Video Article

Generation of Induced Pluripotent Stem Cells from Muscular Dystrophy Patients: Efficient Integration-free Reprogramming of Urine Derived Cells

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Abstract

Dystrophic cardiomyopathy is a poorly understood consequence of muscular dystrophy. Generating induced Pluripotent Stem Cells (iPSCs) from patients with muscular dystrophy is an invaluable cellular source for *in vitro* disease model systems and can be used for drug screening studies. Patient-derived urine cells have been used in successful reprogramming into induced pluripotent stem cells in order to model dystrophic cardiomyopathy[1]. Addressing the safety concerns of integrating vector systems, we present a protocol using a non-integrating Sendai virus vector for transduction of Yamanaka factors into urine cells collected from patients with muscular dystrophy. This protocol generates fully reprogrammed clones within 2–3 weeks. The pluripotent cells are vector-free by passage-13. These dystrophic iPSCs can be differentiated into cardiomyocytes and used either to study disease mechanisms or for drug screening.

Video Link

The video component of this article can be found at https://www.jove.com/video/52032/

Introduction

Cardiomyopathy is the second leading cause of death in patients with Duchenne and Becker muscular dystrophy (MD). Although mutations in the X-linked dystrophin gene occur in 1:3,500 male births, very little is known about the molecular and cellular events leading to progressive cardiac muscle damage. Human induced pluripotent stem cells derived from muscular dystrophy patients have emerged as a novel tool to study the underlying disease mechanisms and to use for drug screening[1,2].

The anticipated discomfort of skin biopsies or blood samples may dissuade young patients and/or their guardians to give consent for study participation. Urine samples are a non-invasive source of somatic cells that are amendable to reprogramming methods. We have recently shown that urine cells collected from muscular dystrophy patients may be cultured and efficiently reprogrammed into iPSCs using retroviral transduction with the Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc; OSKM)[1]. The disadvantage of retroviral gene delivery is the random integration of the reprogramming genes into the host chromosomes. To overcome this limitation, we have used the non-integrating Sendai virus for urine cell reprogramming.

This protocol details the Sendai virus reprogramming of isolated urine cells from muscular dystrophy patients which can then be differentiated into cardiomyocytes or other cell types for further study. This protocol can also be adapted for other patient specific diseases.

Protocol

NOTE: Patients and/or their guardians should give informed consent to participate in an Institutional Review Board approved study.

1. Buffers and Media Preparations

1. Washing buffer: To prepare 100 ml of washing buffer, add 1 ml of 100x pen/strep Solution (100 U/ml penicillin + 100 µg/ml of Streptomycin) to 99 ml of phosphate buffer saline (PBS).
2. Urinary Progenitor Cell (UPC) Medium: To prepare UPC medium, mix equal volumes of Keratinocyte Serum Free (KSF) Medium + Progenitor Cell Medium.
    1. KSF Medium: To prepare keratinocyte medium, add 5 ng/ml of epidermal growth factor (EGF), 50 ng/ml of bovine pituitary extract (BPE), 30 ng/ml of cholera toxin, and pen/strep solution (100 U/ml penicillin, 100 µg/ml streptomycin) to 500 ml KSF medium.

2. Progenitor Cell Medium: Add three quarters of DMEM with one quarter of Ham-F12 media and supplement with 10% FBS, 0.4 µg/ml hydrocortisone, 0.1 nM cholera toxin, 5 ng/ml insulin, 1.8 x 10^{-4} M adenine, 5 µg/ml transferrin, 2 x 10^{-9} M triiodo thyronine, 10 ng/ml EGF, and pen/strep solution.

3. hES Medium: Add 20% KO-serum replacement (K-SR), 1x MEM non-essential amino acids, 2 mM l-glutamine, 100 µM β-mercaptoethanol, 20 ng/ml bFGF and pen/strep to 400 ml DMEM/F12 medium.

2. Urine Sample Collection

1. Instruct the patients to drink fluids 30 min prior to urine collection to ensure that an adequate amount (~30–40 ml) of urine can be collected.
2. Give the patients and/or their guardians a urine collection kit containing the following items: (1) written instructions on how to obtain sterile or clean catch urine sample, (2) moist anti-bacterial toilettes, and (3) a 100 ml sterile specimen collection cup. Instruct patients to collect sample.
3. Immediately place the urine samples on ice and transfer to the laboratory in a cooler. Process the urine for cell isolation immediately. If a delay is unavoidable, store the specimen on ice for up to 4 hr with minimal loss of cell viability.

3. Isolation and Expansion of Urine Cells

NOTE: Perform the following steps under sterile conditions in a BSL2 Biological Safety Cabinet.

1. Using sterile pipettes, transfer urine samples to sterile 50-ml centrifuge tubes. Centrifuge at 400 × g for 10 min at RT.
2. Aspirate the supernatant leaving 1 ml in the tube; be careful not to disturb the cell pellet.
3. Resuspend and combine the pellets from multiple tubes into 7 ml of washing buffer and repeat centrifugation at 400 × g for 10 min at RT.
4. Carefully aspirate the supernatant, leaving the cell pellet and ~0.2–0.5 ml of supernatant. Resuspend in 2–3 ml of Urinary Progenitor Cell (UPC) medium and transfer the suspension into 4–6 wells of an uncoated 24-well plate.
5. After 72 hr, supplement the culture with 0.5 ml of fresh UPC medium per well and change medium every 2–3 days after. The erythrocytes and squamous cells that do not attach to the plate will be removed with the medium changes.
6. Monitor culture after 4–6 days.
   NOTE: Small 2–4 cell colonies will start to appear. Cells will be rounded or elongated which represent type I or type II of renal epithelial (RE) cells respectively[3].
7. Change the medium every 2–3 days since once the cells appear, they will undergo a rapid expansion phase.
8. Once the cells reach 80–90% confluence, around 9–15 days after plating, dissociate and passage the cells (15,000–18,000 cells/cm^2) onto 2–4 wells of 24 well plate for further expansion. Mark this as cell passage 1 (P1).
9. Characterize urine cells for lineage specifications through flow cytometric analysis, immunohistochemical staining or through reverse transcriptase-polymerase chain reaction (RT-PCR).
10. Reprogram isolated urine cells (section 4) or freeze them down in Freezing Medium (DMEM supplemented with 10% serum and 10% DMSO) for long-term cryostorage.

4. Urine Cell Reprogramming Using Sendai Virus

NOTE: The proper handling and the use of PPE (personal protective equipment) is recommended while manipulating the transfecting agents. Perform the following steps under sterile conditions in a BSL2 Biological Safety Cabinet. The proper disposal of transfecting agent and/or transfected cells is recommended to avoid risk of environmental and health hazards. For urine cell reprogramming, use a Sendai Reprogramming Kit with modifications to the manufacturer's feeder-dependent protocol as detailed below.

1. To ensure high efficiency reprogramming using the Sendai virus, use passages 1–5 of urine cells that are rapidly dividing. If the cells do not replicate well or become senescent, discard them as they will not reprogram efficiently.
2. Seed 60,000 cells per well in two wells of a 6 well plate. Mark this as Day -2. Adjust cell seeding density (5 x 10^4 – 9 x 10^4 cells per well) as needed to reach 80–90% confluence by Day 0.
3. On Day 0 (48 hr after plating the cells), prepare the Sendai reprogramming vectors (SeV) containing each of the four OSKM factors. Add each of the vectors to 1 ml of pre-warmed UPC medium. Use an MOI of 1–1.5 (5–7.5 x 10^5 CIU), which is sufficient for reprogramming urine cells. Aspirate the medium and slowly add the 1 ml UPC + SeV to one well of the urine cells. Use the second well as the transduction negative control.
4. On Day 1, replace the UPC + SeV medium with fresh UPC medium. Some cell death is seen after 24 h due to viral cytotoxicity.
5. Depending on efficiency of reprogrammed clone formations and the compact morphology, keep changing the medium daily until day 6 or 7.
6. One day prior to dissociation of transduced cells (day 5 or 6), prepare MEF feeder plates by plating Mitomycin-C (10 µg/ml for 3 hr) treated-MEFs at density of 5 x 10^4 cells/cm^2, on 0.1% gelatin coated 100 mm^2 culture dishes.
7. Next day (day 6 or 7), dissociate the cells with 0.25% trypsin, resuspend the cells in UPC medium and plate 5 x 10^4 – 2 x 10^5 cells/100 mm^2 MEF feeder plate.
8. The following day, switch to hES medium and change the medium every day. Observe the cells under a microscope to monitor transformed cells.
   NOTE: The cells form clonal aggregates with characteristic cobblestone morphology and having higher nucleus to cytoplasmic ratio (12–18 days post transduction).
9. Within two to three weeks after transduction, pick colonies and transfer to new plates for clonal expansion.
10. Perform live cell staining for selecting iPSC clones by incubating cells with TRA-1-81 antibody for 1 hr at 37 °C in CO_2 incubator followed by counter staining with specific florescent dye conjugated secondary antibody for 1 hr at 37 °C in CO_2 incubator.
11. Use a 26 G 1½ inch needle to cross-hatch the larger TRA-1-81 positive iPSC clones into small equal sized pieces (4–6 pieces per clone).
12. Use a sterile pipette-tip to pick and transfer 10–20 cross-hatched pieces from multiple clones into each well of Matrigel (10 µg/cm^2)-coated 24 well plate with hES medium.

NOTE: Pieces from a single clone plated individually into a single well do not expand or maintain pluripotency.

13. Change from hES to iPSC medium 24–48 hr after the reprogrammed clones are attached to the Matrigel surface.
14. During maintenance on Matrigel-coated plates, manually scrape-off any differentiated cells or contaminating MEF feeder cells using a pipette-tip to enrich for fully reprogrammed and pluripotent dystrophic iPSC (MD-iPSC) clones.
15. After individual clones have reached ~100 µm size, expand the clones by dissociating with 0.48 mM EDTA (ethylenediaminetetraacetic acid) solution for 3 min and plating small cell aggregates of MD-iPSCs suspended in iPSC medium (supplemented with 5 µM Y27632, RhO Kinase inhibitor) on fresh matrigel (10 µg/cm^2) coated 12 well plate. Change iPSC medium daily.
16. Full reprogramming of each clone can be determined by both gene expression analysis of OSKM genes and immunohistochemical staining of pluripotency markers (Oct3/4 and TRA-1-81). A rigorous evaluation of pluripotency potential should be employed to confirm fully reprogrammed iPSC lines can differentiate into three germ layers. This can be accomplished either by embryoid body (EB) formation and differentiation assay or a teratoma formation assay.

Representative Results

Most progenitor cells isolated from human urine are positive for uroepithelial progenitor and pericyte markers such as CD44, CD73, and CD146 (97.37%, 97.09%, and 97.3% respectively; **Figure 1A** and **1B**). These cells also expressed other mesenchymal markers such as alpha-smooth muscle actin and vimentin (**Figure 1B**). RT-PCR analysis gives evidence of a mixed population of cells in the cultures in that there is weak expression of Cytokeratin-7 (CK-7) and Uroplakin (UP)-Ia & -IIIa, markers of uroepithelial lineage (**Figure 1C**).

The reprogramming steps are summarized by schematic in **Figure 2A**. Representative images demonstrate the morphology of the cells during different time points throughout the protocol (**Figure 2B**). The urine cells transform from elongated, type II cells (Day 0) into a cobblestone pattern (Day 4) during reprogramming. By Day 12, typical pluripotent clones (iPSC) are seen and are able to maintain their pluripotent morphology under feeder free conditions (iPSC-P2). Live cell staining for TRA-1-81 identifies reprogrammed clones (**Figure 2C**).

To confirm the generation of vector and transgene free pluripotent clones, RT-PCR is performed using primers against the SeV viral genome and each of the exogenous OSKM factors. The up-regulation of endogenous reprogramming factors can also be confirmed at this step. The exogenous gene expression is no longer detected by passage-13, but the up-regulation of endogenous factors is seen (**Figure 3A**). Immunofluorescent staining confirms pluripotency marker expression on iPSCs (Oct3/4 and TRA-1-81; **Figure 3B**). The endogenous reprogramming gene expression seen in urine cells may lead these cells to reprogram at higher efficiencies[1], as compared to other somatic cell sources. The expression of dystrophin gene and protein is verified by immunofluorescence staining, western blot (WB) and RT-PCR analyses (**Figure 3C-E**). Immunofluorescent staining of wild type UCs and iPSCs for dystrophin demonstrate evident nuclear localization[11] in iPSCs, but very few UCs expressed positive (**Figure 3C**). To verify dystrophin expression in the cells, immunoblot analysis for dystrophin revealed that the ubiquitous dystrophin isoform (Dp71) is the predominant isoform produced in wild type iPSCs, while dystrophic iPSCs lacking dystrophin gene expression has undetectable Dp71 expression (**Figure 3D**). The exon deletion mutations of dystrophin can be confirmed in the dystrophic iPSCs using specific primers flanking the deleted exons. No PCR amplification is detected in the dystrophic iPSCs whereas wild type iPSCs demonstrate dystrophin transcript amplification (**Figure 3E**).

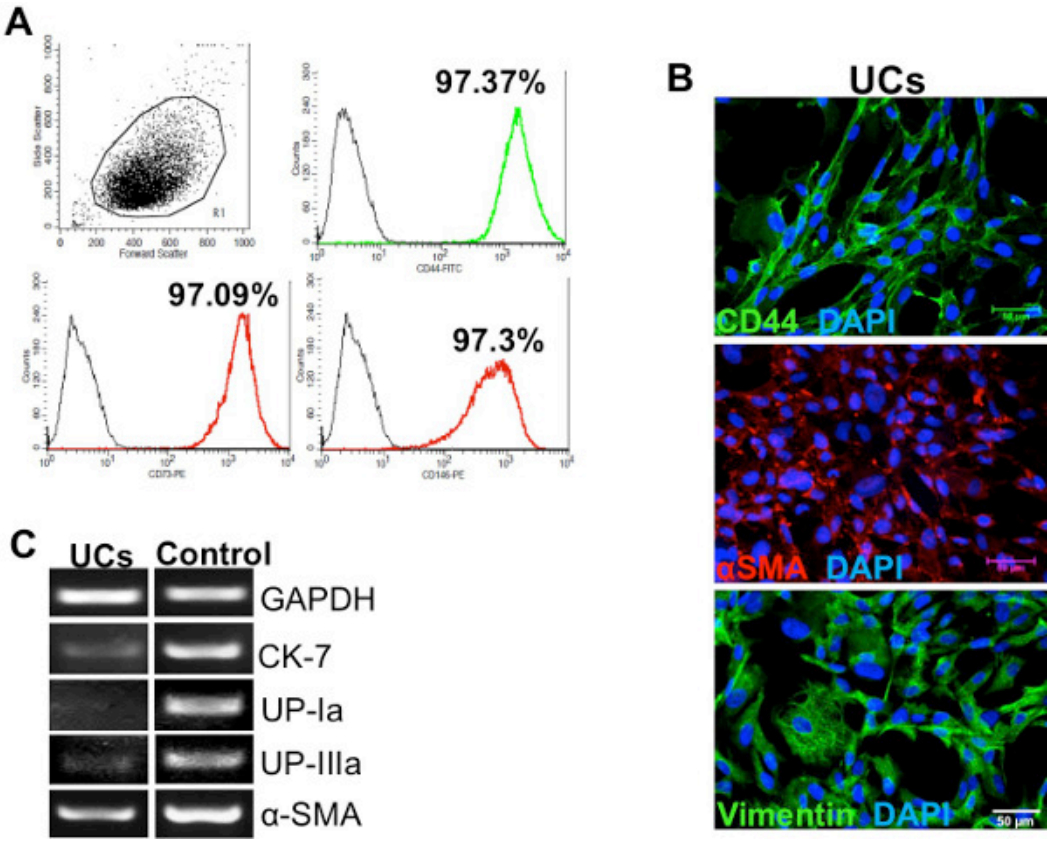

Figure 1. Characterization of isolated Urine Cells (UCs) shows uroepithelial progenitor, mesenchymal and smooth muscle lineages. (**A**) Flow cytometric analysis of UCs probed with conjugated antibodies against uroepithelial and pericyte markers CD44, CD73 and CD146. (**B**) Immunofluorescent staining of UCs with CD44, αSMA and Vimentin. (**C**) Gene expression profile of UCs show weak expression of CK-7 and UP-Ia and UP-IIIa as compared to positive control samples while gene expression of alpha-smooth muscle actin (αSMA) is comparable to positive control. Please click here to view a larger version of this figure.

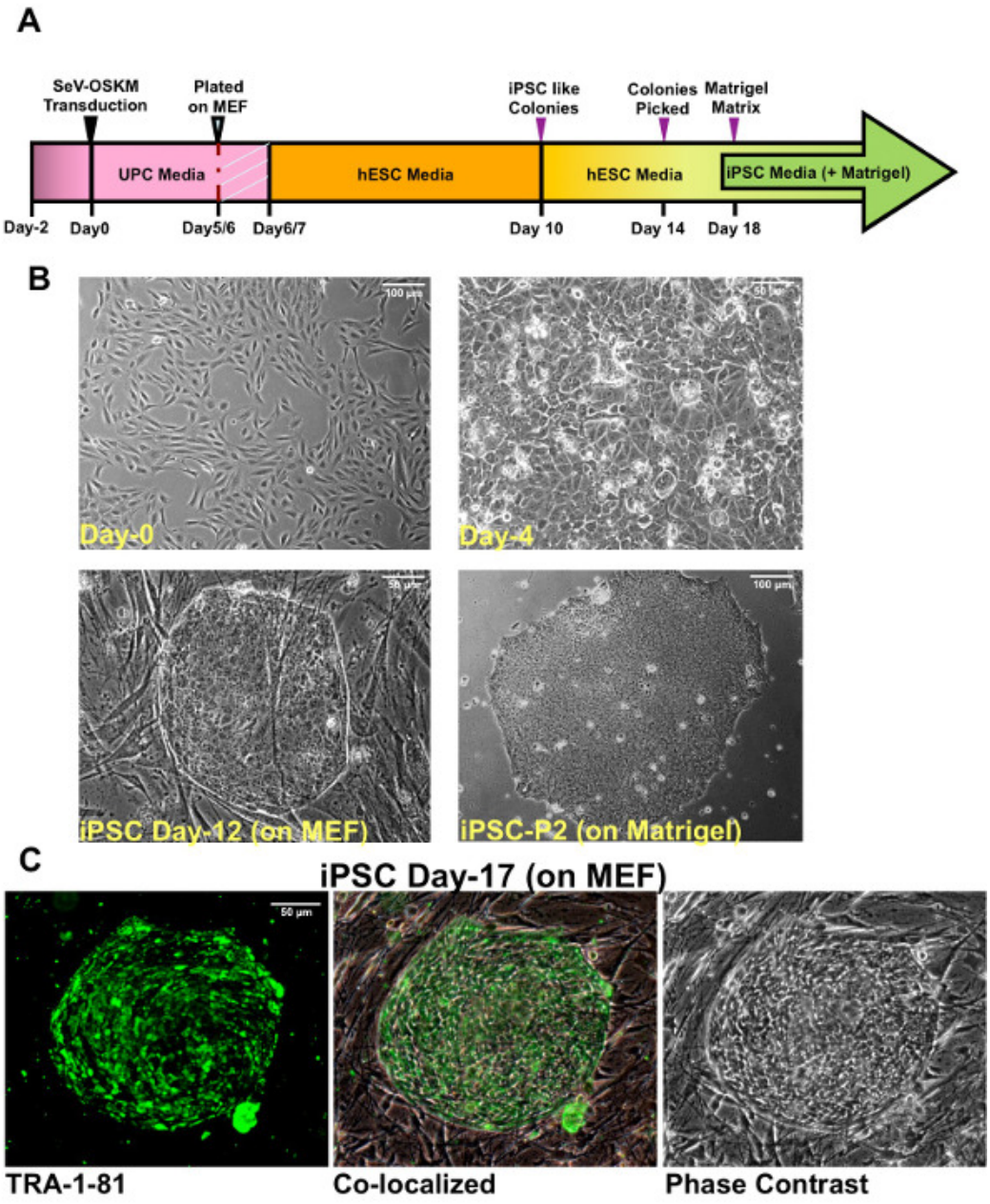

Figure 2. Reprogramming of UC to generate iPSCs. (**A**) Schematic overview of the reprogramming timeline. (**B**) Images representing different morphologies of UCs during the SeV transduction, at Day 0 (SeV transduction), Day 4 (morphological transition), Day 12 (pluripotent clones emerging on MEFs) and the characteristic iPSC clone under feeder-free conditions (iPSC-P2 on Matrigel). (**C**) Live cell imaging for TRA-1-81 to identify pluripotent colonies at Day 17. Please click here to view a larger version of this figure.

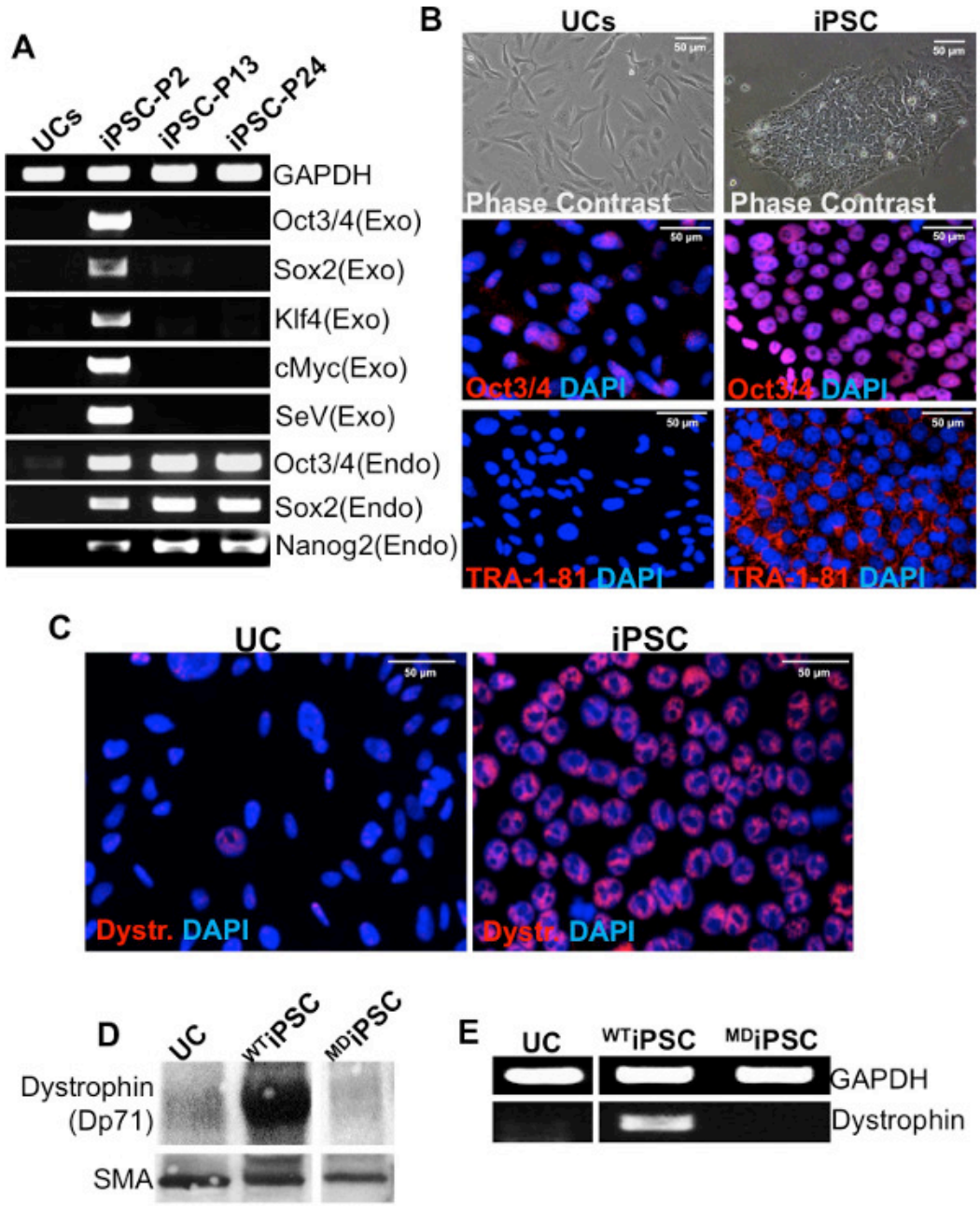

Figure 3. Generation of Viral Genome and Transgene Free iPSCs. (**A**) Gene expression analysis shows the presence of the SeV genome and OSKM transgenes at passage 2 (iPSC-P2) and their disappearance by passage 13 (iPSC-P13) while endogenous gene expression persists throughout. (**B**) Phase contrast images and immunofluorescence comparing Oct3/4 and TRA-1-81 staining in UCs and resultant iPSCs. (**C**) Dystrophin is detected by immunofluorescence in wild-type iPSCs compared to wild-type UCs, and (**D**) the specific dystrophin isoform (Dp71) can be confirmed by WB. (**E**) RT-PCR analysis is used to confirm the specific dystrophin exon deletion in the dystrophic iPSCs compared to wild-type UCs and iPSCs. Please click here to view a larger version of this figure.

Name of Reagent/Material/ Equipment	Company	Catalog Number	Comments
GAPDH-Forward Primer	IDT Inc.	Seq given in comments	GTGGACCTGACCTGCCGTCT
GAPDH-Reverse Primer	IDT Inc.	Seq given in comments	GGAGGAGTGGGTGTCGCTGT
CK7-Forward Primer	IDT Inc.	Seq given in comments	TGGTGCTGAAGAAGGATGTG
CK7-Reverse Primer	IDT Inc.	Seq given in comments	CACGCTGGTTCTTGATGTTG
Up-Ia-Forward Primer	IDT Inc.	Seq given in comments	ACGTCCTACACCCACCGTGA
Up-Ia-Reverse Primer	IDT Inc.	Seq given in comments	ACCCCACGTGTAGCTGTCGAT

Up-IIIa-Forward Primer	IDT Inc.	Seq given in comments	ACAAACAGAGGGTGGGAGGA CAG
Up-IIIa-Reverse Primer	IDT Inc.	Seq given in comments	AGAAGGGCAGGGAGCCCAGG
αSMA-Forward Primer	IDT Inc.	Seq given in comments	ACCCACAATGTCCCCATCTA
αSMA-Reverse Primer	IDT Inc.	Seq given in comments	TGATCCACATCTGCTGGAAG
Oct3/4 (Exogenous)-Forward Primer*	IDT Inc.	*Life Tech-Cytotune kit	CCCGAAAGAGAAAGCGAACCAG
Oct3/4 (Exogenous)-Reverse Primer*	IDT Inc.	*Life Tech-Cytotune kit	AATGTATCGAAGGTGCTCAA
Sox2 (Exogenous)-Forward Primer*	IDT Inc.	*Life Tech-Cytotune kit	ATGCACCGCTACGACGTGAG CGC
Sox2 (Exogenous)-Reverse Primer*	IDT Inc.	*Life Tech-Cytotune kit	AATGTATCGAAGGTGCTCAA
Klf4 (Exogenous)-Forward Primer*	IDT Inc.	*Life Tech-Cytotune kit	TTCCTGCATGCCAGAGGAGCCC
Klf4 (Exogenous)-Reverse Primer*	IDT Inc.	*Life Tech-Cytotune kit	AATGTATCGAAGGTGCTCAA
cMyc (Exogenous)-Forward Primer*	IDT Inc.	*Life Tech-Cytotune kit	TAACTGACTAGCAGGCTTGTCG
cMyc (Exogenous)-Reverse Primer*	IDT Inc.	*Life Tech-Cytotune kit	TCCACATACAGTCCTGGATGAT GATG
SeV (Exogenous)-Forward Primer*	IDT Inc.	*Life Tech-Cytotune kit	GGATCACTAGGTGATATCGAGC
SeV (Exogenous)-Reverse Primer*	IDT Inc.	*Life Tech-Cytotune kit	ACCAGACAAGAGTTTAAGAGA TATGTATC
Oct3/4 (Endogenous)-Forward Primer	IDT Inc.	Seq given in comments	CAGTGCCCGAAACCCACAC
Oct3/4 (Endogenous)-Reverse Primer	IDT Inc.	Seq given in comments	GGAGACCCAGCAGCCTCAAA
Sox2 (Endogenous)-Forward Primer	IDT Inc.	Seq given in comments	CAAGATGCACAACTCGGAGA
Sox2 (Endogenous)-Reverse Primer	IDT Inc.	Seq given in comments	GTTCATGTGCGCGTAACTGT
Dystrophin-Forward Primer	IDT Inc.	Seq given in comments	GGCAAAAACTGCCAAAAGAA
Dystrophin-Reverse Primer	IDT Inc.	Seq given in comments	GACCTGCCAGTGGAGGATTA

Table 1. List of Primer Sequences.

Discussion

Modeling cardiovascular diseases using iPSCs is becoming a common approach to understand the genetic contribution[4-6]. Some unanticipated difficulties of obtaining cell samples from patients, especially young children, can be avoided by offering the option of a non-invasive approach such as a urine collection. In this young patient population, it is often difficult to collect a volume of urine sufficient to yield enough urine cells for reprogramming. Coaching the young patients to drink fluids 30 min prior to the urine collection improves the success of urine cell isolation. However, repeat urine collections may be necessary in this population. We have combined and adapted two different protocols[3,7] in order to optimize the isolation and culture of UCs from patients with muscular dystrophy.

Induced pluripotent stem cells have been generated from either skin fibroblasts or urine cells from muscular dystrophy patients[1,2]. However, both reprogramming protocols relied on lentiviruses to introduce the reprogramming factors into the somatic cells. This integrating virus delivery method can be problematic if the transgene randomly integrates into the host genome and causes either transgene reactivation or mutagenesis (reviewed in 8). Therefore, we improved upon these techniques by using the Sendai virus to transduce the OSKM transgenes into the urine cells in a non-integrating manner[9].

This method using Sendai virus to reprogram UCs offers the advantage of a non-integrating approach with a higher transduction efficiency[9,10]. Urine cells collected from muscular dystrophy patients are reprogrammed within 2–3 weeks post-transduction. These reprogramming kinetics are comparable to UC reprogramming using lentiviral transduction[1]. Although this protocol can be scaled to establish feeder-free method for urine cell reprogramming, being an efficient method using Sendai-virus transduction of urine cells isolated from muscular dystrophy patients. This method described relies on MEFs as a feeder-layer in order to fully establish the pluripotent MD clone prior to transfer to feeder-free conditions at passage 2.

The nuclear distribution of the Dp71 isoform of the dystrophin protein has long been established in different embryonic stage cell models[11,12]. This ubiquitous expression of Dp71 in nuclear matrix fraction of early progenitor/embryonic stage cells suggests their role in nuclear architecture

and cell cycle regulation[12]. Our reprogrammed wild type iPSCs express Dp71; however, its absence in dystrophic iPSCs does not inhibit the reprogramming or pluripotent potential of dystrophic cells. In conclusion, the dystrophic gene mutations are conserved in reprogrammed iPSCs; making it an efficient tool to model dystrophic cardiomyopathy.

Disclosures

The authors declare that they have no competing financial interests.

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