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Dental pulp stem cells differentiation into retinal ganglion-like cells in a three dimensional network

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ABSTRACT

The loss of retinal ganglion cells (RGCs) in majority of retinal degenerative diseases is the first seen pathological event. A lot of studies aim to discover suitable cell sources to replace lost and damaged RGCs. Among them dental pulp stem cells (DPSCs) have a great potential of differentiating into neuronal lineages as well as RGCs. Moreover, three-dimensional (3D) networks and its distribution for growing and differentiation of stem cells as much as possible mimic to native tissue holds great potential in retinal tissue engineering. In this study, we isolate DPSCs from rat incisors and validate them with flow cytometry. Briefly, we differentiated cells using DMEM/F12 containing FGF2, Shh and 0.5% FBS into retinal ganglion-like cells (RGLCs) in two conditions; 3D state in biocompatible fibrin hydrogel and two-dimensional (2D) or conventional culture in polystyrene plates. Immuncytochemical and gene expression analysis revealed the expression of Pax6, Atoh7 and BRN3B increased in 3D fibrin culture compared to 2D conventional culture. In combination, these data demonstrate that using 3D networks can resemble near natural tissue properties for effective generating RGCs which used to treat neurodegenerative diseases such as glaucoma.

1. Introduction

Retinal degeneration which terminates in neuronal cells of retina is the leading cause of blindness in this century. The loss of neurons in the retina is routinely referred to be the inevitable cause of blindness. Age-related macular degeneration (AMD), glaucoma, and diabetic retinopathy are three most common figures of visual impairment and blindness in progressive loss of the neural cells of the eye. Glaucoma, the second leading cause of blindness worldwide, is a chronic degenerative disease that affects only RGCs [1,2]. Enhancing viability and function of RGCs remains a major goal through basic and translational researches in ophthalmology.

Stem cells have been suggested as an alternative therapeutic approach for treating a large spectrum of disorders. There are currently several trials in progress to treat neuronal related diseases of the retina using stem cells [3,4]. The results of these trials have been shown stem cell therapy for retinal degeneration diseases is a viable approach. Partially differentiated stem cells or even stem cells themselves which have the ability to produce trophic factors to rescue the dying cells of the retina can eventually restore their functions [5,6]. In this regard, stem cells derived from umbilical tissue, autologous bone marrow-derived stem cells and fetal stem cells are being transplanted to the eye in an attempt to maintain a healthy retina [7,8].

A major barrier to studying and treating RGCs related diseases is the amount and accessing of RGCs sources. Thus, the generation of RGCs in vitro is crucial to advance research and therapy in optic neuropathies. Dental pulp is a specific tissue originating from the neural crest [9] and it contains dental pulp stem cells (DPSCs) [9]. DPSCs are a heterogeneous population of cells including mesenchymal and ectodermic cells. This population of cells has been shown to possess properties similar to neural stem cells and mesenchymal stem cells. [10]. By isolation and expansion of these stem cells usually for long-term culture in media containing higher concentrations of serum followed by the induction of neuronal lineage with appropriate neurogenic conditions, neuronal differentiation of DPSCs is possible.[11,12]

Thanks to the tissue engineering approach three main components have become available in regenerative medicine; biomaterial

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which act as a scaffold, stem cells and growth factors. This would be very effective to restore cell functions that remain no longer capable to carry out their function or even in damaged tissues. Fibrin is a suitable natural polymer for tissue engineering applications [13–15]. In the formation of the fibrin network, fibrinogen is converted into fibrin through the reaction of thrombin in the presence of a large amount of calcium ions [16]. These reactions make 3-dimensionally organized clot resemble to classic wound healing which accrue in the body [17]. Beside of its biocompatibility, elasticity, it provides suitable mechanical support for cell migration and cell entrapment [15,18].

In this study we use the fibrin network as a three dimensional environment to mimic mechanical properties of the native retina in developing the retina. In this regard, we were used rheological properties of the fibrin and differentiate DPSCs into retinal ganglion-like cells in an aforementioned environment in comparison to two dimensional conventional cell culture.

2. Materials and methods

2.1. Isolation of dental pulp stem cells

Three adult male Sprague–Dawley rats weighing 170–200 g were housed under the ethical principles of Tehran University of Medical Sciences (TUMS) guidelines for animal researches. The dental pulp was removed under sterile conditions and locate in transferring medium containing 1× PBS (TAKARA BIO INC., Japan) and 1% penicillin (100 μ/ml) streptomycin (100 μg/ml) in DMEM (Gibco®, USA) supplemented with 1% amphotericin-B (200 μg/ml) (Gibco®, USA) and then sliced into 1 mm^3 fragments and incubated in 4 ml of 0.25% trypsin–EDTA [21,22] for 120 min at 37 °C. Trypsin was inactivated by adding an equal volume of DMEM containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco®, USA). A single cell population was obtained by passing the cell suspension through a 70 μm cell strainer (BD Biosciences, Oxford, UK), which was centrifuged at 150*g* for 5 min. Cell pellets were resuspended in DMEM containing 1% P/S and 10% FBS and seeded into T25 flasks (Corning, Amsterdam, NL) in a total volume of 5 ml. Cultures were maintained at 37 °C in 5% CO_2. The medium was changed 72 h after seeding and every 4 days thereafter for up to four weeks. Moreover, when cells reached 80% confluent they passaged using 0.05% Trypsin/EDTA Solution (Gibco®, USA).

2.2. Identification of cell phenotypic markers by flow cytometry analyses

Primary antibodies as well as CD44, CD73, CD90, CD31, CD34, CD45 and CD105 and secondary antibodies with fluorescent conjugates were used for assay according to abcam protocol (abcam, Cambridge, UK). At the end, the cells were checked using flow cytometry and data analysis was performed by flowing software (BD Biosciences Inc.).

2.3. Osteogenic differentiation and mineralization assay

Isolated DPSCs were seeded at a concentration of 1×10^4 cells/ml in a 6-well plate (SPL Life Sciences, Korea) with 0.7 ml media per well. The medium was changed to the osteogenic induction media which was contain 50 μg/ml ascorbic acid, 10 μM β-glycerophosphate, 0.1 μM dexamethasone and 10% FBS in low glucose DMEM (Sigma, Germany) for 21 days. Then the cells were stained with alizarin red S (Sigma–Aldrich Co., Germany) for 15 min [19]. The cells were visualized under an inverted phase contrast microscope (LabPro CETI, OXFORD).

2.4. Differentiation to retinal ganglion-like cell

Differentiation of DPSCs into retinal ganglion-like cells was done according the protocol of Jagatha et al. with some modifications in 2D/3D culture systems [20]. Briefly, isolated DPSCs were differentiated on 150 μg/ml poly-D-Lysine and 1 μg/ml Laminin substrate for 11 days in a differentiation medium which was contained DMEM/F12 supplemented with 1% N2 supplement (Invitrogen), 0.5% FBS, 2 μg/ml Heparin and 10 ng/ml FGF2 (Sigma–Aldrich Co., Germany). Afterward, the medium was altered to the growth factor containing media consisted of DMEM/F12 and combinations of 500 ng/ml Shh, 8 ng/ml FGF2, in 0.5% FBS for 16 h with the aim of effective differentiation. N2 supplemented DMEM/F12 with 0.5% FBS was used as control differentiation medium.

2.5. Cell Immobilization in fibrin gel

To prepare fibrin gel, 1.5 mg of fibrinogen (Sigma) was dissolved in 0.5 ml M199 medium (Sigma) and 2×10^5 cells/ml of DPSC were loaded to 500 μl of the prepared fibrinogen solution then added to 24-well culture plate. Then, 15 μl of a thrombin solution (120 U/ml in 1 M $Cacl_2$, Nacl, pH: 7.4 Sigma) and 50 μl of fetal bovine serum (FBS) were added to fibrinogen solution (3 mg/ml). After gelatin occurred at 25 °C, the dish was placed in an incubator at 37 °C and 98% relative humidity for 1 h to form its final three dimensional network structure [21].

2.6. Rheological analysis

Fibrin gel rheometry was done using a Physica MCR 300 rheometer (Anton-Paar, Ashland, VA). Fibrin gels were placed directly onto the parallel plate with 25 mm diameter, and 0.05 mm was a gap between the plates. Plate temperature was set 37 °C at the start of the experiment. A constant stress mode applied to the sample. The tests were done by dynamic method at strain (1%). The storage (G′) and loss moduli (G″) were measured by small-amplitude oscillatory shear measurement at the frequencies whiten 0.1–10 Hz.

2.7. Biocompatibility

500 μl of 5 mg/ml fresh MTT (3-[4,5-dimethyl-2-thia-zolyl]-2,5-diphenyl-2*H*-tetrazolium bromide) (Sigma–Aldrich, Germany) was added to each well and they were incubated in a humidified 5% CO_2 incubator at 37 °C for four hours. Afterward, the formazan salts were dissolved with 200 μl of DMSO and finally the optical density was measured at 575 nm by using a microplate reader (PHOmo, Autobio-labtec, China).

2.8. Immunofluorescent analysis

All antibodies were purchased from abcam (abcam, Cambridge, UK). According to abcam procedure the cells prepared. Primary antibodies as well as Rabbit polyclonal Anti-GFAP antibody, Mouse monoclonal anti-MAP2 antibody and Rabbit polyclonal Anti-BRN3B/POU4F2 antibody was added to the cells and then the secondary antibodies were added to the cells at recommended dilution. Moreover, DAPI (Sigma–Aldrich, Germany) was used to counterstaining the nuclei. Samples were visualized using a fluorescent inverted microscope (Trinocular Epi Flu Inverted microscope, Ceti Microscopes, UK).

2.9. Reverse transcription-polymerase chain reaction (RT-PCR) and real time-PCR

Total RNA from DPSCs derived osteoblast and differentiated Retinal Ganglion-like Cells in 2D and 3D cultures were extracted using the RNeasy Mini Kit (Qiagen, Valencia, USA), according to manufacturer's instructions. RNA quantity was assessed by spectrophotometry (ScanDrop; Analyticjena, Germany). After DNase treatment 2 μg of total RNA was used for Reverse transcription with the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Japan). One microliter of synthesized cDNA used for RT-PCR and for real time-PCR reaction with SYBR® Premix Ex Taq™ (TAKARA BIO, INK, Japan) and the detection was carried out in a CFX Real-Time PCR System (Biorad, USA). The reaction was performed by following program; 5 min of 95 °C for enzyme activation, initial denaturation for 20 s at 95 °C, annealing temperature for 30 s, and extension at 72 °C for 30 s, followed by 43 cycles with a final extension at 72 °C. The final stage comprises the analysis of the melt curve. The primer sequences are shown in Table 1. Levels of mRNA for tested genes were quantified using ΔΔCT method and were normalized against GAPDH.

3. Results

3.1. Undifferentiated DPSC characterization and cell culture

Cultured DPSCs were adherent and showed a heterogeneous phenotype and turned to homogenous following subculture especially after passage two. The majority of cells had fibroblast-like morphology and projections although a minority of the proportion of cells displayed a webbed-like soma (Fig. 1A and B). DPSCs were proliferating rapidly with a doubling time about 40 h. Flow cytometry data displayed they were positive for CD44, CD105, CD90, and CD73 and negative for CD34, CD45 and CD31 (Fig. 2).

3.2. Osteogenic differentiation capacity of DPSCs

The cells were cultured in the presence of dexamethasone and β-glycerophosphate demonstrates osteocyte phenotypes as evidenced by alizarin red staining (Fig. 1G). Furthermore, the positive expression of osteonectin (SPARC), osteopontin (SPP1), and alkalin phosphatase (ALPl) mRNA measured by RT-PCR after 21 days of osteogenic induction, exhibited the potency of DPSCs to differentiate to osteoblast (Fig. 1I).

3.3. Morphology and differentiation of DPSCs into cells expressing retinal ganglion-like cells markers

After day two of treating DPSCs with retinal ganglion cell's differentiation media in the 2D and 3D cultures, cells were induced through retinal ganglion-like cells. Their morphology gradually started to change to neuron-like cell especially after day five and shows a multipolar shape and projections resembling the neuronal axon and dendrite assume as neuronal-like cells. (Fig. 1C and D). In 3D cultures, cells more clamped than conventional 2D cultures (Fig. 1E and F). Immunofluorescence data revealed differentiated cells were expressed neuronal markers as well as *GFAP*, *MAP2* and also *BRN3-B* as one of selective markers of retinal ganglion cell in the both 2D conventional culture and 3D fibrin scaffold (Fig. 3A).

3.4. Gene expression analysis of retinal ganglion-like cells in 2D and 3D cultures

The real-time PCR analysis for neuronal cell markers as well as *MAP2*, *GFAP* and retinal ganglion cell specific marker genes such as *Pax6*, *Atoh7* and *BRN3B* in DPSCs derived retinal ganglion-like cells revealed from the 13th day after treatment with retinal ganglion cell's induction media. The expression of *GFAP*, as an astrocyte marker, demonstrated that differentiated cells over-expressed this early neuronal glial *GFAP* to 0.891-fold. Furthermore, *MAP2* as a specific neuronal marker has been done concurrently for neuronal confirmation after days of retinal ganglion cell induction and its expression increase 1.142-fold in 3D culture than 2D culture (Fig. 3B). The results demonstrating neuronal morphology expected to direct differentiation. According to Fig. 3B mRNA expression level showed that the expression of *Pax6*, *Atoh7* and *BRN3B* was increased significantly in 3D fibrin culture compared to 2D conventional culture 2.307-fold, 1.624-fold, and 3.14-fold respectively, after differentiation. However MAP2 was not detected in DPSCs without retinal ganglion cell induction media, that is, cultured in complete medium as a negative control. Our results indicated that, under all the experimental conditions, the expression of the retinal ganglion-like markers Pax6, Atoh7 and BRN3B is not detectable in DPSCs, while they express in low level the neuronal marker GFAP.

3.5. Cell viability of cell immobilized in gel and cell plating

The MTT data revealed that the cell viability and biocompatibility of fibrin hydrogel after 1, 6, and 12 days after DPSCs seeding in fibrin network and in conventional cell culture. It demonstrated

Table 1
The primer sequences used for RT-PCR and real-time PCR.

Gene symbol	Detected transcript	Primer sequence, 5 > 3	Annealing (°C)	Amplicon length (bp)
SPP1	NM_012881.2	F-CGCATTACAGCAAACACTCAG R-AGTCATCCGTTTCTTCAGAGG	59	114
ALPL	NM_013059.1	F-GGCTGGAGATGGACAAGTTC R-TTCACGCCACACAAGTAGG	59	109
Sparc	NM_012656.1	F-GGCGAGTTTGAAAAGGTGTG R-AGAAGTGGCAGGAAGAGTC	59	61
Gapdh	NM_017008.4	F-AAGGAGTAAGAAACCCTGGAC R-GCAGCGAACTTTATTGATGG	59	202
MAPII	NM_013066.1	F-TTGATGAGAAGGCGGAGGTC R-GAGCGGAAGAGCAGTTTGTC	59	199
GFAP	NM-017009	F-TGGGCAGGTGAGGAAGAAATG R-AAGGTTAGCAGAGGTGACAAGG	59	123
Pax6	NM_013001.2	F-AGTGCCCGTCCATCTTTGC R-TTTCTCCCTGTCCTTCCTGTTG	59	279
BRN3-B	NM_134355.1	F-CAACCAGAGGCAGAAACAGAAG R-AAAGAGGCAGGAGAGACAAGAG	59	124
Atoh7	NM_001170482.1	F-CGCAATCACCCCTACCTC R-GAAGGACTCTGGCTGGAAAC	59	96

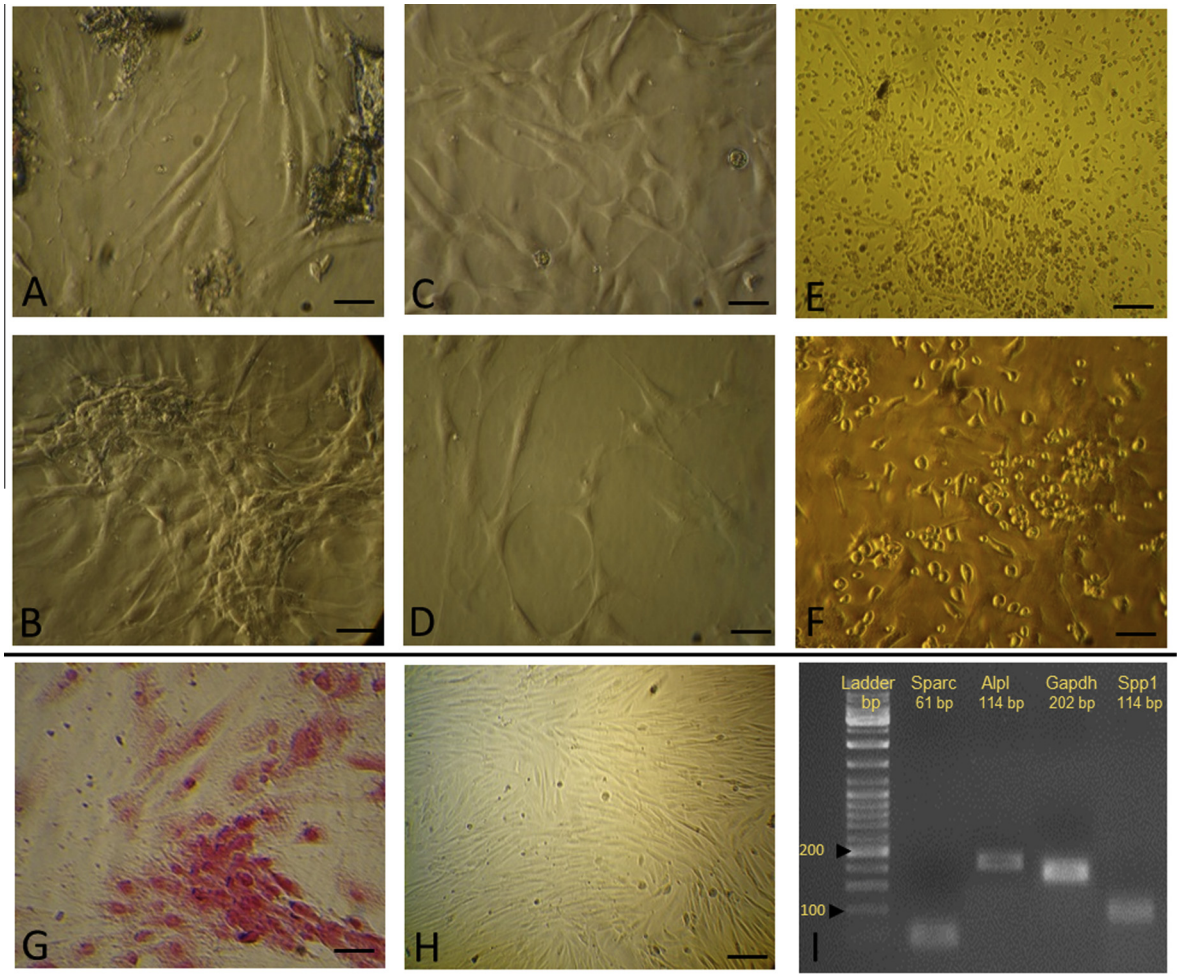

Fig. 1. (A) Fibroblast like morphology of isolated DPSCs and (B) colony forming potential of stem cells. (C) Initial projection of cells and (D) their neuronal like morphology after day 5 of culture. (E) The encapsulated cells on fibrin gel on 2nd days and (F) 5th days of continually culture. (G) Differentiated osteoblasts derived from DPSCs after day 21 of differentiation which stained with alizarin red. Red color demonstrated calcium deposition and (H) the negative control. (I) The RT-PCR result of mRNA expression of SPP1, SPARC and ALPL. The scale bar is 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

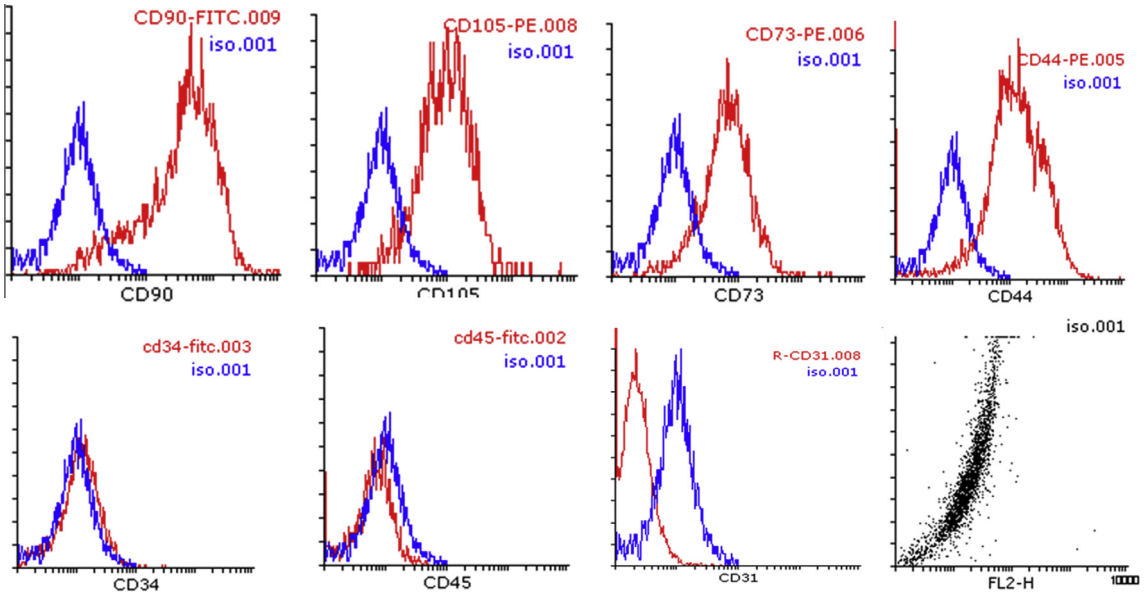

Fig. 2. Flow cytometric analysis; CD90, CD105, CD73, CD44 were positive and CD34, CD45 and CD31 were negative. The red line indicates for positives and blue one for negatives. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

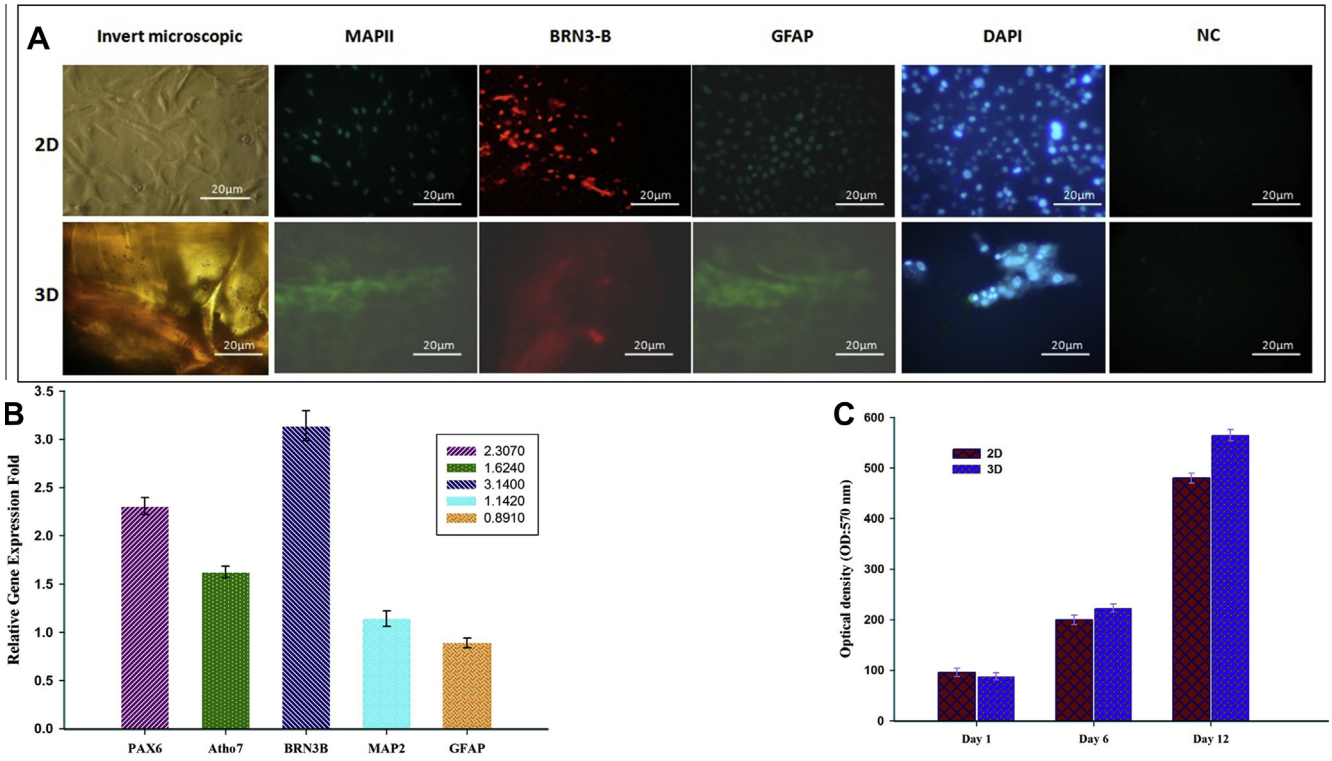

Fig. 3. (A) Immunofluorescent staining for expression of neuronal markers and retinal ganglion cell specific marker in DPSCs after 21 days post-treatment by induction media in 2D and 3D culture. Nuclei are stained with DAPI. (B) Relative gene expression of retinal ganglion cell mRNA and neuronal mRNA in 3D environment compared to 2D. (C) MTT assay for DPSCs encapsulated in fibrin scaffold on days 1, 6 and 12 of culture.

Fibrin gel had not toxic effects on cells and viability of differentiated retinal ganglion-like cell significantly ($P < 0.05$) decrease during cell proliferation and also differentiation in 2D culture compared to 3D cultures (Fig. 3C).

3.6. Fibrin rheology analysis

Rheology was used to assess the mechanical properties of fibrin hydrogel compared with normal rat retina tissue. The storage (G′) and loss modulus (G″) versus frequency are plotted in Fig. 4. As it is shown in the graph, over the range of frequencies from 0.1 to 10 Hz, saves its elastic properties (G′) and G′ curve does not cross loss modulus (G″) curve that means in this range, our fibrin scaffold elastic behavior dominates over the viscous behavior and fibrin gel is stable. G′ value for fibrin with 3 mg/ml concentration of fibrinogen lies between 0.1 and 1 kPa, which indicates that this is a soft gel and is proper for retinal tissue substitution from viscoelastic point of view that is near to normal retina tissues one.

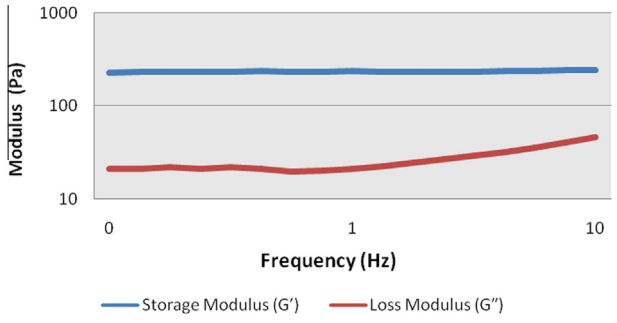

Fig. 4. Rheological properties of fibrin network designed for immobilizing cells. The elastic properties (G′) and loss modulus G″) were examined by small-amplitude oscillatory shear measurement at frequencies ranging from 0 to 10 Hz for the prepared fibrin gel.

4. Discussion

Here, we have developed the generation of retinal ganglion-like cells from DPSCs in the fibrin scaffold as a 3D network which may eventually be a good potential for treating neurodegenerative disease of the eye caused due to the degeneration of RGCs. In order to find out whether or not three dimensional culture systems possess any ability to affect generation of RGC-like cells, we analyzed the effect of fibrin gel providing a 3D network resembling the natural environment of retinal ganglion cell layer on RGC-like cell differentiation.

The investigation of Mead et al. showed that DPSCs significantly secreted 2- to 3-fold higher titers of NGF, BDNF and NT-3 in the cultures. They emphasized neuroprotective and pro-regenerative effects of DPSCs that can be related to their enhanced neurotrophic profile suggesting that they can be a proper candidate to repair CNS and retinal injury [23]. Numerous groups have confirmed the potential of dental pulp stem cells (DPSC) to differentiate into multiple neural crest-lineage cell types as well as some studies on neuronal differentiation from DPSCs [24–26]. We selected DPSCs because of the above properties and potency of them to treat retinal degenerative diseases. In our study the isolated cells analyzed using flow cytometry for validation of DPSCs. To confirm the differentiation of DPSCs to a neuronal phenotype we tested morphological changes and expression of MAP2 and GFAP. After 5 days of culture most of the cells resembled multipolar neuron and the expression of both MAP2 and GFAP after differentiation indicated that the DPSCs gradually had differentiated into neuronal cells. FGF2 plays an important role in RGC differentiation, RGC axon growth and axon guidance [27]. It has been shown that FGF2 stimulate neurite extension of

RGCs in vitro [28] and is a potent stimulator of axon growth during RGC development, so it is crucial for RGC differentiation [29,30]. FGF2 and Shh activate Pax6, a neural/retinal progenitor marker. Pax6 acts as a master switch for activation of ATOH7, an RGC regulator, thereby supposed to initiate the RGC differentiation cascade through three stepwise stages for RGC development; ATOH7 which is specifically required for competence, differentiation, and specification activate firstly [31–33]. Up-regulation of ATOH7 plays an important role in RGC specification. As it is known ATOH7 activates Pou4f2 and Isl1 to final early born ganglion cells [34]. Interestingly, in our investigation the expression of Pax6, Atoh7 and BRN3B increased 2.307-fold, 1.624-fold, and 3.14-fold respectively ($P < 0.05$) in 3D compared to 2D which indicate the affectivity of 3d networks on differentiation of DPSCs into retinal ganglion cells. Furthermore, immunocytochemistry data shows the expression of MAP2, GFAP, and BRN3B in the end days of retinal ganglion cell differentiation. In other words, the results from mRNA expression and immunocytochemistry show three dimensional networks such as fibrin gel scaffolds provided applicable microenvironment for retinal ganglion cells differentiation from DPSCs. The mechanical analysis and results showed that three dimensional network preserve the 3D hydrogel structure under frequency sweep as a viscoelastic material. It seems an environment with elasticity of <900 Pa is appropriate for retinal ganglion-like cells proliferation, differentiation and survival in vitro. The concentrations of fibrin at about 3 mg/ml provide the suitable environment in both durability and similarity to the retinal environment. Although there are efforts for retinal ganglion cell generation from various cell sources [35], to our knowledge it is the first report of differentiation of stem cells as well as DPSCs into RGLS in 3D network. Moreover, MTT assay shows there was not any toxicity regarding to the effect of fibrin on cells. As a result, our data revealed that near mechanical and structural properties to normal retina may help to enhance differentiation potential of DPSCs to Retinal ganglion like cells.

Since in tissue engineering the strategy is growing cells in biomimetic scaffolds then transferring resulting constructs in desired sites to obtain new tissues, we selected fibrin gel as a three dimensional network to growing cells. A lot of studies using fibrin gel for entrapping cells and stimulate differentiation of cells in the three dimensional state which their results emphasis its affectivity on differentiation and proliferation [36–38]. We tried to examine producing flexible and pliable like natural tissues as a result of such 3D cell culture in the case of regeneration of RGC layer.

A broad group of ocular diseases, such as glaucoma, chemical and mechanical trauma characterized by loss of retinal ganglion cells (RGCs) and their axons that comprise the optic nerve [39]. A major issue in studying with the aim of treating eye disease is the difficulty of providing RGCs. Moreover, the generation of RGC in vitro is vital to high tech research in optic neuropathies.

Conflict of interest

The authors have declared that no conflict of interest exists.

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