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DOWN-REGULATED NOTCH SIGNALING IN ARPE-19 CELLS CULTURED ON DENUDED HUMAN AMNIOTIC MEMBRANE.

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ABSTRACT

Denuded human amniotic membrane is widely proposed to be used for retinal pigment epithelial cell transplantation. Notch signaling though important and known to have role in both health and disease of retinal pigment epithelial cells; is not well studied for retinal pigment epithelial cells cultured on denuded human amniotic membrane. The outcome of Notch signals is strictly dependent on the cellular level and can influence differentiation, proliferation and apoptotic cell fates. We have studied the regulation of Notch signaling in human retinal pigment epithelial cells (ARPE-19) cultured on denuded human amniotic membrane using gene expression and immunofluorescence analysis. Results revealed a significant down regulation in the levels of *NOTCH 3, 4* receptors, *DELTA-LIKE 1, 4*, *JAGGED 1, 2* ligands and *HES 3, HES 5* and *HEY 1* downstream targets in cells cultured on denuded human amniotic membrane. It can be concluded that human retinal pigment epithelial cells cultured on denuded human amniotic membrane revealed lower Notch activity in comparison to those cultured on tissue culture dish. These findings might provide an initial insight in the signaling modulations of retinal pigment epithelial cells cultured on denuded human amniotic membrane.

KEY WORDS: **ARPE-19, denuded human amniotic membrane, Notch signaling, degenerative retinal pigment epithelial diseases**

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INTRODUCTION

Age related macular degeneration (AMD) is still one of the leading causes of visual impairment in aging population of the western world. Among the two forms of AMD, dry AMD has a higher prevalence and is characterized by degeneration and atrophy of retinal pigment epithelial (RPE) cells.[1] Various options of treatment though available such as laser photocoagulation, photodynamic therapy, transpupillary thermotherapy and so on, had limited success.[2] More *in vivo* animal models and clinical data has to validate the incorporation of natural drugs in the treatment of AMD.[3] Hence, cellular therapy using transplantation of healthy RPE cells have been on the horizon as one of the potential futuristic treatment strategies, more so with the advent of RPE cells derived from induced pluripotent stem cells or embryonic stem cells. Transplantation of RPE cells in suspension state has resulted in complications like fibrosis, invasion of RPE cells into the retinal layer and vitreous cavity resulting in proliferative vitreoretinopathy.[4, 5] Hence, in order to maintain the cellular orientation and monolayer structure, a carrier for RPE cell transplantation is very much needed. One of the major limitations of cellular therapy is in identifying a suitable cell carrier. Several carriers apart from biodegradable scaffolds have been tried such as Descemet's membrane, lens capsule, Bruch's membrane, blood cryoprecipitates but with limited success.[6] Denuded human amniotic membrane (dHAM) have used as a scaffold for long in ophthalmic clinics for cell transplantation as well as patch for ocular cosmetics.[7-9] RPE cells also have been transplanted using dHAM as well.[2, 6, 10-12] It has been shown that RPE cells maintain their morphology, express RPE specific markers, show higher transepithelial resistance with calcium regulation, secrete vascular endothelial growth factor (VEGF) and other factors, phagocytose, differentiate, and undergo epithelial-mesenchymal transition (EMT).[6, 12-14] Apart from the basic physiological functions, it is important to study the signaling pathways as they are vital both in regeneration as well as integration of the transplanted RPE into the host tissue. But there is sparse information about the underlying signaling pathways and their status in RPE cells cultured on dHAM. Notch signaling is a developmental regulated and highly conserved signaling pathway involved in spatio-temporal regulation of cell physiological functions such differentiation, proliferation, angiogenesis, VEGF secretion, membrane potential, EMT and so on.[15-17] With respect to RPE it has also been shown that Notch signaling is crucial for migration and proliferation.[18] Notch modulates cell fate decision and is composed of membrane bound receptors and ligands. Notch signaling comprises of four receptors (Notch 1, 2, 3, 4) and five ligands (Deltalike (Dll) 1, 3, 4 and Jagged 1, 2). Signaling gets activated on binding of ligand to the receptor and there by activating gamma-secretase enzyme. The enzyme cleaves the membrane bound Notch receptor and release the Notch intracellular domain (NICD). The NICD translocates to the nucleus and binds to the CSL-binding domain thereby activating transcription of Notch downstream targets; such as the basic helix-loop-helix proteins Hes 1, 3, 5 and Hey 1 and 2.[19] These proteins regulate the cellular functions of Notch signaling. Though Notch signaling plays a definitive role in RPE functionality not much is known about its endogenous levels in RPE cells cultured on dHAM. We have investigated the expression profile of Notch receptors, ligands and the downstream targets on human RPE cells cultured on dHAM in comparison to RPE cells cultured on tissue culture dishes (TC).

MATERIALS AND METHODS

Preparation of Human Amniotic Membrane

Placenta is collected after obtaining the required consent. Human amniotic membrane is prepared as per the previously described protocol.[7] Briefly, the placenta is washed with 2X antibiotic solution (200U/ml Pencillin, 200µg/ml Streptomycin and 5µg/ml amphotericin B) (Himedia, Mumbai, India) inside class 2 biosafety cabinet. Then manually amnion is separated from chorion by leaving a small portion of umbilical cord tissue attached to the placenta in order to distinguish the orientation of the membrane (epithelial and stromal surfaces). Then the amnion is spread out with its epithelial side facing downwards. The mucus layer is cleaned with cotton swabs and sterilized nitrocellulose membrane was placed on the membrane without trapping air bubbles. Membranes of appropriate sizes are then cut and stored in vials containing 15ml of 1:1 Dulbecco's Modified Eagle Medium (DMEM): glycerol (Gibco, Life Technologies, Carlsbad, USA) medium at -80^{0}C. The sterility of the processed membrane was checked.

Preparation of dHAM

The amniotic membrane was stored at -80^{0}C in medium constituting 50% glycerol and DMEM in a ratio of 1:1 with antibiotics (100U/ml of penicillin, 100ug/ml of streptomycin (Himedia, Mumbai,India). It has been further de-epithelialized as per the protocol.[7] Thawed membrane was carefully placed on 60 mm tissue culture dish with the epithelial surface facing upwards and rinsed in 1X Phosphate Buffered Saline (PBS). Further 0.25% trypsin (Gibco, Life Technologies, Carlsbad, USA) was added and incubated for 15-20 minutes at 37^{0}C. Then the dissociated epithelial layer of the membrane was scrapped off with sterile glass slides and was rinsed with 1X PBS; observed under phase contrast microscope (Olympus CKX 41, Pennsylvania, USA) to ensure the complete removal of epithelial cells.

Hemotoxylin & Eosin (H & E) staining

The amniotic membrane has been stained with hemotoxylin and eosin before and after de-epithelialization procedure to depict the thorough removal of epithelial cells. The membranes were fixed in 10% formalin (Sigma Aldrich, MO, USA), washed with three changes of xylene (Sigma Aldrich, MO, USA) and rehydrated with graded series of ethanol (MP Biomedicals, CA, USA). The processed membranes were incubated for 5 min in hematoxylin solution, followed by decolorization in acid alcohol and counter stained with eosin for 1 minute (Thermo Fisher Scientific, Mumbai, India). The stained membranes were mounted with Distrene Dibutyl Phtalate Xylene (DPX)

(Merck, Darmstadt, Germany) and documented under light microscope (LeicaDM3000) using Leica DFC-290.

Culture of ARPE-19 cells

Cell culture studies were carried out with adult retinal pigment epithelial cell line (ARPE- 19) (generously gifted from Helder Andre, Karolinska Institute, Stockholm, Sweden) cultured on TC and dHAM. Dulbecco's Modified Eagle Medium: Nutrient mixture F12 (DMEM/ F-12) (Gibco, Life Technologies, Carlsbad, USA), containing 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, USA), and antibiotics (100U/ml of penicillin, 100ug/ml of streptomycin) (Himedia, Mumbai,India) was used for culture and maintenance of the cells for 5 days at 37°C with 5% CO_2. The ARPE-19 of passage 14 has been used for the study.

RNA extraction and cDNA conversion

Total RNA was extracted from the cells grown on different matrix using RNesay Micro Kit, (Qiagen, GmbH, Hilden, Germany) it has been quantified with Nanodrop spectrophotometer 1000 (Thermoscientific, Wilmington, USA) and complementary DNA was generated from those samples using High capacity cDNA Reverse Transcription Kit, (Life Technologies, Foster City, Carlsbad, USA).

Quantitative Real Time -PCR and Reverse Transcriptase -PCR

Quantitative real time-PCR was performed by using KAPPA SYBER FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), and analyzed on Bio-Rad CFX Connect (Bio-Rad, Hercules, CA, USA) for the cells cultured on TC and dHAM.. All experiments were done in triplicate, and data were normalized with the expression of housekeeping gene *GAPDH*. The list of gene specific primers is mentioned in Table 1. An RT-PCR was performed for *GAPDH*; *RPE65* gene to confirm the cell identity of the cultured ARPE-19 cells. Total RNA was extracted and converted to cDNA from cultured cells as mentioned previously. Briefly, equal amounts of extracted RNA was converted to cDNA using High-Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Carlsbad, USA) according to manufacturer's instructions and stored at -20°C. RT-PCR was performed on an Applied Biosystems™ Veriti (Life Technologies, Foster City, CA) with rpe65 and gapdh gene specific primers (Table 1). The PCR was set in a total volume of 25µl. The products were resolved on 1.5% agarose gel and documented on a UV transilluminator (Vilber Lourmat, Mumbai, India).

Immunofluorescence Staining

Cells cultured on TC and dHAM were fixed using 2% paraformaldehyde (Sigma Aldrich, MO, USA) on ice for 10 min, permeabilized with 0.1% Triton X 100 (Thermo Fischer Scientific, Mumbai, India) in 1X PBS for 15min, blocked for 1 h at room temperature with 1% bovine serum albumin/phosphate-buffered saline (Himedia, Mumbai, India). The blocked samples were followed by an overnight incubation at 4^0C with anti-Notch 1 polyclonal primary antibody raised in rabbit (1:100 dilution from 1 mg/ml), anti-Notch 4 primary antibody raised in mouse (1: 100 dilution from 1 mg/ml) (Cell signalling technology, Beverly, USA), anti-dll 1(1:100) and anti-dll4 polyclonal primary antibody raised in rabbit (1:100) (Abcam, Cambridge, United Kingdom). After a brief wash in 1X PBST (0.02% Tween 20 (MP Biomedicals, CA, USA), cells were incubated with dylight 488 anti-rabbit and dylight 488 anti-mouse (1:1000, 0.5 mg/ml; Biolegend, San Diego, USA) for 1 h. The slides were mounted using Vector shield containing 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) in aqueous mounting medium (Vector laboratories, CA, USA). The fluorescent images were documented using Zen software on fluorescent microscope -Olympus BX41 (Olympus America Inc., Melville, New York). Further the fluorescence intensity was quantified using ImageJ 1.48 version software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All the experiments were performed in triplicate and results of three independent experiments were used for statistical analysis. Data are represented as the mean ± SD or SE and were analyzed with Student's t-test. Significance value denoted, p* < 0.05, **< 0.01, ***< 0.005.

RESULTS

Culture and characterization of ARPE-19 cells

The results show a good de-epithelialization of the human amniotic membrane as has been shown with hematoxylin and eosin staining both the intact amniotic membrane (Fig 1A) and the denuded amniotic membrane (Fig 1B). ARPE-19 cells were cultured on TC and dHAM. Morphologically cells showed cluster formation when grown on dHAM compared to well spread out fibroblastic morphology on TC. Cells showed a hexagonal structure on TC as well as on dHAM (Fig 1C-D). Reverse transcriptase PCR showed RPE-65 gene expression confirming the RPE nature of the cells (Fig 1E).

Expression of Notch receptors in ARPE-19 cell

There are four Notch receptors through which the Notch signaling is exerted in cells. Gene expression of Notch receptors was evaluated in cells cultured on TC and dHAM. mRNA levels of *NOTCH 1* and *2* did not show any significant difference in the cells cultured on dHAM in comparison to those cultured on TC. *NOTCH 3* mRNA was expressed at low levels compared to the mRNA of four receptors on cultured ARPE-19 cells. On contrary, *NOTCH 4* gene was expressed in highest level among all the four receptors. Moreover, there was a significant decrease in the mRNA expression of *NOTCH 3* (p=0.01) in cells cultured on dHAM in comparison to those cultured on TC. Additionally, a significant decrease in the expression of *NOTCH 4* (p=0.02) in cells cultured on dHAM in comparison to those in TC (Fig 2A). Immunofluorescence staining with NOTCH 1 and NOTCH 4 antibodies revealed a lower expression in both the receptors in ARPE-19 cells cultured on dHAM in comparison to TC (Fig 2B- M). Quantification of the mean fluorescence intensity detected a significant downregulation of staining of NOTCH 1 and NOTCH 4 in ARPE-19 cells cultured on dHAM in comparison to TC (Fig 2N).

Expression of Notch ligands in ARPE-19 cell

Similar to the receptors, the Notch signalling ligands showed differential expression profile. Jagged 1, 2 and Dll 1, 3 and 4 are members of Notch ligands that play the crucial role of activating Notch signaling by binding to the Notch receptors. mRNA levels of *DLL 1* (p=0.02), *DLL 4* (p=0.004), *JAGGED 1* (p=0.03) and *JAGGED 2* (p=0.001) were significantly reduced in ARPE-19 cells cultured on dHAM compared to cells cultured on TC. Expression of *DLL 3* did not show any difference in cells cultured on dHAM to the cells cultured on TC. (Fig 3A). Immunofluorescence staining of cultured ARPE-19 cells revealed a weaker staining for DLL 1 and DLL 4 in cells cultured on dHAM in comparison to TC (Fig 3B-M). Quantification of the intensity of the images revealed a significant lower mean fluorescence intensity of staining in ARPE-19 cells cultured on dHAM in comparison to TC for DLL 1 *and* DLL 4 (Fig 3N).

Expression of Notch downstream target in ARPE-19 cell

Activation of the downstream targets are regarded as a hallmark for signaling pathway activity. Basic helix-loop-helix proteins such as HES 1, 3, 5 and HEY 1, 2 are the downstream targets of Notch signaling pathway. All the functions of Notch signaling are driven by these proteins. Expression of *HES 3* (p=0.02), *HES 5* (p=0.02) and *HEY 1* (p=0.002) were significantly downregulated in ARPE-19 cells cultured on dHAM in comparison to cells cultured on TC. However, there was no difference in the expression of *HES 1* in cells cultured on dHAM to those cultured on TC (Fig 4A).

Table 1
List of primers used for RT and q-RT PCR.

Serial Number	Primer Name (RT- PCR)	Sequences 5'-3'	Size, bp	Gene Accession No.
1	Gapdh	FP: GCCAAGGTCATCCATGACAAC RP: GTCCACCACCCTGTTGCTGTA	498	NM_001289746.1
2	Rpe-65	FP: TCCCCAATACAACTGCCACT RP: CCTTGGCATTCAGAATCAGG	369	NM_000329.2
Serial Number	**Primer Name (qRT- PCR)**	**Sequences 5'-3'**	**Size, bp**	**Gene Accession No.**
1	gapdh	FP: ACCCACTCCTCCACCTTTGAC RP: TGTTGCTGTAGCCAAATTCGTT	100	NM_001289746.1
2	Notch 1	FP: ATCCAGAGGCAAACGGAG RP: CACATGGCAACATCTAACCC	106	NM_017617
3	Notch 2	FP: GGACCCTGTCATACCCTCTT RP: CATGCTTACGCTTTCGTTTT	150	NM_024408.3
4	Notch 3	FP: GTGTGTGTCAATGGCTGGAC RP: GTGACACAGGAGGCCAGTCT	150	NM_000435.2
5	Notch 4	FP: GAGGACAGCATTGGTCTCAAGG RP: CAACTCCATCCTCATCAACTTCTG	61	NM_004557.3
6	Dll1	FP: GGCTACTCCGGCTTCAACT RP: ATCACCGAGGTCCACACACT	90	NM_005618.3
7	Dll3	FP: AGCTCGTCCGTAGATTGGAA RP: AGCGTAGATGGAAGGAGCAG	81	NM_016941.3
8	Dll4	FP: AAGGCTGCGCTACTCTTACC RP: AAGTGGTCATTGCGCTTCTT	90	NM_019074.3
9	Jagged 1	FP: AAGGCTTCACGGGAACATAC RP: AGCCGTCACTACAGATGCAC	120	NM_000214
10	Jagged 2	FP: GTCGTACTTGCACTCACAATACC RP: GTAGCAAGGCAGAGGGTTGC	51	NM_145159.2
11	Hes 1	FP: GAGAGGCGGCTAAGGTGTTT RP: GTGTAGACGGGGATGACAGG	118	NM_005524
12	Hes 3	FP: GAAAGTCTCCCTGGCTCGTC RP: CCAAATAGGGAGCGCCTTCA	146	NM_001024598
13	Hes 5	FP: AGAGAATGTGTGTGCAGAGTCC RP: GGTCAGACACTTGGCAGAAGA	70	NM_001010926
14	Hey 1	FP: GACCGTGGATCACCTGAAAA RP: TCCCAAACTCCGATAGTCCA	91	NM_012258

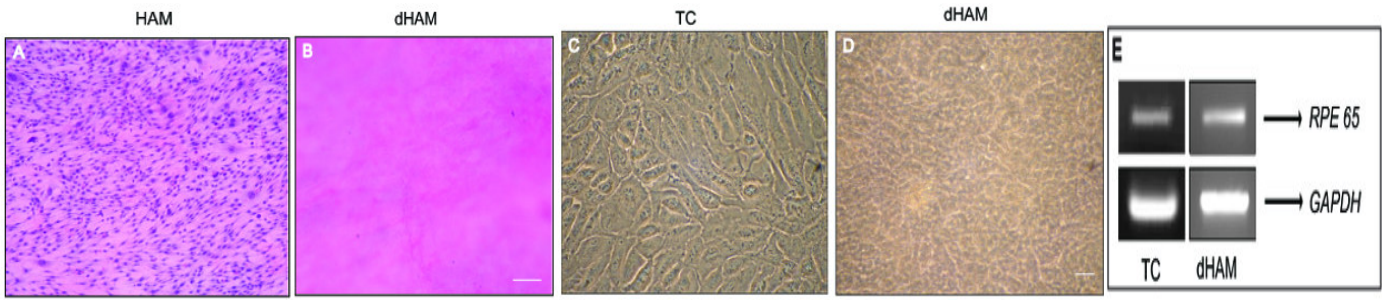

Figure 1
Culture of ARPE-19 cells.

Hematoxylin &eosin stained intact human amniotic membrane (A) and denuded human amniotic membrane after de-epithelialization method (B) . Phase contrast microscopic images of ARPE-19 cells cultured on tissue culture dishes (C) and denuded human amniotic membrane (D). RT-PCR results of RPE-65 gene in cells cultured on tissue culture dishes and denuded human amniotic membrane (E) (Bar: 10µm).

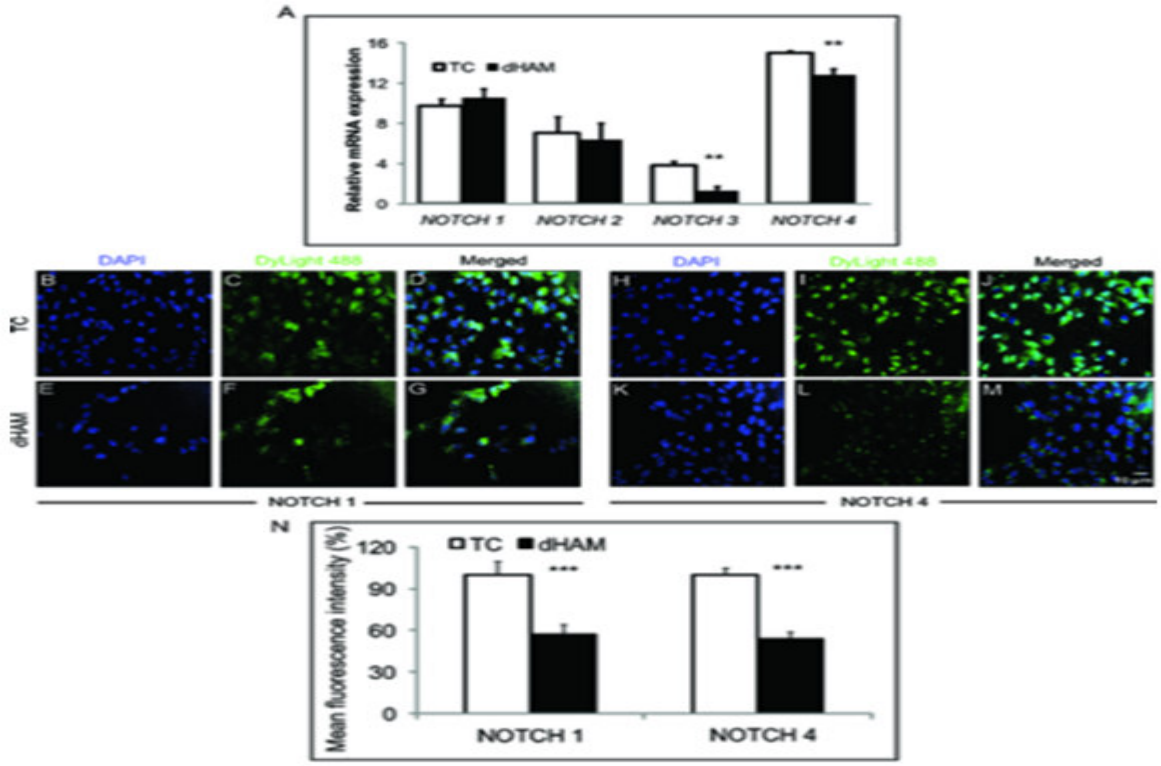

Figure 2

Expression of Notch receptors

Quantitative PCR of Notch receptors (Notch 1, 2, 3, 4) in ARPE-19 cells cultured on tissue culture dishes and denuded human amniotic membrane (A). GAPDH was used as the internal control . Immunofluorescence staining of Notch 1 (B-G) and Notch 4 (H-M) in cells cultured on tissue culture dish and denuded human amniotic membrane. Bar graph represents the mean fluorescent intensity of the images (N). Statistical calculation was performed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.005) (n ≥ 3).

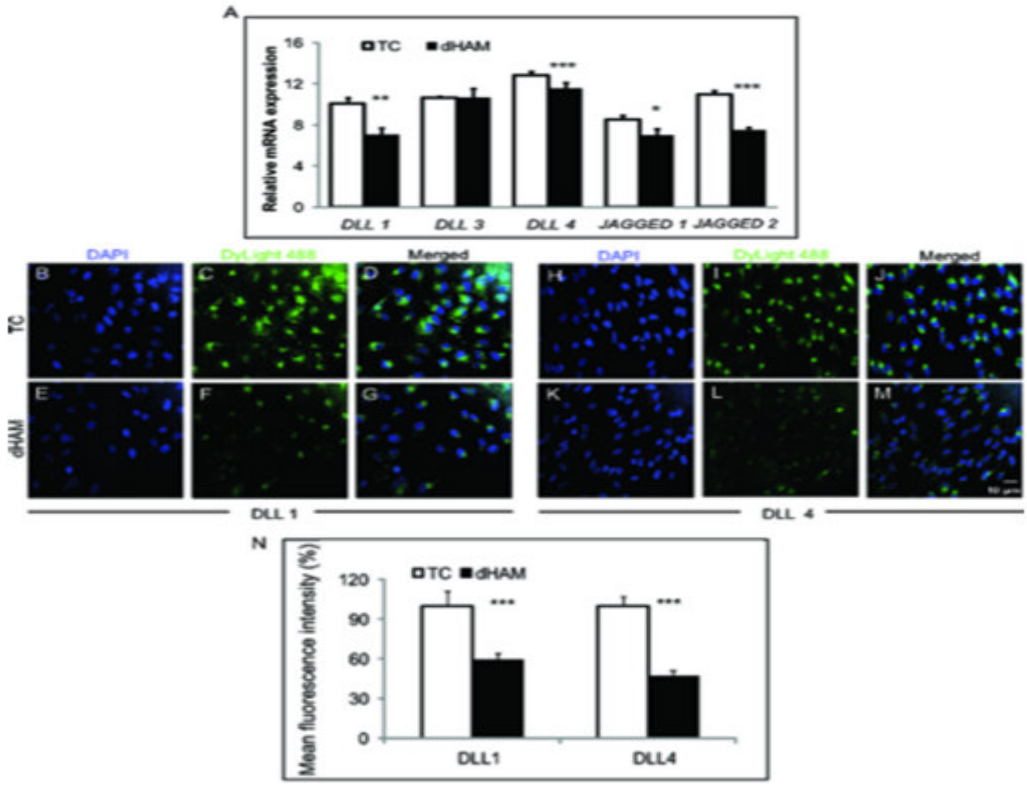

Figure 3

Expression of Notch ligands.

Quantitative PCR of Notch ligands (Dll 1, 3, 4 and Jagged 1, 2) in ARPE-19 cells cultured on tissue culture dishes and denuded human amniotic membrane (A). GAPDH was used as the internal control . Immuno fluorescence staining of Dll 1 (B-G) and Dll 4 (H-M) in cells cultured on tissue culture dishes and denuded human amniotic membrane . Bar graph represents the mean fluorescent intensity of the images (N). Statistical calculation was performed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.005) (n ≥ 3).

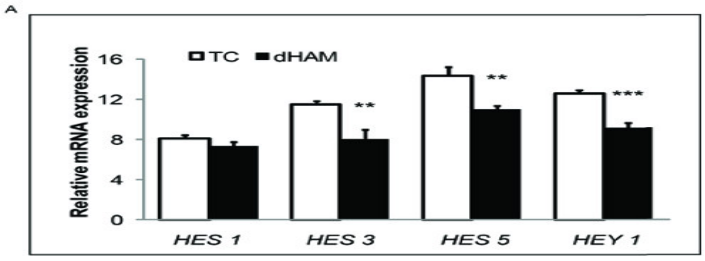

Figure 4

Expression of Notch downstream targets.

Quantitative PCR of Notch downstream targets (Hes 1, 3, 5 and Hey 1) in ARPE-19 cells cultured on tissue culture dishes and denuded human amniotic membrane (A). GAPDH was used as the internal control. Statistical calculation was performed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.005) (n ≥ 3).

DISCUSSION

Age related macular degeneration is one of the leading visions threatening disease caused by degenerative changes in the retinal pigment epithelium of the older population. Though a number of treatment strategies are practiced in the clinics, they have limited therapeutic benefits. There are reports of successful RPE cell transplantation in animal models of AMD.[20, 21] This paved the way for attempting for RPE cell transplantation in patients with AMD. In an effort to pursue cell therapy, denuded human amniotic membrane has been looked upon as a befitting cell carrier for retinal pigment epithelial cell transplantation. Denuded human amniotic membrane has been used as a carrier in ophthalmology for cellular therapy (limbal stem cell deficiency disease).[7, 17, 22] Studies have shown that RPE cells cultured on dHAM showed enhanced physiological activities such VEGF secretion, phagocytosis, trans-epithelial resistance with calcium regulation, EMT regulation and so on.[12] Though all the studies have highlighted the cellular properties of RPE cell cultured on dHAM, there is a lack of studies investigating the underlying signal transduction pathways in RPE cells cultured on dHAM. It is imperative that signaling pathways would be crucial in not only dictating the RPE cellular functions but also aid in proper integration and recovery of degenerative RPE in pathological state. These underlying signaling pathways would also provide insight on the success of cell transplantation in pathological conditions. In an attempt to understand, we have investigated the Notch signaling pathways that are known to have definitive role in RPE cell physiology in health and disease.[23, 24] We have studied the endogenous profile of Notch signaling that has been shown to have a role in EMT as well as in regulating RPE functions in the presence of bevacizumab.[16] Notch is an evolutionarily conserved signaling pathway and is spatio-temporal regulated during developmental stages. It has four receptors and five ligands as its members which are membrane bound proteins. Notch signaling is activated with the receptor-ligand binding, resulting in cleavage of membrane bound Notch receptor releasing the Notch intracellular domain (NICD). NICD then translocates to the nucleus to initiate transcription of basic helix-loop-helix proteins, by binding to the CSL-domain.[15] Notch receptors and ligands are differentially expressed on the ARPE-19 cells cultured on TC and dHAM. Cultured ARPE-19 cells showed highest mRNA levels of *NOTCH 4* and lowest of *NOTCH 3* expression among all the four receptors. There were no detectable differences in the mRNA expression of *NOTCH 1* and *NOTCH 2* in ARPE-19 cells cultured on dHAM and TC. Expression of *NOTCH 3* and *NOTCH 4* genes were significantly lower in ARPE-19 cells cultured on dHAM in comparison to TC. Additionally, a significant downregulation in the protein levels of NOTCH 1 and NOTCH 4 was detected in cells cultured on dHAM compared to those on TC. Though there was no difference in the mRNA levels of NOTCH 1 in cultures, a significant difference in the NOTCH 1 protein levels was detected in cell cultured on dHAM to those on TC. The poor correlation of the NOTCH 1 levels with respect to mRNA and protein suggest a need for a better understanding on the degradation and protein synthesis.[25] Unaffected mRNA levels and lower protein detection also implies a plausible defective protein synthesis machinery.[26, 27] Among the ligands of Notch signaling pathway, *DLL1*, *DLL4, JAGGED 1* and *JAGGED 2* showed significantly lower expression in cells grown on dHAM in comparison to those cultured on TC. Concurrent to the downregulation of the mRNA expression, immunofluorescence staining too revealed a lower staining intensity of DLL1 and DLL4 in ARPE-19 cells cultured on dHAM compared to those on TC. Among the downstream targets of Notch signaling, mRNA levels of *HES 3*, *HES 5* and *HEY 1* showed significant decrease in the cells grown on dHAM in comparison to those cultured on TC. Expression levels of *HES1* remained unaffected in cells cultured on dHAM and TC. This might suggest that *HES1* regulation in the ARPE-19 cells cultured on dHAM could be independent of canonical Notch signaling. It has been shown that *HES 1* can be regulated by Sonic hedgehog signaling in a Notch independent pathway.[28, 29] Notch signaling modulates cell fate determination by spatio-temporal activity. Activated Notch signaling can block neural differentiation, where as it is much needed for corneal as well as skin epithelial differentiation.[17, 30] Notch exerts cell type specific function in context dependent manner.[15] NOTCH 1, 4 and DLL4 have been strongly implicated in vasculogenesis and angiogenesis in retinal vasculature.[31, 32] Lower expression of the protein levels of NOTCH 1, 4 and DLL4 in ARPE-19 cells cultured on dHAM suggest that most likely these cells on transplantation may not support for angiogenesis regulation in the retina. Additionally NOTCH 1 also regulates the proliferation of RPE cells and studies have shown that blocking Notch signaling prevents proliferation and migration.[18] Based on our results of NOTCH 1 regulation it can be interpreted that ARPE-19 cells cultured on dHAM might reveal a defective proliferative and migratory behaviour on a long term

follow-up post transplantation. EMT of RPE cells is a key process in development of proliferative vitreoretinopathy and proliferative diabetic retinopathy.[33] Notch signaling also induces EMT via TGFβ-2 induction in RPE cells through the upregulation of *NOTCH 3* and *JAGGED 1* along with downstream targets.[24, 33, 34] The lower expression of *NOTCH 3* in ARPE-19 cells cultured on dHAM might indicate a lower induction of the receptor. Hence, lower expression *JAGGED 1* and unaffected *NOTCH 3* levels suggest that ARPE-19 cells cultured on dHAM might be resistant to the EMT induced by TGFβ-2. Notch signaling has been also implicated in proliferation and migration as shown by Notch blocking experiments, epigenetic regulation and using transgenic animals.[18, 23, 35] Apart from the basic cellular functions, *NOTCH 4* and *DLL4* modulate the venous and arterial vasculature.[31] Decrease in the expression of *NOTCH 4* and *DLL 4* imply a very likely compromise in vascular regulation with a biasness towards venous vasculature.[16] DLL-1 and DLL-4 have role in arterial specification along with NOTCH 4.[36] DLL-4 and JAGGED 1 have opposing role during vascular sprouting with respect to tip and stalk.[36] JAGGED 1 in conjunction with DLL-1 and NOTCH -1 have been implicated in tissue regeneration.[37, 38] Zhang et al., showed that Notch signaling can modulate pathogenesis of proliferative vitreoretinopathy (PVR) in mouse model by regulating macrophage polarization.[39] The differential expression of Notch signaling suggests its definitive role in retinal pigment epithelial cells. Our results provide the foundation for a detailed study on the outcome of differential Notch pathway molecules in RPE cells. Hence, further studies in understanding the outcome of the differential expression on the RPE functionality with respect to RPE properties and the regenerative potential. It is of utmost value as this would pave way for a better understanding of the cell transplantation outcome. It is feasible that RPE cells could be provided with the necessary Notch regulators (as recombinant proteins) prior to transplantation in host to enhance the transplantation success.

CONCLUSION

Denuded human amniotic membrane are widely proposed to be suitable carrier for retinal pigment epithelial cell transplantation in patients with age related macular degeneration and other retinal pigment epithelial disorders. Notch signaling is known to have regenerative and growth promoting role in retinal pigment epithelial cells. Hence we have looked in regulation of Notch signaling in human retinal pigment epithelial cells cultured on denuded human amniotic membrane. The results show that Notch signaling is reduced in ARPE-19 cells cultured on dHAM compared to those cultured on tissue culture dishes. These results provide an initial insight into the signaling pathway modulation with respect to Notch signaling. Though further studies would be necessary to study the implications with respect to the regeneration and cellular therapy, it can very well be envisaged that for better outcome of the transplantation it might be necessary to culture the cells in the presence of recombinant Notch proteins to enhance the endogenous Notch levels of cells prior to transplantation.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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