]; however, there still exist some laborious points such as multiple RNA transfections and culturing under low-oxygen conditions during RNA reprogramming of human somatic cells.

We previously reported that chemical inhibition of the NOTCH signaling pathway and a DOT1 L inhibitor (DAPT/iDOT1L) promoted the generation of hiPSCs from human keratinocytes under retrovirus transduction of reprogramming factors [8]. Here, we applied two inhibitors, DAPT/iDOT1L, for RNA reprogramming, and found that this approach significantly increased hiPSC reprogramming efficiency in human fibroblasts even under normoxic culture conditions.

Accumulating evidence indicates that MET is essential during early somatic cell reprogramming [12,13,18]. Principal component analysis (PCA) was used to map the samples of the array PCR analysis of MET-related genes in a trajectory that reflected the progression of reprogramming to iPS cells from BJ fibroblasts during conventional RNA and DAPT/iDOT1L-RNA reprogramming. Expressions of epithelial-related genes such as CDH1, DSC2, ERBB3, KRT14, NODAL, and TMEFF1 were significantly upregulated in the samples subjected to DAPT/iDOT1L-RNA reprogramming compared to those subjected to conventional RNA reprogramming at day 10, which implies an accelerated MET process. This tendency was confirmed through qRT-PCR analysis of pluripotency markers. ALPL, FOXH1, LIN28A, NANOG, and POU5F1 expressions were significantly upregulated in the samples subjected to DAPT/iDOT1L-RNA reprogramming compared to those subjected to conventional RNA reprogramming. It is well known that epigenetic status dynamically changes in somatic cells during reprogramming, including DNA methylation and nuclear histone modifications [3,19]. MET events are also epigenetically controlled. Inhibition of chromatin H3K79 methyltransferase DOT1L downregulates mesenchymal gene expression and enhances reprogramming efficiency in human fibroblasts [6,20]. Furthermore, our previous study demonstrated that Notch signaling inhibition increases the reprogramming efficacy of human iPS cells from keratinocytes, and a combination of DOT1L and NOTCH signaling inhibition further improves the efficiency of iPS reprogramming [8]. Here, we found that NOTCH/DOT1L inhibition markedly increased iPSC colony generation from human fibroblasts through RNA reprogramming under normoxic conditions. It is important to note that RNA reprogramming with NOTCH/DOT1L inhibition promoted the reprogramming of human fibroblasts in a p53 tumor suppressor protein-independent manner. It has been reported that human iPSCs reprogramming suppresses p53 activity [16,21], which in turn results in the accumulation of genetic mutations in derived iPS cells [22].

5. Conclusion

Based on this robust and efficient reprogramming protocol, iPSCs were successfully generated from human adult fibroblasts using a single-cell sorting system. This provides a user-friendly iPS reprogramming platform, which is not necessary for skillful manual picking of iPS cell colonies under a microscope, based on the evaluation of colony morphology by well-trained personnel.

Overall, the combined chemical inhibition of NOTCH and DOT1L provides a robust approach to boost the reprogramming potential of human fibroblasts. Our study paves the way for expanding the practical application of personalized iPS cells in an efficient and safe manner. This would be suitable for disease modeling and drug screening studies.

Declaration of competing interest

The authors have no conflict of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.09.002.

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