Integration-free Genetic Reprogramming of Human Umbilical Vein Endothelial Cells Towards Induced Pluripotent Stem Cells

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Abstract:

Induced Pluripotent Stem Cells (iPSCs) have the potential to be differentiated into any cell type and play an important role in regenerative medicine and cell therapy. The objective of this study includes the integration-free genetic reprogramming of human umbilical vein endothelial cells (HUVEC) from the cord towards iPSCs followed by molecular characterization of the obtained iPSCs for pluripotency markers. HUVEC were cultured and characterized by flow cytometry using the cell surface markers CD105 and CD45. HUVEC were subjected to integration-free cellular reprogramming with episomal plasmids carrying OCT-4, SOX-2, KLF-4, L-MYC, and LIN-28, using the Neon Transfection System for electroporation. Molecular characterization of iPSCs was done by immunofluorescence staining for the nuclear marker OCT-4 and the cell-surface marker TRA-1-81. The HUVEC-derived iPSC colonies started appearing after one week and exhibited a flat appearance with well-defined, rounded edges and tightly packed cells in the center. The iPSCs showed immunofluorescence for the positive pluripotency markers OCT-4 and TRA-1-81. Episomal non-viral-based integration-free and transgene-free cellular reprogramming is a suitable and efficient method for reprogramming cord-derived HUVEC towards iPSCs. These iPSCs can potentially be used for investigating molecular mechanisms in *in vitro* disease modeling, drug testing, and drug discovery, in addition to their use in Regenerative Medicine and cell therapy.

Keywords: Cellular reprogramming, episomal plasmids, human umbilical vein endothelial cells, induced pluripotent stem cells.

1. INTRODUCTION

Stem cells are the cells that are defined by their unique properties, that they can renew themselves and can give rise to differentiated cell types [1, 2]. Stem cells may be classified into adult stem cells and embryonic stem cells (ESCs) based on their origin. ESCs are isolated from the inner cell mass of the blastocyst at the 3-5-day stage. There are ethical issues surrounding the use of embryos and strict regulations only allow the use of redundant embryos from *in vitro* fertilization clinics [3]. Disease-specific cells are obtained through either spontaneous mutations or genetic engineering. On the other hand, adult or tissue stem cells reside at specific locations in humans and replicate to make up for cell death but have limited replicative potential. Therefore, human iPSCs generated through the cellular reprogramming of adult somatic cells, help overcome the limitations faced by the former cell types [4]. The somatic cells are genetically reprogrammed back to an ESC-like state, by the expression of genes essential for sustaining ESC properties [5].

These iPSCs are a very useful tool for developmental studies, disease modeling, drug discovery drug testing, toxicology screening, regenerative therapy, and novel cell therapy approaches [6]. Scientists have established iPSCs *in vitro* using a wide range of adult cell types using integrative and non-integrative methods. Although integrative viral methods (Lentivirus and Retrovirus) involving the integration of foreign genetic material into host chromosomes are more efficient, they may cause tumorigenesis and immunogenicity, thereby limiting the therapeutic usefulness of the generated iPSCs [7-10]. Non-integrative methods include the use of non-integrating viruses (Sendai virus and Adenovirus), as well as non-viral episomal plasmids, synthetic RNAs, and recombinant proteins. The episomal method is non-integrating and non-viral and yields safer foot-print-free iPSCs, but its transfection efficiency is not as high as the integrative method [11].

Previous studies have demonstrated the generation of integration-free iPSCs through various methods employing episomal vectors, Adenoviral vectors, Sendai viral vectors, synthetic mRNAs, miRNAs, proteins, and small molecules. Most of these methods have limitations, such as low reprogramming efficiency or a requirement for consecutively repeated application of transfection agents [12, 13]. Episomes are non-integrating plasmids that can serve as vectors for the delivery of genetic material into cells. In this study, we used episomal vectors for delivering reprogramming factors in a simple, reproducible, and efficient manner [14]. A single episode of transfection was sufficient and the risk of insertional mutagenesis and subsequent tumor generation was low [15].

Since, Sendai viral-based reprogramming kits mostly included c-myc as a reprogramming factor which is a proto-oncogene, in this study, we used electroporation as the method of choice for achieving an optimal balance between transfection efficiency and safety of the generated iPSCs that is necessary for downstream applications like regenerative medicine [16].

Electroporation is a technique that increases cell membrane permeability using short high-voltage electrical pulses, enabling the entry of genetic material into the cell. Electroporation is a consistent, efficient, and reproducible method of gene delivery compared to other non-viral methods [17].

2. MATERIAL AND METHODS

Methods:

2.1 Ethical Approval

This experimental *in vitro* study was conducted at the Stem Cells and Regenerative Medicine Lab at Dow Research Institute of Biotechnology and Biomedical Sciences (DRIBBS), Dow University of Health Sciences (DUHS), Pakistan. Ethical approval was taken from the Institutional Review Board of DUHS (IRB-1324/DUHS/Approval/2019).

2.2 HUVEC isolation and Culture

Umbilical cords which are normally discarded after caesarean sections were collected from the Obstetrics wards of Dow University Hospital. The umbilical cord was collected in a sterile falcon and the outer surface of the umbilical cord was cleaned with alcohol swabs to eliminate the excess blood and debris. The umbilical cord was compressed lightly to eliminate any clots that had formed inside the vein. To ensure maximum sterility the cord was cut around 1-2 cm from both ends. A 21-gauge butterfly needle was inserted into the vein, which had the largest opening and thick lumen as compared to the arteries. Once inserted, the butterfly needle was clamped with a hemostat and then a 30cc syringe containing DPBS (1x) was attached to the needle. The umbilical vein was washed with DPBS to eliminate excess blood such that no blood or clot is visible. After washing the 12cm umbilical cord, the 30cc syringe was disconnected and a 10cc syringe was connected containing 10 ml collagenase type IV (0.2% in DPBS) to the butterfly needle. The collagenase solution was injected into the vein and the clamp was used with a surgical clip to stop leakage at the other end. The cord was incubated for 25 mins at 37°C. After incubation, the vein was washed with a culture medium containing 10 ml EBM-2 containing FBS to stop the proteolytic activity of the collagenase solution. The cells were collected in a sterile falcon tube and centrifuged for 5 min at 800 x g. After centrifugation, the supernatant was aspirated off and the pellet was resuspended in 5 ml EBM-2 supplemented with streptomycin (100 µg/ml), penicillin (100 units/ml), and FBS (10%). The cells were plated in a T-25 tissue culture flask and then incubated at 37°C in the presence of 95% humidified air and 5% CO_2.After 24 hours, the T-25 flask was removed from the incubator and the non-adherent cells were removed by changing the culture medium. The cells were washed with DPBS. The spent media was aspirated off from the flask and the cells were washed with 1X DPBS. Trypsin-EDTA was added into the flask and incubated at 37°C. The cell suspension was aspirated and EBM-2 media was added. The cell suspension was centrifuged at 800×g for 5 minutes. The pellet was resuspended in EBM-2 media and cells were plated as P1 cells. Regular phase contrast microscopy was done and cells were culture for 6 days until 70-80% confluence was achieved. The HUVECs were trypsinized and expanded till P5.

2.3 Multicolour Flow Cytometry of HUVEC

Immunophenotyping of HUVEC was done through Multicolour Flow Cytometry (BD FACS Celesta). Fluorochrome labelled antibodies against cell surface antigens were used, with CD105 as positive and CD45 as negative marker (BD PharMingen), in order to confirm the molecular signature of HUVEC. The data analysis was carried out with FACS Diva software 8.0. One million cells per sample were used. The cells were resuspended in 1000µL PBS and transferred to a FACS-labelled tube. Fluorochrome-labelled antibodies CD105 and CD45 were added according to the manufacturer's

instructions and incubated the cells in the dark for 20-30 minutes at room temperature. After incubation, the cells were examined under flow cytometry. The gating strategy was designed using the unstained sample.

2.4 Transfection of HUVEC

HUVEC were transfected with OriP/EBNA-1 (Origin of plasmid replication / Epstein-Barr nuclear antigen-1) based episomal vector in the Epi5 Reprogramming Kit (Invitrogen; A15960); carrying the reprogramming factors OCT-4, SOX-2, KLF-4, L-MYC and LIN-28. Electroporation (Nucleofection) was done using the Neon transfection system (Life Technologies; MPK5000) and a single pulse of 1350V was applied for 30ms.

The HUVEC were centrifuged to obtain a cell pellet which was re-suspended in 10µl of a mix of Re-suspension buffer R containing 1µl each of the episomal vectors in the Epi5 kit's tubes A and B. These transfected HUVEC were then plated on a Geltrex-coated surface (10µg/cm2) in pre-warmed Complete EBM2 Medium with 10% FBS, and incubated in a humidified 5% CO_2 incubator at 37°C for 4 days. Thereafter, the culture media was replaced with mTeSR1 and changed daily. After 10 days, colonies with ESC-like morphology started to emerge. At 3 weeks post-transfection, colonies with iPSCs morphology were picked for molecular characterisation by immunostaining.

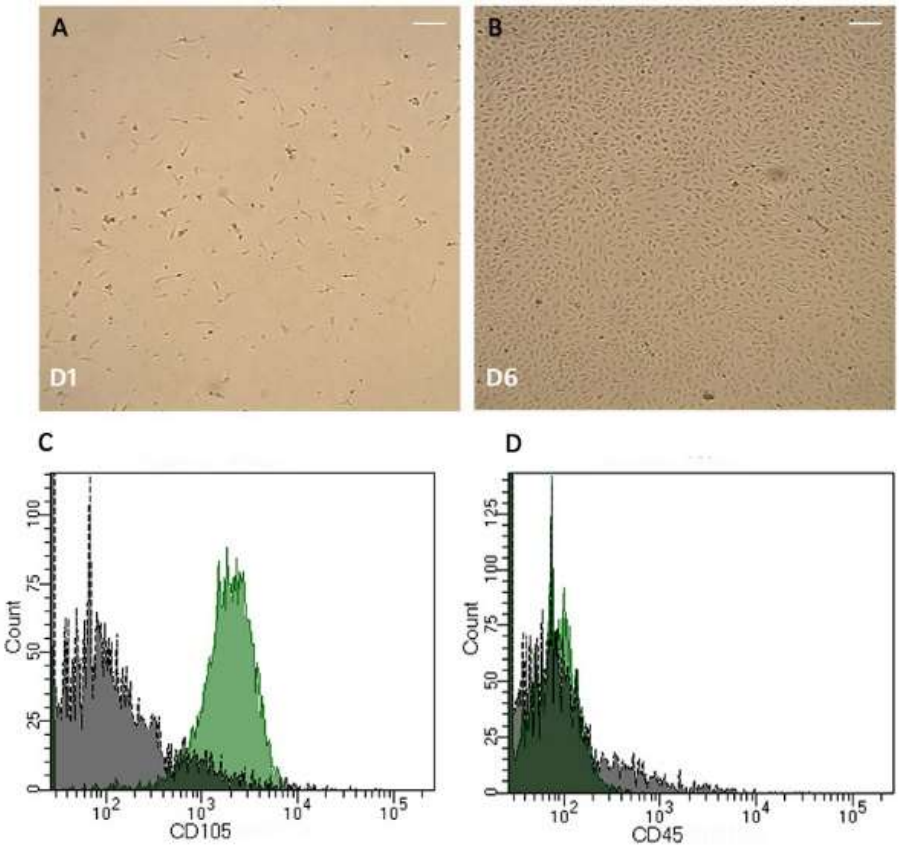
2.5 Immunofluorescence staining of iPSCs

The iPSCs were fixed and permeabilized with 1ml ice-cold acetone and methanol (1:1) for 10 minutes at room temperature. . The fixative solution was removed and the cells were washed with 1X wash buffer (0.05%tween20 in 1X DPBS without $Ca^{2+}Mg^{2+}$) twice and cells were blocked with 2% Bovine Serum Albumin for 30 minutes at room temperature. Incubation with primary antibodies (Mouse IgM Anti-Human TRA-1-81 Antibody and Mouse IgG Anti-Human OCT-4 Antibody at 1:50 dilutions) from the Embryonic Stem Cell Marker Sample Kit (Merck Millipore; SCR002), was done for 60 minutes. Then incubation with fluorescent labelled secondary antibodies (Texas Red labelled Goat IgM Anti-Mouse Antibody and Fluorescein Isothiocyanate (FITC) labelled Goat IgG Anti-Human Antibody at 1:500 dilutions) was done in the dark for 60 minutes. Cellular nuclei were counterstained with 4, 6-Diamidino-2-phenylindole (DAPI) and cells were visualized under a fluorescence microscope (Leica; DMi8).

3. RESULTS

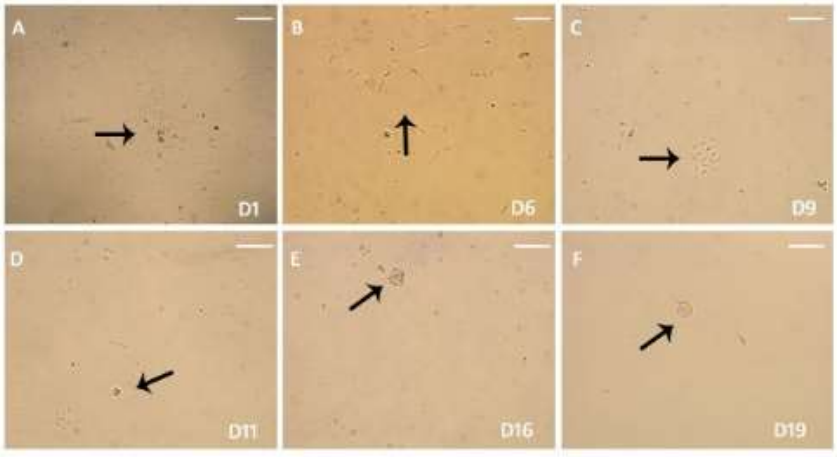
Inverted phase-contrast microscopy showed that on culturing, the HUVEC grew as a monolayer of a homogenous population, and exhibited a large, polygonal spindle shape (cobblestone morphology). Multicolour Flow cytometry of HUVEC showed the expression of the positive marker CD105 and the absence of the negative marker CD45, as shown in Fig. 1.

Fig 1: HUVEC culture and molecular characterization by multi-color flow cytometry. HUVEC culture (A) Day 1: only a few cells seen at 5% confluence; (B) Day 6: the cells are polygonal spindle-shaped, and have proliferated and reached 80-90% confluence; (C-D) Molecular characterization of HUVEC by multi-color flow cytometry, showing positive expression of CD105 and negative expression for CD45.



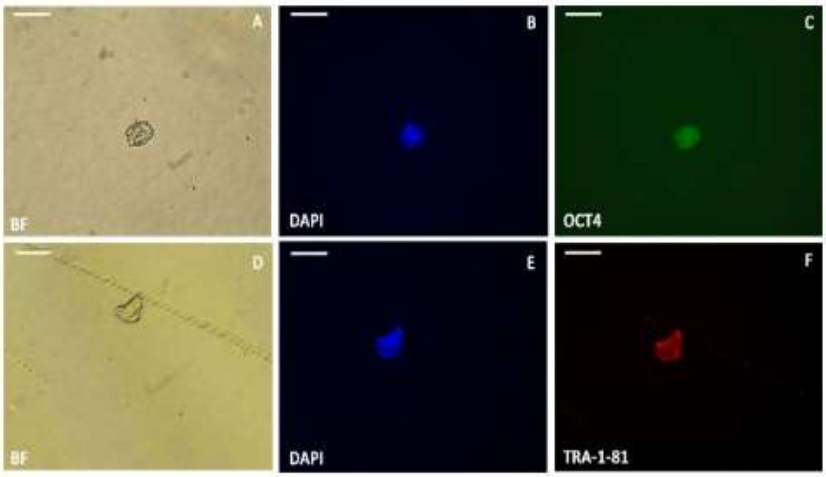
Upon transfection, a lot of cell death was observed but the transfected cells that survived generated iPSC colonies. These proliferated and changed morphologically to become more polygonal and tightly packed. By day 10-11 post-transfection, iPSC colonies exhibiting ESC-like morphology started appearing with distinct regular margins and tightly packed cells inside as shown in Fig. 2.

Fig 2: Generation of iPSCs from HUVEC. (A) Post-transfection Day 1: cell confluence reduced to 4% as most of the cells have died; (B) Day 6: Cells have cobblestone appearance with a few having elongated arms (100×); (C) Day 9: Cells growing closer, forming colony-like appearance (100×); (D) Day 11: iPSC colonies originating; ESC-like colonies having distinct boundaries (100×); (E) Day 16: iPSC colonies, with tightly packed cells inside colonies and having high nuclear-cytoplasmic ratio, are evident at higher magnification (400×); (F) Day19: Mature iPSC colony (400×).



The iPSCs were round with a high nuclear-cytoplasmic ratio and prominent nucleoli. The iPSC colonies stained positive for the expression of the positive pluripotency markers OCT-4 and TRA-1-81. Green immunofluorescence for the nuclear marker OCT-4, red immunofluorescence for the cell surface marker TRA-1-81, and blue immunofluorescence for the nuclear counterstain DAPI, were observed upon fluorescent microscopy, as shown in Fig. 3.

Fig 3: Characterization of HUVEC-derived iPSCs by immunofluorescence. (A-C) Bright-field image of an iPSC colony, followed by its fluorescent microscopy images showing blue immunofluorescence by DAPI for nuclei and green immunofluorescence for the positive pluripotency marker OCT-4; (D-F) Bright-field image of another iPSC colony, followed by its fluorescent microscopy images showing blue immunofluorescence by DAPI for nuclei and red immunofluorescence for the positive pluripotency marker TRA-1-81.



4. DISCUSSION

This study aimed to establish the protocol for our lab for the generation of integration-free iPSCs from HUVEC. We utilized somatic endothelial cells, derived from the umbilical cord of a new born. The ease of collection and reduced risk of genetic mutations compared to adult somatic cells have made HUVEC an attractive source for generating iPSCs for applications in regenerative therapy, disease modelling, drug testing, and discovery [18].

Cultured HUVEC showed polygonal cobblestone morphology upon inverted phase-contrast microscopy, which is consistent with previous studies [19].

The Molecular characterization of HUVEC by Multi Colour Flow cytometry confirmed the expression of the positive marker CD105 for HUVEC and the absence of the negative marker CD45, by previous studies [20]. We used electroporation for the delivery of the episomal plasmids. Electroporation of somatic cells using a neon transfection system is a suitable, safe, easy, and cost-effective method for producing transgene-free iPSCs with high transfection efficiency, as reported previously by others including Sharipova et al. and Grigoreva et al [21].

A previous study by Manzini et al compared the transfection efficiency of several non-integrating methods on human dermal fibroblasts and found the non-integrating Sendai viral method to be the most efficient (0.04%), followed by electroporation, wherein the Nucleofector system was more efficient and robust than the Neon system (0.0166% versus 0.0044%); while a chemical method of delivery like Lipofectamine 3000 was less efficient than the former methods (0.001%) [22].

Other studies by Young et al and Huet et al compared liposomal and non-liposomal reagents for transfection of HUVEC but the results were contradictory and therefore inconclusive in determining if either method yielded a better transfection efficiency. Young et al. found that transfection efficiencies with Effectene and FuGENE6 were 34% and 33% respectively at 48 hours while that with liposomal DOTAP was only 18% [23]. On the other hand, Huet et al found that

liposomal Lipofectamine LTX and Lipofectamine 2000 yielded higher transfection efficiencies of 38% and 23% respectively versus <20% efficiency with non-liposomal FuGENE 6 and Effectene [24].

The earlier study by Matz et al generated HUVEC-derived iPSCs using a non-viral, integration-free method employing episomal plasmid-based delivery of the six reprogramming factors OCT-4, SOX-2, LIN-28, NANOG, c-MYC and KLF-4 [21]. The previous publications have mostly included the transcription factor c-MYC in their reprogramming cocktail and although highly efficient, c-MYC is a proto-oncogene resulting in tumor formation later [25, 26]. Thus, we did not use c-MYC in this study. We used OriP/ EBNA-1-based episomal vectors carrying genes for the five reprogramming factors OCT-4, SOX-2, KLF-4, L-MYC, and LIN-28 along with mp53DD and EBNA-1 for improving the reprogramming efficiency [27]. EBNA-1 codes for the Epstein-Barr Nuclear Antigen-1 protein in the Epstein-Barr virus; which functions as the binding protein for the origin of replication (OriP) of the latent phase of the virus. It activates transcription and initiates DNA replication and mitotic segregation when the cell divides. Thus the strategic presence of OriP on all but the EBNA-I plasmid in the Epi5 kit allows only transient initial EBNA-1 protein production while playing a key role in other plasmids' retention and the increased production of the reprogramming factors carried by them [28].

The p53 gene codes for a protein that normally plays a role in tumor suppression by causing cell cycle arrest and apoptosis whenever the cellular DNA is mutated or damaged. The dominant negative mutant of p53 used prevents programmed cell death, increasing cell survival and the efficiency of iPSC generation [29].

The iPSC colonies started to emerge after day 11 as shown in figure 3, which was consistent with work by Yohannes Haile et al [30]. Morphologically these colonies resembled ESCs and iPSCs as described in past studies [31]. The colonies appeared flat and small with tightly packed cells. Cells within the colonies had a high nuclear-to-cytoplasmic ratio as shown in Figure 4. The presence of immunofluorescence for OCT-4 and TRA-1-81 confirmed the expression of these positive pluripotency-related markers in the generated iPSC colonies, as demonstrated in past studies [32]. This confirmed the isolated clones' identity as iPSCs. This study using episomal plasmids achieved a reprogramming efficiency of approximately 0.01% based on the number of cells plated and several colonies that emerged having ESC-like morphology. This was similar to the past study by Manzini et al. from electroporation of human dermal fibroblasts [23]. A previous study by Hunt et al. showed that Lipofectamine LTX and 2000 were the most efficient in transfection (up to 38% HUVEC transformed) compared to 9 other chemical agents including cationic lipids, polycationic lipids, non-liposomal lipids, cationic polymers of linear PEI, lipids, and polyamine, activated dendrimers [21].

Other studies on HUVEC have reported transfection efficiencies with chemical agents up to 0.45% with Lipofectamine [33], 10.9% with SuperFect [34], 50% with DOTAP [35], and up to 77% with Effectene [36].

During the iPSCs generation, we encountered bacterial contamination in the culture which gradually killed cells that were about to generate iPSCs. The contaminated culture was indicated by the color of the media that changed from red to yellow due to acidic conditions and cloudiness in the media or a thin film on the surface of the media was seen. Several sources can contaminate the cell culture and destroy batches of culture, therefore, for troubleshooting follow cited article [37].

Despite transfection through electroporation being a safe and efficient method, it exhibits limitations as we faced during the study that transfection of cells with 80% viability gives only a few iPSC colonies and most of the cells died. This limitation can only be overcome by optimization of parameters. During the transfection, we transfected cells with two different voltages and pulse widths i.e. 1350 volts with 30ms (PW) and 1200 volts with 40ms (PW). Where microscopy showed that due to a high voltage more debris and less number of attached cells were present whereas transfection at low voltage appeared better as less cell debris and more attached cells were observed. So it's better to use 1200 volts. For more efficient transfection, electric field parameters should be properly chosen [38].

During the cryopreservation of cells when DMSO is added, flash freezes the cells and immediately placed the cells at -80 for short-term storage and at -196 for long-term storage otherwise, DMSO may negatively affect the cells and decrease the viability [39]. We used 10% DMSO for cryopreservation although 5% DMSO can also be used from a safety perspective when cell-based therapies are required for future studies [40].

Since DMSO is toxic to the cells if they are being subjected to infusions, therefore, 10%-20% glycerol is a good cryoprotectant that gives more viability to cells after cryovial revival [41].

Future challenges related to electroporation systems that need to be addressed are that neither capable enough to transfect a large number of cells on large scale nor they can efficient in targeting disease treatments in vivo therefore, modifications in existing electroporation systems are required.

5. CONCLUSION

In this study using a neon transfection system we successfully established a robust feeder-free episomal reprogramming protocol in our lab for umbilical cord-derived HUVEC. HUVEC are an attractive cell source for generating iPSCs, as demonstrated by their successful and efficient reprogramming in this study. Integration-free, transgene-free, and non-viral-based episomal vectors are the most suitable and efficient methods of cellular reprogramming. The neon transfection system is reliable and cost-effective for gene delivery; it has an optimized protocol available for the successful transfection of HUVEC.

Acknowledgment

None.

Conflict of interest

The authors declare no conflict of interest.

Author's Contribution

Rameen Raza carried out all the experiments and data collection; Mohsin Wahid designed the study and conception of the work, supervised the experimental work and final review and approval of the manuscript; Abdul Qadir wrote the manuscript, data analysis and facilitated in HUVEC culture; Fareeha Faizan facilitated the Genetic reprogramming experiment and reviewed the manuscript; Hina Jabeen performed data analysis and contributed towards manuscript writing.

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