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**Original Article**

# Augmented Expression of *NOGO-A* and Its Receptors in Human Retinal Pigment Epithelial Cells Following Treatment with Human Amniotic Fluid

***Bahar Safdari*** [1], ***Mozhgan Rezaei-Kanavi*** [1], ***Mohammad Amir Mishan*** [1], ***Hamid Ahmadieh*** [2], ***Zahra-Soheila Soheili*** [3], ***Iman Salahshourifar*** [4], ****Fatemeh Suri*** [2]

1. *Ocular Tissue Engineering Research Center, Research Institute for Ophthalmology and Vision Science, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
2. *Ophthalmic Research Center, Research Institute for Ophthalmology and Vision Science, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
3. *Department of Molecular Medicine, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran*
4. *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

***Corresponding Author:** Email: fatemehsuri@gmail.com; fatemeh.suri@sbmu.ac.ir

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**Abstract**

**Background:** Nogo-A, a myelin-associated inhibitor for neurite outgrowth, has important role in visual system development. Trans-differentiation ability of human amniotic fluid (HAF) on human retinal pigment epithelial cells (hRPEs) towards neural progenitor cells has been observed in several studies. We aimed to investigate the expression of *NOGO-A* gene and its receptors as a marker of neural differentiation in HAF-treated hRPE cells.

**Methods:** hRPE cells were cultivated and immune characterized via RPE65 and cytokeratin 8/18 protein markers. Also, the cytotoxicity effect of 30% HAF on hRPE cells was evaluated using ELISA cell death assay. Finally, expression of *NOGO-A* and its receptors, *RTN4R* and *LINGO1* was evaluated in the cells treated with HAF in comparison with FBS-treated cells using quantitative real-time PCR.

**Results:** Harvested cells showed immunoreactivity for cytokeratin 8/18 and RPE65, confirming the hRPE cell identity. Besides, HAF had no cytotoxic effect on hRPE cells compared with FBS-treated cells. Results showed that *NOGO-A* and its receptors were expressed in cultured hRPE cells. Besides, comparative gene expression analysis revealed significant increased expression of the investigated genes in HAF-treated hRPE cells compared to FBS-treated cells.

**Conclusion:** Augmented expression of *NOGO-A* and its receptors can support neural differentiation of hRPE when the cells are treated with HAF. Our outcomes provide more evidences on the trans-differentiation ability of HAF on hRPE cells into neural progenitors and retinal neural cells, but further studies are needed to elucidate the exact mechanism.

**Keywords:** Retinal pigment epithelial cells; Human amniotic fluid; Ophthalmology

## Introduction

Retinal pigment epithelium (RPE) with hexagonal cells located between choriocapillaris and neurosensory retina, has pivotal roles in visual functions (1) and its dysfunction is associated with a

wide range of retinal disorders such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) (2-4). Given that current treatments for these types of retinal disorders are challenging, tissue engineering and cell therapy have been introduced as the state-of-the-art promising strategies to cure ocular diseases with dysfunctional RPE cells (5-7). RPE cells can differentiate into neural retinal cells or progenitors following induction by various growth factors or stimuli (8-10).

Reticulon 4 (*RTN4*; OMIM 604475), also known as *NOGO*, belongs to the family of reticulon encoding genes that are associated with the formation and stabilization of the endoplasmic reticulum (ER) tubules (11-13). Alternatively, different transcript variants of the gene have been identified. Reticulon-4 isoform A (NM_020532.5), also known as *NOGO-A*, is the longest transcript, and encodes the longest isoform of the protein. Nogo-A is a myelin-associated inhibitory protein in axonal regeneration processes and neuronal cell growth in central nervous system (CNS) (14, 15). This protein has two determinative roles; one in developing nervous system in form of regulating neurite fasciculation and extension, and another in the adult CNS in forms of negative regulator of axon-axon adhesion and growth, stabilization of wiring and restriction of plasticity (16, 17). On the other hand, it was suggested that Nogo-A degradation can promote cell migration (18) and also axonal regeneration (19).

Nogo-A has three inhibitory domains (20); an amino acid sequence inhibiting fibroblast cell spreading in N-terminal, Nogo-A-Delta 20 located in the central region of the protein that restrict cell spreading and neurite outgrowth and a C-terminal domain called Nogo-66 which induces growth cone collapse. Nogo-66 can bind and activate a receptor complex comprised of reticulon 4 receptor (RTN4R) which also known as Nogo receptor (NgR) (21), a Leucine rich repeat and Immunoglobin-like domain-containing protein 1 (Lingo-1) (22) and p75/Troy (23, 24) leading to actin cytoskeleton disassembly by the small Rho A GTPase (25).

Nogo-A has important role in the visual system development through affecting on optic nerve myelinogenesis, retinal angiogenesis and retinal axon projection formation (26). In normal conditions, *NOGO-A* is expressed in retinal ganglion cells (RGCs) and Müller cells, however it has low expression in vitreous (27). There are not any evidences for expression of NOGO-A and its receptors in RPE cells.

Human amniotic fluid (HAF) as a biological fluid, contain various factors (28, 29). Trans-differentiation ability of HAF on human RPE cells culture has been demonstrated in previous studies, but its mechanistic effect was poorly understood (30, 31). In this study, we investigated the expressions of *NOGO-A* and its receptors at mRNA level in human retinal pigment epithelial cells (hRPEs) following treatment with HAF.

## Materials and Methods

Before conducting the study, ethical approval was obtained from the Ethics Committee of the Research Institute for Ophthalmology and Vision Science, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.ORC.REC.1391.13). The posterior eyecups of donated whole eyes were used for investigation based on the eye bank informed consents and in accordance with the declaration of Helsinki regulations. Moreover informed consents were obtained from all the pregnant subjects that underwent amniocentesis, in order to use the remained HAF samples after karyotype analyses.

### *hRPE cells isolation and culture*

Human posterior eyecups from two donors aged 30 and 50 years with death-preservation time less than 24 h were provided from the Central Eye Bank of Iran (Tehran, Iran). Isolation of the hRPE cells was performed as previously described (32). Briefly, after separating the hRPE layer from the human eyecups and dissecting into small pieces, the tissues were incubated with the dispase I solution (1.1 U/ml) (Cat#17105041,

Gibco, Germany) for 50 min at 37 °C with 5% $CO_2$. Thereafter, the solution was centrifuged at 300g for 5 minutes at 4 °C. Finally, the harvested cells were cultured in 25 $cm^2$ flasks with DMEM: F12 (1:1; Sigma-Aldrich, Germany) supplemented with 20% fetal bovine serum (FBS) containing penicillin (120 µg/ml) and streptomycin (220 µg/ml) at 37 °C with 5% $CO_2$. After that, the culture medium was changed twice a week with 10% FBS-supplemented medium to obtain about 80% confluency. Then, the cells were passaged and cultures in passages 3 to 6 were used for all experiments.

### *hRPE cells morphology and immune characterization*

The morphological features of hRPE cells were observed under an inverted microscope (Olympus IX71, Tokyo, Japan) and the corresponding images were captured by using a digital camera (Olympus U-TV0.63XC; Tokyo, Japan). Immunocytochemistry was performed to characterize the identity of the harvested hRPE cells. The cultivated cells were seeded in 24-well plates at the density of $5\times10^3$ cells/well. After fixation with cold methanol (-10°C) at room temperature for 10 min, the cells were permeabilized using Triton X-100 (0.25%) and blocked with 1% bovine serum albumin (BSA) in PBS at room temperature for 60 min. After removing the blocking agent, the cells were incubated with RPE65 antibody (1:200, rabbit polyclonal IgG; Santa Cruz Biotechnology, Dallas, TX) and cytokeratin 8/18 antibody (1:200, mouse monoclonal IgG2a, Santa Cruz Biotechnology) overnight at 4°C. Then the cells were irrigated with PBS and incubated, respectively, with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100; Santa Cruz Biotechnology Inc., Dallas, USA) and FITC-conjugated goat anti-mouse IgG (1:100; Santa Cruz Biotechnology Inc., Dallas, USA) for 45 min at darkness and room temperature. The immune stained cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 mg/ml; Santa Cruz Biotechnology Inc., Dallas, USA) for 5 min and examined with an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan) with the excitation wavelength ranging from 450 to 490 nm and the corresponding images were captured with the digital camera.

### *Human amniotic fluid preparation and treatment*

Human amniotic fluid samples were provided from 30 pregnant women who underwent amniocentesis in the first trimester of gestation. Amniotic fluid cells were removed for karyotype analysis. The remaining supernatants, in cases with no evidence of chromosomal abnormalities, were pooled and used for experimental procedures. HAF samples were centrifuged at 300 g for 5 min at 4 °C, and finally the supernatants were sterilized using a 0.2-µm filter and stored at −70 °C. hRPE cells were treated with HAF 30% for 24 h in the current experiment.

### *ELISA cell death assay*

Following treatment of the cultured hRPE cells with 30% HAF at a cell density of $1\times10^3$/ml in a 24-well plate for 24 h, cell apoptosis was assessed using ELISAPLUS kit (Cat#11774425001, Roche, Germany), according to the manufacturer's guidelines (Roche, Germany). Absorption of the samples was read at specific wavelengths (405 and 492 nm), using an ELx 808 absorbance reader (BioTek Instruments, VT, USA), and apoptosis of HAF-treated cells were compared with FBS-treated cells as control. The reading results of HAF-treated and control cells were compared with each other and with the positive control (a complex of DNA and histone) included in the kit. The assay was performed for three times.

### *RNA extraction and real-time PCR*

In order to analyze gene expressions of Nogo-A, Lingo-1 and NgR encoding genes, total RNA was extracted from the treated and control hRPE cells by using TRIzol reagent (Cat#15596026, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Concentration and purity of the extracted RNAs was determined with a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). Also, RNA in-

tegrity was evaluated by agarose gel electrophoresis to observe 28S and 18S rRNA bands.
Reverse transcription synthesis of the extracted RNAs was performed using SuperScript reverse transcriptase kit (Cat#18080093 Invitrogen, USA) and oligo dT primers (Cat#18418012, Invitrogen, USA). Afterward an EvaGreen master mix (Cat#08-24-0000S Solis BioDyne, Estonia) was used for quantitative real-time PCR assay by using real-time PCR instrument (Corbett Industries, Sydney, Australia). Specific primers for the investigated genes and *GAPDH*, as a housekeeping gene, were provided from Qiagen QuantiTect Primer Assays (Qiagen, Hilden, Germany). PCR conditions included an amplification cycle of 94°C for 12 min; 40 cycles of denaturation, amplification, and quantification of 95°C for 15 seconds, 58-64°C for 30 seconds, and 72°C for 25 seconds; followed by a melting curve analysis (from 65°C to 95°C with a gradual increase). Every sample was examined in triplicate.

### *Statistical analysis*

Analyses of the genes expression levels were performed by evaluating threshold cycle (Ct) valueshttps://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/quantitative-reverse-transcription-polymerase-chain-reaction using RotorGene 6000 software of the instrument. All the expression curves were confirmed by checking the melting temperatures of the amplicons to omit any misinterpretation of the data. Normalized data against the control gene were used for comparisons between groups by using comparative quantification tool of the software. Results from ELISA cell death assay and real-time PCR were presented as mean ± SD of three separate experiments. Kolmogorov Smirnov test was used to check the normal distribution of data and Mann-Whitney test or t-test whenever appropriate was used to determine statistically significant results. Graphs were drawn using the Prism software package (GraphPad; https://www.graphpad.com/). *P*-Value<0.05 was considered statistically significant.

## Results

### *hRPE cells morphology and immune characterization*

The hRPE cells presented as monolayers of pigmented hexagonal cells with epithelial cells morphology. Furthermore, RPE65 and cytokeratin 8/18 markers were positive in all the cells, confirming the RPE cell identity (Fig. 1).

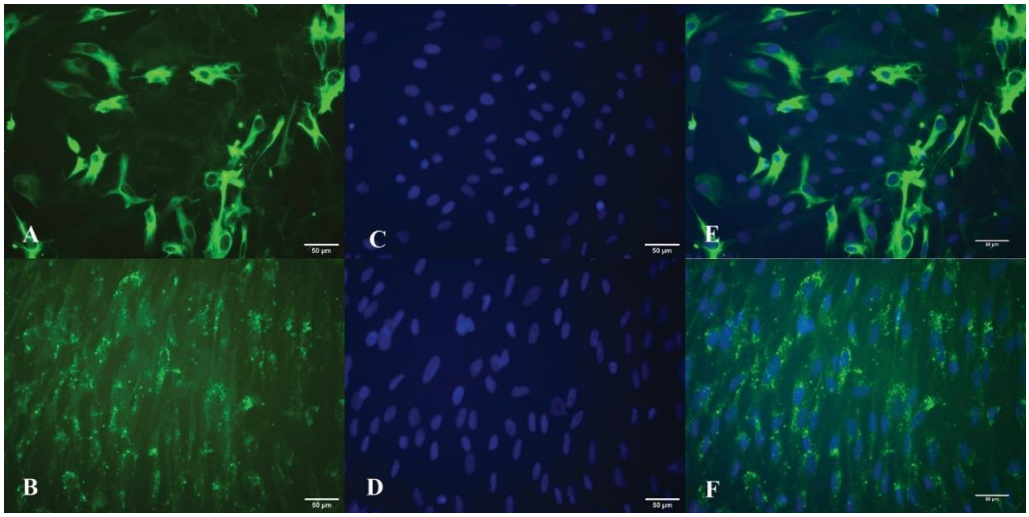


**Fig. 1:** RPE cell characterization. Note the immune reactivity of the cultivated hRPE cells for the FITC-conjugated RPE65 (A) and cytokeratin 8/18 (B). The corresponding dapi-stained (blue) nuclei (C and D) and the merged images (E and F) were illustrated

### *Cell death assay*

The effect of 30% HAF on the cell apoptosis was evaluated by ELISA. HAF in a concentration of 30% had no cytotoxic effect on hRPE cells as compared with FBS-treated control cells (Fig. 2).

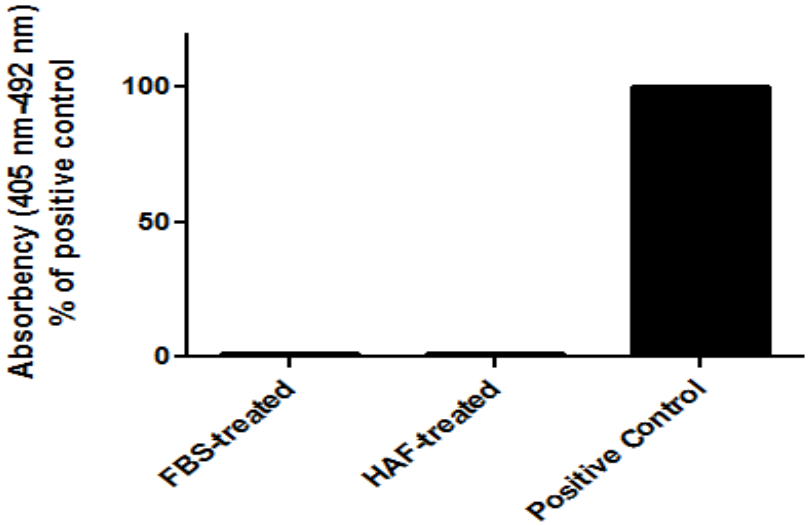


**Fig. 2:** Cytotoxicity analysis. After 24 h of hRPE cells treatment with 30% HAF, cultures were subjected to cell death ELISA assay. There were no cytotoxic effects in the culture of hRPE cells treated with 30% HAF as compared to the FBS-treated cells. The positive control showed a 100% absorbance

### *Gene expression analysis*

The transcriptional investigations indicate that *NOGO-A* and its receptors are expressed in cultured hRPE cells. Significant upregulation of *NOGO-A*, *RTN4R* and *LINGO1* was detected in 30% HAF-treated hRPE cells by 112 ($P < 0.001$), 20 ($P < 0.001$), and 11 ($P = 0.014$) folds in average, respectively, as compared with the cells treated with 20% FBS-treated control cells (Fig. 3).

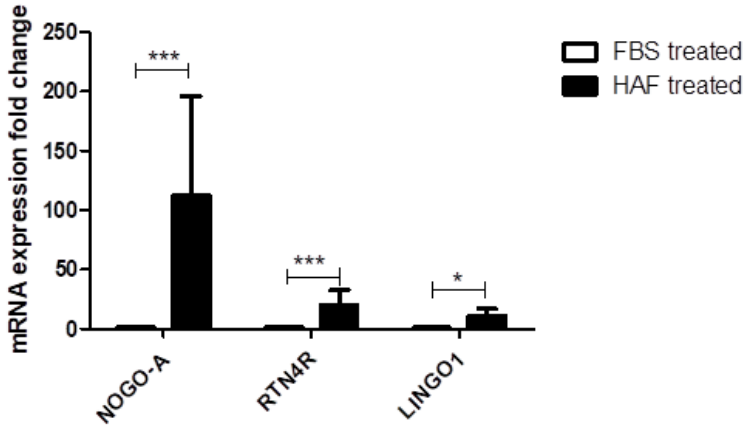


**Fig. 3:** Gene expression analysis. After 24 h of 30% HAF treatment, gene expression analysis was performed by real-time PCR. Expression levels of all the genes were significantly increased in the HAF-treated hRPE cells compared to the control cultures. ***, $P < 0.001$; *, $P < 0.05$

## Discussion

We observed, for the first time, the expression of *NOGO-A* and its receptors, *RTN4R* and *LINGO1*, in normal cultivated hRPE cells and increased expression of the corresponding genes after treatment with HAF. This results can support previous outcomes from the studies on neural trans-differentiation of hRPE cells following treatment with HAF (30, 31), which may stimulate upregulation of Nogo-A and its receptors as the neural growth inhibitors at the early stages of neural growth and regeneration (26, 33).

Evidence showed that *NOGO-A* is expressed at high level in embryonic mice RGCs and their axons (34) in contrast to its downregulation when maturation of the RGC dendrites and axons occurs (35). This process is also associated with an augmented differentiation of Müller glia, the principal glial cells of the retina, and the optic nerve oligodendrocytes with high level of Nogo-A until adulthood (36). High sensitivity to the inhibitory effects of Nogo-A was detected in RGC axons (37, 38). Nogo-A is highly upregulated in RGC axons, when the axons maturate and elongate in the presumptive optic nerve towards the brain (39), that is associated with collateral branches development (16, 40). We observed that *NOGO-A* was strongly elevated in hRPE cells in HAF culture. As HAF has shown neural trans-differentiation effect on hRPE cells (30, 31), the elevated expression of *NOGO-A* in the treated cells can be explained as the regulatory role of Nogo-A and its receptors in neurite branching and extension that occur in the development of nervous system.

Nogo receptor (NgR) has inhibitory effects on neuronal axon growth (21, 40). NgR can interact with integrins, to decrease integrin activity and cell–substrate adhesion and a transactivation of the epidermal growth factor (EGF) receptor involving in cell migration and growth (41, 42). Also, Lingo-1 was detected as a negative regulator of oligodendrocytes differentiation in the CNS (43) and it was observed that Lingo-1 suppression resulted in enhanced regenerative capacity and neuroprotection after spinal cord injury (44). Enhancement in cell migration ability was observed in cortical neural precursors after *NOGO-A* knockout animal models and also in wild-type animal neurons treated with Nogo-A, NgR and Lingo-1 antibodies, suggesting that the important role of Nogo-A at the cell surface for regulating neuronal migration at the CNS early development via an NgR–Lingo-1 receptors (45). This was similar to our observations in the current investigation in which significantly high expressions of *RTN4R* and *LINGO1* transcripts were detected in cultivated hRPE cells after 24 h treatment with HAF.

The limitation of this study was lack of the expression data of the investigated genes at the protein level and it could be considered in the prospective plans.

## Conclusion

*NOGO-A*, *RTN4R* and *LINGO1* genes are expressed in cultivated hRPE cells. High expression of the *NOGO-A* and its receptors could be a pivotal regulatory factor in hRPE cells following HAF treatment. These results may support previous studies on RPE cell neural differentiation following exposure to HAF, but further studies are needed to verify the exact role of Nogo-A in trans-differentiation of RPE cells and recruitment of the RPE cells in retinal regenerative studies. More importantly, it can be suggested that if the treatment time was extended, the expression of these neural growth inhibitors, might be changed. This study also extends our view on the several effects of HAF and its therapeutic role for cell-based therapies in retinal diseases, but further studies are needed to elucidate more signaling pathways.

## Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or fal-

sification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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## Conflict of interest

The authors declare that there is no conflict of interests.

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