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Claudia Aruta, Francesca Giordano, Anna de Marzo, Antonella Comitato, Graça Raposo, et al.. In vitro differentiation of retinal pigment epithelium from adult retinal stem cells. *Pigment Cell & melanoma research*, 2010, 24 (1), pp.233 - 240. 10.1111/j.1755-148x.2010.00793.x . hal-03086757

HAL Id: hal-03086757

<https://hal.science/hal-03086757>

Submitted on 17 Nov 2021

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In vitro differentiation of retinal pigment epithelium from adult retinal stem cells

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KEYWORDS RPE/retinal neurospheres/phagocytosis/EM/melanogenesis

PUBLICATION DATA Received 8 July 2010, revised and accepted for publication 14 October 2010, published online 19 October 2010

doi: 10.1111/j.1755-148X.2010.00793.x

Summary

One of the limitations in molecular and functional studies of the retinal pigment epithelium (RPE) has been the lack of an in vitro system retaining all the features of in vivo RPE cells. Retinal pigment epithelium cell lines do not show characteristics typical of a functional RPE, such as pigmentation and expression of specific markers. The present study was aimed at the development of culture conditions to differentiate, in vitro, retinal stem cells (RSC), derived from the adult ciliary body, into a functional RPE. Retinal stem cells were purified from murine eyes, grown as pigmented neurospheres and induced to differentiate into RPE on an extracellular matrix substrate using specific culture conditions. After 7–15 days of culture, pigmented cells with an epithelial morphology showed a polarized organization and a capacity for phagocytosis. We detected different stages of melanogenesis in cells at 7 days of differentiation, whereas RPE at 15 days contained only mature melanosomes. These data suggest that our protocol to differentiate RPE in vitro can provide a useful model for molecular and functional studies.

Introduction

The retinal pigment epithelium (RPE) is highly specialized and polarized. It has important functions in the retina: absorption of stray light, formation of the blood–retinal barrier, regeneration of visual pigments and phagocytosis of outer segments of photoreceptors (Strauss, 2005). Several inherited and degenerative diseases, such as albinism, retinitis pigmentosa and age-

related macular degeneration, are associated with impaired RPE functions.

The lack of accurate in vitro models for this tissue hampers functional studies on these diseases. Primary cultures of RPE cells exhibit considerable physiological variability based on donor differences. Alternative sources include cell lines spontaneously created during the passaging of primary cells, such as the human RPE cell lines D407 (Davis et al., 1995) and ARPE-19 (Dunn

Significance

In vitro development of retinal pigment epithelium (RPE) is of great interest because several retinal inherited and degenerative diseases are associated with RPE defects. Regenerative medicine can be envisioned as a therapeutic approach for these diseases. Furthermore, lack of an in vitro system retaining all the features of in vivo RPE cells has impaired functional and molecular studies on RPE. We describe here a protocol for differentiation into RPE of retinal stem cells derived from adult eyes. We demonstrate gene expression compatible with progressive maturation of RPE. Ultrastructural analysis and functional assays confirm that this differentiation system will provide an in vitro model for RPE studies and melanogenesis.

et al., 1996), rat RPE cell line BPEI-1 (McLaren et al., 1993) and mouse B6-RPE07 (Chen et al., 2008). Other cell lines have been generated by transformation with the SV40 virus, such as the rat RPE-J cell line (Nabi et al., 1993).

However, all of these cell lines only retain limited characteristics of RPE cells because they lose pigmentation, and show reduced or altered gene expressions, changes in morphology and alteration in metabolic functions (Alge et al., 2003; Campochiaro et al., 1991; Hunt and Davis, 1990; Huotari et al., 1995; Rizzolo, 1990). An innovative and promising method is the use of stem cells that have the ability to differentiate into RPE (Osakada et al., 2008). More interestingly, retinal stem cells (RSC) can be derived from adult murine and human ciliary bodies and cultured in vitro as pigmented neurospheres (RNS) (Coles et al., 2004; Tropepe et al., 2000). These cells have been shown to be able to differentiate in all retinal cell types, including RPE (Tropepe et al., 2000; Vossmerbaeumer et al., 2008). However, in none of the in vitro RPE cultures published so far has a progressive pigmentation, which allows the study of melanosome biogenesis, been reported.

The purpose of this study was to develop culture conditions to differentiate RSC in vitro into a functional RPE to be used as a tool to perform molecular and functional analyses. Melanocytes cannot be considered an appropriate model for RPE melanogenesis because melanocytes and RPE cells have different embryonic origins and show distinctive melanosomal shapes (Smith-Thomas et al., 1996). Furthermore, melanocytes and RPE have different melanogenic gene expression regulation (Bharti et al., 2006, 2008; Murisier et al., 2007). However, little is known about this subject and RPE pigmentation is still not fully understood. The process of melanogenesis in adult RPE is debated because only mature melanosomes can be detected. Although melanosomes are released from melanocytes, the secretion of melanosomes from the RPE has never been reported. Whether this difference is associated to different myosin proteins involved in melanosome transport in the two cell types is still not known. For all of these reasons, we believe that an in vitro model of RPE will be essential for a more detailed characterization of pigmentation and melanogenesis in this tissue.

Results and discussion

Retinal neurospheres (RNS) were derived from adult mouse ciliary body and cells were cultured at low density (20 cells/ μ l). Retinal neurospheres are clones of pigmented cells derived from the pigmented layer of the ciliary body, as described recently (van der Kooy, personal communication; Takabe et al., 2009, abstract). Cells in RNS share some RPE features such as epithelial morphology and tight junctions, and produce pigment epithelium-derived factor (PEDF) (Moe et al., 2009). We

tested different media to induce differentiation of RNS into RPE: Dulbecco's modified Eagle's medium (DMEM)-F12, serum-free medium (SFM), modified Eagle's medium (MEM), DMEM high glucose, and DMEM low glucose with hormone mix (see Methods). Differentiation into RPE was first estimated by morphological analysis. The best protocol for in vitro differentiation of RPE from RNS was as follows: RNS were seeded on extracellular matrix (ECM) and allowed to proliferate for 2 days in SFM with basic fibroblast growth factor (bFGF). At this time, cells migrated out from RNS, lost pigmentation and acquired an elongated shape (Figure 1A,B). The medium was then changed to DMEM low glucose with hormone mix and the culture continued for 7–15 days. When cells reached confluence they started to pigment again. During this time in culture we did not detect dying cells (data not shown). After 7 days of culture, a monolayer of pigmented cells with epithelial morphology could be seen (Figure 1C).

We previously reported that these same RNS could differentiate, upon specific culture conditions, into neural retina cell types, including rod photoreceptors, bipolar neurons and Müller glia (Giordano et al., 2007). Therefore, we analyzed the characteristics of the RPE-like cells by RT-PCR and immunofluorescence. We found that stem cell and neural progenitor markers (*Oct3/4*, *Nanog* and *Nestin*), which are expressed, except for *Oct3/4*, at higher levels in RNS compared to the ciliary body, were downregulated after 7 days of differentiation (Figure 1D). Moreover, the same cells did not express neural retinal markers such as *Pkcx* (bipolar cell marker) or *Rhodopsin* (rod marker) (Figure 1E). Pax6, a marker of retinal progenitor cells and, in the adult retina, of amacrine cells, was strongly expressed in RNS and in progenitors after 3 days in culture (Figure 1F,G), and was downregulated upon RPE differentiation (Figure 1E,H). Similarly, Nestin, a marker of neuronal progenitors, was detectable in RNS and progenitors but not in differentiated RPE (Figure 1I–K).

Different isoforms of the microphthalmia transcription factor (Mitf) are differently expressed in the retina and RPE, and the H-Mitf isoform increases with RPE differentiation (Bharti et al., 2008). Therefore we analyzed A-Mitf, H-Mitf and M-Mitf in our culture isoforms. The three isoforms were all expressed in RNS. A-Mitf and H-Mitf were downregulated in unpigmented progenitors at 3 days of culture (3D), and expression increased again in RPE at 7 and 12 days of differentiation (Figure 1L). Interestingly, the H-Mitf isoform progressively increased during RPE maturation, as found during RPE development (Figure 1L and Bharti et al., 2008). M-Mitf isoform is not expressed in RPE and is specific for neural crest-derived melanocytes (Bharti et al., 2008). M-Mitf was detectable only in RNS but not in RPE differentiated in vitro (Figure 1L), confirming differentiation of an RPE-like cell layer and not of pigmented cells with choroidal characteristics. Changes in

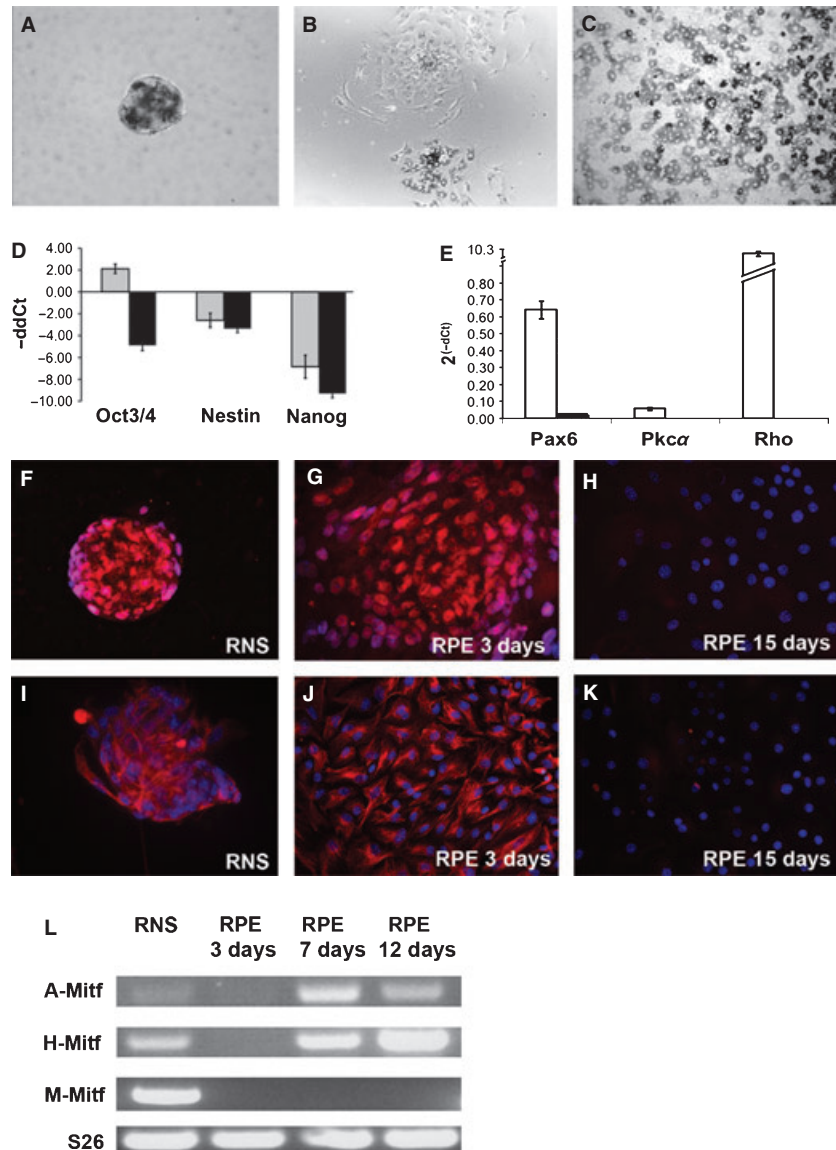


Figure 1. Expression analysis of stem cell and progenitor markers in the in vitro differentiating retinal pigment epithelium (RPE). (A) Pigmented retinal neurosphere (RNS) grown clonally for 7 days. (B) Cells from a RNS, after 2 days on extracellular matrix, gave rise to pigmented and non-pigmented cells with an elongated shape. (C) Retinal pigment epithelium-like cells after 7 days of differentiation formed a monolayer of pigmented cells with a polygonal shape. (D) Real-time PCR analysis of stem cell and progenitor markers (*Oct3/4*, *Nestin*, *Nanog*): relative expression in ciliary body (grey bars) and in RPE-like cells at 8 days of differentiation (black bars) compared to expression in RNS (set as 0) and expressed as log of fold change. (E) Real-time PCR analysis of retinal neuronal markers: *Pax6* (retinal progenitors and amacrine cell marker), *Pkcx* (bipolar cell marker) and *Rhodopsin* (Rho, rod photoreceptor marker) in adult retina (white bars) and RPE-like cells at 8 days of differentiation (black bars). (F–K) Time course of Pax6 (F–H) and Nestin (I–K) proteins in RNS, RPE-like cells after 3 days of differentiation (RPE 3 days) and RPE-like cells after 15 days of differentiation (RPE 15 days). (L) RT-PCR analysis of A-, H- and M-Mitf isoforms in RNS, RPE-like cells after 3, 7 and 12 days of differentiation.

expressions of Pax6 and Mitf in the differentiating RPE culture are consistent with published data showing that Pax6 promotes *Mitf* expression and, in contrast, that Mitf downregulates Pax6 (Baumer et al., 2003; Tsukiji et al., 2009). These data suggested that cells were activating a specific differentiation program under the tested conditions.

RPE-like cells were further characterized by immunofluorescence. We detected expression of the melanin biosynthesis enzymes tyrosinase-related protein 1 (Typr1), dopachrome tautomerase (Dct) and tyrosinase (Figure 2A–C), the pigment cell transcription factor, Mitf (Figure 2D) and the tight-junction epithelial protein ZO-1 (Figure 2E). Expressions of other RPE markers were analyzed at the mRNA level by RT-PCR analysis. We detected expression of other genes that are correlated to different forms of albinism and are important for melanosome biogenesis, such as *Pmel17*, *Matp*, *Oca2*,

Oa1, *Mlana* and *Myo7a* (Figure 2K). *Mlana*, *Pme17* and *Myo7a* were present in RNS, but the other mRNAs were not detectable (Figure 2K). In addition, the pigment epithelium derived factor (*Pedf*), the retinaldehyde-binding protein (*Rlbpl*) and the gap junction connexin 43 (*Cx43*) were detected in RNS and at all in vitro differentiation time points of RPE (Figure 2K).

Expression of markers characteristic of mature RPE required a longer differentiation time. In cells differentiated for 15 days we could detect expression of an enzyme involved in the recycling of the photopigment 11-cis-retinal, the RPE-specific 65-kD protein (Rpe65) (Figure 2F). At this same time of differentiation, cells showed a polarized phenotype typical of RPE. We detected the basolateral membrane localization of Bestrophin 1 (Figure 2G), the apical localization of Ezrin (Figure 2H) and localization at the apical side and in microvilli of $\beta 5$ integrin and Na^+/K^+ ATPase (Figure 2I–J).

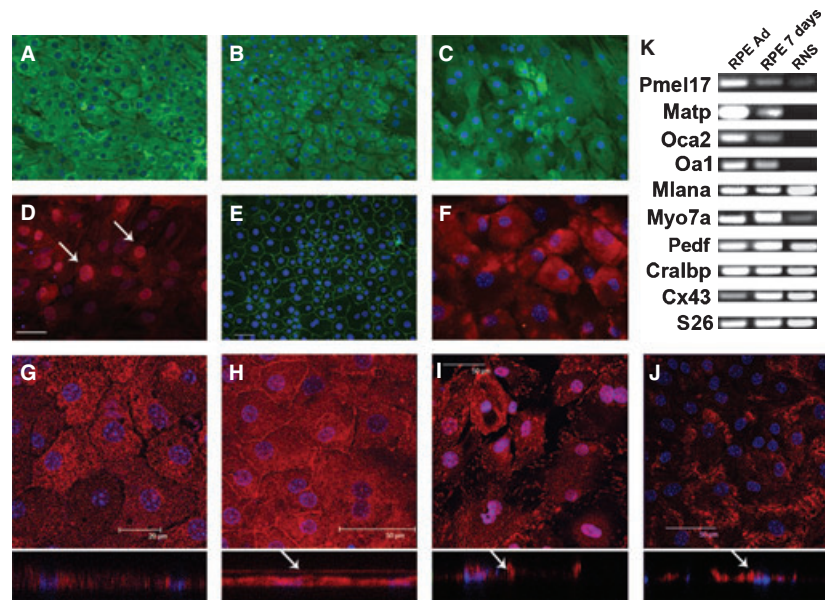


Figure 2. Characterization of retinal pigment epithelium (RPE) markers in RPE-like cells differentiated in vitro. (A–E) Retinal pigment epithelium-like cells after 7 days of differentiation. Expression of enzymes involved in melanin synthesis: Dct in (A), Tyrp1 in (B), Tyr in (C); pigment cell characteristic transcription factor Mitf in (D) (arrows point to nuclear localization) and epithelial tight-junction protein ZO-1 in (E). (F–J) Retinal pigment epithelium-like cells after 15 days of differentiation. Expression of the RPE-specific enzyme: RPE65 in (F). (G–J) Confocal images of RPE-like cells that form a polarized epithelium: basal localization of Bestrophin 1 (G, lower panel shows the z-axis with Bestrophin localized below and at the level of nuclei in blue); intracellular and apical localization of Ezrin (H, lower panel shows the z-axis with Ezrin localized above, arrow, and at the level of nuclei in blue); apical localization of $\beta 5$ integrin (I) and Na^+/K^+ ATPase (J) lower panels show the z-axis with $\beta 5$ integrin (I, arrow) and Na^+/K^+ ATPase (J, arrow) localized above the nuclei in blue. (K) RT-PCR analysis of RPE genes in adult RPE (RPE Ad), RPE-like cells at 7 days of differentiation (RPE 7 days) and retinal neurospheres. Scale bar: 50 μm (D–J); 20 μm (G).

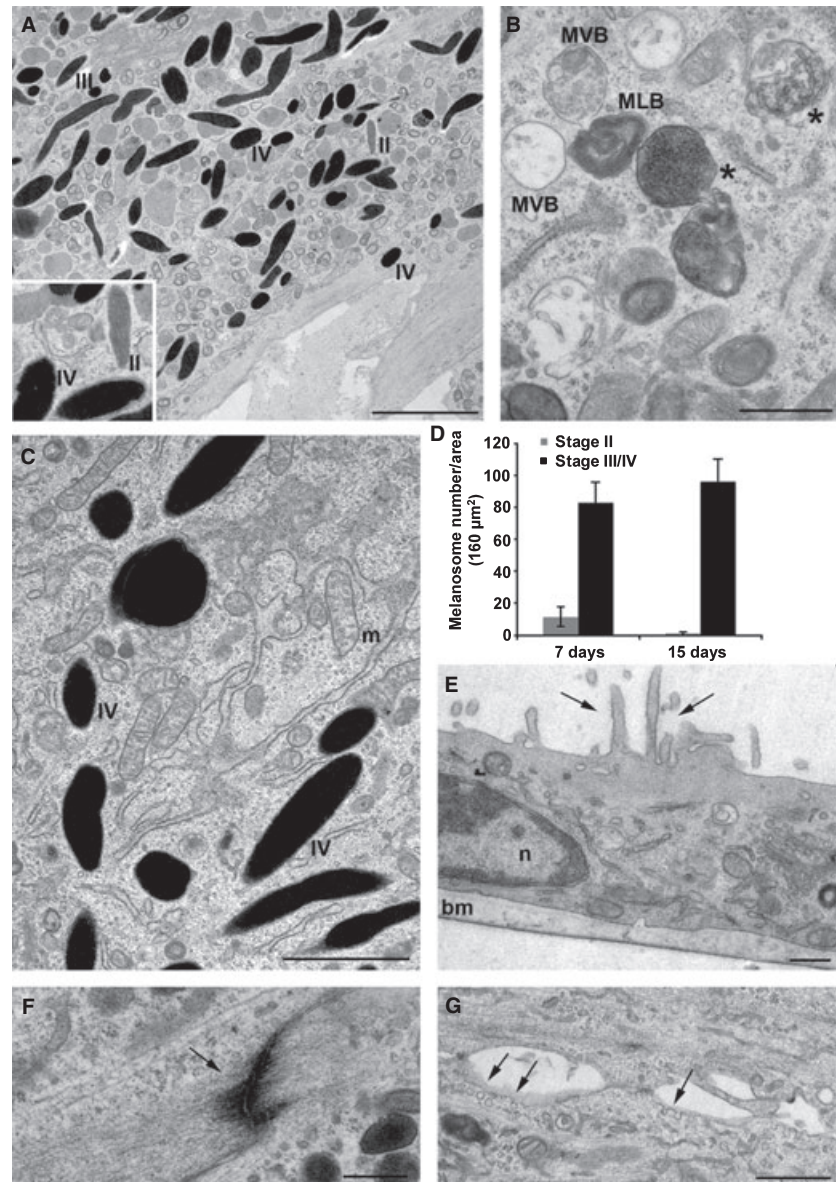
We performed electron microscopy (EM) analysis to define the ultrastructural characteristics of in vitro differentiated RPE-like cells and to analyze melanosomes. After 7 days of differentiation melanogenesis was still progressing in some cells (Figure 3A) and we could identify multivesicular bodies with morphological hallmarks of stage I premelanosomes (Figure 3B), stage II immature melanosomes, and stage III and stage IV mature melanosomes (Figure 3A, insert). After 15 days of differentiation only stage IV mature melanosomes could be observed (Figure 3C). Counts of melanosomes at different stages confirmed that in vitro maturation of RPE-like cells correlated with an increase in stage III and IV melanosomes and a decrease in stage II melanosomes (Figure 3D), similarly to prenatal and postnatal murine RPE (Cortese et al., 2005). Electron microscopy also revealed the presence of microvilli and basement membrane (Figure 3E), adherence junctions (Figure 3F) and caveolae at the plasma membrane (Figure 3G) typical of RPE (Mora et al., 2006).

To confirm that in vitro differentiated RPE was functional, we tested its ability to phagocytose photoreceptor outer segments (POS). Cells, after either 7 or 15 days of differentiation, were exposed to fluorescein-labeled POS. Because $\beta 5$ integrin and Mer tyrosine kinase (Mertk) mediate binding and internalization of POS, respectively (Feng et al., 2002; Finnemann and

Silverstein, 2001), we investigated co-localization of these molecules with POS. Incubation with POS for 1 h was sufficient for the binding with $\beta 5$ integrin receptors localized at the apical RPE microvilli (Figure 4A,B). After 3 h we observed a uniform uptake of POS across the pigmented RPE-like cell monolayer, as revealed by the co-localization of POS and the internalization receptor Mertk (Figure 4C,D). We confirmed that labeled POS were inside the cells by comparing the localization of the lateral tight-junction marker ZO-1 and an apical surface marker Na^+/K^+ ATPase with POS, using z-axis sections (Figure 4E–H). Fluorescein-labeled POS were observed within the apical region of the cell after 1 h of incubation (Figure 4B) and inside the cell, proximal to the 4',6-diamidino-2-phenylindole (DAPI) -stained nuclei, after 3 h of incubation (Figure 4F,H). We observed an increase in internalized POS per cell in RPE-like cells differentiated for 15 days when compared to RPE-like cells differentiated for 7 days (Figure 4I), indicating a direct correlation between POS uptake and in vitro RPE cell maturation.

In this study we provided a differentiation protocol to derive a functional RPE in vitro. By molecular, morphological and functional analyses we showed that pigmented RNS could attach to an ECM substrate, start to proliferate and lose pigmentation. When cells reached confluence, after 7 days of differentiation, we could

Figure 3. Electron microscopy (EM) analysis. Retinal pigment epithelium (RPE)-like cells were analyzed by conventional EM after 7 (A–E) and 15 days of in vitro differentiation (C–G). (A) Retinal pigment epithelium-like cell differentiated for 7 days harbours melanosomes at different stages of maturation. Insert: high magnification showing presence of immature stage II premelanosomes (II), stage III melanosomes (III) and pigmented mature stage IV melanosomes (IV). Bar: 2 μm . (B) Example of immature precursors of melanogenesis in an RPE-like cell. Note the presence of multivesicular bodies (MVB) with hallmarks of stage I premelanosomes, multilamellar bodies (MLB) and pigmented multilamellar bodies (asterisks). (C) Retinal pigment epithelium-like cells differentiated for 15 days contain stage IV mature melanosomes. Bar: 500 nm (B,C). (D) Histogram representing the number of immature (stage II) and mature (stage III, IV) melanosomes per area (160 μm^2) in RPE cells differentiated for 7 (7 days) or 15 days (15 days). (E) Retinal pigment epithelium-like cells show apical villi (arrows) and basement membrane (bm). (F) Example of zonula adherens (arrow). Melanosomes can be found in cells showing microvilli. (G) Example of caveolae at the plasma membrane (arrows). Bars: 500 nm (E–G). n, nucleus; m, mitochondria.



detect melanogenesis; and functionality was observed after 15 days in vitro. Our data suggest that RPE derived from RNS can differentiate in vitro, activating melanogenesis, and therefore can provide an accurate in vitro model for pigmentation studies of RPE that have not been possible so far.

Methods

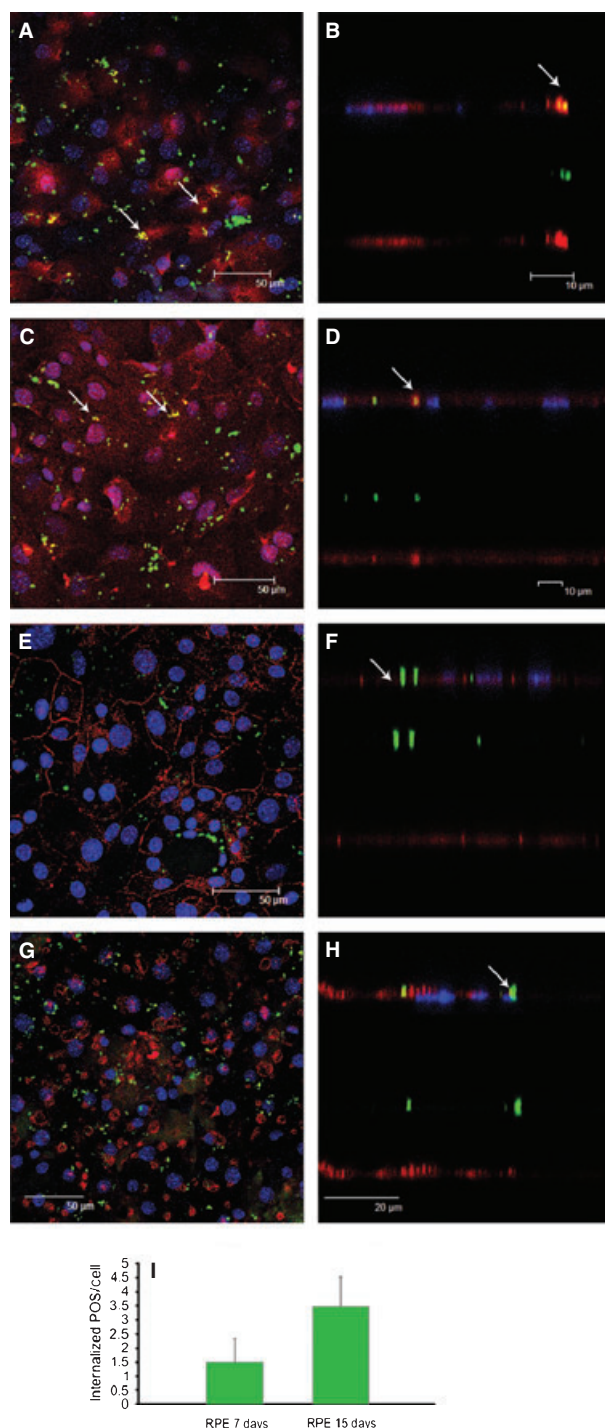
RPE generation from adult retinal stem cell

All procedures on mice (including their euthanasia) were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with institutional guidelines for animal research. We used C57BL/6 and CD-1 mice, purchased from Charles River Italy (Calco, Italy) and housed under standard conditions with a 12-h light/dark cycle. We dissected eyes and

isolated RSC as previously described (Giordano et al., 2007). Retinal neurospheres were picked after 7 days of culture and placed on glass coverslips coated with ECM (Sigma, Milan, Italy) in a serum-free medium (DMEM-F12 with 0.6% glucose, N2 hormone mix, 20 ng/ml bFGF; Invitrogen, Milan, Italy). Two days later, the medium was replaced with differentiating medium (DMEM low glucose with 1% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and hormone mix: 1.4×10^{-8} M selenious acid, 2.8×10^{-8} M hydrocortisone, 3×10^{-7} M linoleic acid, 8.3×10^{-7} M insulin, 6.3×10^{-8} M transferrin, 2.4×10^{-6} M putrescine, 10^{-11} M triiodothyronine). Cell culture was extended for 7–15 days. Cell death was assessed by staining for 1 min with 2 μM of the cell impermeable dye Ethidium Homodimer 2 (Invitrogen).

Immunofluorescence

Immunofluorescence experiments were performed as previously reported (Giordano et al., 2007). The following primary antibodies



were used: anti-Tyr PEP7, anti-Tyrp1 PEP1 and anti-Dct PEP8 (1:300 from Dr Hearing); anti-ZO-1 (1:100; Invitrogen); anti- $\beta 5$ Integrin (1:20; Abcam, Cambridge, UK); anti- Na^+/K^+ ATPase (1:100; Santa Cruz Biotechnology, Heidelberg, Germany); anti-Bestrophin (1:1000; Novus Biologicals, Cambridge, UK); anti-Mertk (1:50; R&D Systems, Minneapolis, MN, USA); anti-Ezrin (1:250; Sigma); anti-Rpe65 (1:200 from Dr Redmond); anti-Pax6 (1:10 from Dr van Heyningen); anti-Nestin (1:400; Chemicon, Vimodrone, Italy). Secondary antibodies were used as follows: 1:1000 Oregon Green® 488 goat anti-mouse (Invitrogen), 1:1000 Alexa Fluor® 568 goat

Figure 4. Phagocytosis assay. Retinal pigment epithelium (RPE)-like cells were exposed to fluorescein-labeled photoreceptor outer segments (POS). Confocal images were taken after immunofluorescence with RPE markers. (A) RPE-like cells bind POS (green) through $\beta 5$ integrin (red) after 1 h of incubation. (B) z-axis showing merged image of co-localization of POS with $\beta 5$ integrin (arrow) at the top; fluorescein-labeled POS in the middle; $\beta 5$ integrin in red at the bottom; (C) RPE-like cells bind POS (green) through Mertk (red) after 3 h of incubation. (D) z-axis showing merged image of co-localization of POS with Mertk (arrow) at the top; fluorescein-labeled POS in the middle; Mertk in red at the bottom. (E) POS internalization is confirmed by immunostaining of ZO-1 (red). (F) z-axis showing no co-localization of POS with ZO-1 localized at the tight junctions (merged image at the top); fluorescein-labeled POS in the middle; ZO-1 in red at the bottom. (G) POS internalization is confirmed by immunostaining of Na^+/K^+ ATPase (red). (H) z-axis showing no co-localization of POS with Na^+/K^+ ATPase localized at the apical membrane (merged image at the top); fluorescein-labeled POS in the middle; Na^+/K^+ ATPase in red at the bottom. (I) Quantification of internalized POS/cell: phagocytosis significantly increases ($P \leq 0.01$) with RPE differentiation from 7 to 15 days in vitro.

anti-rabbit (Invitrogen) and 1:200 Cy3-conjugated mouse anti-goat (Jackson ImmunoResearch, Suffolk, UK). Coverslips were mounted with mowiol 4–88 (Sigma) and analyzed in an Axioskop 40 fluorescence microscope (Zeiss, Arese, Italy) and with a Leica laser confocal microscope system.

RT-PCR and real-time PCR

Total RNA was extracted from adult RPE, RNS and in vitro differentiated RPE using RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. cDNA was synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Milan, Italy). Real-time PCR was carried out with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Monza, Italy) as previously described (Giordano et al., 2007) and normalized using the ribosomal gene *S26*. The primers used for quantitative PCR are listed in Table 1. Primers used for the identification of *Mitf* isoforms were as in Bharti et al. (2008).

Electron microscopy

In vitro differentiated RPE cells grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h and then processed according to the procedure described in Giordano et al. (2009). Melanosomes were counted as described in Giordano et al. (2009).

Binding and phagocytosis of POS

Porcine POS were isolated and fluorescein-labeled as described in Nandrot et al. (2008). RPE cells differentiated in vitro for either 7 or 15 days were incubated with 1×10^7 POS/ml for 1 and 3 h to allow binding and internalization, respectively. Cells were then quenched with trypan blue solution, washed three times in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. Cells were then processed for immunofluorescence and analyzed with a Leica laser confocal microscope system. Quantitative analysis of internalized POS was performed by counting fluorescein-labeled POS in 10 fields per experiment after quenching non-internalized POS with trypan blue. The number of cells in the field was evaluated by nuclear counting. Mean values were reported as POS numbers per cell and P-values were determined by Student's *t*-test.

Table 1. Sequences of primers (F, forward; R, reverse) used for real-time PCR and RT-PCR experiments. We report the official name of the genes and the commonly used names in parentheses

Gene	Primer sequence (5'–3')
Gja1 (Cx43)	F: CTTTGGCGTGCCGGCTTC R: ACCCCAAGCTGACTCAACC
Gpr143 (Oa1)	F: CTGGGAATCGTTATCAGGT R: GACTGGCATCTGAATCTTC
Mlana (Mart1)	F: GACGAAGTGGATACAGAACC R: CTCTTGAGAAGACATCGGC
Myo7a	F: CGCTACCGGGACACCTC R: GGGCATCTCCCCTATCTTC
Nanog	F: GGCAGAAAACAGTGGTTGA R: CTGCAATGGATGCTGGGATAC
Nestin	F: AGCTGGCTGTGGAAGCCC R: CTCTGTAGACCCTGCTTCTC
Oca2	F: CTCTGCTCTGGAATTCATCC R: ATGCATTTTGTCTCCAGGC
Pax6	F: GCGGAAGCTGCAAAGAAATA R: TGGCCTGTCTTCTCTGGTTC
Pkc α	F: TGCCTTCTTCTCAGGAGAATCGA R: TTTCTGCTCCTTGCCACACA
Pou5f1 (Oct3/4)	F: CTGTTCCCGTCACTGCTCTG R: TGGTGCCTCAGTTTGAATGC
Rhodopsin (Rho)	F: AATCTCGAGGGCTTTCTTTCG R: CCACGTAGCGCTCAATGGC
Rlbp1	F: TCTTGAAGAACAAGCTGCTAC R: CTGTGTTCTCAACTTCAGCC
Slc45a2 (Matp)	F: AAGTTTGTGATTCGGAACATT R: GATCGATTCTTAACAACCTTG
S26	F: GCTGTGCTGACTGTCCCC R: CGGGTGATTCAAACAACGAC
Serpinf1 (Pedf)	F: TCATTAATGATGGGCCTACAC R: CATCAGGCTCCTGTGGATAC
Si (Pmel17)	F: CCTCCACTACCATGCCCTCCT R: CCCAGTCTATGGCACCCAAA

Acknowledgements

We are grateful to Dr Hearing, Dr van Heyningen and Dr Redmond for antibodies. We acknowledge the CIGS of the University of Modena for providing confocal microscopy assistance. V.M. was supported by research grants GGP06096 from Fondazione Telethon, 1R01EY015136-01 and Progetti di ricerca a carattere internazionale from Fondazione Cassa di Risparmio di Modena. E.F.N. was supported by Fondation Voir et Entendre and Fondation Bettencourt Schueller (Young Investigator Grants), Centre National de la Recherche Scientifique (CNRS); the Institut de la Vision is funded by Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie-Paris 6, Centre National de la Recherche Scientifique, Département de Paris and Région Ile-de-France.

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