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Stem Cell Discovery

Low level of activin A secreted by fibroblast feeder cells accelerates early stage differentiation of retinal pigment epithelial cells from human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells (hPSC) differentiated to retinal pigment epithelial cells (RPE) provide a promising tool for cell replacement therapies of retinal degenerative diseases. The *in vitro* differentiation of hPSC-RPE is still poorly understood and current differentiation protocols rely on spontaneous differentiation on fibroblast feeder cells or as floating cell aggregates in suspension. The fibroblast feeder cells may have an inductive effect on the hPSC-RPE differentiation, providing variable signals mimicking the extraocular mesenchyme that directs the differentiation *in vivo*. The effect of the commonly used fibroblast feeder cells on the hPSC-RPE differentiation was studied by comparing suspension differentiation in standard RPEbasic (no bFGF) medium to RPEbasic medium conditioned with mouse embryonic (mEF-CM) and human foreskin (hFF-CM) fibroblast feeder cells. The fibroblast secreted factors were found to enhance early hPSC-RPE differentiation. The onset of pigmentation was faster in the conditioned media (CM) compared to RPEbasic for both human embryonic (hESC) and induced pluripotent (iPSC) stem cells, with the first pigments appearing around two weeks of differentiation. After four weeks of differentiation, CM conditions consistently contained higher number of pigmented cell aggregates. The ratio of *PAX*6 and *MITF* positive cells was quantified to be clearly higher in the CM conditions, with mEF-CM containing most positive cells. The mEF cells were found to secrete low levels of activin A growth factor that is known to regulate eye field differentiation. As RPEbasic was supplemented with corresponding, low level (10 ng/ml) of recombinant human activin A, a clear increase in the hPSC-RPE differentiation was achieved. Thus, inductive effect provided by feeder cells was at least partially driven by activin A and could be substituted with a low level of recombinant growth factor in contrasts to previously reported much higher concentrations.

Keywords: Retinal Pigment Epithelial Cell; Human Pluripotent Stem Cell; Conditioned Medium; Human Foreskin Fibroblast; Mouse Embryonic Fibroblast; Activin A; Cell Differentiation

1. INTRODUCTION

Retinal pigment epithelium (RPE) is a highly polarized and specialized monolayer of cells located between the neural retina and choroid at the back of the eye. RPE has several vitally important functions as a part of the blood-retina-barrier and in supporting photoreceptor function and survival [1,2]. RPE degeneration has a major role in pathogenesis of retinal diseases including age-related macular degeneration (AMD) and retinitis pigmentosa. The degeneration of RPE cells leads to the degradation of photoreceptors and as a consequence to either partial or total loss of vision. Currently, functionality of destroyed RPE cells can be restored only with cell transplantation, setting high demands to develop novel cell sources for replacement therapy. Transplantation of RPE cells has been studied extensively in animal models and also in humans [2-5]. Several cell sources have been

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studied for cell therapy but currently human pluripotent stem cells (hPSCs) are considered to be the most promising cell source of differentiating cells for tissue engineering applications due to their differentiation potential and high replicative capacity. Several research groups have reported successful differentiation of RPE cells from hPSCs [6-10] and first clinical studies using human embryonic stem cell (hESC) derived RPE cells are ongoing [11].

During mammalian development, RPE and neural retina are both derived from optic neuroepithelium and share the same progenitor [12]. The neuroepithelium near the anterior part of the neural tube evaginates laterally to form the optic vesicles. Invagination of the distal part of the optic vesicle leads to the formation of the optic cup in a complex environment affected by many external signals [13]. By the sixth or seventh week of development, the optic cup has differentiated into two epithelial sheets. Of these, the distal layer differentiates into the neural retina and the proximal layer develops into the RPE in interactions with the surrounding extraocular tissue, including the extraocular mesenchyme [12,14,15].

In the absence of external signal molecules, the hPSCs choose the neural differentiation pathway as a default [16]. Most of the published hPSC-RPE differentiation protocols rely on spontaneous differentiation in absence of basic fibroblast growth factor (bFGF). The induction of differentiation is based on confluent overgrowth on feeder cells especially mouse embryonic fibroblasts (mEF) or through embryoid body/neurospehere formation [7, 9,10]. Recently, RPE differentiation efficiency has been enhanced with prolonged culture and growth factor/inhibitor based differentiation strategies. Factors, such as activin A, transforming growth factor β1 (TGFβ1), and nodal antagonist SB431542 [17] as well as Wnt signaling inhibitor CKI-7 together with Dkk-1, Lefty-A, FGF antagonist Y-27632 and SB431542 [18,19] have been used. Regardless of these, many groups are using feeder cell (mEF, foreskin fibroblasts, PA6 cells) containing methods with spontaneous differentiation method [20,21] and first clinical studies are conducted with mEF supported and spontaneously differentiated hESC-RPE cells [11]. It is not clear why the removal of FGFs from feeder cell based hPSC cultures [20] or the use of PA6 stromal feeder cell to promote neural differ- entiation [22-24] is sufficient to produce RPE cells but both of the differentiation strategies suggest important function of external signals provided by mesenchymal fibroblasts/stromal cells.

We hypothesized that fibroblast feeder cells used for the culture of undifferentiated hPSC may have an inductive effect on RPE cell differentiation providing mesenchymal signals necessary for the key cellular decision guiding optic cup differentiation and further cell commitment towards RPE cell fate [25,26]. Moreover we hypothesized that different feeder cells types (mEF and human foreskin fibroblast, hFF) may provide variable mesenchymal signals guiding RPE differentiation. In this study, we studied the inductive effects of feeder cells routinely used for hPSC differentiation towards RPE cells. Human PSCs were differentiated using media conditioned by two types of fibroblasts feeder cells (hFF-CM and mEF-CM) and non-conditioned differentiation medium (RPEbasic).

2. MATERIALS AND METHODS

All cells were cultured in 37˚C, 5% CO_2 incubator (Thermo Electron Corp., Waltham, MA, USA) and monitored regularly with Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands).

2.1. Fibroblast Feeder Cell Culture

Human FF (CRL-2429™, American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria) and 0.5% Penicillin/Streptomycin (Lonza Group Ltd, Basel, Switzerland). P-MEF (EmbryoMax®, Millipore, Billerica, MA, USA) were cultured in Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 10% FBS and 1% GlutaMax-I, sterile-filtered prior use. Cell culture flasks for mEF were precoated with 0.1% porcine gelatin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature (RT). Both fibroblast cell lines were purchased as frozen stocks and cryopreserved at early passages with 5% - 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) supplementation.

2.2. Human Pluripotent Stem Cell Culture

The human embryonic stem cell (hESC) line Regea 06/040 was derived at IBT—The Institute of Biomedical Technology (former Regea—Institute for Regenerative Medicine), University of Tampere, Finland. The hESC line was derived on hFF feeder cells and cultured and characterized as described previously [27]. Human induced pluripotent stem cell (iPSC) line FiPS5-7 was established by Professor Otonkoski's research group at University of Helsinki, Finland. It was generated from human fibroblasts using four transcription factors—*OCT*3/4 (*POU5F*1), *SOX*2, *nanog*, and *LIN*28 [28], and transgene silencing was confirmed with qPCR [29]. Prior to the experiments, both pluripotent cell lines were cultured on hFF feeder cells in standard hPSC culture medium consisting of KO-DMEM supplemented with 20%

knock-out serum replacement (KO-SR), 2 mM GlutaMax-I, 0.1 mM 2-mercaptoethanol (all from Life Technologies), 1% Non-Essential Amino Acids (NEAA), 50 U/ml Penicillin/Streptomycin (both from Lonza Group Ltd.) and 8 ng/ml human bFGF (R&D Systems Inc., Minneapolis, MN, USA). The culture medium was changed five times a week and undifferentiated colonies were manually passaged onto new, γ-irradiated (40 Gy) feeder cell layers once a week.

2.3. Collection of Conditioned Media

Both hFF (passage 6 - 11) and mEF (passage 4 - 5) were harvested at confluency with TrypLE™ Select (Life Technologies) at 37˚C, 15 min, and mitotically inactivated with γ-radiation (40 Gy). Irradiated fibroblasts were seeded onto 0.1% gelatin-coated culture dishes (cell density $3.6 \times 10^4/cm^2$) and left to adhere overnight. The cells were adapted to serum-free culture conditions by sequential addition of RPE differentiation medium (RPEbasic) the day after irradiation. RPEbasic included the same reagents as described above for hPSC culture medium, but supplemented with 15% KO-SR and lacking bFGF. For a period of 10 days, 2 ml/cm^2 of RPEbasic was collected daily from the culture dishes and replaced with equal amount of fresh medium. Collected media were centrifuged at 1000 rpm, 4 min, transferred to new tubes and stored at −70˚C. After collection, CM for each fibroblast type was thawed, pooled, and stored at −70˚C in aliquots until used for differentiation experiments. Four different batches of CM were similarly prepared for both fibroblast types.

2.4. Differentiation Culture

Undifferentiated hPSC colonies (Regea06/040 and FiPS5-7) were manually dissected, and the pieces transferred to low cell-bind cell culture plates (Corning Inc., Corning, NY, USA) in RPEbasic, mEF-CM or hFF-CM. The media were changed five times a week. Human ESC line (Regea06/040) was used for differentiation experiments at passage levels 31 - 91 and hiPSC line (FiPS5-7) at passage levels 48 - 117. The differentiation experiments were repeated six times in total. Influence of activin A on RPE differentiation was tested with hESC line (Regea06/040) (passages 37 - 42) using RPEbasic supplemented with 10 ng/ml activin A (Peprotech, London, England). The activin A supplementation test was repeated three times. The workflow of the study and analyses performed are summarized in **Figure 1**.

After six to seven weeks in suspension culture, pigmented areas of cell aggregates were selectively replated to adherent cultures, in order to create purified populations of hPSC-RPE. Pigmented areas were selected, washed with Dulbecco's Phosphate Buffered Saline (DPBS, Lonza Group Ltd.) and dissociated with 1× Trypsin-EDTA (Lonza Group Ltd.) for 20 - 35 min at 37˚C with repeated trituration. Trypsin was inactivated with 10% human serum (PAA Laboratories), and cells collected to appropriate culture medium through 40 µm cell strainers. Dissociated cells were plated either on 24-well plate wells (Corning Cellbind, Corning Inc.) coated with 5 µg/cm^2 human placental collagen IV (Sigma-Aldrich) for 3 h at 37˚C, or to permeable 0.3 cm^2 BD BioCoat™ mouse collagen IV cell culture inserts (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Adherent cultures were maintained using appropriate media that were changed three times a week.

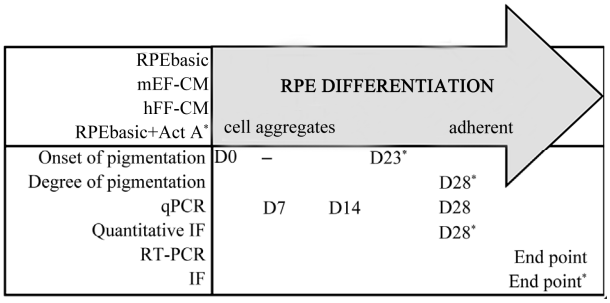

Figure 1. Workflow of the study. RPE differentiation was performed using four test media: RPEbasic, hFF and mEF conditioned media and RPEbasic supplemented with activin A. Analyses performed in different time points are presented.

2.5. Analysis of Pigmentation

The onset of pigmentation was followed daily and the day of appearance of the first pigmented cells in each medium was recorded. The appearance of first pigmentation was recorded from five individual differentiation experiments for hESCs, four experiments for hiPSCs, and three activin A supplementation experiments. To assess the amount of pigmentation after four weeks of differentiation, the ratio of cell aggregates containing pigment in relation to total number of aggregates was counted after 28 days of differentiation. This was done in three individual differentiation experiments for both studied cell lines and for all three activin A supplementation experiments. Results were plotted using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4.

2.6. Quantitative Real-Time Polymerase Chain Reaction

Differences in expression levels of genes related to RPE differentiation: retina and anterior neural fold homeobox (*RAX*), paired box gene 6 (*PAX*6) and microphthal-mia-associated transcription factor (*MITF*), were studied with qPCR. Gene expression was evaluated for hPSCs differentiated in the three test media: RPEbasic, mEF-CM and hFF-CM, after 7, 14, and 28 days in

differentiation culture. Additionally the expression of neural retina markers ceh-10 homeodomain containing homolog (*CHX*10) and cone-rod homeobox protein (*CRX*) was studied after 28 days of differentiation.

Ten to fifteen differentiated cell aggregates were collected from each test medium. In addition, pieces of undifferentiated colonies of both hPSC lines were collected for control material prior to the beginning of the experiment. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, GmbH & Co., Düren, Germany), according to the manufacturer's protocol. The RNA quality and concentration were determined using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 200 ng of each RNA sample, using MultiScribe Reverse Transcriptase in the presence of RNase inhibitor (High-capacity cDNA RT kit, Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. The synthesis of cDNA was carried out in PCR MasterCycler (Eppendorf AG, Hamburg, Germany): 10 min at 25˚C, 120 min at 37˚C, 5 min at 85˚C, and finally cooled down to 4˚C.

FAM-labeled TaqMan® Gene Expression Assays (Applied Biosystems Inc.) were used for qPCR reactions: *RAX* (Hs00429459_m1), *PAX*6 (Hs00240871_m1), *MITF* (Hs01115553_m1), *CRX* (Hs01549131_m1) and *CHX*10 (Hs01584048_m1). Glyceraldehyde 3-phosphate dehydrogenase, *GAPDH* (Hs99999905_m1) was used as endogenous control. Each reaction mixture consisted of 7.5 µl TaqMan® Universal PCR Master Mix (2×), 0.75 µl Gene Expression Assay (20×), 3 µl of cDNA (diluted 1:5 with sterile water) and sterile water to the total volume of 15 µl. All samples and controls were run as triplicate reactions using the 7300 Real-time PCR system (Applied Biosystems Inc.) as follows: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 s at 95˚C, and 1 min at 60˚C. Results were analyzed using 7300 System SDS Software (Applied Biosystems Inc.). Based on the C_T-values given by the software, the relative quantification of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [30] and Microsoft Excel 2003.

The values for each sample were normalized to expression levels of *GAPDH*. The expression level of undifferentiated hPSC sample was set as the calibrator (fold change equals 1). Results were plotted using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4. For visualization of down-regulation, the fold change values <1 are presented as the negative inverse of the value, calculated as –1/(fold change). Standard deviations were calculated for each set of technical replicates, and presented as error bars.

2.7. Immunofluorescence

Differences in protein expression of *PAX*6 and *MITF* after 28 days of differentiation were studied using immunofluorescence. The cell aggregates were dissociated to single cells as described above. Single-cell suspensions containing 1.6 – 3.5 × 10^5 cells/ml were prepared in DPBS, and 150 µl samples were centrifuged onto 15-mm glass cover slips at 600 rpm, 5 min, using Shandon Cytospin 2 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Cells were fixed immediately with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min at RT. Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in DPBS at RT for 10 min, and unspecific binding blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) at RT for 1 h. Incubation with primary antibodies was carried out either overnight at 4˚C or for 1 h at RT with the appropriate antibody: 1:200 dilution of mouse anti-*PAX*6 (Developmental Studies Hybridoma Bank, University of IOWA, Department of Biology, Iowa City, IA, USA) or 1:350 dilution of rabbit anti-*MITF* (Abcam, Cambridge, UK). Secondary antibodies were diluted 1:1500 in 0.5% BSA-DPBS and cells incubated 1 h at RT in either Alexa Fluor 568-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (both from Molecular probes, Life Technologies). Cell nuclei were stained with VectaShield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) containing 4', 6'-diamidino-2-phenylidole (DAPI). Cells were imaged with Olympus BX60 microscope (Olympus, Tokyo, Japan) using a 40× objective. The images were captured using same exposure time within each experiment and imaged areas were selected randomly. Minimum 700 cells were counted from each condition. *PAX*6 and *MITF* expression was quantified using Image J Image Processing and Analysis Software [31]. For each experiment, the threshold for positive expression was set by analysing several randomly selected images. Intensity threshold was adjusted for each image within an experiment and label to normalize the levels of background intensity. Cells below the set threshold level were considered negative. The total number of cells in each image was determined by counting nuclei counterstained with DAPI. The numbers of cells expressing *PAX*6 or *MITF* in relation to the total amount of cells were counted for hESC-RPE from two individual experiments and two activin A supplementation experiments. Results were plotted as bar charts using Microsoft Excel 2003 and figures edited with Adobe PhotoShop CS4.

Monolayers of hESC-RPE maturated on mouse collagen IV cell culture inserts were analyzed with immunofluorescence for the expression and correct localization of RPE-related proteins: *MITF*, cellular retinal-dehyde-binding protein (CRALBP), Bestrophin, and tight junction protein zona occludens-1 (ZO-1). Detailed protocol has been published previously [21]. Images were taken either with Olympus BX60 microscope or LSM 700 confocal microscope (Carl Zeiss, Jena, Germany)

using a 63× oil immersion objective. All images were edited using ZEN 2009 Light Edition (Zeiss) and Adobe Photoshop CS4.

2.8. Reverse Transcriptase PCR

Monolayers of hPSC-RPE maturated on human collagen IV were analyzed for expression of RPE specific genes by reverse transcription polymerase chain reaction (RT-PCR). Expression of the following genes was assessed: RPE precursor markers *MITF* and orthodenticle homeobox 2 (*OTX*2), and mature RPE-specific markers retinal pigment epihelium-specific protein 65 kDA (*RPE*65), bestrophin (*BEST*1), pre-melanosomal protein 17 (*PMEL* 17), pigment epithelium-derived factor (*PEDF*) and tyrosinase (*TYR*). *GAPDH* was used as endogenous control. Total RNA was extracted and 40 ng was reverse- transcribed to cDNA as described above. Genomic control reactions excluding the reverse transcriptase enzyme (-RT) for each RNA sample were performed. RT-PCR was carried out using 1 µl of cDNA as template. Detailed protocol and primer sequences used have been previously published [21].

2.9. Growth Factor Secretion Analysis

The three differentiation media: RPEbasic, mEF-CM and hFF-CM were analyzed for concentrations of TGF-β1, activin A and bFGF growth factors with enzyme-linked immunosorbent assay (ELISA). The following comercial ELISA kits were used: Human TGF-β1 Immunoassay, Human/Mouse/Rat Activin A Immunoassay, human FGF basic Immunoassay (all from Quantikine®, R&D Systems, Minneapolis, MN, USA). The Human TGF-β1 Immunoassay and human FGF basic Immunoassays have been previously shown to detect the growth factor concentrations also from mEF-CM [32]. All assays were performed according to manufacturer's instructions. All standards and samples were tested in duplicates. For the activin A immunoassay, each sample was diluted 1:5 and 1:25, with the diluent supplied in the kit. Optical densities were measured using Wallac Victor^{2TM} 1420 Multilabel counter (Perkin Elmer-Wallace, Norton, OH, USA). Using optical densities of the standard series, standard curves were created using Microsoft Excel 2003 and concentrations of the samples calculated accordingly. Standard deviations were calculated from the concentrations of duplicates of each tested sample, and were presented as error bars. The measurements were repeated twice from two different batches of CM.

2.10. Ethical Considerations

The study of human embryos at University of Tampere has been approved by National Authority for Medicolegal Affairs Finland (TEO) (Dnro 1426/32/300/05). We have a supportive statement of Ethical Committee of Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines from surplus human embryos (Skottman/R05116). No new cell lines were derived for this study.

3. RESULTS

3.1. Appearance of Pigmentation Was Accelerated in the Conditioned Media

Human ESCs and iPSCs were differentiated in suspension as floating cell aggregates in three different media: standard RPEbasic, hFF-CM and mEF-CM. Differentiation rate of hPSC-RPE was monitored by recording the appearance of first pigmented cells in each medium. The appearance of pigmented cells was faster in the CM compared to the RPEbasic for both hESCs and iPSCs. On average, the hESCs pigmented fastest in mEF-CM (day 13), next in hFF-CM (day 15) and slowest in RPEbasic (day 16) (**Figure 2(A)**). Human iPSCs generated pigmented cells on average at day 16 in both CM and at day 18 in RPEbasic (data not shown).

3.2. hPSCs Expressed Marker Genes for Eye Field and RPE Precursor Cells during Differentiation

Gene expression of early eye field markers *PAX*6 and *RAX*, and RPE precursor marker *MITF* was analyzed with relative qPCR after 7, 14 and 28 days of differentiation. The gene expression levels were compared to undifferentiated hPSCs. For hESCs, expression of *PAX*6 increased substantially during differentiation, suggesting that differentiation progressed to eye field direction in all studied media (**Figure 2(B)**). Expression levels of *RAX* increased during the first two weeks of differentiation, and decreased by day 28 in cells differentiated in both CM (**Figure 2(C)**). This decrease in *RAX* expression was accompanied by a 10-fold increase in the expression of RPE-specific *MITF* (**Figure 2(D)**) which indicates progress toward RPE fate. In RPEbasic, expression of *RAX* further increased by day 28, but the pattern of *MITF* expression was similar to that of CM. In addition, the expression of neural retina markers *CHX*10 and *CRX* were analyzed at day 28 and found to be substantially decreased for both CM conditions compared to RPEbasic. Especially the early neural retina marker *CHX*10 expression was 15 times higher in the RPEbasic condition compared to mEF-CM and 9 times higher compared to hFF-CM (data not shown). This expression pattern indicated increased differentiation toward neural retina direction in RPEbasic and toward RPE fate in CM. The studied genes showed a similar expression pattern also in

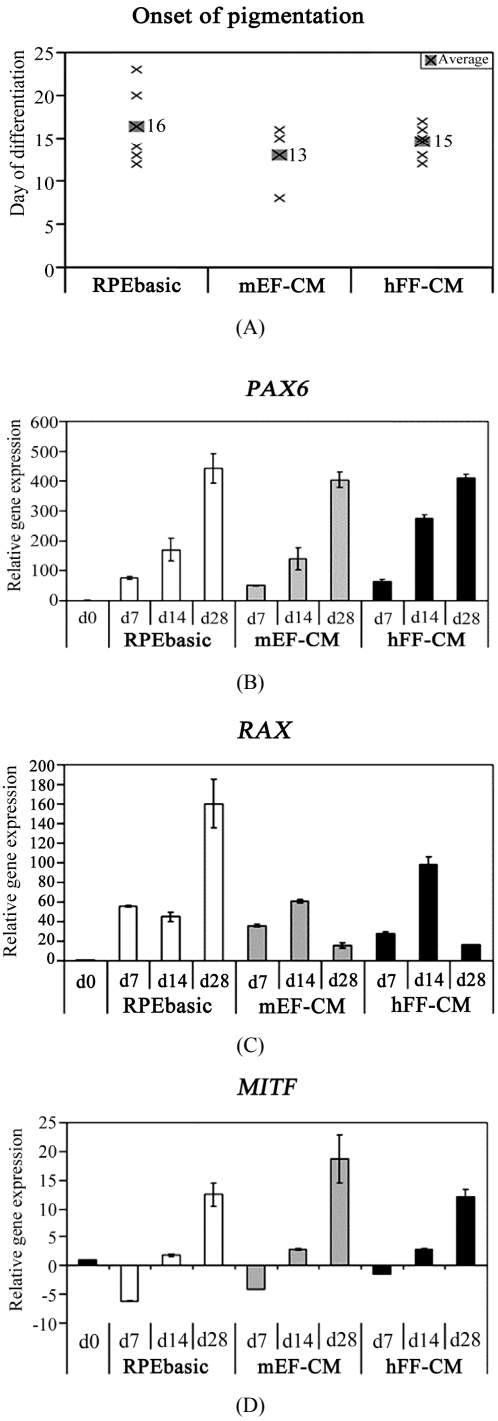

Figure 2. Analysis of early-stage hESC-RPE differentiation. The day of first pigmentation observed in replicate experiments, as well as the average, shown for each medium (A). Gene expression of the early eye-field markers *PAX*6 (B) and *RAX* (C) as well as early RPE marker *MITF* (D), relative to undifferentiated stem cells (d0) was analysed with qPCR.

hiPSC differentiation but with lower relative expression levels (data not shown).

3.3. Conditioned Media Contained More RPE Cells after Four Weeks of Differentiation

After four weeks of differentiation, the number of pigmented cell aggregates to total number of aggregates was calculated for each medium. CM consistently contained higher percentage of pigmented cell aggregates compared to RPEbasic for both hESCs (**Figure 3(A)**) and hiPSCs (data not shown) in each of the three replicate experiments. Typically, the pigmented areas were also larger in CM compared to RPEbasic (**Figure 3(B)**), indicating higher number of pigmented cells within the areas. In addition, the number of *PAX*6 and *MITF* expressing cells in the differentiated cell aggregates in each medium were quantified at 28 day time point. After dissociation to single cells and immunostaining, the number of positive cells was calculated. In two replicate experiments, the ratios of *PAX*6 and *MITF* positive cells were clearly higher in CM compared to RPEbasic (**Figure 3(C)**) with mEF-CM containing highest percentage of positive cells. On average, over 90% of cells expressed *PAX*6 and *MITF* in both CM, whereas in RPEbasic only 61% (±8%) of cells were positive to *PAX*6 and 74% (±8%) to *MITF*. Representative images of cells immunolabeled for *PAX*6 and the same cells counterstained with DAPI are shown in **Figure 3(D)**.

3.4. Mature hPSC-RPE Cells Possessed RPE Morphology and Expressed RPE-Specific Genes and Proteins

After selective plating of pigmented areas to adherent cultures on collagen IV, pigmentation and RPE-like cell morphology were initially lost. Cells acquired fibroblast-like morphology and proliferated to confluence, after which cobblestone morphology and pigmentation began to reappear within two weeks of culture. Mature cells were pigmented and possessed regular hexagonal arrangement typical to RPE (**Figure 4(A)**).

Expression of RPE-specific markers was studied at the protein level with immunofluorescence. Cells co-expressed *MITF* in the nuclei and CRALBP in the cytoplasm and cell membranes (**Figure 4(B)**). Moreover, expression of Bestrophin (**Figure 4(C)**) and tight junction protein ZO-1 (**Figure 4(D)**) confirm the maturity of hESC-derived RPE cells. The maturated hPSC-RPE cells were analysed for RPE-specific gene expression with RT-PCR. Cells in all three test media were shown to express RPE precursor genes *MITF* and *OTX*2, as well as genes specific to mature RPE, namely *RPE*65, *BEST*1, *PMEL*17, *PEDF* and *TYR*, confirming differentiation to RPE fate. The maturated hESC-RPE (**Figure 4(E)**) and hiPSC-RPE (data not shown) cells showed identical gene expression profile.

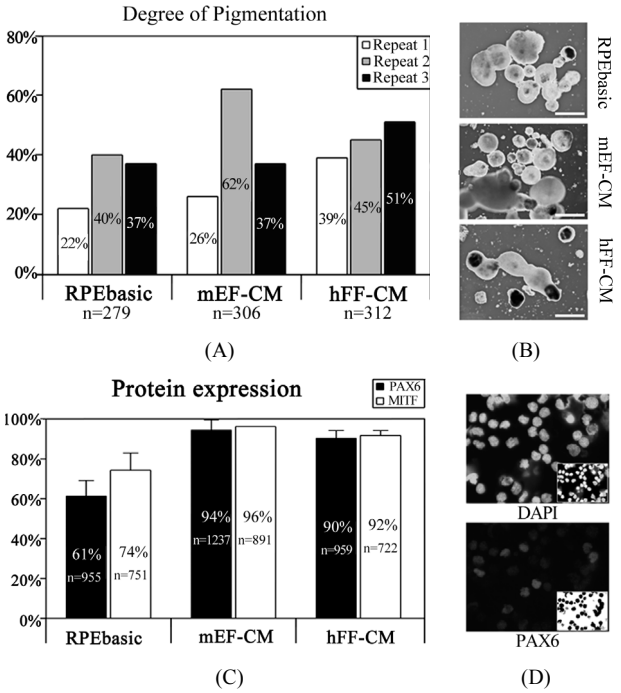

Figure 3. Degree of RPE differentiation at the 28 day time-point. The ratio of pigmented cell aggregates to total number of aggregates in each medium shown for three replicate experiments, n = total number of counted cell aggregates (A). Representative images of pigmented cell aggregates in each medium, scale bars 500 µm (B). Average percentage of cells expressing *PAX*6 and *MITF* in two replicate experiments quantified by cell counting. Standard deviations as error bars, n = total number of counted cells (C). Illustrative images of cells labelled with anti-*PAX*6 for cell counting before and after thresholding (D).

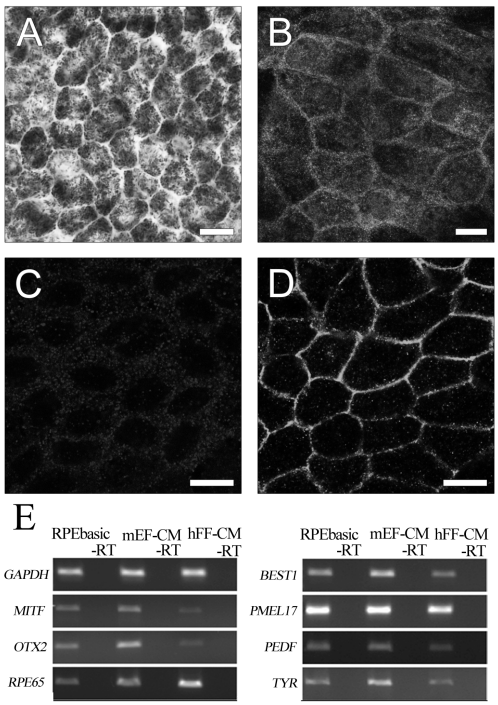

Figure 4. Analysis of mature hESC-RPE cells. Maturated cells possessed appropriate RPE morphology and pigmentation (A). Protein expression of CRALBP (green) and *MITF* (red) (B), Bestrophin (C), and ZO-1 (D) was confirmed with immunofluorescence, Scale bars 10 µm. Similar results were obtained in each test medium. Representative images in (A)-(D) are of cells cultured in mEF-CM. Gene expression profile of several RPE-related genes shown for hESC-RPE differentiated in the three test media, -RT = genomic control (E).

3.5. Feeder Cells Secreted Activin A and TGF-*β*1

Concentrations of bFGF, activin A and TGF-*β*1 were measured in both CM and RPEbasic with ELISA. Concentration of bFGF was undetected in all tested media. Concentration of TGF*β* in RPEbasic (15% KO-SR) was 67 pg/ml. Both fibroblast types secreted low levels of TGF-*β*: mEF-CM contained 207 pg/ml and hFF-CM 549 pg/ml. In addition, mEFs secreted substantially more activin A compared to hFFs—mEF-CM contained 7.1 ng/ml of activin A, whereas hFF-CM contained 1.0 ng/ml. Activin A was undetected in RPEbasic, meaning that practically all the activin A present in CM was secreted by the fibroblasts.

3.6. Activin A Supplementation Accelerated hESC-RPE Differentiation

Based on the results of the growth factor analyses, in ductive effect of activin A was tested by supplementing RPEbasic with 10 ng/ml of recombinant human activin A. In all three separate repeats, addition of activin A had a pronounced effect on the early-stage RPE differentiation. Activin A accelerated the onset of pigmentation from an average of day 16 to day 11 (**Figure 5(A)**), and by day 28 of differentiation enhanced the degree of pigmentation from 30% to 70% of pigmented cell aggregates (**Figure 5(B)**). Furthermore, differentiation cultures treated with activin A showed higher expression of *PAX*6 and *MITF*, quantified from immunofluorescence samples. On average, 96% (±1%) of cells were positive for *PAX*6 and 71% (±14%) for *MITF* after activin A treatment, while corresponding values in RPEbasic were 74% (± 16%) and 57% (±13%) (**Figure 5(C)**). After adherent maturation culture, the cells in RPEbasic supplemented with 10 ng/ml activin A showed mature RPE phenotype with corresponding pigmentation, morphology and protein expression (**Figures 5 (D)-(G)**).

4. DISCUSSION

During early eye development, RPE is surrounded by the

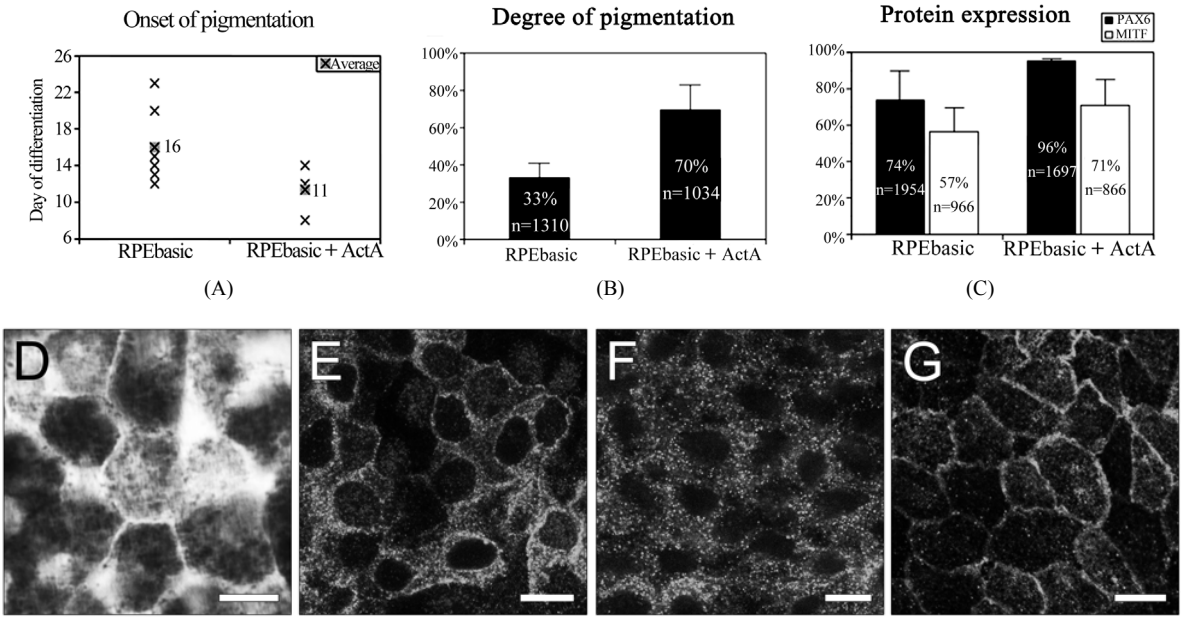

Figure 5. Differentiation efficacy of medium supplemented with activin A. Addition of activin A to the RPEbasic medium had a positive effect on the onset of pigmentation (A). Similarly, ratio of pigmented cell aggregates after 28 days of differentiation was enhanced, n = total number of cell aggregates counted (B). Percentage of *PAX*6 and *MITF* positive cells with and without activin A supplementation (C). Mature hESC-RPE cells cultured in RPEbasic supplemented with activin A possessed appropriate cell morphology and pigmentation (D) and expressed CRALBP (green) and *MITF* (red) (E), Bestrophin (F), and ZO-1 (G). Scale bars 10 µm.

extraocular mesenchyme, while the ectoderm faces the neural retina. RPE cell differentiation is known to be regulated by two key regulatory transcription factors *MITF* and *OTX*2. Expression of these transcription factors is controlled by interactions with the surrounding extraocular tissue, including the extraocular mesenchyime [12].

In the present study we hypothesized that fibroblast feeder cells used for the culture of undifferentiated hPSC may provide variable mesechymal signals having an inductive effect on spontaneous RPE cell differentiation *in vitro*. The results of this study clearly demonstrated the inductive effect of the two most commonly used fibroblast feeder cell types, mEFs and hFFs, on RPE cell differentiation both from hESC and iPSCs. In the presence of soluble factors secreted by feeder cells, both the onset of pigmentation and its rate were clearly enhanced. As expected, there was considerable biological variation in the appearance and amount of pigmentation between the replicate experiments typical for suspension culture methods. However, a clear correlating trend was observed. Along with the appearance of pigmented cells, eye field transcription factor genes *RAX* and *PAX*6 were expressed. After four weeks of differentiation, expression of RPE-specific transcription factor *MITF* was the highest in cells differentiated in mEF-CM, accompanied by decreased expression of *RAX* and a low expression of *CRX* and *CHX*10 demonstrating the early neural precursors' progress towards RPE cell fate instead of neural retina. Similar but moderated effect was seen with cells differentiated in hFF-CM. Most importantly, both of the CM conditions were verified to contain substantially more *PAX*6 and *MITF* expressing cells compared to non-conditioned RPEbasic at the protein level, using quantitative cell counting. After selective plating of pigmented clusters to adherent culture, the cells showed mature RPE morphology and expression of RPE-specific markers, both at gene and protein level. Taken together, the induction of RPE differentiation with feeder cell CM had a positive effect on hPSC-RPE differentiation.

Fibroblast feeder cells in general are known to secrete various factors promoting or inhibiting the growth and differentiation of hPSC cells [33-37]. To elucidate the inductive effect of CM in RPE differentiation, we further studied the secretion of bFGF, TGF-β1 and activin A, known factors regulating eye field differentiation, by the feeder cells. As a result we found that secretion of activin A was substantially higher by mEFs compared to hFFs. In contrast, secretion of TGF-β was higher for hFFs compared to mEFs. This is consistent with our previous studies showing that mEFs secrete more activin A and hFFs secrete more TGFβ [38]. We were not able to detect any measurable levels of bFGF from either CM thus possible effect of difference in bFGF concentration was excluded. Similar trend in fibroblast growth factor secre-

tion has been confirmed by another research group [32]. The extraocular mesenchyme secretes TGF-β1 superfamily growth factors such as activin A, activates the expression of *MITF* and down-regulates *CHX*10 expression directing RPE cell fate differentiation *in vivo*. Similar effects of activin A inducing *MITF* expression have been shown [26]. Activin A has also been shown to induce hESC-RPE differentiation *in vitro,* but only after pretreatment with nicotinamide [17,39]. The superior secretion of activin A by mEF feeder cells could thus be one of the key factors enhancing the early RPE differentiation and reduction of the *RAX*, *CRX*, *CHX*10 expression.

To study the effect of activin A secretion by mEF, we supplemented the RPEbasic medium with 10 ng/ml activin A and concluded that addition of activin A at this low level had a pronounced effect on the early-stage RPE differentiation. In previously published studies, relatively high activin A concentrations of 140 ng/ml between day 14 - 28 of differentiation [17] and 100 ng/ml between day 20 - 40 [40] have been used. On the contrary, we were able to induce early RPE differentiation with substantially lower activin A concentration. However, in addition to activin A both mEF-CM and hFF-CM may contain a pool of other possible factors inducing RPE cell differentiation. Both fibroblast types secrete various ECM components like collagens I and IV, nidogen I, and fibronectin as well as proteins involved in TGFβ, BMP, Wnt and IGF signaling [33]. In addition mEFs secrete the neurotrophic pigment epithelium derived factor (PEDF) [33,34] leaving the field open to identify other important players.

5. CONCLUSION

In this study, we confirmed the inductive effect of commonly used fibroblast feeder cells on hPSC differentiation towards RPE cells. Human PSCs were differentiated using media conditioned by two types of fibroblasts originated from mouse embryos and neonatal human foreskin tissue. Both feeder cell type CM increased RPE differentiation as compared to the non-conditioned medium (RPEbasic). The growth factor activin A, known inductive agent of RPE fate, was concluded to be an important factor present especially in mEF-CM. Consequently, supplementation of RPEbasic medium with a low concentration of activin A increased the differentiation rate of RPE cells to comparative level achieved with CM. Thus, inductive effect provided by feeder cells was at least partially driven by activin A.

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