Retinal Cell Biology

Development of Retinal Pigment Epithelium from Human Parthenogenetic Embryonic Stem Cells and MicroRNA Signature

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Purpose. We investigated the potential of human parthenogenetic embryonic stem cells (hPESCs) to differentiate into RPE cells, and identified development-regulating microRNAs (miRNAs).

Methods. RPE cells were derived from hPESCs. The expression of markers and miRNA expression profiles during differentiation were studied by immunocytochemistry, real-time RT-PCR, and miRNA expression array at three time points. Human fetal RPE (hfRPE) cells also were analyzed. The target genes of candidate miRNAs then were validated.

Results. hPESC-derived RPE cells exhibited similar morphology and pigmentation to hfRPE cells. The expression of markers during differentiation indicated that the hPESC-derived RPE cells were immature. Most specific miRNAs had a role at some time point during the differentiation and maturation of RPE from hPESCs, except for two miRNAs (miR-204 and the miR-302 family). The miR-204 was upregulated and miR-302 was down-regulated throughout the process. Subsequently, pigmented clusters and RPE signature gene expression increased significantly in the miR-204 overexpression group and miR-302 inhibition group compared to the control groups. *CTNNBIP1* and *TGFBR2* were confirmed to be the target genes of miR-204 and miR-302, respectively.

Conclusions. hPESCs can develop into RPE-like cells and, thus, can be additional promising sources of RPE cells in cell therapy. The miR-204, miR-302s, and their targets are involved in regulating directed differentiation during the full course, thereby contributing to the search for a new method of improving differentiation efficiency using miRNAs. (*Invest Ophthalmol Vis Sci.* 2012;53:5334–5343) DOI:10.1167/iovs.12-8303

Age-related macular degeneration (AMD) is the leading cause of visual loss among individuals over 55 years of age. The degeneration and/or dysfunction of the RPE is involved in the two basic forms of AMD, namely, atrophic and exudative.[1] Normal RPE has roles in maintaining the blood-retinal barrier, supplying nutrients to the neural retina, the visual cycle of 11-cis retinal, and outer segment phagocytosis.[2] The impairment and progressive loss of the RPE among AMD patients lead to choroidal neovascularization and/or photoreceptor depletion, which result in irreversible blindness.[3] The transplantation of RPE cells may permit the recovery of visual function.

Pluripotent stem cells have been proposed as attractive alternative cell sources for transplantation. Efficient methods of generating RPE cells from human embryonic stem cells (hESCs) have been developed in recent years.[4–7] RPE cells derived from hESCs reportedly can restore visual function in retinal degeneration rat models.[8–11] Advanced Cell Technology (Marlborough, MA) has received clearance to begin a phase I/II trial of hESC-derived RPE cell therapy involving AMD patients.[12]

Pluripotent stem cells that closely resemble hESCs can be isolated from parthenogenetic blastocysts, which were developed from a single metaphase II (MII) oocyte and contained only the maternal genome.[13–15] Human parthenogenetic embryonic stem cells (hPESCs) have been demonstrated to have broad differentiation potential.[16] The use of hPESC-derived cells avoids immunologic complications and ethical controversies associated with handling hESCs, and may become a platform for personalized medicine by allowing the cells of a female patient to become her own source. However, the potential of hPESCs to differentiate into RPE cells is not known fully. Harness et al. reported that the RPE yield and purity are equivalent in hPESC and hESC cultures, but the gene expression and methylation of imprinted genes vary.[17] The mechanisms that control the differentiation of hPESCs must be understood urgently before hPESCs are used clinically. MicroRNAs (miRNAs) have been demonstrated to have key roles in regulating the "stemness" and various differentiation pathways.[18,19] Comprehensive information on the role of miRNAs during differentiation must be obtained to influence critical gene regulatory networks directly and promote differentiation. In our study, we derived RPE cells from hPESCs and assessed the involvement of possible development-regulating miRNAs during differentiation.

Materials and Methods

Culture of hPESCs and Differentiation into RPE Cells

The hPESC line P-TJ was maintained as described previously.[20] The medium consisted of 80% knockout Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), 20% knockout serum replacement (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1% MEM nonessential amino acids (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 8 ng/mL

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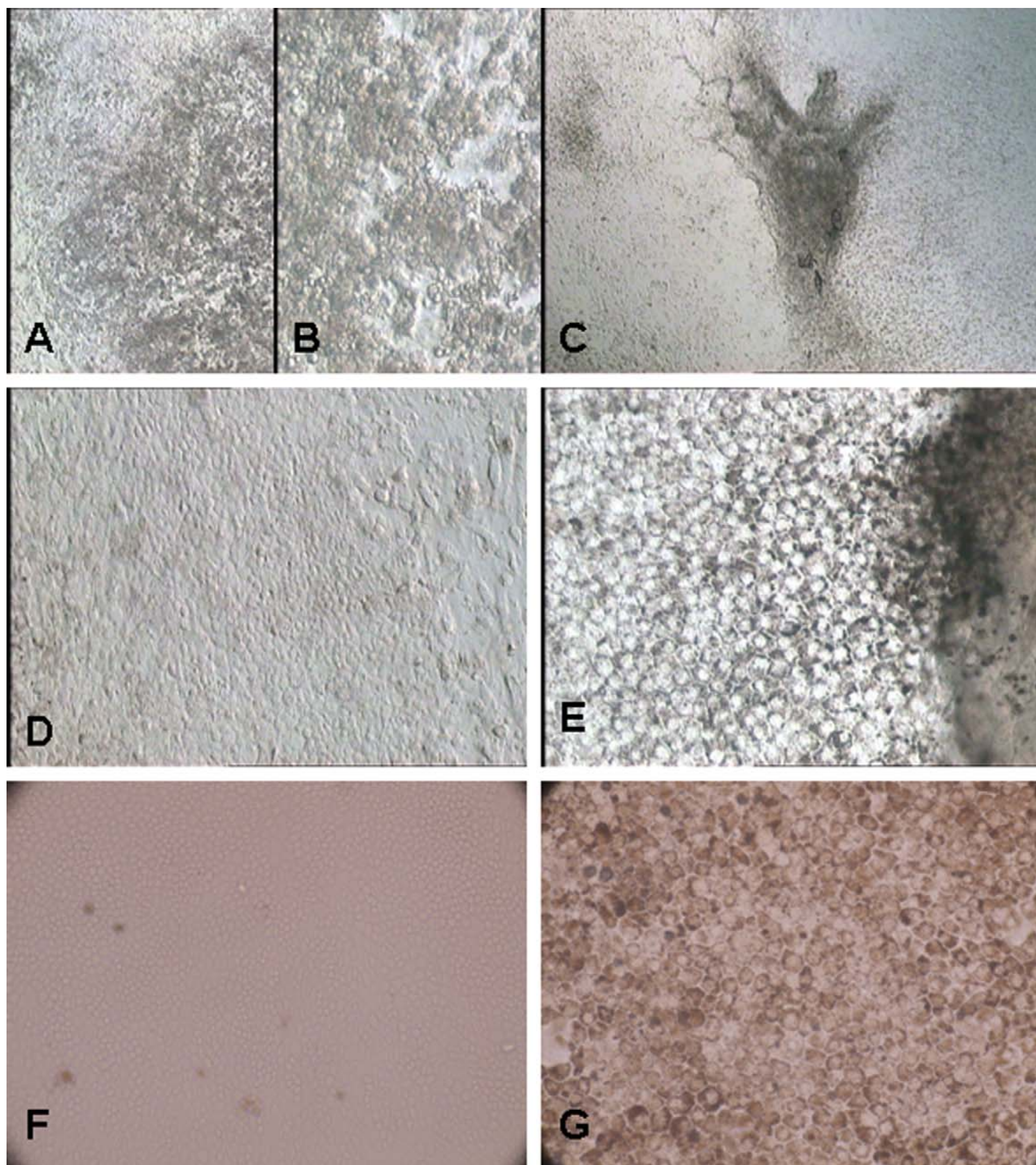

FIGURE 1. Morphology of RPE cells differentiated from hPESCs and hfRPE. (**A**) Pigmented foci from differentiating hPESCs at 3–4 weeks (100×). (**B**) Pigmented foci from differentiating hPESCs at 3–4 weeks (200×). (**C**) Pigmented cluster at 5–6 weeks (40×). (**D**) Surrounding cells of pigmented clusters lost pigmentation and morphology on Matrigel (100×). (**E**) hPESC-derived RPE cells on Matrigel at 4–6 weeks. Cells restored pigmentation and epithelial morphology (200×). (**F**) Primary hfRPE cells lost pigmentation at 3–4 weeks (100×). (**G**) Primary hfRPE cells acquired pigmentation at 6–8 weeks (200×).

bFGF (R&D, Minneapolis, MN). Human foreskin fibroblasts from passages 15–25 were chosen as feeder cells.

All cells were cultured in six-well cell culture plates. Differentiation experiments were performed with hPESCs. When the borders of individual hPESC colonies approached one another at approximately 7–10 days post-passage, the medium was changed daily using basic hPESC medium that lacked bFGF. Once the medium was changed to the bFGF-deficient hPESC medium, the hPESC colonies lost their tight borders and became multilayered, and pigmented foci appeared. These pigmented clusters then were isolated manually using a glass pipette under a dissecting microscope, and seeded onto six-well culture dishes coated with Matrigel (diluted 1:30; BD Biosciences, Bedford, MA). Pigmented foci were allowed to expand in the monolayer, still using bFGF-deficient hPESC medium. Until these reproduced cells exhibited a hexagonal shape and displayed melanin pigments, they can be studied further as hPESC-derived RPE cells.

Human Fetal RPE Cell Isolation and Culture

Fetal eyes were obtained from random donors at 16–18 weeks of gestation. Permission had been given to use the poles for research. The research followed the tenets of the Declaration of Helsinki. Human fetal RPE (hfRPE) cells were isolated and cultured as described previously.[21] Single-cell RPE layers were peeled off in small sheets. The collected cells were washed and seeded onto Primaria flasks with RPE medium. The medium was changed every 2–3 days.

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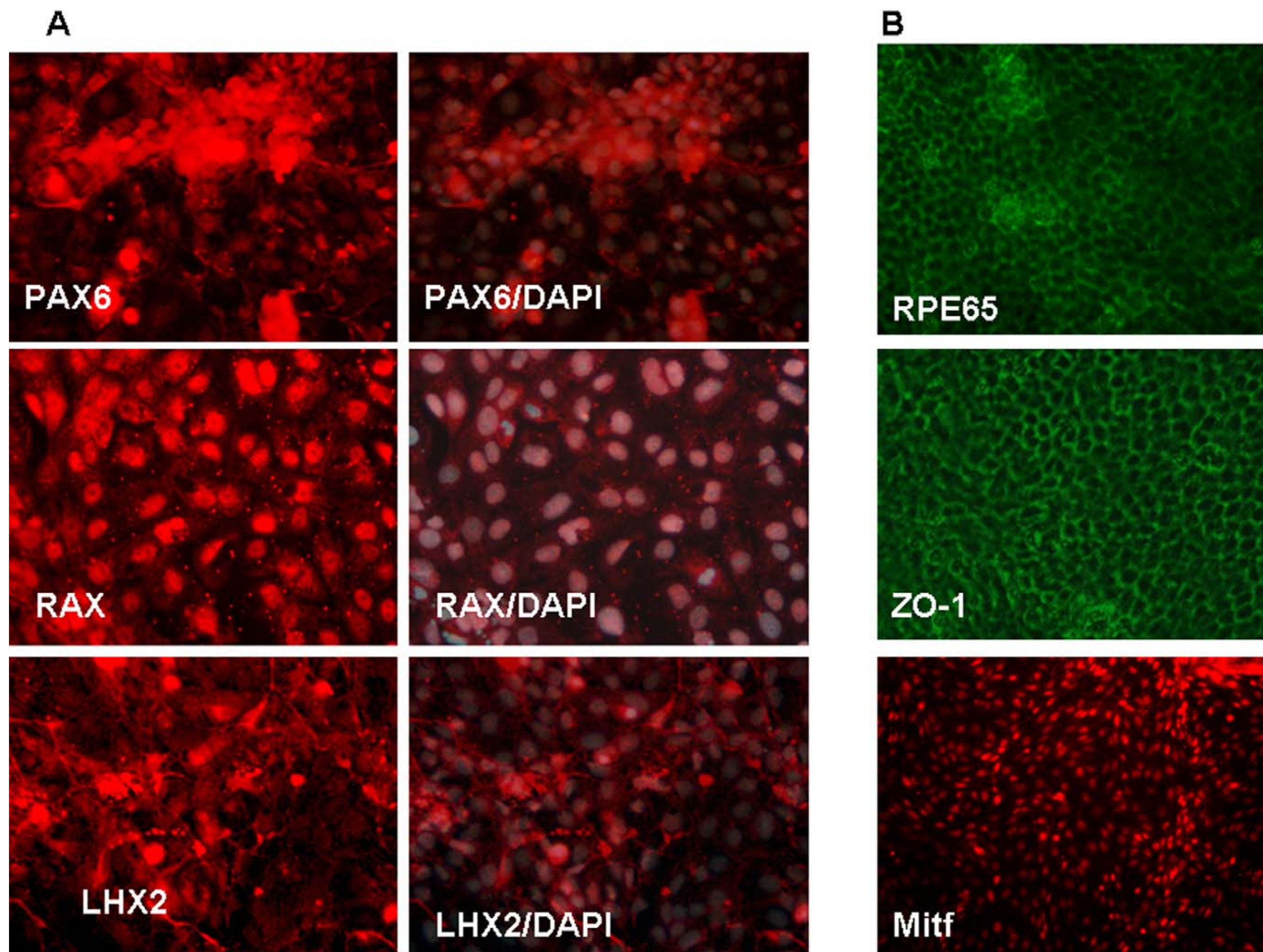

FIGURE 2. Expression of characteristic cell markers during differentiation. (A) Immunocytochemistry of pigmented clusters from differentiating hPESCs at 5 weeks, demonstrating the expression of PAX6 (400×), RAX (400×), and LHX2 (400×). (B) Immunocytochemistry of hPESC-derived RPE cells at 12 weeks, demonstrating the expression of RPE65 (200×), ZO-1 (200×), and Mitf (200×).

Immunocytochemistry

The hPESC-derived cells that contained pigmented foci at 5 weeks and hPESC-derived RPE cells at 12 weeks were fixed for 30 minutes in 4% formaldehyde buffer. Immunocytochemistry was done according to the manufacturer's protocol. The primary antibodies used in hPESC-derived cells containing pigmented foci at 5 weeks were LHX2 (1:500; AB10557; Millipore, Bedford, MA), RAX (0.5 μg/ml; GTX77859; GeneTex, San Antonio, TX), and PAX6 (1:100; 251935; Abbiotec, San Diego, CA). The primary antibodies used in hPESC-derived RPE cells at 12 weeks were RPE65 (1:1000; ab59720; Abcam), ZO-1 (1:100; ab59720; Abcam, Cambridge, UK), and Mitf (4 μg/ml; MS-772-P0; Thermo Scientific).

RNA Extraction and Quality Control

RNA was extracted from hPESCs, hPESC-derived cells containing pigmented foci at 5 weeks, hPESC-derived RPE cells at 12 weeks, and hfRPE cells at 12 weeks using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and quality of RNA samples were determined by an Agilent bioanalyzer. RNA integrity was assessed by observing the 18/28S rRNA peaks and RNA integrity number. RNA concentrations were measured using a nano-drop spectrophotometer (Nano-Drop Technologies, Wilmington, DE). All samples used had 260/280 ratios above 2.0 and 260/230 ratios above 1.7.

miRNA Microarray Analysis

Comprehensive miRNA analysis was performed using the human Affymetrix GeneChip miRNA array platform. The miRNA expression profiles of cells during differentiation from hPESCs into RPE cells were generated from the following groups: hPESC line, hPESC-derived cells containing pigmented foci at 5 weeks, and hPESC-derived RPE cells at 12 weeks. The hfRPE cells at 12 weeks also were analyzed. All procedures were performed according to the manufacturer's instructions. Differentially expressed miRNAs were defined as genes whose expression in the study group consistently showed a two-fold difference compared to the control group.

Validation of Gene Expression Data by Real-Time RT-PCR

Reverse transcription was performed using oligo dT or miRNA and U6 snRNA specific primers at a final concentration of 40 nM each. Reactions were performed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI). Real-time PCR was performed as

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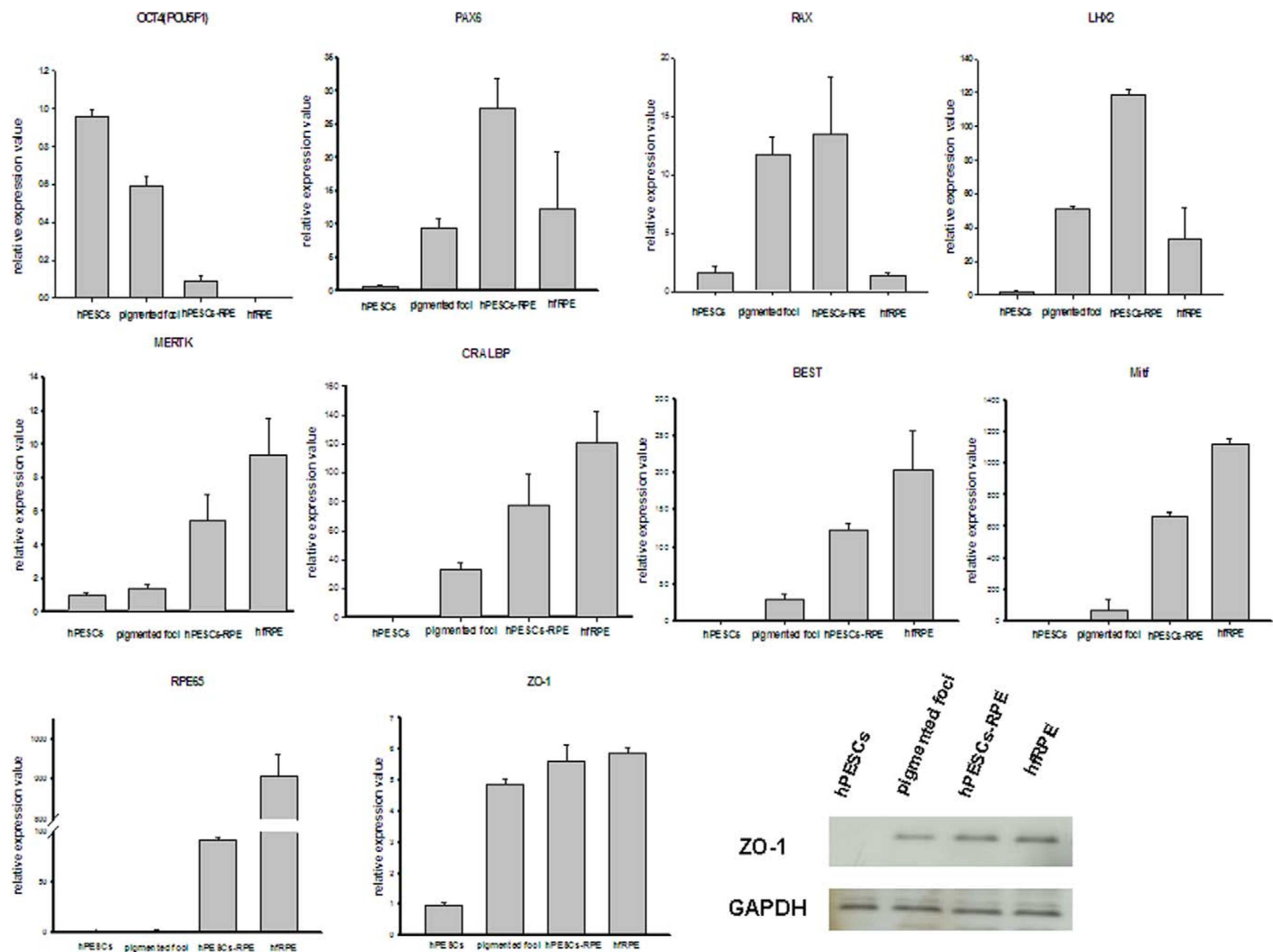

Figure 3. Real-time PCR analysis of the expression of pluripotent gene (*Oct4*), optic genes (*PAX6*, *RAX*, and *LHX2*), and RPE genes (*MERTK*, *CRALBP*, *BEST*, *Mitf*, *RPE65*, and *ZO-1*) during differentiation from hPESCs into RPE cells.

follows: 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 40 seconds. Reactions were done in triplicate in diluted cDNA combined with a Power SyberGreen mix (Takara, Tokyo, Japan) with 1 μM of the appropriate forward and reverse primers, in a final volume of 25 μL using an ABI 7500 sequence detection system. The expression level of a given gene was quantified using the $2^{-\Delta\Delta Ct}$ method. Statistical comparisons were made using the Student's t test. $P < 0.05$ was regarded as significant.

Transfection Assay

MiR-204 expression lentivirals and anti-miR-302 lentivirals were provided by Shanghai Sunbio Medical Biotechnology Co., Ltd. They were transfected into hPESCs at 30%–50% confluence. The cells were harvested 72 hours after transfection for protein analysis. For functional studies examining the effects of miR-204 and anti-miR-302 on differentiation, hPESCs were transfected for 48 hours and cultured with the bFGF-deficient medium described above for 5 weeks. Pigmented foci per well were counted, and the cells that contained pigmented foci were harvested for mRNA analysis.

Target Gene Prediction and Fluorescent Reporter Assay

MiRNA targets predicted by computer-aided algorithms were obtained from Pictar (available in the public domain at http://pictar.mdc-berlin.de/cgibin/), Targetscan (available in the public domain at http://www.targetscan.org), and Mirbase targets (available in the public domain at http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl). From these results, we found one target gene of miR-204, *CTNNBIP1*, and one target of miR-302, *TGFBR2*. The fluorescent reporter construct bearing the *TGFBR2* 3′UTR or *CTNNBIP1* 3′UTR was generated by PCR from a human cDNA library. Both fragments were cloned into the pcDNA3/EGFP vector downstream from the GFP coding region using BamHI and EcoRI. Predicted binding sites of miR-204 and miR-302 were mutated using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). HEK293 cells were transfected with pcDNA3/EGFP-CTNNBIP1 or pcDNA3/EGFP-TGFBR2, and pcDNA3/EGFP vector served as a control along with miR-204 or miR-302 expression vectors or the control vector pcDNA3.1. Approximately 48 hours after transfection, GFP activity was measured using an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Western Blotting

Cells were washed with PBS and lysed with RIPA buffer (Millipore) containing protease inhibitors (Roche, Mannheim, Germany). Proteins (40 μg per sample) were resolved by SDS-PAGE and transferred onto an immobilon-P transfer membrane (Millipore). Membranes were probed with antibodies specific to ZO-1 (1:50, ab59720; Abcam), *TGFBR2* (1:500, ab61213; Abcam), or *CTNNBIP1* (1:500, ab57544; Abcam) with GAPDH as the loading control. The membrane then was incubated

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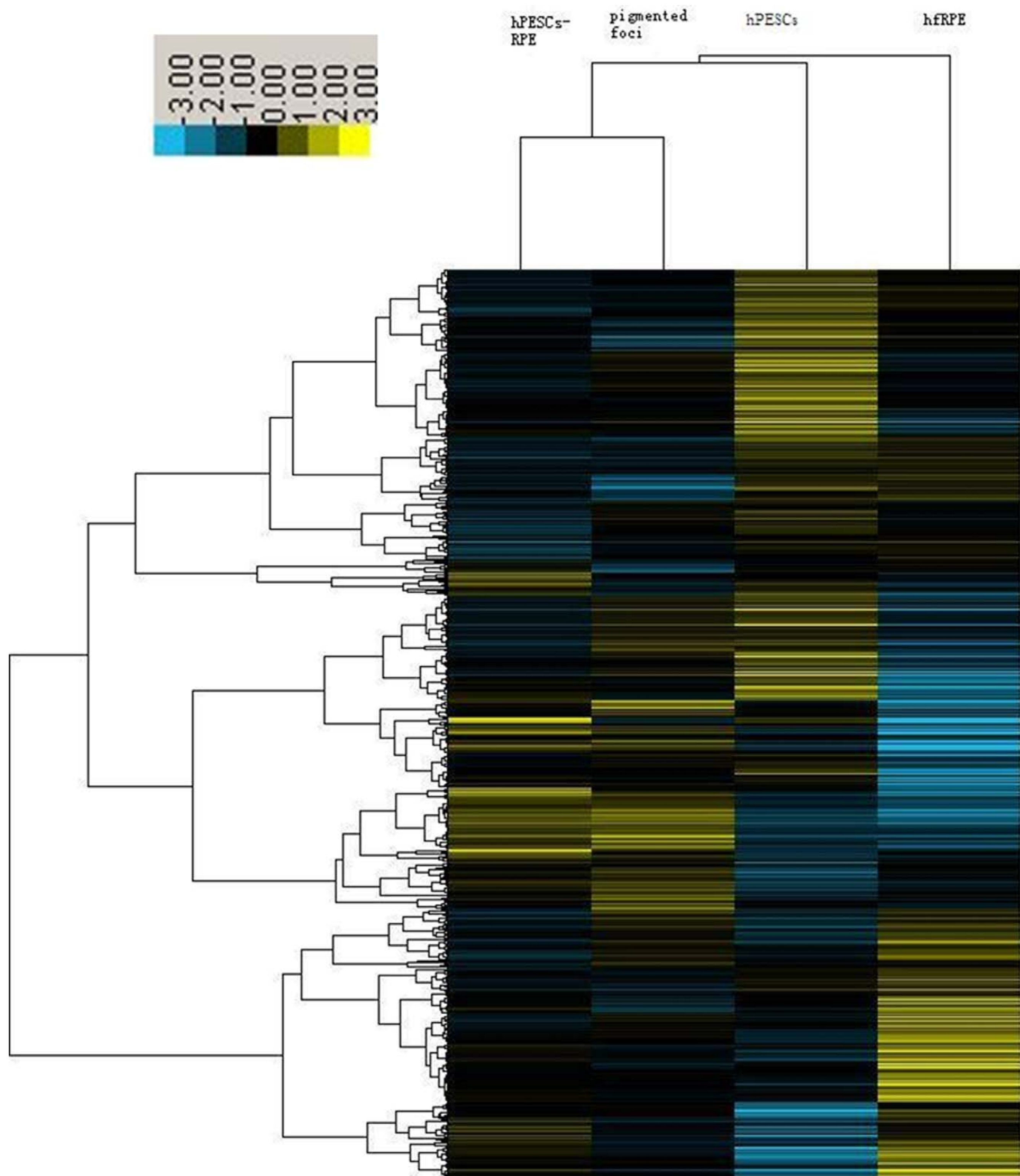

Figure 4. Hierarchical clustering analysis of miRNA expression (hPESC line, hPESC-derived cells containing pigmented foci at 5 weeks, hPESC-derived RPE cells at 12 weeks, and hfRPE cells at 12 weeks).

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TABLE 1. Up-regulated miRNAs throughout the Entire Differentiation Process of hPESC-Derived RPE Cells

Name	Pigmented Foci Compared to hPESCs (Ratio)	hPESCs-RPE Compared to Pigmented Foci (Ratio)
miR-886-5p	9.571	2.867
miR-184	9.006	3.451
miR-204	5.026	3.337
miR-146a	3.728	2.128
miR-10a	2.339	2.774
miR-203	2.287	5.335
miR-194	2.178	2.226

TABLE 2. Down-Regulated miRNAs throughout the Entire Differentiation Process of hPESC-Derived RPE Cells

Name	Pigmented Foci Compared to hPESCs (Ratio)	hPESCs-RPE Compared to Pigmented Foci (Ratio)
miR-187	0.335	0.425
U56_st	0.299	0.484
miR-302a-star	0.08	0.335
miR-302a	0.178	0.173
miR-302b	0.107	0.481
miR-302c-star	0.091	0.324
miR-302c	0.112	0.231
miR-302d	0.152	0.257

with horseradish peroxidase-conjugated secondary antibody, and protein expression was assessed by enhanced chemiluminescence and exposure to a chemiluminescent film.

RESULTS

Differentiation and Expansion of RPE Cells from hPESCs

Pigmented cells emerged spontaneously from differentiating hPESCs after 3–4 weeks of culture in bFGF-free hESC culture medium (Figs. 1A, 1B). Pigmented clusters increased in size and number after an additional 2–3 weeks of culture (Fig. 1C). The P-TJ line produced more than 14 $\pm$ 3 pigmented clusters per well in six-well culture dishes. Pigmented clusters were excised manually and expanded to monolayers on Matrigel. Over time, cells in the pigmented clusters became polygonal shaped and proliferated. The cells lost pigmentation as they divided and migrated (Fig. 1D). The most densely pigmented cells were located in the middle of the clusters, and this dense pigmentation spread gradually to the periphery. Granules of brown pigments appeared in the cytoplasm of the cells along the edges at 4–6 weeks (Fig. 1E). This phenomenon was consistent with the process by which primary hfRPE cells acquired pigmentation (Figs. 1F, 1G). Epithelial morphology and pigment expression suggested the differentiated state of hPESC-derived RPE cells.

hPESC-Derived RPE Cells Expressed Signal Genes during Differentiation

During differentiation, immunofluorescent analysis of pigmented clusters from differentiating hPESCs at 5 weeks revealed the expression of optic markers (PAX6, RAX, and LHX2) associated with eye field specification (Fig. 2A). The RPE-associated tight junction protein ZO-1, visual cycle proteins RPE65, and transcription factor Mitf were expressed in hPESC-derived RPE cells at 12 weeks (Fig. 2B). RT-PCR analyses revealed that hPESCs rapidly lost expression of the pluripotency gene *Oct4*, and acquired expression of the optic and RPE signature genes during this process (Fig. 3). However, lower levels of optic markers (PAX6, RAX, and LHX2) were detected in hfRPE cells than in hPESC-derived RPE cells, in accordance with the development of the fetal eye. The increase in RPE signature genes (CRALBP, BEST, Mitf, RPE65, and a phagocytic gene *MERTK*) was more prominent in hfRPE cells than in hPESC-derived RPE cells. The mRNA level of ZO-1 in hPESC-derived RPE cells was similar to that in hfRPE cells, but the protein level still was lower. The difference in the expression levels of signal genes indicated that hPESC-derived RPE cells were less mature than hfRPE cells.

Identification of Differentiation-Regulated miRNAs during Differentiation from hPESCs into RPE Cells Using miRNA Arrays

To examine the role of the post-transcriptional regulators of RPE cells derived from hPESC, we profiled the hPESC line, hPESC-derived cells containing pigmented foci, hPESC-derived RPE cells, and hfRPE cells using miRNA arrays. The hierarchical clustering analysis is shown in Fig. 4.

There were significant differences between the miRNAs of any two differentiated stages. There were 134 unique miRNAs significantly upregulated and 151 miRNAs down-regulated during the differentiation from hPESCs into cells containing pigmented foci. Of the miRNAs 45 were upregulated and 50 were down-regulated during the differentiation from cells containing pigmented foci into hPESC-derived RPE cells. There were 7 miRNAs continuously upregulated and 8 miRNAs down-regulated throughout the entire differentiation process from hPESCs into cells containing pigmented foci and from cells containing pigmented foci into hPESC-derived RPE cells (Tables 1, 2).

The differences between the miRNA data of hPESC-derived RPE and hfRPE cells, with 154 miRNAs upregulated and 149 miRNA down-regulated, may be responsible for the maturation of RPE cells. We compared miRNAs throughout the entire differentiation process of hPESC-derived RPE cells to those related to the maturation of RPE cells. Only miR-204 showed significant upregulation during differentiation from hPESCs into hfRPE cells, and only the major members of the miR-302 family (302a, 302b, 302c, and 302d) showed strong down-regulation (Table 3).

Validation of RPE "Signature" miRNAs during Differentiation

The miRs-184, -187, -200a, -200b, -204, -211, -221, and -222 reportedly are RPE signature miRNAs based on a validation panel of 20 tissues.[22] During differentiation from hPESCs into RPE cells, the expression levels of these miRNAs were validated by RT-PCR in the hPESC line, hPESC-derived cells containing pigmented foci, hPESC-derived RPE cells, and hfRPE cells (Fig. 5). miR-184, -200b, -222, -204, and -211 increased during the differentiation process of hPESC-derived RPE cells, which may aid specifically the development of RPE cells from pluripotent cells. The change from hPESC-derived RPE cells to hfRPE cells was viewed as a maturation process; miR-204 suddenly increased 30-fold, indicating its important role not only in specific differentiation but also in maturation. Although miR-211 also increased significantly from hPESC-derived RPE cells to hfRPE cells, a nonapparent change was observed between hPESCs and hPESC-derived RPE cells, suggesting that

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TABLE 3. Only Up-regulated or Down-Regulated miRNAs throughout the Entire Differentiation from hPESCs into hfRPE Cells

Name	Pigmented Foci Compared to hPESCs (Ratio)	hPESCs-RPE Compared to Pigmented Foci (Ratio)	hfRPE Compared to hPESCs-RPE (Ratio)
miR-204	5.026	3.337	13.574
miR-302a-star	0.08	0.335	0.336
miR-302a	0.178	0.173	0.192
miR-302b	0.107	0.481	0.265
miR-302c-star	0.091	0.324	0.018
miR-302c	0.112	0.231	0.472
miR-302d	0.152	0.257	0.073

miR-211 may support the maturation of RPE cells. miR-184, -200b, and -222 decreased from hPESC-derived RPE cells to hfRPE cells. Hence, the upregulation of miR-204 may be essential in the entire differentiation process, in accordance with the results from the arrays.

Up-regulation of miR-204 or Down-Regulation of miR-302 Contributed to Differentiation

Based on the findings of miRNA regulation, miR-204 and miR-302 were identified for further study. After culturing in bFGF-deficient medium for 5 weeks, hPESCs with miR-204 expression lentivirals and anti-miR-302 lentivirals produced 20 ± 4 and 22 ± 3 pigmented clusters per well, respectively, in six-well culture dishes. These values were more than those of the miR-negative (14 ± 2) and anti-miR-negative (13 ± 2) lentiviral control groups. After miR-204 overexpression and miR-302 inhibition in hPESCs, the mRNA levels of ZO-1, RPE65, MERTK, and Mitf became significantly higher than those of the control groups at 5 weeks (Fig. 6). This result demonstrated that miR-204 promoted cell differentiation from hPESCs into RPE, but miR-302 had a suppressor role.

Identification of miR-204 and miR-302 Target Genes

miR-204 and miR-302 may regulate hPESC differentiation into RPE cells, but their functions must be reflected by downstream target genes. We identified *CTNNBIP1* and *TGFBR2* as candidate target genes of miR-204 and miR-302, respectively, according to target prediction programs (Fig. 7A). miR-204 overexpression led to decreased protein levels of *CTNNBIP1*,

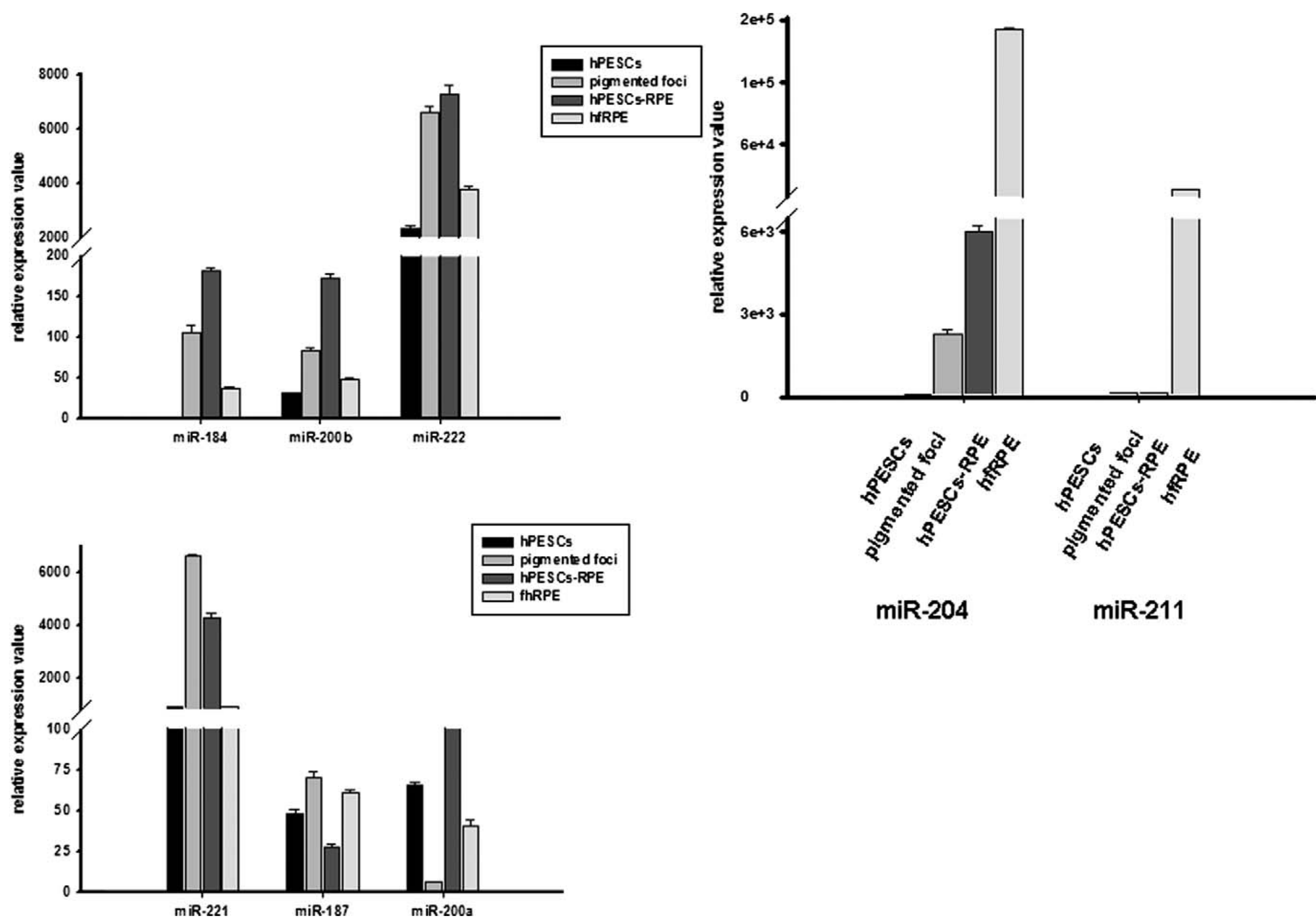

FIGURE 5. Real-time PCR analysis of RPE signature miRNAs (miRs-184, -187, -200a, -200b, -204, -211, -221, and -222) during differentiation from hPESCs into RPE cells.

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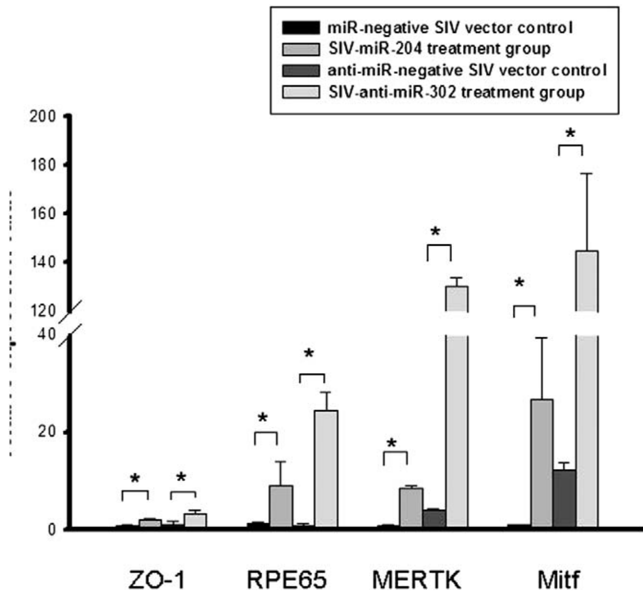
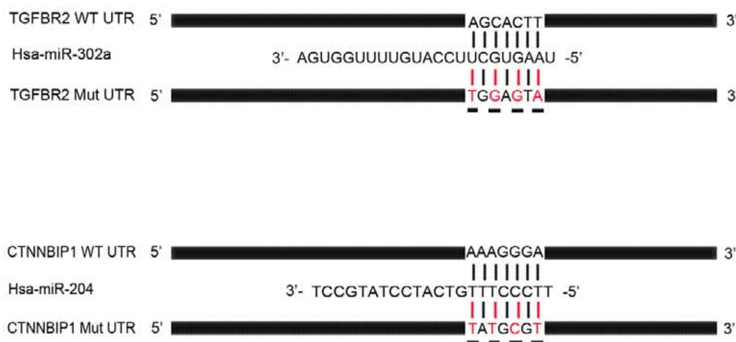


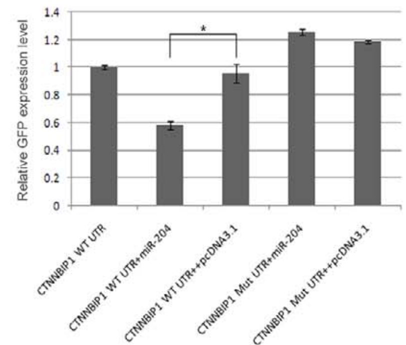
Figure 6. Real-time PCR analysis of RPE markers (*ZO-1, RPE65, MERTK, and Mitf*) in differentiating hPESCs with miR-204 overexpression and miR-302 inhibition at 5 weeks (* $P < 0.05$).

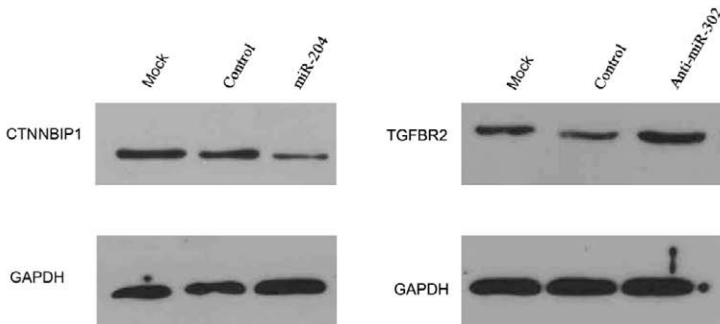
and miR-302 inhibition resulted in increased protein levels of *TGFBR2* in hPESCs (Fig. 7B). Subsequently, we performed a fluorescent reporter assay to confirm the direct regulation of *CTNNBIP1* and *TGFBR2* by miR-204 and miR-302. miR-204 and miR-302 were found to bind directly to the wild type 3′UTR, but not to the mutated *CTNNBIP1* and *TGFBR2* 3′UTR, and to suppress luciferase expression (Fig. 7C). These data indicated that *CTNNBIP1* and *TGFBR2* were the target genes of miR-204 and miR-302, respectively.

Discussion

A significant amount of research has focused on deriving RPE cells from stem cells and the resulting possible therapeutic interventions for macular degeneration diseases.[8–11] One of the best potential stem cell sources are hESCs; however, allogeneic embryonic stem cells (ESCs) are found to elicit vigorous immune response.[23,24] hPESCs isolated from parthenogenetic embryos carry haplotypes identical to those of the egg donor females, indicating that they can provide cells matched genetically with the recipient for clinical applications. The potentially disrupted expression of paternally imprinted genes does not interfere with parthenogenetic ESC (PESC) pluripotency. PESCs are similar to ESCs with respect to the expression of common pluripotency markers, self-renewal, and capacity to generate cell derivatives representative of all three germ layers.[25] In our study, hPESCs appeared to generate less







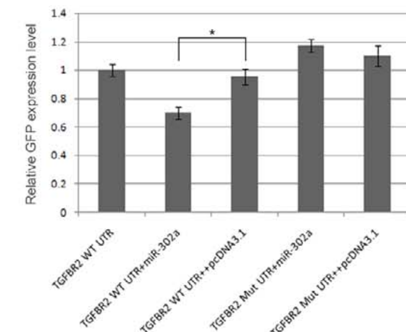


Figure 7. *CTNNBIP1* and *TGFBR2* as candidate target genes of miR-204 and miR-302, respectively. (**A**) The WT 3′UTR and mutated 3′UTR of *CTNNBIP1* and *TGFBR2* mRNA are shown. The red letters represent mutated nucleotides. (**B**) hPESCs were transfected with miR-204/anti-miR-302 lentivirals or control lentivirals, and the protein levels of *CTNNBIP1* and *TGFBR2* were measured by Western blot assay. (**C**) HEK293 cells were transfected with fluorescent reporter plasmids bearing the wild type (wt) or mutated (mut) 3′UTR along with the miRNA expression plasmid or control vector (pri-NC). After 48 hours, the fluorescence value was measured (* $P < 0.05$).

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pigmented clusters than hESCs in the report of Liao,[26] but resembled hESCs in the time-course of differentiation.

The trend of changes in the expression of appropriate markers for differentiation suggested that hPESC-derived RPE cells were in a relatively immature differentiation state. Optic marker expression (PAX6, RAX, and LHX2) was higher and RPE signature gene expression (*CRALBP*, *BEST*, *Mitf*, *RPE65*, *ZO-1*, and *MERTK*) was lower in hPESC-derived RPE cells than in hfRPE cells. Besides, most RPE signature miRNAs (miR-184, -200b, -222, and -204) increased during differentiation from hPESCs into hPESC-derived RPE cells. miR-204 kept on increasing from hPESC-derived RPE cells to hfRPE cells. All these miRNAs may promote differentiation but have different roles in the maturation of RPE cells.

miRNA expression occurred in a stepwise manner with the developmental time course of hPESC-derived RPE cells. Most miRNAs were upregulated or down-regulated only at certain stages of hPESC development. These miRNAs responded to the differentiation and maturation of RPE cells from hPESCs. miRNAs are difficult to control at the right time, so consistent miRNA changes throughout the whole process are good targets for differentiation improvement. In our study, we identified miR-204 and the miR-302 family whose expression showed a single trend.

MiR-204 was found to increase continuously during the entire differentiation process. miR-204 also was relatively enriched in hfRPE cells compared to other normal tissues. miR-204 was detected in the lens and ciliary body.[27–29] All these data suggested that a relatively high expression of miR-204 can drive the differentiation into epithelium and preserve the epithelial phenotype. Meis2 has been proven to be a qualified target of miR-204 activity. The miR-204-mediated regulation of Meis2 modulates the function of the PAX6 transcriptional network, which is an important element of the molecular network that regulates eye development among vertebrates.[30] Fujimura et al. found that Wnt/beta-catenin signaling was highly active in the dorsal RPE during eye development.[31] Using reporter gene assays, we provided evidence that the Wnt/beta-catenin pathway inhibitor *CTNNBIP1* is a direct target of miR-204. These data indicated that miR-204 upregulation can suppress direct targets constantly, which may activate the Meis2/Pax6 and Wnt/beta-catenin pathways. Consequently, the progression of differentiation of RPE cells from hPESCs is facilitated.

MiR-302s decreased sharply throughout the entire process of differentiation from hPESCs into RPE cells. miR-302s are expressed predominantly in hESCs and iPS cells, and are important in the maintenance of pluripotent stem cells.[32–34] miR-302s can suppress lysine-specific histone demethylases (AOF) and methyl CpG-binding proteins (MECP) to induce global demethylation and activate the coexpression of hESC-specific genes required for somatic cell reprogramming.[35] MiR-302s negatively modulate the level of the Nodal inhibitor lefty and become upstream regulators of the TGFβ/nodal pathway, functioning via Smad-2/3 signaling.[36,37] TGFβ superfamily members have been implicated to have a crucial role in directing mesodermal and endodermal fate during early embryogenesis.[38] *TGFBR2* is one of the miR-302 targets predicted by computer-aided algorithms, which we confirmed in our study. However, the ability of miR-302s in mediating *TGFBR2* in hPESCs to maintain the balance between pluripotency and germ layer specification remains unclear. Functional analyses must be conducted in further experiments.

Our experiments showed that hPESCs also can develop into RPE-like cells and, thus, can be promising sources of RPE cells for cell therapy. The upregulation of miR-204 or down-regulation of miR-302 contributed to the differentiation from hPESCs into RPE cells. Future works should analyze the function of miR-211 in the maturation process of hPESC-derived RPE cells to elucidate the use of obtaining more cells with genetic characteristics similar to primary RPE cells. Our findings contributed to the search for a new method of improving the efficiency of the stem-cell-derived RPE differentiation system using miRNAs.

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