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RESEARCH ARTICLE

Differentiation of eye field neuroectoderm from human adipose-derived stem cells by using small-molecules and hADSC-conditioned medium

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ABSTRACT

Recently, stem-cell therapy as a promising therapeutic alternative is considered to treat retinal degenerative diseases. Here, we used small molecules and concentrated conditioned medium selectively enriched with Amicon filter units from human adipose-derived stem cells (hADSC-CM) containing various neurotrophic factors to induce hADSCs toward eye field neuroectoderm (EFN). For induction of stem cells, hADSC-CM and small molecules CKI-7, SB431542 and LDN193189 as inhibitors of Wnt, Nodal and BMP4 signaling pathways were used, respectively. We found the highest expression of β-TUB III as a neural marker in the group in which small molecules and conditioned medium were applied simultaneously. Moreover, EFN markers SIX3, PAX6 and RAX had higher expression in the presence of a conditioned medium. However, the superior expression of ENF marker OTX2 was seen in the small molecules group. Our results indicated that neurotrophic factors present in hADSCs-CM could induce hADSCs into EFN cells. Therefore, a more thorough study of these factors and their effects in hADSC-CM might pave the way for cellular and non-cellular therapy in retinal degenerative diseases.

1. Introduction

Retinal diseases such as retinitis pigmentosa (RP) and age related macular degeneration (AMD) subsequently lead to degeneration of photoreceptors and retinal neurons and visual impairment or blindness (Hartong et al., 2006; Levine and Brivanlou, 2007; Sahni et al., 2011). The neural cells in human retina, like those in the central nervous system (CNS), have limited potential for self-regeneration (Amirpour et al., 2011; Lamba et al., 2009).

Cell therapy could be a therapeutic strategy for treatment of retinal disorders (Hartong et al., 2006). Adult mesenchymal stem cells (ASCs) with neural differentiation potential may represent a suitable source for the treatment of various retinal diseases. Among the ASCs, human adipose-derived stem cells (hADSCs) are considered due to high availability, easy accessibility, active self-renewal, and low immunogenicity (Salehi et al., 2016). Previous studies showed that hADSCs have the potential to differentiate into neural cells such as anterior neuroectodermal cells (Salehi et al., 2016, 2017). According to our knowledge, there are limited studies on the differentiation of hADSCs toward retinal cells (Egashira et al., 2012; Sugitani et al., 2013). It has been shown that hADSCs secrete several neurotrophic factors such as ciliary neurotrophic factor (CNTF), brain derived growth factor (BDNF), neurotrophin-3(NT-3), NT4/5, Glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) (Salehi et al., 2016, 2017). It was demonstrated that hADSC-conditioned media (hADSC-CM) contains the above mentioned neuroprotective and neurotrophic factors (Fontanilla et al., 2015). In the previous decade, the researchers applied some exogenous neurotrophic factors for neural differentiation of stem cells and treatment of neurodegenerative disorders (Chen et al., 2013; Lim et al., 2008; Yeh et al., 2015).

Some signal pathways in a gradient manner regulate vertebrate neural development by their excitatory or inhibitory roles (Spemann and Mangold, 2003). The recent investigations proved that inhibition of bone morphogenetic protein (BMP), Nodal and Wnt signaling pathways plays an essential role in eye field neu-

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Table 1
Antibodies used in this study.

Primary Ab	Species	Clonality	Source
CD44 R-PE	Mouse	Monoclonal	IQ product, IQP118R
CD90 FITC	Mouse	Monoclonal	DakoCytomation, F7274
CD45 FITC/CD14 R-PE	Mouse	Monoclonal	IQ product, IQP-228FR
β-TUB III	Mouse	Monoclonal	Abcam, ab14545
RAX	Rabbit	Polyclonal	LifeSpan, LS-C98140
PAX6	Mouse	Monoclonal	Millipore, MAB5554
BDNF	Rabbit	Polyclonal	Santa Cruz, SC-546
CNTF	Rabbit	Polyclonal	Abbiotec, 251634
pSMAD1/5/8	Rabbit	Polyclonal	Millipore, AB3848-I
pSMAD2	Rabbit	Polyclonal	Millipore, ABE2872
Secondary Ab			
TRITC anti-rabbit IgG	Goat	Polyclonal	Santa Cruz, SC-3841
FITC anti-mouse IgG	Goat	Polyclonal	Abcam, ab97022
HRP anti-rabbit IgG	Goat	Polyclonal	Santa Cruz, SC-2004

ral induction (Levine and Brivanlou, 2007). Recombinant proteins such as Noggin (a BMP antagonist) (Lupo et al., 2013), Lefty (a Nodal antagonist) (Smith et al., 2008) and Dickkopf-1 (Dkk1, a Wnt antagonist) were applied to block these pathways in experimental studies. Previous data showed that the small molecules as non-biological products could be a suitable substitute for recombinant proteins in neural induction (Amirpour et al., 2017; Osakada et al., 2009a). For instance, the small molecules CKI-7 (CKI), SB431542 (SB) and LDN193189 (LDN) can inhibit the signaling pathways Wnt, Nodal and BMP4, respectively (Osakada et al., 2009a). Therefore, in the present study, we used hADSC-CM and CKI, SB and LDN small molecules to induce hADSCs toward retinal progenitor cells.

2. Materials and methods

2.1. Isolation and culture of hADSCs

hADSCs were obtained from subcutaneous tissues of three female donors who underwent elective liposuction surgery (mean age 28.33 ± 6.5 years). hADSCs from each donor were maintained as an independent cell line in all experiment. All patients were informed and gave their approval by written consent. The tissue samples were treated with 0.075% collagenase type I in PBS for 30 min at 37 °C. Afterwards the collagenase I was neutralized with an equal volume of culture medium containing Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The samples were centrifuged for 10 min at 1600 rpm. The pellet was resuspended and cultured in in 75 cm^2 flasks, using culture medium. The medium was changed every 2–3 days. At 70–80% confluency, cells were passaged. In the present study, cells of passages 3–5 were used for trials.

2.2. Characterization of hADSCs

The surface markers of hADSCs were evaluated by flow cytometry. The hADSCs were collected by trypsinization; then the cells were centrifuged and resuspended in culture medium at a density of 1×10^5cells for each test. After that, the cells were washed twice with 1% bovine serum albumin (BSA)/PBS and incubated with antibodies against positive (CD44, CD90, from IQ product and Dako Cytomation, respectively) and negative (CD14/CD45, from IQ product) markers for 30 min as listed in Table 1. Primary antibodies were directly conjugated with fluorescein isothiocyanate (FITC) or R-phycoerythrin (R-PE). Negative control staining was performed using a FITC-conjugated mouse IgG isotype and a PE-conjugated mouse IgG isotype antibody. Flow cytometry was performed using a flow cytometry system (Becton–Dickinson, San Jose, CA).

2.3. Preparation of hADSC-CM

For conditioned medium preparation, hADSCs from passages 3–5 at a seeding density of 1×10^5 cells per cm^2 were maintained in DMEM (FBS-free) medium. After 72 h, the supernatant was harvested and then centrifuged, filtered, and 15-fold concentrated at 3600 g using Amicon_ Ultra-15 centrifugal filter units (Millipore, UFC901024), for 12 min. The fraction (20 kDa < MW < 60 kDa) was used as hADSC-conditioned medium (CM) and stored at −70 °C until use.

2.4. Western Blot

Twenty micrograms of protein from hADSC (STEM) and treated hADSC (after 72 h) lysates and concentrated hADSC-conditioned medium (CM) were collected. The protein concentration was assessed with a Bradford assay (Bio-Rad) kit. After electrophoresis of proteins, the samples transferred on a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Munich, Germany). Members were treated with 10% skim milk overnight and incubated with primary antibodies listed in Table 1 for 90 min. The blots were washed with washing buffer and then incubated with horse radish peroxidase conjugated secondary antibody (Table 1) in blocking solution for 2 h at RT. Afterwards, the immunoblots were visualized with an ECL advanced western blot detection kit (GE Healthcare). All of these steps were done separately for each antibody.

2.5. Induction of hADSCs to eye field neuroectoderm

hADSCs were dissociated with 0.25% trypsin/EDTA and then plated at 4000 cells per well in a 24-well plate. In the present study, the cultivated cells were randomly assigned to five groups. Control group (STEM): hADSCs were seeded on culture medium. The STEM/IM group: the cells were exposed to induction medium (Neurobasal medium supplemented with 25 ng/ml bFGF, 1% none essential amino acid, 1% L-glutamine, 1% penicillin/streptomycin, 2.5 ng/ml IGF-1, 2% N2, 1% B27). The STEM/CM group: the cells were exposed to induction medium with 10% hADSC-CM. The STEM/SM group: hADSCs were seeded in induction medium with small molecules (0.5 μM LDN, 5 μM SB, and 5 μM CKI). The STEM/CM/SM group: the cells were treated with induction medium containing 10% hADSC-CM and above mentioned small molecules. The media were changed every three days, for 21 days.

2.6. Immunocytochemistry

Differentiated cells were fixed for 20 min in 4% paraformaldehyde and permeabilized using PBS including 0.4% Triton X-100 for 30 min. The cells were subsequently treated with a blocking agent. We used serum from the source species for the secondary antibody (goat serum) and bovine serum albumin (BSA) as a blocking agent. The primary antibodies were added overnight. After that, the cells were exposed to a secondary antibody for one hour at 37 °C. Nuclei were stained with 4, 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, D8417). The antibodies used in this experiment are listed in Table 1.

The percentage of positive cells was quantified by ImageJ software ((NIH, MD, USA, Cell counter plugin) in comparison to the total number of cells as indicated by DAPI in the fields. For each staining more than 500 cells were counted.

2.7. RNA isolation and RT-PCR analysis

The high Pure RNA Isolation Kit (Roche) was used for total RNA isolation according to the manufacturer's instructions. The RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis

Table 2
Primer sequences and conditions for RT-PCR and Real-Time PCR.

Gene	Sequence 1 (5′ to 3′)	Sequence 2 (5′ to 3′)	Cycle	Accession no.
GFAP	GAGATGCGGGATGGAGAG	TAGGGACAGAGGAGGGAG	35	NM_002055
C-MYC	AAACACAAACTTGAACAGCTAC	ATTTGAGGCAGTTTACATTATGG	35	NM_002467
OTX2	AACAGCAACAACAGCAGAAT	GGAAGAGGAGGTGGACAAG	40	NM 001270523
SIX3	CGGAATGTGATGTATGATAGC	GGAGAAGGAAGAGGAGGAA	40	NM 005413
RAX	AGCGAAACTGTCAGAGGAGGAACA	TCATGCAGCTGGTACGTGGTGAAA	40	NM_013435
PAX6	AGTGAATCAGCTCGGTGGTGTCTT	TGCAGAATTCGGGAAATGTCGCAC	40	NM_000280
β-TUB III	GCGGATCAGCGTCTACTAC	CCCACTCTGACCAAAGATGAA	40	NM_001197181.1
GAPDH	AAGCTCATTTCCTGGTATG	CTTCCTCTTGTGCTCTTG	40	NM_001256799

Kit (Thermo Scientific) with oligo dT primers (designed by AlleleID 7.60 software and synthesized by Metabion Germany). RT-PCR was carried out with 2 μg total RNA. The used primer sequences are shown in Table 2.

Real-time polymerase chain reaction was carried out using Maxima SYBR Green Rox qPCR master mix kit (Thermo Scientific) and the StepOne PlusTM quantitative Real-time PCR Detection System (Applied Biosystems). PCR reactions were performed in a total volume of 20 μl. The PCR amplification conditions consisted of 10 min at 95 °C followed by 40 cycles of a denaturation step at 95 °C for 15 s and annealing and extension for 1 min at 60 °C. The expression rate of target genes were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression level of each target gene was calculated by $2^{-\Delta\Delta CT}$. Real Time PCR was run at least in three technical replicates for each sample (triplicate).

2.8. MTT assay

To determine viability of differentiated cells, hADSCs (4000cells/well) were cultured for 1, 7, 14 and 21 days. After removing induction media, 400 μl DMEM containing 40 μl MTT(3-

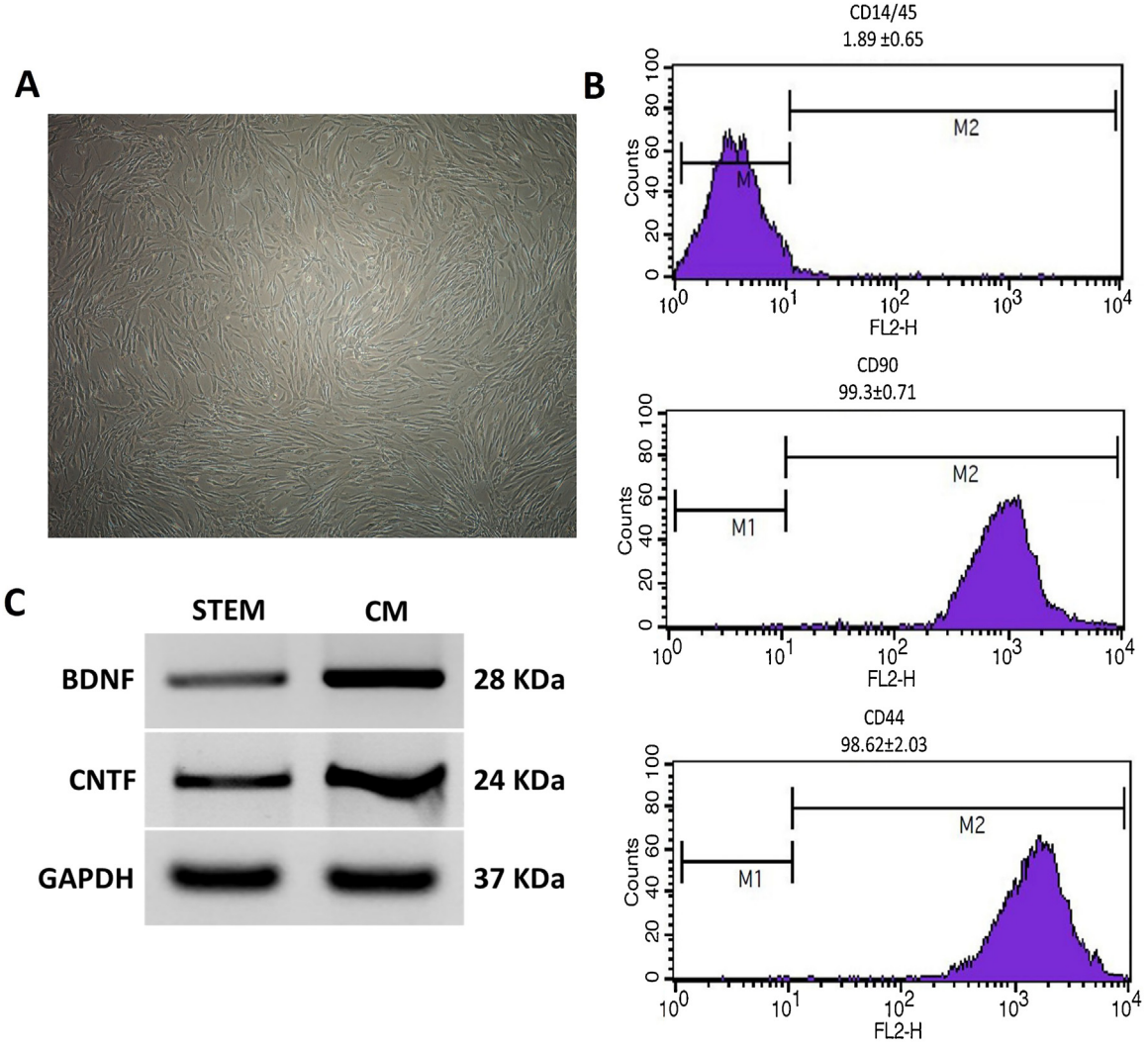

Fig. 1. Characterization of hADSCs: Phase contrast photomicrograph of hADSCs passage 3 (original magnification 400×), A. Flow cytometry histograms of CD14/45, CD44, and CD90 for hADSCs, B. The presence of neurotrophic factors, BDNF and CNTF in hADSCs and their conditioned medium, C. Marker M1: The negative peak of the subclass control. Marker M2: designate positive events ($CD14/45^+$, $CD44^+$, $CD90^+$). The X axis is intensity (of scatter or fluorescence), and the Y axis shows how many cells had each intensity.

[4,5-dimethythiaziazol-2yl]-2,5-diphenyl tetrazolium bromide) was added and incubated for 4 h at 37 °C. The medium was discarded, and 400 μl dimethyl sulfoxide (DMSO) was added to each well. The optical density measurement was performed at 540 nm by a microplate reader (Hyperion MPR 4+, Germany).

2.9. Statistical analysis

All values were presented as mean ± SEM (Standard Error of Mean). The data collected from MTT, real time RT-PCR, and immunocytochemistry were analyzed by one-way ANOVA followed by Tukey's test with a significance threshold of $P < 0.05$. All experiments were independently repeated at least three times.

3. Results

3.1. Characterization of hADSCs

hADSCs from 3rd to 5th passage with fibroblast-like cell morphology were used in this experiment (Fig. 1A). Flow cytometry assessment was conducted to confirm the stemness feature and mesenchymal origin of hADSCs. Flow cytometry analysis demonstrated that the cells were negative for the hematopoietic stem cell marker CD14/45 (1.89 ± 0.65%) and positive for the mesenchymal stem cell markers CD90 (99.3 ± 0.71%) and CD44 (98.62 ± 2.03%) (Fig. 1B). The conditioned medium of hADSCs was harvested after 72 h. Then, hADSCs-CM was concentrated and selectively enriched by Amicon Ultra-centrifuged. The presence of two important neurotrophic factors, BDNF and CNTF was assessed by western blot

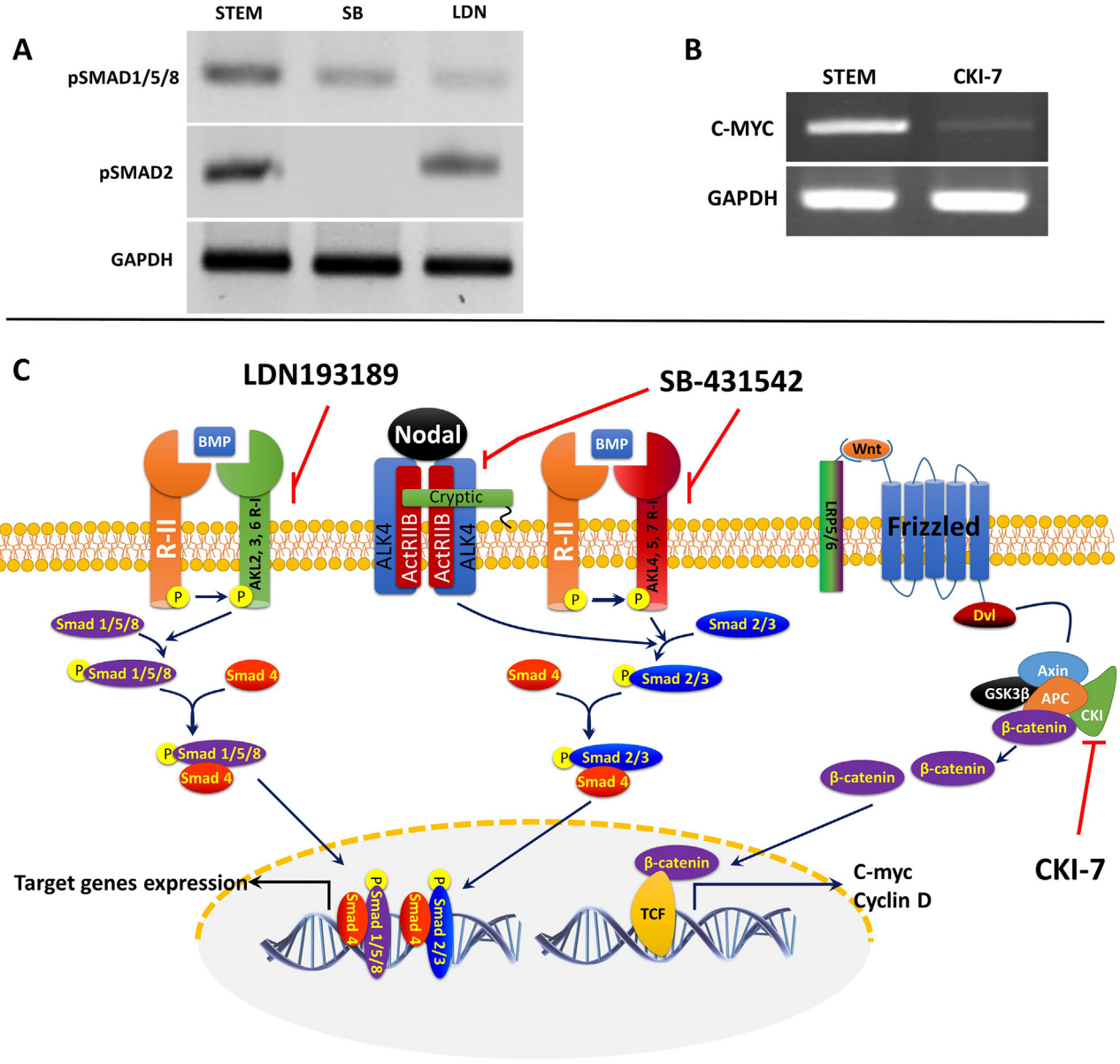

Fig. 2. The inhibitory effects of small molecules: western blot results revealed that the phosphorylation of SMAD 1/5/8 was reduced in presence of LDN and the SMAD 2 phosphorylation was blocked by SB, A. RT-PCR data indicated downregulation of C-MYC by CKI, B. The experimental model of small molecules which inhibits Wnt, Nodal, and BMP signaling pathways: after binding of Wnt ligand to a Frizzled/LRP-5/6 receptor, β-catenin is stabilized. Meanwhile, the small molecule CKI, blocks Wnt signaling through casein kinase I. Therefore, β-catenin is degraded and consequently C-MYC is downregulated. SB and LDN inhibit activin receptor-like kinase receptors (ALKs) and block Smad mediated-signaling transduction. In Smad signaling, Smad 4 heteromerizes with activated R-Smads (Smad2/3 and Samd1/5/8) and subsequently enters into the cell nucleus, C.

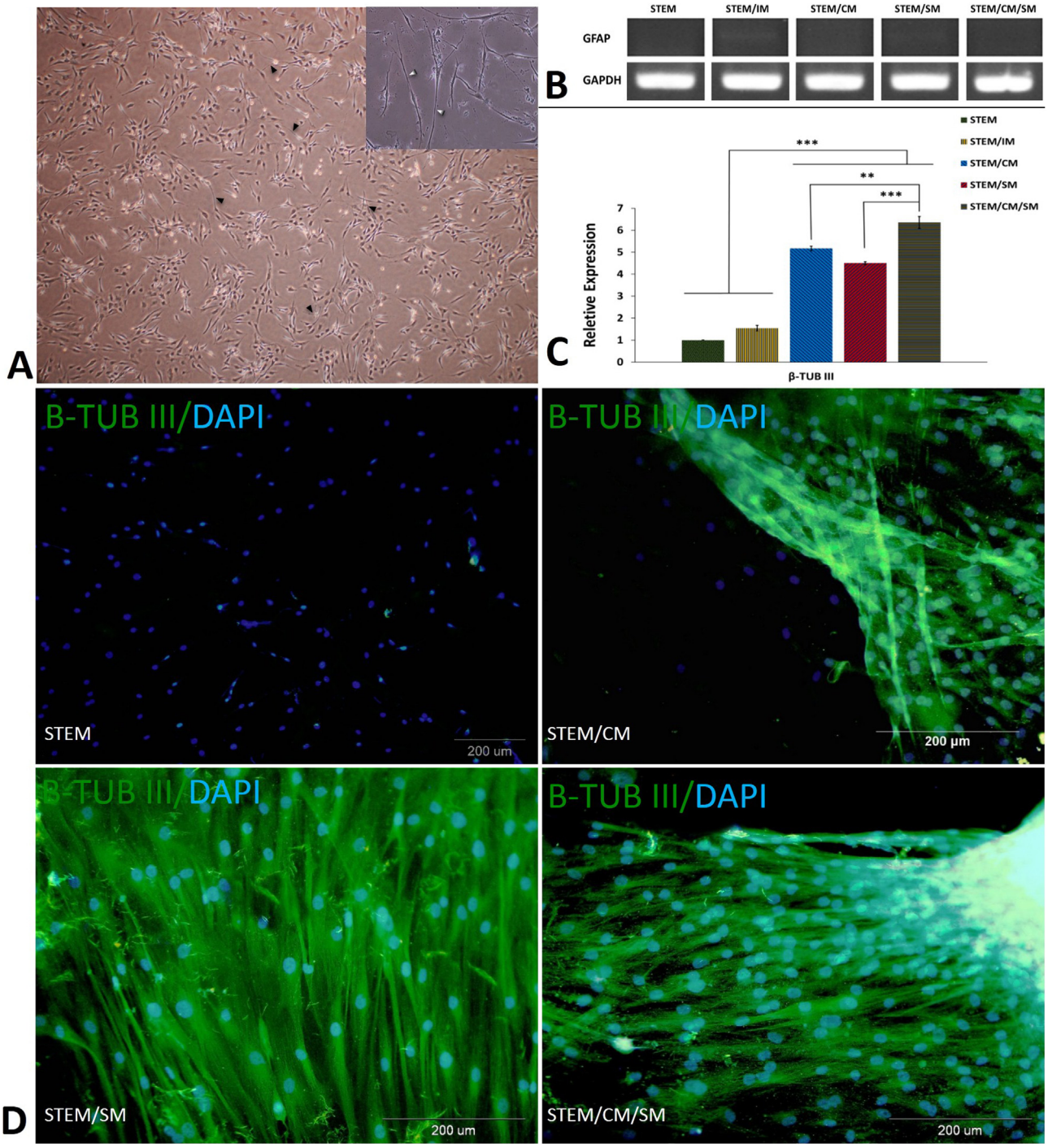

Fig. 3. Neural differentiation of hADSCs: Phase contrast photomicrograph of differentiated hADSCs with neurites (arrowheads) (original magnification 40X and 400X), A. RT-PCR analysis showed the lack of expression of GFAP in the groups at day 21, B. Quantitative RT-PCR, and immunocytofluorescence images of neural differentiated cells for β-TUB III in the experimental groups at day 21, C-D. **:$P < 0.01$ and ***:$P < 0.001$.

method. The western result indicated the existence of these factors in concentrated hADSCs-CM (Fig. 1C).

3.2. Differentiation of human adipose-derived stem cells toward eye field neuroectoderm

For eye field neuroectodermal (EFN) differentiation of hADSCs, the isolated cells were exposed to the induction medium for 21 days. We used three small molecules (CKI, SB and LDN) in induction medium to block the signaling pathways Wnt, Nodal and BMP4, respectively. In order to investigate the blocking effects of these small molecules we determined the expression of pSMAD1/5/8 and pSMAD2 (as a downstream of activated BMP and Nodal signaling pathways, respectively) and C-MYC (as a target gene of Wnt signaling pathway) in our experiment. Our data indicated that SB inhibited the phosphorylation of SMAD2. In addition, LDN attenuated the phosphorylation of SMAD1/5/8. The expression of C-MYC was also decreased by CKI (Fig. 2A–C).

After induction, the fibroblast-like morphology of the cells changed to a typical neuronal morphology with extended neurites

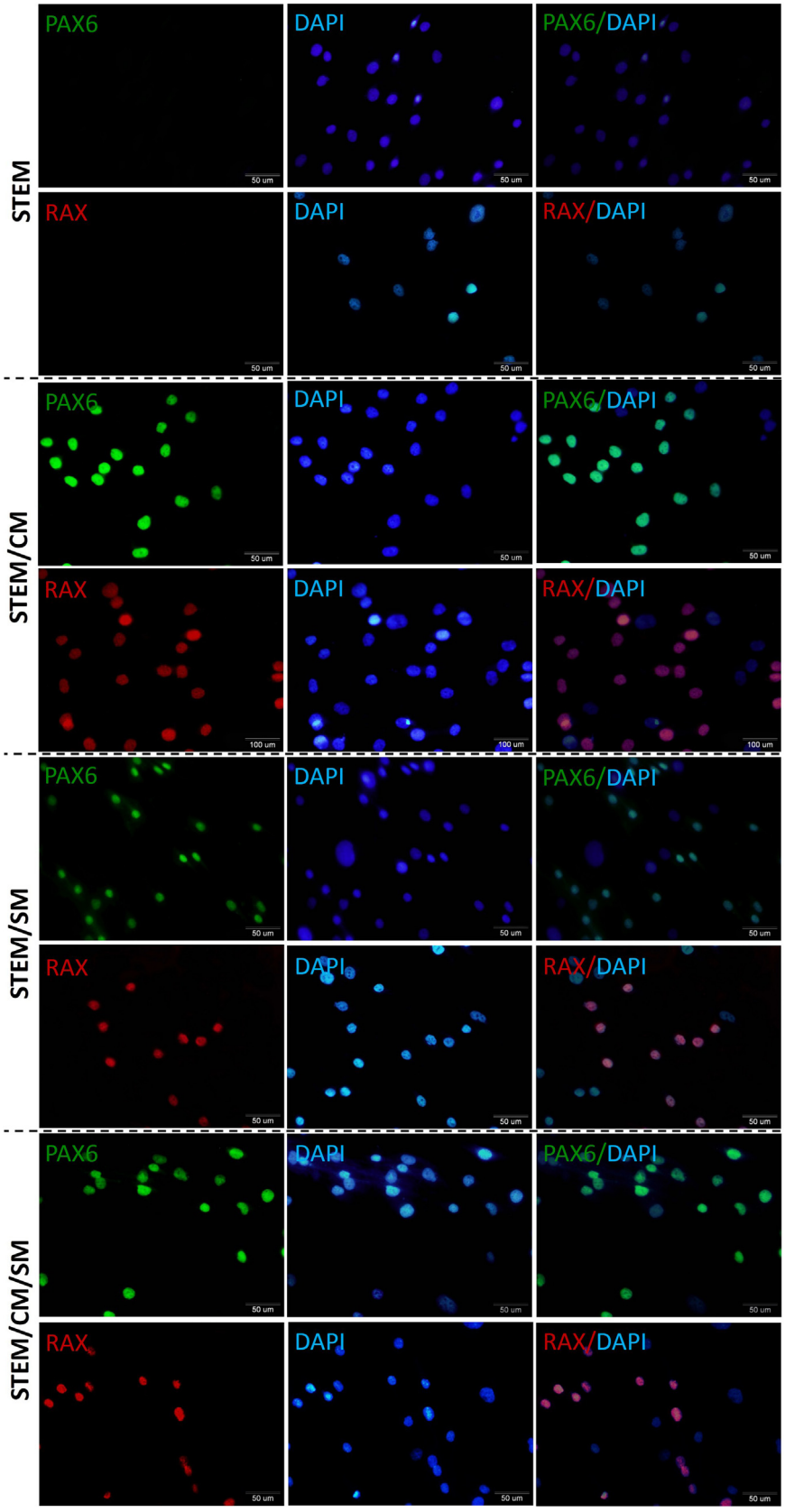

Fig. 4. Characterization of differentiated hADSCs: Immunocytofluorescence images of hADSC-derived EFN cells indicated the expression of EFN markers (PAX6 and RAX) at day 21 in the experimental groups.

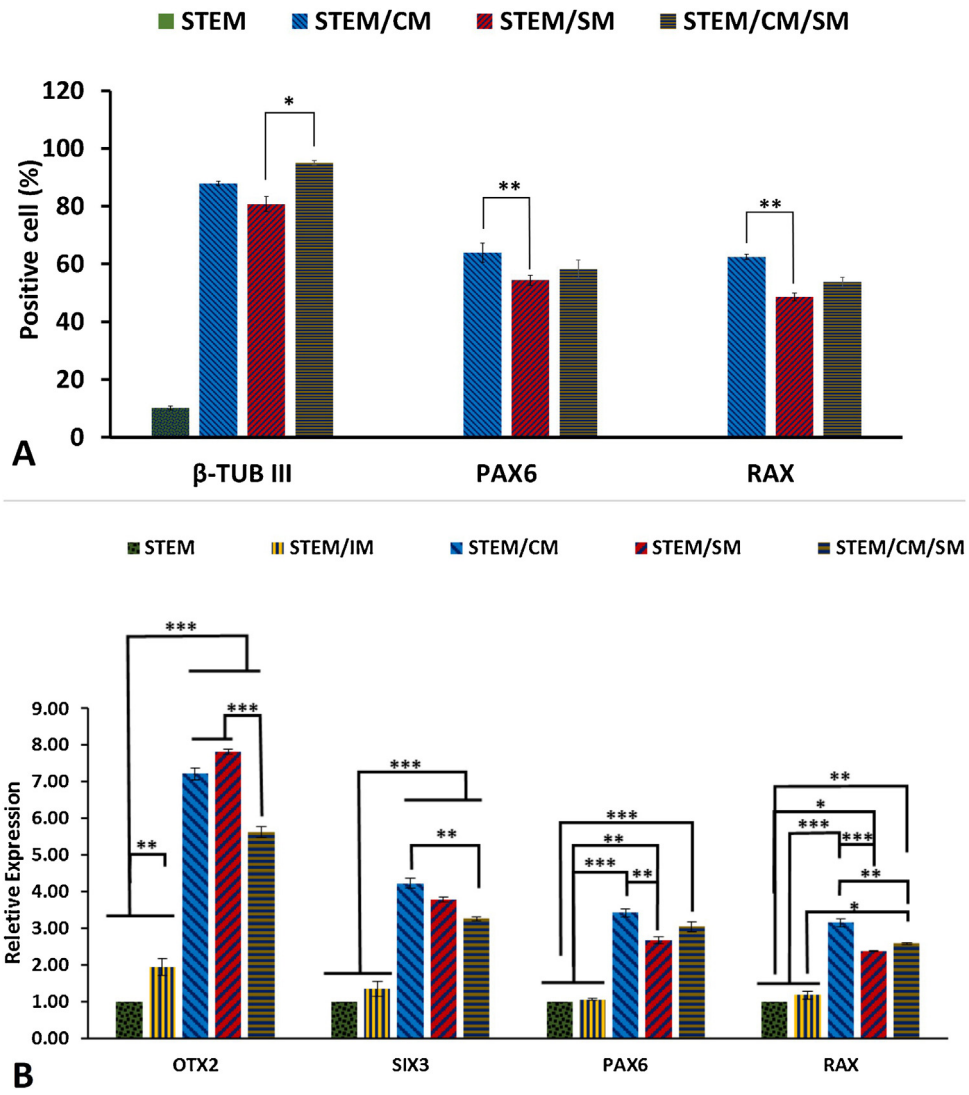

Fig. 5. Quantification of immunofluorescence staining and quantitative RT-PCR: the data showed β-TUB III, PAX6 and RAX significantly upregulated in the treated groups compared to the STEM group (More than 500 cells were counted in each group), A. The expression of OTX2 was significantly superior in the STEM/SM group and SIX3, PAX6, and RAX in the STEM/CM group, B. *: $P < 0.05$, **:$P < 0.01$ and ***:$P < 0.001$.

(Fig. 3A). A potential differentiation of hADSCs into astrocytes was evaluated by RT-PCR. The results indicated that expression of GFAP (astrocyte marker) was not detected in all groups except a negligible expression in the STEM/IM and the STEM/SM groups (Fig. 3B). Furthermore, quantitative RT-PCR and immunocytochemical (ICC) analyses confirmed that significant expression of the neuronal marker (β-TUB III) in the treated groups was present compared to the control group at day 21 (Fig. 3C-D). After 21 days, immunostaining results indicated the expression of EFN markers PAX6 and RAX in treated groups (Fig. 4). Quantitative immunostaining showed the highest expression of β-TUB III (95.06 ± 0.68) in the STEM/CM/SM group, and PAX6 (63.91 ± 1.31) and RAX (62.77 ± 3.31) in the STEM/CM group compared to others (Fig. 5A). The expression of PAX6 and RAX was not detected in the STEM group. Additionally, quantitative RT-PCR confirmed that the expression of EFN markers OTX2, SIX3, PAX6 and RAX were significantly upregulated in treated groups compared to the STEM group. The highest expression of OTX2 was seen in the STEM/SM while the superior expression of SIX3, PAX6, and RAX was detected in the STEM/CM group compared to others (Fig. 5B).

3.3. Assessment of cell viability

The viability of differentiated hADSCs was evaluated by MTT assay according to the mentioned protocol. For this purpose, hADSCs (4000 cells/well) were cultured and differentiated for 1, 7, 14 and 21 days. The optical density for each treatment group was converted to a percentage of the control value (day 1 of each group). As shown in Fig. 6A, a significant increase in cell survival was observed in all groups after 21 days. The highest increment was detected in the STEM/CM and the STEM/SM groups at day 21. However, there was a decrease of cell viability in the STEM/CM/SM group at day 21 compared to day 14 ($P < 0.01$) (Fig. 6A). In comparison between the groups, there was a significant acceleration in cell viability in the STEM/CM at day 21, while there was not any significant difference at day 14 (Fig. 6B).

4. Discussion

The retinal degenerative diseases are the major cause of irreversible loss of retinal neural cells and eventually blindness in the world (Daiger et al., 2007; Rattner and Nathans, 2006). In retinal

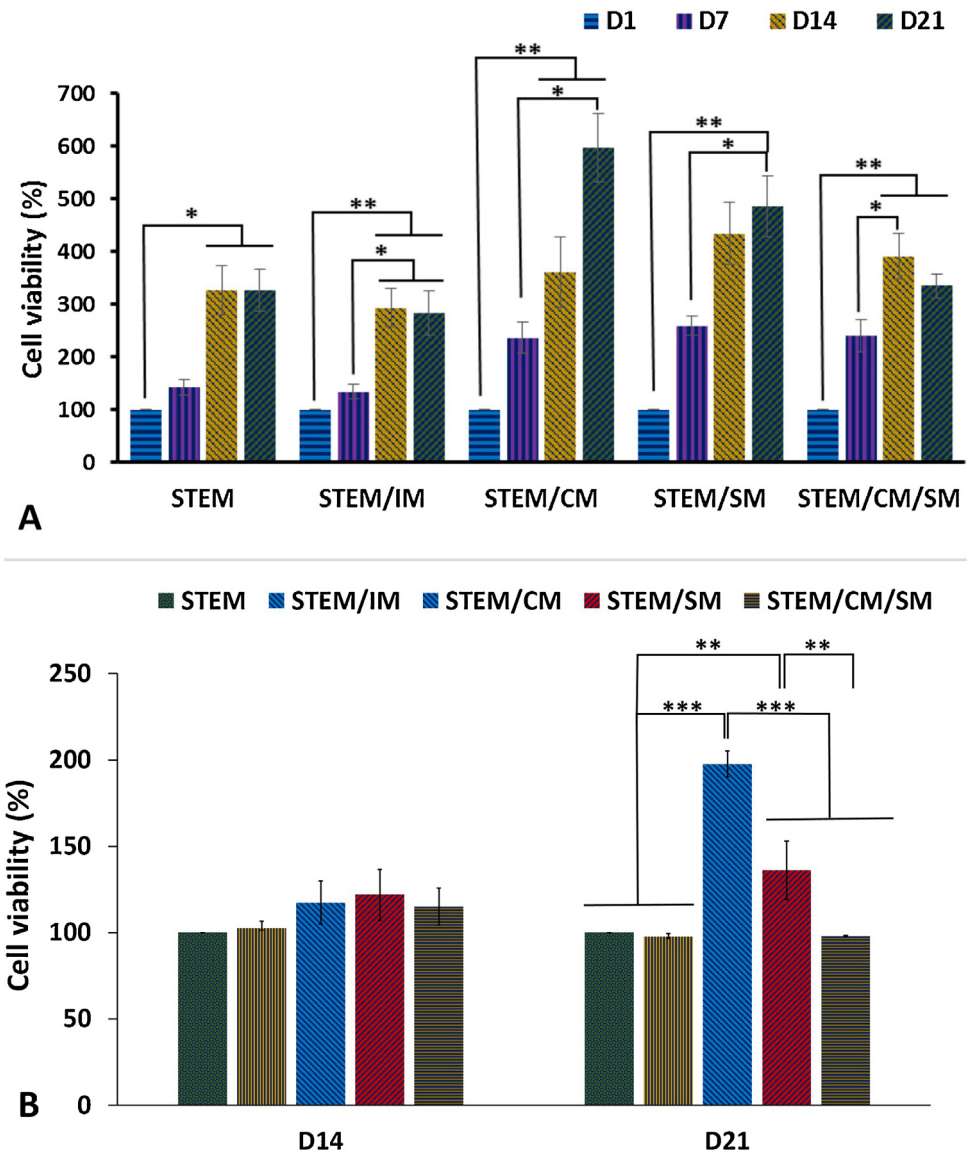

Fig. 6. To monitor cell viability, MTT assay was carried out at days 1, 7, 14 and 21. The differences between the days of each group, A. Comparison of all groups at day 14 and 21, B. *: P < 0.05, **: P < 0.01, ***: P < 0.001.

disorders, cell therapy as an appropriate treatment is considerably noted. It seems that the autologous mesenchymal stem cells are a promising therapeutic strategy between the different cell sources (Moviglia et al., 2012). Among the MSCs, hADSCs have potential to differentiate into retinal neural cells in vitro (Salehi et al., 2017). On the other hand, hADSCs through paracrine secretion of various cytokines in their conditioned medium have neuroprotective and neurotrophic effects (Bollini et al., 2013; Oses et al., 2017). In line with these studies, our data indictaed the existance of two important neurotrophic factors, BDNF and CNTF in hADSCs and their conditioned medium.

During neuroectodermal development, excitation and suppression of some signaling pathways are necessary for patterning the neuroectoderm. For instance, head area and eye field development from neuroectoderm need to suppress Wnt, BMP, and Nodal signaling pathways (Shen, 2007; Stern et al., 2006; Teraoka et al., 2009). Previous reports revealed small molecules (SB, LDN and CKI) inhibit the above-mentioned signaling pathways (Inman et al., 2002; Osakada et al., 2009b; Price, 2006; Surmacz et al., 2012). Although, the inhibitory effects of these small molecules had been shown (Shen et al., 2013), we confirmed their suppression effects in our experiment. Inconsistently with our previous studies, in the present study SB and LDN prevented and decreased phosphorylation of SMAD2 and SMAD1/5/8, respectively. Moreover, CKI downregulated the expression of C-MYC. Consequently, these small molecules inhibited the BMP, Nodal and Wnt signaling pathways.

In the current study, we evaluated the differentiation of hADSCs into EFN cells using small molecules and their conditioned medium. First of all, we characterized the neural differentiation of these cells by expression of β-TUB III and the lack of GFAP expression. In line with our study, it was demonstrated that hADSCs have the potential to differentiate into neural cells (Amirpour et al., 2017; Guilak et al., 2006; Kompisch et al., 2010). Our findings revealed that the highest expression of β-TUB III was in the STEM/CM/SM group compared to the other groups. In the STEM/CM/SM group, we simultaneously used CM and SMs for 21 days. Our previous data indicated that hADSCs can be differentiated toward neuroectodermal cells by using these small molecules (Amirpour et al., 2017). Furthermore, CM of hADSCs synergically enhanced the effect of SM on neural differentiation of hADSCs. The possible reason for this enhancement can be due to the role of CM neurotrophic factors. Other researchers have confirmed that neurotrophic factors have proliferative and neural

inductive effects on stem cells. For example, Zhang and Li (2005) identified cytokines such as bFGF can to improve activity of BMP blockers (Zhang and Li, 2005; Di-Gregorio et al., 2007; Yang and Klingensmith, 2006).

To assess EFN differentiation of hADSCs, we confirmed the expression of EFN markers OTX2, SIX3, PAX6 and RAX, by ICC and real-time PCR. We found that EFN differentiation was enhanced by the presence of CM compared to others. Previous studies approved that MSCs secret different neurotrophic factors such as BDNF, CNTF, NTF, and NT3 in their conditioned medium. Some of these factors have growth, proliferation, differentiation, and neuroprotective effects on retinal cells (Johnson et al., 2014). For instance, the results from a research study indicated that BMSC-CM can upregulate the expression of retinal cell markers in treated cells, which may be mediated by BMSC CM-derived CNTF and bFGF (Xia et al., 2013). Similarly, Razavi et al. proved the secretion of neurotrophic factors, CNTF, and BDNF by hADSCs (Razavi et al., 2013). CNTF and BDNF can regulate differentiation of retinal progenitor cells (Turner et al., 2006; Zahir et al., 2005). Thus, these factors may induce differentiation and lineage selection in hADSCs toward EFN cells, too. Recently, Zhang and collogues promoted the differentiation and proliferation of retinal progenitor cells by using hADSC-CM (Zhang et al., 2017). In the case of the highest expression of OTX2 in the STEM/SM groups, our previous study indicated that the use of SMs improves anterior neural differentiation of hADSCs by expression of OTX2 (Amirpour et al., 2017). Meanwhile, according to embryonic neural tube development, the portion of anterior neuroectodermal cells contribute to eye field formation (Stern et al., 2006; Teraoka et al., 2009). It seems that in the process of eye field induction, CM was more efficient than SM and CM/SM.

In this study, MTT assay showed that the percentage of cell viability increased in all groups after 14 and 21 days, whereas it decreased in the STEM/CM/SM group at day 21. This decrease might be related to increased differentiation of stem cells because the highest expression of β-TUB III (neural differentiation marker) was seen in the STEM/CM/SM group. It was demonstrated that neuronal differentiation occurs when the cell growth and proliferation are turned off (Theocharatos et al., 2013). Meanwhile, the rate of cell viability in the STEM/CM group was significantly the highest compared to the other groups at day 21. This increase may potentially be related to secreted neurotrophic factors and extracellular matrix components like laminin, fibronectin and collagen by hADSCs in CM. Previous studies have shown that neurotrophic factors such as BDNF, NGF, and NT3 promote cell survival and proliferation (Feng et al., 2012; Xu et al., 2016). Moreover, it was indicated that extracellular matrix components promote neural cells adhesion and growth so improve their survival (Yu et al., 2008).

5. Conclusions

In summary, we report hADSCs-CM can differentiate hADSCs into ENF cells. It seems this effect can maybe mediated by neurotrophic factors present in hADSCs-CM. Therefore, better recognition of these factors or by overexpression of the same in hADSCs might pave the way for cellular and non-cellular therapy in retinal degenerative diseases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.08.002.

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