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Original Article

Plasticity of adult human retinal pigment epithelial cells

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Abstract: Retinal pigment epithelium (RPE) cell damage underlies many degenerative, dystrophic, and proliferative diseases of the human retina. Primary adult human RPE cells can be used for *in vitro* modeling of RPE cell behavior under pathological conditions. We demonstrated previously that Wnt7a inhibits adult human RPE cell proliferation, and initiates the spread and polarization of dedifferentiated RPE cells on a plastic surface. Here, we investigated the role of Wnt7a in the regulation of RPE cell plasticity, at cellular and molecular levels. Protein expression and localization were examined by immunofluorescence analysis, while mRNA expression was quantified by real-time PCR. The obtained results showed that Wnt7a influences the expression of a range of neural differentiation markers (nestin, MAP1B, β-tubulin III, synapsin I, 200 kDa and 68 kDa neurofilaments), and increases the expression of various RPE differentiation markers (RPE65, MITF, OTX2, and Pax6). Our findings indicate that Wnt7a exerts a pleiotropic effect on RPE cells *in vitro*, stimulating redifferentiation (RPE properties), while maintaining neural differentiation indicators. Although this effect was shown to be reversible, Wnt7a may act as a regulator of RPE cell plasticity, and may represent a potential therapeutic target for the prevention of RPE transformation.

Keywords: Human retinal pigment epithelium, cell culture, recombinant human protein Wnt7a, immunocytochemistry, signal paths, transdifferentiation

Introduction

Retinal pigment epithelium (RPE) cell damage underlies many degenerative, dystrophic, and proliferative diseases of human retina [1, 2]. RPE cells are essential for retinal function and they are highly specialized and do not proliferate under normal conditions. Their behavior changes dramatically in response to pathological stimuli. Processes similar to dedifferentiation or transdifferentiation are activated in RPE cells and are accompanied by changes in cellular phenotype, such as distortion of intercellular contacts, proliferation, loss of pigmentation, and cell migration [3]. Altered RPE cells damage the retina and other eye structures, often impairing vision or leading to blindness [3-7].

Recently, it has become clear that RPE is formed by heterogeneous cells in mammals and humans [8, 9], which is obvious in a cell culture [10]. Human RPE cells can enter the cell cycle *in vitro*, grow while dedifferentiated, and demonstrate behavioral differences and the signs of dedifferentiation and transdifferentiation. Some of these cells possess multipotent cell properties, but are not typical pluripotent or neural stem cells. They represent a special type of RPE stem cells, which can generate both neural-like and mesenchymal-like cells [10-12]. Mesenchymal differentiation of RPE stem cells would result in their migration, which could cause various pathological changes in the retina. The ability for neural differentiation *in vitro* leads to the conclusion that certain factors may block transdifferentiation of RPE cells into neural retinal cells in the adult human eye [13]. The introduction of specific genes into mammalian and human RPE cells demonstrated that higher vertebrate RPE cells possess an unusual reprogramming potential, and form photoreceptors [14] and other retinal neurons [15], and that it is possible to activate neuronal properties in human RPE cells.

Regulation of RPE plasticity and differentiation under pathological conditions is interesting for both basic and applied research [5, 16-18]. The activation of the neuronal properties of RPE cells may be promising for the suppression of mesenchymal plasticity [2, 13], and the restoration of damaged retina [17]. Therefore, the effects of exogenous factors on signaling pathways [17] involved in the regulation of neuronal cell differentiation should be elucidated.

RPE and neural retina originate from the same neuroepithelial stem cells of the anterior cerebral vesicle. The differentiation of precursor cells into the neural and pigment cells of the neuroepithelium involves the TGF-β/BMP, Wnt, Notch, FGF, and Shh signaling pathways [2, 19-21]. BMPs released from the surface ectoderm, primarily BMP4 and BMP7, play a role in the early RPE specialization [22, 23]. They maintain the polarity of epithelial cells via intracellular BMP receptor signaling, which occurs on the basolateral surface of the cell membrane [24]. BMP signaling pathway proteins prevent the neuronal differentiation of neuroepithelial cells, which leads to their specialization into RPE cells. This effect is facilitated by the activation of Notch signaling, which is involved in the maintenance of the neural stem cell pool [25-27]. Notch and BMP signaling pathways are involved in a cross-talk with Wnt signaling pathway [28]. Activation of the canonical Wnt/β-catenin signaling cascade by exogenous Wnt proteins stimulates healing after various injuries, including bone fractures, retinal lesions, skin wounds, and myocardial infarction, and this has been demonstrated in several animal studies [29]. Osakada et al. [30] have shown that Wnt3a-induced activation of the Wnt signaling pathway, following retinal damage or degeneration, facilitates the proliferation of Müller glial cells and regeneration of neural progenitor cells in mice. The addition of Wnt3a to the culture medium of the spontaneously transformed RPE cell line ARPE19, improved the viability of these cells in the presence of cytotoxic agents, compared with the control cells [31]. Studies using animal models have shown that the activation of the canonical Wnt/β-catenin signaling pathway in RPE cells stimulates pathological processes in age-related macular dystrophy and diabetic retinopathy [32], and leads to the formation of contractile membranes by dedifferentiated RPE cells in the proliferative vitreoretinopathy [33]. Blocking of the Wnt/β-catenin signaling pathway in genetically modified mice leads to the transdifferentiation of RPE to neural retina, which is confirmed by a downregulation of RPE-specific markers, such as MITF and OTX2, and ectopic expression of neural retina-specific markers [21]. The effects of different Wnt ligands on RPE cells have not been characterized to date.

The effects of Wnt7a, a Wnt protein that maintains neural differentiation, should be investigated in this context. Wnt7a is involved in the regulation of neural stem and progenitor cells, the development of their dendrites and synapses, and axon growth [29, 34-36]. It induces human mesenchymal stem cell transdifferentiation to neural cells, by stimulating the expression of synapsin I, a mature neuron marker, during the formation of cholinergic and dopaminergic neurons [29]. However, very little is known about the role of Wnt7a in the determination of the fate of dedifferentiated human RPE cells. We previously showed that Wnt7a inhibits the proliferation of adult human RPE cells, and initiates the spreading and polarization of dedifferentiated RPE cells on a plastic surface [37]. Here, we aim to elucidate further Wnt7a roles in the regulation of RPE cell plasticity, at cellular and molecular levels. Our results show that Wnt7a changes the expression of BMP isoforms, Notch signaling pathway proteins, a range of neural differentiation markers (nestin, MAP1B, β-tubulin III (TUBB3), synapsin I, 200 kDa and 68 kDa neurofilaments (NFs)), and increases the expression of various RPE differentiation markers (RPE65, MITF, OTX2, and Pax6). Our findings indicate that Wnt7a exerts a pleiotropic effect on RPE cells *in vitro*, stimulating redifferentiation and maintaining neural differentiation. Although this effect was shown to be reversible, possibly due to the microenvironmental factors, Wnt7a may act as a regulator of RPE cell plasticity.

Materials and methods

Cell culture

Donor eyes were obtained from the Moscow Helmholtz Eye Bank, Moscow, Russia. The design of this study and all experimental procedures have been approved by the Clinical Research Ethics Board of the Moscow Helmholtz Research Institute of Eye Diseases, Moscow, Russia. Written informed consent was obtain-

ed for all specimens, in accordance with the principles outlined in the Declaration of Helsinki. Human eyes from three donors were delivered to a scientific laboratory within 15-20 h following the donors' death. Donor age ranged from 41 to 59 years, with a mean age of 50. RPE cells were isolated from the eyeballs as described previously [10]. Cells were washed, collected by centrifugation, and resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, GE Healthcare, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Pan-Biotech, Aidenbach, Germany). RPE cells were cultured to a confluent monolayer in an atmosphere containing 5% CO_2 at 37°C; the medium was changed two or three times per week. Confluent cultures were passaged using trypsin-EDTA solution (Paneco, Moscow, Russia). RPE cells obtained after three passages were used in the experiments. Cells were maintained in the same medium, but with a lower FBS content (2%), and treated with 60 ng/mL recombinant human Wnt7a (Sigma-Aldrich). Cells cultured in the absence of Wnt7a were used as a control.

To assess morphological changes, RPE cells were seeded in CELLSTAR® Standard Cell Culture Flasks with growth areas of 25 cm^2 (Greiner Bio-One, Germany). Cellular behavior was observed using an Olympus CKX31 inverted microscope (Japan) at 24, 48, and 72 h after seeding, in a medium with or without (control) Wnt7a. Cell images were obtained using an Olympus microscope with a DP70 digital camera. During the incubation, the medium was not changed for 72 h.

Cell morphology analysis

The analysis of cell morphology was performed by processing JPEG format images with ImageJ 1.48 software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA), which computed the projected area of a cell onto the support (hereafter referred to as area), the cell perimeter (perimeter), and the major (M) and minor (m) axis lengths of an ellipse best fitting the cell shape [38, 39].

The spreading coefficient (Rp/Ra) is ratio of radii calculated from the known perimeter (Rp) and area (Ra) of the measured cell [39]. The spreading (Rp/Ra) and polarization (M/m) coefficients [38, 39] and statistical parameters were calculated using Excel 2007 (Microsoft Corporation, USA). Additionally, the percentage of spread cells in the total number of attached cells was determined by counting spread and non-spread cells. Cells were considered to be non-spread if they had a regular round shape, a shiny surface, and no protrusions; cells were considered to be spread when they were irregular in shape (including elongated and polygonal ones) and had a number of protrusions. Three independent field images, with total magnification of 100× (objective 10×, eyepiece 10×), were examined in each of the investigated groups. The results are expressed as mean ± standard deviation (SD); statistical significance (Student's t-test) was accepted at *P< 0.01 and **P<0.05.

Immunocytochemistry

Immunocytochemistry (ICC) was performed using RPE cells subcultured on 4-well culture slides (BD Falcon™, BD Biosciences, USA). RPE cells were seeded at $4.5\times10^4/cm^2$ or $2.5\times10^4/cm^2$ in DMEM/F12 containing 2% FBS with or without (control) 60 ng/mL Wnt7a, cultured for 24 or 72 h, respectively, and fixed with cold acetone for 5 min. The prepared samples were stored at -70°C until further analysis.

The differentiation of RPE cells was assessed by immunofluorescence, using Abcam (Cambridge, UK) anti-HES1 (ab87395, mouse monoclonal [4H1], 1:100), anti-HES5 (ab107593, rabbit polyclonal, 1:100), anti-Hey1 (ab22614, rabbit polyclonal, 1:300), anti-BMP2 (ab14933, rabbit polyclonal, 1:250), anti-BMP7 (ab54904, mouse monoclonal, 1:250), anti-nestin (ab22035, mouse monoclonal [10C2], 1:100), anti-β-tubulin III (TUBB3, ab7751, mouse monoclonal [TU-20], 1:100), anti-MAP1B (ab3095, mouse monoclonal [3G5], 1:100), anti-200 kDa and 68 kDa neurofilaments (NFs, ab15234, mouse monoclonal [SPM145], 1:20), anti-synapsin I (ab8, rabbit polyclonal, 1:125), anti-GFAP (ab7260, rabbit polyclonal, 1:150), anti-E-cadherin (ab76055, mouse monoclonal [M168], 1:75), anti-β-catenin (ab32572, rabbit polyclonal [E247], 1:250), and anti-FAK (focal adhesion kinase, ab40794, rabbit polyclonal [EP695Y], 1:250) primary antibodies, and Sigma-Aldrich anti-BMP4 (SAB2700755, rabbit polyclonal, 1:100), and anti-Wnt7a (HPA015719, rabbit poly-

Table 1. List of primers used for qPCR

Gene (Ref_Seq)	Primer sequence or assay_id	Amplicon size, bp
RPE65 (NM_000329)	F-5'-GCCTACAACATTGTAAAGATCCCA-3'	63
	R-5'-ACGTAAGATGGCTTGAATCGGT-3'	
	Probe-5'-FAM-CACTGCAAGCAGACAAGGAAGATCCA-BHQ1-3'	
MITF (NM_198159)	F-5'-CACCAGCCATAAACGTCAGTGT-3'	87
	R-5'-CGAGGTGGGTCTGCACCTT-3'	
	Probe-5'-FAM-CCACCACCCTTCCCTCTGCCAC-BHQ1-3'	
OTX2 (NM_001270525, NM_021728)	F-5'-ATGGACTTGCTGCACCCCT-3'	109
	R-5'-CAAACAGTGCTTCCAGCACATC-3'	
	Probe-5'-VIC-GGGCTACCCGGCCACCCC-BHQ1-3'	
Pax6 (NM_001604)	F-5'-CAATTCCACAACCCACCACAC-3'	88
	R- 5'-CTGTAGGTGTTTGTGAGGGCTGT-3'	
	Probe-5'-FAM-TCCTCCTTCACATCTGGCTCCATGT-BHQ1-3'	
TUBB3 (NM_006086)	F-5'-GGGCCAAGTTCTGGGAAGTC-3'	71
	R-5'-CGAGTCGCCCACGTAGTTG-3'	
	Probe-5'-FAM-ATGAGCATGGCATCGACCCCAGC-BHQ1-3'	
NES (NM_006617.1)	Hs04187831_g1	58
NOTCH1 (NM_017617.3)	Hs01062014_m1	80
JAG1 (NM_000214.2)	Hs01070032_m1	72
HEY1 (NM_001040708.1, NM_012258.3)	Hs01114113_m1	82
HES1 (NM_005524.3)	Hs00172878_m1	78
BMP2 (NM_001200.2)	Hs00154192_m1	60
BMP4 (NM_001202.3, NM_130850.2, NM_130851.2)	Hs00370078_m1	58
BMPR2 (NM_001204.6)	Hs00176148_m1	69
GAPDH (NM_002046)	GAPDH_oligos	220

clonal, 1:100) antibodies. All antibodies were diluted with 0.1% BSA (Sigma-Aldrich).

The treated and control RPE cell samples were treated in parallel for subsequent comparisons, with an Image-iT™ FX signal enhancer, obtained from the SFX kit (Molecular Probes, USA) for 30 min, in order to block nonspecific binding. Furthermore, they were incubated with a primary antibody at 4°C overnight, treated with the appropriate secondary antibody labeled with Alexa Fluor® 488 (A31619) or Alexa Fluor® 594 (A31631) fluorochrome (1:300, Molecular Probes) at room temperature for 40 min, and mounted in a medium containing a nuclear dye, 4',6-diamidino-2-phenylindole (DAPI, Vectashield®, Vector Laboratories, USA).

The ICC samples were observed and imaged using an Olympus microscope with a DP70 or Keyence BZ-9000E (Japan) digital camera.

Quantitative real-time PCR (qPCR)

Total RNA was obtained from the freshly isolated RPE cells (from the cells isolated from an eyeball, a RPE tissue sample) or from the cultured cells, with the TRI® reagent (Sigma-Aldrich), according to the manufacturer's instructions. For cultured cells, a lysing solution was added to T25 flasks 24, 48, and 72 h after seeding of the cells in a medium with or without (control) Wnt7a. cDNA synthesis was performed in a reaction volume of 20 µL with 1 µg of DNase-treated total RNA using a RevertAid H Minus kit (Fermentas, Lithuania).

The changes in mRNA expression of the following transcription factors, signaling proteins, and differentiation markers were determined by qPCR: *RPE65*, *MITF*, *OTX2*, *Pax6*, *TUBB3* (primers and probes, DNK-Sintez, Moscow, Russia), *NES*, *NOTCH1*, *JAG1*, *HEY1*, *HES1*, *BMP2*, *BMP4*, *BMPR2* (primers and probes, Applied Biosystems, Thermo Fisher Scientific, USA). The qPCR mixture (25 µL) contained 2 mM $MgCl_2$ (Fermentas), 200 µM dNTPs (Fermentas), 1 unit of Maxima HS Taq polymerase (Fermentas), 2.5 µL of diluted cDNA (equivalent to 4 ng of the original total RNA), 250 nM primers, and 250 nM probe (**Table 1**). The reaction was performed using the ABI Prism

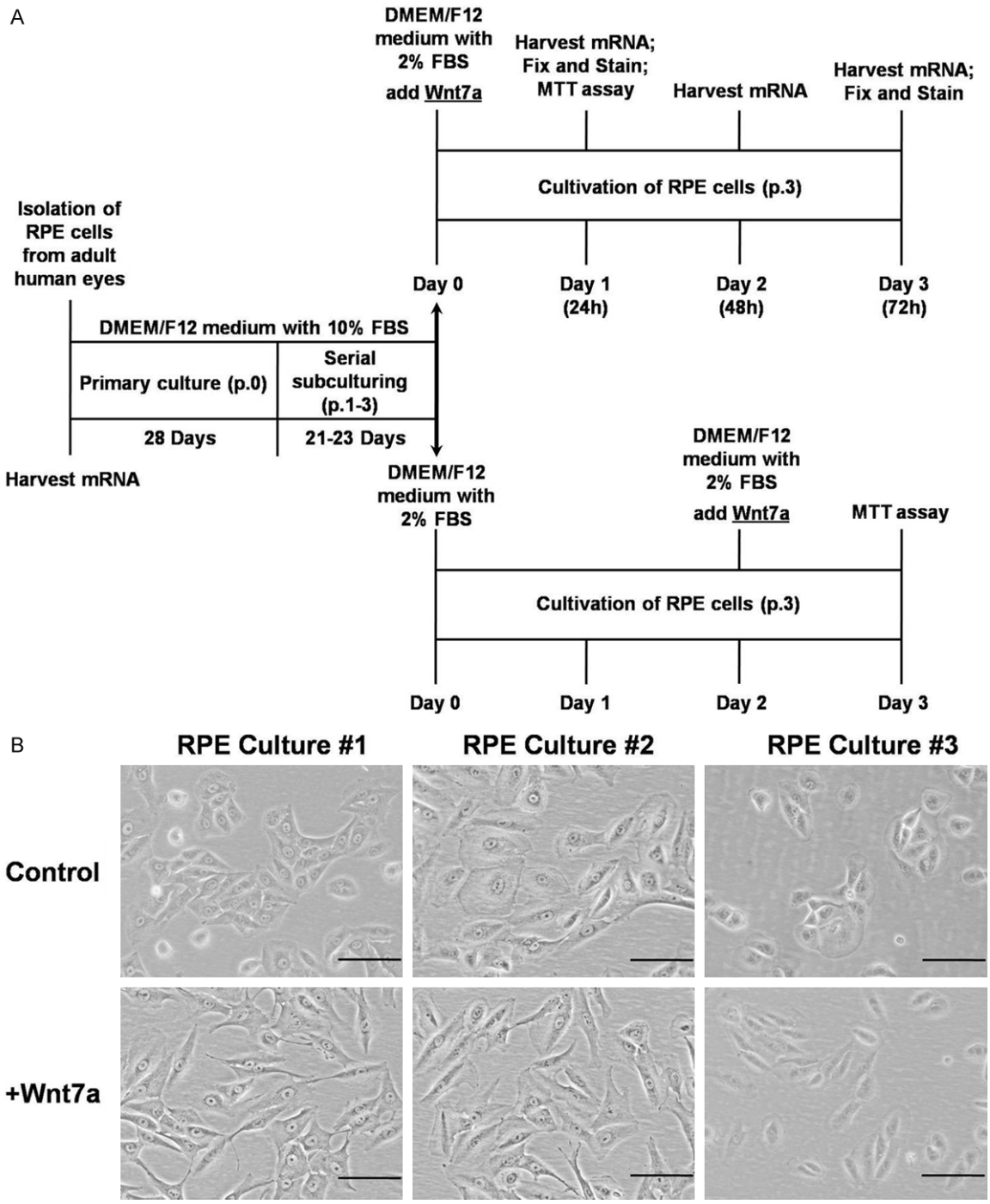

Figure 1. Wnt7a treatment changes RPE cell morphology. A: A schematic illustration of the experimental design (p, passage; h, hours). B: Morphological changes of the adult human RPE cells, compared with control cells 24 h after exposure to 60 ng/mL Wnt7a. Phase contrast microscopy. Bar, 100 *μm*.

7500 PCR system. PCR program included an initial denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. The threshold cycle (Ct) was determined using 7500 Software v2.0.1 (Applied Biosystems). All PCR reactions were performed independently two times in duplicate. The Ct values were averaged, and relative expression was estimated for each sample with the ΔΔCt method, using *GAPDH* (DNK-Sintez) levels for normalization. The results were expressed as mean ± SD. The

Table 2. Morphometry of RPE cells in control and Wnt7a-treated cultures after 24 h incubation

Culture	Area[1], pixels	Perimeter[1], pixels	Spreading coefficient Rp/Ra	Polarization coefficient M/m
Control	3028±2239	249±108	1.3	1.9
+Wnt7a	3837±1869*	325±116*	1.5*	2.3*

[1]Data are expressed as mean ± SD, *P<0.01 compared with the control.

results (Student's t-test) were considered statistically significant when P<0.05.

Cell proliferation assay (MTT assay)

RPE cells were seeded in DMEM/F12 supplemented with 2% FBS in 96-well plates (Greiner, Germany) and incubated in the absence (control) or presence of Wnt7a used in two concentrations (30 and 60 ng/ml). In one series of experiments, RPE cells were seeded directly in a Wnt7a-containing medium at 4×10^4 cells/cm^2. In another series, RPE cells were seeded at 2×10^4 cells/cm^2 and let to attach, spread, and reach 70-75% confluence for 48 h, and then the medium was changed to a Wnt7a-containing medium. Incubation with Wnt7a was carried out in an atmosphere containing 5% CO_2 at 37°C for 24 h. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml, Amresco, United States) was added to each well at a solution-to-medium ratio of 1:10 (v/v) 3 h before the end of incubation. After incubation, the supernatant was carefully removed and the pellet resuspended in 100 µL of DMSO (Applichem, Germany). Optical density (OD) of the eluate at 545 nm was measured using a StatFax 2100 plate reader (Awareness Technology, United States) with a 630 nm correction wavelength filter. Mean OD (for n=16 repetitions) and standard deviation were calculated using Excel 2007 (Microsoft Corporation). The significance of difference in mean OD between treated and control cultures in the MTT assay was assessed by Student's t-test. The cell proliferation (%) was expressed as follows: cell proliferation (%) = $([OD]_{Wnt7a}/[OD]_{control})\times100$, where OD is the mean OD of the respective sample at 545 nm.

Statistical analysis

Results of cell morphology analysis, qPCR tests, and MTT assay are expressed as mean ± SD. Statistical significance (Student's t-test) was accepted at *P<0.01 and **P<0.05 for cell morphology analysis and MTT assay , and at P<0.05 for qPCR tests. An inhibitory effect of Wnt7a on cell proliferation was considered to be detected when the difference was significant at P<0.01.

Results

Morphometric analysis of adult human RPE cells

A schematic illustration of the experimental design is presented in **Figure 1A**. RPE cells in cultures intensely proliferated, lost pigmentation, and produced epithelioid colonies. Morphological differences were observed in Wnt7a-treated RPE cells compared with control cells, in all samples (**Figure 1B**). The spread cells with an elongated or polygonal shape and distinct protrusions were the most prevalent cells in Wnt7a-treated cultures, 24 h after RPE cells were collected with trypsin and seeded on a solid support (92±3.6%, **P<0.05). In the control sample, round cells were more abundant, and spread cells (79±5.3%) were polygonal in shape and lacked protrusions at the same time point.

Additionally, higher mean values of cell area, perimeter, and spreading and polarization coefficients were obtained for Wnt7a-treated cells (**Table 2**). Wnt7a treatment led to the increase of mean area and mean perimeter by 27 and 30%, respectively. The spreading coefficient, which characterizes the extent of cell spreading on the support, increased by 15%, and the polarization coefficient, which characterizes the cell elongation, increased by 17%.

No significant differences in cell shape and distribution were observed between RPE cells cultured in the presence of Wnt7a and the control cells, 48 and 72 h after seeding. At these time points, the cells treated with Wnt7a were morphologically similar to control cells.

Wnt7a inhibits adult human RPE cell proliferation

The MTT assay was used to study how Wnt7a affects proliferation of RPE cells. Wnt7a was found to inhibit RPE cell proliferation compared with control cells. Inhibition of cell proliferation was observed regardless of whether Wnt7a

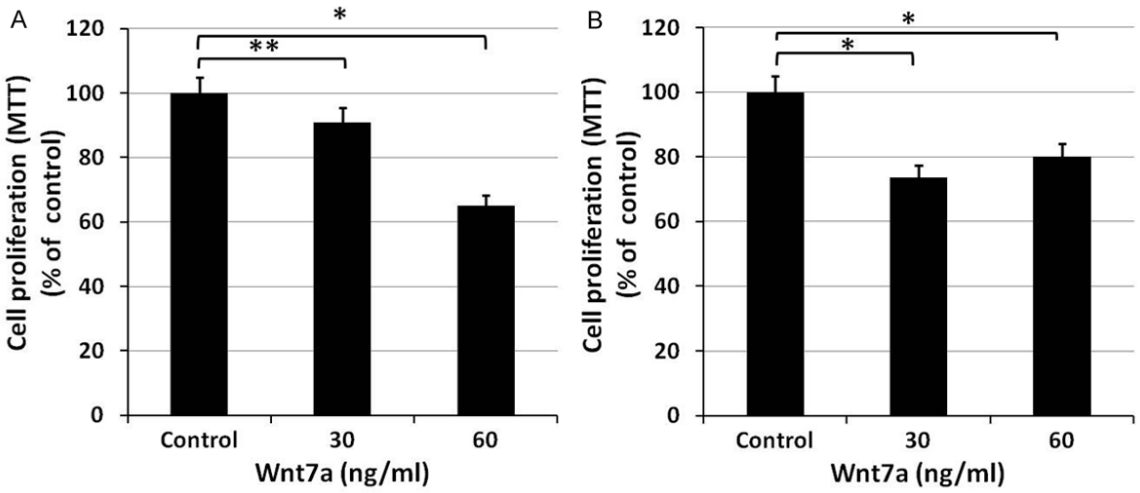

Figure 2. Wnt7a suppresses human RPE cell proliferation. A: Cells were reseeded in a Wnt7a-containing medium. B: Wnt7a was added after RPE cells were let to attach, spread, and reach 70-75% confluence for 48 h. Data are expressed as mean ± SD (n=16) *P<0.01 versus control, **P<0.05 versus control.

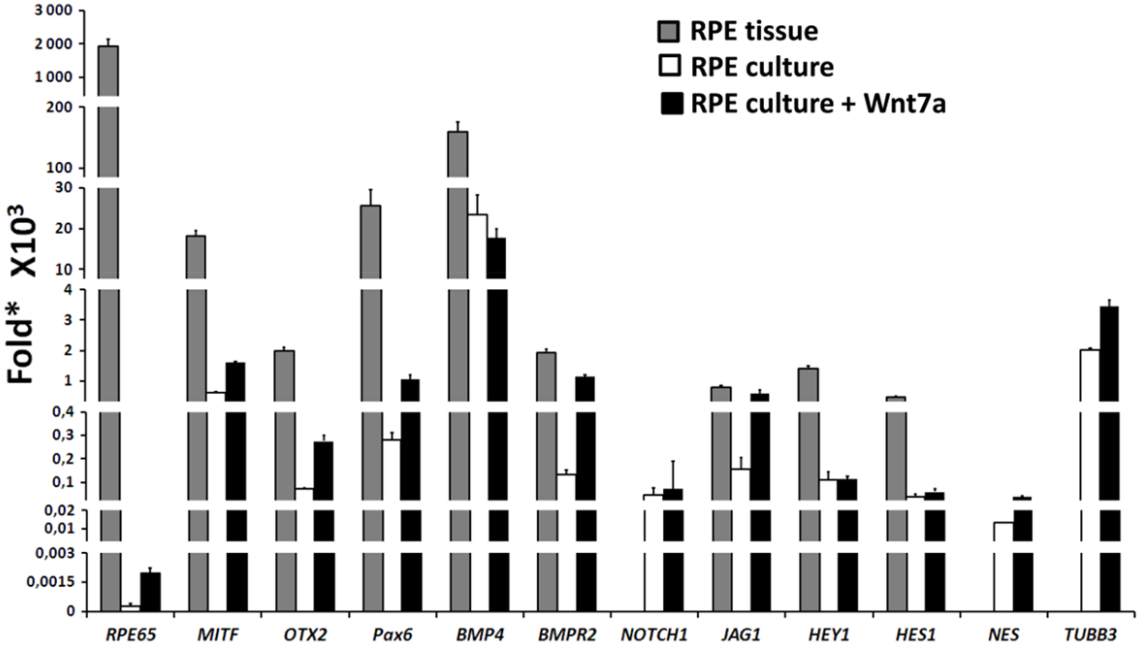

Figure 3. mRNA expression profiles of RPE tissue, control culture, and culture treated with Wnt7a. The expression profiles of *RPE65, MITF, OTX2, Pax6, BMP4, BMPR2, NOTCH1, JAG1, HEY1, HES1, NES,* and *TUBB3* in RPE tissue, RPE control culture and RPE culture treated with Wnt7a, estimated by qPCR. The expression for *NOTCH1*, *NES*, and *TUBB3* in RPE tissues was below the detection level. *GAPDH* level was used for the normalization of the expression levels of the investigated genes. These levels were averaged over two independent experiments performed in duplicate; the error bars represent standard deviation.

was added at cell reseeding or after culturing cells for 48 h (**Figure 1A**). A significant (*P<0.01) Wnt7a-induced decrease in cell proliferation after disrupting cell contacts with trypsin was observed at 60 ng/ml Wnt7a; the MTT result was 35% lower than that of control cells (**Figure 2A**). When Wnt7a was added to an established RPE cell culture 48 h after seeding, a significant (*P<0.01) decrease in cell proliferation was observed at both 30 and 60 ng/ml Wnt7a,

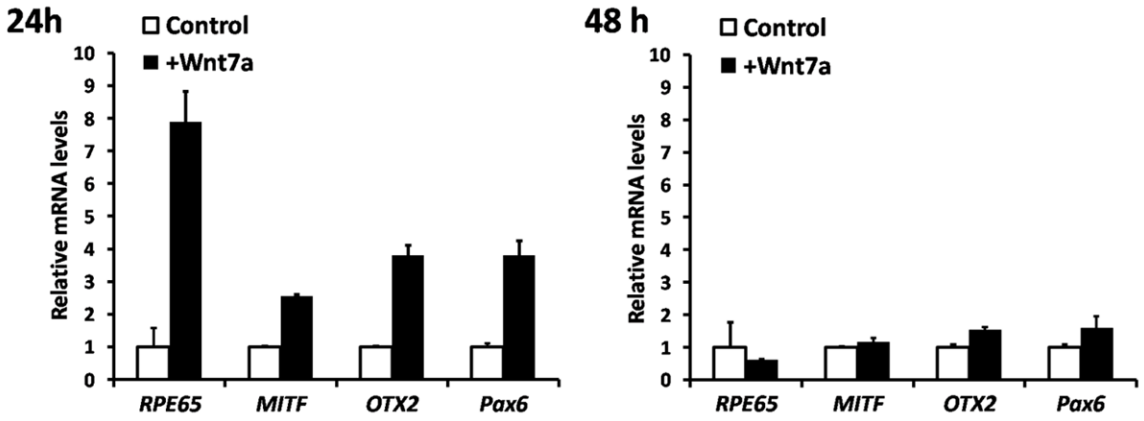

Figure 4. Wnt7a upregulates the expression of RPE differentiation markers in adult human RPE cells. Third-passage RPE cells were incubated with 60 ng/mL Wnt7a. The relative expression of *RPE65, MITF, OTX2,* and *Pax6* in cells exposed to Wnt7a for 24 and 48 h was assessed by qPCR. mRNA levels of these genes in control cells were considered as one. The results were averaged over two independent experiments performed in duplicate; the error bars show standard deviation. The differences in mRNA expression between the treated and control cells were tested for significance by Student's t-test, and considered significant at $P<0.05$.

the MTT result being 26 and 20% lower than the control, respectively (**Figure 2B**).

Wnt7a upregulates a number of differentiation markers in adult human RPE cells

In order to investigate whether the differentiation of RPE cells changes in response to Wnt7a, the expression of RPE-specific genes, *RPE65, MITF, OTX2,* and *Pax6,* was determined by qPCR. RPE cells examined before seeding had increased levels of *RPE65, Pax6*, and *MITF* mRNAs, and we were able to detect *OTX2* mRNA (**Figure 3**). Third-passage RPE cells (the control sample) displayed an approximately 10^6-fold decrease in *RPE65* mRNA levels, a more than 90-fold decrease in *Pax6* mRNA levels, an approximately 28-fold decrease in *MITF* mRNA, and a 26-fold decrease in *OTX2* mRNA levels, compared with RPE tissue (**Figure 3**), which is consistent with the cell dedifferentiation *in vitro*.

In the same passage RPE cells, treated with Wnt7a for 24 h, mRNA levels of the investigated genes increased significantly. Fold increase was 7.9 for *RPE65*, 3.8 for *Pax6*, 2.5 for *MITF*, and 3.8 for *OTX2*, compared with the control (**Figure 4**). In the cells exposed to Wnt7a for 48 h, the expression levels of these genes were determined to be lower, and they showed no significant difference compared with the control levels (**Figure 4**).

Wnt7a changes the expression of BMP isoforms in adult human RPE cells

ICC analysis using anti-BMP2, anti-BMP4, and anti-BMP7 antibodies showed that these proteins were expressed in both the control and treated RPE cells (**Figure 5A**). While the staining intensity was low in the case of BMP4, *BMP4* expression was higher compared with *BMP2* mRNA level, as determined by qPCR (**Figure 5B**).

It was shown that the localization of the investigated proteins changed from nuclear to cytoplasmic, BMP2 and BMP7 staining intensity increased, and the perinuclear BMP4 staining intensity decreased in RPE cells incubated with Wnt7a for 24 h, compared with the control cells. BMP2 was detected mostly in the perinuclear space in the Wnt7a-treated cells and in the nucleus of the control cells (**Figure 5A**). BMP7 localized at different spots at the plasma membrane in the treated cells, and predominantly in the nucleus of the control cells (**Figure 5A**). An increase in BMP2 staining in Wnt7a-treated RPE cells was confirmed using qPCR analysis, which showed that the expression of *BMP2* mRNA increased 2.8-fold, compared with the control (**Figure 5C**). An 8.7-fold increase in *BMPR2* mRNA level compared with the control level was observed together with the increase in *BMP2* mRNA. However, the expression levels of the investigated genes decreased after 48 h

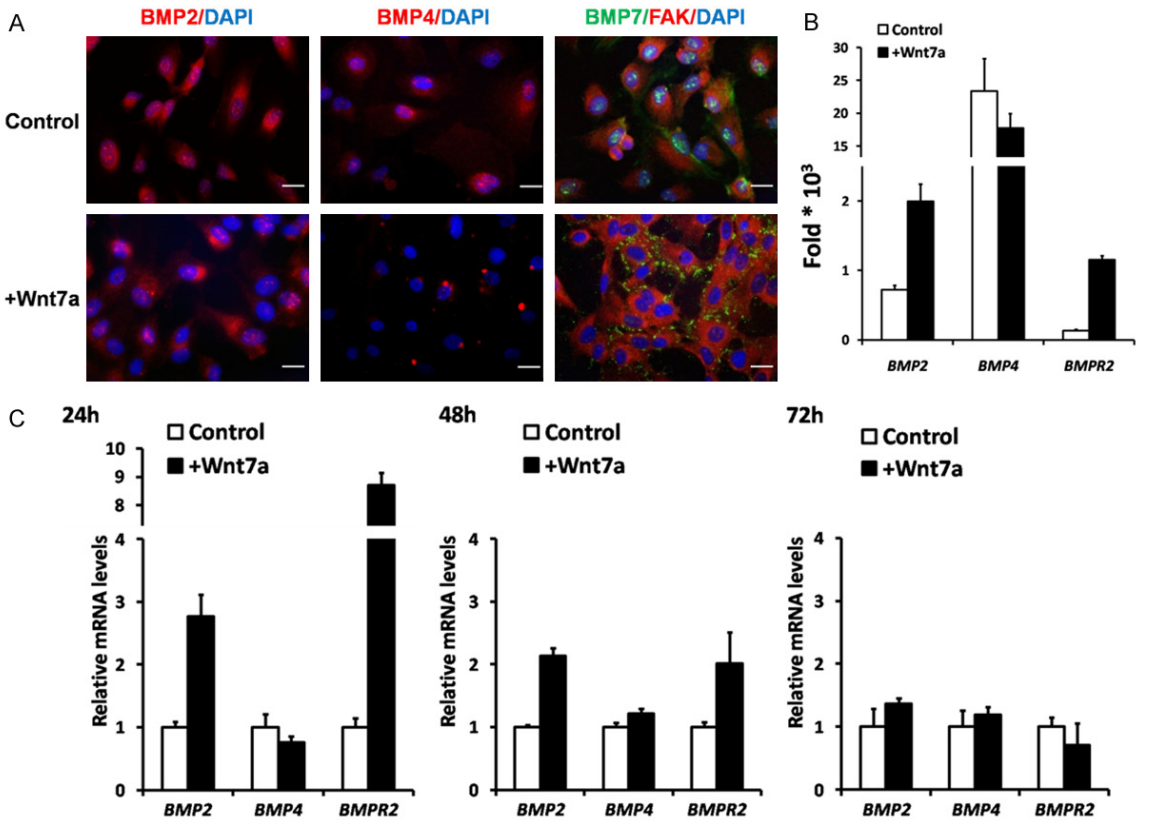

Figure 5. Wnt7a changes the expression of BMP isoforms in adult human RPE cells. Third-passage RPE cells were incubated with 60 ng/mL Wnt7a. A: ICC analysis using anti-BMP2, anti-BMP4, and anti-BMP7 antibodies. Bar, 20 *μm*. B: The expression of *BMP2, BMP4,* and *BMPR2* in RPE cells in the absence (the control sample) or presence of Wnt7a 24 h after seeding, determined by qPCR. *GAPDH* level was used for the normalization of results. C: Relative expression of *BMP2, BMP4,* and *BMPR2* in cells exposed to Wnt7a for 24, 48, and 72 h, determined by qPCR; mRNA levels obtained in the control cells were taken as one. The results were averaged over two independent experiments performed in duplicate; the error bars show standard deviation. Differences in mRNA expression between the treated and control cells were tested for significance using Student's t-test, and they were considered significant at $P<0.05$.

of incubation with Wnt7a, and showed no significant difference compared with the control after 72 h of incubation (**Figure 5C**).

The effects of Wnt7a on the expression of the Notch signaling pathway proteins in adult human RPE cells

ICC analysis of the protein products of Notch target genes (*HES1*, *HES5*, and *HEY1*) showed Hey1- and HES5-positive cells in both treated and control cultures (**Figure 6A**). The immunofluorescence staining was perinuclear in the case of Hey1 and HES5. HES1 staining intensity was at the level of background staining. The expression of Notch signaling pathway target genes in RPE cells was analyzed by qPCR and confirmed for *HEY1* and *HES1* (**Figure 6B**). *HEY1* and *HES1* mRNA levels in Wnt7a-treated cells did not significantly differ from the control sample (**Figure 6C**). Higher *HEY1* mRNA levels were observed in comparison with *HES1* levels, both in the treated and control cells (**Figure 6B**). Double staining of Wnt7a-treated cells for Hey1 and nestin proteins was performed, and Hey1 expression was determined to be present in the nestin-positive cells. A double HES5 and NF (200- and 68-kDa) staining showed that these proteins are expressed in different cells, and their colocalization was not detected.

A significant increase in the level of *JAG1* was observed following the exposure to Wnt7a for 24 h, with 3.9-fold increase compared with the control (**Figure 6C**). The expression level of this gene decreased to the control level after 48 h of exposure to Wnt7a (**Figure 6C**).

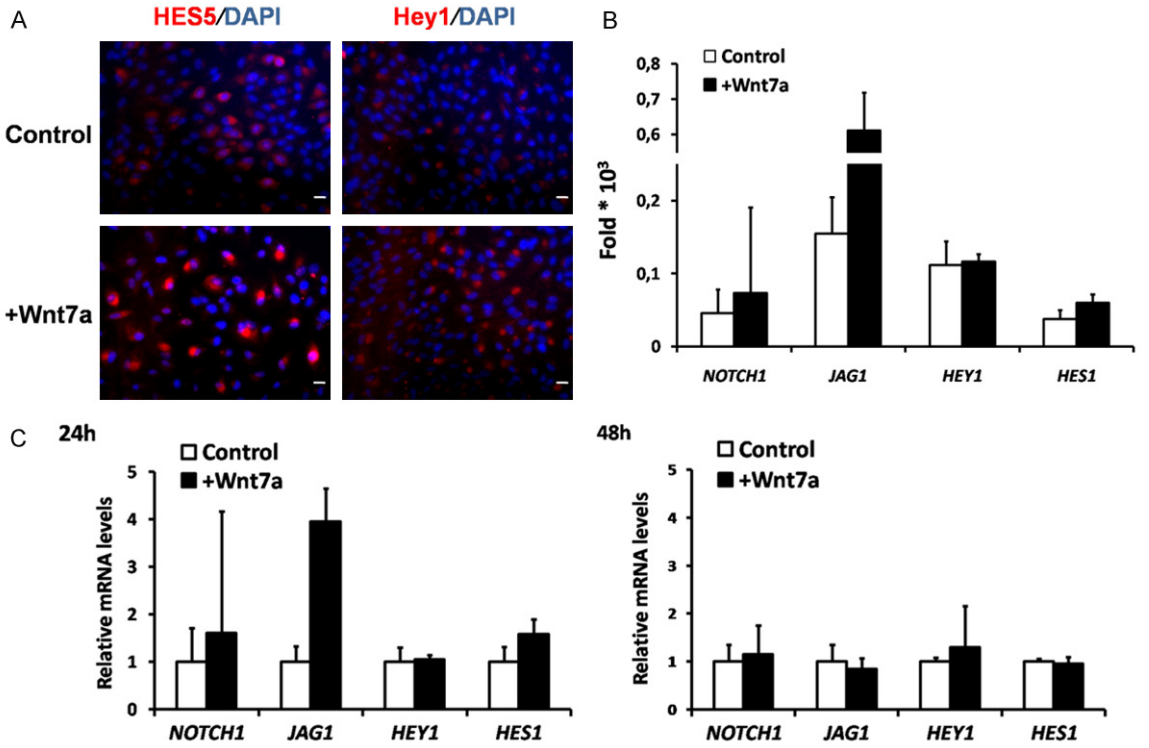

Figure 6. The effect of Wnt7a on the expression of the Notch signaling pathway proteins. Adult human RPE cells were incubated with 60 ng/mL Wnt7a. A: ICC analysis using HES5 and Hey1 antibodies. Bar, 20 *μm*. B: The expression of *NOTCH1, JAG1, HEY1,* and *HES1* in RPE cells in the absence (the control sample) or presence of Wnt7a 24 h after seeding, determined by qPCR. *GAPDH* level was used for the normalization of results. C: Relative expression of *NOTCH1, JAG1, HEY1,* and *HES1* in the cells exposed to Wnt7a for 24 and 48 h, determined by qPCR; mRNA levels of the investigated genes in the control cells were taken as one. The results were averaged over two independent experiments performed in duplicate; the error bars show standard deviation. The differences in mRNA expression levels between the treated and control cells were tested for significance using Student's t-test, and considered significant at $P<0.05$.

Wnt7a changes the expression of several neural differentiation markers in adult human RPE cells

MAP1B- or TUBB3-positive cells were not detected in RPE cultures treated with Wnt7a for 24 h, while distinct staining was observed in the control cells (**Figure 7A**). Although TUBB3 was not observed after 24 h of the exposure to Wnt7a, a 1.7-fold increase in *TUBB3* mRNA was detected in Wnt7a-treated cells compared with the control, as determined by qPCR. However, *TUBB3* mRNA level decreased to the control level after 48 h of incubation, and decreased below the control level after 72 h of exposure (**Figure 7B**).

Following the incubation of cells with Wnt7a for 24 h, cell clusters positive for the neural stem cell marker nestin and neuron markers, NFs (200- and 68-kDa), were observed in the cell cultures using ICC (**Figure 7A**). Single nestin- and NF-positive cells were detected in the control. A 3-fold increase in *NES* mRNA level was determined in the cells exposed to Wnt7a for 24 h, compared with the control cells (**Figure 7C**). However, *NES* expression level decreased after 48 h of treatment (**Figure 7C**), and no significant difference compared with the control was found after 72 h of incubation.

Additionally, synaptic contact protein synapsin I was detected in the cytoplasm of Wnt7a-treated cells, while predominantly localizing in the nucleus of the control cells (**Figure 7B**).

Discussion

Our preliminary study [37] has been the first to implicate Wnt7a in the transformation of human RPE cells *in vitro*. It showed that Wnt7a exerts antiproliferative effects and initiates the spread

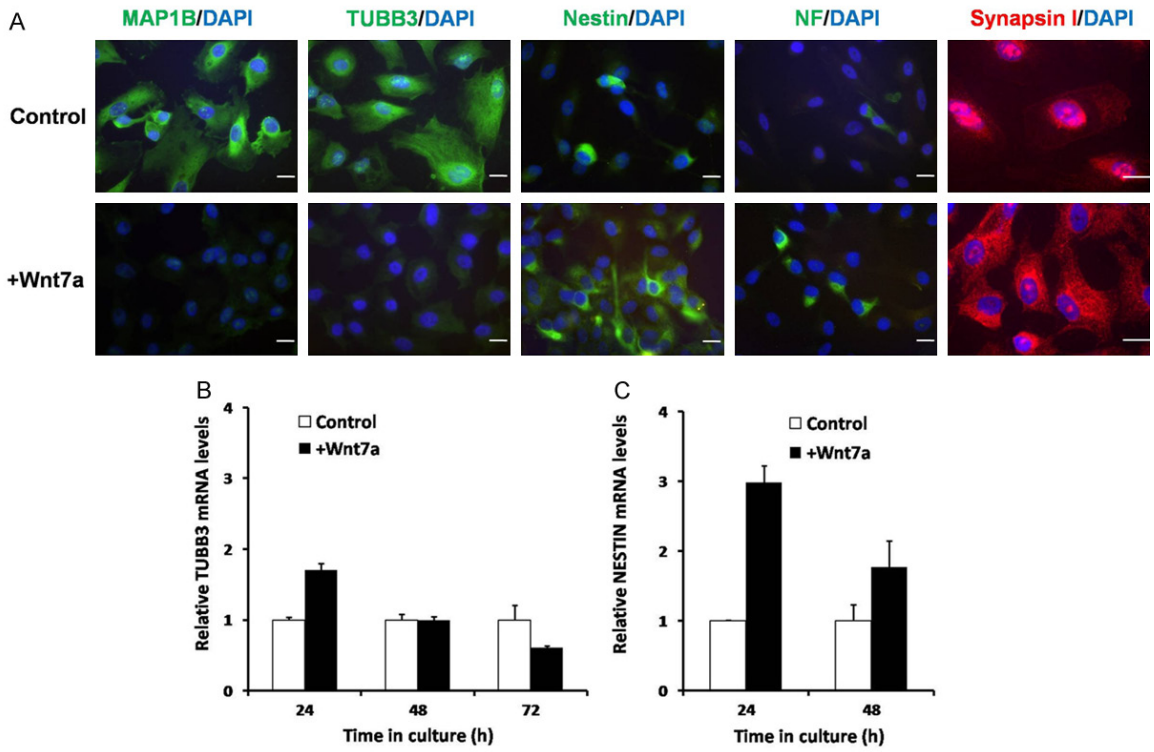

Figure 7. Wnt7a changes the expression of several neural differentiation markers. Third-passage human RPE cells were incubated with 60 ng/mL Wnt7a. A: ICC analysis using the neural marker antibodies MAP1B, TUBB3, nestin, NFs (200- and 68-kDa), GFAP, and synapsin I levels after 24 h of incubation with Wnt7a. Bar, 20 *μm*. B: The relative expression of *TUBB3* after 24, 48, and 72 h exposure to Wnt7a, determined by qPCR. C: The relative expression of *NES* after 24 and 48 h incubation with Wnt7a, determined by qPCR. B, C: mRNA levels of the investigated genes determined in the control cells were taken as one. The results were averaged over two independent experiments performed in duplicate; the error bars show standard deviation. Differences in *TUBB3* and *NES* mRNA expression between the treated and control cells were tested for significance by Student's t-test, and considered significant at $P<0.05$.

and polarization of dedifferentiated adult human RPE cells on a plastic surface. Here, these results were confirmed by morphometric analysis of the spread RPE cells. In addition, using the MTT assay, we demonstrated that Wnt7a suppresses cell proliferation whether it been added during cell reseeding or after cells culturing for 48 h. Further, we investigated the role of Wnt7a in the regulation of RPE cell plasticity, at cellular and molecular levels. Wnt7a was shown to upregulate the expression of E-cadherin, which was tightly associated with β-catenin, in RPE cells [37]. The activation of E-cadherin and β-catenin at the plasma membrane suggests adhesion contact formation and stabilized cell adhesion, indicating that the epithelial properties of RPE cells are enhanced in culture [40, 41]. All-trans retinoic acid exerted similar effects on E-cadherin activation, restoring the epithelial properties of guinea pig primary RPE cells *in vitro* [41]. Cell polarization is regulated through the noncanonical Wnt/PCP signaling pathway, and the Wnt7a ligand may play a role in the activation of noncanonical Wnt signaling branches in RPE cells *in vitro*. The activation of noncanonical Wnt/Ca^{2+} pathway leads to the redifferentiation of RPE cells [42]. Wnt7a significantly upregulates the expression of the RPE marker genes *RPE65, MITF, OTX2,* and *Pax6*, which represents an additional confirmation of the enhanced epithelial properties of these cells. Pax6 and MITF have been shown to cooperate during RPE development [3, 43]. During embryonic development, Pax6 regulates MITF and, together with it, activates the expression of melanogenesis-inducing genes [44]. MITF and Wnt/β-catenin signaling pathway interact during RPE cell specification and differentiation. Therefore, Wnt7a acts as a trigger during RPE cell redifferentiation, as evidenced by the ICC-detected distribution of the cell contact proteins, β-

catenin and E-cadherin, and the upregulation of the *RPE65, MITF, OTX2* and *Pax6* mRNAs in the primary human RPE cells.

We demonstrated here that Wnt7a modulates the activity of BMP signaling pathway in dedifferentiated adult human RPE cells. Dramatic downregulation of *BMP4* and *BMPR2* was observed in the dedifferentiated adult human RPE cells *in vitro,* compared with RPE tissues. A predominantly nuclear localization of BMP2 and BMP4 was observed, which may be related to the activation of the cell cycle [45]. Wnt7a significantly upregulated the expression of *BMP2* and *BMPR2*, but did not have any effect on *BMP4* expression. BMP2 was shown to change the localization from nuclear to cytoplasmic, and the staining of this protein increased in intensity, while BMP4 staining intensity decreased in Wnt7a-treated RPE cells, compared with the control cells. BMP2 and BMP4 have been shown to suppress the proliferation of ARPE19 cells, acting as negative growth regulators [46]. Similar effects have been observed in neural progenitor cells *in vitro* [47]. Upregulation of *BMP2* and the increase in cytoplasmic BMP2 staining in response to Wnt7a indicates that endogenous BMP2 is involved in the suppression of cell proliferation, and/or that redifferentiation is initiated in RPE cells. BMP2 was previously shown to play a role in the differentiation of various adult human cells [48, 49]. The cytoplasmic intensity of BMP7 staining increased in Wnt7a-treated RPE cells. Plasma membrane localization suggest a role of BMP7 in cell polarization [50].

This study is the first to demonstrate the expression of the components of the Notch signaling pathway in primary human RPE cells, including the expression of *NOTCH1* receptor gene, *JAG1* ligand gene, and *HEY1* and *HES1* target genes, as well as the expression of Hey1 and HES5 target proteins. Liu et al. [16] have studied the functions of Notch signaling pathway in ARPE19 cells, and observed a differential expression of its components. Jagged1 was shown to have the highest expression levels among all Notch ligands in ARPE19 cells. *NOTCH1* knockdown substantially downregulates *HES1, HEY2, MYC,* and *SOX9* genes and suppresses cell proliferation and migration of ARPE19 cells. We have observed a dramatic decrease in the expression of *JAG1* ligand and *HEY1* and *HES1* proneural gene repressors [51], and a minor increase in *NOTCH1* mRNA levels in primary human RPE cells dedifferentiated *in vitro,* compared with RPE tissue. Wnt7a caused substantial (almost 4-fold) upregulation of the *JAG1* mRNA, and its levels in the treated cells were comparable with the levels in RPE tissues. However, Wnt7a did not affect the expression levels of *NOTCH1*, *HEY1*, and *HES1*. This suggests that neural differentiation is possible for primary RPE cells *in vitro* both in the presence and absence of Wnt7a [52]. *HEY1* expression at mRNA and protein levels was higher than *HES1* expression in the treated and control RPE cells. *HES1* is known to play a role in the development of the lens, ocular vesicle, and RPE in mice [53], and therefore, the Hey1 and Nestin coexpression observed in some cells cultured *in vitro* indicates that RPE dedifferentiation yields stem-like cells, whose self-maintenance involves the Notch signaling pathway. A Wnt7a-induced increase in *JAG1* expression suggests potential regulatory effects of Wnt7a on the dedifferentiated RPE cells. It was shown that, *in vitro*, endogenous Jagged1 ligand interacts with Notch1, affecting neural stem cells, their self-maintenance, and their multipotent character [54], and that Jagged1 activates neural specialization in pluripotent embryonic stem cells [55].

Our results demonstrate for the first time that Wnt7a changes the expression of several neural differentiation markers in human RPE cells. MAP1B- and TUBB3-positive cells were not observed in Wnt7a-treated cultures, while synapsin I- and NF-positive cells were detectable, suggesting their further neuronal differentiation. Our findings agree with a previous study by Horn et al. [56]. A study with transgenic mouse embryos showed that Wnt7a overexpression delays TUBB3 expression, contributing to the control of neuronal progenitor maturation. We observed groups of nestin-positive cells in primary RPE cell cultures following Wnt7a treatment, together with a 3-fold increase in *NES* mRNA, compared with the control, indicating that Wnt7a is capable of maintaining stem-like cell status [12]. Wnt7a is known to regulate many steps of neurogenesis [36], facilitates self-renewal of neural stem cells [36, 57], and increases the portion of neural marker-expressing cells in a population of neural stem and progenitor cells *in vitro* [58]. Wnt7a stimulates the development of neuron presynaptic com-

ponents during transdifferentiation of mesenchymal stem cells, increasing synapsin I expression in them [29]. Our results agree with these data, and, taken together, indicate that Wnt7a differentially affects the neuronal development of RPE cells. Wnt7a maintains stem cell status of some cells and stimulates neuronal differentiation in others, which may be explained by the differences in the differentiation stages of RPE cells *in vitro* that consequently may lead to the variation of their response to Wnt7a.

Conclusion

Our study showed that Wnt7a triggers redifferentiation and neural differentiation. These Wnt7a-induced morphological and molecular changes in adult RPE cells are transient, which may be explained by a lack of a microenvironment suitable for the extended transformation. Further studies of the Wnt signaling pathway and its regulatory mechanisms will improve our understanding of the processes occurring in RPE cells under pathological conditions, and enable us to design new-generation drugs based on this knowledge.

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Disclosure of conflict of interest

None.

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