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GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED CARDIOMYOCYTES AND MESENCHYMAL STEM CELLS FROM AN INDIAN CONTROL LINE

Medicine

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ABSTRACT

The spectacular progress in stem cell research has laid the foundation for a new era of disease modelling, drug discovery and regenerative medicine. Over the past two decades, numerous studies have reported robust protocols for developing various induced pluripotent stem cell-based models, as well as applications of these cellular models in basic and clinical research. Indeed, several studies have focused on generation of induced pluripotent stem cells from individuals belonging to a certain ethnicity, providing the potential to study ethnic related stem cell applications. However, the availability of induced pluripotent stem cells or derived cell types generated from Indian ethnic population is limited. Here we report the generation of cardiomyocytes and mesenchymal stem cells from D149, a control human induced pluripotent stem cell line of Indian origin. Early cardiomyocytes and mesenchymal stem cells generated from D149 exhibited marked expression of corresponding cell type specific markers at the transcriptional and protein levels. These Indian control iPSC derived cellular models will represent a valuable resource with potential to advance future research in academic and private sectors in India and around the world.

KEYWORDS

Indian control line, induced pluripotent stem cell derived cardiomyocytes, induced pluripotent stem cell derived mesenchymal stem cells, disease modelling, regenerative medicine

INTRODUCTION

Recent advances in the field of induced pluripotent stem cells (iPSCs) has revolutionized the world of stem cell biology and biomedical research. The ability of iPSCs to self-renew and differentiate into any specialized cell type have made them a powerful tool with applications in basic stem cell research and clinical applications. iPSCs are being hailed as a promising alternative to embryonic stem cells (ESCs) as they eliminate the bioethical, legal and social issues associated with ESCs . Patient derived iPSCs represent the molecular and cellular phenotypes of specific diseases and serve as a reliable in vitro model for studying disease onset and progression, outperforming conventional animal models and in-vitro cell expression models.

iPSC models provide a suitable platform for developing novel therapies, , high-throughput screening of drug candidates, and toxicity prediction of diverse chemical compounds, drugs and environmental factors[1,2,3].iPSC- derived cell types are promising prospects for cell therapy and regenerative medicine [4,5].The transplantation of autologous iPSC-derived cell products is expected to overcome the risk of immune rejection complications [6].Acknowledging the advantages and applications of iPSC models, researchers are constantly working to develop rapid and efficient protocols for generating different cell types from iPSCs, which is a prerequisite to demonstrate more functionality and expand the scope of stem cell applications. Cardiomyocytes and mesenchymal stem cells have garnered substantial interest and attention among the several cell types produced from iPSCs due to their enormous basic and translational applications.

iPSC derived cardiomyocytes (iPSC-CMs) represents a physiologically relevant invitro system that displays key characteristics of human cardiomyocyte biology. Numerous protocols have been established over the last two decades to generate iPSC-CMs, which indeed mimics the embryonic cardiogenesis by modulating cardiac specific signalling pathways [7]. iPSC-CMs have been demonstrated to exhibit molecular, mechanical, electrophysiological, metabolic and ultrastructural properties similar to primary human cardiomyocytes, but also share many functional and structural characteristics to fetal cardiomyocytes [8].Researchers are currently adopting numerous strategies to optimize the differentiation protocols to promote robust, reproducible, scalable and cost-effective generation of iPSC-CMs of high purity, maturity and subtype specificity, thus broadening the scope of applicability of these cells for disease modeling and clinical applications.

iPSC- CMs have successfully been used to describe a large number of inherited arrhythmogenic syndromes and channelopathies including Long QT Syndrome (LQTSs), Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT), arrhythmogenic right ventricular dysplasia (ARVC) and Brugada syndrome [9,10].Cardiomyopathies such as familial hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and Barth syndrome have been modelled using patient-specific iPSC-CMs [11].iPSC-CMs have also been used to model diseases with cardiac traits such as LEOPARD syndrome, Duchenne muscular dystrophy, Myotonic Dystrophy Type 1, Friedreich's ataxia, Barth syndrome, familial transthyretin amyloidosis (ATTR), abetalipoproteinemia (ABL), and storage diseases such as Pompe, Fabry, and Danon disease [12,13].Furthermore, iPSC-CM models have been established for acquired cardiac diseases such as diabetic cardiomyopathy, Takotsubo cardiomyopathy and infectious diseases affecting the heart such as viral myocarditis and endotoxin-induced inflammation in cardiomyocytes [14]. These iPSC-CM models not only help in understanding molecular mechanisms underlying the disease but also in identifying disease targets for developing new therapeutic strategies.Several studies have demonstrated that iPSC-CMs are potential platforms for drug toxicity screening for a wide range of drugs including chemotherapeutic agents [15]. iPSC-CMs are also appealing candidates for use in high throughput drug screening, drug validation and metabolism studies [16,15,17]The use of iPSC-CMs aids in clinical trial optimization by reliably predicting responders and non-responders [18].

A recent study demonstrated that iPSC-CMs can serve as a platform for understanding the mechanisms of cardiac-specific infection by SARS-CoV- 2 in vitro and could potentially be employed to develop antiviral compounds [19].Disease and patient specific iPSC-CMs offers an unprecedented opportunity for the development of precision medicine , such as personalized cardiovascular drugs and therapeutic testing [20].As iPSC-CMs serve as an inexhaustible source of cardiac cells, they can be used in cardiovascular regenerative therapies to repair and/or replace injured heart [21].A vast number of proof-of-principle studies in small and bigger animal models have been conducted to investigate the feasibility, efficacy, and safety of the approach [22,23]. Unfortunately, no successful iPSC-CM transplantation has been reported in humans to date.

PSC derived mesenchymal stem cells (iPSC-MSC) are yet another fascinating in vitro model system with potential applications in basic and translational research. Mesenchymal stem cells (MSCs) are a subset of heterogeneous fibroblast-like multi-potent cells that have the potential to differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes and skeletal myocytes [24]. MSCs can be isolated from a variety of adult tissues, including bone marrow, umbilical cord blood, placenta, adipose tissue and a range of dental tissues. MSCs have been defined by three minimal criteria proposed by the International Society for Cellular Therapy (ISCT): (1) Must be plastic adherent when maintained in standard culture conditions (2) MSCs must express CD105, CD90 and CD73, and must not express CD45, CD19, CD34, CD14, CD11b, CD79α and HLA-DR. (3) Must have the capability to differentiate into osteoblasts, chondroblasts and adipocytes in vitro [25]. MSCs have been shown to

exhibit immunomodulatory effects on a wide range of immune cells in both innate and adaptive immune systems [26]. Furthermore, MSCs secrete a multitude of soluble factors such as growth factors, hormones, chemokines and cytokines , which in turn can modulate several actions of MSCs [27]. Owing to their diverse biological properties, MSCs have been recognized as a potential tool for various clinical applications.

MSCs represent a promising cell resource for regenerative medicine and tissue engineering. MSC-based treatment has been proven to be successful in the treatment of several immune mediated diseases including diabetes, graft versus host disease, cancer, multiple sclerosis, Crohn's disease, and systemic lupus erythematosus [28]. MSCs are also employed in the treatment of cardiovascular diseases, liver disorders and skin problems [29,30,31]. MSCs offer potential for use in tissue regeneration for bone, muscle, tendon and cartilage [32]. MSC-derived extracellular vesicles can serve as an excellent alternative to cell-based therapy [33]. MSCs can be used as a valuable tool to study genotoxicity, mutagenicity, carcinogenicity, and pharmacokinetics [34].

The extensive clinical use of MSCs has been hampered by the lack of an easily accessible cell source and strategies to produce sufficient amounts of cells. However, with the advancement of stem cell technology, iPSCs have been identified as a potential and reliable source for derivation of MSCs [35]. Several protocols have been described to differentiate iPSC into MSC employing various approaches [36,37,38,39,40,41].Human iPSC-derived MSCs have been shown to share genetic and functional characteristics with mature MSCs [42]. For clinical applications, iPSC-derived MSCs have several advantages over human tissue-derived MSCs, including easy accessibility, high proliferative capability, improved genetic stability, lower immunogenicity, and increased potential for therapeutic efficacy regardless of donor age [43]. Numerous studies are investigating the applications of iPSC derived MSCs in regenerative therapies, disease modelling, drug screening, and toxicity testing [44,45,46] . A recent phase 1 clinical trial using CYP-001, an iPSC-derived MSC reported promising results for treating patients with a steroid-resistant graft-versus-host disease [47,48,49].

With recent advancements in stem cell biology, it is becoming increasingly evident that factors like age, gender and ethnicity of donors can have a substantial impact on the application aspects of iPSC models. The development of iPSCs from different ethnic background, in particular, would provide a unique opportunity to evaluate the contributions of the ethnic origin to various clinical applications. Several recent studies have demonstrated the generation of iPSCs from diverse ethnic backgrounds [50,51,52].There is also a growing demand for ethnically distinct control lines in basic research and clinical application. A few studies in India have reported the generation of iPSC lines from healthy individual belonging to Indian ethnicity [53,54,55].However, no studies describing the generation and characterization of iPSC-derived cell types from Indian donors have been reported.

The present study aims to generate cardiomyocytes and mesenchymal stem cells from a human iPSC line of Indian origin. These iPSC-models of Indian origin are expected to be a potential resource for stem cell research both in India and around the world.

MATERIALS AND METHODS

Human Pluripotent Stem Cell Culture Maintenance And Characterization

Human iPSC used in this study was D149 (ADBSi002-A), a reference control line of the Indian population generated at ADBS [The Accelerator program for Discovery in Brain disorders using Stem cells] [53]. The iPSC line was cultured in Essential 8 medium (Gibco #A1517001) on Matrigel (Corning #354277) coated dishes with daily media changes and, incubated in a humidified incubator at 37°C with 5% CO2. Cells were passaged by enzymatic dissociation with Stempro Accutase (Gibco # A1110501) and recovered post-split using E8 medium supplemented with the rock inhibitor, 1X RevitaCell™ supplement (Gibco #A2644501). The rock inhibitor was removed on the next day after passage. For cryopreservation, iPSCs were frozen at a density of 1×10^6 in 500 µL PSC Cryopreservation media (Gibco #A2644601).

Presence of mycoplasma was assessed on cell culture supernatants using MycoAlert™ Mycoplasma Detection kit (Lonza #LT07-318). Prior to differentiation, pluripotency was assessed for pluripotency using Alkaline Phosphatase Detection Kit (Millipore # SCR004) and PSC immunocytochemistry kit (Invitrogen #A24881) as per manufacturer's instructions. Gross chromosomal integrity was checked by karyotype analysis by a qualified service provider (Anand Diagnostics Laboratory, Bengaluru) using standard G-banding methods.

Cardiomyocyte Differentiation And Characterization

A state-of-the-art differentiation protocol for iPSC-CM employing heparin based monolayer differentiation protocol utilizing small molecule modulators of Wnt signalling was adapted from Lin et al.,2017 [56].Briefly, when D149 line had grown to 85-90% confluency, E8 complete medium was replaced with differentiation basal medium, containing E8 basal medium,1X chemically defined lipid concentrate (Gibco #11905031) and 1X penicillin streptomycin (Gibco # 15140122). At day 0, 5µM CHIR99021 (Sigma #SML1046) was added to the differentiation basal medium for 24 hours. From day 1, 3µg/ml Heparin (Sigma# H3149) was added to the differentiation basal medium. From day 2 to day 5, 3µg/ml IWP2 (Sigma# I0536) was added to differentiation media along with 3µg/ml Heparin. From day 5, cells were maintained in heparin containing basal medium until beating was observed. Once beating was observed, cells were maintained in differentiation basal media containing 20 mg/ml insulin (Sigma# I9278) and thereafter media change was done every 2 days. All small molecules were solubilized in DMSO (Sigma #D2650).

Mesenchymal Stem Cell Differentiation

Mesenchymal stem cell differentiation was performed in D149 employing protocol adapted from Zeng et al.,2017 [39]. Briefly, iPSCs at 70-80% confluency, were treated with Mesoderm Induction Medium consisting of B27 medium (1:1 mixture of DMEM/F12 (Gibco # 11320033) with 1% Glutamax (Gibco # 35050061) and 1% B27 (Gibco #12587010) and 1% Penicillin-Streptomycin with 8 µM CHIR99021 and 25 ng/ml BMP4 (Sigma# H4916). After three days, the Mesoderm induction medium was replaced with Mesenchymal cell differentiation medium A consisting of 1:1 mixture of DMEM/F12 with 1% Glutamax ,1% B27 and 1% Penicillin-Streptomycin with 2ng/ml activin A (Gibco # PHG9014) and 10 ng/ml PDGFBB (Sigma# P3201). After two days, Mesenchymal cell differentiation medium A was replaced with Mesenchymal cell differentiation medium B consisting of 1:1 mixture of DMEM/F12 with 1% Glutamax and 1% B27 with 10 ng/ml FGF2 (Sigma# SRP4037) and 12 ng/ml BMP4 for additional two days. After passaging onto gelatin - coated dish, iPSC-MC were maintained in MSC-GM medium (Lonza # PT-3001). At day 10 of differentiation, MSCs were examined for the expression of MSC specific markers.

Quantitative Real Time PCR

Total RNA extraction was performed using the TRIzol reagent (Invitrogen), according to manufacturer's instructions. The concentration and purity of the RNA were determined using an ND-1000 spectrophotometer (Nanodrop) .2 µg of total RNA was used for cDNA synthesis using PrimeScript 1st strand cDNA Synthesis Kit (Takara #6110A) as per the manufacturer's instruction. SYBR Green primers were designed using QuantPrime (http://www.quantprime.de/) and were purchased as crude oligo nucleotides from Sigma-Genosys **(Table 1)**. YWHAZ and GAPDH were used as the endogenous control. Reactions were carried out in QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) following the thermal cycling conditions: 50^0C for 2 min, 95^0C for 10 min and 40 cycles of 95^0C for 30 sec 60^0 C for 30 sec and 72^0C for 45 sec. The real-time PCRs were run in triplicate. Relative quantification of gene expression levels of the target genes was determined by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Oligonucleotide primers used for PCR.

Gene	Forward primer (5'- 3')	Reverse primer(5'- 3')
Cardiomyocyte differentiation		
TBXT	TCCCGTCTCCTTCA GCAAAGTC	TGCAAGGAGTTCAGCAT GATCTGG
MESP1	TCGAAGTGGTTCCT TGGCAGAC	CCTCCTGCTTGCCTCAA AGTGTC
MESP2	TACTAAATGGCCTC GGCTTCCC	TAAGGAGGCACCCAAGC TACAG
GATA4	GTGTCCCAGACGT TCTCAGTC	GGGAGACGCATAGCCTT GT

GATA6	TGTAGAGCCCATCTTGACCC	TCCCCCACAACACAACCTAC
ISL1	TACAAAGTTACCAGCCACC	GGAAGTTGAGAGGACATTGA
MEF2C	TCCGAGTTCTTATTCCACC	ATCCTCCCATTCCTTGTC
NKX2.5	CCCTGACCGATCCCACCTCAAC	GGCGGGCGACGGCGAGATAGC
cTnI	TTCACCAAAGATCTGCTCCTCGCT	TTATTACTGGTGTGGAGTGGGTGTGG
cTnT	CACCAAGAACATCACGGAGATTGC	CGCTTAAACTTGCCTCGAAGGTC
ACTN1	GAAGAGGAGAGAAGCCCTAGAGAG	CTTGGCAAACTCCAGGTGAAGC
ACTN2	GAAGAGGAGAGAAGCCCTAGAGAG	CTTGGCAAACTCCAGGTGAAGC
α-MHC	GCATTCTCCTGCTGTTTCCTT	TGGATTCTCAAACGTGTCTAGTGA
β-MHC	GATCACCAACAACCCCTACG	GAAGCCCAGCACATCAAAAG
MLC2a	AGAAGCTCAATGGGACAGAC	CTTGTCTGCCTGGGTCAG
MLC2v	AGAACAGGGATGGCTTCATTGAC	TTTCACGTTCACTCGCCCAAG
Mesenchymal stem cell differentiation		
CD90	CACCCTCTCCGCACACCT	CCCCACCATCCCACTACC
ALCAM	AATGCTTAGGGAATGGCAACCC	TCTTATTCCTTCGGGCTGTCCTG
FOXF1	CAGCCGTATCTGCACCAGAA	ACTCCTTTCGGTCACACATGCT
HLX1	AGTTCCAAGACACGTTTCCAGGTC	TCCTCTGCAGGTTGGAGAACAC
EPHA2	CTGCCAGTGTCAGCATCAAC	TCTTGCGGTAAGTGACCTCG
TMEM4J	TCACAGCTGACCACCAGTACTACC	AGCAATCTGCCAATCGCTGCTG
NECTIN2	AAGTGGAGCATGAGAGCTTCGAG	ATGGACACTTCAGGAGGGTAGC
Pluripotency markers		
SOX2	GCCAGACGCCATGTTAAGTAAGTC	TGCCCGTTCTAGGCTTCTTGAG
NANOG	TAGCAATGGTGTGACGCAGAAG	TCTGGTTGCTCCACATTGGAAGG
Housekeeping genes		
GAPDH	TCCTGCACCACCAACTGCTTAG	TGGTCATGAGTCCTTCCACGATAC
YWHAZ	TCATCTTGGAGGGTCGTCT	GACTTTGCTCTCTGCTTGTG

Immunofluorescence Staining

Immunostaining for cardiomyocytes was performed using the Human Cardiomyocyte Immunocytochemistry Kit (Thermo Fisher Scientific, A25973) and additionally using following antibodies from Abcam: Anti-Sarcomeric Alpha Actinin antibody (ab9465), Anti-Cardiac Troponin I antibody (ab47003), Anti-heavy chain cardiac Myosin antibody (ab50967), Anti-Myosin Light Chain 2 antibody (ab79935) and Anti-MYL7 antibody (ab68086). Immunofluorescence analysis of MSC specific markers was performed using Mesenchymal Stromal Cell Marker (CD44, CD45, CD90 CD29, and CD105) Antibody Panel (Abcam # ab93758). Briefly, cells were fixed, permeabilized, blocked, successively incubated with a primary and a fluorescently-labelled secondary antibody (Alexa Fluor® 594 donkey anti-rabbit and Alexa Fluor® 488 donkey anti-mouse) and counterstained with DAPI nuclear DNA stain (Invitrogen # R37606). Imaging was performed using Olympus 1X73 inverted microscope and analysis was performed using Image J software.

Statistical Analysis

Data were presented as means ± EM. The significance of differences was tested by t-test. All statistical tests were performed with GraphPad Prism 6 (San Diego, CA, USA). P values of <0.05 were considered statistically significant.

RESULTS

Characterization of D149

To ensure the quality of starting iPSCs prior to differentiation experiments, we examined the pluripotency status and genomic integrity of our resource. Characterization of D149 was performed by alkaline phosphatase assay, immunocytochemistry and karyotype analysis. D149 stained positive for alkaline phosphatase activity and showed the expression of pluripotency markers including OCT4, SOX2, TRA-1-60 and SSEA-4, thus confirming pluripotency. Karyotype analysis showed normal karyotype (46, XY) without any apparent chromosomal abnormalities. Results are presented in **Figure 1**. D149 was tested for mycoplasma and other bacterial or fungal contamination at routine time point across multiple passage numbers and found to be contamination free.

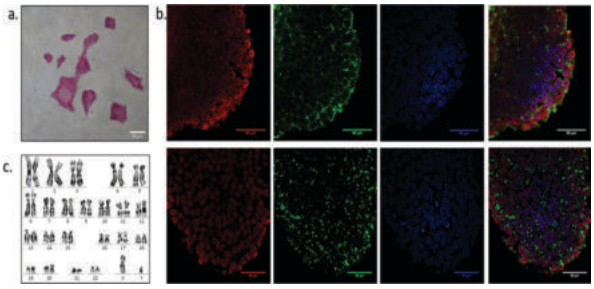


Figure 1. Characterization of D149

a) D149 iPSC colonies displayed high expression of alkaline phosphatase, characterized by reddish pink cells, indicating undifferentiated cells with self-renewal potential .Scale bar 50 µm b) Immunofluorescence staining showing presence of pluripotency markers OCT4, SOX2, TRA-1-60 and SSEA-4 in D149. Scale 50 µm c) Karyotype analysis of D149 showed a normal karyotype (46, XY).

Generation and characterization of D149 derived cardiomyocytes

The invitro cardiac differentiation process recapitulates step-wisely the early embryonic cardiogenesis process proceeding through sequence of events starting from mesoderm induction, patterning of mesoderm to cardiac mesoderm, differentiation to cardiac progenitors and further to functional cardiomyocytes. Each of these stages is characterized by distinct gene expression patterns consistent with in vivo cardiac development.

In this study, D149 was differentiated into cardiomyocytes for a period of 14 days under conditions described on the established protocol [56].A timeline of the cardiomyocyte differentiation protocol is represented in **Figure 2a**. This protocol is based on the temporal modulation of regulatory elements of Wnt/β-catenin signalling.The undifferentiated iPSCs were induced into mesoderm differentiation by treating with the Wnt activator CHIR99021 and subsequent cardiomyocyte differentiation by treating with the Wnt signaling inhibitors IWP2 and heparin. The progressive induction of cardiac mesoderm and cardiac-committed cells to cardiomyocytes was characterized by assessing expression of corresponding molecular markers.

Morphological analysis of D149 derived cardiomyocytes

During the differentiation of D149 to cardiomyocytes, significant changes in the cell morphology was observed upon addition of Wnt signalling modulators from day 0 until day 14. At day 1, following CHIR treatment, considerable amount of cell death was observed, which is a normal and essential step during the differentiation. From day 2, some cells started fusing formed branch- like structures and from day 5, differentiating cells began to form several clusters. The first beating was observed on day 10 within several patches. In the following days, gradual expansion of cell cluster sizes was observed with more synchronized beating sheets formed at day 14 (approximately 20-25 beats per minute). Representative light microscopy images demonstrating cell morphology changes in the induction of iPSCs to CMS is shown in **Figure 2b**.Representative video of beating cardiomyocytes derived from D149 after 14 days of differentiation is shown in Video1.

Characterization of D149-derived cardiomyocytes by real time PCR

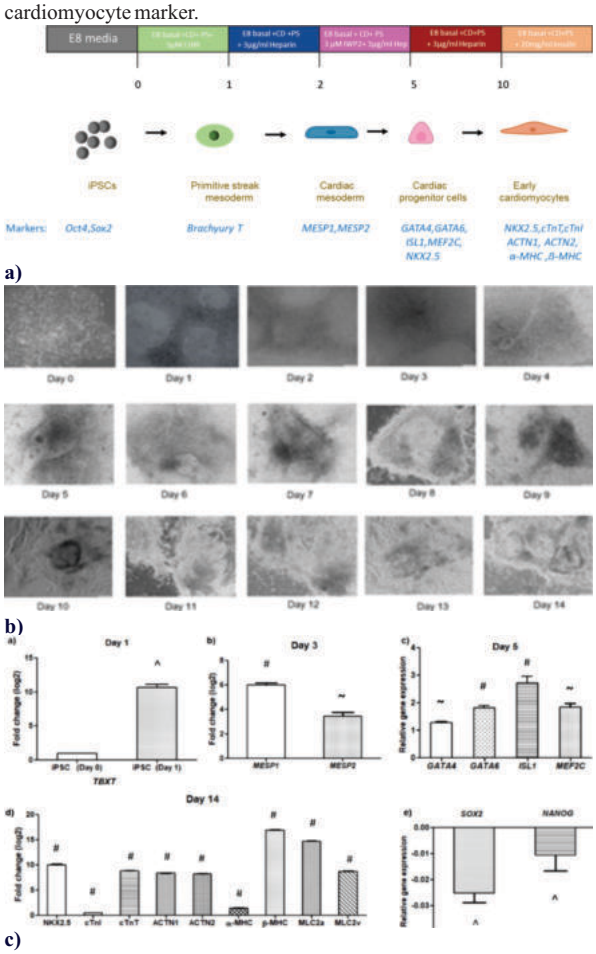
The gene expression status of distinct sets of cardiac-related transcription factors and cardiac- specific marker genes expressed in specific stages of iPSC differentiation to cardiomyocytes were assessed. The expression of markers for mesoderm (*TBXT*), early

cardiac mesoderm (*MESP1* and *MESP2*) and cardiac progenitor (*GATA 4*, *GATA 6*, *ISL1, MEF2C)* was determined at day 1,3 and 5 respectively. At day 14, the expression of cardiac markers was determined including cardiac homeobox transcription factor (*NKX2.5)* ,cardiac troponins (*cTnI* and *cTnT*), and cardiac structural proteins including cardiac actinins (*ACTN1* and *ACTN2*), cardiac-specific myosin heavy chain isoforms (α-MHC and β-MHC) and myosin light chain (MLC) 2 isoforms- *MLC2a* (atrial isoform of MLC2) and *MLC2v* (ventricular isoform of MLC2).In addition, the expression of pluripotent marker genes *SOX2* and *NANOG* was also assessed at day 14.

Results have shown that differentiating cells displayed significantly increased expression of *TBXT* (Day 1), *MESP1* and *MESP2* (Day 3), *GATA 4, GATA 6, ISL1, MEF2C* (Day 5), *NKX2.5, cTnT, cTnI, ACTN1, ACTN2*, α-*MHC*, β-*MHC*, *MLC2a* and *MLC2v* (Day 14) compared to undifferentiated iPSCs (Day 0). Also, *SOX2* and *NANOG* expression was significantly low in iPSC-CMs, compared to the corresponding undifferentiated iPSCs. **(Figure 2c)** Thus, our gene expression studies clearly indicated the presence of cardiac transcription factors and cardiac genes at each stage of cardiomyocyte differentiation. Increase in *TBXT* gene expression in differentiating D149 line indicated mesoderm induction. Increase in the expression of *MESP1* and *MESP2* indicated the formation of cardiac committed mesoderm and the expression of *GATA4, GATA6, ISL1* and *MEF2C* indicated the formation of cardiac progenitor cells. The increased expression of *NKX2.5*, *cTnT, cTnI, ACTN1, ACTN2, α-MHC, β-MHC, MLC2a* and *MLC2v* suggested the formation of early cardiomyocytes.

Characterization of D149 derived cardiomyocytes by immunofluorescence staining

At day 14, immunofluorescence staining was used to assess the expression of cardiac markers NKX2.5, cTnT, cTnI, -actinin, -MHC, MLC2a, and MLC2v in D149 derived early cardiomyocytes **(Figure 2d)**. Results have shown evident expression of markers NKX2.5, MYH7, cTnT, cTnI, α-actinin, and MLC2a in early cardiomyocytes, thus confirming the cardiac identity of D149 derived cardiomyocytes. However, the expression of late cardiomyocyte marker, MLC2v was not observed at day 14, which could be because MLC2v is a late cardiomyocyte marker.



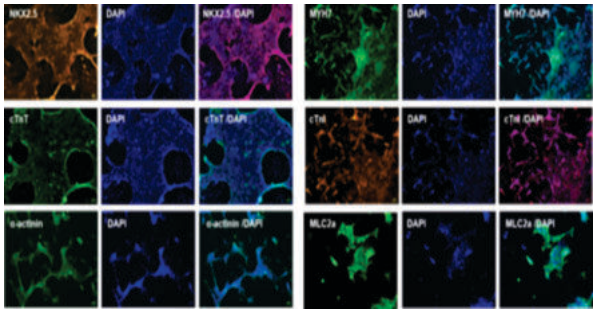


Figure 2. Differentiation of D149 into cardiomyocytes and characterization

a) Schematic illustration of experimental pipeline for the differentiation of iPSCs into early cardiomyocytes. b) Representative bright field images demonstrating cell morphology changes during differentiation of D149 iPSCs to early cardiomyocytes c) Real time PCR analyses of D149 derived early cardiomyocytes for mesoderm marker TBXT, cardiac mesoderm marker MESP1, MESP2, cardiac progenitor cell marker *GATA 4, GATA 6, ISL1, MEF2C* and the cardiac-specific marker genes *NKX2.5*, *cTnT, cTnI, ACTN1, ACTN2, α-MHC, β-MHC, MLC2a* and *MLC2v* . Symbols indicate significance from control (undifferentiated iPSC at day 0) with P value thresholds of <0.0001 (^), <0.005 (#), <0.01 (~), <0.05 (*) .d) Immunofluorescence staining for cardiac-specific markers NKX2.5, cTnT, cTnI, α-actinin, β-MHC, MLC2a. Scale bar shows 50 µm.

Representative video of beating cardiomyocytes derived from D149 after 14 days of differentiation. (as separate attachment)

Generation and characterization of D149 derived mesenchymal stem cells

To induce mesenchymal stem cell differentiation, D149 were cultured as described previously [39]. A timeline of the mesenchymal stem cell differentiation protocol is represented in **Figure 3a.**

Morphological analysis of D149 derived mesenchymal stem cells

During the differentiation state, the morphology of the cells rapidly changed from day 1 and continued until day 17. Cells were sub cultured on day 7 and were maintained up to passage 4. After day 7, the cells exhibited plastic adherence properties, which is a characteristic of mesenchymal stem cells. From day 10, cells started showing fibroblast like morphology with spindle shaped cells. As the number of passages increased, mesenchymal stem cell population displayed large and flattened cell morphology. Representative light microscopy images demonstrating cell morphology changes in D149 differentiation to MSCs are given in **Figure 3b**.

Characterization of D149 derived mesenchymal stem cells by real time PCR

D149 derived MSCs were subjected to characterization of mesenchymal markers (*ALCAM, CD90, EPHA2, FOXF1, HLX1, NECTIN2, TMEM47)* and pluripotency markers (*SOX2* and *NANOG)* at day 17 using real time PCR**(Figure 3c)**. Real time PCR data showed that the genes related to MSCs markers such as *CD90, EPHA2, FOXF1, HLX1* and *NECTIN2* were expressed at higher levels in D149 derived MSCs compared to undifferentiated state ,while no change in expression was observed in *ALCAM* and *TMEM47.*This was coupled with a reduction in expression of iPSC markers such as *SOX2* and *NANOG* in D149 derived MSCs. Decreased expression of pluripotency markers and increased expression of mesenchymal stem cell markers indicated that D149 iPSCs were successfully differentiated to mesenchymal stem cells.

Characterization of D149-derived mesenchymal stem cells by immunofluorescence staining

D149 derived mesenchymal stem cells were further interrogated by immunofluorescence for the presence of a panel of MSC markers including CD44, CD90, CD105 and CD29 as positive markers and CD45 as negative marker **(Figure 3d)**. Our results revealed that the D149-MSCs at day 17 were positive for the typical mesenchymal markers CD44, CD90, CD105 and CD29 but negative for the expression of hematopoietic marker CD45 thereby verifying the MSC identity.

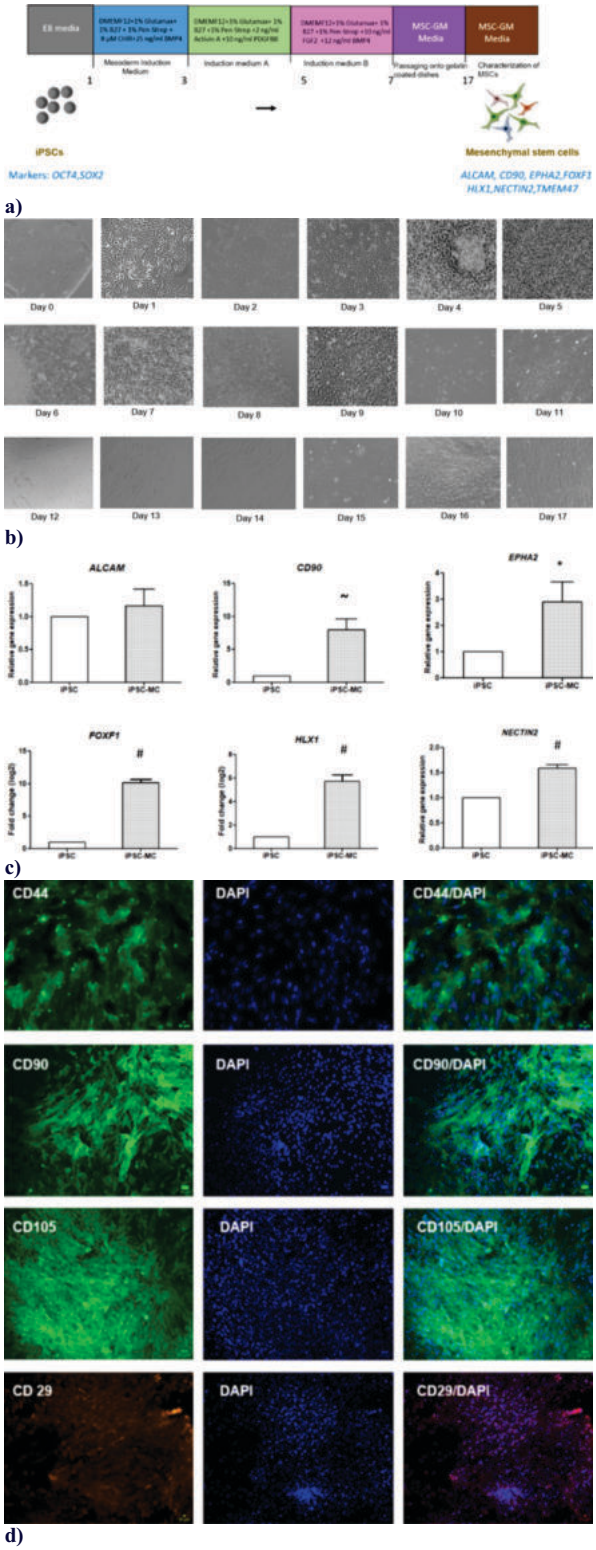


Figure 3: Differentiation of D149 into mesenchymal stem cells and characterization

a) Schematic illustration of experimental pipeline for the differentiation of D149 into MSCs b) Representative bright field images demonstrating cell morphology changes during differentiation of D149 iPSCs to MSCs c) Real time PCR analyses of D149 derived MSCs for specific marker genes(*ALCAM, CD90, EPHA2, FOXF1, HLX1, NECTIN2, TMEM47)* and pluripotency markers *SOX2* and *NANOG* .Symbols indicate significance from control (undifferentiated iPSC at day 0) with P value thresholds of <0.0001 (^), <0.005 (#), <0.01 (~), <0.05 (*) .d) Immunofluorescence staining for MSC markers (CD44, CD90, CD105 and CD29). Scale bar shows 50 µm.

DISCUSSION

In our present study, we report the generation and characterization of CMs and MSCs from D149, a well-characterized Indian control reference human iPSC line. For the generation of cardiomyocytes and mesenchymal stem cells from D149, we adopted established monolayer differentiation protocols based on chemical induction strategy. We performed preliminary characterization studies for the D149 derived early cardiomyocyte and mesenchymal stem cells.

For cardiomyocyte differentiation, we performed a heparin-based cardiac differentiation method, which is one of the simplest, efficient and cost-effective protocols reported till date. A recent study has identified heparin as a novel factor that significantly promotes cardiomyocyte differentiation efficiency. Heparin is known to inhibit Axin2, a downstream target of canonical Wnt signalling, at the later cardiac specification stage [56].We validated the role of heparin in cardiac specification by measuring Axin2 gene expression, and the inhibition of Axin2 by heparin was confirmed at the early cardiomyocyte stage (data not shown). Consistent with previous reports from the monolayer cultures of iPSC-CMs, our study showed that the early cardiomyocytes demonstrated spontaneous beating behaviour and the expression of several human CM-specific markers at mRNA and protein level.

To drive D149 iPSCs towards mesenchymal stem cell lineage, we adopted a well-established standard differentiation protocol based on application of growth factors and small molecules.Consistent with the minimal criteria for defining multipotent mesenchymal stromal cells by ISCT, D149 derived MSCs exhibited plastic adherent properties and expression of a subset of MSC specific markers.

Our studies showed that D149 iPSCs can be efficiently differentiated into multiple cell types and our choice of differentiation methodology is promising. To the best of our knowledge this is the first study to report the derivation of iPSC derived cell types from an Indian control line. Detailed characterization is required to confirm the functionality of D149 derived cell types. Strategies for cardiomyocyte maturation need to be adopted for D149 derived CMs and more functional studies need to be performed.The differentiation potential of D149 derived MSCs to form adipogenic, chondrogenic and osteogenic lineage must be evaluated. More functional characterization investigations of D149 derived cardiomyocytes and mesenchymal stem cells are now underway. Efforts are also being made to replicate the differentiation studies in more Indian control lines. We believe that future studies in these lines will shed valuable insights into the clinical application of these cell types.

D149 iPSC line is derived from a healthy donor belonging to Indian ethnicity, hence cell types derived from this line could serve as a standard reference for basic and translational stem cell research particularly in Indian population-based studies. D149 derived cell types can serve as suitable experimental controls for disease modeling studies. These cell models will undoubtedly aid the drug discovery and development efforts of Indian pharmaceutical, biotechnology, and contract research organizations. Furthermore, they can be used to develop customized therapies that successfully target the Indian population. In addition, iPSC derived CMs and MSCs can be used in high-throughput drug screening and assessing drug induced toxicity at the population level in preclinical and clinical development.

In conclusion, our study demonstrated generation and characterization of cardiomyocytes and mesenchymal stem cells from an Indian control iPSC line. The generated iPSC derived cell models represent a new resource for basic and translational research by the national and international stem cell research communities.

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Author Contributions

Conceived and designed the experiments: Mahendra Rao. Performed the experiments, analyzed the data and wrote the paper: Swathy Babu.

Conflicts of Interest

The authors declare no conflict of interest.

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