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TITLE:

Generation of induced pluripotent stem cells from Turner Syndrome (45XO) fetal cells for downstream modelling of neurological deficits associated with the syndrome

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SUMMARY:

This protocol describes the generation of integration free iPSCs from fetal tissue fibroblasts through delivery of episomal plasmids by nucleofection followed by description of methods used for iPSC characterization and neuronal differentiation.

ABSTRACT:

Chromosomal aneuploidies cause severe congenital malformations including central nervous system malformations and fetal death. Prenatal genetic screening is purely diagnostic and does not elucidate disease mechanism. Although cells from aneuploid fetuses are valuable biological material bearing the chromosomal aneuploidy, these cells are short lived, limiting their use for downstream research experiments. Generation of induced pluripotent stem cell (iPSC) models is an effective method of cell preparation for perpetual conservation of aneuploid traits. They are self-renewing and differentiate into specialized cells reminiscent of embryonic development. Thus, iPSCs serve as excellent tools to study early developmental events. Turner syndrome (TS) is a rare condition associated with a completely or partially missing X chromosome. The syndrome is characterized by infertility, short stature, endocrine, metabolic, autoimmune and cardiovascular disorders and neurocognitive defects. The following protocol describes isolation and culturing of fibroblasts from TS (45XO) fetal tissue, generation of integration free TSiPSCs through delivery of episomal reprogramming plasmids by nucleofection followed by characterization. The reprogramming TSiPSCs were initially

screened by live cell alkaline phosphatase staining followed by extensive probing for pluripotency biomarkers. Selected colonies were mechanically dissected, passaged several times and stable self-renewing cells were used for further experiments. The cells expressed pluripotency transcription factors OCT4, NANOG, SOX2, cell surface markers SSEA 4 and TRA1-81 typical of pluripotent stem cells. The original 45XO karyotype was retained post reprogramming. The TSiPSCs were able to form embryoid bodies and differentiate into cells of endoderm, mesoderm and ectoderm expressing lineage specific biomarkers ((SRY BOX17), (MYOSIN VENTRICULAR HEAVY CHAINα/β), (βIII TUBULIN)). The exogenous episomal plasmids were lost spontaneously and not detected after passage15 in cells. These TSiPSCs are a valuable cellular resource for modelling defective molecular and cellular neurodevelopment causing neurocognitive deficits associated with Turner syndrome.

INTRODUCTION:

Aneuploidies lead to birth defects/congenital malformations and pregnancy loss in humans. ~50%–70% of specimens from pregnancy losses show cytogenetic abnormalities. Aneuploid embryos lost early in pregnancy cannot be easily obtained for experimental analysis raising the need to develop other models closely representing human embryogenesis. Induced pluripotent stem cells (iPSCs) derived from cells diagnosed with genetic disorders have been used to model the representative genetic irregularities and their consequence on fetal development[1–4]. These iPSCs resemble epiblast cells of the developing embryo and can recapitulate the early events of embryo formation. They allow understanding and characterization of the developmental program of cell lineages and patterning in early mammalian embryos. iPSCs derived previously from skin fibroblasts and amniocytes from prenatal diagnostic tests of aneuploidy syndromes like monosomy X (Turner syndrome), trisomy 8 (Warkany syndrome 2), trisomy 13 (Patau syndrome) and partial trisomy 11; 22 (Emanuel syndrome) have provided valuable insights regarding failed development[4].

Turner syndrome (TS) is a rare condition characterized by female infertility, short stature, endocrine and metabolic disorders, an increased risk of autoimmune disease, and a predisposition to cardiovascular disease[5]. Though it is the only survivable monosomy syndrome it is also lethal to the developing embryo causing spontaneous abortions[6]. Surviving individuals with TS present with degrees of alteration of X-chromosomal material in their cells. Karyotypes range from complete loss of one X chromosome (45,XO) to mosaics like 45,XO/46,XX; 45,XO/47,XXX, the presence of ring chromosomes, the presence of Y-chromosomal material, etc[5].

Diagnosis of the syndrome is generally done by karyotyping blood of symptomatic individuals and chorionic villi sampling (CVS) to detect early aneuploidy syndromes. Since aneuploidy syndromes account for ~30% of spontaneous abortions, it is routine to karyotype the product of conception (POC) upon a spontaneous abortion. These fetal cells including the chorionic villi possessing the cytogenetic abnormality and iPSCs derived from them provide a valuable source of biological material to study aneuploidy syndromes[4,6]. TS iPSCs have been previously established from amniocytes via retroviral reprogramming[4] and fibroblasts of chorionic villi via retroviral reprogramming[6] obtained through prenatal diagnosis, from blood mononuclear cells[7] via Sendai virus reprogramming and from skin fibroblasts of TS individuals via lentiviral reprogramming[4]. Since the primary focus of our lab is to understand developmental failure, we have generated TS iPSCs from POC, specifically the chorionic villi component of a

spontaneous abortion[8]. All the cells isolated from this fetal tissue had a 45XO karyotype and yielded iPSCs with the same karyotype. These iPSCs are unique as they are the first to be generated from an aborted fetus and provide a valuable resource to study aneuploidy related pregnancy failures. This article provides a detailed methodology of the generation of iPSCs from this unique cell source via episomal reprogramming.

The early methods of iPSC generation used viral transduction and transposons to deliver the reprogramming factors. Methods of inducing cells to pluripotency have evolved from using integrating retroviral vectors[9], excisable lentiviral vectors[10,11] and transposon-based methods[12] to non-integrating adenoviral vectors[13] and Sendai virus based vectors[14]. Retroviral and lentiviral based reprogramming, although efficient, involve integration of the reprogramming factors into the host chromosomes, causing insertion mutations which have unforeseen effects in the iPSCs. Furthermore, viral-based reprogramming prevents translational application of iPSCs. RNA-based systems[15] and direct protein delivery[16] have been explored to completely eliminate the potential risks associated with the use of viruses and DNA transfections. However, these methods have proven inefficient.

In 2011, Okita et al. reported improved efficiency of reprogramming by episomal plasmids augmented with TP53 suppression via shRNA. They also replaced cMYC with non-transforming LMYC (small cell lung carcinoma associated MYC) to enhance safety of the hiPSCs. These episomal plasmids express 5 reprogramming factors: OCT4, LIN28, SOX2, KLF4, LMYC and shRNA for *TP53*[17,18]. These vectors are maintained extra-chromosomally and lost from the reprogrammed cells upon continuous culture, thus making the lines transgene-free within 10-15 passages. Nucleofection is a specialized form of electroporation that delivers nucleic acids directly into the nucleus of host cells. It is an efficient method for delivery of the reprogramming plasmids into various cell types. Episomal plasmids are cost effective and compensate the high costs of nucleofection. This method is efficient and reproducible under optimized conditions yielding stable iPSCs from a variety of somatic cells. In this protocol, we describe the method for generation of iPSCs from fibroblasts isolated from fetal tissue by nucleofection of episomal reprogramming plasmids. Here are the detailed protocols for fibroblast isolation from fetal chorionic villi, plasmid purification, nucleofection, picking of colonies from the reprogramming plate and establishment of stable iPSCs.

It is essential to confirm the presence of pluripotency traits in the newly generated iPSCs. This includes demonstration of pluripotency related factors (e.g., alkaline phosphatase expression, NANOG, SSEA4, Tra 1-80, Tra 1-81, E-cadherin; usually shown with immunofluorescence or gene expression assays), identification of the three germ layers by *in vitro* differentiation assays to validate their differentiation potentials, karyotyping to determine chromosomal content, STR typing to establish identity with parent cells, verify loss of exogenous genes, and more stringent *in vivo* assays such as teratoma formation and tetraploid complementation. Here we describe characterization protocols of karyotyping, live cells alkaline phosphatase staining, detection of pluripotency related biomarkers by immunofluorescence, *in vitro* differentiation assays and method to demonstrate loss of exogenous genes[19].

PROTOCOL:

FCV were obtained from Manipal Hospital, Bengaluru, under Ethics Committee of Manipal Hospitals approval.

NOTE: See **Table 1** for composition of all buffers and solutions.

1. Isolation of fibroblasts from fetal chorionic villi (FCV)

1.1 Sample collection and tissue disintegration in collagenase

1.1.1 Collect FCV under sterile conditions in phosphate buffered saline (PBS) and transport (at room temperature) to the cell culture facility.

1.1.2 Transfer the villi to a 60 mm Petri dish and wash several times (minimum 4 times) in PBS containing 1x Antibiotic Antimycotic solution (PBS-AA). Remove PBS-AA completely by pipetting.

1.1.3 Treat the chorionic villi with 1 mL collagenase blend (5 mg/mL) for 5 min at 37 °C.

1.1.4 Neutralize with cell culture medium containing 10% fetal bovine serum (FBS), transfer digest to a 15 mL tube and centrifuge at 225 x g for 5 minutes to collect the disintegrated villi and released cells as a pellet.

1.2 Subculture and stock expansion

1.2.1 Plate disintegrated villi along with released cells in a T25 culture flask containing 5 mL of complete media (e.g., AmnioMAX) and grow till a confluent fibroblast culture is obtained.

1.2.2 Expand fibroblasts in culture to prepare stocks for use in subsequent transfection and characterization experiments as follows:

1.2.2.1 Add 2 mL of 0.05% trypsin to T25 flask containing FCV fibroblasts and incubate at 37 ˚C for 3-5 min to dissociate the cells.

1.2.2.2 After incubation, neutralize trypsin by adding FBS (at the same volume as trypsin).

NOTE: Culture medium containing FBS can also be used to neutralize trypsin, when added at a 1:3 trypsin: media ratio.

1.2.2.3 Collect the dissociated cells in a 15 mL tube and centrifuge at 225 x g for 5 min to obtain cell pellet.

1.2.2.4 Decant supernatant and resuspend cell pellet in 1 mL of complete media.

1.2.2.5 Transfer 500 µL each to 60 mm tissue culture-treated dishes and make up the volume to 5 mL. This splitting ratio of 1:2, was also used for subsequent passages.

1.3 Cryopreservation

1.3.1 Perform enzymatic dissociation using 0.05% trypsin as described in steps 1.2.2.1 – 1.2.2.3 and obtain cell pellet.

1.3.2 Discard the supernatant and resuspend the cell pellet in 1 mL of freezing mix, comprising 1:9 dimethyl sulfoxide (DMSO): FBS.

1.3.3 Transfer contents to a sterile cryovial and place the vial in a freezing container.

1.3.4 Freeze overnight at -80 ˚C and then transfer vials to liquid nitrogen (-196 ˚C) the next day.

2. Plasmids DNA Isolation and verification

2.1 Bacterial Cell Preparation

2.1.1 Streak glycerol stocks of *E. coli* containing the three individual plasmids pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL (from Addgene) on separate Luria Bertani-ampicillin agar plates.

2.1.2 Inoculate single colonies into starter cultures of 5 mL of Luria Bertani-ampicillin medium. Incubate for 8 hours at 37 °C with shaking (10 x g).

2.1.3 Inoculate 200 µL of this starter culture into 100 mL of Luria Bertani-ampicillin medium. Incubate overnight at 37 °C with shaking.

2.1.4 Harvest overnight bacterial culture by centrifuging at 6000 x g for 15 min at 4 °C.

2.2 Plasmid isolation with Midi Plasmid purification kit

2.2.1 Resuspend bacterial pellet in 4 mL resuspension buffer.

2.2.2 Add 4 mL of lysis buffer and mix thoroughly by vigorously inverting 4–6 times and incubate at room temperature for 5 min.

2.2.3 Add 4 mL of pre-chilled neutralization buffer and invert tube 4-6 times to mix thoroughly. Incubate on ice for 15 min.

2.2.4 Centrifuge at ≥20,000 x g for 30 min at 4 °C. Collect supernatant in a fresh tube and re-centrifuge at ≥20,000 x g for 15 min at 4 °C.

2.2.5 Equilibrate the column by applying 4 mL of equilibration buffer.

2.2.6 Apply the supernatant to the column.

2.2.7 Wash the column twice with 10 mL of wash buffer.

2.2.8 Elute DNA with 5 mL of warm (65 °C) elution buffer.

2.2.9 Precipitate DNA by adding 3.5 mL of isopropanol to the eluted DNA. Mix well. Centrifuge at ≥15,000 x g for 30 min at 4 °C. Decant supernatant carefully.

2.2.10 Wash the DNA pellet with 2 mL of 70% ethanol and centrifuge at ≥15,000 x g for 10 min. Decant supernatant carefully.

2.2.11 Air-dry pellet for 5–10 min and dissolve DNA in a suitable volume of PCR-grade water to obtain a final concentration of 1 µg/mL.

NOTE: Do not dissolve DNA in buffers as it is not suitable for electroporation. Old plasmid DNA preparation do not yield reprogrammed colonies.

2.3 Plasmid Verification by EcoRI restriction digestion

2.3.1 Combine 15 µL of nuclease-free water, 2 µL of 10x buffer, 1 µg of plasmid DNA and 1 µL of EcoRI enzyme. Mix gently.

2.3.2 Incubate the mixture at 37 °C for 15 min in a heat block.

2.3.3 Mix the digested plasmid samples with 6x DNA gel loading dye and electrophorese on 1% agarose gel in 1x TAE buffer with 0.5 µg/mL of ethidium bromide. Include standard DNA ladder. Image the gel after the DNA has resolved appropriately. Expected EcoRI band sizes of pCXLE-hOCT3/ 4-shp53-F are 6,834 bp, 3,758 bp, and 1,108 bp; pCXLE-hUL are 10,200 bp and 1,900 bp; pCXLE-hSK are 10,200 bp and 2,500 bp.

3. Nucleofection

3.1 Cell pelleting

3.1.1 Culture the isolated fetal chorionic villi fibroblasts in T25 flask in 5 mL of complete media till 80-90% confluency.

3.1.2 Wash cells twice with PBS and trypsinize as described in steps 1.2.2.1 - 1.2.2.3.

3.1.3 Remove the supernatant, resuspend pellet in 5 mL of reduced serum media (e.g., Opti-MEM). Count cells with hemocytometer and take 10^6 cells for nucleofection. Centrifuge at 225 x g for 5 min. Remove supernatant completely.

3.2 Reagent preparation and nucleofection

3.2.1 Prepare nucleofector reagent by mixing 0.5 mL of supplement and 2.25 mL of nucleofector solution (both provided in the kit).

3.2.2 Add 100 µL of nucleofector solution in a 1.5 mL tube. Add 1µg each of pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL to the tube. Gently resuspend 10^6 cells (from step 3.1.2) in this mix.

3.2.3 Transfer the cell-DNA suspension into cuvette, ensuring that the sample covers the bottom of the cuvette (provided in kit) without any air bubbles. Cap the cuvette and insert into the holder. Select the nucleofector Program U-23 (for high efficiency) and apply.

3.2.4 Remove cuvette out of the holder and add 1 mL of complete media. Transfer the contents gently into a 60 mm tissue culture treated Petri dish filled with 4 mL of complete media (to a total of 5 mL of media). Incubate the cells in a humidified CO_2 incubator at 37 °C.

3.2.5 After 24 h, check if the cells have attached. Replace the medium completely.

NOTE: The rate of cell death is high in nucleofection leaving few viable cells which attach.

3.2.6 Maintain the cells in complete media for 10 days and shift to pluripotency media for the next 20 days.

NOTE: Visualize cells regularly to follow morphological changes occurring in the reprogramming cells (like epithelial morphology and compact colony formation) to confirm if the experiment is working. Around 25 iPSC colonies can be seen after 20 days of culture in pluripotency media.

4. Picking and propagation of iPSC colonies

4.1 Picking colony from reprogramming plate

4.1.1 Manually dissect the embryonic stem cell-like colonies formed in the reprograming dish using pulled glass pipettes or 1 mL syringe needles and transfer to previously prepared plate with inactivated mouse embryonic fibroblast feeders with pluripotency medium or establish feeder free cultures on Matrigel coated plates with mTESR medium.

NOTE: Mouse embryonic fibroblasts (MEFs) were derived using enzymatic isolation from mouse embryos (dissected from 13-14 days pregnant female mice) and were mitotically inactivated by mitomycin C treatment. Establish single clone populations by growing single colonies from reprogramming plate in separate dishes or mixed clone populations by transferring many colonies from reprogramming plate to a single dish.

4.2 Mechanical transfer of emerging colonies to fresh feeders and passaging to establish stable iPSCs

4.2.1 Propagate iPSCs in pluripotency medium by feeding every second day and split 1:3 every 5-7 days. Prepare stocks by cryopreserving in a freezing mix of KnockOut Serum Replacement and DMSO in the ratio 9:1.

NOTE: KnockOut Serum Replacement is used in the freezing mix for cryopreservation of iPSCs instead of FBS as components in the FBS could induce differentiation of the pluripotent cells during long term preservation.

5. Characterisation of iPSCs

NOTE: Characterization studies including PCR and immunostaining for pluripotency biomarker were done after the fifth passage number. Karyotyping was performed at a later passage number.

5.1 Karyotyping

5.1.1 Treat a confluent 60 mm Petri dish of iPSCs with colcemid for 45 min in humidified CO_2 incubator at 37 °C.

5.1.2 Harvest by 0.05% trypsin treatment and centrifuge. Remove the supernatant and pipette the leftover traces of medium to loosen the cell pellet.

5.1.3 Add 5 mL of hypotonic solution. Mix by inverting tube and incubate for 20 minutes at 37 °C. Centrifuge at 225 x g for 5 min.

NOTE: The obtained pellet should appear fluffy.

5.1.4 Add 2.5 mL of Carnoy's fixative solution slowly, while tapping to loosen the pellet.

5.1.5 Prepare spreads for karyotyping by dropping the cell suspension on clean glass slides.

5.1.6 Treat the slides with 0.15% trypsin for 1 minute, and wash once with PBS. Then stain with Giemsa solution for 4 min and end with a distilled water wash. Acquire and process with appropriate software.

5.2 Demonstration of transgene free status

5.2.1 Genomic DNA Isolation

5.2.1.1 Pipet 20 µL of protease into the bottom of a 1.5 mL microcentrifuge tube.

5.2.1.2 Add 200 µL of TSiPSCs resuspended in PBS to the microcentrifuge tube.

5.2.1.3 Add 200 µL of Buffer AL to the sample and mix for 15 s by pulse-vortexing.

5.2.1.4 Incubate for 10 min at 56 °C.

5.2.1.5 Briefly centrifuge the microcentrifuge tube to remove drops from the inside of the lid.

5.2.1.6 Add 200 µL of ethanol (96–100%) to the sample, and mix again for 15 s by pulse-vortexing. After mixing, briefly centrifuge the tube to remove drops from the inside of the lid.

5.2.1.7 Carefully apply the mixture from the previous step to the mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min. Discard the tube containing the filtrate and place the mini spin column in a clean 2 mL collection tube.

5.2.1.8 Carefully open the mini spin column and without wetting the rim, add 500 µL of Buffer AW1. Close the cap and centrifuge at 6000 x g for 1 min. Place the mini spin column in a clean 2 mL collection tube and discard the collection tube containing the filtrate.

5.2.1.9 Carefully open the mini spin column and add 500 µL of Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g) for 3 min.

5.2.1.10 Place the mini spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min to eliminate the chance of possible Buffer AW2 carryover.

5.2.1.11 Place the mini spin column in a clean 1.5 mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the mini spin column and add 200 µL Buffer AE or distilled water. Incubate at room temperature (15–25 °C) for 5 min, and then centrifuge at 6000 x g for 1 min.

5.2.2 **Transgene-Free Status PCR (Using KAPA HiFi PCR Kit KR0368)**

5.2.2.1 Ensure that all reagents are properly thawed and mixed.

5.2.2.2 Prepare a PCR master mix containing the appropriate volume of all reaction components based on **Table 2** (set up reactions on ice).

5.2.2.3 Transfer the appropriate volumes of PCR master mix, template and primer to individual PCR tubes.

5.2.2.4 Cap individual reactions, mix and centrifuge briefly.

5.2.2.5 Perform PCR following **Table 3**.

5.3 Pluripotency biomarker identification

5.3.1 **Alkaline phosphatase (AP) staining**

5.3.1.1 Prepare a 1x AP live stain working solution by diluting 3 µL of 500x stock solution in 1.5 mL DMEM/F-12 for every 10 cm^2 of culture area.

5.3.1.2 Remove the medium from the iPSC culture dish. Wash the culture with DMEM/F-12 once. Add the 1x AP live stain solution onto the iPSCs. Incubate at 37 °C for 45 min.

5.3.1.3 Remove the AP stain and wash twice with DMEM/F-12. Add fresh DMEM/F-12 and image under fluorescent microscope using a standard FITC filter within 30–90 min of staining.

5.3.2 **Immunostaining for pluripotency biomarkers**

5.3.2.1 Fix confluent iPSC cultures with 4% paraformaldehyde overnight at 4 °C. Wash thrice with PBS Tween 20 (PBST), each wash for 5 min.

5.3.2.2 Permeabilize the cells with 0.3% Triton X-100 in PBST for 15 minutes at room temperature. Wash thrice with PBST.

NOTE: Permeabilization should be done only for intracellular antigens.

5.3.2.3 Block cells with 3% bovine serum albumin (BSA) in PBST for 30 min at room temperature. Stain the cells with primary antibodies (diluted 1:1000 in 1% BSA) overnight. After primary antibody incubation, wash thrice with PBST.

5.3.2.4 Incubate cells with the secondary antibody (diluted 1:1000 in 1% BSA) for 5 h at room temperature. Wash thrice with PBST.

5.3.2.5 Label the nuclei with 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) for 1 minute. Wash the cells once with PBST.

5.3.2.6 Capture images under fluorescent microscope.

6. *In vitro* differentiation assays

6.1 Embryoid Body (EB) Differentiation

6.1.1 Cut the iPSC colonies into small pieces, collect and plate in low attachment Petri dishes in embryoid body medium. Grow the cells for 15 days by replacing medium every 3 days.

NOTE: The day 15 EBs can be used directly for detection of the three germ layer biomarkers. Alternatively, specific cell lineages can be induced with growth factors, followed by biomarker detection.

6.2 Endoderm (Hepatocyte) differentiation

6.2.1 Grow the iPSCs in monolayer cultures in pluripotency medium.

6.2.2 Once confluent, shift to RPMI 1640 media with 1x Insulin Transferrin Selenite and 100 ng/mL activin A for 2 days, followed by growth in RPMI 1640 media with 30 ng/mL bFGF and 20 ng/mL BMP4 for 9 days. Replace medium every 2 days.

6.2.3 From day 10 onwards, supplement media with 0.1 μM dexamethasone. Terminate the experiment on day 20.

6.3 Mesoderm (Cardiomyocyte) differentiation

6.3.1 Plate day 8 EBs on 0.5% Matrigel-coated plates in embryoid body medium. Allow the EBs to attach and collapse.

6.3.2 Supplement media with 20 ng/mL BMP4 and grow for 20 days. Replace medium every 2-3 days. Terminate the experiment on day 20.

6.4 Ectoderm (Neuronal) differentiation

6.4.1 Plate day 4 EBs on 2 μg/cm^2 collagen type IV-coated plates in embryoid body medium. Allow the EBs to attach and collapse.

6.4.2 Next day, shift medium to DMEM F-12 with 2mg/mL glucose, 1x Insulin Transferrin Selenite, and 2.5μg/mL fibronectin. Terminate the experiment on day 15.

6.5 Formation of cerebral organoids

6.5.1 Grow TSiPSCs in a 35 mm tissue culture dish on MEFs till 70–80% confluent. Cut the colonies and collect in a 15 mL tube. Centrifuge the cells at 225 x g for 5 min. Discard the supernatant.

6.5.2 Wash the pieces of colonies by resuspending in 2 mL of PBS and centrifuge to remove the supernatant.

6.5.3 Add 1 mL of 0.05% trypsin and tap the tube to dislodge the cells. Incubate the tube at 37 ˚C for 3-4 min to dissociate the colony pieces into a single cell suspension.

6.5.4 Neutralize the trypsin by dilution with 4 mL of pluripotency media containing 10 μg/mL rho-associated protein kinase (ROCK) inhibitor Y-27632 dihydrochloride (ROCKi) to prevent dissociation induced cell death.

6.5.5 Centrifuge to obtain a pellet. Discard the supernatant and resuspend the cells in 2mL embryoid body medium containing 10 μg/mL ROCKi.

6.5.6 Remove 10 μL of cell suspension for cell counting. Add 10 μL of Trypan blue to detect dead cells. Count the cells using a hemocytometer.

6.5.7 Add appropriate volume of embryoid body medium with ROCKi to the cell suspension to obtain 9,000 live cells per 150 μL.

6.5.8 Plate 150 μL in each well of a low-attachment 96-well plate and incubate in a humidified CO2 incubator at 37 °C. Check the plates for aggregation after 24 hours. On day 2 gently remove the medium and replace with fresh embryoid medium without ROCKi.

6.5.9 On day 6, transfer EBs to wells of a low attachment 24 well plate containing 500 μL of neural induction medium composed of DMEM-F12 with 1% N2 supplement, 2 mM GlutaMAX

supplement and 1 mM non-essential amino acids and 1 µg/mL heparin. Change the medium every 2 days.

6.5.10 After 5 days in neural induction medium embed the neuroepithelial aggregates in Matrigel by layering a 2 cm x 2 cm square of parafilm over an empty tip tray of 200 µL tips. Press parafilm with gloved fingers over each hole in the tip tray to make small dents. Clean parafilm with 70% ethanol to sterilize.

6.5.11 Transfer the parafilm square into a 60 mm dish. Use cut 200 µL tips to transfer the neuroepithelial aggregates onto the dents in parafilm. Remove excess medium by pipetting.

6.5.12 Add 30 µL of thawed Matrigel on the neuroepithlial aggregates and position the aggregate to the center of the Matrigel using a pipette tip. Place the 60 mm dish for 20-30 min in a 37 °C incubator for the Matrigel to polymerize.

6.5.13 Add 5 mL of cerebral organoid differentiation medium composed of 1:1 DMEM-F12: Neurobasal medium, 0.5% N2 supplement, 2.5 µg/mL of insulin, 2 mM GlutaMAX supplement, 0.5 mM NEAA, 1% B27 supplement and 2.5 mL of penicillin-streptomycin.

6.5.14 Using a sterile forceps turn the parafilm sheet over and agitate the dish until the Matrigel embedded aggregates fall off the sheet into the medium. Grow the embedded aggregates in a humidified CO_2 incubator at 37 °C for 4 days giving media changes on alternate days.

6.5.15 After 4 days of static culture place the 60 mm dishes onto an orbital shaker installed inside the incubator shaking at 50 rpm. Culture the organoids for 3 months giving complete media changes with cerebral organoid differentiation medium every 3 days.

7. Demonstration of transgene free status

7.1 Extraction of genomic DNA

7.1.1 Collect iPSC colonies in 15 mL tubes. Centrifuge and remove supernatant completely. Resuspend the cell pellet in 200 µL of PBS.

7.1.2 Add 20 µL of proteinase K to the cell suspension and mix. Add 200 µL of lysis buffer and mix by pulse-vortexing for 15 seconds till the solution appears homogenous. Incubate the lysate at 56 °C for 10 minutes in a heating block.

7.1.3 Add 200 µL of ethanol to the lysate and mix by pulse-vortexing for 15 seconds. Spin to collect the sample to the bottom of the tube.

7.1.4 Apply the lysate to spin column (in 2mL collection tubes) and centrifuge at 3,500 x g for 1 min. Discard flow through.

7.1.5 Add 500 µL of wash buffer to column without wetting the rim and centrifuge at 3,500 x g for 1 min. Discard flow through. Repeat the wash and centrifuge at 10,000 x g for 1 min. Discard flow through.

7.1.6 Place spin column in clean 1.5 mL micro centrifuge tube and add 200µL of elution buffer. Incubate at room temperature for 5 minutes and then centrifuge at 3,500 x g for 1 min to collect the genomic DNA.

7.2 ORI P AND EBNA1 genomic DNA PCR

7.2.1 Prepare a high-fidelity PCR mix with primers specific for ORIP and EBNA1 sequences present on the episomal plasmids and iPSC genomic DNA as template according to the manufacturer's instructions.

7.2.2 Set a positive control reaction with any one of the episomal reprogramming plasmid.

7.2.3 Amplify the PCR mix for 30 cycles. Analyze the PCR products by electrophoresis on 2% agarose gel in 1x TAE buffer. For ORI P, the expected band size is 524 bp and for EBNA1, 646 bp band size is expected.

REPRESENTATIVE RESULTS:

Generation of integration-free iPSCs from a spontaneously aborted fetus with 45XO karyotype

We isolated fibroblasts from FCV with a Turner syndrome (TS) specific 45XO karyotype and nucleofected them with episomal reprogramming plasmids to generate TSiPSCs which can be used for downstream modelling of the syndrome, specifically the associated neurological deficits (**Figure 1a&b**). We used nonintegrating episomal vectors and nucleofection for the transfection experiments (**Figure 1 c&d**). We followed morphological changes of cells to monitor the success of reprogramming. The shift from the fibroblast to epithelial morphology, followed by a delineated compact colony formation was observed (**Figure 2a**). TSiPSCs acquired human embryonic stem cell like morphology with distinct edges and a high nucleus-to-cytoplasm ratio around day 20 post transfection (**Figure 2b**). In contrast, incompletely reprogrammed cells acquire epithelial morphologies but fail to form compact colonies. (**Figure 2c**).

Characterization of TSiPSCs

Karyotyping of TSiPSCs revealed the 45XO karyotype associated with Turner Syndrome (Figu**re 3a**). Immunofluorescence of TSiPSCs showed expression of pluripotency transcription factors OCT4, NANOG, SOX2, and cell surface markers SSEA4, E-Cadherin, and TRA-1-81. Human embryonic stem cells are the gold standards of pluripotent stem cells. We simultaneously performed immunofluorescence of HUES 1 which was used as positive control for comparison of pluripotency biomarker expression by TSiPSC (**Figure 3b**). Transgene free status of the TSiPSCs was demonstrated by a genomic DNA PCR for episomal plasmid markers OriP and EBNA. By passage 15, OriP and EBNA gene were lost in the TSiPSCs. The episomal genes OriP and EBNA were amplified and showed bands in passage 9 TSiPSCs indicating the presence of the episomal plasmids at this stage. However, these genes were not amplified in passage 15

TSiPSCs indicating a loss of the episomal plasmids and hence a transgene free state (Figure 3c).

***In vitro* differentiation assays**

The differentiation potential of TSiPSC lines was demonstrated *in vitro*. TSiPSCs upon aggregation in low attachment plates formed embryoid bodies (**Figure 4a**). Growth factor induced differentiation of TSiPSCs was used to generate cell types of the three germ layers. Immunofluorescence analysis using lineage specific biomarkers confirmed that TSiPSCs differentiated into representative derivatives of endoderm (SOX17), mesoderm (MYOSIN VENTRICULAR HEAVY CHAINα/β) and ectoderm (βIII TUBULIN) (**Figure 4b**).

Cerebral organoid differentiation.

TSiPSCs were differentiated as cerebral organoids in a stage wise manner. Single cell suspensions of TSiPSCs were aggregated into embryoid bodies to stimulate development of germ layers for initial 6 days followed by induction of neuroepithelial development for 5 days. The neuroepithelial aggregated were them embedded in Matrigel which provided the extracellular matrix and basement membrane components which support proper apicobasal orientation, outgrowth of neuroepithelial buds which expand and form lumens. Immunofluorescence with neuroepithelial marker NESTIN was performed to observe the overall morphology of the organoids (**Figure 5b**). The neuroepithelium surrounds a ventricle like cavity (**Figure 5c** - white line). The organoids morphologically display ventricular zones (VZ), sub ventricular zone (SVZ) and cortex like regions (**Figure 5c** - red, orange and yellow lines respectively)

FIGURE AND TABLE LEGENDS:

Figure 1: Fibroblast isolation and reprogramming via nucleofection

(**a**) Microscopic image of fetal chorionic villi prior to collagenase treatment. (**b**) Fibroblasts isolated from fetal chorionic villi for reprogramming experiments. (**c**) Verification of reprogramming plasmids by EcoRI restriction digestion. (**d**) Schematic diagram of transfection protocol employed for iPSC generation from fetal chorionic villi fibroblasts using episomal reprogramming plasmids via nucleofection.

Figure 2: Establishment of Turner Syndrome induced pluripotent stem cells

(**a**) Cell morphology changes observed during the time course of reprogramming. (**b**) A fully reprogrammed TSiPSC colony. (**c**) A representative image of a colony with improperly reprogrammed cells.

Figure 3: Characterisation of TSiPSCs

(**a**) Karyotype of TSiPSCs. (**b**) Immunofluorescence analysis of pluripotency biomarkers OCT4, NANOG, SOX2, SSEA-4 and TRA-1-81 in TSiPSCs compared with embryonic stem cell HUES1. Nuclei are stained with 4', 6-diamidino-2-phenylindole. 3c. Demonstration of transgene free status of TSiPSCs. Lane 1- DNA ladder, Lane 2- OriP positive control with pCXLE-hSK, Lane 3-EBNA positive control with pCXLE-hSK, Lane 4-OriP with TSiPSCs, Lane 5-EBNA with TSiPSCs.

Figure 4: *In vitro* differentiation potential of TSiPSCs

(**a**) TSiPSC differentiated to Embryoid Bodies. (**b**) Immunofluorescence analyses of TSiPSCs for endodermal marker SOX17, mesodermal marker myosin ventricular heavy chain α/β and

ectodermal markers βIII tubulin and SOX2. Nuclei are stained with 4', 6-diamidino-2-phenylindole.

Figure 5: Neuronal and cerebral organoid differentiation of TSiPSCs

(**a**) To understand cytoarchitecture of differentiated neurons, phalloidin staining of Actin was done. TSiPSC-derived neurons displayed pyramidal shaped neuronal soma (arrowhead) with dendrites and axons (arrows). Nuclei are stained with 4', 6-diamidino-2-phenylindole. Immunostaining. (**a**) Immunostaining for Nestin and actin to observe gross morphology of the organoids. (**c**) Staining for Nestin to visualize the apically and basally organized neuronal layers. Nuclei are stained with 4', 6-diamidino-2-phenylindole.

Table 1: Composition of media, buffers, and solutions

Table 2: PCR Reaction Mix

Table 3: PCR Cycling Program

DISCUSSION:

Generation of stable cellular models of cytogenetically abnormal fetal tissue is necessary for perpetuating defective phenotype. The iPSC route is the most effective method of cell preparation for perpetual conservation of defective properties [20].

Pluripotent stem cells (PSC) display properties of self-renewal and differentiation into specialized cells reminiscent of early cleavage embryos[21]. Hence, PSCs can serve as excellent models to study early molecular, cellular and developmental defects in prematurely aborted fetuses.

In this article we have described human iPSC generation using nucleofection combined with the improved episomal vectors. The results show that this combination comprises a robust method for generating integration-free human iPSC lines as evidenced by the fact that single transfections were sufficient for successful reprogramming. We tracked the progressive conversion of FCV fibroblasts to pluripotent cells microscopically. 20 days post transfection we observed colonies of reprogrammed TSiPSCs surrounded with non-reprogrammed FCV fibroblasts. Morphologically, the derived human iPSCs resembled embryonic stem cells grown alongside in the lab. Typically, the cells aggregated as compact colonies with shiny borders. The cells of the colonies had large nuclei and tightly packed suggesting close membrane contact between the cells. The non-reprogrammed fibroblasts arched and surrounded these colonies. Upon transfer to iMEFs they continue proliferate in culture for over 30 transfers demonstrating the property of continued self-renewal.

As TSiPSCs were generated from 45XO fibroblasts we karyotyped the cells to check if they retained the chromosomal composition. The TSiPSCs maintained the 45XO karyotype in cell continuous culture suggesting a stable 45XO chromosome genetic makeup. To be useful as cellular resource representing 45XO aneuploidy the TSiPSCs should be free of exogenous DNA used in the reprogramming experiments. We checked to the presence of residual episomal plasmids by performing a genomic DNA PCR for episomal specific markers-OriP and EBNA. We

found no trace of these markers in TSiPS cells after 15 passages suggesting that the TSiPSCs progressively lost episomal reprogramming vectors in prolonged culture.

The hallmark of a pluripotent cells is its potential to differentiate to cells of three germ lineages both *in vitro* and *in vivo.* To test this capability in the derived TSiPSCs we subjected them *in vitro* to embryoid body formation and differentiation assays directed by lineage specifying cytokines and growth factors. TSiPSCs formed embryoid bodies and differentiated into ectodermal cells expressing neuronal markers, mesodermal cells expressing cardiac markers and endodermal cells expressing SOX17 a biomarker of endoderm fate. We also tested the ability of TSiPSCs to differentiate into higher order 3D cerebral organoids using previously established protocols[22]. TSiPSCs progressively self-organise due to their own intrinsic developmental programs into mini tissues called organoids. TSiPSCs yielded cerebral organoids showing a cytoarchitecture similar to brain tissue with neuroepithelium surrounding a ventricle like cavity. However these organoids have to be further characterised extensively to reveal the exact cell types and compared with normal iPSCs to distinguish the intrinsic neural tissue patterning properties of TSiPSCs. These cerebral organoids and other types of brain organoids generated from TSiPSCs can be used to model developmental and functional inconsistencies that may contribute to the symptoms of neurological deficiencies of TS individuals. TSiPSCs exhibited biomarker characteristics of pluripotency as well as the hallmark trait of differentiation thus highlighting the success of reprogramming to induced pluripotency.

The above-described method has work efficiently in reprogramming dermal fibroblasts and mesenchymal cells derived from various sources in our lab (data of other lines not shown). In our experience, the following steps are critical for the success of the reprogramming experiment:

a) Quality of plasmid preparation: old preparations do not yield iPSCs.

b) Quality of cells used for transfections: proliferating cells are essential for iPSC generation. 0.5 to 1 million cells per transfection yielded a reproducible reprogramming efficiency.

c) Freshly reconstituted nucleofector reagents: reconstituted nucleofector reagents stored for over a month did not yield iPSCs.

d) Maintenance of master cell bank by mechanical subculture of the iPSCs yielded stable lines. Enzymatic dissociation was used as per experiment requirement.

The future aim of the lab is to establish a panel of chromosomally abnormal iPSCs for downstream development, functional and disease modelling using this efficient method. Fetal aneuploidies cause pregnancy loss and organ malformations in live births. Aneuploid iPSCs derived from tissues of spontaneous abortuses are a valuable resource to model and study failed embryonic developmental events. *In vitro* 2D and 3D culture systems including embryoid bodies and tissue specific organoids[22] will enable researchers to understand molecular and cellular irregularities such as aberrant cell proliferation and cell death in lineage specific cells that could manifest as developmental anomalies and pregnancy failures associated with aneuploidy syndromes.

ACKNOWLEDGMENTS:

Financial support for the above research was provided by Manipal Academy of Higher Education. Characterization of the line was conducted partially in the laboratory of M. M. Panicker at NCBS. We thank Anand Diagnostic Laboratory for assistance with karyotyping.

DISCLOSURES:

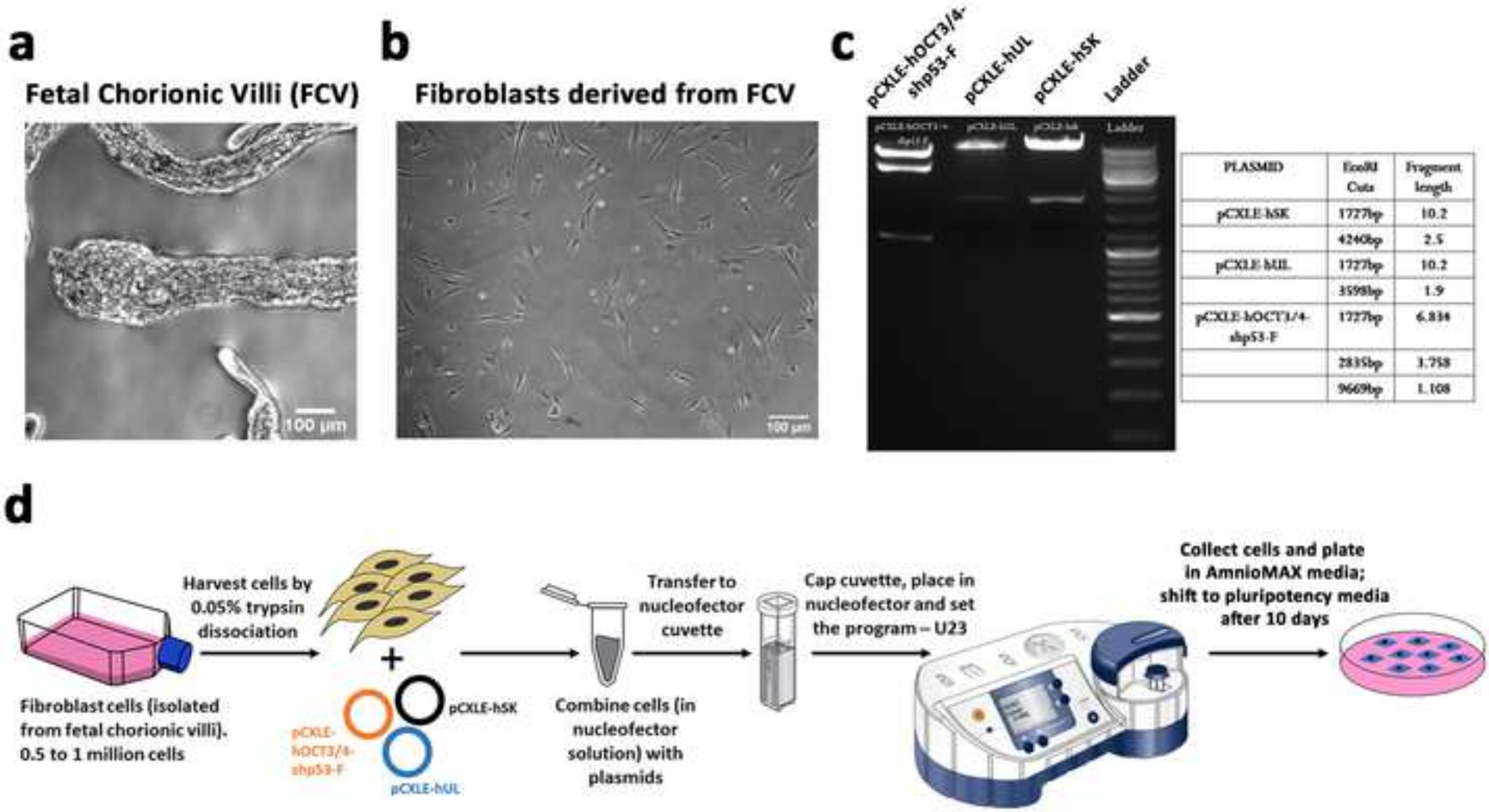
The authors have nothing to disclose.

REFERENCES:

1. Verlinsky, Y. *et al.* Human embryonic stem cell lines with genetic disorders. *Reproductive BioMedicine Online*. **10**, 105–110 (2005).
2. Eiges, R. *et al.* Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos. *Cell Stem Cell*. **1**, 568–577 (2007).
3. Biancotti, J.-C. *et al.* Human Embryonic Stem Cells as Models for Aneuploid Chromosomal Syndromes. *STEM CELLS*. **28**, 1530–1540 (2010).
4. Li, W. *et al.* Modeling abnormal early development with induced pluripotent stem cells from aneuploid syndromes. *Human Molecular Genetics.* **21**, 32–45 (2012).
5. Gravholt, C. H., Viuff, M. H., Brun, S., Stochholm, K., Andersen, N. H. Turner syndrome: mechanisms and management. *Nature Reviews Endocrinology.* **15**, 601–614 (2019).
6. Luo, Y. *et al.* Uniparental disomy of the entire X chromosome in Turner syndrome patient-specific induced pluripotent stem cells. *Cell Discovery.* **1**, 15022 (2015).
7. Luo, Y. *et al.* Generation of an induced pluripotent stem cell line from an adult male with 45,X/46,XY mosaicism. *Stem Cell Research.* **27**, 42–45 (2018).
8. Parveen, S., Panicker, M. M., Gupta, P. K. Generation of an induced pluripotent stem cell line from chorionic villi of a Turner syndrome spontaneous abortion. *Stem Cell Research.* **19**, (2017).
9. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*. **131**, 861–872 (2007).
10. Soldner, F. *et al.* Parkinson's Disease Patient-Derived Induced Pluripotent Stem Cells Free of Viral Reprogramming Factors. *Cell*. **136**, 964–977 (2009).
11. Somers, A. *et al.* Generation of Transgene-Free Lung Disease-Specific Human Induced Pluripotent Stem Cells Using a Single Excisable Lentiviral Stem Cell Cassette. *STEM CELLS*. **28**, 1728–1740 (2010).
12. Woltjen, K. *et al.* piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. **458**, 766–770 (2009).
13. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., Hochedlinger, K. Induced Pluripotent Stem Cells Generated Without Viral Integration. *Science*. **322**, 945 LP – 949 (2008).
14. Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., Hasegawa, M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proceedings of the Japan Academy, Series B*. **85**, 348–362 (2009).
15. Warren, L. *et al.* Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell*. **7**, 618–630 (2010).
16. Kim, D. *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell*. **4**, 472–476 (2009).

17. Yu, J. *et al.* Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. *Science*. **324**, 797 LP – 801 (2009).
18. Okita, K. *et al.* A more efficient method to generate integration-free human iPS cells. *Nature Methods*. **8**, 409–412 (2011).
19. Martí, M. *et al.* Characterization of pluripotent stem cells. *Nature Protocols.* **8**, 223–253 (2013).
20. Avior, Y., Sagi, I., Benvenisty, N. Pluripotent stem cells in disease modelling and drug discovery. *Nature Reviews Molecular Cell Biology.* **17**, 170–182 (2016).
21. Ambartsumyan, G., Clark, A. T. Aneuploidy and early human embryo development. *Human Molecular Genetics.* **17**, R10–R15 (2008).
22. Lancaster, M. A., Knoblich, J. A. Generation of cerebral organoids from human pluripotent stem cells. *Nature Protocols.* **9**, 2329–2340 (2014).

Figure 1



PLASMID	EcoRI Cuts	Fragment length
pCXLE-hSK	1727bp	10.2
	4240bp	2.5
pCXLE-hUL	1727bp	10.2
	3598bp	1.9
pCXLE-hOCT3/4-shp53-F	1727bp	6.838
	2835bp	3.758
	9669bp	1.108

Figure 2

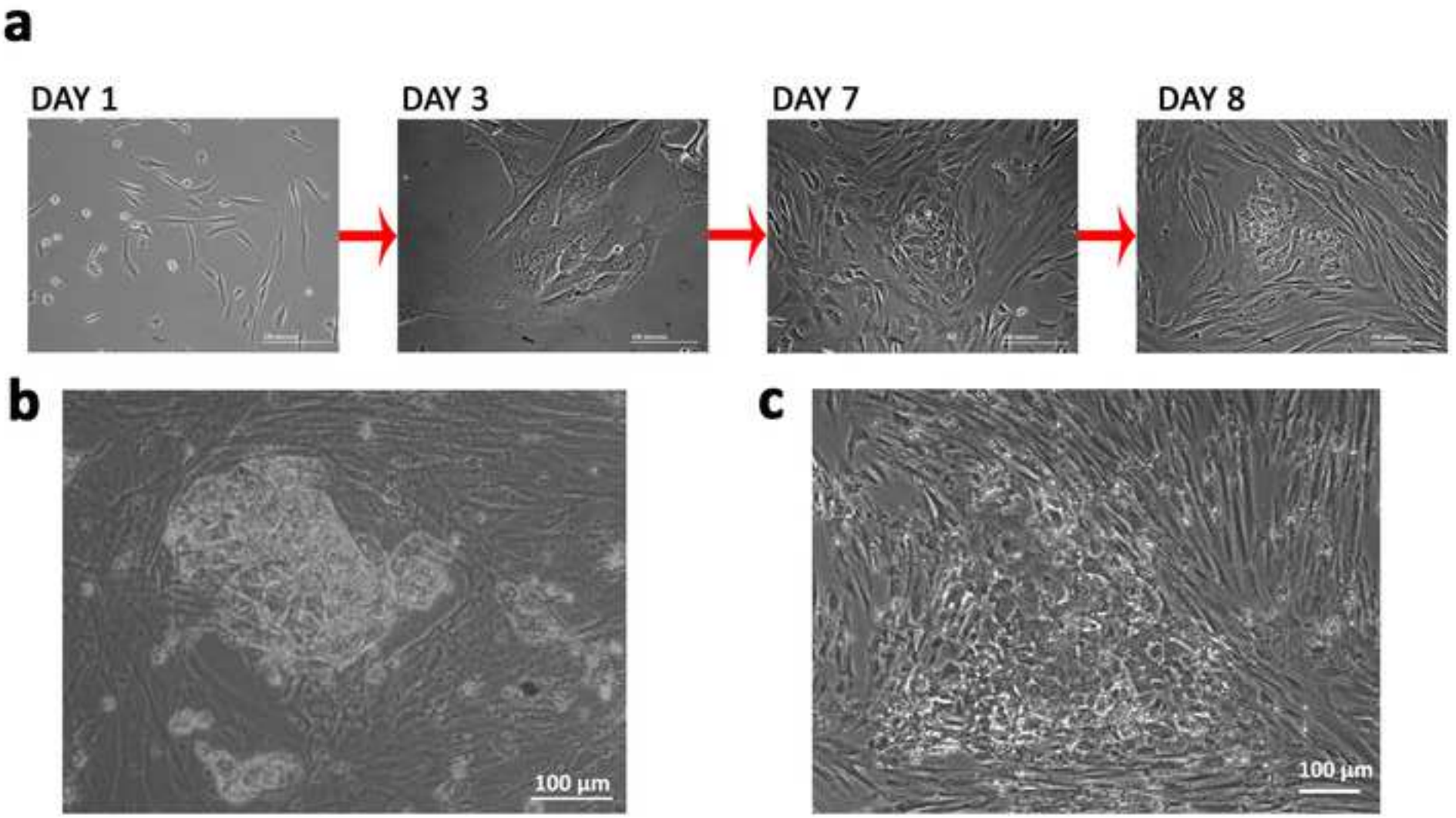


Figure 3

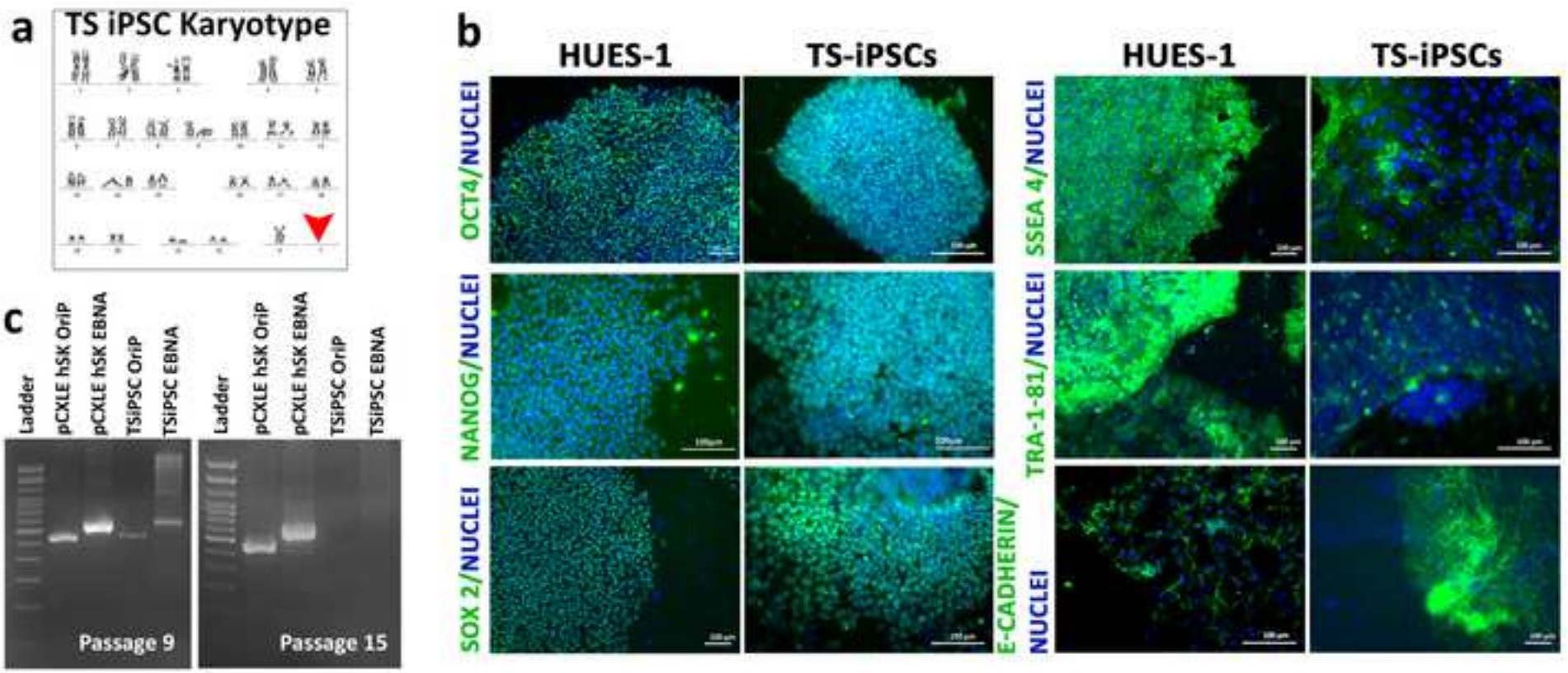


Figure 4

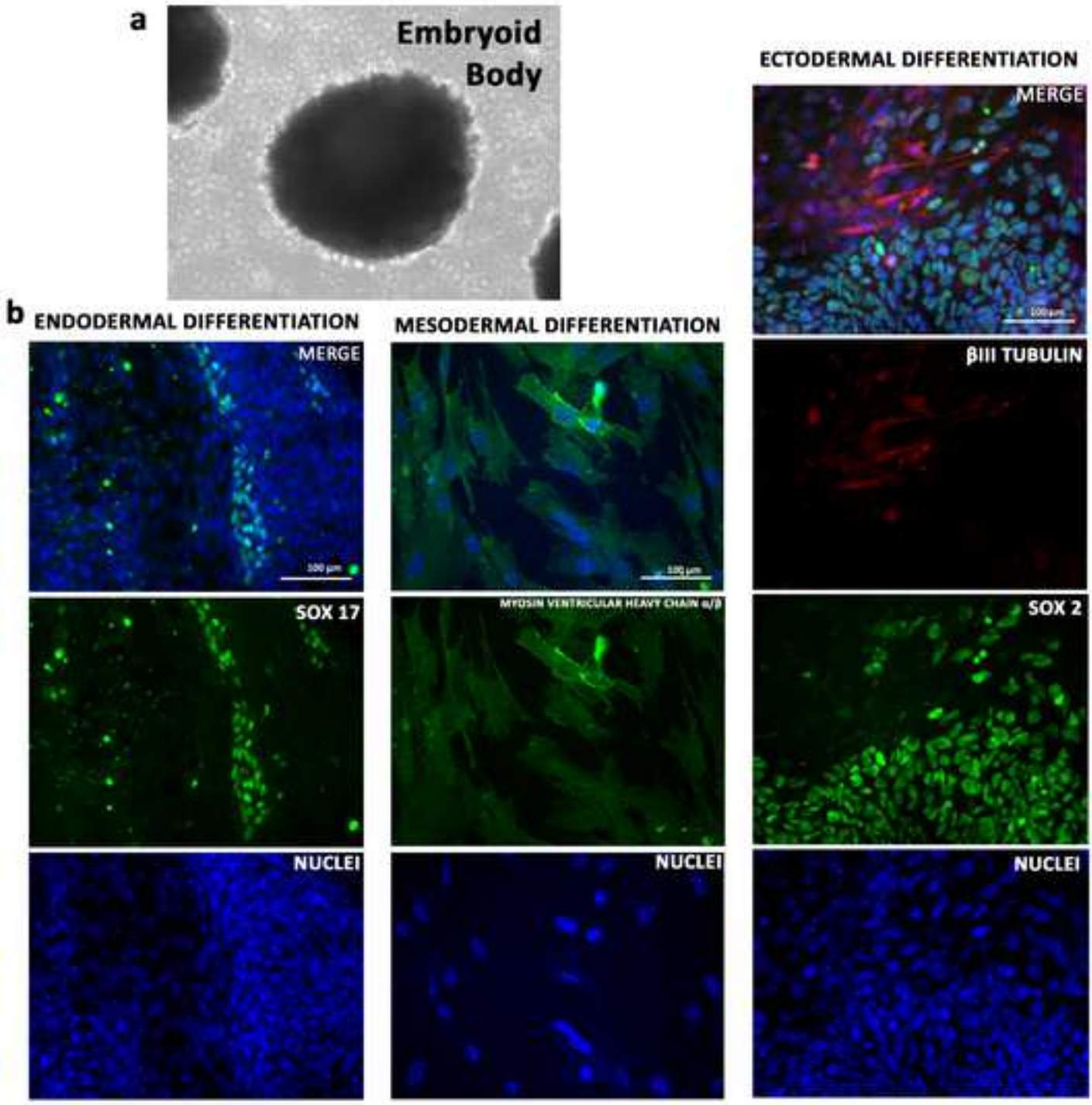


Figure 5

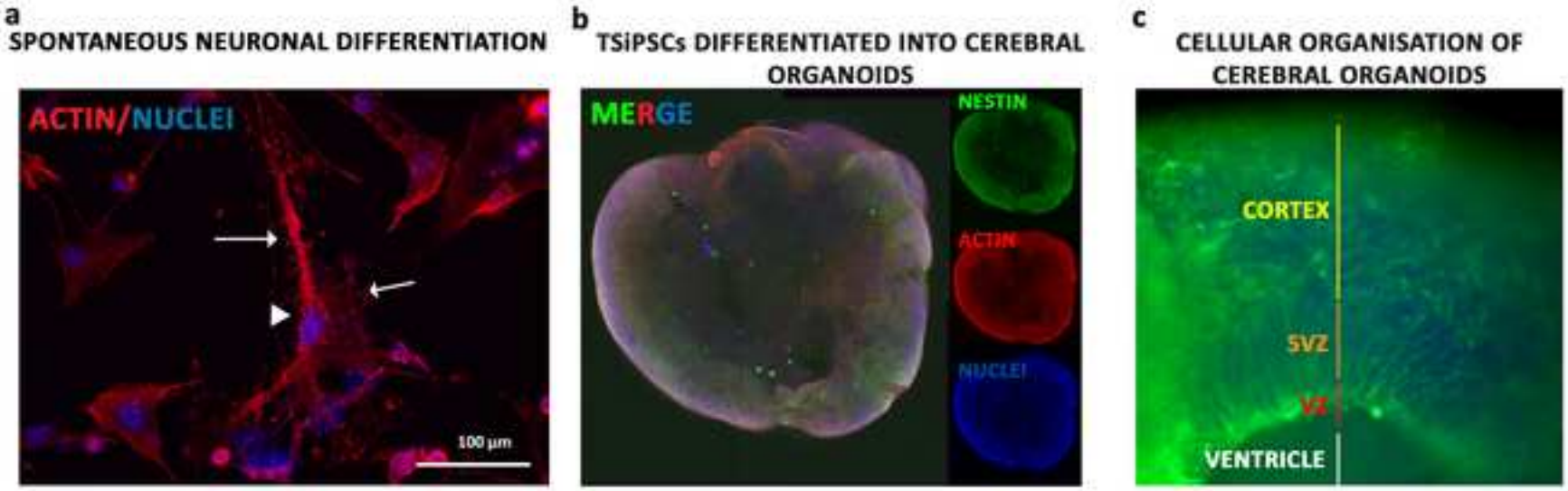


Table 1

Media, Buffers etc	Composition
Pluripotent Stem Cell Medium	Knock out DMEM Knock out serum replacement 200mM L-glutamine(100X) 10mM Non-essential amino acids (100X) Penicillin Streptomycin 55mM 2- Mercaptoethanol Basic fibroblast growth factor (100ng /µL)
Embryoid Body Medium	Knock out DMEM Knock out serum replacement 200mM L-glutamine (100X) 10mM Non-essential amino acids (100X) Penicillin Streptomycin 55mM 2- Mercaptoethanol
Tris Acetate EDTA Buffer (TAE) (50X)	Tris base Glacial acetic acid EDTA (5M,pH8) Storage: Room temperature.
Hypotonic Solution	Potassium chloride solution Sodium citrate
Carnoy's Fixative	Glacial acetic acid :Methanol
Resuspension buffer	Tris·Cl (pH 8.0) EDTA RNase A
Lysis buffer	NaOH SDS
Neutralization buffer	Potassium acetate (pH 5.5)
Wash buffer	NaCl MOPS (pH 7.0) Isopropanol
Elution buffer	NaCl Tris·Cl (pH 8.5) Isopropanol

Amount	Final concentration	Comments
38.75mL 10 mL 0.5mL 0.5mL 0.25mL 2.5 µL 5µL **Total volume:50mL**	 20% 2mM 0.1mM 50U/mL and 50µg/mL 0.1mM 10 ng/mL	For pluripotent stem cell culture
38.75mL 10 mL 0.5mL 0.5mL 0.25mL 2.5 µL **Total volume:50mL**	 20% 2mM 0.1mM 50U/mL and 50µg/mL 0.1mM	For embryoid body culture
242 g 57.1 mL 100mL **Total volume: 1000 mL**	50X	For electrophoresis
0.56 g 1g **Total volume: 100 mL**	0.075 M 1%	For karyotyping
1:3 v/v		For karyotyping
	50mM 10mM 100 µg/ml	For plasmid isolation
	200 mM 1% (w/v)	For plasmid isolation
	3.0 M	For plasmid isolation
	1.0 M 50 mM 15% (v/v)	For plasmid isolation
	1.25 M 50 mM 15% (v/v)	For plasmid isolation

Table 2

Component	**25 µL reaction**	**Final conc.**
PCR-grade water	Up to 25 µL	N/A
5X KAPA HiFi Buffer	5.0 µL	1X 10 mM
KAPA dNTP Mix	0.75 µL	0.3 mM each
10 µM Forward Primer	0.75 µL	0.3 µM
10 µM Reverse Primer	0.75 µL	0.3 µM
Template DNA	As required	As required
1 U/µL KAPA HiFi DNA Polymerase	0.5 µL	0.5 U

Table 3

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	98°C	20 sec	15- 35
Annealing	60 – 75°C	15 sec	
Extension	72°C	15 – 60 sec/kb	
Final extension	72°C	1 min/kb	1

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Table of Materials

TableofMaterials.xlsx

RESPONSE TO REVIEWERS COMMENTS

Serial No.	COMMENT	RESPONSE
Reviewer 1		
1	Never addressed some of my concerns from before. They don't present text discussing how their lines compare to others in field.	The comparison included in the revised manuscript (lines 70 to 85)
2	They also never did any basic genomic stability testing, yes they did karyotyping but that was it.	Since the line was karyotypically stable, we decided that further testing was not needed.
3	Also the cells only get to P5, for any stable iPSC it should be a lot more than that, so these lines only seem to be partially reprogrammed based on their findings and descriptions of text. Given the lack of genomic stability testing, I'd be concerned that these cells are even stable enough for research applications.	In the manuscript we have mentioned the passage number in which the experiment was done. The lines are stable and still in culture in the lab.
4	New data was added on cerebral organoids, but it was not overly convincing. Staining was poor and not sure why they didn't stain for one neuronal marker like MAP2. They did stain for Nestin, but it's clear they get a neuronal ball of cells, whether this is cerebral organoid I am not sure. I don't think it adds to paper, and it is unclear why they don't simply show some 2D neurons.	Since 2D differentiation was already shown in the characterisation we decided to put up the organoid data. The Figure 5 clearly shows the organisation of cortical, subventricular, ventricule like zones in the organoid.
Reviewer 4		
5	A revision of literature on iPSCs generated from fetal cells like FCV and methods used for reprogramming should be mentioned in the Introduction and/or discussed in the Discussion to better understand the benefits of the protocol presented.	The revised literature included in introduction (lines 62 to 85)
6	In the Introduction, 2nd paragraph - The authors state "Though viral transductions are efficient, they integrate the reprogramming factors into the host chromosomes, causing insertion mutations which have unforeseen effects in the iPSCs and could pose risks for the translational application of iPSCs." Adenoviruses and Sendai viruses are used as non-integrative agents for reprogramming in contrast to lentivirus and other retroviruses. Therefore it is incorrect to say:" "Though viral transductions are efficient, they integrate the reprogramming factors into the host chromosomes,…". To my knowledge, these viruses are not any more prone to integration than the episomal vectors. This	The sentence has been changed to "Retroviral and lentiviral based reprogramming, although efficient, involve integration of the reprogramming factors into the host chromosomes, causing insertion mutations which have unforeseen effects in the iPSCs".

	should be corrected or the authors should provide more information that I am not aware.	
7	In point 4.1.1. of the "Protocol" indicate how MEFs were inactivated. This information is missing.	Inactivation method has been mentioned as Mitomycin C treatment.
8	The immunofluorescence images in Fig. 2 (the authors mention immunocytochemistry, but this looks immunofluorescence to me) for SSEA4, TRA-1-81 and E-Cadherin in TS-iPSCs are quite disappointing and differ from the control HUES1 ESC line. Better images are required to show correct staining of these surface markers. Also E-cadherin is not mentioned in "Characterization of TSiPSCs" of the "Representative Results" section.	The word immunocytochemistry has been changed to immunofluorescence in the revised manuscript. E-Cadherin staining has been mentioned in the legend in the revised manuscript.
9	The authors should refer whether they perform mycoplasma testing and the method used. This is a requirement for newly generated hiPSCs and should therefore be performed.	Mycoplasma testing has been performed. Data not shown in this manuscript. Previously published in *Parveen, S., Panicker, M. M. & Gupta, P. K. Lab resource: Stem cell line Generation of an induced pluripotent stem cell line from chorionic villi of a Turner syndrome spontaneous abortion. Stem Cell Res. 19, 12–16 (2016).*
10	The authors mention a section in the protocol called "Demonstration of transgene free status". Unfortunately, these data are not provided. A gel with appropriate controls should be shown.	The gel picture with appropriate controls has now been included in the revised manuscript.
11	In the introduction, last paragraph, the authors discuss the requirements needed to characterize newly generated iPSCs. I would add the importance of microbiology and virology testing, namely for mycoplasma to the list of requirements.	For this revision we are unable to do virology tests as we need to purchase reagents for the same.
12	"EcoR1" should be corrected "EcoRI" throughout the text.	Correction has been made in the revised manuscript.
13	All the figures are incorrectly labelled. The actual Figure 1 is not mentioned in the manuscript and it should, while Fig.2 is labelled as Fig.1, Fig.3 as Fig.2, etc. A small paragraph in the "Representative Results" section is needed for the actual Fig.1.	Figures are labelled correctly in the revised manuscript.
14	The analysis of cerebral organoid differentiation is rather minimal and could be improved, but, I agree, it is out of the scope of this article.	Yes. It is out of the scope of the article hence we did not elaborate in the manuscript.

15	Legend of Figure 4 - Chance "immunocytochemistry" for "immunofluorescence". The SOX2 staining is shown but not referred in the text or in the legend.	The changes have been made in the revised manuscript.
16	Legend of Figure 5 - Fig. 5b does not show Phalloidin, it shows Actin. Please correct in the legend. Also "oranoids" should be "organoids".	The corrections have been made in the revised manuscript.
Reviewer 5		
17	The authors mentioned FCV in Line 110 but the abbreviation of FCV was explained later (Line 113), which should be corrected.	Abbreviation and full form has been mentioned already in the heading of the protocol (line 98).
18	The authors used human embryonic stem cells to compare the characterization of TSiPSCs, however, they only mentioned it in the figure legend. They should mention it in the results and discuss the source and reasons for using these cells in this study.	Mentioned in discussion in revised manuscript
19	Figure 4 should be rearranged with the same size of both individual markers and merged images. Individual markers, BIII tubulin, Sox2, and Nuclei in the lower panel represent the merged image (upper panel) of ectodermal differentiation, which seem to be unclear because of smaller size and poor image quality. It should be corrected.	The image has been rearranged in the revised manuscript.
20	In this study, TSiPSCs were generated by delivering episomal reprogramming plasmids and the original paper was published elsewhere. The advantage of using this approach is that iPSC lines can be free from transgene after 10-15 pages. However, the authors did not clarify why they used the lower passage of TSiPSC lines (after 5-passage) to characterize and compare with human embryonic stem cells.	Since the line could be stably passaged the characterisation experiments were started. Since the cells were expressing NANOG we assumed the cells to be fully reprogrammed and did not wait for the line to become transgene free. Repeated characterisations have been performed and the cell still express pluripotency markers. We have put up representative images in the figures.
21	The discussion seems incomplete because of lacking the accuracy of cohesion and coherence in the texts and insufficient information. Methods and results should be discussed precisely in the discussion.	Discussion modified in revised manuscript.

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LIFE'S ON

ETHICS COMMITTEE

Ethics Committee of Manipal Hospitals, Bangalore

17th Oct 2013

To,

Ms. Shagufta Parveen,
Senior Grade Lecturer,
School of Regenerative Medicine,
Bangalore -65

Dear Ms. Shagufta Parveen,

The Institutional Ethics Committee/ Independent Ethics Committee **"Ethics Committee of Manipal Hospitals", Bangalore** reviewed and discussed your application to conduct the study entitled **"Generation & study of induced Pluripotent Stem Cells from Placental MSCS, Chorionic Villus samples and B Thallasemia Blood"**

The following documents were reviewed & approved:-

1. Protocol
2. Investigators protocol agreement
3. Patient Information sheet and informed consent form

The following documents were reviewed:

1. Protocol signature page
2. CV of the Investigator
3. Scientific Committee Approval

The following members of the Ethics Committee were present at the meeting held on 10th Oct 2013 at 3.00 pm, at Manipal Hospital Bangalore-17. (Quorum as per ICMR guidelines was met)

Name	Qualification	Designation/ Title	Affiliations as to the Institution Yes/No
Dr. Ananya Chakraborthy	MD (Pharmacology)	Pharmacologist/ Voting Member	No
Rev Fr. Arokianathan	MA (Sociology), MA (Journalism) B Ed, B.Th	Theologian/ Voting Member	No
Mrs. Rekha. K	M.C.S	Lay Person/ Voting Member	No
Dr. S. S. Iyengar	DM (Cardiology)	Member Secretary Non-Voting Member	Yes
Dr. Vishwanath Siddini	MD (Nephrology)	Assistant Member Secretary/Voting Member/	Yes
Dr. Ravi Shankar. S	FRCPCH (Pediatrics)	Clinician/ Voting Member	Yes
Dr. Shivaram. C	MBBS,MHA DCP (Pathology)	Clinician Voting Member	Yes
Mr. Vasantha Krishna. K	BA (Law) L.L.B	Voting Member/ Legal	Yes
Dr. Rita Mhaskar	MD (Obst & Gynae)	Voting Member/ Clinician	Yes
Dr. Pratibha Pereira	Diploma (Geriatrics)	Voting Member/ Clinician	No

NOTE: In absence of the Chairman Justice S R Bannurmath, Dr. Ananya Chakraborty was designated as Chairperson for this Meeting.

Please find enclosed a letter for the same

We approve the study to be conducted in its presented form

We hereby confirm that the **Ethics Committee of Manipal Hospitals, Bangalore** is organized and operates as per the ICH-GCP and Schedule Y guidelines.

Yours sincerely,

Dr. S. S. Iyengar
Member Secretary,
Ethics Committee of Manipal Hospitals

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ETHICS COMMITTEE

Ethics Committee of Manipal Hospitals, Bangalore

08th Nov 2016

To,

Ms. Shagufta Parveen,
Senior Grade Lecturer,
School of Regenerative Medicine,
Manipal University
Bangalore -65

Ref: "Generation & study of induced Pluripotent Stem Cells from Placental MSCS, Chorionic Villus samples and B Thallasemia Blood"

Sub: Informed Consent Waiver

Dear Ms. Shagufta Parveen,

This is with reference to your request to waive off the Informed Consent Form for the above referred study. EC noted that the study was approved by the EC on 17th Oct 2013 & as per ICMR guidelines for Biomedical Research on Human Participants the Informed Consent can be waived if it is justified that the research involved not more than minimal risk or when the participants and the researcher do not come in contact. If such studies have protection in place for both privacy and confidentiality, and do not violate the rights of the participants the IECs may waive off the requirement for informed consent in the following instances:

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ETHICS COMMITTEE

- Research on **anonymised biological sample for deceased individual, left over samples after clinical investigations.**

EC also noted that the samples collected by you for the study are of aborted fetuses (deceased) and left over samples after clinical investigation were collected & no invasive procedure was used to collect the sample. Hence EC gives waiver of informed consent for the above study & also waiver for the previous samples.

We hereby confirm that the **Ethics Committee of Manipal Hospitals, Bangalore** is Organized and operates as per the ICH-GCP and Schedule Y guidelines.

Yours sincerely,

Dr. Vishwanath Siddini
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