• Basic Research •

A novel xeno-free culture system for human retinal pigment epithelium cells

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Abstract

• AIM: To find out an animal-free, xeno-free culture method for human fetal retinal pigment epithelium (fRPE) cells aiming for cell-replacement therapy.

• METHODS: Human AB serum, knock-out serum replacement (KSR) and B27 were supplemented as a substitute of fetal bovine serum (FBS) in culture medium of human fRPE cells. Cell morphology was examined by light microscope and transmission electron microscope. Proliferation ability was detected by cell cycle analysis and examination of KI67 expression. Apoptosis was analyzed using FACS. The expression of RPE-specific markers was demonstrated by quantitative real-time polymerase chain reaction (qPCR), Western blot (WB) and immunocytochemistry. Paracrine function was determined using enzyme-linked immunosorbent assay method.

• RESULTS: Our results indicated that the optimum concentration of KSR was 15%, the optimum concentration of B27 was 2%, and the optimum concentration of human AB serum was 10%. fRPE cells cultured in 15% KSR and 2% B27 media showed repressed propagation and differentiation ability, and gradually lost epithelial morphology and RPE function. While fRPE cells cultured in 10% human AB serum media showed a typical cobblestone morphology with pigmentation, elevated proliferation ability, satisfying paracrine function and expressed RPE-specific markers.

• CONCLUSION: Our study indicates that culturing fRPE cells in 10% human AB serum-supplemented medium is more favorable compared with KSR, B27 and traditional FBS-supplemented mediums when fRPE cells are to be applied in cell-based therapy.

• KEYWORDS: retinal pigment epithelium; human AB serum; cell-based therapy

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INTRODUCTION

Retinal degeneration diseases (RDDs), including retinitis pigmentosa, age-related macular degeneration, glaucoma, diabetic retinopathy and Stargardt disease, are leading causes of irreversible blindness worldwide. So far, the treatments available for RDDs showed limited efficacy. Because destruction of retinal pigment epithelium (RPE) cells are their common pathogenesis, recent studies have focused on RPE transplantation. Tremendous preclinical and clinical trials have demonstrated that RPE cell replacement therapy is a promising approach[1-2]. Human fetal retinal pigment epithelium (fRPE) cells seems to be the best bet for RPE cell replacement therapy, because they show full function of native RPE with no tumorigenicity[3-4]. However, traditional culture medium of fRPE contains fetal bovine serum (FBS)[4], which will not only bring about risk of zoonose and xenogeneic immune rejection after transplantation[5], but also lead to laboratory culture instability. Therefore, traditional cultured fRPE cells do not meet Good Manufacturing Practice (GMP) standard and cannot be used in clinical RPE transplantation. A propitiate animal-free culture system is needed for clinical application of fRPE cells transplantation.

Human AB serum is produced from blood bank-derived plasma, and is suggested to be an alternative to FBS in supporting mesenchymal stem/stromal cells (MSC) growth[6-7]. Knock-out serum replacement (KSR) was originally developed to establish a feeder-free culture system of embryonic stem cells (ESCs)[8]. KSR is demonstrated to outperforms FBS as a growth media supplements for culturing immature spermatogonial tissue[9]. B27 has been reported to propagate and sustain human RPE *in vitro*[10]. Nowadays, B27, human AB serum and KSR has been put into mass production and can be easily purchased. So we wondered which is the best addictive in supporting fRPE propagation and differentiation *in vitro*.

In this study, an xeno-free culture system for human fRPE was established by replacing FBS-supplemented media with human

Table 1 Information on reagents in xeno-free culture system

Name	Vender, catalog	Storage
MEM α modification	Sigma, M-4526	+4℃
N1 supplement	Sigma, N-6530	+4℃
Glutamin-penicillin-streptomycin	Sigma, G-1146	-20℃
Non essential amino acids	Sigma, M-7145	+4℃
Taurine	Sigma, T-0625	Room temperature
Hydrocoortisone	Sigma, H-0396	Dissolved in DPBS to 1000× solution and storage at -20℃
Triiodo-thyronin	Sigma, T-5516	Dissolved in DPBS (without Ca or Mg) to 10000× solution and storage at -20℃
B27	Gibco, 12587-001	-20℃
Fetal bovine serum, heat inactivated	Thermo scientific, SH3007003HI	-20℃
Knock-out serum replacement	Gibco, A3181501	-20℃
Human AB serum	Sigma, H6914	-20℃
DPBS (with Ca and Mg)	Gibco, 14040-133	+4℃
Tryple select enzyme	Gibco, A12859-01	+4℃
Cell start	Invitrogen, A10142-01	+4℃
Cryo-SFM	Promo cell, C-29912	+4℃

AB serum-supplemented media. fRPE cells which cultured in 10% human AB serum-supplemented medium exhibited morphology and function of native RPE. Our study provided a candidate cell source for clinical RPE replacement therapy.

MATERIALS AND METHODS

Ethical Approval Approval of permission to work with human fRPE was granted from the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. This study has been registered with ClinicalTrials.gov (No. NCT02868424), and Chinese Clinical Trial Registry (No. ChiCTR-OPC-15006757). All the aborted fetus were 11^{th} and 12^{th} weeks of gestation. Informed consents were signed by donors to ensure that donations were voluntary with no commercial purpose.

Human Fetal Retinal Pigment Epithelium Cells Culture Preparation and culture of human fRPEs cells were performed as described previously with modification[4]. In brief, the intact globes were curved out in Dulbecco's phosphate buffered saline (DPBS). Then eyes were dissected in a laminar flow hood with the aid of a microscope. The anterior segment, vitreous content and retina were removed. The RPE monolayer was carefully dissected and digested in tryple select enzyme for 7min before seeding. RPE cells were inoculated at the density of 3×10^5 per well in cell start-coated 6-well culture plates at 37℃ in a humidified atmosphere with 5% CO_2. Media was changed every three days. fRPE cells were expanded, examined and frozen after reaching confluence. Detailed information of medium contents and culture related reagents is shown in Table 1.

Western Blot Human fRPE and human adult RPE cell line (ARPE-19) were lysed in RIPA buffer (Sigma-Aldrich, St.Louis, Mo, USA) containing a proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein extracts (10-15 μg) were electrophorese using 4%-12% BisTris NuPAGE gel and blotted onto nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). The blots were incubated with antibodies against human RPE-specific 65-kDa protein (RPE65), pigment epithelium derived factor (PEDF), bestropohin 1 (BEST1) or cellular retinaldehyde-binding protein (CRALBP). β-actin were used as inner reference. A minimum of three experiments were performed.

Enzyme-Linked Immunosorbent Assay Three-day culture media were extracted when fRPE cells reaching confluence at P2. Protein levels of vascular endothelial growth factor (VEGF), PEDF, fibroblast growth factor-basic (FGF2), transforming growth factor beta (TGF-β) and beta nerve growth factor (β-NGF) were qualified by enzyme-linked immunosorbent assay (ELISA) kit (Qiaoyi, Anhui, China; USCN, Wuhan, China) using the manufacturer's protocol. Protein levels of cytokines in blank medium with no cells were also measured to exclude medium content influence. The plated RPE cells from each well were then dissociated and counted using a hemocytometer. A minimum of three assays were performed.

Quantitative Real-Time Polymerase Chain Reaction Total RNA was extracted using the Rneasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. To measure the expression of CRALBP, RPE65, BEST1 and PEDF, quantification was performed with a two-step reaction process: reverse transcription (RT) and polymerase chain reaction

Table 2 Primer information for quantitative real-time PCR

Gene	Sequence from (5'-3')	Product length (bp)
RPE65 (H) Fw	TACAGAAAGCACTGAGTTGAGC	154
RPE65 (H) Rev	CCATTTAGTAAGTCCACATTCATTTCC	
CRALBP (H) Fw	CAAAGCCATCCACTTCATCCACCA	162
CRALBP (H) Rev	AAGTCAGAGGGCAGGATGTTCTCA	
PEDF/SERPINF1 (H) Fw	TTATGAAGGCGAAGTCACCAAGTCCC	143
PEDF/SERPINF1 (H) Rev	CATCCTCGTTCCACTCAAAGCCA	
BEST1 (H) Fw	GTTTGCCAACCTGTCAATG	109
BEST1 (H) Rev	GTGTCCACACTGAGTACG	
GAPDH (H) Fw	GGAAGCTTGTCATCAATGGAAATC	168
GAPDH (H) Rev	TGATGACCCTTTTGGCTCCC	

Table 3 Information on the first fluorescent antibody

Antibody	Vender, Catalog	Concentration	City, State, Country	Host
RPE65	LifeSpan BioSciences, LS-C671938	1/100	Seattle, WA, USA	Rabbit
CRALBP	Abcam, ab15051	1/250	Cambridge, MA, USA	Mouse
BEST1	Abcam, ab2182	1/500	Cambridge, MA, USA	Mouse
MERTK	Abcam, ab5110108	1/100	Cambridge, MA, USA	Goat

(PCR). Real-time PCR was performed using LightCycler® 480 II real-time PCR instrument (Roche, Swiss). At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The primer sequences were designed in the laboratory and synthesized by Generay Biotech (Generay, PRC) based on the mRNA sequences obtained from the NCBI database as shown in Table 2. The expression levels of mRNAs were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method. Three samples were analyzed in each group.

Fluorescence-Activated Cell Sorting Approximate 2×10^5 of human fRPE cells were digested with tryple select enzyme. Then cells were fixed and permeated with Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Next, cells were stained with KI67 anti-bodies (BioLegend, San Diego, CA, USA). Negative control was set up by staining corresponding isotype control. The cells were washed twice before being analyzed on a fluorescence-activated cell sorter (FACS; Beckman, S.Kraemer Boulevard Brea, CA,USA). Fluorescence intensity was expressed as arbitrary units on a log scale. Histogram overlays were made with the use of the analyze software Flow Jo. Cell cycle analysis was conducted with a cell cycle assay kit (Keygen Biotech, Nanjing, Jiangsu, China). Cell apoptosis assay using Annexin V/PI staining (BD, Franklin Lakes, NJ, USA) was conducted following kit protocols. A minimum of three assays were performed.

Immunocytochemistry The expression of CRALBP, MER proto-oncogene tyrosine kinase (MERTK), BEST1, RPE65 were determined by indirect immunofluorescence. Briefly, cells were cultured on cell start-coated cell chamber slides (Thermo Fisher Scientific, Lab-Tek, Waltham, Massachusetts, USA) at the density of 3×10^4 per well for more than three weeks until pigment was shown. Then slides were washed with phosphate buffer solution (PBS) for three times and fixed in 4% paraformaldehyde at room temperature for 30min. After being washed with PBS three times again, slides were treated with PBST (PBS with 0.1% tween) on ice for 10min. Then slides were blocked in 5% bull serum albumin (BSA) for 1h at room temperature. Incubate in first antibody for 2-3h at 37℃. Wash thoroughly with PBS then incubate in second antibody for 1h at room temperature, followed by the addition of DAPI-Fluoromount-G (Southern Biotech, Birmingham, Alabama, USA) to preserve immunofluorescent labels. Information on the primary antibody is provided in Table 3.

Transmission Electronic Microscope Cells were fixed in 2.5% glutaraldehyde solution, then epoxy resin-embedded after dehydration. Samples were sliced before examined by a transmission electronic microscope (JEOL, Japan). Selected areas were trimmed for ultrathin sectioning and stained with uranyl acetate before electron microscopy observation.

Statistical Analyses Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc, La Jolla, CA, USA). The values are expressed as the mean±standard deviation (SD), with $P<0.05$ being considered statistically significant. Significance was assessed using the paired and unpaired Student's *t*-test (Prism 7.0). One-way ANOVA followed by Scheffe's test was performed.

RESULTS

Optimum Concentration of B27, KSR and Human AB Serum fRPE cells were cultured in 10% FBS from passage 0 (P0). Culture mediums were changed into mediums

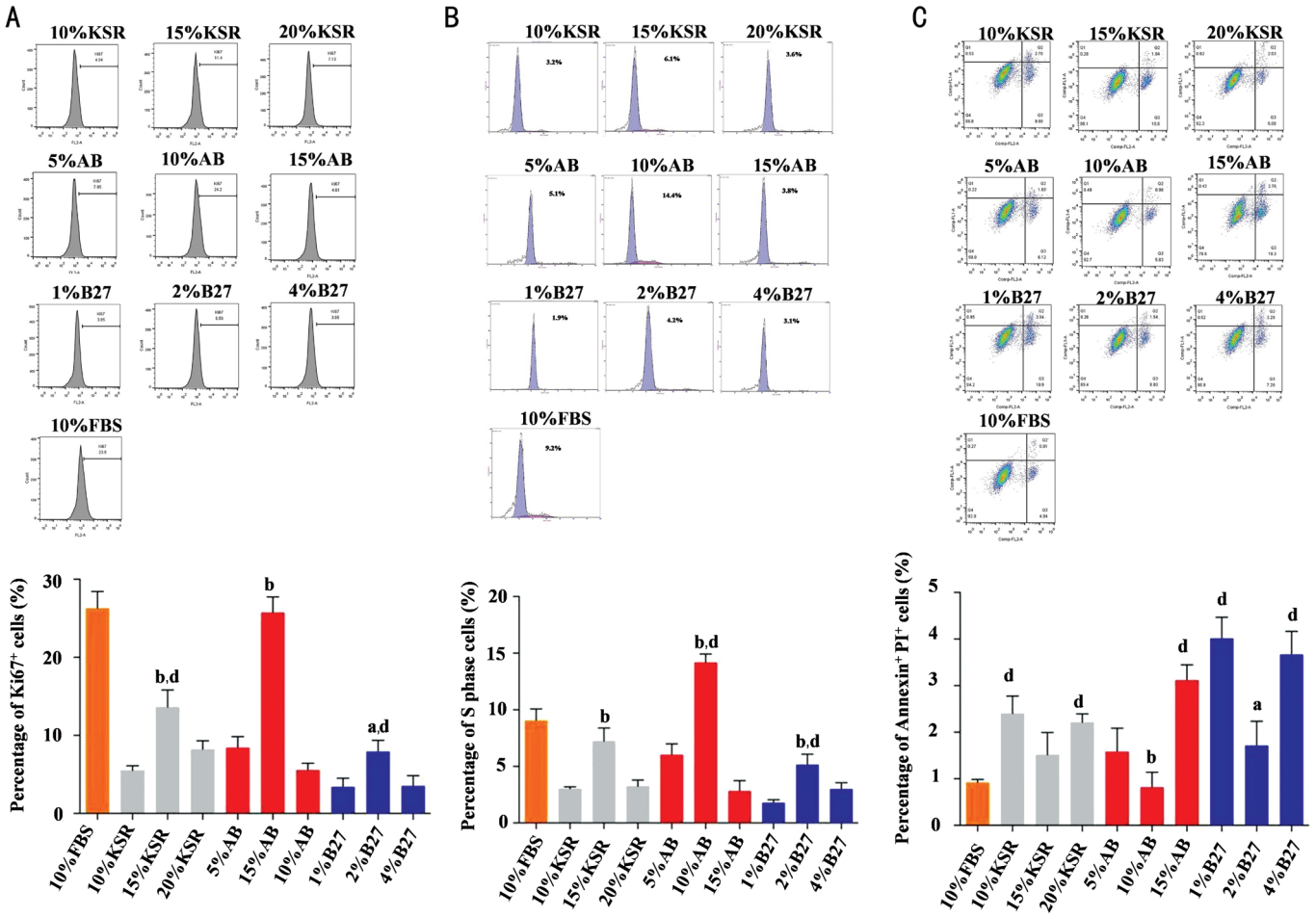

Figure 1 Optimum concentration of B27, KSR and human AB serum A: FACS outcomes showed the KI67$^+$ cells was significantly increased in the 10% AB serum group 15% KSR group, and 2% B27 group. The KI67$^+$ cells was significantly reduced in the 15% KSR group and 2% B27 group compared to 10% FBS group. B: The S phase cells was significantly increased in the 10% AB serum group, 15% KSR group, and 2% B27 group. Besides, the S phase cells was significantly increased in 10% AB serum group and significantly reduced in 2% B27 group compared to 10% FBS group. C: The apoptotic cells was significantly reduced in the 10% AB serum group, and 2% B27 group. The apoptotic cells was significantly elevated in the 10% KSR, 20% KSR, 15% AB serum, 1% B27 and 4% B27 groups when compared to 10% FBS group. [a]P<0.01, [b]P<0.001 *vs* corresponding concentration of KSR, B27 and AB serum groups respectively; [d]P<0.001 *vs* 10% FBS group. All experiments were repeated three times; error bars indicate SD.

supplemented with different concentrations of B27, human AB serum and KSR after seeding at P2. The optimum working concentration of B27, human AB serum and KSR was determined by cell cycle, apoptosis and the expression of KI67 (Figure 1). Our results showed that the percentage of KI67$^+$ cells was significantly increased in the 15% KSR group (13.6%) 10% AB serum group (25.7%), and 2% B27 group (7.95%). The percentage of KI67$^+$ cells was significantly reduced in 15% KSR group and 2% B27 group when compared to 10% FBS group (26.3%). There was no significant difference in the percentage of KI67$^+$ cells between 10% AB serum group and 10% FBS group (Figure 1A). The percentage of cells in S phase was significantly increased in the 15% KSR group (7.2%), 10% AB serum group (14.2%), and 2% B27 group (5.17%). The cells in S phase was significantly reduced in 2% B27 group and significantly increased in 10% AB serum group compared to 10% FBS group (9.06%). There was no significant difference in S phase cells between 15% KSR group and 10% FBS group (Figure 1B). FACS results indicated that the proportion of apoptotic cells which positively stained with Annexin and PI was significantly decreased in 10% AB serum group (1.34%), and 2% B27 group (1.71%). Additionally, the apoptotic cells was significantly increased in 10% KSR group (2.4%), 20% KSR group (2.21%), 15% AB serum group, 1% B27 group and 4% B27 group (3.67%) compared to 10% FBS group (0.92%; Figure 1C).

Taken together, the optimum concentration of KSR was 15%, the optimum concentration of human AB serum was 10%, and the optimum concentration of B27 was 2%. What's more, the proliferation ability of fRPE cells was elevated in 10% AB serum group and repressed in 15% KSR and 2% B27 groups when compared to 10% FBS group.

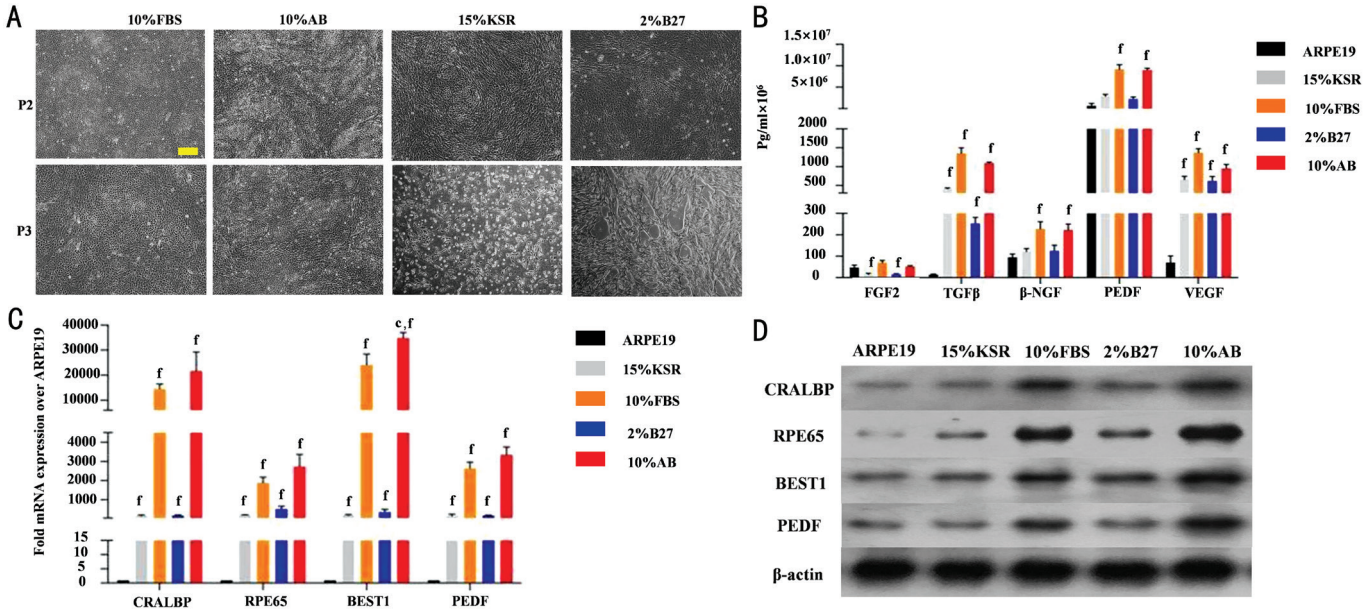

Figure 2 Differentiation and function of fRPE cells in B27, KSR and human AB serum A: Light microscope images of fRPE cells cultured in 2% B27, 15% KSR, 10% human AB serum and 10% FBS-supplemented mediums at P2 and P3. Scale bar=20 μm. B: ELISA results showing the secreted protein level of fRPE-derived trophic factors (FGF2, TGFβ, β-NGF, PEDF and VEGF). C, D: qPCR and Western blot analyses of RPE-specific markers (CRALBP, RPE65, BEST1, PEDF) in ARPE19 cells as well as fRPE cells cultured in 2% B27, 15% KSR, 10% human AB serum and 10% FBS-supplemented mediums at P2. $^{f}P<0.001$ *vs* ARPE19 cells; $^{c}P<0.01$ *vs* 10% FBS group. All experiments were repeated three times; error bars indicate SD.

Differentiation and Function of fRPE Cells in B27, KSR and Human AB Serum fRPE mediums were changed into 10% FBS, 10% AB serum, 15% KSR and 2% B27-supplemented mediums respectively at P1 to investigate the differentiation potential of fRPE cells. Light microscope images showed fully differentiated fRPE cells with typical cobblestone morphology and pigmentation in all groups at P2. However, many cells in 15% KSR group stretched and dedifferentiated into fibroblast-like cells. Many cells in 2% B27 group did not show hexagon morphology and pigmentation. Additionally, fRPE cells in 10% AB serum group packed more closely and the morphology of fRPE cells in 10% AB serum group were more uniform than in 10% FBS group. At P3, fRPE cells in 15% KSR group were detached and fRPE cells in 2% B27 serum group failed reaching confluence. While fRPE cells in 10% AB serum group and 10% FBS group exhibited a typical epithelial morphology with pigmentation (Figure 2A).

We examined the paracrine function of fRPE cells in all groups by detecting the protein level of RPE-secreted growth factors (VEGF, PEDF, FGF2, TGF-β and β-NGF) in culture mediums at P2. ARPE19 was examined as control. ELISA results showed that the secretion of FGF2, TGFβ, β-NGF, PEDF and VEGF were significantly reduced in 15% KSR group and 2% B27 group compared to 10% FBS group ($P<0.05$) as well as 10% AB serum group ($P<0.05$). There was no significant difference of FGF2, TGFβ, β-NGF, PEDF and VEGF between 10% FBS group and 10% AB serum group (Figure 2B). Then we examined fRPE cell function by detecting the expression level of RPE signature markers (CRALBP, BEST1, PEDF and RPE65). The expression of CRALBP, BEST1, PEDF and RPE65 were significantly elevated in 10% FBS group and 10% AB serum group compared to 15% KSR group ($P<0.05$) as well as 2% B27 group ($P<0.05$). Besides, the expression of CRALBP, BEST1, PEDF and RPE65 were slightly increased in 10% AB serum group than 10% FBS group. ARPE19 cells were examined as control (Figure 2C, 2D).

In summary, fRPE cells were fully differentiated and showed native RPE morphology and function in 10% human AB serum-supplemented medium at P2 and P3. While fRPE cells in 15% KSR medium and 2% B27 medium gradually lost RPE morphology and function, and could only be passaged to P2.

Xeno-free Culture System-Generated fRPE Cells In order to verify the efficacy of 10% human AB serum culture system, we cultured fPRE cells in 10% AB serum-supplemented xeno-free culture medium from P0. Pigmented cobblestone-like fRPE cells can be produced from P0 to P3. At P4, fRPE cells dedifferentiation into spindle-shaped cells resembling fibroblasts and failure to reach confluency (Figure 3A). Electron micrograph of xeno-free culture system-generated fRPE cells showed RPE-specific structures at P3: apical microvilli, melanosomes and tight junctions (Figure 3B). Immunocytochemical staining demonstrated that fRPE cells can fully differentiated and expressed RPE-specific markers CRALBP, RPE65, MERTK and BEST1 at P3 (Figure 3C).

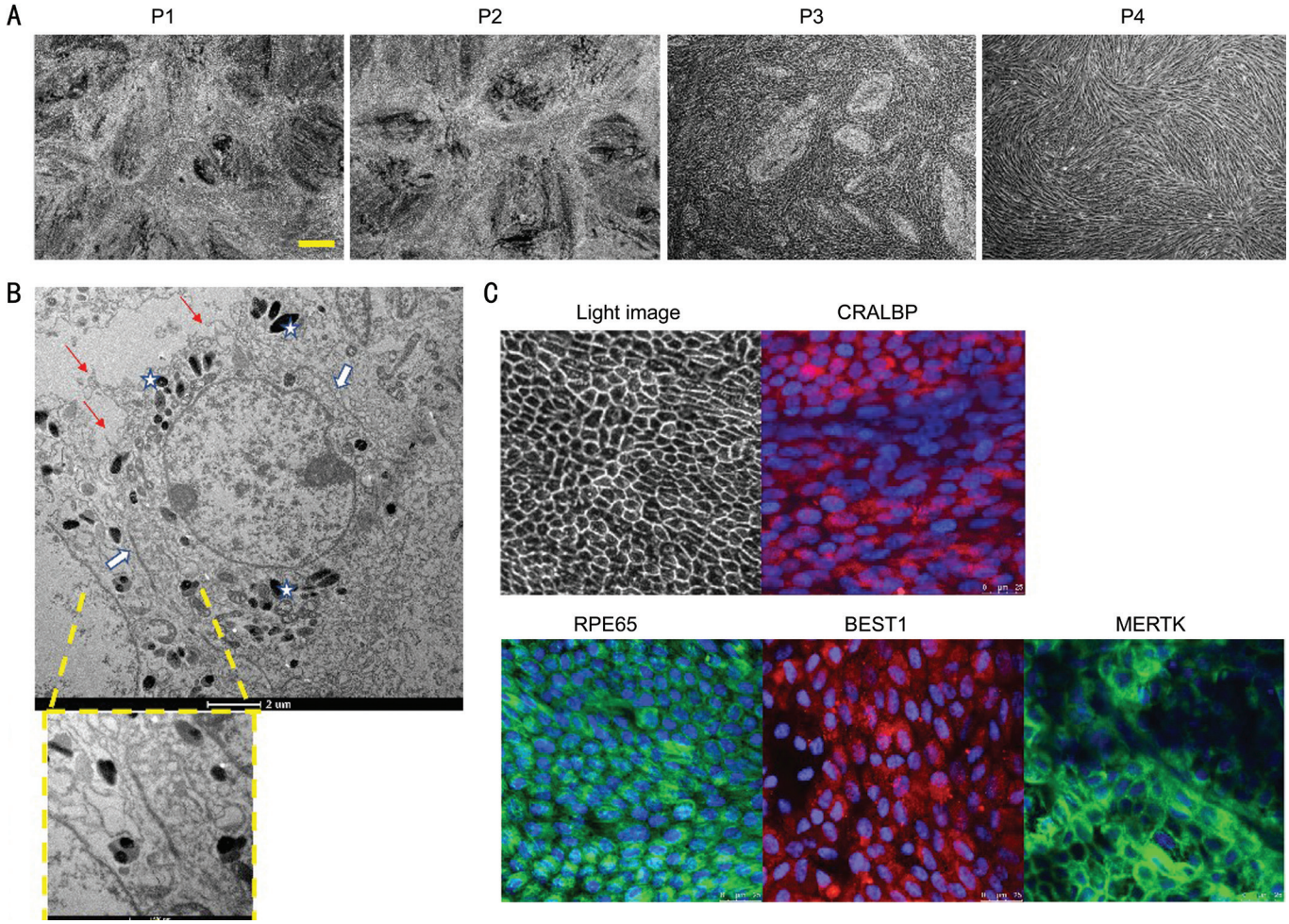

Figure 3 Characterization of xeno-free culture system-generated fRPE cells A: Light microscope images showing the morphology and density of fRPE cells cultured in 10% AB serum mediums for three weeks at P2, P3 and P4. Scale bar=20 μm. B: Electron micrograph showing cell morphology and structure of xeno-free culture system-generated fRPE cells. RPE-specific structures are highlighted: apical microvilli (red arrows), melanosomes (white stars) and tight junctions (white arrows). Scale bars represent 2 μm and 500 nm respectively. C: Fluorescence microscope view showing the expression of RPE-specific markers: CRALBP, RPE65, BEST1 and MERTK. Scale bar=25 μm.

DISCUSSION

Our findings provided a novel xeno-free culture system for human fRPE cells to meet GMP standard, so that fRPE cells can be transplanted to treat RDDs patients. To replace FBS, B27, human AB serum and KSR were added into culture medium as substitutes. We first screened out the optimum working concentration of B27, AB serum and KSR by examining proliferation ability and apoptosis of fRPE cells (Figure 1). Then we compared morphology and function of fRPE cells between FBS group and B27, AB serum, KSR groups. Paracrine function is known as one of RPE critical functions, because many functions of RPE cells attribute to RPE trophic factors secretion: TGF-β plays a pivotal role in immune privilege[11], PEDF, β-NGF and FGF2 promote RPE proliferation and protect RPE from oxidative stress[12-14], PEDF, TGF-β and VEGF regulate angiogenesis[15], PEDF, FGF2 and β-NGF support photoreceptor[16-17]. What's more, studies have demonstrated photoreceptor rescue effect by exogenous trophic factor administration in RDDs animal models: FGF2 injection preserved degenerated photoreceptors[18]; NGF regressed retinal degeneration by stimulation of other endogenous biological mediators[19]; PEDF showed antiapoptotic effect on photoreceptors[20]. Other functions of fRPE cells were detected by examining the expression of typical RPE markers: the expression of RPE65 and CRALBP associates with retinoid cycle, the expression of BEST1 associates with chloride channels, the expression of PEDF associates neurotrophic and anti-angiogenic effect. Our results revealed that fRPE cells cultured in 15% KSR and 2% B27 media showed repressed propagation and differentiation ability. These fRPE cells gradually lost epithelial morphology and RPE function, and could only survive to P2 (Figure 2).

Finally, we generated fRPE cells using 10% human AB serum-supplemented culture medium from P0. A typical cobblestone morphology with pigment was shown in fRPE cells from P0 to P3. At P4, fRPE cells underwent epithelial to mesenchymal transition (EMT) by dedifferentiation into spindle-shaped cells resembling fibroblasts and failure to reach confluency. EMT

can also be observed in intraocular fibrotic diseases (such as epiretinal membrane), traditional cultured fRPE cells and RPE cells generated from pluripotent sources (including embryonic stem cells and pluripotent stem cell)[21-23]. This transition is result from wound-healing response and higher oxidative stress *in vitro* culture progress, as well as unknown hormones, cytokines and growth factors in culture mediums[24-25]. Studies have elucidated that epithelial-mesenchymal transition (EMT) can be repressed by adding TGF-β receptor inhibitor and Rho-associated and coiled-coil protein kinase (ROCK) inhibitor into RPE culture medium[22,26]. Additionally, the expression of RPE-specific markers was demonstrated and functional RPE structure can be observed: apical microvilli help phagocytosing shed photoreceptor outer segments, tight junctions help building blood-ocular barrier (Figure 3).

In summary, our study indicated that culturing fRPE cells in 10% human AB serum medium was more favorable compared with KSR medium, B27 and traditional FBS medium when fRPE cells are to be applied in cell-based therapy. Further study will be needed to confirm the safety and effectivity of xeno-free culture system-generated fRPE cell transplantation in treating RDDs.

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