Tropepe, V., Hitoshi, S., Sirard, C., Mak, T. W., Rossant, J., and van der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* **30,** 65–78.

Ying, Q. L., Nichols, J., Chambers, I., and Smith, A. (2003a). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115,** 281–292.

Ying, Q. L., and Smith, A. G. (2003). Defined conditions for neural commitment and differentiation. *Methods Enzymol.* **365,** 327–341.

Ying, Q. L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003b). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nature Biotechnol.* **21,** 183–186.

[11] Retinal Pigment Epithelium

By IRINA KLIMANSKAYA

Abstract

Retinal pigment epithelium (RPE) arises from neuroectoderm and plays a key role in support of photoreceptor functions. Several degenerative eye diseases, such as macular degeneration or *retinitis pigmentosa*, are associated with impaired RPE function that may lead to photoreceptor loss and blindness. RPE derived from human embryonic stem (hES) cells can be an important source of this tissue for transplantation to cure such degenerative diseases. This chapter describes differentiation of hES cells to RPE, its subsequent isolation, maintenance in culture, and characterization.

Introduction

Human embryonic stem (hES) cells bear a promise for cellular therapy of many ailments because of their unique ability to differentiate into the derivatives of all three germ layers. It is considered that in the absence of other inductive cues, embryonic stem (ES) cells choose a ''default'' neural pathway for differentiation (Smukler *et al.*, 2006; Ying *et al.*, 2003; reviewed by Muñoz-Sanjuán and Brivanlou, 2002), and to date different types of derivatives of this lineage have been isolated (reviewed by Kania *et al.*, 2004; Wei *et al.*, 2005; Teramoto *et al.*, 2005; Olsen *et al.*, 2006; Peschle and Condorelli, 2005; Ben-Hur, 2006; Reubinoff *et al.*, 2001). Among them is retinal pigment epithelium (RPE), a derivative of neuroectoderm, which progenitor it shares with neuronal retina in early development. Transplantation of RPE has been studied extensively in animal models (reviewed by Lund *et al.*, 2001) and in a few human trials (Binder *et al.*, 2004; van Meurs

METHODS IN ENZYMOLOGY, VOL. 418 0076-6879/06 $35.00
Copyright 2006, Elsevier Inc. All rights reserved.
DOI: 10.1016/S0076-6879(06)18011-8

et al., 2004; Radtke *et al*., 2004; Weisz *et al*., 1999) as a potential treatment for retinal degenerative diseases, such as macular degeneration or *retinitis pigmentosa.* Several cell sources have been considered for such therapy: fetal RPE (Radtke *et al*., 2004; Weisz *et al*., 1999), autologous RPE (Binder *et al*., 2004; van Meurs *et al*., 2004), or established RPE cell lines (Lund *et al*., 2001). However, each source is not perfect. With all human donor tissue there is batch-to-batch variation and safety issues; in addition, fetal tissue as a cell source raises ethical concerns. Autologous RPE may already have an impaired function due to the developing disease. Cell lines such as ARPE-19 and h1RPE7 were used by Lund and coauthors (2001) in the Royal College of Surgeons (RCS) rat model of retinal dystrophy and showed preservation of the photoreceptor. These or similar cell lines could be a good source, if they can prove to maintain stable karyotype and RPE functions over multiple passages. However, it could be challenging to generate multiple lines that meet these criteria with minimal batch-to-batch variation if donor tissue is used as a source. Generation of RPE from hES cells has numerous advantages, as it can be done from pathogen-free cell lines under good manufacturing practices (GMP) conditions and with minimal variation among batches. Such cells can be characterized extensively prior to preclinical studies or for clinical applications, and large numbers of cells can be generated from a virtually unlimited supply of each hES cell line. With the future development of technologies such as somatic cell nuclear transfer- or parthenote-generated ES cells, banks of RPE cell lines can be established for future selection of cell lines more closely immune matched with a patient.

There are currently several reports on producing RPE from ES cells. In 2002, in the same experiments when primate ES cells differentiated into dopamine neurons, RPE was also observed and isolated in the same cultures (Kawasaki *et al*., 2002), and more extensive characterization of such cells was done 2 years later (Haruta *et al*., 2004), which showed that these cells express mRNA for RPE-specific markers RPE65 (Redmond *et al*., 1998) and CRALBP (Saari *et al*., 2001), perform phagocytosis with latex beads, and attenuate the loss of visual function after transplantation into the subretinal space of RCS rats. Mouse ES cells were differentiated into RPE (among other retinal structures) in the experiments of another group (Hirano *et al*., 2003). In all these experiments, coculture with mouse skull stromal cell line PA6 was used, and this differentiation was attributed to the stromal cell-derived inducing activity.

Experiments with differentiating hES cells showed that no such coculture is required for efficient and reliable differentiation of RPE. In the model system used in our laboratory, such differentiation occurs spontaneously when overgrown hES cells are maintained in the same plates, with or without feeders, until clusters of pigmented epithelia appear and can be

harvested (Klimanskaya *et al.*, 2004). Alternatively, embryoid bodies (EB) that are produced from hES cells and cultured for 6 to 8 weeks show pigmented areas on the surface; such EBs can be plated for outgrowth and produce primary cultures of RPE. Such differentiation was observed in medium supplemented with fetal bovine serum (FBS) or Serum Replacement (Invitrogen), with or without basic fibroblast growth factor (bFGF), on feeder cells, mouse embryonic fibroblasts (MEF), and in feeder-free systems: on MEF-derived extracellular matrix (Klimanskaya *et al.*, 2005), on fibronectin, collagens I and IV, and laminin. The differentiating cultures of hES cells are diverse, showing the presence of various cell types, and the sequence of events leading to the formation of RPE is still unclear. One of the possible models is that the earliest step is ''default'' neural lineage commitments of ES cells and formation of neuroectoderm or similar retinal progenitor cells. In eye formation during early mammalian development, the dorsal part of the optic vesicle adjacent to the mesoderm receives RPE-inductive signals, such as activin A expressed by extraocular mesenchyme (Chow and Lang, 2001; Feijen *et al.*, 1994; Fuhrmann *et al.*, 2000), which promote RPE formation, while the distal part receiving the FGF signals from surrounding ectoderm becomes neural retina. It is likely that similar events leading to RPE specification occur in differentiating hES cultures in response to and as a result of cues produced by the differentiating derivatives of hES cells that surround clusters of neuroectoderm.

Differentiation of hES Cells to RPE

Our method of producing RPE from hES cells mostly relies on long-term spontaneous differentiation of hES cells in serum-free medium. The cells are grown (Fig. 1A) on mitomycin C-treated primary mouse embryonic fibroblasts (PMEF) until they ''overgrow'' (usually 7 to 10 days; Fig. 1B), and then the medium is replaced with FGF-free differentiation medium. As an alternative, embryoid body cultures can be set up. Our experiments isolated RPE cells from 15 different hES cell lines: 6 lines derived at Harvard University in the laboratory of Dr. Douglas Melton (Cowan *et al.*, 2004), 3 lines from Wicell (Thomson *et al.*, 1998), and 6 lines established at Advanced Cell Technology, Inc.

After 5 to 7 days in culture, we usually see signs of differentiation, when the typical ES cell morphology is lost and various differentiated cell types appear (Fig. 1B and C). Most colonies usually show signs of neural lineage commitment, including cells that stain positive for tubulin βIII, pax6, and GFAP. These observations are in agreement with numerous observations in the literature that ES cells in culture select the neuronal pathway of differentiation most readily, which could be chosen by default (Smukler

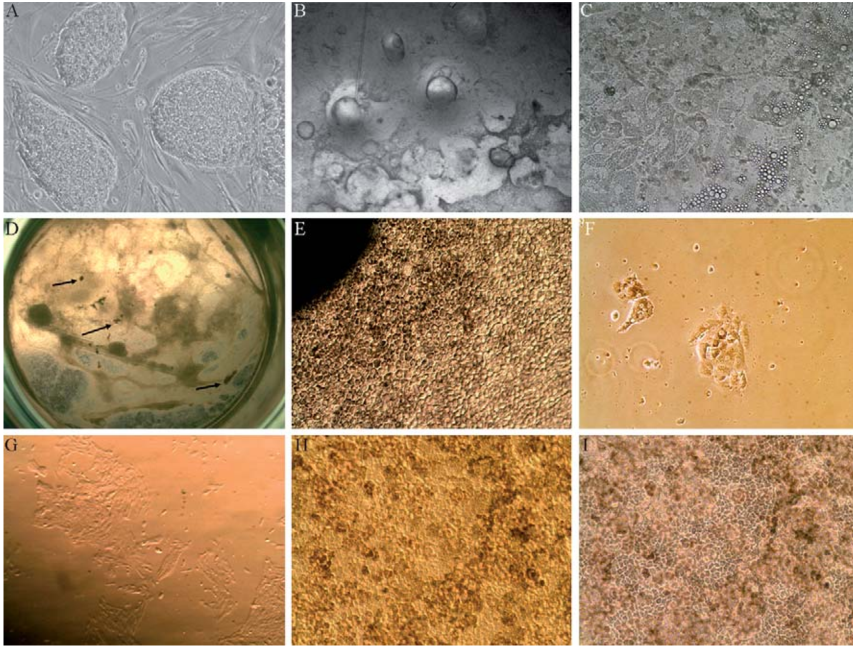

FIG. 1. Differentiation of hES cells to RPE. (A) Undifferentiated hES cell colonies. (B and C) Differentiating three-dimensional structures and various cell types from hES cells. (D and E) Appearance of pigmented cell clusters in long-term differentiating cultures of hES cells. (F and G) Growth of isolated RPE cells. (F) The next day after isolation. (G) Five days after isolation. (H and I) Mature hES–RPE culture, the same field is shown with (H) HMC and (I) phase contrast. Original magnification: A, C, E, F, H, I, 200×; B, 50×; D, 7.5×; G, 50×.

et al., 2006; Tropepe *et al.*, 2001), in response to the activity of FGF (Bouhon *et al.*, 2005; Ying *et al.*, 2003), or as a result of elimination of other inductive signals (Ying *et al.*, 2003). The plates are then cultured until clusters of pigmented epithelial cells begin to appear, which usually happens in 6 to 8 weeks (Fig. 1D and E). Such clusters keep slowly increasing in size, while new clusters continue to emerge. The same process can be initiated in conventional embryoid body culture (EB), in which case pigmented epithelial cells would appear on the surface of EBs and then this transition of nonpigmented cells to pigmented epithelium would slowly take over the whole EB. The cell lines were used at various passages, and the visible efficiency of RPE formation was higher at earlier passages. While on average the first pigmented epithelial clusters appeared around 6 to 8 weeks after the cells were subcultured, at early passages such pigmented cells were observed

after 3 weeks. This is possibly happening because the lines were either passaged only mechanically or just adapted to trypsinization and had not undergone multiple passaging with trypsin, which could be removing cell surface molecules and thus causing a certain degree of selection of such cells.

Of note, clusters of cells stained positively for neural lineage markers Pax6 and/or tubulin βIII, often in close conjunction with pigmented epithelium, were found in such differentiating systems. Cells of various types, still unidentified, are also found in the same differentiating cultures of hES cells, surrounding the clusters of RPE and their presumptive progenitors. It is possible that cells producing signals promoting RPE specification in clusters of Pax6-positive progenitors, similar to the signaling of ocular mesoderm in patterning ocular tissues, could be found among such differentiated cells next to Pax6-positive clusters.

These weeks-old cultures are composed of several layers of cells with a lot of extracellular matrix deposition, which makes it difficult to disperse them into a single cell suspension to select the desired cell type using FACS or magnetic beads. Instead, we use an approach in which the multilayer of cells is loosened with trypsin or collagenase and the pigmented cells are picked under the dissecting microscope using a glass capillary. Collected cells are plated on laminin or gelatin in RPE culture medium containing Serum Replacement and FBS with optional bFGF; within 24 to 48 h, clusters of cells begin to proliferate. Proliferating cells lose pigment and acquire a fibroblastic phenotype (Fig. 1F, G, Fig. 2A and B), strongly resembling the transdifferentiated RPE, which dedifferentiate as they proliferate and return to typical RPE morphology after they establish a monolayer (Fig. 1H and I), which usually takes 2 to 3 weeks (Chen *et al.*, 2003; Reh *et al.*, 1987; Sakaguchi *et al.*, 1997; Vinores *et al.*, 1995). Such RPE transdifferentiation has been shown to result in the formation of neuronal, amacrine, and photoreceptor cells (Zhao *et al.*, 1995), glia (Sakaguchi *et al.*, 1997), neural retina (Galy *et al.*, 2002), and neuronal progenitors (Opas and Dziak, 1994). bFGF accelerates transdifferentiation and RPE proliferation (Fig. 2), thus allowing the cells to reach confluence and begin to revert to the RPE phenotype much sooner. hES-derived RPE (hES–RPE) in the transdifferentiated state express the neural lineage markers Pax6 and tubulin βIII (Fig. 3), strongly resembling immature neural cells, and our comparative gene expression profiling showed their similarity to neural stem cells (Klimanskaya *et al.*, 2004).

Materials and Equipment

Unless the manufacturer and model are specified, most brands are acceptable.

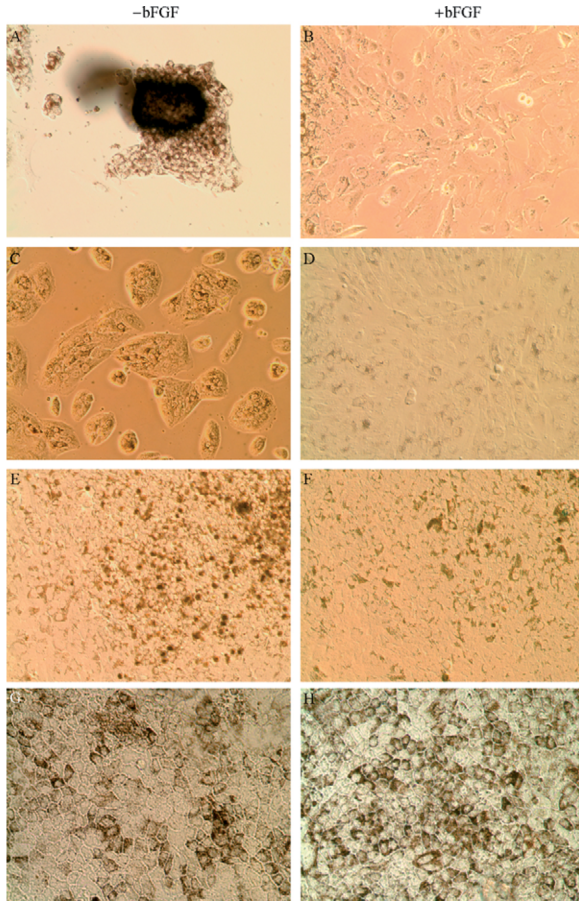

Fig. 2. Proliferation of hES–RPE in culture and transdifferentiation. (Left) No FGF; (right) 10 ng/ml bFGF. (A and B) Initial outgrowth after isolation of RPE from hES cells, 5 days. (C and D) Three days after passaging of hES–RPE. (E and F) Seven days after passaging. (G and H) Twenty-five days after passaging. Note that in the presence of bFGF transdifferentiation is more prominent and the monolayer is established faster (C and D). Cells in mature cultures of hES–RPE of the same age are more pigmented (G and H). Original magnification: A–F, 200×; G, H, 400×.

Equipment and Cell Culture Disposables

Stereomicroscope for microdissection (we use Nikon SMZ-1500)
Inverted microscope (we use Nikon TE 300 and TS 100) with phase (4, 10, 20, 40×) and Hoffman modulation Optics (HMC, 20, 40×) objectives

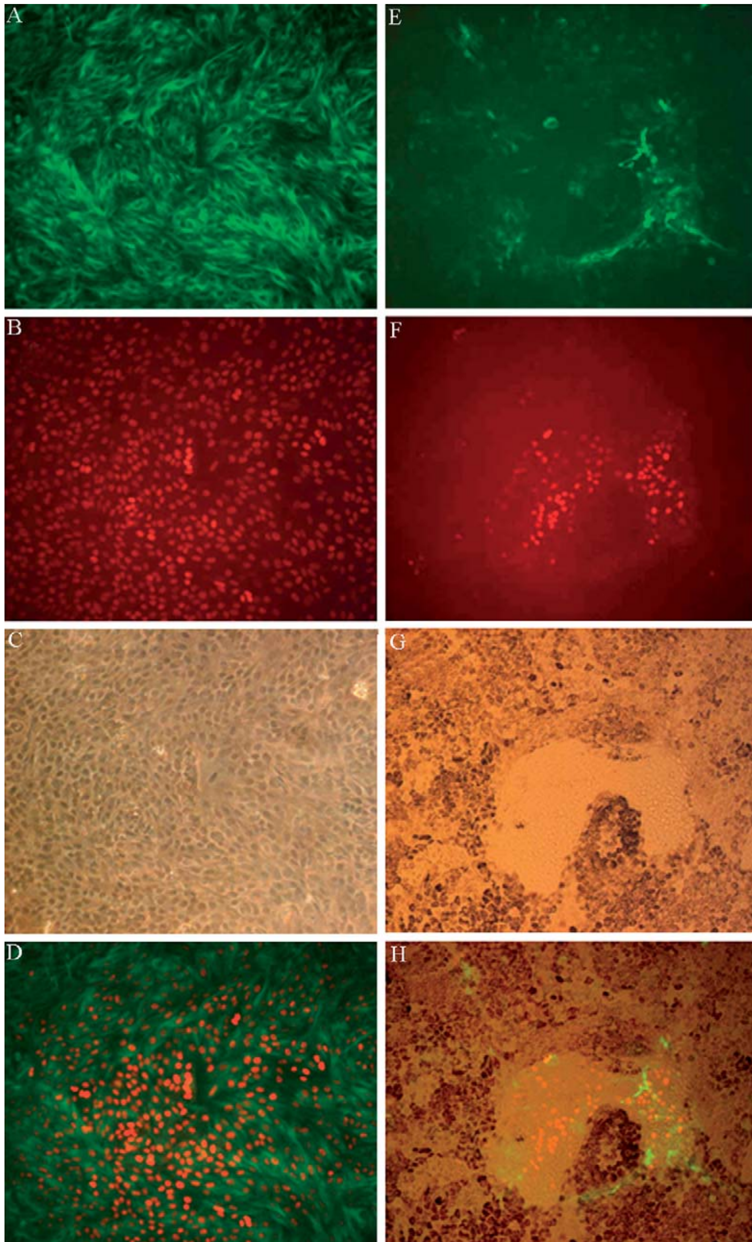

FIG. 3. Transdifferentiated (A–D) and "mature" (E–H) hES–RPE stained with antibodies to tubulin βIII (A and E) and Pax6 (B and F). (C and G) Phase contrast of the same fields. (D and H) Merged images of the first figures. Original magnification: 200×. Reproduced from Klimanskaya *et al.* (2004) with permission.

Bench-top biological safety cabinet (Terra Universal, Anaheim, CA) or micromanipulation workstation (MidAtlantic Diagnostics, Mount Laurel, NJ). The dissecting microscope is set up in this biosafety cabinet/workstation.
Biosafety cabinet (laminar flow hood) for cell culture
CO_2 incubator
Cell culture centrifuge
Automatic pipettors P1000, P200, P20
Six-well tissue culture plates
Four-well tissue culture plates
100-mm tissue culture dishes
Ultralow attachment cell culture plates or flasks (Corning)
Tissue culture flasks
15- and 50-ml conical centrifuge tubes
Glass capillaries flame pulled from Pasteur pipettes. *Note:* Pasteur pipettes need to be autoclaved or otherwise sterilized using biological indicators for quality control. We use spore strips from Steris (Mentor, OH).
Pipette-aid, cell culture disposables, etc.

Media Components and Other Reagents

Knockout Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)
DMEM high glucose (Invitrogen)
Serum Replacement (Invitrogen)[1]
Plasmanate (Bayer)[1]
FBS (Hyclone)
β-Mercaptoethanol, 1000× solution (Invitrogen)
Nonessential amino acids (NEAA), 100× solution (Invitrogen)
Penicillin/streptomycin, 100× solution (Invitrogen)
Glutamax-I, 100× solution (Invitrogen)
bFGF (Invitrogen)
Human LIF (Chemicon International)
0.05% trypsin/0.53 mM EDTA (Invitrogen)
Collagenase type IV (Invitrogen)
Gelatin from porcine skin (Sigma)
Laminin from human placenta (Sigma)
PBS, Ca^{2+}, Mg^{2+} free (Invitrogen)

[1] Each lot of these reagents needs to be tested for quality before it is used for hES cell medium preparation. For more detailed quality control protocols, see Klimanskaya and McMahon (2004).

Normocin (Invivogen, San Diego, CA): combination of three antibiotics active against mycoplasma, both positive and negative Gram bacteria and fungi. Usually well tolerated by the cells.

Mitomycin C (Sigma): the mitomycin C water solution normally has a deep purple color. Some batches have an insoluble precipitate forming the next day, which is reflected in the color of the solution becoming very light, and the mitomycin becomes less effective in arresting the cell division.

Primary Mouse Embryo Fibroblast (PMEF) Feeders[1]

PMEF are prepared from E12.5 fetuses of CD1 mice, expanded 1:5, and frozen at passage one; passage two is treated with 10 μg/ml mitomycin C for 3 h at 37° (for more detailed procedures, see Klimanskaya and McMahon [2004, 2005]). PMEF are plated at a density of 50,000 to 60,000 cells/cm^2 and used within 3 days.

hES Cell Culture and Differentiation Culture Setup

Basal medium (BM): knockout DMEM, supplemented with 1:100 NEAA, 1:100 penicillin/streptomycin, 1:100 glutamax, 1:1000 β-mercaptoethanol

hES cell growth medium (GM): BM, supplemented with 8% Serum Replacement, 8% plasmanate, 8 ng/ml human bFGF, 10 ng/ml human LIF

Differentiation medium (DM): BM, supplemented with 15% Serum Replacement

Note: to prevent contamination in long-term cultures, Normocin can be added.

hES cells are grown in GM, and are passaged routinely every 4 to 6 days using trypsin or mechanical dispersion (for more detailed procedures, see Klimanskaya and McMahon [2004] and Cowan *et al.* [2006]). For adherent differentiation the cells are allowed to overgrow on MEF until dome-like structures begin to appear, usually 7 to10 days. The medium is then changed to DM and replaced every day or every other day, depending on the volume of the medium per well. *Note:* because medium containing NEAA and Serum Replacement generally looks more yellowish than orange/pink, a slight color change toward yellow is acceptable before the medium needs to be changed. The frequency of medium change usually depends on the number of the cells per well and the well size/shape. Usually, four-well plates that can only hold 0.5 to 0.8 ml of medium need change every day, whereas six-well plates with 5 to 7 ml of medium can be changed every 36 to 48 h. These are

approximate guidelines, and the frequency of medium change for other sizes of tissue culture dishes needs to be established empirically.

For differentiation as embryoid bodies, hES cultures are treated with 2 to 4 mg/ml collagenase IV in GM for 5 to 10 min or until the colonies begin to detach from PMEF. The colonies are then collected by gentle pipetting and centrifuged at 1000 rpm in a standard cell culture centrifuge for 5 min (about 160*g*). The medium is aspirated, and the cell clumps are plated into ultralow attachment plates/flasks in DM. The medium is changed as required (see earlier discussion) by careful aspiration from the top, leaving a layer of the medium on the bottom to prevent disturbing of the clumps/EBs. If the EBs become hollow and rise to the surface, a cell culture pipette is used to remove the medium carefully. *Note 1:* spreading the EB to multiple wells reduces the frequency of medium change. *Note 2:* Ultralow attachment plates/flasks cannot be substituted with nontissue culture Petri dishes because many EBs will eventually attach and begin to grow out.

Pigmented clusters usually become visible within 6 to 8 weeks and will continue to grow slowly, and more of them may appear (Fig. 1D and E). To harvest more cells, the cultures need to continue, usually 2 to 3 months. In some experiments, we had 9-month-old cultures of EBs that produced passageable RPE cultures after being plated on gelatin. *Note:* when cells are cultured for such a long time, the medium is replaced only partially, and the wells are filled almost to the top; extra care needs to be exercised to prevent contamination. Normocin can be used as a wide-spectrum agent (fights Gram-positive and Gram-negative bacteria, fungi, and mycoplasma without any noticeable effect on hES cell performance). The plates need to be stacked carefully and be aligned properly to prevent accidental sliding and medium spills. If this happens, the spills need to be aspirated immediately. Setting up long-term cultures in flasks may reduce the risk of contamination.

Observe the cultures under a stereoscope at low power and at higher power under an inverted microscope (preferably using HMC objectives) for the appearance of pigmented cell clusters with cobblestone morphology. Usually when they appear, they can be seen clearly without any microscope by simply putting a dish against a white surface, appearing as little "freckles" (Fig. 1D) on the bottom of the plate or on EBs. However, to confirm that these are the anticipated cells, microscopic observation is required. *Note:* HMC observation is highly desirable for seeing both pigment and three-dimensional cell shapes (Fig. 1H and I), but if it is unavailable, using the "wrong" phase match for the regular phase-contrast objective allows one to see pigmentation better and adds some depth to the picture.

Harvesting RPE Cells

After several weeks of differentiation, a lot of extracellular matrix is deposited by the cells and it becomes very difficult to dissociate them into single cells to collect RPE by FACS or another cell-sorting method. However, its unique appearance allows one to handpick the cells of the right phenotype. We use two approaches: (1) handpicking pigmented cells under the stereomicroscope after the monolayer has been loosened with collagenase IV or trypsin or (2) outgrowth of pigmented EB.

Media and Reagents

RPE growth medium (RPE-GM)
BM supplemented with 7% Serum Replacement and 4% FBS. A concentration 10 ng/ml bFGF is optional.
0.05% trypsin/0.53 m*M* EDTA (Invitrogen)
PBS, Ca^{2+}, Mg^{2+} free
Collagenase IV, 20 mg/ml in DMEM stock solution (sterilize by 0.22-μm filtration and keep frozen in 1- to 2-ml aliquots)
Gelatin 0.01% (1 mg/ml) solution in PBS, sterilize by 0.22-mm filtration
Laminin from human placenta, 10 μg/ml solution in PBS, sterilize by 0.22-mm filtration

Coating Tissue Culture Plates with Gelatin or Laminin

Add solution of gelatin (0.1 to 0.2 mg/ml) or laminin (5 to 10 $\mu g/cm^2$) to cell culture plates.

Method 1. Handpicking Pigmented Cells

Method 1 can be performed on multiwell plates or tissue culture dishes. Cell culture flasks with a detachable side can also be used.
Trypsin is only used to assist removing the cells; it could be done without any enzymes, if the cells detach easily, so the cultures need to be probed first. Collagenase IV at a concentration of 5 to 10 mg/ml can be used instead of trypsin.
RPE-GM used for culture of isolated cells can be used with or without bFGF: in the presence of bFGF cells will grow faster, so the monolayer will be reached faster and reacquisition of the RPE phenotype after transdifferentiation will happen faster (Fig. 2).

1. Rinse the plate with PBS two or three times, add 0.05% trypsin/ 0.53 m*M* EDTA. Incubate for a few minutes, checking frequently under the

microscope and probing the culture with a flame-pulled capillary. If using collagenase, expect to wait longer, that is, 1 to 2 h.

2. The technique is simple gentle ''scraping'' off of the pigmented clusters and aspirating the removed cells; this should be done as soon as the monolayer has loosened enough to allow the cells to be collected easily. Keep scraping the pigmented clusters gently and transfer the collected cells to another plate or tube with RPE medium. This should be done quickly because the cells remain in trypsin and cell damage will occur after prolonged exposure. Using collagenase allows more time for the procedure.

3. Rinse RPE cells either by transferring them through two to three changes of medium in four-well plates (for larger clumps) or by centrifugation at 1000 rpm for 5 min in 5 to 10 ml RPE medium.

4. If large clumps of cells are collected, they can be transferred into a conical centrifuge tube, washed with PBS by centrifugation, and treated with 0.05 trypsin/0.53 m*M* EDTA for several minutes in a water bath. *Note:* agitate the tube by gently tapping it frequently and observe under a dissecting microscope; add RPE medium to quench trypsin immediately after large clumps are broken into desired smaller-sized clumps and single cells.

5. Plate into one or two wells of a four-well plate on gelatin or laminin in RPE medium.

Note: because this technique is designed for isolation of very small numbers of RPE cells, one or two wells of a four-well plate are recommended. Usually, even several hundred cells will fill such wells in 2 to 3 weeks and can be passaged after that by regular methods. See Fig. 1F and G for initial stages of growth of such manually isolated RPE cells.

Method 2. Outgrowth of RPE from EBs

Note: Use for EB cultures that show pronounced RPE areas on the surface and for large clumps of RPE cells that sometimes spontaneously detach from the cellular multilayer in adherent differentiating cultures of hES cells.

1. Plate EBs or RPE clumps onto cell culture dishes coated with gelatin or laminin in RPE medium.

2. After 2 to 3 days outgrowth of RPE cells should be visible.

3. Collect any large clusters of RPE cells that remain loosely attached after 1 to 2 weeks using a glass capillary or a P20/P200 automatic pipette under a dissecting microscope, wash by transferring large clumps through two to three wells of a four-well plate filled with PBS, and incubate in 0.05% trypsin/0.53 m*M* EDTA (in a drop or in another well) for several minutes, checking frequently under the microscope. Quench trypsin with

RPE medium when the desired small cell clump/single cell suspension is obtained.

4. Collect the cells in a centrifuge tube, centrifuge at 1000 rpm for 5 min, remove the supernatant, resuspend the cells in RPE growth medium, and plate onto gelatin- or laminin-coated tissue culture plates.

Note: proliferating cells lose their RPE morphology, turning into lightly or nonpigmented elongated cells. After the confluent monolayer is established, they will begin to revert to RPE morphology. Adding bFGF to growth medium after isolation will accelerate formation of the confluent monolayer so the cells will reacquire the RPE morphology faster.

Culture and Properties of hES-Derived RPE

Proliferating RPE will transdifferentiate and then begin to redifferentiate upon formation of the confluent monolayer (Fig. 2). The full cycle usually takes about 2 to 3 weeks, but even after that RPE will continue to "mature," becoming more pigmented. However, because very "mature" cells do not survive trypsinization and freezing well, we prefer to subculture them every 2 to 4 weeks. Passaging RPE too soon before they can fully reacquire RPE morphology results in a reduced life span of such cultures: the cells do not revert to RPE morphology and stop growing. RPE are relatively "slow" cells: even in the presence of bFGF, which accelerates their transdifferentiation and proliferation, it may take up to 2 to 3 weeks at each passage at a 1:3 ratio before they "mature" and regain the RPE phenotype. Such cell behavior requires slow propagation, and two or three confluent wells of a four-well plate can be produced in 3 to 4 weeks from several clusters of RPE cells usually found in one 35-mm plate of differentiating ES cells (each cluster usually has several hundred cells; some large older ones may have several thousand). After that the cells are usually subcultured at a 1:3 to 1:6 ratio at 2- to 3-week intervals. However, high numbers of RPE can be obtained from large-scale differentiating cultures of hES cells.

RPE Culture Protocols

Media and Reagents

RPE-GM, with and without bFGF
0.05% trypsin/0.53 m*M* EDTA (Invitrogen)
Laminin- or gelatin-coated tissue culture plates
For passaging, 0.05% trypsin/0.53 m*M* EDTA is usually sufficient but may require incubation for several minutes at 37°.

Medium Change

For freshly isolated or passaged cultures it is best to use RPE growth medium supplemented with 10 ng/ml bFGF, which we replace with bFGF-free medium after the monolayer is established. Because they differentiate faster in the presence of PEDF secreted by RPE, it may be beneficial to only replace two-thirds to one-half of the medium in a well. On average, the medium is changed once or twice a week. The monolayer is usually established within the first week after a 1:3 to 1:6 split, and the RPE phenotype is regained in 2 to 3 weeks. After that the cells can be maintained without significant loss of properties for several months; however, after 6 to 8 weeks it would require extended time in trypsin to passage them, so the viability can be decreased. On average, our usual passaging time is between 2 and 8 weeks.

Characterization of hES–RPE Cells

After the culture of hES–RPE is established, the next important step is to characterize the cells at the molecular and functional level. The RPE markers used in our studies are bestrophin, a 68-kDa product of the Best vitelliform macular dystrophy gene (Marmorstein *et al*., 2000), CRALBP, a water-soluble 36-kDa cellular retinaldehyde-binding protein (CRALBP), which is found in apical microvilli of RPE and in Muller glia (Bunt-Milam and Saari, 1983; Saari *et al*., 2001), RPE65, a 65-kDa cytoplasmic protein involved in retinoid metabolism (Hamel *et al*., 1993; Ma *et al*., 2001; Redmond *et al*., 1998), and PEDF (Jablonski *et al*., 2000; Karakousis *et al*., 2001; Steele *et al*., 1993). Pax6, although seen by some authors as a molecular marker of RPE (Kawasaki *et al*., 2002), is normally downregulated in mature RPE, so it could rather indicate the presence of immature cells. CRALBP, PEDF, and bestrophin can be detected by Western blot or immunofluorescence (see Fig. 4 for localization of bestrophin and CRALBP in differentiated hES–RPE by immunostaining), and PEDF can be measured conveniently by ELISA in conditioned medium and/or lysed cells. Translationally controlled RPE65 has been reported previously to be absent from cultured RPE at the protein level (Nicoletti *et al*., 1995), although real-time RT-PCR has detected high levels of RPE65 mRNA, and we found the same thing happening with our hES–RPE cells. Interestingly, the level of its expression correlated with the differentiation: in more mature cultures its expression was several times higher than in recently passaged cells (Klimanskaya *et al*., 2004). Therefore, RT-PCR can be used for RPE65 detection.

Because every culture of hES–RPE has variable proportions of more and less “mature” RPE cells, for more comprehensive characterization of

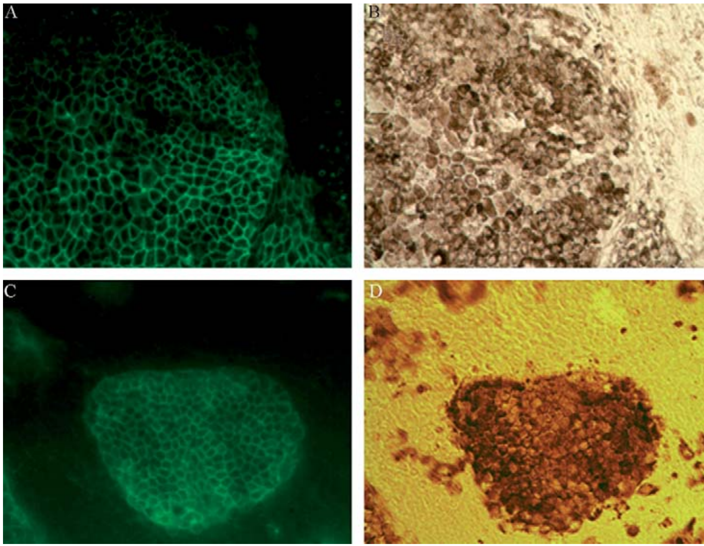

FIG. 4. Localization of bestrophin (A and B) and CRALBP (C and D) in hES–RPE. Original magnification: A, B, 400×; C, D, 200×. Parts of the figure are reproduced from Klimanskaya *et al.* (2004) with permission.

different populations/batches of RPE, real-time quantitative PCR can be used. Additionally, gene expression profiling allows a thorough comparison of hES–RPE with their *in vivo* counterparts, such as primary cultures of fetal and adult RPE, retinal tissues, or established lines (Klimanskaya *et al.*, 2004).

Immunofluorescence and Western Blot

The following antibodies are used.

- Anti-bestrophin antibody from Novus Biologicals (Littleton, CO)
- CRALBP antibody (we used a gift from Dr. John Sari, University of Washington; antibody is now available commercially)
- PEDF ELISA kit (Chemicon)
- Pax6 (Chemicon AB 5409)
- Secondary antibodies conjugated with FITC, rhodamine red, or biotin from Jackson Immunoresearch (West Grove, PA)
- Streptavidin-FITC/streptavidin-Texas red from Amersham
- Blocking solution: PBS with 10% goat serum, 10% donkey serum (Jackson Immunoresearch)
- Mounting medium Vectashield with DAPI (Vector Laboratories, Burlingame, CA)

For immunofluorescence staining, we fix the cells with 2% freshly made PBS-buffered paraformaldehyde for 10 to 20 min at room temperature and then permeabilize with 0.1% PBS-buffered NP-40 for 10 to 15 min at room temperature. *Note:* we do not have any particular preference for manufacturer of paraformaldehyde or NP-40; however, it is *crucial* (as with any cell/tissue sections staining) that freshly prepared paraformaldehyde is used. Freshly made PBS-buffered 4% paraformaldehyde can be stored in frozen aliquots. See Appendix 1 for a simple procedure for making the paraformaldehyde solution.

Fixed (but not yet permeabilized) cells can be stored for several weeks at 4° before staining, if necessary. Blocking is done for 1 h at room temperature, primary antibodies are added overnight at 4°, and secondary antibodies are added for 1 h at room temperature; fluorescently labeled streptavidin, if desired, is added for 20 min. The specimens are mounted in Vectashield with DAPI and observed/photographed under a fluorescent microscope.

For Western blot the cells are lysed in 4× Laemmli buffer (Laemmli, 1970) without reducing agents (proteinase inhibitors are optional), frozen, and stored at −20°. Reducing agents (β-mercaptoethanol or dithiothreitol) can be added before boiling the samples.

For PEDF ELISA, collect the medium conditioned by RPE cells for several days so it becomes yellow. The cells can also be lysed according to the instructions of the PEDF ELISA kit (Chemicon).

Primers Used in RT-PCR and Quantitative PCR (QPCR)

Gene-specific primer pairs are given for the following genes: RPE65, bestrophin, CRALBP, PEDF, Pax6, β-actin, and GADPDH:

RPE65-F
ATGGACTTGGCTTGAATCACTT
RPE65-R
GAACAGTCCATGAAAGGTGACA
Bestrophin-F
TAGAACCATCAGCGCCGTC
Bestrophin-R
TGAGTGTAGTGTGTATGTTGG
CRALBP-F
AAATCAATGGCTTCTGCATCATT
CRALBP-R
CCAAAGAGCTGCTCAGCAAC
PEDF-F1
TCTCGGTGTGGCGCACTTCA

PEDF-R1
GTCTTCAGTTCTCGGTCTATG
Pax6 F1
GTTTCAGCACCAGTGTCTAC
Pax6 R1
TATTGAGACATATCAGGTTCAC
β-actin-F
GCGGGAAATCGTGCGTGACA
β-actin-R
GATGGAGTTGAAGGTAGTTTCG
GADPH-F
CGATGCTGGCGCTGAGTAC
GADPH-R
CCACCACTGACACGTTGGC

For RNA isolation and RT-PCR/real-time QPCR conditions, see Appendix 2. For more details on gene expression profiling of hES RPE, including data analysis, see Klimanskaya *et al*. (2004) and Hipp and Atala (2006).

Functional Tests

One of the major RPE functions in supporting the photoreceptor is phagocytosis of the shed photoreceptor fragments, and *in vitro* assays for phagocytosis include assays with latex beads and, more specific for RPE, with labeled photoreceptor segments. Briefly, a monolayer of differentiated RPE grown in plastic chamber slides is incubated with 10^8/ml latex beads for up to 24 h, fixed with 2.5% glutaraldehyde in PBS for 30 min, rinsed three times with PBS, and then processed for electron microscopy as described elsewhere. Electron microscopy shows the presence of latex beads in the cytoplasm of the RPE cells. RPE-specific phagocytosis of rod outer segments is described in detail by Finnemann and coauthors (1997; http://www.pnas.org/cgi/content/full/94/24/12932).

Freezing ES–RPE

We use basic freezing medium and techniques. Briefly, trypsinized cells are centrifuged in DMEM with 10% FBS, the pellet is resuspended in cold (stored on ice or at 4°) 90% FBS, 10% DMSO, and the suspension is dispensed in prelabeled cryovials. For larger quantities (more than four to five vials) the vials are kept on ice during the cell-dispensing process. The vials are then sandwiched between two Styrofoam racks from 15-ml centrifuge tubes, and the racks are taped together and put into a –80° freezer overnight. The vials are transferred to liquid nitrogen storage next

morning or within 1 to 2 weeks. *Note*: because strongly pigmented cells do not recover well after thaw, we prefer to freeze hES–RPE before they become too pigmented, usually between 1 and 2 weeks after passaging.

Concluding Remarks

In our experiments, all hES cell lines ever handled reliably produced RPE cells (the total number of such hES cell lines is 18) in multiple experiments (over 80). We are currently investigating the *in vivo* performance of some of these hES–RPE cell lines in animal models (such as RCS rat), and preliminary data indicate that there can be a variation between lines or cultures of hES–RPE due to their transdifferentiation abilities and thus differences in the proportion of more and less differentiated cells across the cultures at any time point. This unique ability of RPE to transdifferentiate into cells of the neural lineage creates certain challenges for research and production of FDA-compliant cells for therapeutic applications. At the very minimum, a quantitative evaluation of markers of differentiated RPE versus neural lineage needs to be performed by real-time PCR and by FACS with different populations of cells used for experiments. For any preclinical studies or phase I clinical trials, batch-to-batch variation has to be minimized and the optimal level of differentiation needs to be found. Approaches to generating a suitable RPE cell population are currently being developed in our laboratory and are beyond the scope of this chapter.

Acknowledgments

I appreciate the hard work of Rebeca Ramos-Kelsey on optimization of hES–RPE culture and PCR/QPCR conditions and preparing RNA isolation and PCR protocols. My deep gratitude goes out to Sandy Becker for critical reading of the manuscript. I thank Advanced Cell Technology, Inc. for supporting this work.

Appendix 1

Preparation of 4% Paraformaldehyde Solution

1. Weigh 2 g of paraformaldehyde into a 50-ml centrifuge tube.
2. Add 3.5 ml of Milli-Q or similar quality water.
3. Add 10 μl of 10 *N* KOH.
4. Bring to a boiling point in a microwave.

Because microwaves vary, times will be different for each microwave. Watch the tube carefully *all the time* to avoid the paraformaldehyde

solution boiling over and producing toxic fumes! Ideally, do it under a fume hood; if this is not available, follow these steps:

1. Close the cap on the tube tightly; mix the contents by vortexing or shaking.
2. Unscrew the cap slightly, put the tube into a small (100 to 200 ml) glass beaker or plastic container, and place in the middle of the rotary table in the microwave.
3. Start the microwave and *watch the tube closely* until the liquid *begins* to boil and rise in the tube.
4. *Before* it reaches the top of the tube, press the door button to open. *Note:* when the liquid begins to boil and rise, it takes 1 s or less for it to overflow, so watch carefully and open the door promptly!
5. Screw on the cap tightly, shake the tube to mix the remaining particles (if everything is already dissolved, skip this step), unscrew the cap slightly, put the tube back, and repeat steps 3 and 4. Usually, after that everything is dissolved; if not, repeat step 5.
6. Prepare a 15-ml centrifuge tube and 5-ml syringe with a syringe filter (acrodisc), pour contents into the syringe, and filter into the prepared tube. Do this under the fume hood; if a fume hood is not available, immediately close the tube and rinse the syringe, filter, and original tube with plenty of *cold* running water.
7. Dilute the paraformaldehyde solution with PBS 1:10. This produces approximately 3.7 to 4% paraformaldehyde solution. Make aliquots and freeze for storage or use fresh.
8. Discard the tubes, syringe, and filter according to the chemical waste disposal procedures used in your laboratory.

Appendix 2

RT-PCR and Quantitative Real-Time PCR

Total RNA Isolation Using the RNeasy Minikit

1. Grow cells in a four- or six-well plate to almost confluency. Remove media and add 350 or 600 μl of buffer RLT with 1% BME.
2. Transfer sample to a Qiashredder column and spin for 2 min at maximum speed.
3. Toss the column and add an equal volume of 70% ETOH to the sample and transfer all to a RNeasy column. Spin for 15 to 30 s at maximum speed.
4. Add 350 μl of RW1 buffer. Spin for 15 to 30 s at maximum speed.

5. Add 70 μl of RRD buffer to 10 μl of reconstituted DNase and mix gently. Add 80 μl of diluted DNase to column. Incubate for 15 min.
6. Add 350 μl of RW1 buffer. Spin for 15 to 30 s at maximum speed.
7. Change column to a new collection tube. Add 500 μl of buffer RPE and spin for 15 to 30 s at maximum speed. Do this wash twice.
8. Spin for 1 min at maximum speed to dry the column.
9. Put column in a microcentrifuge tube and add 50 μl of RNase-free water. Spin for 1 min at maximum speed.

Note: for highly differentiated (pigmented) cells it is advisable to start with the Trizol purification step.

RT-PCR Using Qiagen One-Step RT-PCR Kit

Setup for a 25-μl Reaction

5 μl of RT-PCR buffer
5 μl of Q buffer
1.5 μl of 10 μM forward primer
1.5 μl of 10 μM reverse primer
1 μl of dNTP mix
1 μl of enzyme mix
5 units of RNase inhibitor
10 ng^{-1} μg of template
RNase-free water up to 25 μl

RT-PCR Program

50° for 30 min/reverse transcription
95° for 15 min/inactivation of RT/activation of *Taq*
Cycles (25–40×)
94° for 30 s
55 to 60° for 30 s
72° for 1 min/kb
72° for 10 min/final extension

First-Strand cDNA Synthesis (Promega Kit)

1. Incubate 2 or 10 μl of RNA ($\sim$1 μg) at 70° for 10 min.
2. Spin down sample and place on ice.
3. To 8.25 or 41.25 μl of RNase-free dH_2O add 4 or 20 μl 25 mM $MgCl_2$, 2 or10 μl 10× RT buffer, 2 or 10 μl 10 mM dNTP, 0.5 or 2.5 μl RNase inhibitor, 0.75 or 3.75 μl AMV RT (15 μg), 0.5 or 2.5 μl oligo(dT) (0.5 μg), and 2 or 10 μl of denatured RNA.
4. Incubate at 42° for 15 to 60 min.

5. Heat samples at 95° for 5 min and place on ice for 5 min.
6. Dilute sample with 80 or 400 μl of water.
7. Store sample at –20°. Use 2 to 5 μl for PCR.

PCR Using Amplitaq Gold

Setup for a 25-μl Reaction

2.5 μl of 10× buffer II
0.5 μl of 10 μ*M* forward primer
0.5 μl of 10 μ*M* reverse primer
2 μl of 2.5 m*M* each dNTP mix
1.5 μl of 25 m*M* $MgCl_2$
0.25 μl Amplitaq Gold
1 μl of DNA (100 to 200 ng)
16.75 μl of water

Final Concentrations

1× PCR buffer II
0.2 μ*M* forward primer
0.2 μ*M* reverse primer
200 μ*M* dNTP mix
1.5 m*M* $MgCl_2$
1.25 units Amplitaq Gold

PCR Program

95° for 10 min/activation of Amplitaq Gold
Cycles (25 to 35×)
94° for 30 s
55 to 60° for 30 s
72° for 1 min/per kb
72° for 5 min/final extension

Quantitative Real-Time PCR

Primers for RPE65, Bestrophin, PEDF, CRALBP, Pax6, and β-actin are optimized using various concentrations of 50, 100, 150, 200, 250, and 300 n*M*. The optimal concentration that gives the lowest Ct value and the highest fluorescence value is used to generate standard curves using various concentrations of the positive control. Optimal standard curves should fall between 90 and 110% efficiency. Except for Bestrophin, whose highest efficiency achieved was 86.8%, all other efficiencies fall within the acceptable limits

Once standard curves are generated, comparative quantification is possible using β-actin as the normalizer gene. Amplification plots should

display Ct values that fall within the acceptable range of 15 to 30 cycles. All amplification plots generated fall within this acceptable range.

Real-Time PCR Using SYBR Green

Dilute the passive reference dye 1:500 and keep protected from light. Thaw the master mix and keep on ice protected from light.

For 150 n*M* Final Primer Concentration

12.5 μl of 2$\times$ master mix
0.375 μl of 10 μM forward primer
0.375 μl of 10 μM reverse primer
0.375 μl of diluted reference dye
2 to 5 μl of cDNA
Water up to 25 μl

For 200 n*M* Final Primer Concentration

12.5 μl of 2$\times$ master mix
0.5 μl of 10 μM forward primer
0.5 μl of 10 μM reverse primer
0.375 μl of diluted reference dye
2 to 5 μl of cDNA
Water up to 25 μl

For 250 n*M* Final Primer Concentration

12.5 μl of 2$\times$ master mix
0.625 μl of 10 μM forward primer
0.625 μl of 10 μM reverse primer
0.375 μl of diluted reference dye
2 to 5 μl of cDNA
Water up to 25 μl
Mix gently and spin down plate.

Real-Time PCR Program

Amplification Curve

95° for 10 min
Cycles (40$\times$)
95° for 30 s
55 to 60° for 1 min
72° for 1 min

Dissociation Curve

95° for 1 min
Ramping down to 55°
Ramp up from 55° to 95° at 0.2°/s.

References

Akutsu, H., Cowan, C., and Melton, D. (2006). Human embryonic stem cells. *Methods Enzymol.* **418**(this volume).

Ben-Hur, T. (2006). Human embryonic stem cells for neuronal repair. *Isr. Med. Assoc. J.* **8**(2), 122–126.

Binder, S., Krebs, I., Hilgers, R. D., Abri, A., Stolba, U., Assadoulina, A., Kellner, L., Stanzel, B. V., Jahn, C., and Feichtinger, H. (2004). Outcome of transplantation of autologous retinal pigment epithelium in age-related macular degeneration: A prospective trial. *Invest. Ophthalmol. Vis. Sci.* **45**(11), 4151–4160.

Bouhon, I. A., Kato, H., Chandran, S., and Allen, N. D. (2005). Neural differentiation of mouse embryonic stem cells in chemically defined medium. *Brain Res. Bull.* **68**(1-2), 62–75.

Bunt-Milam, A. H., and Saari, J. C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J. Cell Biol.* **97**(3), 703–712.

Chen, S., Samuel, W., Fariss, R. N., Duncan, T., Kutty, R. K., and Wiggert, B. (2003). Differentiation of human retinal pigment epithelial cells into neuronal phenotype by N-(4-hydroxyphenyl) retinamide. *J. Neurochem.* **84**(5), 972–981.

Chow, R. L., and Lang, R. A. (2001). Early eye development in vertebrates. *Annu. Rev. Cell Dev. Biol.* **17,** 255–296.

Cowan, C. A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J. P., Wang, S., Morton, C. C., McMahon, A. P., Powers, D., and Melton, D. A. (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N. Engl. J. Med.* **350**(13), 1353–1356.

Feijen, A., Goumans, M. J., and van den Eijnden-van Raaij, A. J. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* **120**(12), 3621–3637.

Finnemann, S. C., Bonilha, V. L., Marmorstein, A. D., and Rodriguez-Boulan, E. (1997). Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha(v)-beta5 integrin for binding but not for internalization. *Proc. Natl. Acad. Sci. USA* **94**(24), 12932–12937.

Fuhrmann, S., Levine, E. M., and Reh, T. A. (2000). Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Development* **127**(21), 4599–4609.

Galy, A., Neron, B., Planque, N., Saule, S., and Eychene, A. (2002). Activated MAPK/ERK kinase (MEK-1) induces transdifferentiation of pigmented epithelium into neural retina. *Dev. Biol.* **248**(2), 251–264.

Hamel, C. P., Tsilou, E., Pfeffer, B. A., Hooks, J. J., Detrick, B., and Redmond, T. M. (1993). Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated *in vitro*. *J. Biol. Chem.* **268**(21), 15751–15757.

Haruta, M., Sasai, Y., Kawasaki, H., Amemiya, K., Ooto, S., Kitada, M., Suemori, H., Nakatsuji, N., Ide, C., Honda, Y., and Takahashi, M. (2004). *In vitro* and *in vivo* characterization of pigment epithelial cells differentiated from primate embryonic stem cells. *Invest. Ophthalmol. Vis. Sci.* **45**(3), 1020–1025.

Hirano, M., Yamamoto, A., Yoshimura, N., Tokunaga, T., Motohashi, T., Ishizaki, K., Yoshida, H., Okazaki, K., Yamazaki, H., Hayashi, S., and Kunisada, T. (2003). Generation of structures formed by lens and retinal cells differentiating from embryonic stem cells. *Dev. Dyn.* **228**(4), 664–671.

Jablonski, M. M., Tombran-Tink, J., Mrazek, D. A., and Iannaccone, A. (2000). Pigment epithelium-derived factor supports normal development of photoreceptor neurons and opsin expression after retinal pigment epithelium removal. *J. Neurosci.* **20**(19), 7149–7157.

Kania, G., Blyszczuk, P., and Wobus, A. M. (2004). The generation of insulin-producing cells from embryonic stem cells—a discussion of controversial findings. *Int. J. Dev. Biol.* **48**(10), 1061–1064.

Karakousis, P. C., John, S. K., Behling, K. C., Surace, E. M., Smith, J. E., Hendrickson, A., Tang, W. X., Bennett, J., and Milam, A. H. (2001). Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues. *Mol. Vis.* **7,** 154–163.

Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., Haruta, M., Takahashi, M., Yoshikawa, K., Nishikawa, S., Nakatsuji, N., and Sasai, Y. (2002). Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc. Natl. Acad. Sci. USA* **99**(3), 1580–1585.

Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M. D., and Lanza, R. (2005). Human embryonic stem cells derived without feeder cells. *Lancet* **365**(9471), 1636–1641.

Klimanskaya, I., Hipp, J., Rezai, K. A., West, M., Atala, A., and Lanza, R. (2004). Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. *Cloning Stem Cells* **6**(3), 217–245.

Klimanskaya, I., and McMahon, J. (2004). Approaches for derivation and maintenance of human ES cells: Detailed procedures and alternatives. *In* ‘‘Handbook of Stem Cells’’ (R. Lanza, *et al*., eds.),. Academic Press, San Diego.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259), 680–685.

Lund, R. D., Adamson, P., Sauve, Y., Keegan, D. J., Girman, S. V., Wang, S., Winton, H., Kanuga, N., Kwan, A. S., Beauchene, L., Zerbib, A., Hetherington, L., Couraud, P. O., Coffey, P., and Greenwood, J. (2001). Subretinal transplantation of genetically modified human cell lines attenuates loss of visual function in dystrophic rats. *Proc. Natl. Acad. Sci. USA* **98**(17), 9942–9947.

Lund, R. D., Kwan, A. S., Keegan, D. J., Sauve, Y., Coffey, P. J., and Lawrence, J. M. (2001). Cell transplantation as a treatment for retinal disease. *Prog. Retin. Eye Res.* **20**(4), 415–449.

Ma, J., Zhang, J., Othersen, K. L., Moiseyev, G., Ablonczy, Z., Redmond, T. M., Chen, Y., and Crouch, R. K. (2001). Expression, purification, and MALDI analysis of RPE65. *Invest. Ophthalmol. Vis. Sci.* **42**(7), 1429–1435.

Marmorstein, A. D., Marmorstein, L. Y., Rayborn, M., Wang, X., Hollyfield, J. G., and Petrukhin, K. (2000). Bestrophin, the product of the Best vitelliform macular dystrophy gene (VMD2), localizes to the basolateral plasma membrane of the retinal pigment epithelium. *Proc. Natl. Acad. Sci. USA* **97**(23), 12758–12763.

Muñoz-Sanjuán, I., and Brivanlou, A. H. (2002). Neural induction, the default model and embryonic stem cells. *Nature Rev. Neurosci.* **3**(4), 271–280.

Nicoletti, A., Wong, D. J., Kawase, K., Gibson, L. H., Yang-Feng, T. L., Richards, J. E., and Thompson, D. A. (1995). Molecular characterization of the human gene encoding an abundant 61 kDa protein specific to the retinal pigment epithelium. *Hum. Mol. Genet.* **4**(4), 641–649.

Olsen, A. L., Stachura, D. L., and Weiss, M. J. (2006). Designer blood: Creating hematopoietic lineages from embryonic stem cells. *Blood* **107**(4), 1265–1275.

Opas, M., and Dziak, E. (1994). bFGF-induced transdifferentiation of RPE to neuronal progenitors is regulated by the mechanical properties of the substratum. *Dev. Biol.* **161**(2), 440–454.

Peschle, C., and Condorelli, G. (2005). Stem cells for cardiomyocyte regeneration: State of the art. *Ann. NY Acad. Sci.* **1047,** 376–385.

Radtke, N. D., Aramant, R. B., Seiler, M. J., Petry, H. M., and Pidwell, D. (2004). Vision change after sheet transplant of fetal retina with retinal pigment epithelium to a patient with retinitis pigmentosa. *Arch. Ophthalmol.* **122**(8), 1159–1165.

Redmond, T. M., Yu, S., Lee, E., Bok, D., Hamasaki, D., Chen, N., Goletz, P., Ma, J. X., Crouch, R. K., and Pfeifer, K. (1998). Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nature Genet.* **20**(4), 344–351.

Reh, T. A., Nagy, T., and Gretton, H. (1987). Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330**(6143), 68–71.

Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T. (2001). Neural progenitors from human embryonic stem cells. *Nature Biotechnol.* **19**(12), 1134–1140.

Saari, J. C., Nawrot, M., Kennedy, B. N., Garwin, G. G., Hurley, J. B., Huang, J., Possin, D. E., and Crabb, J. W. (2001). Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. *Neuron* **29**(3), 739–748.

Sakaguchi, D. S., Janick, L. M., and Reh, T. A. (1997). Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: Generation of retinal neurons and glia. *Dev. Dyn.* **209**(4), 387–398.

Smukler, S. R., Runciman, S. B., Xu, S., and van der Kooy, D. (2006). Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences. *J. Cell Biol.* **172**(1), 79–90.

Steele, F. R., Chader, G. J., Johnson, L. V., and Tombran-Tink, J. (1993). Pigment epithelium-derived factor: Neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc. Natl. Acad. Sci. USA* **90**(4), 1526–1530.

Teramoto, K., Asahina, K., Kumashiro, Y., Kakinuma, S., Chinzei, R., Shimizu-Saito, K., Tanaka, Y., Teraoka, H., and Arii, S. (2005). Hepatocyte differentiation from embryonic stem cells and umbilical cord blood cells. *J. Hepatobiliary Pancreat. Surg.* **12**(3), 196–202.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**(5391), 1145–117. Erratum in *Science* **282**(5395), 1827 (1998).

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T. W., Rossant, J., and van der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* **30**(1), 65–78.

van Meurs, J. C., ter Averst, E., Hofland, L. J., van Hagen, P. M., Mooy, C. M., Baarsma, G. S., Kuijpers, R. W., Boks, T., and Stalmans, P. (2004). Autologous peripheral retinal pigment epithelium translocation in patients with subfoveal neovascular membranes. *Br. J. Ophthalmol.* **88**(1), 110–113.

Vinores, S. A., Derevjanik, N. L., Mahlow, J., Hackett, S. F., Haller, J. A., deJuan, E., Frankfurter, A., and Campochiaro, P. A. (1995). Class III beta-tubulin in human retinal pigment epithelial cells in culture and in epiretinal membranes. *Exp. Eye Res.* **60**(4), 385–400.

Wei, H., Juhasz, O., Li, J., Tarasova, Y. S., and Boheler, K. R. (2005). Embryonic stem cells and cardiomyocyte differentiation: Phenotypic and molecular analyses. *J. Cell. Mol. Med.* **9**(4), 804–817.

Weisz, J. M., Humayun, M. S., De Juan, E., Jr., Del Cerro, M., Sunness, J. S., Dagnelie, G., Soylu, M., Rizzo, L., and Nussenblatt, R. B. (1999). Allogenic fetal retinal pigment epithelial cell transplant in a patient with geographic atrophy. *Retina* **19**(6), 540–545.

Ying, Q. L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nature Biotechnol.* **21**(2), 183–186.

Zhao, S., Thornquist, S. C., and Barnstable, C. J. (1995). *In vitro* transdifferentiation of embryonic rat retinal pigment epithelium to neural retina. *Brain Res.* **677**(2), 300–310.

[12] Mesenchymal Cells

By TIZIANO BARBERI and LORENZ STUDER

Abstract

Human embryonic stem cells (hESC) provide a potentially unlimited source of specialized cell types for regenerative medicine. Nonetheless, one of the key requirements used to fulfill this potential is the ability to direct the differentiation of hESC to selective fates *in vitro*. Studies have reported the development of culture strategies to derive multipotent mesenchymal precursors from hESCs *in vitro*. This chapter reviews the techniques that allow the selective derivation of such precursors and their differentiation toward various mesenchymal cell types. It also discusses current limitations and future perspectives on the use of hESC-derived mesenchymal tissues.

Introduction

The isolation of human embryonic stem cells (Thomson *et al.*, 1998) has led to renewed focus on developing *in vitro* differentiation strategies to control embryonic stem cell (ESC) fate. Such strategies may yield specialized cell types suitable for cell therapy in degenerative diseases. Understanding the differentiation steps directing human ESCs (hESCs) toward mesenchymal fates will also provide an important tool to study developmental biology and the molecular mechanism controlling mesodermal and mesenchymal fate specification in humans. It is established that spontaneous differentiation of hESCs in immunocompromised hosts *in vivo* leads to the formation of teratomas, tumor masses that typically include a wide variety of mesenchymal tissues, including cartilage, bone, and striate muscle in addition to ectodermal and endodermal derivatives (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998). These data suggest that hESCs have the intrinsic ability to generate mesenchymal tissues and lead

METHODS IN ENZYMOLOGY, VOL. 418 0076-6879/06 $35.00
Copyright 2006, Elsevier Inc. All rights reserved.
DOI: 10.1016/S0076-6879(06)18012-X