Derivation, characterization and retinal differentiation of induced pluripotent stem cells

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Millions of people world over suffer visual disability due to retinal dystrophies which can be age-related or a genetic disorder resulting in gradual degeneration of the retinal pigmented epithelial (RPE) cells and photoreceptors. Therefore, cell replacement therapy offers a great promise in treating such diseases. Since the adult retina does not harbour any stem cells, alternative stem cell sources like the embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) offer a great promise for generating different cell types of the retina. Here, we report the derivation of four iPSC lines from mouse embryonic fibroblasts (MEFs) using a cocktail of recombinant retroviruses carrying the genes for Oct4, Sox2, Klf4 and cMyc. The iPS clone MEF-4F3 was further characterized for stemness marker expression and stable reprogramming by immunocytochemistry, FACS and RT-PCR analysis. Methylation analysis of the nanog promoter confirmed the reprogrammed epigenetic state. Pluripotency was confirmed by embryoid body (EB) formation and lineage-specific marker expression. Also, upon retinal differentiation, patches of pigmented cells with typical cobble-stone phenotype similar to RPE cells are generated within 6 weeks and they expressed ZO-1 (tight junction protein), RPE65 and bestrophin (mature RPE markers) and showed phagocytic activity by the uptake of fluorescent latex beads.

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1. Introduction

Retinal degenerations are progressive disorders resulting in gradual loss of photoreceptors and RPE cells of retina leading to blindness or severe visual impairment (Verhoeff 1930; Klein *et al.* 1997; Weleber and Gregory-Evans 2001; Dandona and Dandona 2001) and the disease onset can be congenital or age-related. Mutations in several genes involved in photo transduction pathways, retina-specific transporters, transcription factors and vitamin A metabolism are linked to Mendelian forms of retinal dystrophy. However, the exact physiological effects of these mutations leading to cell death are poorly understood. Since the adult retina does not have any stem cells, the cells that are gradually lost are not regenerated. The existing treatment modalities employing gene therapy aims to treat the disease by gene replacement and thereby preventing or delaying further cell death. However, the non-functional cells should remain viable for the gene therapy to be effective. But, in congenital forms of retinal dystrophy and in cases where the majority of photoreceptors are lost, cell replacement therapy holds a great promise in treating such conditions. Studies have shown that the retina is amenable to cell replacement therapy with retinal progenitors or post-mitotic photoreceptor precursors

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(Klassen *et al.* 2004; MacLaren *et al.* 2006), and embryonic stem (ES) cells (Lund *et al.* 2006) are a valuable stem cell source that are capable of forming neural retinal progenitors and RPE cells (Ikeda *et al.* 2005; Lamba *et al.* 2006; Osakada *et al.* 2008; 2009a; 2009b; Idelson *et al.* 2009; Meyer *et al.* 2009). Also, ES-cell-derived retinal cell types have been shown to rescue disease phenotype to some extent when transplanted in animal models (Maclaren *et al.* 2006; Lamba *et al.* 2009). Also, a recent study has demonstrated the safety of subretinal transplantation of hESC-derived retinal pigment epithelium (RPE) in patients with Stargardt's macular dystrophy and reported no signs of tumour formation or immune reaction after 4 months of transplantation (Schwartz *et al.,* 2012). However, the lack of sufficient well-characterized and clinical grade ES lines for HLA matching of diverse ethnic groups and the associated ethical concerns pose a significant obstacle for their clinical use even in case of allogenous applications. This has initiated a search for possible adult stem cell sources and several reports have shown that adult somatic cells can be directly reprogrammed to a pluripotent state very similar to ES cells by the ectopic expression of transcription factors like Oct4, Sox2 and Klf4 with/without cMyc to generate induced pluripotent stem (iPS) cells (Takahashi and Yamanaka 2006; Takahashi *et al.* 2007; Yu *et al.* 2007; Wernig *et al.* 2007; 2008; Nakagawa *et al.* 2008; Park *et al.* 2008). It was also shown that the iPS cells are capable of giving rise to retinal cells when differentiated under suitable culture conditions (Osakada *et al.* 2009b; Buchholz *et al.* 2009; Carr *et al.* 2009; Hirami *et al.* 2009; Meyer *et al.* 2009; Lamba *et al.* 2010; Eiraku *et al.* 2011; Kokkinaki *et al.* 2011; Tucker *et al.* 2011). These adult somatic cell derived pluripotent stem cells could therefore become a valuable stem cell alternative for developmental studies and possibly for cell-based therapies.

Here we report the establishment and characterization of a mouse iPS cell line, MEF-4F3, and also discuss a protocol for differentiating these cells into RPE cells by supplementing the differentiating medium with a RPE-cell-line-conditioned medium.

2. Materials and methods

2.1 *Maintenance of cell cultures*

The primary MEFs were cultured in DMEM containing 10% FBS, 50 units of penicillin and 50 mg/mL streptomycin and the irradiated MEFs were seeded on 0.1% gelatin-coated dishes in MEF medium and were used as feeders for iPSCs within 3 days. The ecotropic retroviral packaging cell line, Plat-E (Morita *et al.* 2000), were maintained in DMEM containing 10% FBS, 50 units of penicillin, 50 mg/mL streptomycin, 1 mg/mL puromycin and 100 mg/mL blasticidin S (Sigma). Mouse iPS cells were maintained on irradiated MEF feeders or 0.1% gelatin-coated dishes and cultured with ES medium that consisted of DMEM/F-12 (Invitrogen, Life Technologies Corporation, Carlsbad, USA), supplemented with 15% ES-Qualified FBS (Hyclone, Thermo Fisher Scientific Inc.), 1% nonessential amino acid solution, 1 mM L-glutamine (Invitrogen), 0.1% β-mercaptoethanol and 10 ng/mL mouse leukemia inhibitory factor (Sigma-Aldrich, Inc) and maintained at 37°C with 5% CO_2. The iPS cells were passaged by manual cutting or by enzymatic digestion with 0.25% Trypsin/1 mM EDTA for 5 min at 37°C.

2.2 *Preparation of mouse embryonic fibroblasts and irradiated feeders*

Embryos from 13.5 day pregnant CD1 mice were collected and washed with sterile PBS containing double-strength antibiotics. The head and visceral tissues of embryos were removed and the remaining tissues were washed, minced thoroughly and suspended in 10 mL of MEF medium and transferred to a 50 mL falcon tube. The contents were passed 2–3 times through a 16-gauge needle fitted to 20 mL syringe. The larger tissue pieces were allowed to settle down briefly for a minute and the suspension was transferred to a fresh tube, centrifuged at 100*g* for 3 min and the cell pellet was suspended in MEF medium and plated in a 100 mm dish. The larger tissue pieces were then washed once with PBS, incubated with 10 mL of 0.25% trypsin/1 mM EDTA for 10 min at 37°C. After trypsinization, 20 mL of MEF medium was added and triturated by pipetting few times using a 10 mL pipette to enable tissue dissociation. The larger tissue pieces were allowed to settle down for a minute and the suspension was transferred to a fresh tube, centrifuged at 100*g* for 3 min and the cell pellet was suspended in MEF medium and plated into four 100 mm dishes and the cultures were maintained at 37°C with 5% CO2 and are passaged after 3 days. Stock vials were frozen from passage P_0–P_3. MEFs at passage one were used for reprogramming experiments. For preparing feeders, MEFs at passage three was mitotically inactivated by applying 5000 rad of radiation in a gamma irradiator, cryopreserved or seeded at a density of 1×10^4 cells/cm^2 in MEF medium and used within 3 days. All experiments involving animals were conducted in adherence to the ARVO statement for use of animals in ophthalmic and vision research and with the approval of Institutional Review Board (IRB) and ethics committee.

2.3 *Retroviral preparation and derivation of mouse miPSCs*

For preparing recombinant retroviruses, Plat-E cells (Morita *et al.* 2000) were seeded at a density of 0.75×10^6 cells per well of a collagen coated 6-well plate. The cells were then

transfected with pMXs retroviral vectors for mouse Oct3/4, Sox2, klf4 and cMyc ((Addgene Nos. 13366, 13367, 13370, 13375) using Fugene 6 (Roche Ltd., Basel, Switzerland) according to the manufacturer's instructions. Transfected cells were then cultured at 37°C with 5% CO2 and after 12 h; the spent medium was replaced with fresh medium supplemented with 10 mM sodium butyrate to boost viral production and further cultured for not more than 12 h. After sodium butyrate treatment, the cells were washed with PBS three times and fresh MEF medium was added and the cells were further incubated 37°C with 5% CO2. After 48 h of transfection, the retrovirus-containing culture supernatants are collected, passed through 0.45 μm cellulose acetate filters (Millipore Corporation, MA, USA) and supplemented with 10 μg/mL polybrene (Millipore). MEFs at passage one were seeded 12 h before transduction at a density of 1×10^5 cells per well of a 6-well plate. Equal volumes (0.5 mL each) of retroviral/polybrene-containing supernatants of all four genes (approximate MOI of 2) were added to the MEFs and incubated overnight at 37°C with 5% CO2. Fresh MEF medium was added after removing the spent medium next day. After 3 days of transduction, the cells were split and transferred to a 100 mm dish containing irradiated MEF feeders seeded at a density of 1×10^4 cells per cm^2 and cultured in mouse ES medium at 37°C with 5% CO_2, with fresh media changes on every alternate day. The clones that emerged after 2 to 3 weeks of culture were picked based on colony morphology for further expansion and characterization.

2.4 *Immunocytochemistry and imaging*

The cells grown on glass coverslips were fixed for 10 min with 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X-100 for 10 min with three PBS washes after each step. The permeabilization step was skipped for staining surface antigens. The cells were then blocked with 1% BSA at room temperature for 1 h and then sequentially incubated with specific primary and fluorescent dye conjugated secondary antibodies at appropriate dilutions (refer to table 1) at room temperature for 1 h each with three PBS washes after each incubation steps. Propidium iodide or DAPI was used as counter stains. Alkaline phosphatase staining was done using a kit as per the manufacturer's instruction (Chemicon, Millipore Corporation, MA, USA). The cells were then washed and mounted on a glass slide and imaged using an epifluorescence microscope (Olympus IX71, Japan) or a confocal microscope, LSM 510 (Carl Zeiss, Inc., NY, USA), and the images were analysed using the Image Pro Express and LSM 510 Meta, Version 3.2 softwares, respectively, and the composites were prepared using Adobe Photoshop CS.

2.5 *Gene expression by RT-PCR*

Total RNA was isolated from cell samples by Trizol method and cDNAs were prepared by reverse transcription using SuperScript® II RT kit (Invitrogen, Life Technologies Corporation) as per the manufacturer's instruction. PCRs were performed for all the genes tested using cDNAs as the template. Table 2 summarizes the primer details. The amplicons were resolved on 1% (w/v) agarose gels, stained with ethidium bromide (0.5 μg/mL), imaged under UV light and documented using Gel Doc™ XR+ System (BioRad, USA).

2.6 *FACS analysis*

The cells harvested by enzymatic digestion were fixed and/or premeabilized as described above. Staining was done by sequential incubation with specific primary and fluorescent dye conjugated secondary antibodies at appropriate dilutions (refer to table 1) for 1 h each with three PBS washes after each incubation steps. Unstained cells and cells stained with secondary antibody alone or isotype antibody were used as negative controls. The stained samples were then analysed using FACS Aria I cell sorter and analysis was done using FACS Diva software (BD Biosciences, NJ, USA).

2.7 *Methylation analysis by bisulphite sequencing*

Genomic DNA isolated from the MEFs and miPS clones were bisulphate-treated using the Bisulphite conversion kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, USA) according to the manufacturer's instructions. The bisulphate-converted DNA was then used for PCR amplification of Nanog gene promoter region (refer to table 1 for primer details). The primers were designed to be specific for converted DNA by choosing a C-rich region. They do not have CpGs in their sequence but flank 6 CpGs in the amplified region. Therefore, they can be used to amplify both the methylated and non-methylated bisulphate-converted DNA samples. The resulting amplicon was cloned into pMOS-Blue vector (Amersham, GE Healthcare Ltd., UK) and screened for positive transformants by blue-white screening. Plasmid DNA was isolated from six randomly selected transformants for each cell type and methylation patterns of the inserts were analysed by sequencing using T7 and U19 universal primers.

2.8 *RAPD genotyping*

For RAPD genotyping, PCR reactions were carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Life Technologies Corporation, Carlsbad,

Table 1. Primary and secondary antibodies

S. No.	Antigen	Antibody	Supplier	Dilution
1	Oct4	Mouse monoclonal	Chemicon,	1:50
2	SSEA1	Mouse monoclonal	Chemicon	1:50
3	Nanog	Rabbit polyclonal	Abcam	1:50
4	Bestrophin	Rabbit polyclonal	Abcam	1:50
5	ZO-1	Rabbit polyclonal	Invitrogen	1:100
6	RPE65	Mouse monoclonal	Chemicon	1:50
7	Anti-mouse IgG, Biotin conjugated	Goat polyclonal	Invitrogen	1:300
8	Anti-rabbit- IgG, Biotin conjugated	Goat polyclonal	Invitrogen	1:300
9	Streptavidin-Alexa 488	NA	Invitrogen	1:700
10	Streptavidin-Alexa 594	NA	Invitrogen	1:700

USA) using genomic DNA templates along with no templates controls. Each 20 µL reaction contained about 50 ng of DNA template, 1X PCR Buffer (10 mM Tris Hcl pH 8.3; 50 mM KCl), 2.5 mM MgC_{12}, 0.2 mM dNTP mix (Fermentas, Thermo Fisher Scientific Inc.), 10 pM of single decamer primer (Eurofins Genomics India Pvt. Ltd, Bangalore, India) and 0.2 U of Taq DNA polymerase (Fermentas, Thermo Fisher Scientific Inc). Thermocycler was programmed for initial denaturation for 3 min at 94°C, followed by 30 cycles of 45 s at 94°C denaturation, 1 min at 37°C annealing, 1 min at 72°C extension and final extension at 72°C for 7 min and kept on hold at 4°C thereon. The amplicons were resolved on 1% (w/v) agarose gels, stained with ethidium bromide (0.5 µg/mL), imaged under UV light and documented using Gel Doc™ XR+ System (BioRad, USA).

2.9 *Lineage differentiation of iPSCs*

To initiate differentiation of iPS into different germ layers, the cells grown on gelatin-coated dishes were manually cut into small pieces consisting of 100–150 cells and induced to undergo aggregation or embryoid body formation in suspension cultures on non-adherent bacteriological dishes with mouse ES medium devoid of LIF (differentiating medium, DM) for 7 days. To differentiate iPS cells into RPE cells, we followed a directed differentiation protocol. For this, the iPS cells were grown on Matrigel-coated dishes (BD Biosciences) and were directly allowed to differentiate for about 4–6 weeks in two stages without EB formation. In stage 1, the adherent cultures were allowed to differentiate for 4 days in DM in the absence of LIF. In stage 2, from day 5 onwards, the cells were cultured in the presence of DM supplemented with equal volumes of conditioned medium obtained from confluent cultures of the human RPE cell line, D407. The conditioned medium was passed through 0.45 µm cellulose acetate filters before use.

2.10 *Phagocytosis assay*

miPS-derived RPE cells grown on 35 mm Matrigel-coated dishes were incubated with 1-µm-sized green fluorescence latex beads (Sigma-Aldrich, Inc) at a concentration of 1.0×10^6 beads/mL for 6 h at 37°C. The cells were then washed thoroughly with PBS for five times to remove the free extracellular and floating beads. The cells were then fixed and processed for fluorescence imaging with DAPI as counter stain for the nucleus.

3. Results

3.1 *Derivation and characterization of miPSCs*

We used the retroviral method of reprogramming the MEFs to generate mouse iPSC lines. Primary cultures of MEFs were established and about 1×10^5 cells in their first passage were transduced with freshly prepared cocktail of viral supernatants (Oct3/4, Sox2, Klf4 and cMyc) to generate mouse iPSC lines (figure 1A). Out of the total 40–50 colonies that emerged after 3 weeks of transduction (0.05%), we picked up 12 early-emerged colonies (0.012%) based on their embryonic stem-cell-like morphology (figure 1B) for immediate expansion and cryopreservation. Four of these iPS clones, named 4F1–4F4, were successfully expanded beyond 20 passages and were further characterized. RT-PCR analysis of all the four clones for the expression of stem cell factors, Oct3/4, Sox2, Klf4 and cMyc, using specific primer sets (refer to table 2) revealed that the endogenous copies of all the four genes were successfully activated in all the clones (figure 2A). However, the ectopically introduced transgenes were completely silenced in clone 4F3, while some of them continued to express albeit at low levels in clones 4F1 (Klf4), 4F2 (Oct3/4) and 4F4 (Sox2 and Klf4) (figure 2A). Silencing of ectopically expressed transgenes

Table 2. Gene-specific primer sets for PCR amplification

Genes	Primer Sequence	Annealing Temperature °C	Product size (bp)	Accession number
	Pluripotency markers			
Oct3/4 Endo	N-S: F: TCTTTCCACCAGGCCCCCGGCTC N-AS: R: TGCGGGCGGACATGGGGAGATCC	63	224	NM_013633
Oct3/4 Transgene	F: pMX-S1811- GACGGCATCGCAGCTTGGATACAC N-AS: R: TGCGGGCGGACATGGGGAGATCC	61	319	
mSox2 Endo	C-S: F: TAGAGCTAGACTCCGGGCGATGA C-AS: R: TTGCCTTAAACAAGACCACGAAA	53	297	NM_011443
Sox2 Transgene	F: pMX-S1811- GACGGCATCGCAGCTTGGATACAC N-AS: R: GACCATACCATGAAGGCGTTCATG	60	287	
Klf4 Endo	C-S: F: GCGAACTCACACAGGCGAGAAACC C-AS: R: GAATCGCTTCCTCTTCCTCCGACAC	57	714	NM_010637
Klf4 Transgene	F: pMX-S1811- GACGGCATCGCAGCTTGGATACAC N-AS: R: GTCTGTGGCCACCGTCGCCGCCAG	61	312	
cMyc Endo	C-S: F: TGACCTAACTCGAGGAGGAGCTGGAATC C-AS: R: AAGTTTGAGGCAGTTAAAATTATGGCTG	55	170	NM_010849
cMyc Transgene	F: pMX-S1811- GACGGCATCGCAGCTTGGATACAC N-AS: R: CAGCTCGCTCTGCTGTTGCTGGTG	59	245	
	Ectodermal markers			
Sox1	F: GGATCTCTGGTCAAGTCGGAG R: CTGGCGCTCGGCTCTCCAGAG	61	381	NM_009233
Pax6	F: AACCAGCTCCAGCATGCAGAA	58	304, 262	NM_001244198
	R: AACTTCTGGAGTCGCCACTCT			NM_001244201
	Mesodermal markers			
α-SMA	F: GAGAAGAGCTACGAACTGCCTGAC R: CACATCTGCTGGAAGGTAGACAG	57	359	NM_007392
Bra	F: GTTCCTGGTGCTGGCACCCTCTGC R: CAGACCAGAGACTGGGATACTG	58	316	NM_009309
	Endodermal markers			
Sox17	F: CACAGCAGAACCCAGATCTGCAC R: CATGTGCGGAGACATCAGCGGAG	61	338	NM_011441
AFP	F: GTGAGCATTGCCTCCACGTGCTG R: GTGACAGCCGCCAGCTGCTCCTC	59	353	NM_007423
	Loading control			
ACTB	F: GTTGCCGGTCCACACCCGCCAC R: GTTGAAGGTCTCAAACATGATC	57	415	NM_007393
	Early eye field markers			
Pax6	F: AACCAGCTCCAGCATGCAGAA	58	304, 262	NM_001244198
	R: AACTTCTGGAGTCGCCACTCT			NM_001244201
Six3	F: GCGAGCAGAAGACCCATTGCTTC R: CTATCATACATCACATTCCGAGTC	59	386	NM_011381
	RPE transcription factors			
mMitf	F: GCGAACCGGCACCTGCTGCTCAG R: CATGCTCCGTTTCTTCTGCGCTC	59	424	NM_008601
Otx2	F: CTGTCCACTCCAGGCGAATCGAG R: CAGGCCTCACTTTGTTCTGACCTC	59	412	NM_144841
	Pigment biosynthesis			
Tyr	F: GTAGCATGCACAATGCCTTAC R: CTGAAGGCATAGCCTACTGC	55	428	NM_011661
	Phagocytosis			
Mertk	F: GAGAGCCTGGCGGACCGAGTCTAC R: GCAACTGGGTATTGATGTAGATG	56	325	NM_008587

Table 2 (continued)

Genes	Primer Sequence	Annealing Temperature °C	Product size (bp)	Accession number
	Primers for methylation analysis by bisulfite sequencing			
Met-Nanog	F: GATTTTGTAGGTGGGATTAATTGTGAATTT R: ACCAAAAAAACCCACACTCATATCAATATA	53 (6 CpGs)	367	NC_000072
	RAPD primers			
OPP-10	TCCCGCCTAC	37	NA	NA
OPZ-10	CCGACAAACC	37	NA	NA
OPP-02	TCGGCACGCA	37	NA	NA
OPAH-01	TCCGCAACCA	37	NA	NA

and the activation of the endogenous pluripotency genes are the hallmarks of stable reprogramming. Therefore, we proceeded with the clone 4F3 for further characterization and differentiation experiments.

Immunocytochemistry with primary antibodies against the pluripotent stem cell markers like the Oct4, SSEA1 and Nanog and the alkaline phosphatase (ALP) enzyme assay confirmed the expression of these antigens in the iPS clone 4F3 (figure 2C). Also, FACS analysis of the cells grown on gelatin-coated dishes with antibodies against Oct4 and SSEA1 revealed that majority of them (99% and 94% respectively) expressed these stem cell antigens and retained their pluripotent state even after being in culture for 20 passages (figure 2B). Unstained cells and the isotype antibody stained cells were used a negative controls. These observations confirm the long term stability of reprogrammed cells.

In order to confirm successful genomic reprogramming, we analysed the methylation patterns of the mouse Nanog gene promoter region, both in the parental MEFs and in the iPS clone 4F3 by bisulphite conversion method as described above. Sequence analysis of the cloned promoter region revealed that majority of the CpGs analysed remained methylated in MEFs while they became unmethylated in the iPS clone 4F3 (Figure 3A). This suggests that the iPS clone 4F3 has undergone stable epigenetic reprogramming at this genomic locus.

To verify the genetic identity of the iPS clone 4F3 with that of the parental MEF cells, we performed RAPD fingerprinting. RAPD profiling was done using four different random decamers (refer to table 2). Out of the four primers used, OPP10 and OPZ10 primers showed distinct PCR product profiles, which confirmed that the MEFs and the iPS clone were genetically identical as opposed to a genetically distant human HCE cell line (figure 3B).

3.2 *Differentiation of mouse iPS cells*

In order to check for the pluripotency of the mouse iPS clone 4F3, the cells were induced to undergo EB formation for 7 days and were analysed for different germ layer specific marker expression by RT-PCR, using specific primer sets listed in table 2. The results reveal that upon differentiation, the cells have down-regulated the expression of stem cell markers like the Oct4 and Sox2 and up-regulated the expression of all three germ layer specific markers like the ectodermal markers (Pax 6 and Sox1), mesodermal markers (α-SMA and Brachyury) and the endodermal markers (Sox 17 and AFP) (figure 4A), thus confirming their pluripotent state.

3.3 *Derivation of RPE cells from mouse iPS cells*

To differentiate the iPS cells to RPE lineage, we have established a two-step differentiation protocol by directly culturing the cells grown on matrigel in ES medium devoid of LIF for the first 4 days, and subsequently from day 5 onwards, the cultures were maintained in differentiating medium supplemented with RPE conditioned medium as described above. RT-PCR analysis of day 30 differentiated cultures have revealed that, upon differentiation, the expression of pluripotent stem cell marker Oct4 is down-regulated and the expression of the neuroectodermal commitment marker (Pax 6), eye field specification markers (Six3 and Otx2), RPE progenitor cell marker (Mitf) and the mature RPE markers involved in phagocytosis (Mertk) and pigment biosynthesis (Tyrosinase) were up-regulated (figure 4B), thus confirming retinal lineage differentiation.

Upon light microscopic examination of these differentiated cells after 6 weeks of differentiation, we could observe several pigmented and distinct epithelial cell patches with typical cobble-stone phenotype similar to RPE cells. These cells expressed ZO-1 (a tight junction protein) and also had phagocytic activity as observed by the uptake of 1 μm sized green fluorescence latex beads, which were localized in the cytoplasm by confocal microscopy (figure 4C). Also, FACS analysis of these differentiated cultures with primary

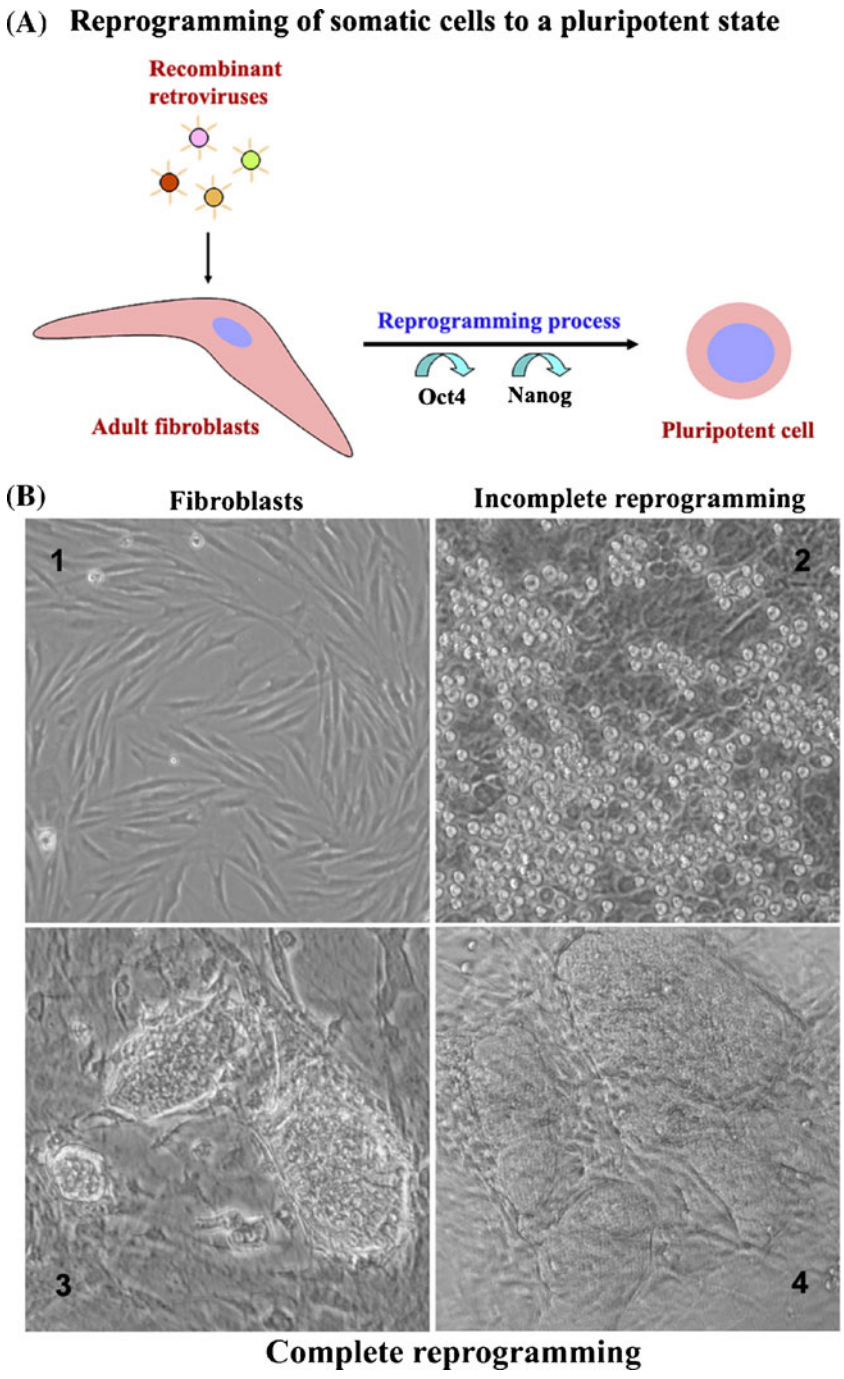


Figure 1. Generation of mouse iPSCs. (**A**) Cartoon representing the reprogramming process. Briefly, the somatic cells are transduced by a cocktail of retroviruses expressing Oct3/4, Sox2, Klf4 and cMyc. This in turn triggers the activation of endogenous copies of these genes, resulting in epigenetic reprogramming of the entire genome and the establishment of a pluripotent state. (**B**) (**1**) Phase contrast image showing the morphology of primary MEFs. (**2**) Partially reprogrammed clusters of MEF cells showing changes in cell morphology after 1 week of retroviral transduction. (**3, 4**) Completely reprogrammed ES-like colonies of iPS cells that emerged after 3 weeks of transduction. Magnification 10×.

antibodies against RPE65 and bestrophin revealed that 13.6% and 8.5% of the analysed cell population expressed these antigens respectively, thus confirming the differentiation of iPS clone 4F3 into RPE cells.

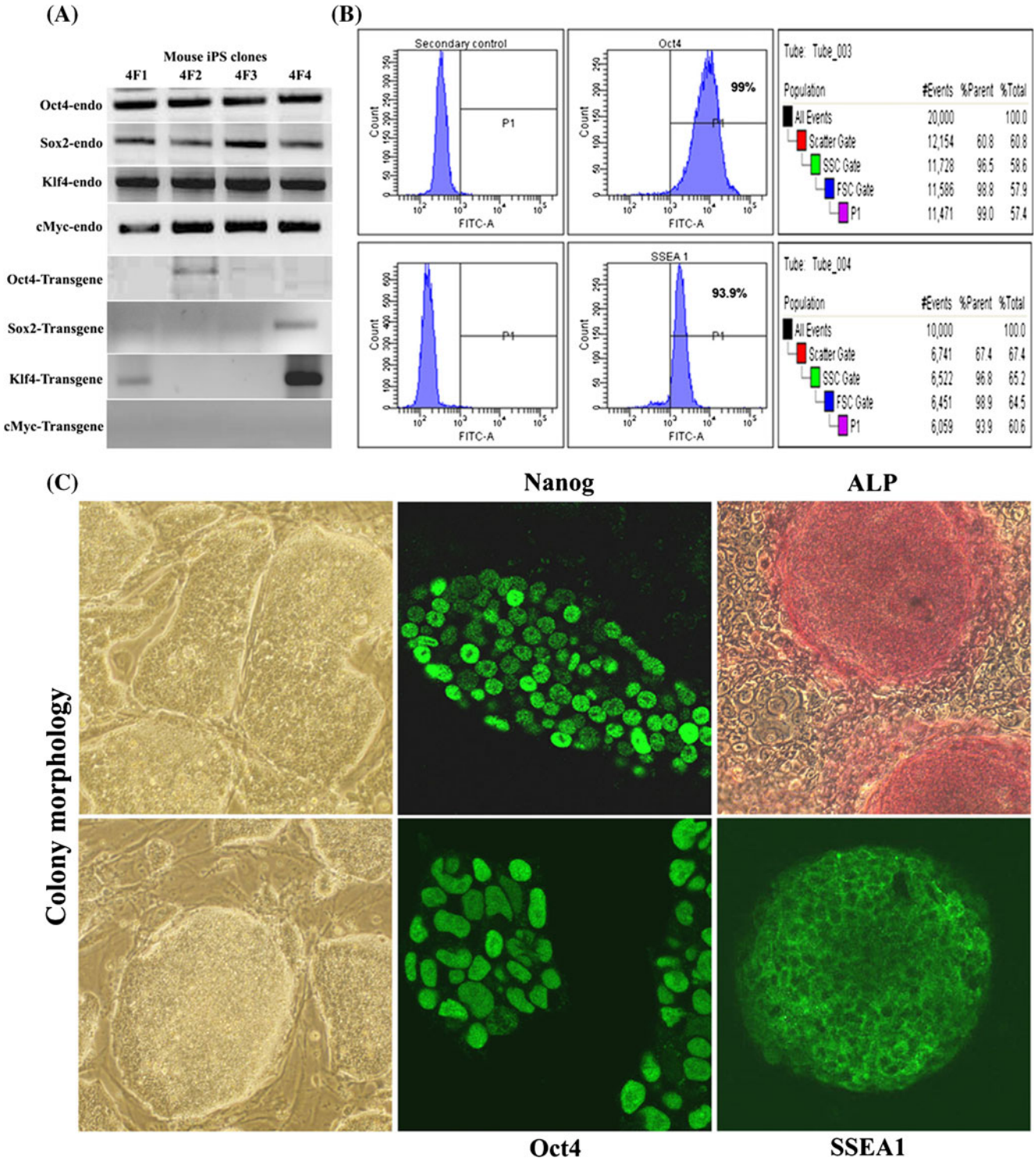


Figure 2. Characterization of miPSC lines for stem cell marker expression. (**A**) Total RNA isolated from mouse iPSC clones were used in RT-PCR experiment to check for the expression of both the endogenous and ectopically transduced copies of the genes Oct3/4, Sox2, Klf4 and cMyc. All the four iPS clones (4F1–4F4) expressed high levels of endogenous genes while some of the transgenes were either weekly expressed (4F1,4F2, 4F4) or completely silenced (4F3). (**B**) FACS profile of Oct3/4 and SSEA1 expressing cells (right panels) in clone 4F3 cultures which indicates 99% and 93.9% positives, suggesting their pluripotent stem cell state. Left panel shows the event profile of secondary antibody control and the gates were set to exclude the unstained cells. (**C**) Phase and immuno fluorescence pictures of iPS clone 4F3 showing proper colony morphology and the expression of stem cell antigens like Oct4, Nanog, SSEA1 and alkaline phosphatase (ALP). Magnification 10×.

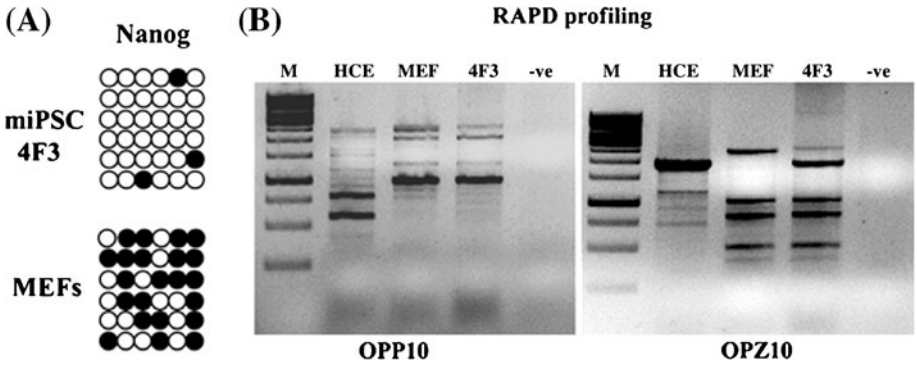
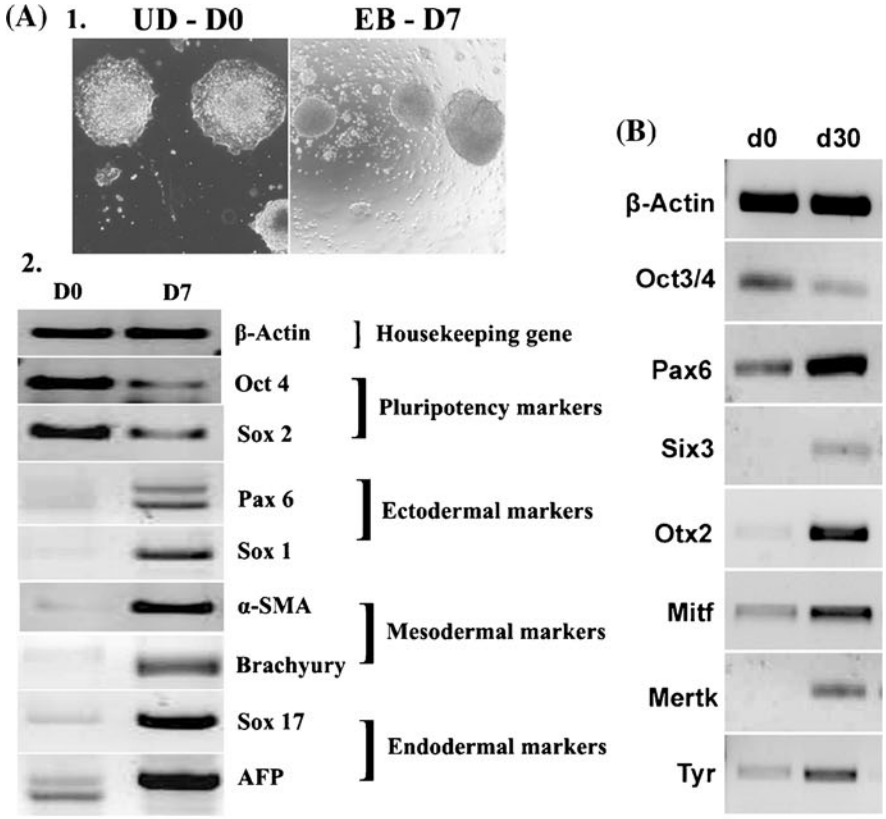


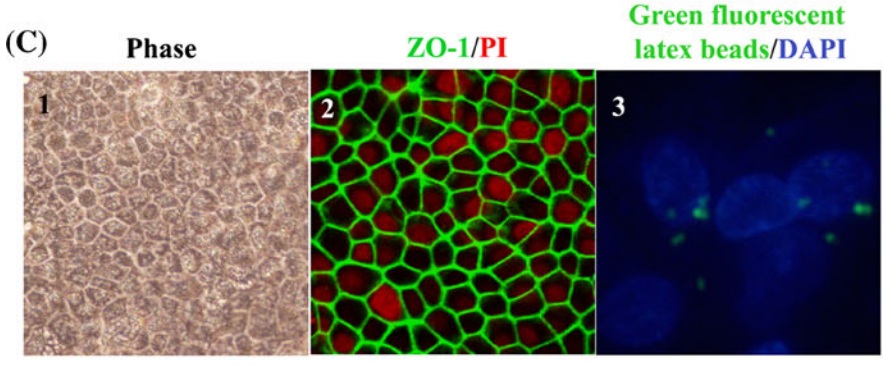
Figure 3. Assessment of epigenetic reprogramming and genotyping of miPSCs. (**A**) Methylation status of the 6 CpGs analyzed (each column) of six randomly sequenced clones (6 rows) in the promoter region of nanog gene is represented as 6X6 matrix for both the iPSC clone 4F3 and the parental MEFs. Open circles indicate the unmethylated state and dark and filled circles indicate the methylated state and the overall pattern indicates that the loci tested is highly methylated in MEFs, while they got reprogrammed to unmethylated state in the iPS clone. (**B**) RAPD profiling of genomic DNA with random decamer primers, OPP10 and OPZ10 shows similar amplicon profiles for both the MEFs and the iPSCs (lanes 3,4) while it is different for the unrelated human corneal epithelial cells (lane 2). A 100 bp ladder (M) and a no template PCR control (negative) are loaded in lanes: 1, 5 respectively.

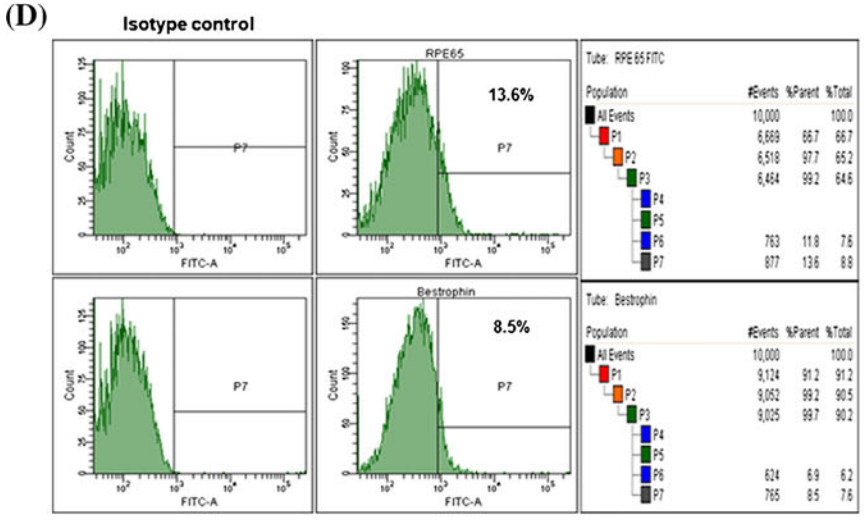
4. Discussion

Induced pluripotent stem cells offer a very good *in vitro* model system to address several basic questions related to development and cellular differentiation as well as disease-related changes and they can be derived using methods that are not technically demanding as opposed to deriving embryonic stem cell lines. In this study, we report the derivation of mouse iPS lines using the retroviral method reported earlier by Takahashi and Yamanaka (2006). We used all four factors (Oct4, Sox2, Klf4 and cMyc) to induce reprogramming in MEF cells and could derive mouse iPSCs at an efficiency of about 0.05% after 3 weeks of transduction, which was comparable to those reported earlier using similar methods (Wernig *et al.* 2007; 2008; Nakagawa *et al.* 2008). Also, we could expand four of these clones beyond 20 passages, both on MEF feeders as well on 0.1% galatin coated plates (data not shown). Although all the four clones retained their stemness after passaging several times, three of them continued to express some of the transgenes as reported earlier (Park *et al.* 2008). However, the hypomethylated status of the nanog promoter region of clone 4F3, which did not express any of the transgenes, suggests that epigenetic reprogramming of the genome may be important for the generation of stable iPS clones. Although improved methods of iPS derivation protocols were reported to generate zero footprint iPS lines (Huangfu *et al.* 2008; Kaji *et al.* 2009; Kim *et al.* 2009a, b; Okita *et al.* 2011; Narsinh *et al.* 2011) at a much higher efficiency and using lower number of factors, the retroviral method still offers a simple, definite and one-shot approach to derive iPS lines for non-clinical and basic research purposes.

The stable iPS clone 4F3 also retained its pluripotency and could differentiate into cell types of all three germ layers in EB cultures. Upon differentiation, the stem cell markers were down-regulated and the lineage-specific markers were activated within 7 days of differentiation. While the iPSCs are capable of spontaneous differentiation towards different cell types in the absence of LIF, we have used RPE-conditioned medium to initiate directed differentiation of iPS cells towards the RPE lineage. With this differentiation protocol, the iPS clone 4F3 gave rise to rosette-like differentiating cell clusters resembling retinal progenitors, which gave out epithelial outgrowths predominantly and non-pigmented RPE-like patches of cells with typical cobble-stone phenotype, could be seen as early as 2–3 weeks after differentiation (data not shown). They continued to expand further to generate larger sheets of RPE-like cells that gradually acquired pigmentation over a period of 4–6 weeks of differentiation, expressed some of the progenitor and mature cell markers and also showed some level of phagocytic activity as discussed in the results. We also found that the cells were not heavily pigmented at this time point and continued to proliferate when they were manually passaged by selective picking. However, long-term cultures (3–4 months) might result in terminally differentiated cells showing increased pigmentation and the expression of high levels of mature cell markers as reported by some of the earlier studies (Buchholz *et al.* 2009; Carr *et al.* 2009; Osakada *et al.* 2009b; Hirami *et al.* 2009). We also found that after the initial passage they lost their cobble-stone morphology and became fibroblastic. However they regained their typical epithelial morphology upon reaching confluency and also developed pigmentation (data not


(A)
1.
UD - D0
EB - D7
2.
D0
D7
β-Actin
Housekeeping gene
Oct 4
Sox 2
Pluripotency markers
Pax 6
Sox 1
Ectodermal markers
α-SMA
Brachyury
Mesodermal markers
Sox 17
AFP
Endodermal markers
(B)
d0
d30
β-Actin
Oct3/4
Pax6
Six3
Otx2
Mitf
Mertk
Tyr


(C)
Phase
ZO-1/PI
Green fluorescent latex beads/DAPI
1
2
3


(D)
Isotype control
RPE65
13.6%
Bestrophin
8.5%
P7
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Count
Tube: RPE65 FITC
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#Events
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P1
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P3
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P6
P7

◀ **Figure 4.** Differentiation and characterization of iPSC-derived RPE cells. (**A**) (**1**) Morphology of growing culture of iPSCs (day zero, D_0) and susension cultures of EBs (day seven, D_7). Magnification 4×. (**2**) RT-PCR profiles showing comparative gene expression levels of pluripotent stem cell markers (Oct3/4, Sox2), ectodermal markers (Pax6, Sox1), mesodermal markers (alpha smooth muscle actin, brachyury) and the endodermal markers (Sox17, alpha fetoprotein) in day zero and day seven EB cultures. (**B**) RT-PCR profiles showing comparative gene expression levels of neuro ectodermal commitment marker (Pax6), eye field specification markers (Six3 and Otx2), RPE progenitor cell marker (Mitf) and the mature RPE markers involved in phagocytosis (Mertk) and pigment biosynthesis (Tyrosinase)) in day zero, D_0 and day thirty, D_{30} retinal differentiation cultures. Beta actin expression level is used as a loading control. (**C**) (**1**) Phase image of RPE-like pigmented cells differentiated from iPSCs. Magnification 10×. (**2**) Confocal picture of iPSC-derived RPE cells stained with primary antibody against tight junction protein, ZO-1 (green) showing typical cobble stone phenotype. The nuclei of the cells are counted stained with PI (red). Magnification 40×. (**3**) Confocal picture of iPSC-derived RPE-like cells showing phagocytosed and internalized 1 μm sized fluorescence latex beads (green) in the cytosol which confirms their phagocytic activity. The nuclei of the cells are counted stained with DAPI (blue). Magnification 63×. (**D**) FACS profile of RPE65 and bestrophin expressing cells (right panels) in 6 weeks old differentiating cultures indicates 13.6% and 8.5% positives, which confirms the retinal potential of mouse iPS clone 4F3. Left panel shows the event profile of isotype control and the gates were set to exclude the unstained cells.

shown). Therefore, subsequent passaging of proliferating progenitors might enable further cell expansion and enrichment. However, it may be important to check as to how long these proliferating immature cells could be passaged and maintained in culture before they get terminally differentiated to form mature and functional RPE cells. While the long-term cultures are required for generating mature cell sheets for drug testing and for transplantation studies, committed retinal progenitors could be derived as early as 1–2 weeks of differentiation. Recent reports have shown that retinal progenitors and non-pigmented, proliferating RPE cells are amenable for cryopreservation and further cell expansion *in vitro* (Schwartz *et al.* 2012). Therefore, it may be important to identify suitable culture conditions to maintain them in the progenitor state to enable large scale cell expansion without terminal differentiation. Also, since the differentiated cell population remains heterogeneous, it may be important to identify suitable cell surface markers for high-efficiency isolation of these committed retinal progenitors that can be cryopreserved to establish master cell banks meant for clinical applications. To conclude, the mouse iPS line (MEF-4F3) generated and characterized by us behaved like ES cells in terms of the stemness and pluripotent properties and was capable of differentiating into RPE cells *in vitro*.

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References

Buchholz DE, Hikita ST, Rowland TJ, Friedrich AM, Hinman CR, Johnson LV and Clegg DO 2009 Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. *Stem Cells* **27** 2427–2434

Carr AJ, Vugler AA, Hikita ST, Lawrence JM, Gias C, Chen LL, Buchholz DE, Ahmado A, *et al.* 2009 Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. *PLoS One* **4** e8152

Dandona R and Dandona L 2001 Review of findings of the Andhra Pradesh Eye Disease Study: policy implications for eye-care services. *Indian J. Ophthalmol.* **49** 215–234

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T and Sasai Y 2011 Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472** 51–56

Hirami Y, Osakada F, Takahashi K, Okita K, Yamanaka S, Ikeda H, Yoshimura N and Takahashi M 2009 Generation of retinal cells from mouse and human induced pluripotent stem cells. *Neurosci. Lett.* **458** 126–131

Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W and Melton DA 2008 Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat. Biotechnol.* **26** 1269–1275

Idelson M, Alper R, Obolensky A, Ben-Shushan E, Hemo I, Yachimovich-Cohen N, Khaner H, Smith Y, *et al.* 2009 Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell Stem Cell* **5** 396–408

Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, Miyoshi H, Kamiya D, Honda Y, *et al.* 2005 Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **102** 11331–11336

Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P and Woltjen K 2009 Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* **458** 771–775

Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, *et al.* 2009a Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* **4** 472–476

Kim JB, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, *et al.* 2009b Oct4-induced pluripotency in adult neural stem cells. *Cell* **136** 411–419

Klassen HJ, Ng TF, Kurimoto Y, Kirov I, Shatos M, Coffey P and Young MJ 2004 Multipotent retinal progenitors express developmental markers, differentiate into retinal neurons, and preserve light-mediated behavior. *Invest. Ophthalmol. Vis. Sci.* **45** 4167–4173

Klein R, Klein BE, Jensen SC and Meuer SM 1997 The five-year incidence and progression of age-related maculopathy: the Beaver Dam Eye Study. *Ophthalmology* **104** 7–21

Kokkinaki M, Sahibzada N and Golestaneh N 2011 Human induced pluripotent stem-derived retinal pigment epithelium (RPE) cells exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE. *Stem Cells* **29** 825–835

Lamba DA, Gust, J and Reh TA 2009 Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. *Cell Stem Cell* **4** 73–79

Lamba DA, Karl MO, Ware CB and Reh TA 2006 Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **103** 12769–12774

Lamba DA, McUsic A, Hirata RK, Wang PR, Russell D and Reh TA 2010 Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. *PLoS One* **5** e8763

Lund RD, Wang S, Klimanskaya I, Holmes T, Ramos-Kelsey R, Lu B, Girman S, Bischoff N *et al.* 2006 Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. *Cloning Stem Cells* **8** 189–199

MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, Akimoto M, Swaroop A, Sowden JC and Ali RR 2006 Retinal repair by transplantation of photoreceptor precursors. *Nature* **444** 203–207

Meyer JS, Shearer RL, Capowski EE, Wright LS, Wallace KA, McMillan EL, Zhang SC and Gamm DM 2009 Modeling early retinal development with human embryonic and induced pluripotent stem cells. *Proc. Natl. Acad. Sci. USA* **106** 16698–16703

Morita S, Kojima T and Kitamura T 2000 Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* **7** 1063–1066

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, *et al.* 2008 Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* **26** 101–106

Narsinh KH, Jia F, Robbins RC, Kay MA, Longaker MT and Wu JC 2011 Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. Nat. Protoc. **6** 78–88

Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, *et al.* 2011 A more efficient method to generate integration-free human iPS cells. *Nat. Methods* **8** 409–412

Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, Yoshimura N, Akaike A, Sasai Y and Takahashi M 2008 Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat. Biotechnol.* **26** 215–224

Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, Sasai Y and Takahashi M 2009a In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. *J. Cell. Sci.* **122** 3169–79

Osakada F, Ikeda H, Sasai Y and Takahashi M 2009b Stepwise differentiation of pluripotent stem cells into retinal cells. *Nat. Protoc.* **4** 811–24

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW and Daley GQ 2008 Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451** 141–146

Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, *et al.* 2012 Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* **379** 713–720

Takahashi K and Yamanaka S 2006 Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126** 663–676

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S 2007 Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131** 861–872

Tucker BA, Park IH, Qi SD, Klassen HJ, Jiang C, Yao J, Redenti S, Daley GQ and Young MJ 2011 Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. *PLoS One* **6** e18992

Verhoeff FH 1930 Microscopic observations in a case of retinitis pigmentosa. *Trans. Am. Ophthalmol. Soc.* **28** 176–195

Weleber RG and Gregory-Evans K 2001 Retinitis pigmentosa and allied disorders; in *Retina* (eds) SJ Ryan (St. Louis: Mosby) pp 362–470

Wernig M, Lengner CJ, Hanna J, Lodato MA, Steine E, Foreman R, Staerk J, Markoulaki S and Jaenisch R 2008 A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat. Biotechnol.* **26** 916–924

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE and Jaenisch R 2007 *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448** 318–324

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, *et al.* 2007 Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318** 1917–1920

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