Chapter 9
Spontaneous Generation of Patient-Specific Retinal Pigment Epithelial Cells Using Induced Pluripotent Stem Cell Technology

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Abstract Stem cell technology has a number potential uses when it comes to the eye, particularly disease and developmental modelling, and as potential therapeutic source. A variety of protocols have been developed that facilitate the generation of the different cell types found within the eye, as well as those that produce a facsimile of the developing eye *in vitro*. This chapter introduces the importance of the Retinal Pigment Epithelium (RPE) in maintaining visual function. We then focus on methods developed by our group to produce RPE from patient skin samples using human induced pluripotent stem cell technology (iPSC).

Keywords Human induced pluripotent stem cells • Retinal pigment epithelium • Transplantation

9.1 Introduction

The Retinal pigment epithelium (RPE) is the monolayer of epithelium found at the back of the eye, behind the retina. The RPE performs a support and maintenance role for the retina and is involved in many processes crucial to the health of the overlying photoreceptive cells (Strauss 2005). The transport of nutrients, water and ions (Reichhart and Strauss 2014) from the blood supply to photoreceptor cells is

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regulated by the RPE monolayer. The RPE is involved in the phagocytosis of photoreceptor outer segments (POS) discarded daily by the retina; being responsible for binding (mediated by Integrins and CD36 and 81), engulfing (FAK and MERTK) and breaking down the shed POS (Kevany and Palczewski 2010). The RPE is also important role for the visual cycle and produces various proteins, including RPE65 and LRAT, which play crucial roles in retinoid recycling (Kiser et al. 2012; Wright et al. 2015).

The RPE is a highly pigmented monolayer, with each cell containing many melanosomes that aid vision by absorbing any stray light within the eye (Boulton 1998). Within the monolayer, individual RPE cells are tightly packed together to give a 'cobblestone'-like morphology. The formation of tight junctions between cells is crucial for the role of the RPE as a component of the blood:retina barrier (Rizzolo 2014). The development of tight junctions enables the establishment of cell polarity, with concentration gradients maintained through ion pumps/channels such as Na^+/K^+-ATPase and Bestrophin at the apical and basolateral surface respectively (Lehmann et al. 2014), and the polarized secretion of growth factors, such as Pigment epithelium-derived growth factor (PEDF) and Vasoactive Endothelial Growth Factor (VEGF) (Sonoda et al. 2009).

The RPE is vital for the maintenance of vision, therefore the deterioration of, or deficiencies in the functional performance of the RPE can result in various forms of blindness (Sparrow et al. 2010). RPE-specific rare inherited genetic disorders (Table 9.1) and a number of degenerative conditions, such as age-related macular degeneration (AMD) arise when the RPE is lost or dysfunctional. AMD is the leading cause of sight loss in the developed world (de Jong 2006); there are over half a million people diagnosed with late-stage AMD in the UK, and around half of these are registered as visually impaired. Clinically, there are two forms of AMD, the slowly progressing non-exudative form, known as dry AMD, and the rapidly developing exudative or "wet" AMD, which results from the infiltration of new, leaky blood vessels through the RPE barrier. A number of pharmaceutical products are available to treat wet AMD, namely Anti-VEGF drugs e.g. Ranibizumab (Lucentis) and Bevacizumab (Avastin) (Martin et al. 2011). New drugs in the anti-VEGF class are being developed but these treatments tend only to arrest decline in vision rather than inducing any significant improvement. Anti-VEGFs are ineffective in some patients with wet AMD and are of no benefit to the patient population with dry AMD. Over recent years, focus has turned to using a cell therapy for the treatment of AMD, however, although it is possible to utilise RPE from an extra-macula area of a patients own eye (Chen et al. 2009), it is technically quite difficult to do so, and the cells/tissue yielded are fragile and limited in amount. Human primary RPE is also difficult to obtain due to reliance on post-mortem tissues. These sources are inconsistent and limited, which makes planning studies for transplantation therapeutics very difficult. This makes non-donor derived RPE a highly desirable research and clinical resource for cell transplantation.

With the advent of the regenerative medicine era, much effort has been directed to using stem cells as a potential source to generate new RPE (Klimanskaya et al. 2004, Vugler et al. 2008, Carr et al. 2013). These cell therapies have the potential to

Table 9.1 Commonly used markers to identify retinal pigment epithelial cells

Gene	Description	Role	Notes	Reference
RPE65	RPE specific protein 65kDA	Visual cycle	Mutations lead to RP, LCA, FA	Wright et al. (2015), den Hollander et al. (2008)
LRAT	Lecithin retinol acyltransferase	Visual cycle	Mutations lead to early onset RD, LCA	Dev Borman et al. (2012), den Hollander et al. (2008)
RLBP1	Retinaldehyde binding protein 1	Visual cycle	Mutations lead to severe RCD, FA	Maw et al. (1997), Eichers et al. (2002)
FAK	Focal adhesion kinase	Phagocytosis	POS binding and engulfment	Finnemann (2003)
MerTK	Mer tyrosine protein kinase	Phagocytosis	Mutations leads to RP, LCA	den Hollander et al. (2008)
ITGAV	Alpha V integrin	Phagocytosis	Extracellular interactions with POS	Finnemann et al. (1997)
ITGB5	Integrin beta 5	Phagocytosis	Extracellular interactions with POS	Finnemann et al. (1997)
CD36	Cluster of differentiation 36	Phagocytosis	Extracellular interactions with POS	Sun et al. (2006)
CD81	Cluster of differentiation 81	Phagocytosis	Extracellular interactions with POS	Chang and Finnemann (2007)
CTSD	Cathepsin D	Phagocytosis	Lysosomal protease—degrades internalised POS	Bosch et al. (1993)
GAS6	Growth arrest specific 6	Phagocytosis	MerTK interactions	Hall et al. (2001), (2005)
MFGE8	Milk fat globule EGF factor 8	Phagocytosis	Interacts with integrins	Nandrot et al. (2007)
BEST1	Bestrophin 1	Ion transport	Expressed basolaterally, mutations lead to BVMD	Marmorstein et al. (2000)
ATP1A	Na+/K+-ATPase	Ion Transport	Expressed apically	Hu and Bok (2001)
TYR	Tyrosinase	Melanogenesis	Pigment production	Murisier and Beermann (2006)
PMEL17	Pre-melanosome protein 17	Melanogenesis	Biogenesis of pre-melanosomes	Vugler et al. (2008)
MITF	Micropthalmia-associated transcription factor	RPE development	Transcription factor for RPE specification	Martínez-Morales et al. (2004)

(continued)

Table 9.1 (continued)

Gene	Description	Role	Notes	Reference
OTX2	Orthodenticle homolog 2	RPE development	Transcription factor for RPE specification	Martínez-Morales et al. (2004)
PAX6	Paired box protein 6	Transcription factor	Early eye field marker—associated with eye defects	Martínez-Morales et al. (2004)
COL4A1	Collagen IV	Extracellular matrix	Secreted component of RPE extracellular matrix	Campochiaro et al. (1986)
PEDF	Pigment epithelium derived factor	Growth factor	Anti-angiogenic factor	Dawson et al. (1999)
VEGF	Vascular epithelium growth factor	Growth factor	Pro-angiogenic factor—associated with AMD	Witmer et al. (2003)
KRT8	Keratin 8	Intermediate filament	Marker of proliferative RPE	Vugler et al. (2008)

RPE retinal pigment epithelium, *RP* retinitis pigmentosa, *LCA* Leber's congenital amaurosis, *RD* retinal dystrophy, *RCD* rod-cone dystrophy, *FA* Fundus albipunctatus, *POS* photoreceptor outer segments, *AMD* age-related macular degeneration, *BVMD* Best's vitteliform macular dystrophy

replace lost or damaged cells and therefore improve the recipients' visual acuity. Human embryonic stem cells (hESC) have been used to generate RPE for treatment of AMD and Stargardt's Macular dystrophy in on-going clinical trials (Schwartz et al. 2012, 2014), however, due to the origin of these cells, there may be concerns with immune rejection. Induced pluripotent stem cells (iPSC) offer an alternative source of tissue for transplantation therapies. Somatic cells isolated from patient tissues, such as skin and blood, can be reprogrammed into iPSCs, which can then be used to generate any tissue within the body (Takahashi et al. 2007; Yu et al. 2007). Development of new methods to reprogram cells, e.g. using episomal vectors, rather than the original retroviral constructs, make generation of iPSC safer for use in humans (Okita et al. 2011; Goh et al. 2014). Many groups have described methods to differentiate RPE from iPSC, these range from protocols describing spontaneous differentiation (Carr et al. 2009a), differentiation via embryoid body/neural differentiation (Meyer et al. 2009) and directed differentiation (Westenskow et al. 2012). Studies have shown that these iPSC-derived RPE are morphologically similar to human RPE, perform many of the functions required to maintain the health of retinal cells (Carr et al. 2009a; Meyer et al. 2011; Vaajasaari et al. 2011; Kokkinaki et al. 2011; Westenskow et al. 2012) and do not form teratomas upon transplantation in the subretinal space (Kanemura et al. 2014, Kamao et al 2014). The possibility of producing stem cells from a patient's own tissue, and differentiating these cells into an autologous source of functional RPE, may alleviate rejection issues associated with HESC-derived tissues.

The generation of RPE from induced pluripotent cells also offers other areas of possibility for the novel treatment of diseases. Genetic disorders affecting the RPE may be rare in terms of overall incidence, but still affect thousands of people world-

wide. In many of these conditions there is no sufficiently analogous animal model available. Using iPSCs derived from patients to generate the required tissue in the laboratory presents an opportunity to study these conditions at the cellular and molecular levels (Yvon et al. 2015). Furthermore, the generation of RPE from a patients own cells provides a unique opportunity to investigate new medicinal products, using the patients own cells as a model platform to screen for novel therapeutics in human diseased cells (Meyer et al. 2011; Schwarz et al. 2015). Additionally, the generation of RPE from pluripotent stem cells provides a new arena in which to understand the development of the RPE.

9.2 Materials

9.2.1 Solutions and Chemicals

- 70 % Alcohol solution.
- Fibroblast cell media: Remove 55 ml of DMEM:F12 medium (Life Technologies, Cat. No. 31331-028) from the bottle. Add 50 ml of fetal bovine serum (Life Technologies, Cat. No 16000-044) and 5 ml penicillin/streptomycin (Life Technologies, Cat. No. 15140-122). Store at 4 °C for up to 4 weeks.
- Sodium butyrate (Tocris, Cat. No. 3850): Dilute 50 mg in 4.54 ml H_2O and store aliquots at −20 °C. Dilute 1:200 in cell culture medium for a final concentration of 0.5 mM.
- DPBS, no calcium no magnesium (Life Technologies, Cat. No. 14190-94).
- TrypLE Select Solution (Life Technologies, Cat. No. 12563-011).
- Cell freezing solution: For 50 ml of solution, combine 30 ml DMEM (Life Technologies, Cat. No 41966-029) with 15 ml fetal bovine serum and 5 ml DMSO (Life Technologies, Cat. No. D12345). Store at 4 °C.
- Gelatin-coated plates: Add 1 g gelatin (Sigma-Aldrich, Cat. No. G9136) to 500 ml of ddH_2O. Autoclave the solution to dissolve and sterilise. Add 5 ml of the solution to a 10 cm^2 tissue culture treated dish in the laminar flow hood. Incubate at room temperature for 1 h. Remove the excess gelatin prior to use. Plates can be wrapped in parafilm and stored at 4 °C for up to a week.
- Amaxa Cell Line Nucleofector® Kit R (Lonza, Cat. No. VCA-1001).
- Yamanaka episomal reprogramming plasmids (Okita et al. 2011—Addgene, pCXLE-hOCT3/4-shp53-F (Cat. No.27077); pCXLE-hUL (Cat. No. 27080); pCXLE-hSK (Cat. No. 270778); pCXLE-GFP (Cat. No. 27082)).
- HESC qualified Matrigel-coated plates: All tissue cultureware, plastics and media should be ice-cold to prevent gelling of the Matrigel. Defrost the BD Matrigel hESC-qualified matrix solution (BD Biosciences, Cat. No. 354277) overnight in an ice bucket within a fridge (4 °C). Dilute 1:1 with DMEM, prepare 1 ml aliquots in 1.5 ml microcentrifuge tubes and store at −20 °C. Slowly thaw aliquots overnight as described above and dilute in cold DMEM to the final ratio suggested according manufacturers certificate of analysis. Coat tissue culture

plastic (1 ml/well of a 6-well plate, 3 ml/T25 flask) and incubate at room temperature for at least 1 h. Aspirate off excess Matrigel immediately prior to use.

- mTeSR1 Media Complete Kit (Stem Cell Technologies, Cat. No. 05850). Prepare according to the manufacturers instructions.
- Stainalive Tra-1-60 Antibody (DyLight 488), mouse anti-human (STEMGENT, Cat. No. 09-0068).
- Stainalive Tra-1-81 Antibody (DyLight™ 488), mouse anti-human (STEMGENT, Cat. No. 09-0069).
- iPSC-RPE differentiation media: All components are supplied from Life Technologies. Remove 111.2 ml of media from a bottle of Knockout DMEM (Cat. No. 10829-018), then add 100 ml of KnockOut Serum Replacement (Cat. No. 10828010), 5 ml of 200 mM L-Glutamine (Cat. No. 25030-081), 5 ml of 100× MEM Non-essential amino acids (Cat. No. 11140-035), 909 μl of 55 mM β-mercaptoethanol (31350-010) and 300 μl of 50 mg/ml Gentamicin (Cat. No. 15750-037) to the bottle. Store at 4 °C for up to 4 weeks.
- Accutase® Solution (Sigma-Aldrich, Cat. No. A6964).
- BD Matrigel Growth Factor reduced (GFR) matrix (BD Biosciences, Cat. No. 356230): Thaw, dilute 1:1 with DMEM, aliquot and store as described above. Dilute 1:15 with DMEM to coat plates for a final dilution of 1:30, incubate at 37 °C for at least 2 h, place at room temperature for 1 h and aspirate excess Matrigel immediately prior to use.
- 30 % sucrose cryopreservation solution: Dissolve 30 g of sucrose (Sigma, Cat. No. S0389) in 0.01 M PBS (Sigma, Cat. No. P4417) to a final volume of 100 ml.
- OCT embedding compound (CellPath, Cat. No. KMA-0100-00A).
- Immunocytochemistry cell permeabilization solution: For a 50 ml solution, dilute 1.5 ml of 10 % Triton-X solution (Sigma-Aldrich, Cat. No. 93443) in 0.01 M PBS.
- Immunocytochemistry blocking solution: Add 0.5 ml of normal donkey serum (Jackson ImmunoResearch Laboratories Ltd.) to 0.3 g of Bovine serum albumin and make up to 10 ml with PBS.
- RPE specific antibodies: Pmel17 Mouse monoclonal antibody (Dako, Cat. No. M0634, 1:500 final dilution), MerTK Rabbit monoclonal antibody (Abcam, Cat. No. ab52968, 1:50 final dilution), Bestrophin Mouse monoclonal antibody (Abcam, Cat. No. a2182, 1:1000 final dilution).
- Secondary antibodies: Donkey Anti-mouse IgG Alexa Fluor® 488 (Abcam, Cat. No. ab150105) and Donkey Anti-Rabbit IgG Alexa Fluor® 555 (Abcam, Cat. No. ab150074).
- VECTASHEILD anti-fade mounting medium with DAPI (Vector Laboratories, Cat. No. H-1200).

9.2.2 Disposables

- 7 ml Bijou tube.
- Conical tubes (15 and 50 ml).
- 1.5 ml microcentrifuge tube.

- Cryovial cryogenic preservation tubes.
- 6-Well tissue culture-treated plate.
- 10 cm^2 tissue culture-treated plastic dish.
- 25 cm^2 (T25) tissue culture-treated plastic flask.
- 160 cm^2 (T60) tissue culture-treated plastic flask.
- Extended fine tip Pasteur pipette (Alpha Labs, Cat. No. LW4636).
- Sterile serological pipette (5, 10 and 25 ml).
- 40 μm Cell Strainer (Corning, Cat. No. 352340).
- 22×22 mm glass cover slip (VWR, Cat. No. 631-0124). Sterilise the coverslips overnight by immersing in 100 % ethanol. Allow to dry thoroughly before placing on cells.
- 22×50 mm glass coverslip (VWR, Cat. No. 631-0094).
- Sterile cell scraper (Greiner Bio-one, Cat. No. 541070).
- 11 mm Crescent blade microknife (Interfocus, Cat. No 10317-14).
- Superfrost Plus glass slides (VWR, Cat. No. 631-0447).

9.2.3 *Equipment*

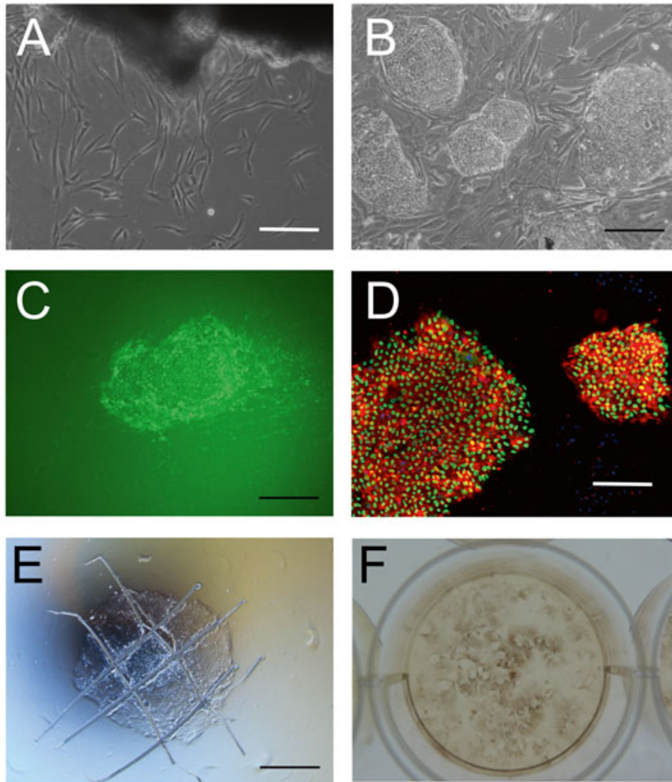
- Tissue culture incubator (humidified to 95 % with 5 % CO_2 maintained at 37 °C).
- Laminar flow hood (Class I and II).
- Centrifuge.
- Media Aspirator.
- Sterile dissection forceps.
- Sterile scalpel blades.
- Dissection teasing needle.
- Water bath.
- Inverted Microscope with LCD screen (2, 4, 10 and 20× objectives) contained inside a laminar flow hood.
- Cell counter (automated or haemocytometer).
- CoolCell SV2 cell freezing container (Biocision Cat. No. BCS-172).
- Liquid nitrogen storage tank with cryovial storage racking.
- Amaxa Nucleofector device.

9.3 Methods

9.3.1 *Generation of Fibroblast Cells from a Patient Dermal Skin Biopsy*

1. A 5-mm skin biopsy should be obtained from a patient with informed consent under aseptic conditions by a trained physician. The protocol should be approved by the appropriate research ethics committee and review board.

2. Collect the biopsy and transfer to a sterile 7 ml Bijou tube containing fibroblast cell media and transport on ice to the laboratory;
3. Remove the biopsy sample from the tube using sterile forceps and place into a 10 cm^2 sterile culture dish containing DPBS to wash the sample;
4. Remove the epidermal layer of the skin using a sterile scalpel blade;
5. Place the biopsy into a fresh 10 cm^2 dish and dissect the biopsy into small pieces (approx. 1 mm) using sterile scalpel blades;
6. Transfer the biopsy fragments to two wells of a 6-well tissue culture-treated plate and overlay with a sterile 22 × 22 mm glass coverslip;
7. Carefully add 500 µl of fibroblast media to the well so that sufficient media is drawn underneath the coverslip;
8. Culture the sample overnight in a humidified incubator at 37 °C, 5 % CO_2;
9. The following day add 1.5 ml of fibroblast media and culture the tissue for 2–3 weeks to permit primary fibroblast cell emergence (Fig. 9.1a);
10. When sufficient fibroblast cell outgrowth has occurred (approx. 70 % confluence) the cells are ready to be passaged;
11. Aspirate the media and transfer the coverslip to a fresh well, inverting the coverslip so that the side which has been in contact with cells is uppermost (see ***Note 1***);
12. Wash the coverslip and cells with sterile DPBS and add 500 µl of TrypLE Select solution to each well. Incubate at room temperature until cells have detached (approx. 10 min);
13. Add 2 ml of fibroblast medium to each well. Collect the media and pool in a 15 ml conical tube. Remove clumps of tissue by placing the media through a cell strainer (see ***Note 2***);
14. Resuspend the cells in an appropriate volume of media (2 ml per well of a 6-well plate or 6 ml per T25) and passage at a ratio of 1:3 (see ***Note 3***);
15. Replace the fibroblast medium twice weekly and passage the cells using TrypLE solution as described above when the cells reach approx. 75 % confluency, maintaining a 1:3 split ratio (see ***Note 4***);
16. Patient fibroblast cells can be cryopreserved long-term in liquid nitrogen. Dissociate cells using TrypLE solution as above and resuspend cells in fibroblast media. Centrifuge the cells at 250 × *g* for 5 min and aspirate the supernatant leaving a small miniscus of media (approx. 50 µl) in which to resuspend the cells. Add 1 ml of Cell Freezing solution and ensure cells are fully dispersed. Aliquot the cells into cryo-preservation vials (cells from 1 well of a 6-well plate aliquoted into one vial, from a T25 into thee vials). Place the cryovials inside a CoolCell SV2 controlled rate freezing container and freeze overnight in a −80 °C freezer. Vials should be transferred into liquid nitrogen storage the following day.
17. Cells can be recovered from cryopreservation prior to reprogramming. Warm the vial in a 37 °C water bath until most of the tube has thawed. Wipe the tube with 70 % alcohol before adding 1 ml of prewarmed fibroblast cell media.



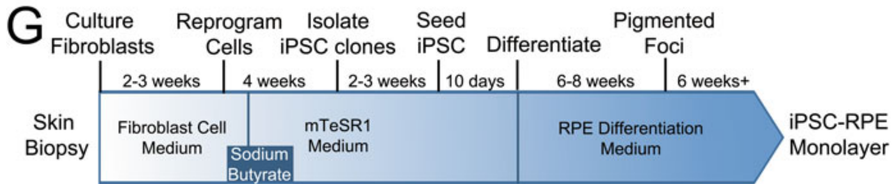


Fig. 9.1 Generation of iPSC-RPE from a patient skin biopsy. (**a**) Fibroblast outgrowth from a skin biopsy. (**b**) Emergence of iPSC colonies in reprogrammed cultures (**c**) stained with Stainalive Tra-1-60 antibody. (**d**) Oct4 (*green*) and Tra-1-81 (*red*) staining of iPSCs. (**e**) Dissection of iPSC colony for clonal expansion. (**f**) Appearance of pigmented foci from patient iPSC cultures. (**g**) Timeline of iPSC-RPE generation from a patient skin biopsy. Scale bars—All 200 μm

Carefully transfer the contents of the cryovial to a 15 ml conical tube containing 5 ml of warm fibroblast media. Centrifuge the cells at 250 × *g* for 3 min and aspirate the supernatant. Resuspend the cells in 2 ml of fibroblast media and transfer to a well of a 6-well plate. The cells should be cultured as described above.

9.3.2 Reprogramming of Patient Fibroblast Cells into Induced Pluripotent Stem Cell Lines

1. On the day of the transfection prepare gelatin-coated 10 cm^2 culture dishes;
2. Pre-warm the Nucleofector reagent and DPBS to room temperature. Warm the fibroblast media to 37 °C;
3. Dissociate fibroblast cells from a T160 using TrypLE solution as described above (see ***Note 5***);
4. Resuspend in the cells in 1 ml of DPBS and centrifuge at $250 \times g$ for 3 min;
5. Count cells using an automated cell counter or haemocytometer;
6. Aliquot 1×10^6 cells into a sterile 1.5 ml microcentrifuge tube, centrifuge at $250 \times g$ for 3 min. Remove the supernatant and resuspend the cells in 100 μl of Nucleofector solution (prepared according to the manufacturers instructions). Add 1 μg of each of the thee Yamanaka episomal reprogramming plasmids (pCXLE-hUL, pCXLE-hOCT3/4-shp53-F and pCXLE-hSK). A control transfection reaction to examine transfection efficiency can be prepared by adding 3 μg of pCXLE-GFP to cells to electroporate in parallel with the reprogramming cells;
7. Pipette the reaction gently to mix, then transfer the reaction into an Amaxa electroporation cuvette ensuring minimal bubble formation;
8. Electroporate the cells using an Amaxa *Nucleofector* device using program U-023;
9. Remove the cuvette from the device and add 1 ml of warm fibroblast cell medium;
10. Remove the gelatin solution from the 10 cm^2 plates and add 8 ml of warmed MEF media;
11. Transfer the cell suspension from the cuvette into the prepared 10 cm^2 dish;
12. Add an additional 1 ml of medium to the cuvette to collect any remaining cells and transfer to the 10 cm^2 dish;
13. Incubate the cells overnight at 37 °C and 5 % CO_2;
14. Over the following 6 days replace the media daily with 10 ml fibroblast media containing 0.5 mM Sodium butyrate (see ***Note 6***);
15. Prepare HESC qualified Matrigel-coated plates on the 7th day following the transfection;
16. Dissociate the transfected patient fibroblast cells using TrypLE solution as previously described and resuspend in 5 ml of fibroblast media;
17. Count the cells and plate at a concentration of 2×10^5 cells/well of the coated 6-well plate in a final volume of 2 ml media per well. Culture the cells the overnight 37 °C and 5 % CO_2;
18. Remove the medium from each well on the following day and replace with 2 ml of mTeSR1 medium supplemented with 0.5 mM Sodium butyrate. The cell media should be replaced daily, supplementing with sodium butyrate for the first 5 days only;

19. The appearance of pluripotent colonies in reprogrammed cultures can vary between patients however ESC-like colonies should begin to emerge within 3 weeks of the initial transfection. Under a x2 microscope objective the cells grow within tightly compacted colonies that have a phase positive appearance with defined edges (Fig. 9.1b). Cells have a high nucleus:cytoplasm ratio.

9.3.3 Isolation of Clonal iPSC Colonies

1. Pluripotent colonies can also be identified using StainAlive stem cell antibodies for the stem cell surface proteins Tra-1-60 and Tra-1-81. Aspirate the medium from the cells and add fresh mTeSR1 medium containing 3 μg/ml StainAlive antibody. Incubate the cells at 37 °C, 5 % CO_2 for 30 min. Remove the medium, and wash twice with cell medium. Add fresh mTeSR1 and image the cells using a fluorescent microscope (Fig. 9.1c, see ***Note 7***).
2. On the day of picking, prepare the required number of 6-well plates by coating wells with HESC-qualified Matrigel Basement membrane matrix (1 ml of solution per well). For clonal iPSC lines prepare 1 well per colony;
3. Transfer the plate to laminar flow hood containing an EVOS XL or equivalent LCD display microscope;
4. To pick clonal iPSCs, gently score criss-cross over an iPSC colony with the edge of a fine tip plastic Pasteur pipette (See Fig. 9.1E). Use the tip of the pipette to gently lift the colony fragments away from the surrounding cells (see ***Note 8***). Collect the free-floating colony fragments using the Pasteur pipette and transfer to a well of the Matrigel coated 6-well plate and add 1 ml of medium;
5. Replace the medium on the original plate containing the reprogrammed cells with fresh mTeSR1 medium cell. Repeat the picking procedure for any remaining iPSC colonies;
6. Incubate the cells at 37 °C, 5 % CO_2, taking care not to disturb the plate for 24 h in order to permit colony attachment. Next, cells should be fed daily with fresh mTeSR1 medium. Pluripotency can be confirmed by immunostaining for pluripotent stem cell markers e.g. Oct4 and Tra-1-81 (Fig. 9.1d);
7. iPSC colonies will be ready to passage within 4–7 days. To maintain and expand the iPSC line it is important to removal any differentiated cells prior to passaging; this can be achieved by scrapping across any areas of differentiation with the tip of a Pasteur pipette to detach cells. The well should then be washed with media before adding fresh mTeSR1 medium prior to passaging. Fragment and detach the cells by scoring across the clonal colonies within a well and as described above (Fig. 9.1e) and replate onto HESC qualified Matrigel-coated tissue culture plastic at a split ration of 1:3–1:6.

9.3.4 Spontaneous Differentiation of Patient iPSCs into RPE

1. Passage the iPSCs onto Growth Factor reduced Matrigel-coated plates or flasks as described above using mTeSR1 medium, incubate the cells at 37 °C, 5 % CO_2;
2. Replace the medium daily with mTeSR1 until the individual iPSC colonies merge and become confluent over the dish/flask; this will occur approximately 8–10 days post-seeding;
3. Once cells have become confluent, replace the medium with a 1:1 mix of mTeSR1:iPSC-RPE Differentiation medium;
4. The following day replace the medium with Differentiation medium only and culture the cells at 37 °C, 5 % CO_2. From this point change the media twice weekly;
5. Within a 4–6-week period, pigmented foci of retinal pigment epithelial cells, which are detectable by eye, should begin to appear within the cultures (Fig. 9.1f).

9.3.5 Isolation of Pigmented Foci from iPSC Cultures and Culture as a Monolayer

1. Once pigmented foci have reached a sufficient size (>1 mm diameter) dissect around the foci using a crescent blade. Place the foci into a fresh dish containing differentiation medium and carefully remove any non-pigmented tissue using sterile forceps and scalpel blades;
2. Place the pigmented foci into a 15 ml conical tube containing Accutase solution and incubate at 37 °C for 2–3 h to dissociate cells;
3. Pellet the cells by centrifugation at 250 × *g* for 3 min and resuspend in differentiation medium;
4. Pass the cells through a 40 μm cell strainer to create a single cell suspension of retinal pigment epithelial cells;
5. Count the cells using a haemocytometer;
6. Seed out the pigmented cells in differentiation medium (See ***Note 9***) at a minimum density of 50,000 cells/cm^2 on Growth factor reduced Matrigel-coated tissue culture plastic dishes. Incubate cells at 37 °C, 5 % CO_2;
7. The media should be replaced with differentiation media twice weekly for approximately 6 weeks, until a pigmented monolayer of cells forms (see ***Note 10***);
8. iPSC-derived RPE can be maintained in culture for several months to permit further maturation of the cell monolayer (see ***Note 11***).

A schematic overview of the whole process is shown in Fig. 9.1g.

9.3.6 Immunocytochemical Staining of iPSC-Derived RPE

1. Remove the medium from the cells and wash twice with PBS;
2. Fix the cells in 4 % paraformaldehyde in 0.01 M phosphate buffer at 4 °C for 30 min. Wash twice with PBS;

3. Using a cell scraper, slowly but firmly scrape off the RPE cells from the dish in one sweep, so that an intact sheet of cells is lifted;
4. Carefully transfer the sheet to a bijou tube containing 30 % sucrose using a teasing needle. Cryopreserve the sheet overnight at 4 °C;
5. The following day, cut the RPE sheet into smaller pieces (approx. 1 cm^2) for embedding. Fill a Cryomold with OCT compound being careful to exclude large air bubbles. Transfer the sheets into the OCT cryomolds and gently orientate the sheet into a vertical, on-edge position, using teasing needles;
6. Place the bottom of the cryomold into a dry ice/acetone slurry bath and slowly freeze the block, ensuring the sheet maintains its vertical orientation;
7. Store the blocks at −80 °C;
8. Section the tissue at 14 μm on a cryostat and collect tissue sections on warm Superfrost Plus slides;
9. Air dry the sections and use immediately or store at −80 °C;
10. Permeabilise the tissue in 0.3 % Triton in PBS for 10 min at 4 °C;
11. Remove the permeabilization solution and incubate in blocking solution for a minimum of 30 min;
12. Place the slides inside an immunostaining moisture chamber, add RPE specific antibodies diluted in blocking solution to the sections and incubate at 4 °C overnight;
13. The following day wash the slides five times in PBS;
14. Pipette the secondary antibodies diluted in blocking solution onto the sections and incubate for 1 h inside a moisture chamber;
15. Wash the slides five times in PBS;
16. Mount the cells by adding a drop of VECTASHIELD anti-fade mounting medium with DAPI over the tissue sections. Cover with a clean 22 × 50 mm coverslip and seal with nail polish.

9.3.7 Characterization of the Patient iPSC-Derived RPE

1. iPSC-RPE cells should be characterised to ensure their similarity to human RPE. iPSC-derived RPE cells can be examined initially by their morphological appearance. By eye, an even layer of brown/black cells should be visible on the tissue culture plastic (Fig. 9.2a). The RPE form a monolayer of hexagonal cells arranged in a regular pattern (cobblestone-like appearance Fig. 9.2b), are highly pigmented and appear brown/black under a standard microscope (Fig. 9.2c);
2. The intricate ultrastructure of the RPE cell can be analysed by electron microscopy (Fig. 9.2d). RPE cells are highly polarised with prominent microvilli and coated pits apparent on the apical surface, densely packed melanosomes within the apical cytoplasm and a basal nucleus. The RPE possess cell-cell adhesion structures including adherins junctions, tight junctions and desmosomes, and secrete their own basement lamina;
3. Gene and protein expression can be examined for a panel of RPE markers (Table 9.1) using RT-PCR, immunostaining (see ***Note 12***) and Western blot.

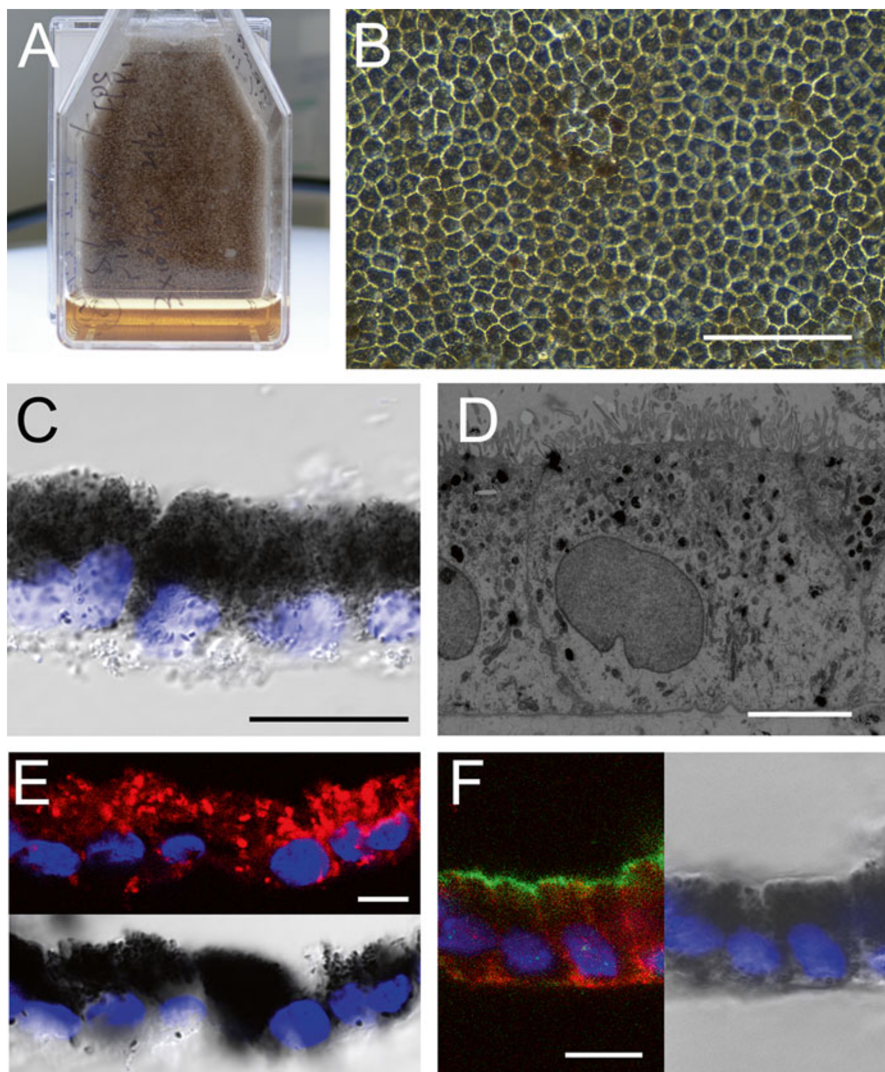


Fig. 9.2 Characterization of patient iPSC-derived RPE. (**a**) A T25 tissue culture flask containing purified RPE. (**b**) Cobblestone-morphology of RPE monolayer. RPE cells are highly polarised epithelial cells with (**c**) a basal nucleus (*blue*) and are packed with melanosomes (*black* granules). (**d**) Electron microscopy of the RPE cell ultrastructure. Immunostaining of cells with (**e**) the pre-melanosome marker Pmel17 (*red*) and (**f**) apical expression of MerTK (*green*) and basolateral localisation of Best1 (*red*). Scale bars—(**b**) 100 μm, (**c**) 20 μm, (**d**) 5 μm, (**e**) 10 μm, (**f**) 10 μm

RPE cell markers should be expressed in the correct cellular compartment. For example, Pmel17 is observed with punctate staining of the cytoplasm (Fig. 9.2e), MerTK should be expressed on the apical surface, whilst Bestrophin should be localised basolaterally (Fig. 9.2f);

4. RPE cells secrete a number of factors crucial for the survival and maintenance of photoreceptor cells, including Vasoactive Endothelial Growth Factor (VEGF) and Pigment epithelium-derived growth factor (PEDF). To analyse secretion of these factors iPSC-RPE should be grown on permeable culture membrane inserts. Apical and basal media can then be collected for cells and growth factor secretion measured using an ELISA kit;
5. The RPE is a tight epithelium and is a component of the blood:retina barrier separating the retina from the choriocapillaris in vivo. The barrier function of iPSC-RPE cells can be assessed by measuring the transepithelial resistance. iPSC-RPE cells should be seeded onto permeable culture membrane insert and development of a functional barrier measured weekly over the course of RPE cell maturation using an epithelial voltohmmeter;
6. An important function of the RPE in the eye is the phagocytosis of the outer segment debris shed daily by the retinal photoreceptor cells. Functional phagocytosis can be examined in patient iPSC-RPE by exposing cells to fluorescently labelled photoreceptor outer segments isolated from porcine, bovine or ovine eyes. Alternatively, an isolated sheet of retina can be co-cultured above the RPE monolayer; RPE cells should then be assessed for ingestion of rhodopsin positive material by immunostaining;
7. The RPE cell is vital for the recycling of retinoids in the visual cycle. In the photoreceptor cells the detection of light results in the isomerisation of the 11-cis-retinal chromophore into all-trans-retinal, after further reduction the by-product of the visual cycle is released from the photoreceptor cell as all-trans-retinol. The RPE is responsible for the recycling of all-trans-retinol into 11-cis-retinal, which can then be transported back to the photoreceptor cells to form a new light-absorbing photopigment molecule. Retinol derivatives of the visual cycle can be measured in cell lysates and cell media by High Performance Liquid Chomatography (HPLC) or Liquid Chomatography-Mass Spectrometry (LC-MS);
8. The functional properties of iPSC-RPE cells can be assessed by transplantion of cells into the subretinal space of a retinal degenerate animal e.g. the Royal College of Surgeons (RCS) rat. Preservation of visual responses in these animals over time is the ultimate test of cell function.

9.4 Notes

1. Patient fibroblast cells can grow on the underside of the coverslip; these can easily be collected for alongside those on the plate for expansion.
2. Any clumps of tissue from the patient skin biopsy can be reseeded onto fresh tissue culture plates as described above for further cell growth if required.
3. Cells from a single well of a 6-well plate can be passaged into one T25 flask for expansion. With fibroblast cells it is important not to split for passage at a high ratio (>1:3) as the seeding density will be too low for the cells to survive.

4. For reprogramming 1×10^6 fibroblast cells are required. A sufficient number of fibroblast cells can be achieved by expanding the cells and preparing a culture for the reprogramming in a T160 flask.
5. For reprogramming cells should be at low passage (<p15) and still be in a proliferative phase, approximately 2–3 days following passage.
6. To establish the efficiency of transformation examine the ratio of GFP expressing:non expressing cells on the control GFP transfected plates the day after electroporation.
7. For ease of picking, the position of Stainalive™ verified pluripotent colonies can be marked on the underside of the plate using an objective stamp attached to the fluorescent microscope.
8. When picking the colonies for the first time it is important to avoid disturbing any untransformed fibroblast cells surrounding each colony as these will be carried over in the sub-culture.
9. A variety of media can be used to culture iPSC-RPE cells; commonly used media include RPE medium (Klimanskaya et al. 2004) and Human Fetal RPE medium (Maminishkis et al. 2006).
10. The iPSC-derived retinal pigment epithelial cells will initially de-pigment after passage, undergoing an EMT-like transformation, re-pigmentation and development of the epithelial cell morphology will occur over the 6-week culture period.
11. iPSC-RPE derived cells can be expanded for a limited number of passages by dissociation using Accutase® solution. It is not advisable to continue passage of cells beyond p4 as the EMT phenotype prevails and cells fail to re-establish the RPE cell morphology.
12. Immunostaining of iPSC-derived RPE cells can be masked due to the high levels of pigmentation in cells. We recommend using the protocol described in Sect. 9.3.7 to prepare cell sections for staining.

9.5 Potential Use of iPSC-Derived RPE

There has been considerable success in generating human RPE *in vitro* from a number of human pluripotent stem cell sources including HESC (Klimanskaya et al. 2004, Vugler et al. 2008) and iPSC (Carr et al. 2009b; Vaajasaari et al. 2011; Kokkinaki et al. 2011). As a single layer of cells responsible for a number of eye diseases, the RPE is an ideal target for regenerative medicine, using stem cell derived RPE as a potential clinical source of replacement tissue. HESC-derived RPE are currently being assessed as a treatment for RPE-based diseases such as AMD and Stargardt's macular dystrophy (Schwartz et al. 2012) and in other clinical trials worldwide. More recently, as part of a first-in-human iPSC clinical study, autologous iPSC-derived-RPE have been made from, and transplanted back into a patient with AMD in Japan. However, the long-term success of these transplants the eye has yet to be evaluated. iPSC-derived RPE cells also offer a powerful new source of tissue for the modelling of RPE disorders, replacing animal models as a

disease-in-a-dish tool to examine the development and pathogenesis of inherited and degenerative RPE disease (Yvon et al. 2015). As patient iPSC-derived RPE also contain the genetic background responsible for RPE disease, the cells will also be a viable tissue source for investigating potential therapeutics in high throughput drug discovery screens (Schwartz et al. 2014; Meyer et al. 2009).

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