Chapter *n*: Spontaneous generation of patient-specific retinal pigment epithelial cells using induced pluripotent stem cell technology.

David A. Carter[1], Britta Nommiste[1], Pete J Coffey[1, 2, 3,] Amanda-Jayne F. Carr[1*]

[1]Division for Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, 11-43 Bath Street, LONDON, EC1V 9EL.

[2] BMRC (NIHR), Moorfields Eye Hospital, 162 City Rd, London, EC1V 2PD

[3]Center for Stem Cell Biology and Engineering, NRI, UC, Santa Barbara, USA

[*]Corresponding author. Email: a.carr@ucl.ac.uk Tel: +44 2076084061

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.

Key Words Human Induced pluripotent stem cells, retinal pigment epithelium, transplantation.

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 from the blood supply to photoreceptor cells is regulated by the RPE. The RPE is involved in the phagocytosis of photoreceptor outer segments (POS) discarded daily by the retina. The RPE is responsible for binding (mediated by Integrins and CD36 and 81), engulfing (FAK and MERTK) and breaking down the shed POS. The RPE also plays an important role in the visual cycle and produces various proteins, including RPE65 and RLBP1, which play crucial roles in retinoid recycling (see Tables 1A and 1B). The RPE is a highly pigmented monolayer, with each cell containing many melanosomes that aid vision by absorbing any stray light inside the eye. 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. The development of tight junctions is also crucial for establishing the polarity of the RPE, which results in the expression of ion pumps/channels such as Na^+/K^+ ATPase and Bestrophin at the apical and basolateral surface respectively and the polarized secretion of growth factors, such as Pigment epithelium-derived growth factor (PEDF) and Vasoactive Endothelial Growth Factor (VEGF).

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. RPE-specific rare inherited genetic disorders (Table 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; 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 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). 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, whilst it is possible to utilise RPE from an extra-macula area of a patients own eye, 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 (Carr et al. 2013). These cell therapies have the potential to 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; Schwartz et al. 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 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). 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 worldwide. 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 level 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.

2. Materials

2.1 Solutions and chemicals

1. 70% alcohol solution.
2. Fibroblast cell media: Remove 55ml of DMEM:F12 medium (Life Technologies, Cat. No. 31331-028) from the bottle. Add 50ml of fetal bovine serum (Life Technologies, Cat. No 16000-044) and 5ml penicillin/streptomycin (Life Technologies Cat. No. 15140-122). Store at 4^{o}C for up to 4 weeks.
3. Sodium butyrate (Tocris, Cat. No. 3850). Dilute 50mg in 4.54ml in H_2O and store aliquots at -20^{o}C. Dilute 1:200 in cell culture medium for a final concentration of 0.5mM.
4. DPBS, no calcium no magnesium (Life Technologies, Cat. No. 14190-94)
5. TrypLE™ Select Solution (Life Technologies, Cat. No, 12563-011).
6. Cell freezing solution: For 50ml of solution combine 30ml DMEM (Life Technologies, Cat. No 41966-029) with 15ml fetal bovine serum and 5ml DMSO (Life Technologies D12345). Store at 4^{o}C.
7. Gelatin-coated plates. Add 1g gelatin (Sigma-Aldrich, Cat. No. G9136) to 500ml of ddH_2O. Autoclave the solution to dissolve and sterilise. Add 5ml of the solution to a 10cm^2 tissue culture treated dish in the laminar flow hood. Incubate at room temperature for an hour. Remove the excess gelatin prior to use. Plates can be wrapped in parafilm and stored at 4^{o}C for up to a week.
8. Amaxa™ Cell Line Nucleofector® Kit R (Lonza, Cat. No. VCA-1001.

9. 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)).
10. 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). Dilute 1:1 with DMEM, prepare 1ml aliquots in 1.5ml microcentrifuge tubes and store at -20^{o}C. Thaw aliquots overnight as described above and dilute in DMEM to the final ratio suggested according manufacturers certificate of analysis. Coat tissue culture plastic (1ml/well of a 6-well plate, 3ml/T25 flask) and incubate at room temperature for at least an hour. Aspirate off excess Matrigel immediately prior to use.
11. mTeSR™1 Media Complete Kit (Stem Cell Technologies, Cat. No. 05850). Prepare according to the manufacturers instructions.
12. Stainalive™ Tra-1-60 Antibody (DyLight™ 488), mouse anti-human (STEMGENT, Cat. No.09-0068).
13. Stainalive™ Tra-1-81 Antibody (DyLight™ 488), mouse anti-human (STEMGENT, Cat, No, 09-0069).
14. 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) and add 100ml of KnockOut™ Serum Replacement (Cat. No. 10828010), 5ml of 200mM L-Glutamine (Cat. No. 25030-081), 5ml of 100x MEM Non-essential amino acids (Cat. No. 11140-035), 909μl of 55mM β-

mercaptoethanol (31350-010) and 300μl of 50mg/ml Gentamicin (Cat. No. 15750-037). Store at 4^{o}C for up to 4 weeks.

15. Accutase® Solution (Sigma-Aldrich, A6964).
16. 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^{o}C for at least 2 hours, place at room temperature for 1 hour and aspirate excess Matrigel immediately prior to use.
17. 30% sucrose cryopreservation solution: Dissolve 30g of sucrose (Sigma, Cat. No.S0389) in 0.01M PBS (Sigma, Cat. No. P4417) to a final volume of 100ml.
18. OCT embedding compound (CellPath, Cat. No. KMA-0100-00A).
19. Immunocytochemistry cell permeabilisation solution: For a 50ml solution, dilute 1.5ml of 10% Triton-X solution (Sigma-Aldrich, Cat. No.93443) in 0.01M PBS.
20. Immunocytochemistry blocking solution: Add 0.5ml of normal donkey serum (Jackson ImmunoResearch Laboratories Ltd.) to 0.3g of Bovine serum albumin and make up to 10ml with PBS.
21. 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).
22. 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).

23. VECTASHEILD anti-fade mounting medium with DAPI (Vector Laboratories, Cat. No. H-1200).

2.2 Disposables

1. 7ml Bijou tube.
2. Conical tubes (15ml and 50ml).
3. 1.5ml microcentrifuge tube.
4. Cryovial cryogenic preservation tubes.
5. 6-well tissue culture-treated plate.
6. 10 cm^2 tissue culture-treated plastic dish.
7. 25 cm^2 (T25) tissue culture-treated plastic flask.
8. 160 cm^2 (T60) tissue culture-treated plastic flask.
9. Extended fine tip Pasteur pipette (Alpha Labs, Cat. No. LW4636).
10. Sterile serological pipette (5ml, 10ml, 25ml).
11. 40μM Cell Strainer (Corning, Cat. No. 352340).
12. 22 x 22mm 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.
13. 22 x 50 mm glass coverslip (VWR, Cat. No. 631-0094)
14. Sterile cell scraper (Greiner Bio-one, Cat. No. 541070).
15. 11mm Crescent blade microknife (Interfocus, Cat. No 10317-14).
16. Superfrost® Plus glass slides (VWR, Cat. No. 631-0447).

2.3 Equipment

1. Tissue culture incubator (humidified to 95% with 5% CO2 maintained at 37^{o}C).
2. Laminar flow hood (Class I and II).
3. Centrifuge.
4. Media Aspirator
5. Sterile dissection forceps.
6. Sterile scalpel blades.
7. Dissection teasing needle.
8. Water bath.
9. Inverted Microscope with LCD screen (2, 4, 10 and 20x objectives) contained inside a laminar flow hood.
10. Cell counter (automated or haemocytometer).
11. CoolCell SV2 cell freezing container (Biocision Cat. No. BCS-172).
12. Liquid nitrogen storage tank with cryovial storage racking.
13. Amaxa Nucleofector device.

3. Methods

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 7ml 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 10cm^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 10cm^2 dish and dissect the biopsy into small pieces (approx. 1mm) 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 x 22mm glass coverslip.
7. Carefully add 500μl 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^{o}C, 5% CO_2.
9. The following day add 1.5ml of fibroblast media and culture the tissue for 2-3 weeks to permit primary fibroblast cell emergence (Figure 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 500μl of TrypLE™ Select solution to each well. Incubate at room temperature until cells have detached (approx. 10 min).

13. Add 2ml of fibroblast medium to each well. Collect the media and pool in a 15ml 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 (2ml per well of a 6-well plate or 6ml 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 x g for 5 min and aspirate the supernatant leaving a small miniscus of media (approx. 50μl) in which to resuspend the cells. Add 1ml 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 three vials). Place the cryovials inside a CoolCell SV2 controlled rate freezing container and freeze overnight in a -80^{o}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^{o}C water bath until most of the tube has thawed. Wipe the tube with 70% alcohol before adding 1ml of pre-warmed fibroblast cell media. Carefully transfer the contents of the cryovial to a 15ml conical tube containing 5ml of warm fibroblast media. Centrifuge the cells for 3 min at 250 x g and aspirate the

supernatant. Resuspend the cells in 2ml of fibroblast media and transfer to a well of a 6-well plate. The cells should be cultured as described above.

3.2 Reprogramming of patient fibroblast cells into induced pluripotent stem cell lines

1. On the day of the transfection prepare gelatin-coated 10cm^2 culture dishes.
2. Pre-warm the Nucleofector reagent and DPBS to room temperature. Warm the fibroblast media to 37^{o}C .
3. Dissociate fibroblast cells from a T160 using TrypLE™ solution as described above (see Note 5).
4. Resuspend in the cells in 1ml of DPBS and centrifuge at 250 x g for 3 min.
5. Count cells using an automated cell counter or haemocytometer.
6. Aliquot 1x10^6 cells into a sterile 1.5ml microcentrifuge tube, centrifuge at 250 x g for 3 min. Remove the supernatant and resuspend the cells in 100μl of Nucleofector solution (prepared according to the manufacturers instructions). Add 1 μg of each of the three 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 3μg 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 1ml 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 10cm^2 dish.
12. Add an additional 1ml of medium to the cuvette to collect any remaining cells and transfer to the 10cm^2 dish.
13. Incubate the cells overnight at 37ºC and 5% CO_2
14. Over the following six days replace the media daily with 10 ml fibroblast media containing 0.5mM Sodium Butyrate (see Note 6).
15. Prepare HESC qualified Matrigel-coated plates on the 7^{th} day following the transfection.
16. Dissociate the transfected patient fibroblast cells using TrypLE™ solution as previously described and resuspend in 5ml of fibroblast media.
17. Count the cells and plate at a concentration of 2 x 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 37ºC and 5% CO_2.
18. Remove the medium from each well on the following day and replace with 2ml of mTeSR™1 medium supplemented with 0.5mM sodium butyrate. The cell media should be replaced daily, supplementing with sodium butyrate for the first five days only.
19. The appearance of pluripotent colonies in reprogrammed cultures can vary between patients however ESC-like colonies should begin to emerge within three 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. (Figure 1B) Cells have a high nucleus:cytoplasm ratio.

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 mTeSR™1 medium containing 3μg/ml StainAlive™ antibody. Incubate the cells at 37^{o}C, 5% CO_2 for 30 min. Remove the medium, and wash twice with cell medium. Add fresh mTeSR™1 and image the cells using a fluorescent microscope (Figure 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 (1ml 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 iPSCs, gently cut the iPSC colony by scoring criss-cross over the colony with the edge of a fine tip plastic Pasteur pipette. 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 1ml of medium.
5. Replace the medium on the original plate containing the reprogrammed cells with fresh mTeSR™1 medium cell. Repeat the picking procedure for any remaining iPSC colonies.

6. Incubate the cells at 37^{o}C, 5% CO_2, taking care not to disturb the plate for 48 hours in order to permit colony attachment. Following this cells should be fed daily with fresh mTeSR™1 medium. Pluripotency can be confirmed by immunostaining for pluripotent stem cell markers e.g. Oct4 and Tra-1-81 (Figure 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 mTeSR™1 medium prior to passaging. Fragment and detach the cells by scoring across the clonal colonies within a well and as described above (Figure 1E) and replate onto HESC qualified Matrigel -coated tissue culture plastic at a split ration of 1:3-1:6.

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 mTeSR™1 medium, incubate the cells at 37^{o}C, 5% CO_2..
2. Replace the medium daily with mTeSR™1 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 mTeSR™1:iPSC-RPE Differentiation medium.

4. The following day replace the medium with Differentiation medium only and culture the cells at 37^{o}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 (Figure 1F).

3.5 Isolation of pigmented foci from iPSC cultures and culture as a monolayer.

1. Once pigmented foci have reached a sufficient size (>1mm 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 15ml conical tube containing Accutase® solution and incubate at 37^{o}C for 2-3 hours to dissociate cells.
3. Pellet the cells by centrifugation at 250 x g for 3 mins and resuspend in differentiation medium.
4. Pass the cells through a 40μm 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 at a minimum density of 50,000 cells/cm^2 on Growth factor reduced Matrigel-coated tissue culture plastic dishes. Incubate cells at 37^{o}C, 5% CO_2.

7. The media should be replaced with differentiation media twice weekly for approximately 6 weeks when a pigmented monolayer of cells forms (see Note 9).
8. iPSC-derived RPE can be maintained in culture for several months to permit further maturation of the cell monolayer (see Note 10).

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 for in 0.01M phosphate buffer for 30 min at 4^{o}C . 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^{o}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^{o}C.
8. Section the tissue at 14μm on a cryostat and collect tissue sections on warm Superfrost® Plus slides.

9. Air dry the sections and use immediately or store at -80^{o}C
10. Permeabilise the tissue in 0.3% triton in PBS for 10min at 4^{o}C .
11. Remove the permeabilisation solution and incubate in blocking solution for a minimum of 30 mins.
12. Place the slides inside an immunostaining moisture chamber, add RPE specific antibodies diluted in blocking solution to the sections and incubate at 4^{o}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 hour 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 x 50mm coverslip and seal with nail polish.

3.7 Characterisation 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 (Figure 2A). The RPE form a monolayer of hexagonal cells arranged in a regular pattern (cobblestone-like appearance Figure 2B), are highly pigmented and appear brown/black under a standard microscope (Figure 2C).
2. The intricate ultrastructure of the RPE cell can be analysed by electron microscopy (Figure 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 1) using RT-PCR, immunostaining (see Note 11) and Western blot. RPE cell markers should be expressed in the correct cellular compartment. For example, Pmel17 is observed with punctate staining of the cytoplasm (Figure 2E), MerTK should be expressed on the apical surface, whilst Bestrophin should be localised basolaterally (Figure 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 Chromatography (HPLC) or Liquid Chromatography-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.

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 $1x10^6$ 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 two to three 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 on 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. 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 six week culture period.
10. 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.
11. 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 section 3.7 to prepare cell sections for staining.

5. Potential uses 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 [Park et al. 2008]. 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 [Schwarz et al. 2014, Meyer et al. 2009].

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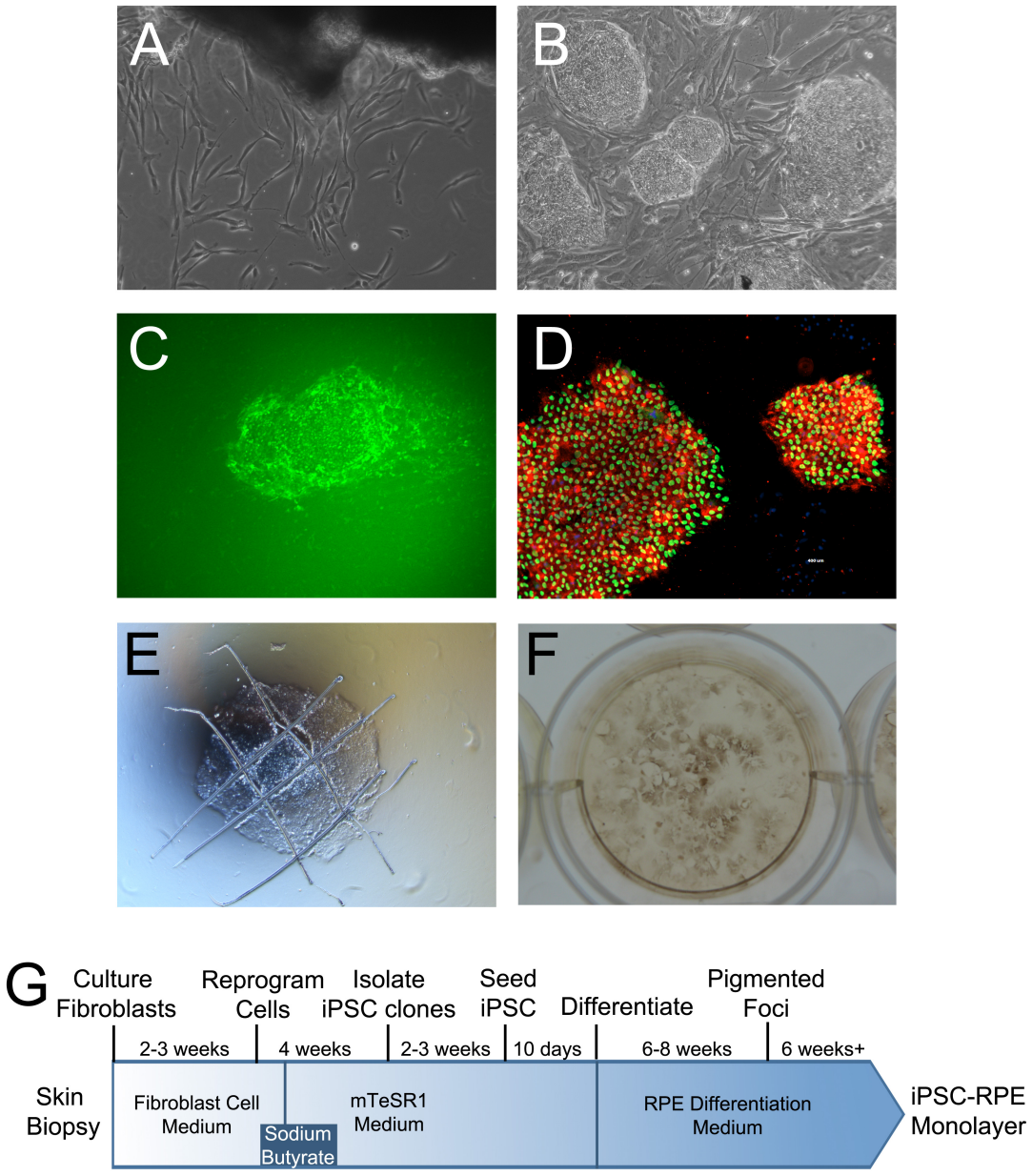

Figure 1. Production of iPSC-RPE from a patient skin biopsy A) Fibroblast outgrowth from a skin biopsy. B) Emergence of iPSC colonies in reprogrammed cultures C) stained with Stainalive™ Tra-1-60 antibody. D) Oct4 (green) and Tra-1-81 staining of iPSCs. E) Dissection of iPSC colony for clonal expansion. F) Appearance of pigmented foci from patient iPSC cultures. G) Timeline of iPSC-RPE generation from a patient skin biopsy.

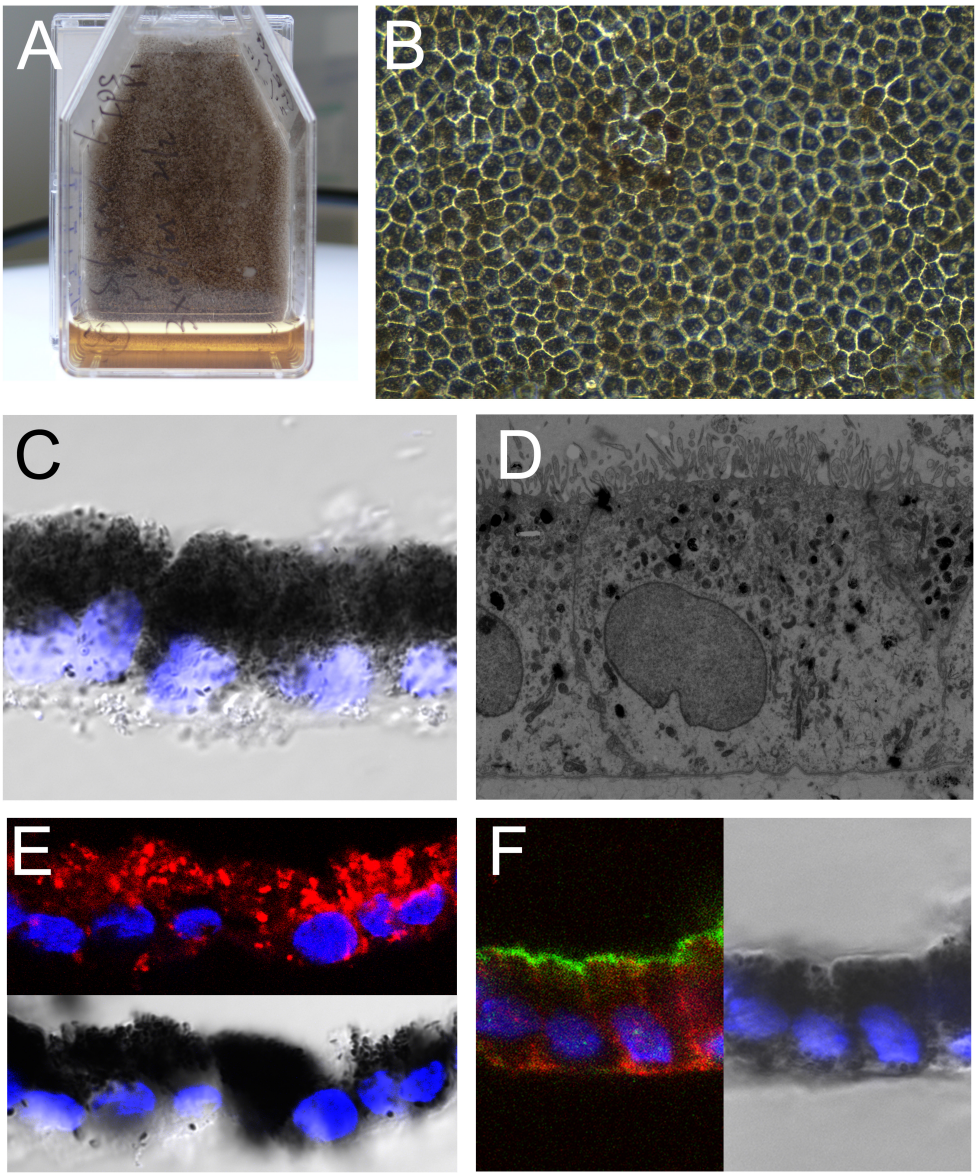

Figure 2 Characterisation of patient iPSC-derived RPE. A) Flask containing purified RPE. B) Cobblestone-morphology of RPE monolayer. RPE cells are highly polarised epithelial cells with C) a basal nucleus (blue) and are packed with melanosomes (Black granules). D) Electron microscopy of the RPE cell 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).

Gene	Description	Role	Notes	Reference
RPE65	RPE specific 65kDA	Visual Cycle	Mutations lead to RP or LCA	Wright et al (2015), den Hollander (2008)
LRAT	Lecithin retinol acyltransferase	Visual Cycle	Mutations lead to early onset RD	Dev Borman (2012), den Hollander (2008)
RLBP1	Retinaldehyde binding protein 1	Visual Cycle	Mutations lead to severe RCD, FA	Maw (1997), Eichers (2002)
FAK	Focal adhesion kinase	Phagocytosis	Cell adhesion and motility	Finneman (2003)
MerTK	Mer tyrosine protein kinase	Phagocytosis	Mutations leads to RP	den Hollander (2008)
ITGAV	Integrin alpha 5	Phagocytosis	Extracellular interactions with POS	Finneman (1997)
ITGBV	Integrin beta	Phagocytosis	Extracellular interactions with POS	Finneman (1997)
CD36	Cluster of differntiation 36	Phagocytosis	Extracellular interactions with POS	Sun (2006)
CD81	Cluster of differntiation 81	Phagocytosis	Extracellular interactions with POS	Chang and Finneman (2007)
CTSD	Cathespin D	Phagocytosis	Lysosomal protease – degrades internalised POS	Bosch (1993)
GAS6	Growth arrest specific 6	Phagocytosis	MerTK interactions	Hall (2001, 2005)
MFGE8	Milk fat globule EGF factor 8	Phagocytosis	Interacts with integrins	Nadrot (2007)
PROS1	Protein S	Phagocytosis	Interacts with MeRTK	
BEST1	Bestrophin 1	Ion transport	Expressed basolaterally – implicated in Best's disease	Marmorstein (2000)
ATP1A	Na+/K+ ATPase	Ion Transport	Expressed apically	Hu and Bok (2001)
TYR	Tyrosinase	Melanogenesis	Oxidase	Murisier and Beerman (2006)
PMEL17	Premelanosome protein 17	Melanogenesis	Biogenesis of pre-melanosomes	Vugler (2008)
MITF	Micropthalmia associated transcription factor	RPE development	Regulates transcription of RPE specific genes – associated with microphthalmia and anophthalmia	Martinez-Morales (2004)
OTX2	Orthodenticle homolog 2	RPE development	Expressed in developing head and RPE - eoRD	Martinez-Morales (2004)
PAX6	Paired box protein 6	Transcription Factor	Early eye field marker – associated with eye defects	Martinez-Morales (2004)
COL4A1	Collagen IV	Extracellular matrix	Secreted component of RPE basement membrane	Campochiaro (1986)
PEDF	Pigment epithelium derived factor	Growth Factor	Anti-angiogenic factor	Dawson (1999)
VEGF	Vascular epithelium growth factor	Growth Factor	Pro-angiogenic factor - associated with wet AMD	Witmer (2003)
KRT8	Keratin 8		Marker of proliferative RPE	Vugler (2008)

Table 1. Commonly used markers to identify retinal pigment epithelial cells. (abbreviations: retinal pigment epithelium (RPE), retinitis pigmentosa (RP), Leber's congenital amaurosis (LCA), retinal dystrophy (RD), rod-cone dystrophy (RCD), Fundus albipunctatus (FA), photoreceptor outer segments (POS), age-related macular degeneration (AMD).