Amniotic fluid promotes the appearance of neural retinal progenitors and neurons in human RPE cell cultures

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Purpose: Retinal pigment epithelial (RPE) cells are capable of differentiating into retinal neurons when induced by the appropriate growth factors. Amniotic fluid contains a variety of growth factors that are crucial for the development of a fetus. In this study, the effects of human amniotic fluid (HAF) on primary RPE cell cultures were evaluated.
Methods: RPE cells were isolated from the globes of postnatal human cadavers. The isolated cells were plated and grown in DMEM/F12 with 10% fetal bovine serum. To confirm the RPE identity of the cultured cells, they were immunocytochemically examined for the presence of the RPE cell-specific marker RPE65. RPE cultures obtained from passages 2–7 were treated with HAF and examined morphologically for 1 month. To determine whether retinal neurons or progenitors developed in the treated cultures, specific markers for bipolar (protein kinase C isomer α, PKCα), amacrine (cellular retinoic acid–binding protein I, CRABPI), and neural progenitor (NESTIN) cells were sought, and the amount of mRNA was quantified using real-time PCR.
Results: Treating RPE cells with HAF led to a significant decrease in the number of RPE65-positive cells, while PKCα- and CRABPI-positive cells were detected in the cultures. Compared with the fetal bovine serum–treated cultures, the levels of mRNAs quantitatively increased by 2-, 20- and 22-fold for NESTIN, PKCα, and CRABPI, respectively. The RPE cultures treated with HAF established spheres containing both pigmented and nonpigmented cells, which expressed neural progenitor markers such as NESTIN.
Conclusions: This study showed that HAF can induce RPE cells to transdifferentiate into retinal neurons and progenitor cells, and that it provides a potential source for cell-based therapies to treat retinal diseases.

The retinal pigment epithelium (RPE) is located in the outer retina between the photoreceptor cells of the neurosensory retina and choroid. The RPE consists of a monolayer of highly pigmented, cuboidal, and specialized non-neural cells [1]. Moreover, it performs numerous specialized functions to maintain the homeostasis of the neural retina. These functions include supplying nutrients and oxygen, taking waste from the photoreceptors, phagocytizing the outer segments of the photoreceptors, secreting a variety of growth factors, and participating in the visual cycle, in which all-trans-retinol is transported to the RPE cells, re-isomerized to 11-cis-retinal, and then returned back to the photoreceptors [2,3]. A failure in one or more of these functions can lead to a retinal degenerative disease; consequently, RPE dystrophy causes a dysfunction in the photoreceptors and other neurons in the retina and leads to irreversible blindness if left untreated [2,4]. Hence, studies are under way that investigate methods to produce retinal neurons from different sources of stem/progenitor cells, such as embryonic [5,6] or retinal stem cells [7,8]. Recently, RPE cells have been considered a promising source with a potential capability of generating retinal neurons.

In amphibians, and especially urodeles, RPE cells exhibit a remarkable ability to regenerate an injured retina [9,10]. After the removal of the neural retina, RPE cells begin to proliferate and completely renew the retinal layers [9-11].

In mammals, the regenerative ability of RPE cells is restricted to a certain period during embryogenesis. However, following retinal damage, RPE cells can proliferate in adult mammals, including humans, but not transdifferentiate into neural cell types found in the retina [12-14].

In vitro, the de- and transdifferentiation of RPE cells into other cell types in the retina has been well established

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[15]. Many studies have demonstrated that RPE cells differentiate into neural retinal cells or progenitors after induction through various stimuli, including growth factors such as the basic fibroblast growth factor [16] or bHLH genes, including neuroD [16,17] and ash1 [18]. Several published papers have reported an enhanced potency in RPE cells that makes them an interesting candidate for regeneration of tissue for direct clinical use [19-21].

Human amniotic fluid (HAF) is a complex biological fluid surrounding the fetus that provides mechanical protection and nutrients required for the development of the fetus. HAF contains water, proteins, peptides, carbohydrates, hormones, lipids, and uric acid [22,23]. Several proteomics analyses have identified a wide range of growth factors in HAF up to the third month of pregnancy. These growth factors include IGF-I, IGF-II, EGF, TGF-α, TGF-β, erythropoietin (EPO), G-CSF, M-CSF [22], vascular endothelial growth factor (VEGF) [24], FGF-2 [25], and NGF [26]. After the third month, concurrent with fetal growth, the growth factor components in HAF decrease. From the 14th to 16th weeks of gestation, HAF consists of multiple trophic factors essential for fetal growth and embryonic cell proliferation and differentiation [22,27]. For example, Hirai et al. (2002) found that multiple growth factors in HAF promote the growth of human fetal small intestinal cells in culture [28]. It also seems that some HAF components, such as fibronectin (the 3rd most abundant protein in HAF in the 16th week of gestation [23]), plays a pivotal role in cell attachment, polarity, and migration [29]. Recently, we showed that HAF was able to promote transdifferentiation of RPE cells into rod photoreceptors and retinal ganglion cells [21]. The present study examined the effect of HAF as a source of growth factors to evaluate the ability of RPE cells to de- and transdifferentiate into neural progenitors and retinal neurons.

METHODS

Isolation and cultivation of RPE cells: Enucleated eye globes were obtained from newborn human cadavers, up to one year of age, less than 24 h after death at the Central Eye Bank of Iran. To isolate the RPE cells, the initial surrounding tissues, including the fat tissue and optic nerves, were removed. Then, a circular incision was made at the anterior region of the globe next to the iris. The anterior segment and its contents, including the iris, cornea, lens, and vitreous humor, were discarded. The remaining part of the globe was opened, and the neural retina was peeled off. The rest of the tissue, including the RPE layer and choroid, were washed several times with PBS. (1X; was prepared by dissolving 8 g NaCl, 0.2 g KCl, 2.68 g Na_2HPO_4-$7H_2O$ and 0.24 g KH_2PO_4 in 800

ml ddH_2O and then pH was adjusted to 7.4 and final volume was adjusted to 1 l).The RPE tissue was then gently separated from the sclera, chopped into small pieces, and incubated in 1 U/ml of dispase I solution (Invitrogen, Brussels, Belgium) at 37 °C for 50 min. Subsequently, the cells were centrifuged at 300 g and 4 °C for 5 min. The pellet was resuspended in DMEM/F-12 (Sigma, Munich, Germany). The suspension was incubated in DMEM/F12 supplemented with 20% FBS; Gibco, Canyon, Australia), 120 µg/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml amphotericin B (the latter was used only for freshly isolated cells) in a 25 cm² flask, which was precoated for at least 2 h at 37 °C with FBS in a humidified incubator containing O_2 95% and CO_2 5% at 37 °C. The medium was changed every 3–4 days. When the RPE cultures reached confluence, the cells were subcultured with a solution of Trypsin 0.25% (w/v; Sigma)–1 mM EDTA (Merck, Darmstadt, Germany) in PBS, and the detached cells were seeded at a density of 5 ×10³ cells/cm². Confluent RPE cultures between passages 3 and 7 were used for all examinations.

Human amniotic fluid preparation: Human amniotic fluid (HAF) samples were obtained from pregnant women between the 14th and 16th weeks of pregnancy. The women had been advised to undergo an amniocentesis procedure for a genetic evaluation, irrespective of the current study. Researchers received the extra quantity of HAF to treat RPE cultures. After centrifuging the HAF at 300 ×*g* at 4 °C for 5 min, the HAF cells were removed for karyotype analysis; the remaining supernatants that were devoid of chromosome abnormalities were pooled, passed through a 0.2 µm filter for sterilization, and preserved at −70 °C. The ethics committee of the National Institute of Genetic Engineering and Biotechnology and Ophthalmic Research Center approved the collection process for these samples. Amniotic fluid samples were obtained from thirty pregnant women who underwent amniocentesis for the assessment of genetic deficiencies in the first trimester of gestation. Amniotic fluid cells were removed for karyotype analysis. The remaining supernatants, in cases with no evidence of chromosomal abnormalities, were pooled and used in our downstream experimental procedures. The collection of these samples was approved by the ethics committees of the NIGEB and the Ophthalmic Research Center. The AF samples were centrifuged at 300 ×g for 5 min at 4 °C, and the resulting supernatants were then sterilized using a 0.2 µm membrane filter (OrangeScientific, Brussels, Belgium) and stored at −70°C until the time of analysis.

Treating RPE cultures with HAF: To examine the effects of HAF on the morphology of the RPE cultures, 1×10⁵ RPE cells/well were cultured in 1 ml of DMEM/F12 supplemented

with HAF 10% (v/v) or FBS 10% (v/v) in a 24-well microplate (Nunc, Roskilde, Denmark). As a control, the same number of cells were cultured in DMEM/F12. Cultures were examined daily by phase contrast microscopy (Axiovert; ZEISS, Germany) for more than 30 days, and morphological changes were regularly surveyed and documented with photographs.

Cell proliferation and death enzyme-linked immunosorbent assays: Cell proliferation and death enzyme-linked immunosorbent assay (ELISA) were performed to investigate whether HAF could induce proliferation or even unfavorable apoptosis in RPE cultures. To evaluate the effect of HAF on either cell proliferation or apoptosis of the cultured RPE cells, the cells were plated at a density of 1×10^4 cells/well in 200 μl of DMEM/F12 supplemented with HAF 10, 20, or 30% (v/v) or FBS 10% (v/v) in a 96-well microplate. The control cultures received DMEM/F12 instead of the HAF- or FBS-supplemented media. Twenty-four hours after incubation, the medium was replaced with fresh medium containing BrdU (for cell proliferation assay; concentration of BrdU stock was 1000X. BrdU stock was diluted 1 to 1000 in each reaction mixture.) and incubated for another 24 h. Then, the treated and control cultures were assayed for cell proliferation (based on the measurement of BrdU incorporation during DNA synthesis) and cell death (qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA fragments) according to the protocols recommended by the manufacturer (cell proliferation ELISA, BrdU colorimetric, and cell death detection ELISA kits, Roche, Grenzach-Wyhlen, Germany). The absorbance values for the proliferation (at 450 nm and 690 nm) and cell death (at 405 nm and 492 nm) assays were recorded using a scanning multiwell spectrophotometer (Titertek multiscan ELISA reader, Labsystems Multiscan, Roden, Netherlands).

Immunocytochemistry: The harvested RPE cells were seeded onto FBS- or HAF-coated glass coverslips at a density of 1×10^5 cells/well in 1 ml of DMEM/F12 supplemented with HAF 30% (v/v), FBS 10% (v/v), or DMEM/F12 (as a control) in 24-well microplates. Seven days later, a standard immunocytochemistry (ICC) procedure was performed, as described by Ghaderi et al. [21]. Briefly, the medium was removed, and the cells were washed with PBS and fixed with cold methanol (−10 °C) for 5 min at room temperature (RT). The air-dried cells were washed with PBS three times and then blocked with bovine serum albumin (BSA) 1% (w/v; Sigma) in PBS for 20 min at RT. The cells were subsequently rinsed with PBS and incubated for 1 h at RT with primary antibodies that had been diluted 1:50 in bovine serum albumin (BSA 1% [w/v])/PBST 1% (v/v). To prepare 1% BSA in PBST, PBST was prepared by dissolving 1 ml Triton X-100 in 99 ml PBS

buffer (1X). Then 1 g of BSA was dissolved in each 100 ml of PBST. The primary antibodies used were the rabbit polyclonal anti-human NESTIN antibody (Santa Cruz, Carlsbad, CA), goat polyclonal anti-CRABPI (Santa Cruz, Carlsbad, CA), rabbit polyclonal anti–PKCα (Santa Cruz), rabbit anti-human RPE65 polyclonal antibody (Santa Cruz), and mouse anti-human cytokeratin 8/18 (Santa Cruz). After three washes with PBS for 5 min each, the cells were incubated with secondary antibodies that had been diluted 1:100 in BSA 1.5% (w/v) / PBST 1% (v/v) in the dark for 45 min at RT. The secondary antibodies were FITC-conjugated goat anti-rabbit IgG (1:100, Santa Cruz), FITC-conjugated goat anti-mouse IgG (1:100, Santa Cruz), and FITC-conjugated donkey anti-goat IgG (1:100, Santa Cruz). Finally, the cell nuclei were stained with 1 mg/ml of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Santa Cruz) for 10 min in the dark at RT. The coverslips were mounted onto slides using fluorescence mounting media (glycerol 90%, PBS 10%, and phenyl diamine 10% [w/v]). The negative controls were processed as described above but without any primary antibody. An Axiophot Zeiss fluorescence microscope equipped with a 460 nm filter for DAPI and a 520 nm filter for the FITC was used to observe and image the cells.

RNA extraction and cDNA synthesis: Total RNA was extracted (using the RNeasy kit; Qiagen, Hilden, Germany) from RPE cultures that were treated for 24 h with 10, 20, or 30% (v/v) HAF and/or 10% (v/v) FBS treated cultures as a control, to normalize the obtained data. Subsequently, the first strand of cDNA was synthesized using the cDNA synthesis kit (Qiagen) according to the manufacturer's instructions.

Real-time quantitative PCR analysis: For real-time PCR assays using SYBR Green I, the primers for all the genes of interest were designed using the Beacon Designer software 7.00. A BLAST search was performed to check the specificity of the primer sequences (for primer details see Table 1). To quantitatively measure the expression of the NESTIN, PKCα, CRABPI, CRABPII, pigment epithelium-derived factor (PEDF), and VEGF genes, real-time PCR was performed using Light Cycler apparatus version 3.5 (Roche). The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. The real-time PCR program included the following three main steps: an initial denaturation step at 95 °C for 10 min; 40 cycles of amplification that consisted of a denaturation step (10 s at 95 °C), an annealing step (40 s at 57 °C for NESTIN, PKCα, CRABPII, and GAPDH, 30 s at 60 °C for PEDF, 30 s at 52 °C for VEGF, and 50 s at 57 °C for the CRABPI gene); and finally, an extension step (15 s at 72 °C). To draw the melting curve and confirm the precision of the reactions, the

Gene	Sequence 5′ -3′	Annealing temperature	Product length
NESTIN	F: GCAGCACTCTTAACTTACG	57 °C	175
	R: ACTTAGCCTATGAGATGG		
PKCα	F: TATGGCGTCCTGTTGTATG	57 °C	171
	R: GCTTGGCTGGGTGTTTGG		
CRABPI	F: TATGTCCGAGAGTGAAAGG	60 °C	197
	R: ATACAAGAGGCACCAAGG		
CRABPII	F: ACAGGAGGGAGACACTTTC	57 °C	126
	R: TTCACCAGGCTCTTACAGG		
PEDF	F: TGCAGGCCCAGATGAAAGGG	60 °C	342
	R: TGAACTCAGAGGTGAGGCTC		
VEGF	F: CGGCGAAGAGAAGAGACACA	52 °C	196
	R: GGAGGAAGGTCAACCACTCA		
GAPDH	F: ACAGTCAGCCGCATCTTC	57 °C	77
	R: CTCCGACCTTCACCTTCC		

TABLE 1. PRIMER SEQUENCES USED FOR REAL-TIME QUANTITATIVE PCR.

All primers were designed using the Beacon Designer Software 7.00

following steps were performed: step 1, 95.0 °C for 0 s; step 2, 65.0 °C for 15 s; step 3, 95.0 °C for 0 s; step 4, 40.0 °C for 30 s. The size of the PCR product for each gene was confirmed using gel electrophoresis. To determine the PCR efficiency of the amplifications, a standard curve was prepared by making serial dilutions of the cDNA samples (a twofold dilution series for NESTIN, CRABPI, CRABPII, PKCα, VEGF, and GAPDH and a fivefold dilution series for PEDF). Relative expressions of all the genes of interest under the treatment of three different doses of HAF measured according to the $2^{-\Delta\Delta Ct}$ method, which yields a normalized, relative gene expression value with respect to FBS-treated cultures, using the Bio-Rad software. Experiments were replicated two or three times.

Statistical analysis: Results of the ELISA assays for cell proliferation and cell death were based on at least two different experiments with two replicates in each studied group. The averages of the absorbance measurements for all the repetitions were calculated and statistically with the *t* test. To calculate the percentage of cells that were immunopositive for PKCα and CRABPI, the number of cells in three different fields of vision was counted under the fluorescent microscope, and the average of the counted immunoreactive cells was reported. To evaluate the expression of the genes of interest, real-time PCR was performed for two series of treated and control cDNA samples obtained from distinct experiments. Minimally, each sample was assessed in duplicate.

RESULTS

The establishment of RPE cells as a monolayer culture: Freshly isolated human RPE cells were seeded in DMEM/F12 supplemented with 20% FBS. During the first week of cultivation, the cells were small and had various shapes, including triangular, star-like, and branching (Figure 1A,B). Afterward, they became elongated and had a fibroblast-like morphology (Figure 1C). Regardless of the morphological features, the RPE cells were heavily pigmented in the developing cultures, although their pigment content became more diluted with consecutive passages. When the cultures formed a monolayer and reached full confluence, the cells became hexagonal and organized into "cobblestone" structures (Figure 1D). More interestingly, we observed the development of colonies consisting of several hundred cells in the established cultures (Figure 1E,F). When the cultures reached the fifth passage, the RPE cells gradually flattened and their ability to proliferate decreased (Figure 1G,H). These alterations implied that the cultures were undergoing progressive senescence [30]. An immunocytochemical examination of the newly isolated cells showed that almost all cells were labeled by the anti-RPE65 and anti-cytokeratin 8/18 antibodies, confirming the identity and the purity of the culture as epithelial cells, and particularly RPE cells (Figure 2A,B). The presence of RPE65 and cytokeratin 8/18 were tracked in the cultures over a 6-week period. We found that the number of RPE65-immunoreactive cells decreased significantly, while a large number of cells strongly expressed cytokeratin 8/18. As negative controls,

secondary antibody–only control was also included; goat anti-rabbit and goat anti-mouse secondary antibodies were used for RPE65 and cytokeratin 8/18, respectively (Figure 2 E,F).

The RPE–derived cells formed neurosphere-like colonies when treated with HAF: To determine the effect of HAF on the morphology of the RPE cells, the cultures from the middle passages (passage 3 was chosen as the middle point of the exponential phase for RPE cultures, data not shown) were
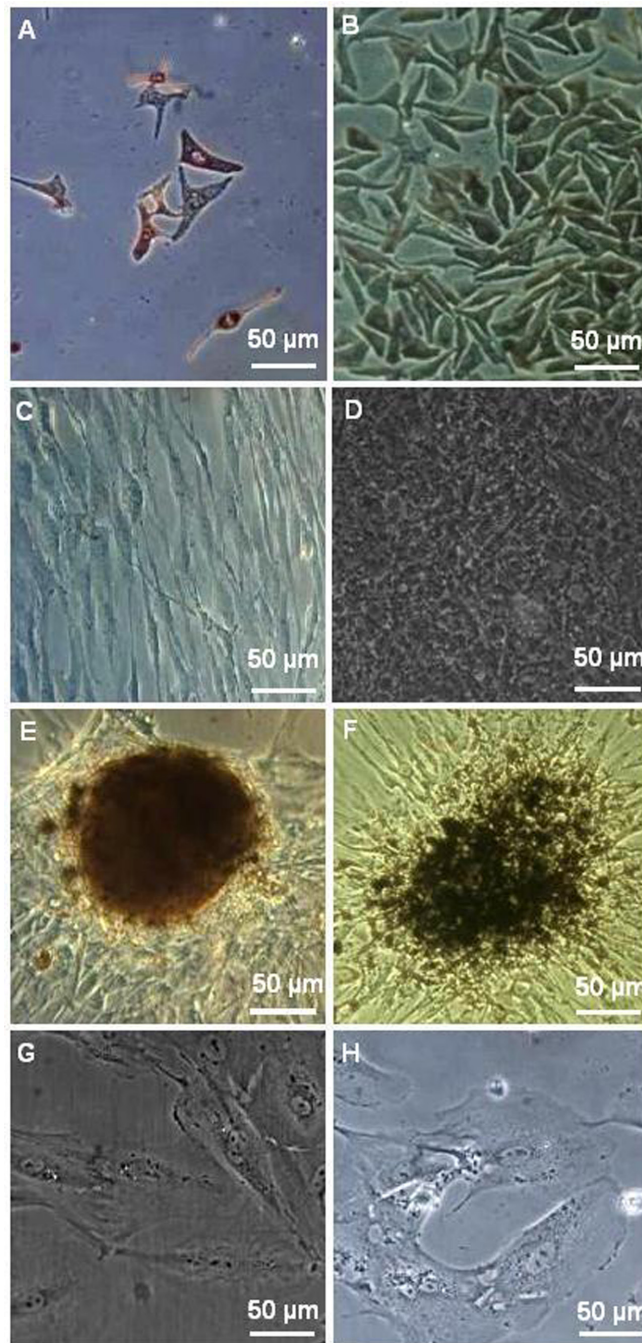

Figure 1. The different stages of retinal pigment epithelium (RPE) cell growth in culture. **A**, **B**: RPE cells freshly isolated from a human eye. **C**: Up to passage number 5, RPE cells have an elongated morphology containing several long processes. **D**: Confluent RPE cells in culture organized into cobblestone structures. **E**, **F**: RPE-derived colonies under normal culture conditions. **G**, **H**: The appearance of flattened cells in an aged culture (over passage 5) suggests that senescence is occurring.
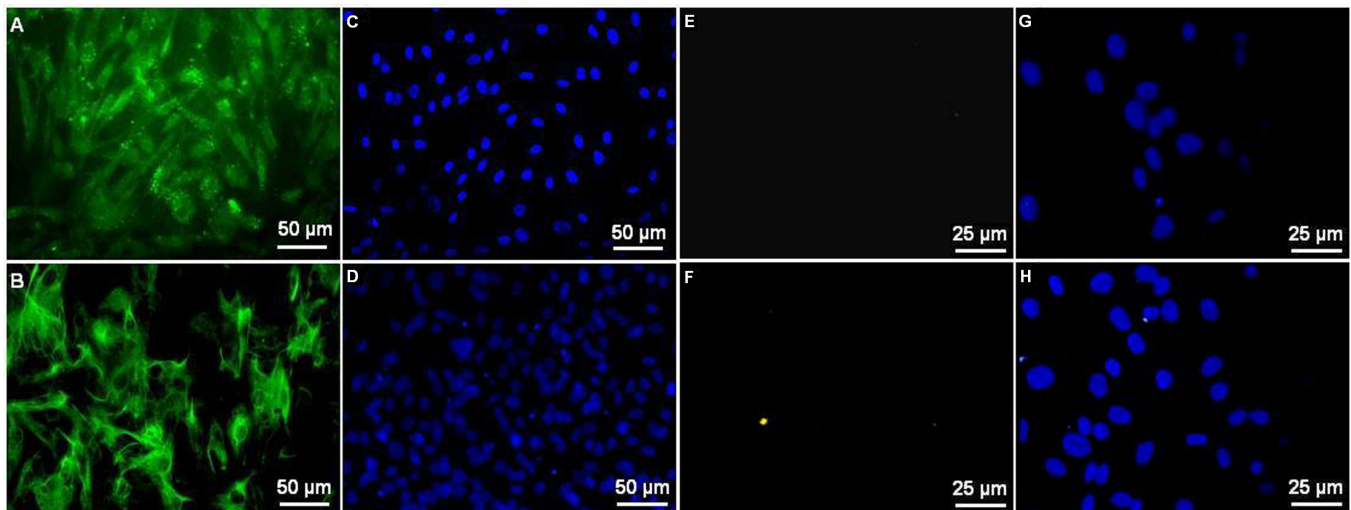
Figure 2. Immunocytochemistry was performed to ensure retinal pigment epithelium (RPE) identity of the isolated cells. **A**: A large population of RPE65-positive cells (almost all) in early primary cultures confirmed the RPE origin of the isolated cells. **C**: The nuclei of RPE cells in (**A**) were stained with : 4′,6-diamidino-2- phenylindole dihydrochloride (DAPI). **B**: The RPE cells were immunostained for cytokeratin 8/18, confirming the epithelial identity of the isolated cells. **D**: The nuclei of RPE cells in (**B**) were stained with DAPI. **E**,**F**: As negative controls, RPE cultures were simultaneously immunostained without using primary antibodies. **G**,**H**: The DAPI-stained nuclei of RPE cells in control cultures.

treated with three doses of HAF (10%, 20%, and 30%) for 30 days. Twenty-four hours after the treatment, the cultures formed a well-adherent monolayer of cells that had a uniform morphology and that were arranged in parallel to each other (Figure 3A). After 7 days, the cells began to aggregate and formed clusters of a few cells in the peripheral area of the vessels. Upon subsequent observation, the clusters of cells grew in number and size and formed sphere-like colonies containing both pigmented and nonpigmented cells (Figure 3B,C). The RPE-derived spheres were consequently detached from the base of the plate and floated into the medium (Figure 3D). The sphere formation rate was associated with HAF concentration, that is, higher doses caused more sphere-like colony formation in a shorter period of time (data not shown). In contrast, sphere-like colony formation was delayed considerably in the control cultures.

The RPE–derived colonies consisting of neural progenitor cells: The examination for the development of progenitor cells was based on tracing the NESTIN-positive cells. Neural progenitor cells were found in the neurospheres, but not in monolayer-associated cells. In cultures treated with 30% HAF, nearly all cells forming neurosphere-like colonies expressed NESTIN, as did neural progenitors (Figure 4). This result suggests that HAF stimulates the RPE cultures to dedifferentiate into cells that phenocopy neural progenitor cells.

HAF–promoted proliferation of RPE cells: The proliferation of RPE cells in the presence of HAF, FBS, and DMEM/F12 was evaluated. We found that the proliferation of the cultures was promoted when the HAF concentration in the DMEM/F12 medium was increased (Figure 5). The rate of cell death for the RPE cells in the HAF-containing media remained almost the same as for the cells in the FBS-containing media (Figure 6). This result is consistent with those obtained from the cell proliferation assay.

The RPE–derived cells treated with HAF proceeded toward retinal neurons: Seven days after HAF treatment, we used immunocytochemistry to characterize the cellular components of the cultures. The number of cells that were immunoreactive for the RPE markers cytokeratin 8/18 and RPE65 decreased significantly in the treated cultures, while a few cells expressed interneuron-specific markers, including PKCα (Figure 7A) and CRABPI (Figure 7B). Approximately 19% and 25% of cells in 30% HAF-treated cultures were positive for PKCα (bipolar cell marker) and CRABPI (amacrine cell marker), respectively. As control, FBS-treated cultures showed 5% and 12% of cells positive for PKCα (Figure 7E) and CRABPI (Figure 7F), respectively. These results suggest that the HAF-treated RPE cultures transdifferentiate into retinal neurons similar to the interneurons located in the inner nuclear layer.

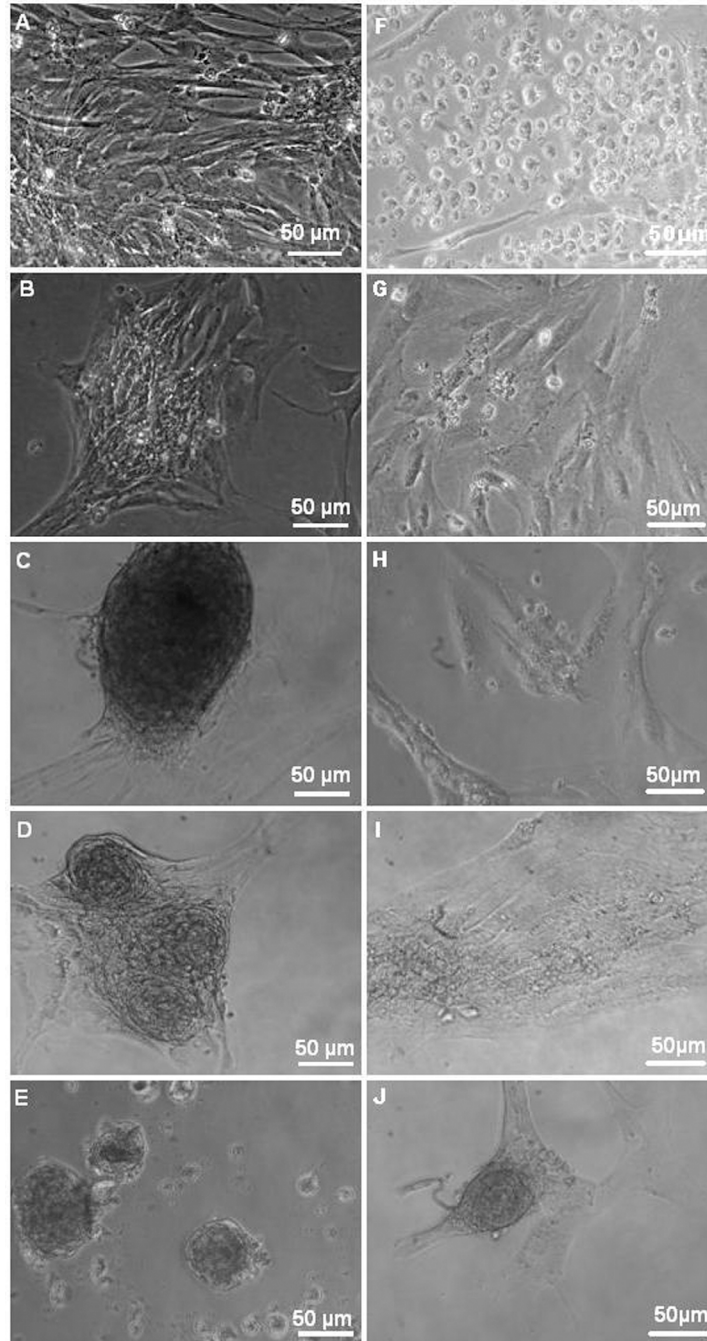An evaluation of the transcript levels of *NESTIN*, *CRABPI*, *PKCα*, and *GAPDH,* as well as *CRABPII*, *PEDF*,

Figure 3. The morphological changes observed in retinal pigment epithelium (RPE) cultures treated with human amniotic fluid (HAF) for over 30 days. **A**: HAF-treated RPE culture after 3 days. The cells grew as a monolayer and were arranged in parallel to each other. **B**: After 7 days, the cells began to form cell clusters. **C, D**: The RPE-derived neurosphere-like colonies formed and increased in number during the 2nd and 3rd weeks. **E**: Consequently, the RPE-derived colonies detached from the base of the plate and floated into the medium. **F, G, H, I, J**: The control cultures (RPE cells cultured in Dulbecco's modified Eagle medium: Nutrient mixture F-12 [DMEM/F12]) had a considerable delay in forming neurosphere-like colonies.

and *VEGF* verified the data obtained from the immunocytochemistry and demonstrated that the expression of NESTIN, PKCα, and CRABPI in HAF-treated cultures were enhanced 2-, 20-, and 22-fold, respectively. Nevertheless, the expression levels of these genes in cells treated with 10 and 20% HAF were the same or less than those of the cells treated with FBS.
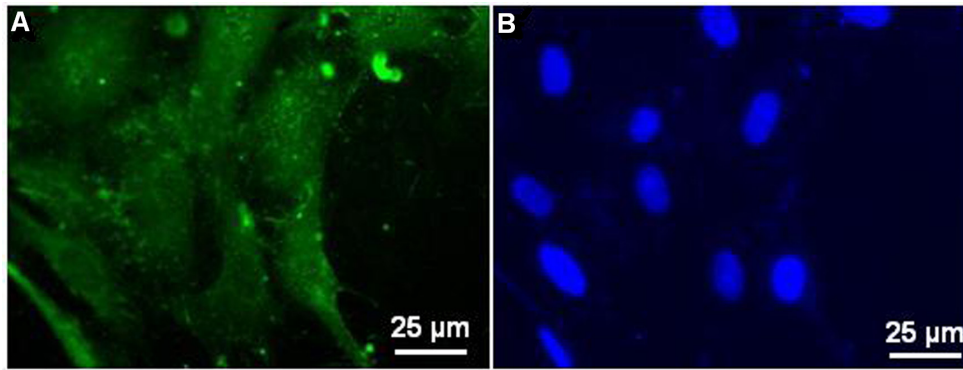
Figure 4. Immunocytochemistry was performed to identify the cell types that formed the colonies. **A**: A developing colony derived from a human amniotic fluid (HAF)-treated retinal pigment epithelium (RPE) culture strongly expressed the neural progenitor marker, NESTIN. **B**: The nuclei of the cells in the colony were stained with 4′,6-diamidino-2- phenylindole dihydrochloride (DAPI).

Although the transcript levels of *PEDF*, *VEGF*, and *CRABPII* indicated increased expression of these genes (7.5-, 5.5-, and 2.7-fold, respectively) in cells treated with 10% HAF, further increasing in concentration of HAF significantly decreased expression levels for genes of interest, especially for *PEDF*. The amounts of mRNA for *CRABPI*, *CRABPII*, and *VEGF* genes were also decreased in the samples treated with 20% HAF (Figure 8).

DISCUSSION

In this study, HAF was used as an invaluable source of growth factors in order to induce human RPE cells to produce interneuron cell types, such as bipolar and amacrine cells. HAF promoted human RPE cells to proliferate further until forming confluent cultures. Under the influence of HAF, near-confluent RPE progeny cells underwent transitions in morphology and gene expression patterns. Through immunocytochemistry, we demonstrated that there was a significant decrease in the expression of RPE65 in cultures treated with HAF; this result is consistent with observations made by Engelhardt and colleagues that implicate a reduction in gene expression of RPE65 under conditions that allow for differentiation [31]. In treated cultures, the RPE-derived cells developed into spheres and most of the cells expressed NESTIN. This observation concurs with Engelhardt et al. [31], who showed that the RPE progeny cells could differentiate into neural progenitors under the conditions required to maintain neural stem cells. For confirmation, we also applied more specific markers indicating neural and retinal progenitors, including Pax6 and CHX10, and detected the increasing expression of the corresponding markers in HAF-treated RPE
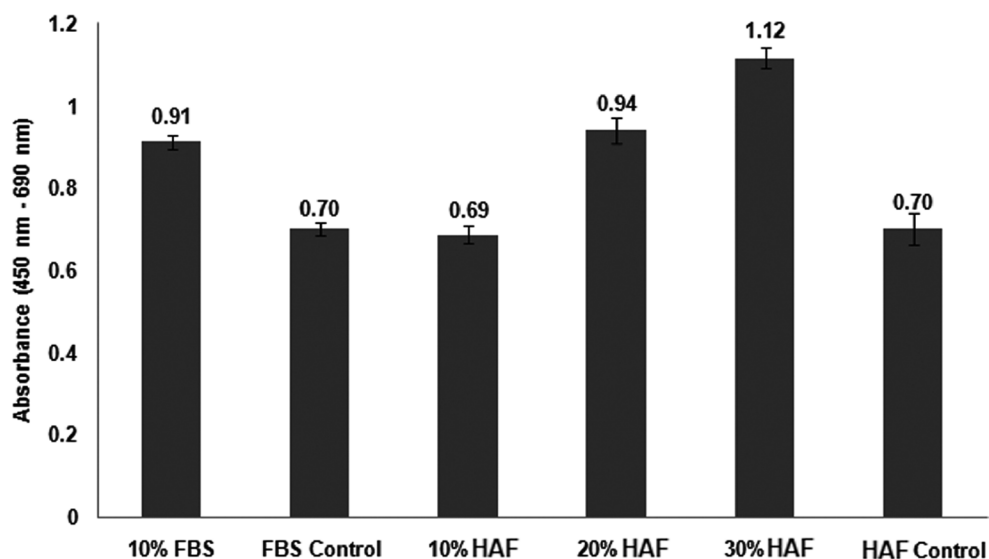

Figure 5. An enzyme-linked immunosorbent assay was used to examine the proliferation of human retinal pigment epithelium (RPE) cells. The cells were first incubated for 24 h in fetal bovine serum (FBS)- or human amniotic fluid (HAF)-containing media and cell proliferation was assessed using the enzyme-linked immunosorbent assay (ELISA) kit. Each bar represents the mean±standard error of the mean (SEM) of at least two independent experiments performed in triplicate. As seen, proliferation rate of HAF-treated RPE cells was increased through a dose-dependent manner.
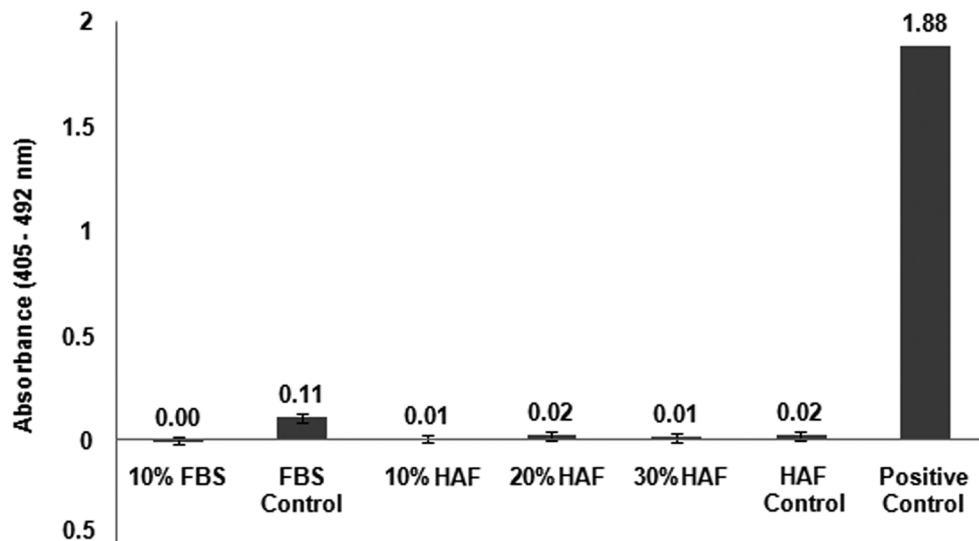
Cell Death ELISA Assay

Figure 6. An enzyme-linked immunosorbent assay (ELISA) was used to examine the death of the human amniotic fluid (HAF)–treated retinal pigment epithelium (RPE) cultures. Bar graphs showing the apoptotic status of human RPE cells treated with 10%, 20%, or 30% (v/v) HAF compared with 10% (v/v) FBS. The human RPE cells were first incubated for 24 h in serum - or AF–containing media, and the cell death assay was performed according to the instructions accompanying the cell death detection ELISA kits. Positive control was the cytoplasmic histone-associated DNA fragments in a solution supplied in the kit contents. Each bar represents the mean±standard error of the mean (SEM) of two independent experiments in at least triplicate.

cultures using both immunocytochemistry and real-time PCR [32].

In cultures treated with 30% HAF, a small population of cells was immunopositive for PKCα and a few cells were immunostained with CRABPI antibody. Among cells positive for PKCα, cells rarely exhibited bipolar-like morphology.

It seems that growing cells on polystyrene influences their exact morphological characteristics. A considerable increase in the expression of NESTIN, PKCα, and CRABPI was found in the treated cultures compared with the controls. These results are consistent with earlier studies by Amemiya et al. [15] and further confirm the competence of RPE cells to
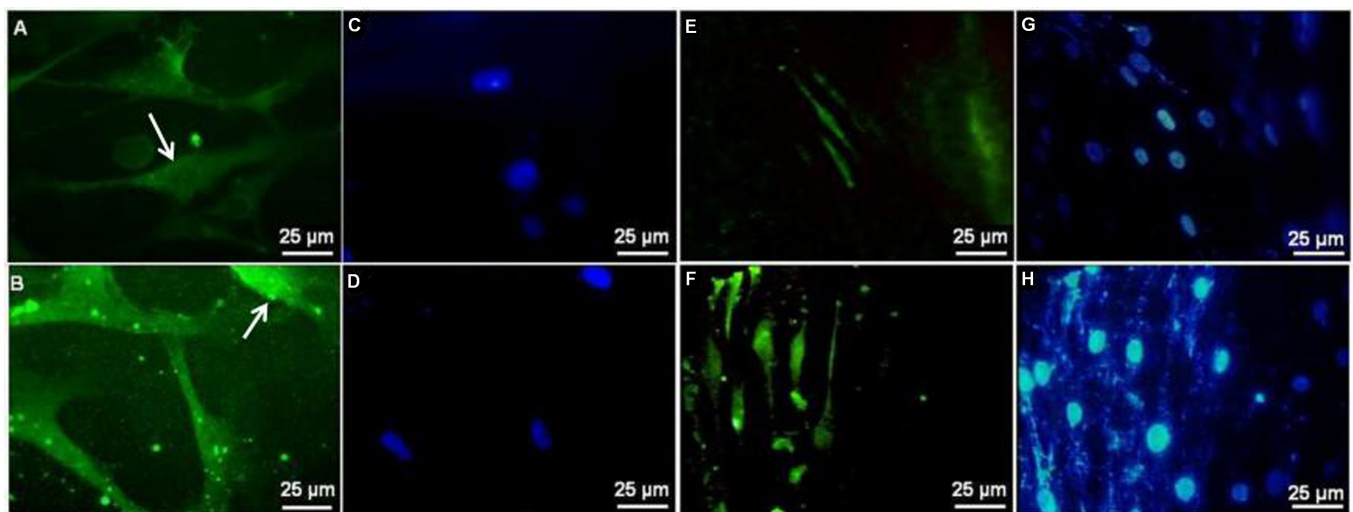

Figure 7. The immunocytochemistry results of the human amniotic fluid (HAF)–treated retinal pigment epithelium (RPE) cultures. By immunofluorescence staining, 19% and 25% of cells in the HAF-treated RPE cultures expressed protein kinase C isomer α (PKCα ; **A**) and cellular retinoic acid-binding protein I (CRABPI; **B**), respectively. Arrows refer to cells exhibiting bipolar-like morphology in panel **A** and both nuclear and cytoplasmic expression of CRABPI in panel **B**. As control, fetal bovine serum (FBS)-treated RPE cultures revealed that only 5% and 12% of cells expressed PKCα (**E**) and CRABPI (**F**), respectively. **C,D, G, H**: The nuclei of the cells in the corresponding cultures were stained with 4´,6-diamidino-2- phenylindole dihydrochloride (DAPI).
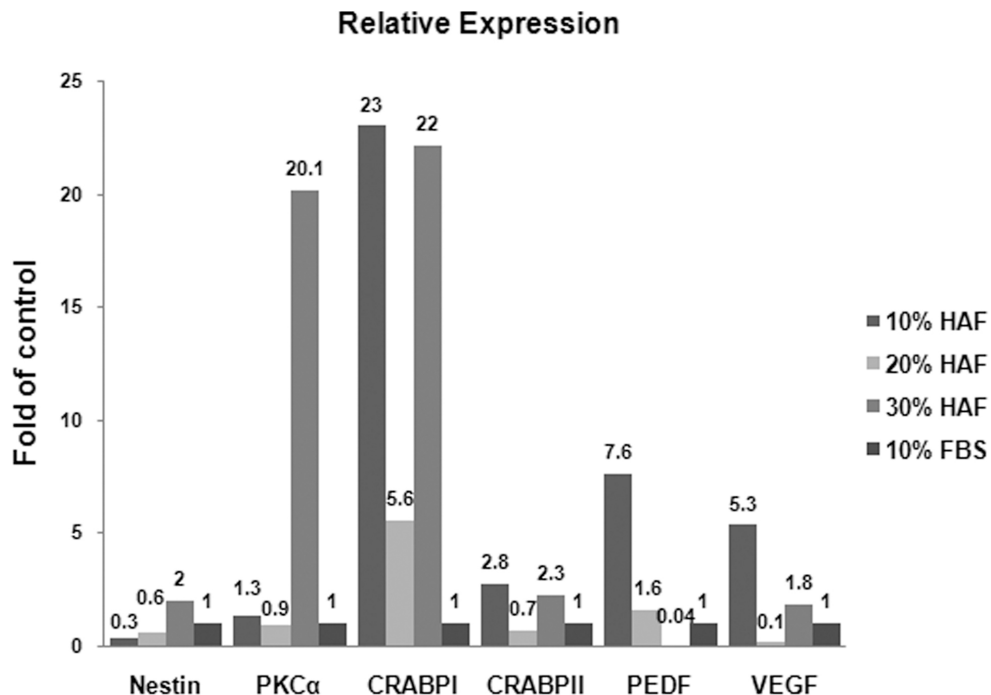
Figure 8. The neural-specific markers were upregulated in human amniotic fluid (HAF)–treated retinal pigment epithelium (RPE) cultures. Bar graphs showing the relative gene expression value of the NESTIN, protein kinase C isomer α (PKCα), cellular retinoic acid-binding protein I (CRABPI), CRABPII, pigment epithelium-derived factor (PEDF), and vascular endothelial growth factor (VEGF) genes in RPE cultures treated with 10%, 20%, or 30% (v/v) HAF with respect to 10% (v/v) fetal bovine serum (FBS). The total RNA was isolated, and the relative gene expression for each of the specific markers was analyzed with real-time PCR. The increased expression of NESTIN and PKCα in cells treated with 30% HAF (v/v) was significant (2- and 20-fold, respectively). For CRABPI, the same level of expression (approximately 22-fold) was found in cells treated with either 10% or 30% HAF (v/v). For the CRABPII, PEDF, and VEGF genes, the levels of mRNA expression were enhanced in cells treated with 10% HAF (v/v; 7.5-, 5.5- and 2.7-fold, respectively), though it decreased with increasing concentrations of HAF. The experiment was repeated twice, with each repetition executed in duplicate.

transdifferentiate into neurons. Furthermore, multiple studies have revealed the competence of RPE cells to be reprogrammed to differentiate into retinal neurons by principal genes such as *NeuroD* [6] and *Sox2* [33] during the development of the retina.

In immunocytochemical investigations, CRABPI had both a cytoplasmic and nuclear subcellular localization, which is consistent with many previous studies [34,35]. According to the results of this study and those by Martin and coworkers [36], the cytoplasmic CRABPI has an uneven distribution throughout the cytoplasm and is localized to the perinuclear region of the cytoplasm [36]. Despite previous reports indicating that *CRABPII* (one of the CRABPI isoforms) is not expressed in the mature retina and that it is only found in tissues exhibiting ongoing growth [37], this study demonstrated that the *CRABPII* gene is expressed in both HAF-treated RPE and control cultures.

Based on our real-time PCR studies, we found that 30% HAF could significantly increase the expression of the NESTIN and *PKCα* genes, suggesting that this concentration of HAF has the highest potential to induce RPE cells to differentiate into neural progenitor and retinal bipolar cells. The expression of the *CRABPI* gene was elevated by all three doses of HAF but occurred mostly with 10% and 30% HAF, suggesting that HAF guided RPE cells to form amacrine cells.

Along with the increase in the expression of NESTIN, PKCα, and CRABPI, which are indicators of neural progenitor cells and mature retinal neurons (such as bipolar and amacrine cells), the expression of the *PEDF* and *VEGF* genes (indicative of RPE cells) was significantly diminished in the HAF-treated cultures. Notably, concurrent with an increase in the concentration of HAF, the expression of the *PEDF* gene dropped to its lowest level. These results demonstrate that under the influence of HAF, RPE cells exhibit characteristics of neural progenitor cells and retinal neurons while losing their specific functional features, such as the expression of the *RPE65* and *PEDF* genes. Nevertheless, there may be another explanation for the decreased expression of *PEDF* and *VEGF*; it may be due to feedback regulation of synthesis due to intrinsic existence of PEDF and VEGF in amniotic fluid. Still, this would not represent all that happened. Other data confirmed that reduced RPE65 expression and increased expression of neural markers implicated HAF-induced changes in the cellular and molecular programs of RPE cells into a new identity.

In this study, we observed a decrease in the transcript levels of the *CRABPI*, *CRABPII*, and *VEGF* genes in cells treated with HAF 20%. According to the results from Willerth et al. [38], the observed decline in the expression of the *CRABPI*, *CRABPII*, and *VEGF* genes in cultures treated with 20% HAF may be due to interference between functions of multiple growth factors found in HAF. HAF is a complex biologic fluid composed of a variety of growth factors that have multifaceted and unforeseeable connections with each other. It has been suggested that there is crosstalk between different growth factors in a complex, meaning that growth factors influence the functional effects of other growth factors so that they exert either stimulatory or inhibitory effects on each other, depending on the combination of content they include [38]. At 20% HAF, the growth factors that increase the expression of the *CRABPI*, *CRABPII*, and *VEGF* genes could be inhibited by other growth factors present in the HAF; hence, the expression of these three genes may be minimized. At 10% HAF, the concentrations of the inhibitory growth factors may be too low to inhibit and block the signaling pathways regulated by the stimulatory growth factors, resulting in the expression of the desired genes. On the other hand, at 30% HAF, the concentrations of the stimulatory growth factors may be dominant enough to induce high levels of expression of the genes of interest, and the inhibitory growth factors may be restrained by the other growth factors, consequently causing the expression of *CRABPI*, *CRABPII*, and *VEGF* to increase again.

We evaluated the expression ratio of the *PEDF* and *VEGF* genes (PEDF/VEGF) under different concentrations of HAF (10%, 20%, and 30%). As previously demonstrated in normal eyes, a balance between the angiogenic factor VEGF and the anti-angiogenic factor PEDF impedes the neovascularization process. Overexpression of the VEGF gene or downregulation of the PEDF gene leads to the activation of angiogenesis [39]. We showed that at 20% HAF, the *VEGF* gene is downregulated, and the expression ratio of PEDF/VEGF is increased. Therefore, 20% HAF may be considered an antiangiogenic factor and may be potentially useful to study the balance of *PEDF* and *VEGF* gene expression.

In conclusion, this study showed that HAF may be a source of growth factors to stimulate the proliferation, differentiation, and transdifferentiation of RPE cells to allow the cells to produce other retinal cellular lineages, including retinal neurons. RPE cells are intrinsically capable of forming sizable colonies and continue to proliferate through several passages [40]. Moreover, RPE cells exhibit a great potential to dedifferentiate into neural progenitors and transdifferentiate into retinal neurons such as interneurons, which include bipolar and amacrine cells. Considering that RPE cells possess a multipotential capability to produce various retinal neurons, further investigations are needed to employ them in therapeutic applications to treat retinal degenerative diseases.

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