

Episomal reprogramming of Duchenne muscular dystrophy patients derived CD3⁺ T cells towards induced pluripotent stem cells

Mehwish Zehravi¹, Mohsin Wahid², Junaid Ashraf³

ABSTRACT

Objective: To derive Duchenne muscular dystrophy patient specific induced pluripotent stem cells (iPSCs) from CD3⁺T cells of DMD patients using episomal reprogramming and characterization of these DMD-iPSCs using immunofluorescence to confirm their pluripotent state.

Methods: DMD patients were selected based upon their clinical history and examination. Peripheral blood mononuclear cells were isolated from peripheral blood of DMD patients (n=3) by density gradient centrifugation and were used to generate DMD patient specific T cells (DMD-T cells) using rhIL-2, plate bound anti CD3 antibody and T cell specific media along with specific culture conditions that promote T cell expansion. CD3⁺ T cells were characterized by flow cytometry and reprogrammed using episomal plasmid to generate DMD-iPSCs. These DMD-iPSCs were characterized using immunofluorescence. The study was carried out at Dow Research Institute of Biotechnology and Biomedical Sciences during August 2017- July 2018 for a period of approximately 12 months.

Results: The peripheral blood mononuclear cells (PBMNC) derived T cells appeared as suspended cell clumps morphologically were positive for the expression of CD3 and negative for CD31. The DMD patient specific iPSCs appeared as round, compact and flat colonies with well-defined edges morphologically and were positive for the expression of pluripotency markers OCT4, SSEA-4 and TRA-1-81 on immunofluorescence.

Conclusion: CD3⁺ T cell derived DMD-iPSCs were obtained under feeder free and xeno-free culture conditions using episomal reprogramming.

KEYWORDS: Induced pluripotent stem cells, Episomal reprogramming, Duchenne muscular dystrophy.

doi: <https://doi.org/10.12669/pjms.37.2.3388>

How to cite this:

Zehravi M, Wahid M, Ashraf J. Episomal reprogramming of Duchenne muscular dystrophy patients derived CD3⁺ T cells towards induced pluripotent stem cells. *Pak J Med Sci.* 2021;37(2):432-438. doi: <https://doi.org/10.12669/pjms.37.2.3388>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

- Mehwish Zehravi,
Dow Research Institute of Biotechnology and Biomedical Sciences,
 - Mohsin Wahid,
Dow Research Institute of Biotechnology & Biomedical Sciences and
Department of Pathology, Dow International Medical College,
 - Junaid Ashraf (Rtd)
Department of Neurosurgery,
- 1-3: Dow University of Health Sciences, Karachi, Pakistan

Correspondence:

Dr. Mohsin Wahid, MBBS, MPhil (Eng), PhD Eng)
Tenure Track System, Assistant Professor,
Department of Pathology,
Dow International Medical College,
Dow University of Health Sciences, Karachi, Pakistan.
E-mail: mohsin.wahid@duhs.edu.pk

- * Received for Publication: July 29, 2020
- * 1st Revision Received: August 11, 2020
- * 2nd Revision Received: November 27, 2020
- * Final Revision Accepted: December 5, 2020

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal disorder with out of frame mutations in dystrophin gene which impairs the production of dystrophin protein responsible for muscle strength and contractility. It is inherited in an X linked recessive manner and particularly affects boys. Deficiency of dystrophin protein results in inefficient linkage between intracellular actin and extracellular laminin resulting in muscle fragility, inefficient contractile ability, contraction induced damage, necrosis and inflammation at the site of damage. DMD is fatal amongst all muscular dystrophies. The muscle weakness begins early

between two to five years of age and the child becomes wheel chair bound by 10 - 14 years of age. These children suffer from respiratory and cardiac complications during the second decade of life. Death occurs around second or third decade due to left ventricular dysfunction and respiratory failure.¹

The development of stem cell research has been a major breakthrough in the field of biomedical research. Induced pluripotent stem cells (iPSCs) are obtained by genetic reprogramming of adult cells and tissues to an embryonic stem cell like state by forced expression of several genes and transcription factors. Yamanaka proposed the concept of induced pluripotency to generate stem cells similar to embryonic stem cells without utilizing egg or embryo thus avoiding ethical concerns. This revolutionary experiment was first performed in mice in 2006 and later was replicated in humans using fibroblast as cell source.^{2,3} The major shortcoming of both these experiments was the requirement of surgical intervention for isolating human fibroblast which makes it impractical source for reprogramming. In contrast to it, T cell obtained by peripheral blood is a most suitable approach to derive induced pluripotent stem cells as venipuncture is a non-invasive technique and is routinely carried out as a blood sampling procedure. In our study we have used T cells as a cell source for generating iPSCs. T cells are comparatively abundant in peripheral blood (6.5×10^5 - 3.1×10^6 /ml).⁴ Using the T cells as a cell source for generation of induced pluripotent stem cells would circumvent the issue of obtaining punch biopsies from patients.

Integration free iPSCs derived by episomal reprogramming are comparatively safer alternative and can serve as disease models and as clinical grade cells for cell therapies in future. Integration free reprogramming is also referred to as foot print free reprogramming. Since it is devoid of genomic integration there is a lower incidence of genomic aberrations and the safety concerns with the use of viral vectors are minimized.⁵ Our study aimed to derive iPSCs from peripheral blood T-cells of DMD patients using episomal plasmid in a feeder free and xeno free cell culture system followed by their characterization using immunofluorescence. The derivation of iPSCs through this approach will open many avenues for future therapeutic applications.

METHODS

The study was started after getting approval from the Institutions Review Board of Dow University of Health Sciences (IRB-771/DUHS/Approval/2016/293, Dated: September 21st, 2016). Informed consent was signed by patient's guardian before sample collection. The study was carried out at Dow Research Institute of Biotechnology and Biomedical Sciences during August 2017- July 2018 for a period of approximately 12 months.

Sample Collection: The study participants were recruited from various hospitals and rehabilitation centers in Karachi. Patients between 2-20 years of age with clinical suspicion of DMD, raised CPK levels and myopathic potential on EMG were included in the study.

Isolation of peripheral blood mononuclear cells from peripheral blood: Peripheral blood mononuclear cells (PBMNC) were separated from whole blood by density gradient centrifugation. PB was mixed with Phosphate buffered saline (PBS, AM9625, Invitrogen) in equal quantity and layered very slowly on ficoll (GE17-5442-02, GE health care) in ratio 1:1:1. The sample was then centrifuged (Eppendorff, 5810R) at 800g for 20 min without brakes at room temperature. This resulted in layers of separation having PBMNCs. This was followed by another round of centrifugation for washing. The cell pellet containing PBMNC was resuspended in complete AIM-V media (12055-091, Invitrogen). The AIM-V media has been formulated for the growth of T cells and contains L- glutamine, streptomycin sulfate at 50 µg/ ml and gentamycin sulfate at 10 µg/ml. Cell viability analysis of PBMNC suspension was done using cell viability analyzer (Beckman Coulter Vi Cell XR).

Culture of PBMNC to derive T cells: PBMNC were grown in culture at 5 % CO₂ at 37°C in incubator (New Brunswick Galaxy 170 R CO₂ Incubator) for five days for T cell derivation. Approximately, 10×10^5 cells were cultured in freshly prepared AIM-V media (12055-091, Invitrogen) with 300 IU/ml rhIL-2 (10799068001, Sigma Aldrich) and 10ng/ml plate bound anti CD3 antibody (OKT-3 clone, 16-0037-81, eBioscience)⁶ The cells were observed daily under microscope (Leica DMI1 microscope, LAS v 4.6.1) and allowed to grow in the above mentioned culture condition for 5 days. On day 5, T cells were characterized by flow cytometry and then used for reprogramming.

Characterization of T-cells using flow cytometer: Immunophenotyping of PBMNC derived T cells was done on day 5 using B.D flow cytometer (B.D FACS Celesta, FACS Diva software version-8). Approximately 1×10^6 PBMNC derived T cells were used for immunophenotyping. CD3 antibody (561800, BD Pharmingen) was used as a positive marker and CD31 antibody (560984, BD Pharmingen) was used as a negative marker for characterization of PBMNC derived T cells.

DMD patient specific iPSCs generation and culture: Approximately 2.5×10^5 T- cells were transfected with Epi5 episomal reprogramming vectors (A15960, Invitrogen) using neon transfection system (MPK5000, Thermo Fisher Scientific). Post transfection the sample was transferred into a vitronectin coated culture plate containing pre warmed complete AIM-V media. Post transfection microscopy was done and images were captured. The plate was kept in 5 % CO₂ incubator at 37°C. The next day media was switched to N2B27 media containing DMEM/F12 (11330-032, Life technologies), N2 supplement (17502-048, Life technologies), B27 supplement (17504-044, Life technologies), MEM Non- Essential Amino Acid (11140-050,

Life technologies), GlutaMAX-I (35050-061, Life technologies), B-Mercaptoethanol (21985-023, Life technologies) with freshly added 100 ng/ml bFGF (PHG0264, Life technologies). From day 2 post transfection N2B27 media was changed every day until day 8 post transfection and then replaced with Complete E8 media (A1517001, Gibco) every day until the iPSC colonies grew to an appropriate size for transfer. The Complete E8 media consists of Essential 8 Basal medium and E8 supplement (A1517001, Gibco).

Characterization of iPSC colonies using Immunofluorescence: The iPSC colonies were fixed using a combination of Acetone & Methanol (1:1), blocked in 2 % BSA (15260037, Gibco) in PBS for 30 minutes. Cells were stained with primary antibodies provided in ES cell marker kit (SCR002, Chemicon). Primary antibodies included OCT4 (MS x OCT4, IgG, clone 10H11.2, Chemicon, USA), SSEA4 (MS x SSEA-4, IgG, clone MC-813-70, Chemicon, USA) and TRA-1-81 (MS x TRA-1-81, IgM, clone TRA-1-81, Chemicon, USA). Primary antibody dilutions were made in blocking solution in the ratio of 1: 50 and dispensed onto the marked iPSC colony. The primary antibodies were incubated for one hour at room temperature in the dark. After

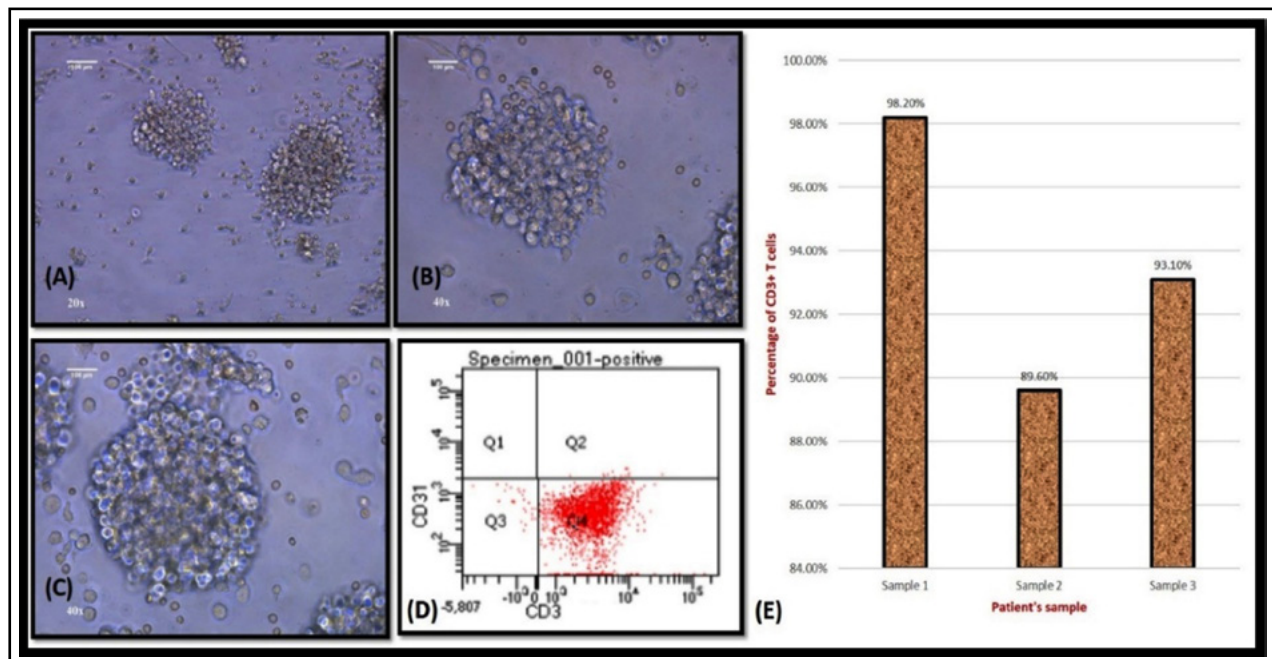


Fig.1: showing the morphology of PBMNC derived T cells using Leica DMi1 Inverted phase contrast microscope, immunophenotyping results using flowcytometry and the percentage of CD3⁺ T cells in n = 3 samples. (A) T cell morphology at day 5 post plating (20 X) (B) T cell morphology at day 5 post plating (40 X). (C) T cell morphology at day 5 post plating (40 X). Scale bar indicates 100 μ m. (D) The dot plot shows that the PBMNC derived T cells were positive for the expression of CD3 and negative for the expression of CD31. (E) The percentage of CD3⁺ T cells in n = 3 samples.

incubation with primary antibody washing was done thrice with 1X rinse buffer 10 minutes each. This was followed by incubation with secondary antibodies. The secondary antibody used for OCT4 and SSEA4 was FITC labelled anti human IgG secondary antibody (goat) (AF102-0115, Euroimmun US) and the secondary antibody used for TRA-1-81 was Texas Red goat anti mouse IgM secondary antibody (NBP1-73626, Novas). The fixed and stained iPSC colonies were visualized using inverted fluorescent microscope Leica DMI-8 (LAS v 4.8) and images captured.

RESULTS

Culture of PBMNC to derive T cells: Under specific culture conditions as mentioned above the T cells enlarged and formed clumps. By day 3 visible T cell clumps were seen. Morphologically, the T cell

clumps appeared as suspended cell clumps and were weakly attached to the bottom of the plate (Fig.1 A-C). Inverted phase contrast microscopy using Leica DMI1 was done every day to assess the growth and proliferation of T cells. The number of T cell clumps increased during the next two days. On day 5, the number of T cell clumps were counted and used for downstream experiments.

Characterization of PBMNC derived T- cells using flow cytometry: The PBMNC derived T cells were found to be positive for the expression of CD3 and negative for the expression of CD31 (Fig.1 D). By day 5, the percentage of CD3 positive T cells had increased significantly. The percentage of CD3⁺ T cells was 98.2% in sample 1, 89.6% in sample 2 and 93.1% in sample 3. The average percentage of CD3⁺ T cells was between 89.6% - 98.2% in n = 3 samples (Fig.1 E).

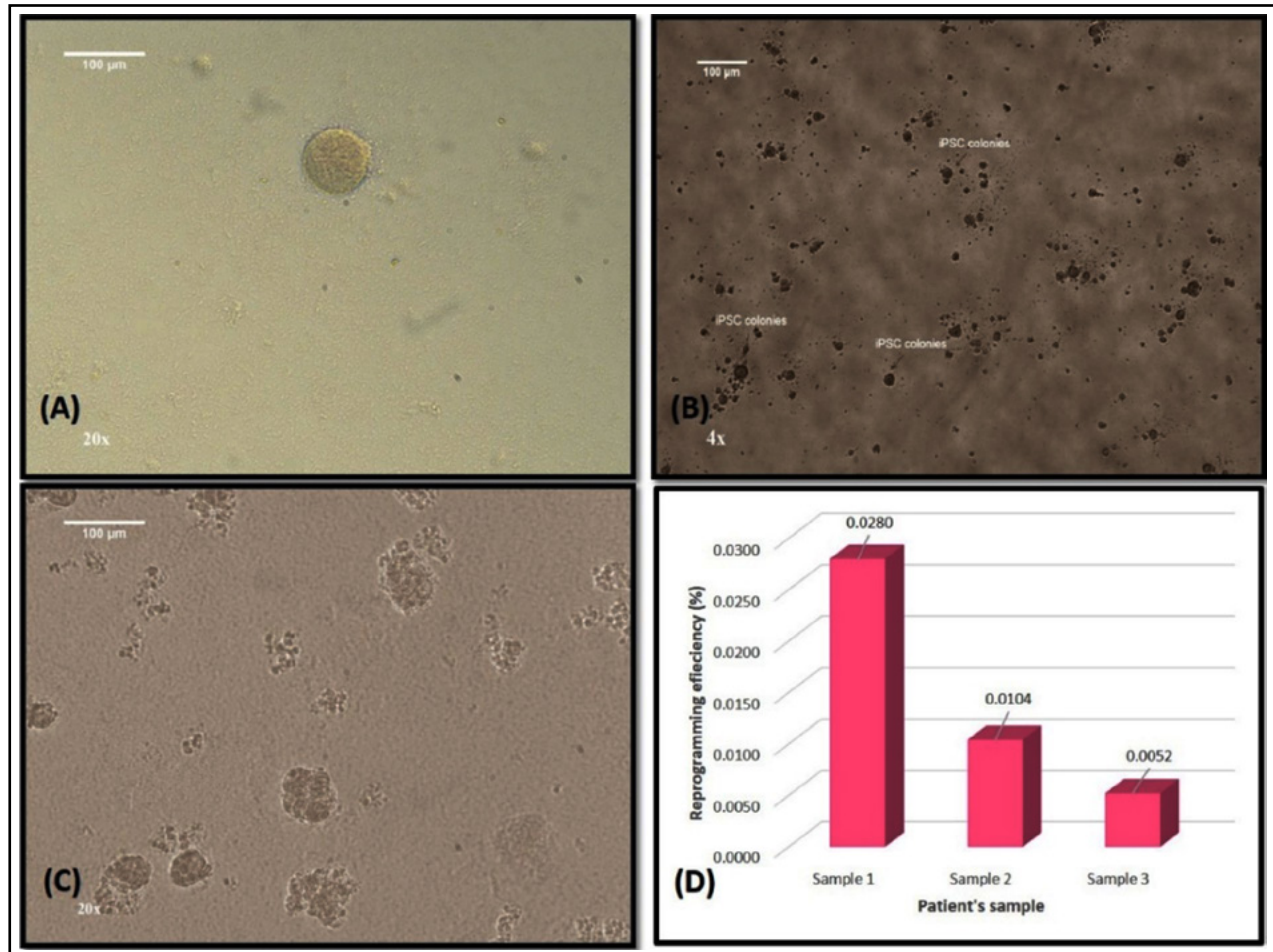


Fig.2: Showing characteristic morphology of human T cell derived iPSCs using Leica DMI1 Inverted phase contrast microscope and reprogramming efficiency in n=3 samples. (A) iPSC colonies at 20X magnification. (B) Representative images of human iPSCs morphology after colony picking 4X and (C) 20X respectively. Scale bar 100 µm. (D) Reprogramming efficiency in n = 3 samples.

DMD patient specific iPSCs generation and culture: The iPSCs resemble embryonic stem cells in morphology. The iPSCs appeared as round, compact and flat colonies with well-defined edges (Fig.2 A). The cellular features include small, round cells with high nucleus: cytoplasm ratio, prominent nucleoli and scant cytoplasm. The iPSCs colonies first appeared on D27 post transfection in sample 1. In sample 2, iPSCs colonies were first seen on D16 post transfection. However, in sample 3, iPSCs colonies were seen on D9 post transfection. Therefore, in n = 3 samples, the iPSCs colony appearance varied from D9- D27 post transfection. The scoring of iPSC colonies was not done as it was not required. However, the reprogramming efficiency was calculated as shown in Figure 2D by dividing all the iPSCs colonies appeared by the number of cells transfected. In n = 3 samples, the average reprogramming efficiency was 0.016 % which is consistent with the literature (Fig.2 D). The iPSCs

colonies that were picked were all undifferentiated. E8 media was used specifically for this purpose, so that the iPSCs do not differentiate and can be expanded in undifferentiated state.

Characterization of DMD patient specific iPSCs through immunofluorescence: The T cell derived iPSCs colonies were positive for the expression of pluripotency markers OCT4, SSEA4 and TRA-1-81. DAPI was used as a nuclear stain to stain the nucleus (Fig.3 A-I).

DISCUSSION

The patient specific iPSCs are a new therapeutic option for patients as they can escape immune surveillance and are a better therapeutic alternative to embryonic stem cells. The primary goal of our research was to generate DMD patient specific iPSCs with a hope to utilize these iPSCs and its derived cells for future personalized medical remedies.

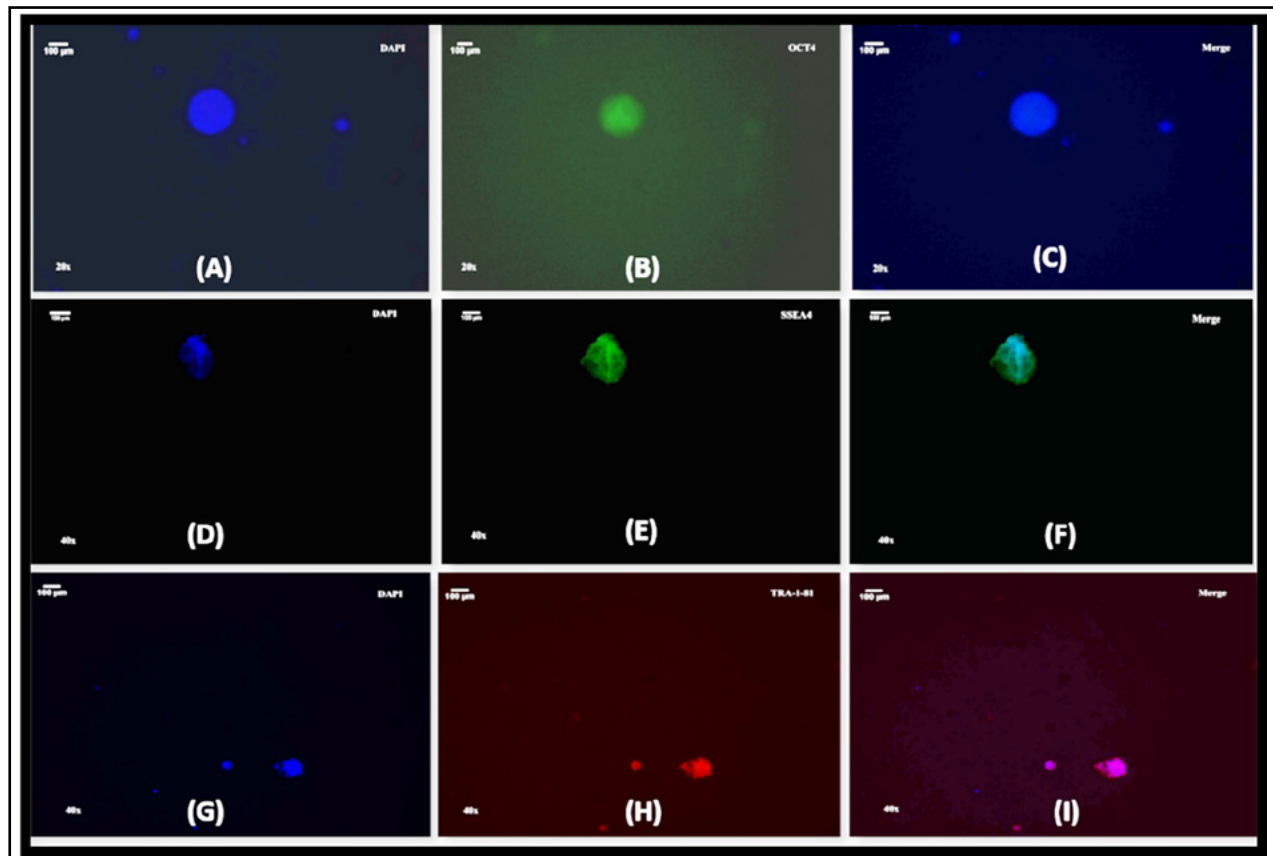


Fig.3: showing immunofluorescence staining depicting protein expression of pluripotency markers in iPSCs colonies (A) DAPI used to stain nuclei (blue). (B) The iPSCs colonies were stained positive for pluripotency markers OCT4, (green). (C) merge image for OCT4 staining. (D) DAPI used to stain nuclei (blue). (E) The iPSC colonies were stained positive for pluripotency markers SSEA4 (green). (F) merge image for SSEA4 staining. (G) DAPI used to stain nuclei (blue). (H) The iPSCs colonies were stained positive for pluripotency marker TRA-1-81 (red). (I) merge image for TRA-1-81 staining. Scale bar indicates 100µm.

Peripheral blood T cells have been labeled as a most convenient, suitable, minimally invasive approach to generate iPSCs as compared to other cell sources. It would increase the likelihood of patients giving consent for sampling due to minimal sample requirement. We successfully isolated sufficient T cells from only five ml peripheral blood of DMD patients and reprogrammed these T cells to iPSCs. The ease of in vitro proliferation makes it an ideal cell source for reprogramming. In contrast to it, skin fibroblast and dental pulp cells take around 3-4 weeks to establish primary culture.⁶ Considering these factors we have used a better alternative to generate iPSCs as compared to other cell sources. Peripheral blood T lymphocytes of healthy individuals have been used to generate iPSCs using Sendai virus.⁷ Induced pluripotent stem cells have been generated from peripheral blood mononuclear cells (PBMNC) using non integrating Sendai virus in normal individuals.⁸

As per our literature review, there have been no studies on derivation of human iPSCs in Pakistan. One study identified deletions in dystrophin gene of Pakistani DMD patients⁹ In another study mutation analysis was performed on 2 families in Baluchistan to identify mutations in the distal hotspot region of DMD gene using Sanger sequencing¹⁰ Retrospective studies have been conducted on dystrophinopathy patients at Aga Khan University Hospital Karachi utilizing MLPA to screen dystrophin gene exons for deletions/duplications.^{11,12} But no study so far has focused on derivation of iPSCs from these patients for future invitro disease modeling.

EBNA1 based episomal vectors have been used for reprogramming different cell types including fibroblasts¹³, urinary epithelial cells¹⁴, amniotic fluid cells¹⁴, CD34⁺ cells¹⁵, adult peripheral blood mononuclear cells^{16,17}, dental pulp cells¹⁸ and T cells.^{6,19}

To overcome this issue we have generated iPSCs in a feeder free and xeno free culture conditions. We have utilized human recombinant vitronectin and E8 medium for generation of iPSCs eliminating the need for feeder cells and animal derived proteins. Vitronectin is a xeno free cell culture matrix that provides a defined surface for feeder free culture of human pluripotent stem cells.^{16,20}

The criteria for characterization of iPSCs is based upon their morphology and expression of pluripotency markers. The iPSCs appear as round, compact and flattened tightly packed colonies

containing cells with high nucleus to cytoplasmic ratio. The colonies have distinct borders and sharp edges.^{3,15,17} The derived iPSCs should be positive for the expression of pluripotency markers like OCT4, SOX2, NANOG, LIN28, SSEA-3, SSEA-4, TRA-1-60.²¹ The iPSCs derived by the episomal reprogramming of T cells from Pakistani Duchenne Muscular dystrophy patients were morphologically similar to the previously published data.

Limitation of the Study: For characterization of PBMNC derived T cells antibodies other than CD3 antibody could have been used but due to time and budget constraints it was not possible.

CONCLUSION

We were successful in developing a baseline strategy for deriving DMD patient specific iPSCs from peripheral blood T cells of DMD patients using a feeder free and xeno free cell culture system. The method will be helpful in future for developing clinical grade iPS cell lines for therapeutic application in patients suffering from DMD and other diseases. The iPSCs models can also be used for drug screening to develop personalized therapy for each patient in future.

Conflict of Interest: None.

Grant Support & Financial Disclosures: None.

REFERENCES

1. Govoni A, Magri F, Brajkovic S, Zanetta C, Faravelli I, Corti S, et al. Ongoing therapeutic trials and outcome measures for Duchenne muscular dystrophy. *Cell Mol Life Sci.* 2013;70(23):4585-602. doi: 10.1007/s00018-013-1396-z
2. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126(4):663-676. doi: 10.1016/j.cell.2006.07.024
3. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-872. doi: 10.1016/j.cell.2007.11.019
4. Brown ME, Rondon E, Rajesh D, Mack A, Lewis R, Feng X, et al. Derivation of induced pluripotent stem cells from human peripheral blood T lymphocytes. *PLoS One.* 2010;5(6):e11373. doi: 10.1371/journal.pone.0011373
5. Yu J, Chau KF, Vodyanik MA, Jiang J, Jiang Y. Efficient feeder-free episomal reprogramming with small molecules. *PLoS One.* 2011;6(3):e17557. doi: 10.1371/journal.pone.0017557
6. Tsai PH, Chang YC, Lee YY, Ko YL, Yang YH, Lin CF, et al. Differentiation of blood T cells: reprogramming human induced pluripotent stem cells into neuronal cells. *J Chinese Med Assoc.* 2015;78(6):353-359. doi: 10.1016/j.jcma.2015.03.007

7. Netsrithong R, Promnakhon N, Boonkaew B, Vatanashevanopakorn C, Pattanapanyasat K, Wattanapanitch M. Generation of two induced pluripotent stem cell lines (MUSi011-A and MUSi011-B) from peripheral blood T lymphocytes of a healthy individual. *Stem Cell Res.* 2019;39:101487. doi: 10.1016/j.scr.2019.101487
8. Vlahos K, Sourris K, Mayberry R, McDonald P, Bruveris FF, Schiesser JV, et al. Generation of iPSC lines from peripheral blood mononuclear cells from 5 healthy adults. *Stem Cell Res.* 2019;34:101380. doi: 10.1016/j.scr.2018.101380
9. Hassan MJ, Mahmood S, Ali G, Bibi N, Waheed I, Rafiq MA, et al. Intragenic deletions in the dystrophin gene in 211 Pakistani Duchenne muscular dystrophy patients. *Pediatr Int.* 2008;50(2):162-166. doi: 10.1111/j.1442-200X.2008.02538.x
10. Zeeshan M, Khan SZ, Ajmal N, Asif M, Shehzad P, Shah SA, et al. DMD Gene Mutation identification in Duchenne Muscular Dystrophy Patients of Quetta, Balochistan, Pakistan. *BioCell.* 2019;43(5).
11. Ahmed Z. Spectrum of dystrophin gene mutations observed in patients suspected of Duchenne muscular dystrophy in Pakistan. *Clin Med.* 2019;19(Suppl3):32. doi: 10.7861/clinmedicine.19-3s-s32
12. Ansar Z, Nasir A, Moatter T, Khan S, Kirmani S, Ibrahim S, et al. MLPA Analyses Reveal a Spectrum of Dystrophin Gene Deletions/Duplications in Pakistani Patients Suspected of Having Duchenne/Becker Muscular Dystrophy: A Retrospective Study. *Genet Test Mol Biomarkers.* 2019;23(7):468-472. doi: 10.1089/gtmb.2018.0262
13. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science.* 2009;324(5928):797-801. doi: 10.1126/science.1172482
14. Drozd AM, Walczak MP, Piaskowski S, Stoczynska-Fidelus E, Rieske P, Grzela DP. Generation of human iPSCs from cells of fibroblastic and epithelial origin by means of the oriP/EBNA-1 episomal reprogramming system. *Stem Cell Res Ther.* 2015;6(1):122. doi: 10.1186/s13287-015-0112-3
15. Mack AA, Kroboth S, Rajesh D, Wang WB. Generation of induced pluripotent stem cells from CD34+ cells across blood drawn from multiple donors with non-integrating episomal vectors. *PloS One.* 2011;6(11):e27956. doi: 10.1371/journal.pone.0027956
16. Chou B-K, Gu H, Gao Y, Doweiy SN, Wang Y, Shi J, et al. A facile method to establish human induced pluripotent stem cells from adult blood cells under feeder-free and xenofree culture conditions: a clinically compliant approach. *Stem Cells Transl Med.* 2015;4(4):320-332. doi: 10.5966/sctm.2014-0214
17. Doweiy SN, Huang X, Chou B-K, Ye Z, Cheng L. Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. *Nat Protoc.* 2012;7(11):2013-2021.
18. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, et al. A more efficient method to generate integration-free human iPS cells. *Nature Methods.* 2011;8(5):409-412.
19. Matsumoto T, Fujimori K, Andoh-Noda T, Ando T, Kuzumaki N, Toyoshima M, et al. Functional neurons generated from T cell-derived induced pluripotent stem cells for neurological disease modeling. *Stem Cell Rep.* 2016;6(3):422-35. doi: 10.1016/j.stemcr.2016.01.010
20. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, et al. Chemically defined conditions for human iPSC derivation and culture. *Nature Methods.* 2011;8(5):424-429.
21. Haase A, Olmer R, Schwanke K, Wunderlich S, Merkert S, Hess C, et al. Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell.* 2009;5(4):434-441. doi: 10.1016/j.stem.2009.08.021

Author's Contribution:

MW; Designed and supervised the MPhil Student project. All work including cell culture, Flowcytometry, Cellular reprogramming using Episomal Plasmids and immunofluorescence was done under his supervision.
MZ: MPhil student who carried out the experimental work under supervision.
JA: Co-supervisor for the MPhil Project.