Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;50:538-551
DOI: 10.1159/000494167
Published online: 11 October 2018
Accepted: 2 October 2018

Original Paper

Generation of a Urine-Derived Ips Cell Line from a Patient with a Ventricular Septal Defect and Heart Failure and the Robust Differentiation of These Cells to Cardiomyocytes via Small Molecules

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Key Words
Ventricular septal defect • Heart failure • Urine-derived cells • Induced pluripotent stem cells • Differentiation • Cardiomyocytes • Small molecules • Autophagy

Abstract
Background/Aims: Ventricular septal defects (VSDs) are one of the most common types of congenital heart malformations. Volume overload resulting from large VSDs can lead to heart failure (HF) and constitutes a major cause of pediatric HF with a series of often-fatal consequences. The etiology of VSD with HF is complex, and increasing evidence points toward a genetic basis. Indeed, we identified an L2483R mutation in the ryanodine receptor type 2 (RyR2) in a 2-month-old male patient with VSD with HF. ***Methods:*** We generated integration-free induced pluripotent stem cells from urine samples (UiPSCs) of this patient using Sendai virus containing the Yamanaka factors and characterized these cells based on alkaline phosphatase activity, pluripotency marker expression, and teratoma formation. Then, we induced the derived UiPSCs to rapidly and efficiently differentiate into functional cardiomyocytes through temporal modulation of canonical Wnt signaling with small molecules. Real-time PCR and immunofluorescence were used to verify the expression of myocardium-specific markers in the differentiated cardiomyocytes. The ultrastructure of the derived myocardial cells was further analyzed by using transmission electron microscopy. ***Results:*** The established UiPSC lines were positive for alkaline phosphatase activity, retained the RyR2 mutation, expressed

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pluripotency markers, and displayed differentiation potential to three germ layers *in vivo*. The UiPSC-derived cells showed hallmarks of cardiomyocytes, including spontaneous contraction and strong expression of cardiac-specific proteins and genes. However, compared with cardiomyocytes derived from H9 cells, they had a higher level of autophagy, implying that autophagy may play an important role in the development of VSD with HF. ***Conclusion:*** The protocol described here yields abundant myocardial cells and provides a solid platform for further investigation of the pathogenesis, pharmacotherapy, and gene therapy of VSD with HF.

Introduction

A ventricular septal defect (VSD) is an abnormal opening in the ventricular septum and one of the most common types of congenital heart disease (CHD)[1]. The hemodynamic significance of a VSD is principally determined by its size, the pressure gradient between the left and right ventricular chambers, and pulmonary resistance. The volume overload resulting from a large VSD can lead to heart failure (HF) and is a major cause of HF in children [2]. HF has many consequences, including high mortality, long-term hospitalization, and a heavy family financial burden [3]. Therefore, early detection and diagnosis are crucially important to prevent VSD with HF. There is no doubt that research into the pathogenesis of this disease will be invaluable in accomplishing this goal.

VSD with HF involves many complex factors, among which genetics is an undeniably important component. It is difficult to establish *in vitro* animal models to replicate specific patient characteristics. To better understand the molecular mechanisms of VSD with HF on a personalized basis, it is necessary to acquire pathologic cells similar to the damaged cells of a specific individual [4]. Induced pluripotent stem cell (iPSC) technology makes this possible [5]. Human iPSCs have been successfully established from different tissues, particularly blood cells [6], extra-embryonic tissues [7], and skin fibroblasts [8, 9]. Human urine-derived cells (UCs) are an ideal source for iPSC generation because they are simply and noninvasively obtained [10, 11]. Particularly in the pediatric population, parents are more receptive to this method of cell collection. The traditional virus-based approaches used in several studies have raised many safety concerns, which hamper their further application. For iPSC technology to be adopted in clinical practice, it is crucial to develop a practical approach to the generation of iPSCs using a virus-free method. The Sendai virus vector system is another option that is currently available, with the advantage that the it does not integrate into the genome or enter the nucleus. The genomic integrity of reprogrammed cells can be conserved by the use of this nonintegrating vector system. Due to their relatively high efficiency and little hands-on time required [12, 13], Sendai virus vectors were chosen for this protocol.

Although more and more progress has been made in generating cardiomyocyte-like lineages from iPSCs, the differentiation of human iPSCs toward functional cardiomyocytes still faces enormous challenges. Biochemical approaches to regulate stem cell fate with readily available and inexpensive synthetic bioactive molecules may rectify these problems [14]. Many small molecules regulate the cellular processes of cardiomyocyte differentiation by modulating signal transduction pathways, including the canonical Wnt/β-catenin pathway [15-17].

Ryanodine receptor 2 (RyR2) plays an important role in HF progression [18]. Thus, in this study, we generated a human cellular VSD with HF model from a patient carrying a RyR2 mutation (c.7448T>G, p.L2483R) by using an efficient integration-free urine-derived cell reprogramming method to generate urine-derived iPSCs (UiPSCs). Then, we used a fully chemically defined, small molecule-mediated directed differentiation protocol to drive the differentiation of the UiPSCs toward cardiomyocytes, effectively inducing myocardial differentiation in a short time frame.

Materials and Methods

Ethics statement

Animal experiments were performed on CF1 mice and nonobese diabetic severe combined immunodeficient (NOD-SCID) mice, which were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Beijing, China). All animal experiments carried out in this study adhered to the guidelines of Chinese legislation on the care and use of laboratory animals. All experiments were approved by the Institutional Review Board of the Children's Hospital of Fudan University (approval no. 043). The patient's family provided signed written informed consent prior to this study.

Urine sample collection and UCs isolation

Clean-catch midstream urine samples (about 100 ml) from the donor were collected using sterile containers, which were immediately placed on ice and then transferred to the laboratory. After centrifugation at 400 × *g* for 10 min at room temperature, the supernatant of the urine samples was discarded and the cell pellets were washed with phosphate-buffered saline (PBS). The viability of all counted cells was measured with a hemocytometer and trypan blue exclusion. The cells were then seeded in 24-well plates at about 500 cells per well with mixed medium consisting of keratinocyte serum-free medium (Gibco, Gaithersburg, MD) and progenitor cell medium in a 1:1 ratio. All 24-well plates were incubated at 37°C and 5% CO_2 and the medium was changed every 2 days. The keratinocyte serum-free medium was supplemented with 5 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO) and 30 ng/ml cholera toxin (Sigma-Aldrich). Progenitor cell medium was supplemented with 10% Dulbecco's modified eagle medium (DMEM), 0.4 μg/ml hydrocortisone (Sigma-Aldrich), 30 ng/ml cholera toxin (Sigma-Aldrich), 5 ng/ml insulin, 1.8×10^{-4} M (24.3 μg/ml) adenine (Sigma-Aldrich), 5 μg/ml transferrin (Sigma-Aldrich), 2×10^{-9} M (1.302 ng/ml) 3, 3',5'-triiodo-L-thyronine (Sigma-Aldrich), and 10 ng/ml epidermal growth factor (Sigma-Aldrich). Medium (1 ml) was obtained to detect fungal and bacterial contamination with a qualitative fluorescence Grocott's Methenamine Silver detection kit.

Non-integrative reprogramming of UCs into iPSCs

According to the manufacturer's protocol (CytoTune™-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific, Waltham, MA), UCs were infected with Sendai virus containing human transcription factors (Oct4/Sox2, c-Myc, Klf4) at multiplicities of infection of 5:5:3 (KOS:hc-Myc:hKlf4) at the recommended transduction confluency for UCs of about 30–60%. The 24-well plates were replaced with fresh UC medium 24 h post-transfection, and all cells were cultured for 6 more days with medium changes every 2 days. Seven days after transduction, UCs were harvested and plated on a mitotically inactivated CF1 mouse embryonic fibroblast (MEF) feeder layer. On the eighth day, the medium was changed to iPSC medium and the spent medium was replaced every day. Starting on day 8, the 24-well plates were observed every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells. Note that the iPSC medium contained DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% KnockOut Serum Replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1% β-mercaptoethanol (Sigma-Aldrich), 4 ng/mL basic fibroblast growth factor, and 1% penicillin/streptomycin.

Genotyping by sequencing analysis

DNA sequencing was used to confirm the mutation in patient-derived iPSCs. Genomic DNA from patient-derived iPSCs was isolated using the DNeasy Blood & Tissue Kit (#69504, Qiagen, Germantown, MD). The region encoding the mutation site in RyR2 was amplified by polymerase chain reaction (PCR). The PCR product was purified using an Agarose Gel Extraction Kit (Thermo Fisher Scientific), followed by direct Sanger sequencing in an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

Teratoma formation from UiPSCs

iPSC cultures were digested by accutase and preplated onto untreated culture plates to remove feeders and differentiating cells. The flanks of NOD-SCID mice were injected with 5×10^6 UiPSCs to form teratomas. After 9 weeks, the tumors were dissected and processed for histological analysis.

Short tandem repeat analysis

To confirm and verify that the derived iPSC line originated from UCs, short tandem repeat (STR) analysis was performed for both UCs and iPSCs. The general process was as follows. First, the parental genomic DNA of peripheral blood, UCs, and UiPSCs was extracted with a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's recommendations. Second, the 19 STR loci were co-amplified using the commercial identifier kit from Applied Biosystems, with which the sex-typing marker amelogenin was labeled with four different dyes (Applied Biosystems). Third, the data were collected using a multi-capillary ABI 3100 Genetic Analyzer (Thermo Fisher Scientific) and converted into the appropriately colored peaks. Finally, the STR genotype information was determined by the GeneScan and Genotyper programs (Applied Biosystems).

Feeder-free culture of human iPSCs

Matrigel-coated 6-well plates were taken from 4°C storage and placed at room temperature for 15 min before use. With mTESR1 complete medium, human iPSCs were preprocessed in the Matrigel-coated plate before differentiation and were passaged approximately every 5–6 days. Note that these colonies were not allowed to touch each other and that the medium was changed every day except for the day after passaging.

Directed cardiac differentiation of human iPSCs via small molecules

One of the most crucial differentiation steps was the digestion of cultured human iPSCs into single cells. First, when the human iPSCs reached 80–90% confluency, each well of the Matrigel-coated 6-well plates was emptied of medium and 1 ml accutase (Life Technologies) was added. The plate was incubated at 37°C and 5% CO_2 for 8–10 min. Second, using a sterile 1-ml pipette, the cells were gently transferred from the 6-well plate and combined in a sterile 15-ml conical tube containing 4 ml of mTeSR1 medium at room temperature. The 10-ml cell suspension was mixed and centrifuged at 200 × *g* for 5 min at room temperature. The cell pellet was resuspended in mTeSR1 medium + 5 μM Y27632 after removal of the supernatant. Third, 10^6 iPSCs were seeded onto a new Matrigel-coated 6-well plate (day-3) and cultured in mTeSR1 medium + 5 μM Y27632 for 72 h (day-3-day -1). At day 0, the cells were treated with 2 μM BIO (Calbiochem, San Diego, CA) and 6 μM CHIR99021 (Selleck, Houston, TX) in RPMI medium for strictly 24 h. At day 1, the medium was aspirated and replaced with fresh RPMI 1640. At day 3, the medium was replaced with fresh RPMI medium with 2.5 μM IWP2 (Tocris Biosciences, Bristol, UK) and 8 μM KY02111 (Selleck) for 2 days. At day 5, the medium was replaced again with fresh RPMI medium. From day 7, the cell culture medium was changed to cardiomyocyte maintenance medium. The cardiomyocyte maintenance medium contained RPMI supplemented with 10 μg/ml insulin (Sigma-Aldrich) and 200 μg/ml L-ascorbic acid 2-phosphate (AA2-P) sesquimagnesium salt hydrate (Sigma-Aldrich).

Alkaline phosphatase staining and immunofluorescence microscopy

Based on the manufacturer's instructions, alkaline phosphatase (AP) activity was measured using an Alkaline Phosphatase Detection Kit (Sidansai, China). The detailed process for immunofluorescence staining was as follows: cells grown on chamber slides (Cellvis, Mountain View, CA) were washed with PBS, fixed with 4% paraformaldehyde for 15–20 min, permeabilized with 0.2% Triton X-100 for 30 min, blocked with PBS containing 3% bovine serum albimin for 2 h, and incubated overnight with primary antibodies at 4°C. The primary antibodies and their dilution rates were: NANOG (1:100, Bioworld, Dublin, OH), SSEA3 (1:150, Bioworld), SSEA4 (1:150, Santa Cruz Biotechnology, Santa Cruz, CA), tumor-related antigen (TRA)-1-60 (1:150, Santa Cruz Biotechnology), TRA-1-81 (1:150, Santa Cruz Biotechnology), troponin I (1:800, Sigma-Aldrich), sarcomeric α-actinin (1:100, Bioworld), GATA4 (1:150, Bioworld), myosin light chain (MLC)-2v (1:200, Synaptic Systems, Goettingen, Germany), and myosin light chain (MLC)-2a (1:200, Synaptic Systems). The secondary antibodies and their dilution rates were as follows: rhodamine-conjugated AffiniPure goat anti-mouse IgM (1:200, Bioworld), goat anti-mouse IgG (1:200, Bioworld), and goat anti-rabbit IgG (1:200, Bioworld). The cell nucleus was stained with DAPI (1:1000, Sigma-Aldrich). After staining, we examined the slides with an epifluorescence microscope (DM IRB; Leica Microsystems, Wetzlar, Germany).

Analysis of marker genes

Total RNA was extracted by using Trizol reagent (Invitrogen). Using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan), 5000 ng RNA was reverse-transcribed to cDNA. Real-time quantitative PCR (RT-PCR) was performed using the 7500 Real Time PCR System (Applied Biosystems) using SYBR Premix EX Taq (Takara) to measure the expression of endogenous pluripotency genes and cardiac-specific genes. Gene-specific primers are listed in Table 1, and GAPDH was used as an endogenous reference gene, as we previously described [19].

Table 1. Primers for RT-PCR analysis

Gene	Primer	
	Forward (5'- 3')	Reverse (5'- 3')
OCT4(total)	AGCTCTGCAGAAAGAACTCG	GCTTCCTCCACCCACTTCT
OCT4(Endo)	GGGAGGAGCTAGGGAAAGAAAACCT	GAACTTCACCTTCCCTCCAACCAGT
SOX2(total)	CCAAGATGCACAACTCGGAG	GGGCAGCGTGTACTTATCCT
SOX2(Endo)	TACGGTAGGAGCTTTGCAGG	ACTGTCCTAAATTTCAGCTGCA
NANOG(total)	ATAGCAATGGTGTGACGCAG	CTGTTCCAGGCCTGATTGT
NANOG (Endo)	ATGGAGGGTGGAGTATGGTTGG	AGGCTGAGGCAGGAGAATGG
CRIPTO(total)	AATTTGCTCGTCCATCTCGG	GACCCACAGTTCTCTTTGCG
CRIPTO(Endo)	CTTCTTTGCCCTGCCCTCT	GCAGGAGAATGGCTTGAACC
MLC-2a	AAGCCCCTCCTCAATGACTC	TGTCACCAAGGTCACAAGGA
MLC-2v	CCATGTTTGAGCAGACCCAG	TGGTCTCTTCAGGATCAGCC
ACTN2	GTCATCTCAGGGGAAAGGCT	CAGCAGACCTTCTTTGGCAG
GATA4	TCCTACTCCAGCCCCTACC	CAGATAGTGACCCGTCCCAT
IRX4	TTCCGTTCTGAAGCGTGGTC	TGAAGCAGGCAATTATTGGTGT
cTnT	TTCACCAAAGATCTGCTCCTCGCT	TTATTACTGGTGTGGAGTGGGTGTGG
cTnI	CTCCAACTACCGCGCTTATG	ACAAGTCCTGCAGCTCCG
GAPDH	CACCCACTCCTCCACCTTTG	ACCACCCTGTTGCTGTAGCC

Transmission electron microscopy

For transmission electron microscopy (TEM), the H9 human embryonic stem cell (hESC) line or UiPSC-derived cardiomyocytes grown in differentiation medium were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in cacodylate buffer for 24 h at 4°C. Then, the samples were sent to the electron microscope room at Shanghai Medical College of Fudan University for subsequent processing and testing.

Results

Acquisition of UCs from a patient with an L2483R mutation in the RyR2 gene

As described in the Materials and Methods, we isolated and amplified UCs from urine samples obtained from a 2-month-old male patient with VSD with HF. An L2483R mutation was identified in RyR2 of the patient. The UCs displayed a high proliferation rate *in vitro*. No fungal or bacterial contamination was detected in any of the cultured urine samples. With trypan blue staining, the average total number of living cells in these samples was 5.5×10^3/100 ml urine,

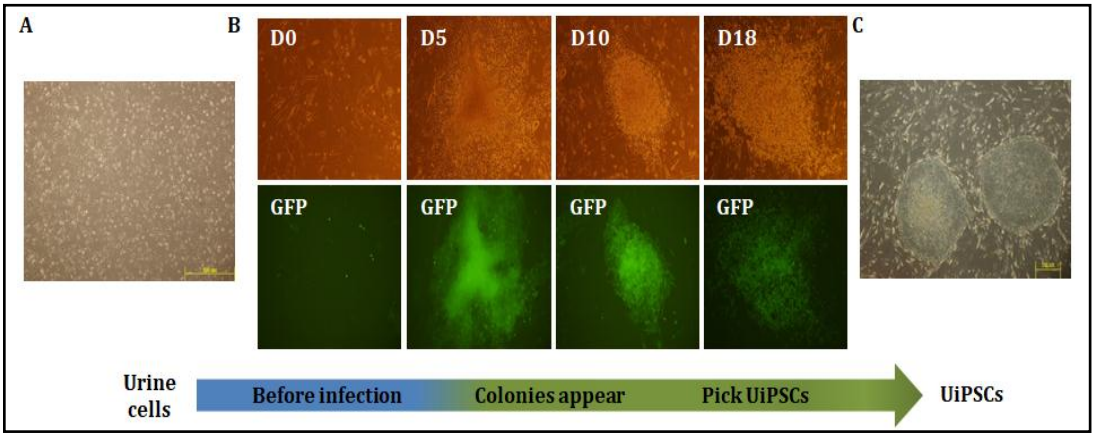

Fig. 1. Successful generation of iPSCs from the UCs of a patient with VSD with HF. (A) Primary cultured urine cells. (B) Schematic representation of UC reprogramming. Representative phase-contrast and immunofluorescence photographs of UCs at different points after infection with GFP Sendai viruses containing the Yamanaka factors (Oct4/Sox2, c-Myc, Klf4). (C) The established UiPSC line. Scale bars as indicated.

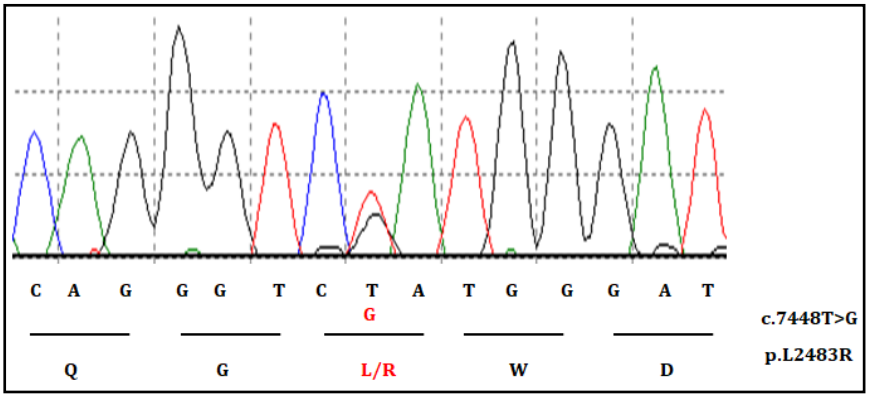

Fig. 2. Sequencing analysis showed that the UiPSCs maintained the RyR2 mutation (c.7448T>G, p.L2483R).

with a range of 4.0–7.0 × 10^3. With regards to morphologic characteristics, 99% of these cells were epithelial-like, large, and flat, which indicated that they were terminally differentiated. These cells grew with adherence on days 3–5 and were expanded on days 5–7. They were able to grow for more than 5 passages *in vitro* (Fig. 1A).

Integration-free iPSCs were successfully generated from the donor's UCs using Sendai virus vectors

To obtain UiPSCs, the UCs from the patient with VSD with HF were infected with the optimized Sendai viruses containing Oct4/Sox2, c-Myc, and Klf4 and were plated onto Matrigel-coated plates. Seven days after transduction, cells were transferred to 0.1% Matrigel-coated dishes containing irradiated CF1 MEF. On approximately day 12 post-transduction, we observed some atypical granulated colonies, which were unlike hESCs.

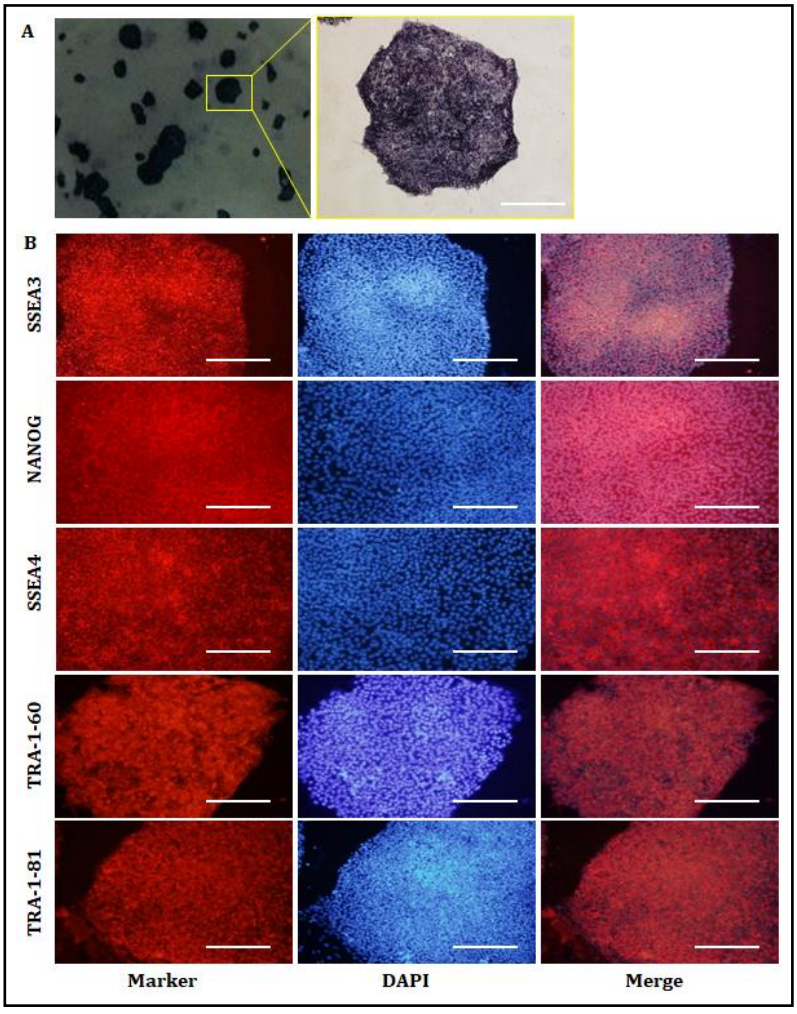

Fig. 3. Positive expression of pluripotency markers in UiPSCs. (A) UiPSCs strongly displayed AP activity. (B) Representative immunofluorescence staining patterns of UiPSCs using antibodies against SSEA3, NANOG, SSEA4, TRA-1-60, and TRA-1-81. Nuclei were counterstained with DAPI. Scale bars: 200 μm.

After the first infection, typical UiPSC colonies with morphologies similar to those of hESCs appeared around 3–4 weeks. These colonies were selected and amplified by subculture (Fig. 1B and C). The undifferentiated iPSC colonies were manually picked and transferred to Matrigel-coated 24-well plates with mTeSR medium for further feeder-free expansion and analysis. H9 hESCs were used as the control.

Genotyping analysis showed that our established UiPSCs retain the RyR2 mutation

As discussed above, the UCs collected from the patient were successfully differentiated to iPSCs. Our sequencing work revealed that the established UiPSCs also retained the RyR2 mutation (Fig. 2).

Reprogrammed UiPSCs strongly express embryonic-specific proteins and marker genes

We assessed the pluripotent properties of UiPSCs (UiPS-1 and UiPS-2) *in vitro* by analyzing AP activity, immunofluorescence staining, and RT-PCR. As shown in Fig. 3, the UiPSCs strongly displayed AP activity (Fig. 3A), and the expression of many hESC-specific surface antigens (NANOG, SSEA3, SSEA4, TRA-1-60, and TRA-1-81) was confirmed by immunofluorescence staining (Fig. 3B). We then measured the gene expression of four pluripotency genes (SOX2, OCT4, NANOG, and CRIPTO/TDGF1), as well as the expression of endogenous transcripts. The differential expression between total and endogenous genes confirmed the expression of the transgenes. RT-PCR results showed that the pluripotency-associated genes were clearly upregulated in the UiPSCs relative to the parental UCs. In the H9 hESC line, there was no significant difference between the total and endogenous transcripts of these four pluripotency-associated genes, which might indicate a lack of transgene expression. In the UiPSCs, the total levels of pluripotency genes exceeded the endogenous levels, indicating residual transgene expression (Fig. 4).

Reprogrammed UiPSCs form teratomas containing three germ layers in vivo

To determine the pluripotency of the UiPSCs *in vivo*, NOD/SCID mice were injected with UiPSCs and, after 9 weeks, the resulting teratoma was dissected for hematoxylin and eosin staining. The results indicated that the UiPSCs gave rise to teratomas containing derivatives of all three embryonic layers, including neuroepithelium (ectoderm), gut-like structures (endoderm), and cartilage (mesoderm) (Fig. 5).

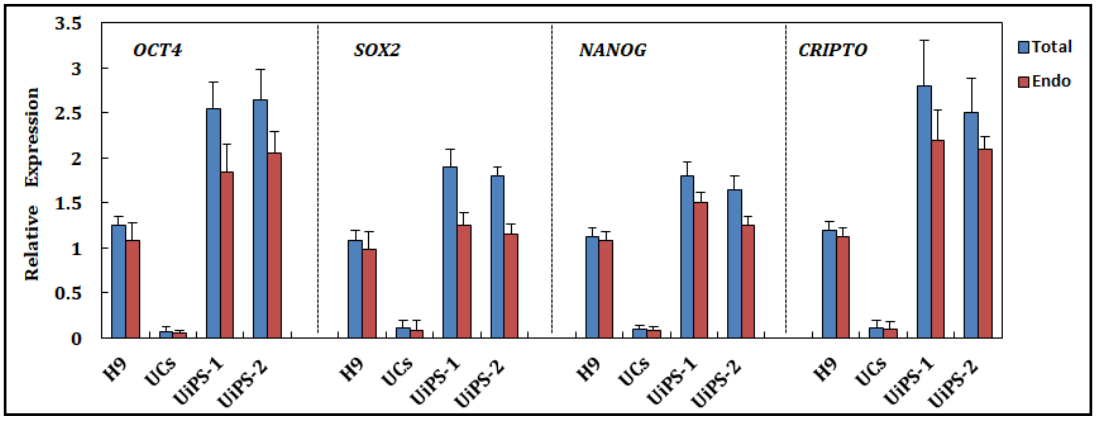

Fig. 4. Expression of hESC marker genes in UiPSCs. RT-PCR showed that the pluripotency-associated genes expressed in UiPS-1 and UiPS-2 were clearly upregulated relative to UCs. In the H9 hESC line, there was no significant difference in the total and endogenous transcripts of these four genes, which might indicate a lack of transgene expression. The UiPSCs exhibited higher expression of total pluripotency genes than of endogenous pluripotency genes.

Fig. 5. Teratoma formation of UiPSCs. Hematoxylin and eosin staining showed that UiPSCs gave rise to teratomas in vivo containing derivatives of the three embryonic layers, including neuroepithelium (ectoderm), gut-like structures (endoderm), and cartilage (mesoderm).

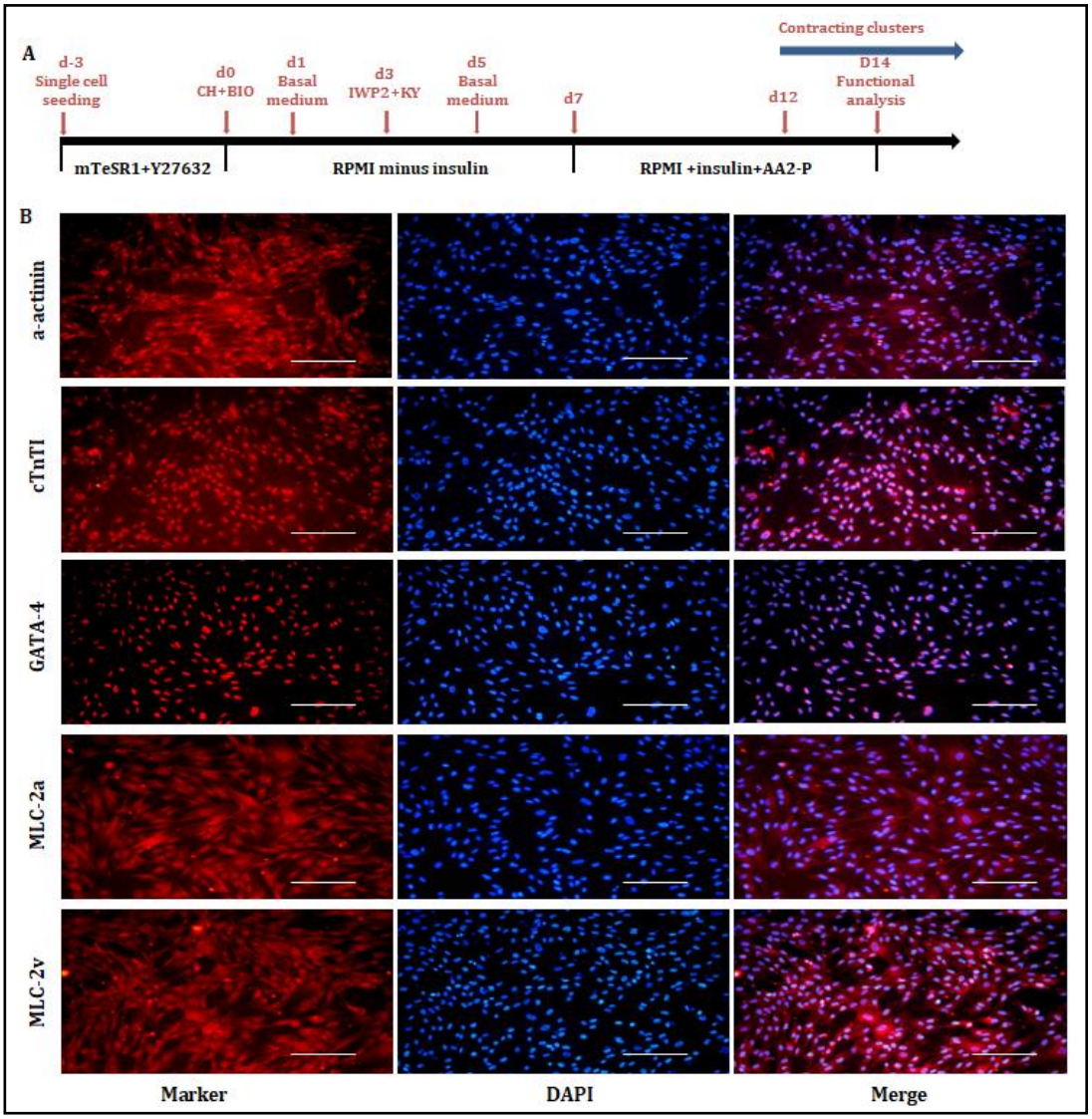

Fig. 6. (A) Schematic of cardiomyocytes generated from UiPSCs via small-molecule modulation of Wnt signaling. (B) Expression of specific cardiac markers (α-actinin, cTnI, MLC-2a, MLC-2v, and GATA4) in UiPSC-derived cardiomyocytes. Nuclei were counterstained with DAPI (blue). Scale bars: 200 μm.

Established iPSCs are derived from the donor's UCs and not cross-contamination

Parental DNA samples from peripheral blood, UCs, and UiPSCs were analyzed by STR analysis to verify that the iPSCs originated from the UCs of the patient and were free of contamination from laboratory hESCs. The patterns of 19 STRs between the iPSCs and the parental UCs were matched. The selected UiPSC clone was genetically matched to the parental UCs, excluding the possibility of cross-contamination from external cultured pluripotent cells (Fig. S1 - for all supplemental material see www.karger.com/10.1159/000494167/).

UiPSCs can be directed differentiated into cardiomyocytes through temporal modulation of canonical Wnt signaling with small molecules

Prior studies [20, 21] indicated that early induction of canonical Wnt signaling and suppression of canonical Wnt signaling at later stages of differentiation synergistically enhance the cardiomyocyte yield. In this study, we applied the Wnt activator CHIR99021 and 6-bromoindirubin-3-oxime (BIO) 3 days before the addition of the Wnt inhibitors KY02111 and IWP2 in the middle differentiation phase, producing up to 75–80% functional cardiomyocytes from human UiPSCs in cytokine-, serum-, and xeno-free defined medium (Fig. 6A). At approximately day 12 after differentiation, the differentiated cells showed hallmarks of cardiomyocytes, including spontaneous contraction and cardiac-specific gene and protein expression.

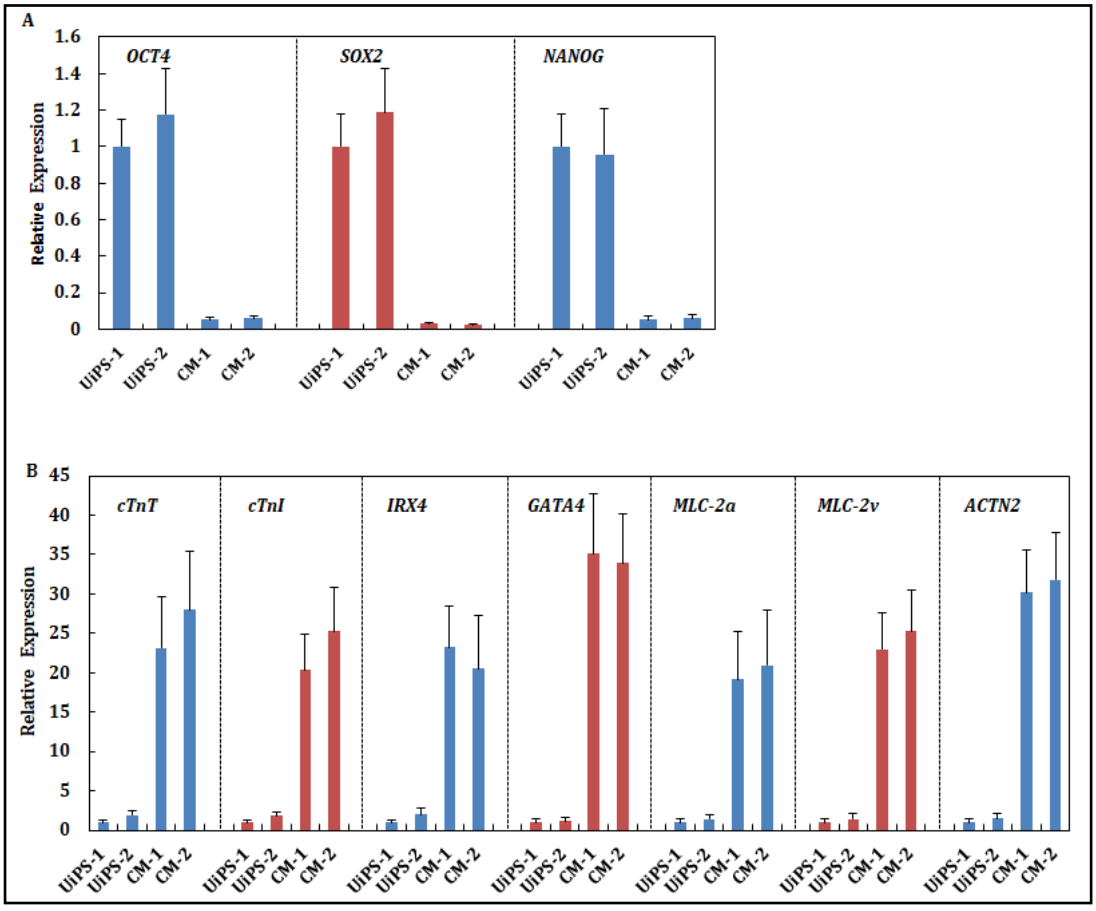

Fig. 7. RT-PCR results indicated that cardiomyocytes derived from UiPSCs expressed high levels of cardiac markers, including cTnT, cTnI, IRX4, GATA4, MLC-2a, MLC-2v, and ACTN2, and barely expressed the hESC marker genes OCT4, SOX2, and NANOG.

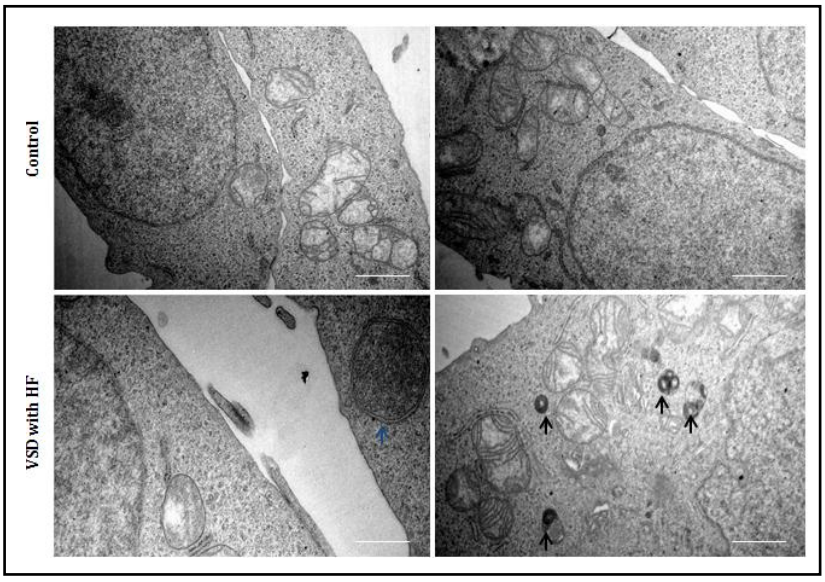

Fig. 8. Electron micrographs of derived myocardial cells. Compared with control, many autophagic vacuoles (black arrow) and double membrane structures (blue arrow) were found in cardiomyocytes derived from the iPSCs of the VSD with HF patient.

Characterization of UiPSC-derived cardiomyocytes

To characterize the differentiated cardiomyocyte cultures (CM1 and CM2) derived from the two iPSC clones (UiPS-1 and UiPS-2), respectively, we performed immunofluorescence staining with antibodies against GATA4, sarcomeric α-actinin, troponin I, MLC-2a, and MLC-2v using enzymatically dissociated cells from the contracting areas. The immunofluorescence staining analysis clearly revealed the distribution and localization of these cardiac markers in the differentiated cells. GATA4, a transcription factor involved in cardiac differentiation, showed specific nuclear localization, whereas sarcomeric α-actinin, troponin I, MLC-2a, and MLC-2v, as myofilament proteins, were mainly distributed in the cytoplasm (Fig. 6B). These results suggested that contracting areas derived from UiPSCs had some features similar to those of human cardiac myocytes.

We also quantified the expression levels of marker genes present in contracting regions from UiPS-1 and UiPS-2 via RT-PCR. The results showed that cardiac-specific markers, such as the zinc-finger transcription factor GATA4, ventricular myocardium marker IRX4, and the myofilament protein genes cardiac troponin T (cTnT), cardiac troponin I (cTnI), a-actinin (ACTN2), MLC-2a, and MLC-2v, were highly expressed in the differentiated CM1 and CM2 cultures (Fig. 7B). As expected, the hESC markers (SOX2, OCT4, and NANOG) were not expressed in the differentiated cells (Fig. 7A).

Clear autophagy is found in UiPSC-derived cardiomyocytes upon ultrastructural analysis with transmission electron microscopy

We further analyzed the ultrastructure of the differentiated cardiomyocytes using transmission electron microscopy (TEM). We found that the mitochondria were organized and aligned with the nucleus in both H9- and UiPSC-derived cardiac myocytes, but the myofilaments were not arranged in a mature manner to form myofibrils. Compared with cardiomyocytes derived from H9 cells, a large amount of vacuoles with low electron densities were found in the cardiomyocytes from the patient with VSD with HF (Fig. 8). The ultrastructural changes implied that autophagy may play an important role in the development of VSD with HF.

Discussion

Heart development is a multistep process involving multiple genes. Disruption at any time during primary cardiac morphogenesis results in any of the wide spectrum of CHDs seen today [1, 22]. VSD is the most frequent form of CHD and its pathogenesis is still unclear due to its genetic complexity and phenotypic variability. Prolonged shunting from left to right through a large VSD may result in Eisenmenger Syndrome and eventually lead to HF [23]. HF is a progressive intractable disease and often occurs due to volume overload secondary to CHDs such as VSD in children, and is thus a major cause of mortality and morbidity worldwide, despite intensive medical and interventional treatments [24]. In-depth research into the mechanisms of VSD with HF is crucial for the early detection and prevention of serious complications.

The method of disease modeling with patient-specific iPSCs can be applied to all types of chromosomal inherited diseases, from monogenetic to complex polygenetic disorders [25-28]. iPSCs hold great promise for disease modeling and regenerative therapies. Theoretically, all human somatic cells can be reprogrammed back into a pluripotent state as iPSCs. Urine samples from patients and healthy donors are convenient to retrieve and to reprogram, and thus represent an attractive cell source [29]. The generation of human iPSCs traditionally depends on retroviral vectors, which randomly integrate into the genome with potentially disruptive effects on genomic integrity [30, 31]. Indeed, the permanent integration of the vector DNA into the cell genome may induce mutagenesis [32]. In addition, the differentiation of iPSCs may be disturbed by insufficient inactivation of reprogramming factors [33]. In the present study, we chose Sendai virus vectors because of their robustness, preservation of genomic integrity, relatively high efficiency, and shorter time required for reprogramming. The rebuilt vectors delivered through Sendai virus are diluted through cell expansion and eventually discharged from the culture, providing a footprint-free reprogramming method [34]. In the present study, we infected UCs at passage 2 to 3 with Sendai virus containing Yamanaka factors, and the generated UiPSCs were demonstrated to have properties similar to those of hESCs.

Genotyping by sequencing analysis was performed on our VSD with HF patient, and an L2483R mutation in the RyR2 gene was identified. Our generated UiPSCs retained this mutation, which suggested that the UiPSCs acquired in this method preserved the genetic basis of the host and would be a unique cell resource for molecular pathology studies as well as the development of treatments for VSD with HF. To increase the potential utility of established iPSC lines for clinical applications, the colonies were adapted to feeder- and xeno-free conditions and directed differentiated into functional cardiomyocytes via small molecules. The original protocols to differentiate hESCs into cardiomyocytes used embryoid bodies in medium with fetal calf serum; however, this approach is not efficient and is inconsistent across different human iPSC lines [10]. During the past 10 years, human iPSCs have been used to produce cardiomyocytes via several differentiation protocols, such as through the addition of defined growth factors, including activin A, bone morphogenic factor 4, and Dickkopf-1, which can further enhance cardiomyocyte differentiation in embryoid bodies [35]. Because of the potential risk for adverse reactions and infections, serum is not used for clinical applications and recombinant cytokines are not cost-effective for large-scale production. However, small molecules are suitable for the production of defined media for large-scale culture because they have considerable potential to act as substitutes for recombinant cytokines and unknown factors in serum [36]. The Wnt signaling pathway plays an important role in cardiogenesis and Wnt signaling can enhance and modulate signaling to enable or disable the process of heart development [37, 38].

In our study, we treated the UiPSCs of our patient with a combination of the Wnt activators BIO and CHIR99021 in the early phase of cardiac differentiation and then used the Wnt inhibitors KY02111 and IWP2 in the middle phase, producing up to 75%–80% cardiomyocytes in cytokine-, serum-, and xeno-free defined medium. This strategy also shortened the differentiation cycle compared with the embryoid body system, with

spontaneous contraction clusters appearing as early as day 12. Enzymatic single-cell passaging with ROCKi Y27632 could maintain cells in a karyotypically normal, pluripotent, and ready-to-differentiate state while at the same time avoiding heterogeneity [39]. TEM showed that the differentiated cardiomyocytes have a higher level of autophagy consistent with what we witnessed in the patient's myocardial tissue.

These UiPSCs gave rise to cardiomyocytes that would carry the precise hallmark of genetic information associated with the pathology of our patient. This method will allow for research into cardiomyocytes derived from patients with VSD with HF with unknown genetic lesions, offering different perspectives into disease modeling and enabling the study of properties possibly playing an important role in the pathogenesis of VSD and HF. Additionally, it can provide a rich source of myocardial cells for regenerative therapies in HF.

Conclusion

We successfully generated integration-free iPSCs from urine samples of a patient with VSD with HF harboring an L2483R mutation in the RyR2 gene using a Sendai virus vector. These UiPSCs were successfully differentiated into functional cardiomyocytes rapidly and efficiently via temporal modulation of canonical Wnt signaling using small molecules. The derived cardiomyocytes carried the genetic mutation and possessed features associated with the pathology of the patient. The protocol described here allows for inexpensive and reproducible generation of human cardiomyocytes in completely chemically defined conditions, accelerating the translation of iPSCs to high-throughput screening applications or regenerative therapeutic methods.

Acknowledgements

This study was supported by the National Research and Development Program of China (2016YFC1000500), the National Natural Science Foundation of China (81670281 and 81570283), the Shanghai Municipal Commission of Science and Technology Research Project (18140900304), and the Outstanding Plan of Fudan University.

Disclosure Statement

The authors declare no conflicts of interest.

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