Research Article

PAX6 (+5a) Expression in Adipose Tissue-Derived Stem Cells Induces Retinal Ganglion Cells

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Abstract

Glaucoma remains one of the major causes of blindness in today's world. The progressive field of stem cell proposes an exciting potential for discovering novel therapies. Here, we report the development of an easy and high throughput method for differentiation of retinal ganglion cells (RGC) and bipolar cells from human adipose tissue-derived mesenchymal stem cells (hADSCs) using PAX6 (+5a) gene expression, a master gene in development of the vertebrate visual system. HADSCs was isolated from fat tissues and confirmed by their surface markers and differentiation potential into adipocytes and osteocytes lineages. Then, the coding region of human PAX6 (+5a) gene was cloned and lentiviral particles were produced. HADSCs differentiation was characterized by morphological characteristics, qRT-PCR and immunocytochemistry (ICC). The hADSCs were isolated successfully with high yield and purity (99%). After 30 hours post transduction by pLEX-pax6- pur lentiviral vectors in fibronectin supplemented medium, cells gradually showed the characteristic morphology of neuronal cells. QRT- PCR and ICC confirmed deriving of mainly RGC and marginally bipolar cells. The current investigation demonstrates the feasibility of differentiation of RGCs and bipolar cells from hADSCs using expression of PAX6 (+5a) in the medium supplemented by fibronectin.

***Keywords*:** human Pax6 gene; adipose tissue-derived mesenchymal stem cells; retinal ganglion cell

Introduction

Glaucoma, a chronic retinal neurodegenerative disease, is the second cause of worldwide blindness in developed countries (Quigley and Broman, 2006). It has been estimated that 80 million people worldwide would have been by this disease within 2020. Glaucoma is characterized by the degeneration of axons in the optic nerve and apoptosis in the retinal ganglion cells (RGCs) (Kuehn et al., 2005). RGCs are the first differentiating retinal cells in all vertebrates that are induced from retinal progenitor cells in embryonic development (Marquardt and Gruss, 2002). The inability of the central nervous system to regenerate new cellular components in response to damage leads to a limited capacity of structural and functional repair in the retina (Cao et al., 2002).

One of the strategies to restore vision in glaucoma patients is the functional replacement of RGCs using autologous or heterologous transplantation (Baker and Brown, 2009; MacLaren et al., 2006; Moshiri et al., 2004; Wallace, 2007; Wong et al., 2011). For the generation and transplantation of RGCs and their precursors the potential of different sources of stem cells, for instance human embryonic stem cells (hESC), bone marrow-derived stem cells, umbilical cord-derived cells, induced pluripotent stem cells (iPS), adult human Müller stem cells and fetal stem cells, have been reported earlier (Baker and Brown, 2009; Buchholz et al., 2013; Jayaram et al., 2011; John et al., 2013; Ramsden et al., 2013; Wallace, 2007).

Adipose tissue represents an abundant and accessible source of adult stem cells (Jurgens et al., 2008; Kokai et al., 2005). A growing body of experimental evidence, from in vitro and in vivo studies, demonstrates the multipotentiality of adipose-tissue derived stem cells (ADSCs) (Gimble et al., 2007).

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While ADSCs are similar to bone marrow stem cells in differentiation and therapeutic potential, they are much easier and safer to obtain in large quantities which make them an ideal and reliable source for regenerative medicine (Rasmussen et al., 2012). The Paired box (PAX) genes are members of the family of tissue specific transcription factors. This family plays a critical role in the formation of tissues and organs during embryonic development and it has vital functions in certain tissues in adults (Hever et al., 2006; Pichaud and Desplan, 2002). PAX6 along with other genes, such as SOX2 and OTX2, control each stage of eye development and it has been called the master gene for eye development (Hever et al., 2006). PAX6 mutations in Drosophila, mouse, rat, and human demonstrate its requirement for the development of eye (Philips et al., 2005; Yasuda et al., 2002). PAX6 (-5a) and PAX6 (+5a) are two isoforms of the conserved PAX6 gene that have different DNA binding specificities and functions (Azuma et al., 2005). It has been reported that PAX6 (-5a) plays important roles in both embryogenesis and adult body homeostasis and PAX6 (+5a) is the most critical isoform that promotes the neuronal differentiation of murine embryonic stem cells (Shimizu et al., 2009).

Based on these findings, it was considered that PAX6 (+5a) transfection into hADSCs may induce retinal neurons, including RGCs and/or their precursors. In so doing, the expression of human PAX6 (+5a) by lentiviral expression vectors was employed in hADSCs under culture medium supplemented with fibronectin. This study offers an effective method for in vitro induction of RGCs like cells that can be used in stem cell based therapy.

Materials and Methods

Adipose tissue Sampling

Human adipose tissue was obtained from abdominal subcutaneous tissues of patients undergoing abdominoplasty procedures, in the Tehran Medical University (N=6, age range: 20-35 years). Before the surgical procedure, informed consent was obtained from the patients. About 150 ml of lipoaspirate was gathered in a sterile bottle, filled with 0.1 M phosphate-buffered saline (PBS) or DMEM-F12 (Sigma, Germany), in order to achieve enough number of cells.

Isolation and Cell Culture of hADSCs

HADSCs isolation was done according to the method as discussed elsewhere (Estes et al., 2010) with some modifications. Up to 2×107 adipose stromal stem cells with more than 98% purity were isolated from 150 ml of lipoaspirate; however, yields varied among patient's samples. Briefly, in order to remove the majority of erythrocytes and leukocytes, the lipoaspirate was washed five times with sterile PBS containing 120 µg/ml of penicillin (Fluka, China), 220 µg/ml of streptomycin (Fluka, China). Then, 0.1% (wt/vol) collagenase type I (Invitrogen, USA) was used to digest the extracellular matrix. Enzyme activity was neutralized with fetal bovine serum 10% (Gibco, Germany) and centrifuged at 1500 rpm for 10 min to obtain a high density pellet. The cell pellet was re-suspended and extensively washed with PBS. Finally, remaining cells were cultured in 25 cm2 flasks (Nunc, Denmark). DMEM-F12 medium supplemented with 10% FBS, 5 ng/ml human epidermal growth factor (Roche, Germany), 1 ng/ml human fibroblastic growth factor (Roche, Germany), 100 U/ml penicillin and100 mg/L streptomycin was used as culturing and expansion medium. The flasks were then incubated at 37 °C with 5 % CO2 at humidified atmosphere. The medium was changed after 16 hours, and then twice a week. Adherent cells were harvested with 0.25% trypsin-0.02% EDTA, and re-plated at a dilution of 1:3 when the confluency was more than 80%.

Flowcytometric surface marker expression analysis

To characterize the phenotype of the cultured cells with flow cytometry, fluorescein isothiocyanate (FITC)-conjugated primary antibodies for human CD44, CD45, CD73, CD90 and CD105 (BD Biosciences, USA) were used. The hADSCs were trypsinised and washed 3 times with cooled PBS containing 2% FBS and sodium azide. Cells (5×104) were incubated with aforementioned antibodies. All antibodies were diluted 1:1000 and incubated with cells for 45 min at 4 °C. Then the cells were washed with PBS containing 2% FBS. After two washing steps, cells were re-suspended in 500 µl paraformaldehyde 2% containing 1% FBS for profile characterization and analyzing by fluorescein-activated cell sorting (FACS) system (Partec II, Germany).

Analysis of multipotent differentiation capacity

When over 80% confluence was reached, cells were incubated in the osteogenic and adipogenic differentiation media for four and three weeks, respectively. Osteogenic differentiation medium consisted of DMEM-F12, 20% FBS with osteogenic supplement: 100 nM dexamethasone (Sigma-Aldrich, Germany), 50 µg/ml ascorbate-2-

phosphate (Sigma-Aldrich, Germany) and 10 mM β-glycerolphosphate (Sigma-Aldrich, Germany). Adipogenic differentiation media consisted of DMEM-F12 supplemented with 20% FBS, 100 nm dexamethasone (Sigma-Aldrich, Germany), 50 µg/ml indomethacin (Sigma-Aldrich, Germany) and 50 µg/ml ascorbate- 2 phosphat (Sigma-Aldrich, Germany). In general, the culture media were changed every 4 days. After induction, alkaline phosphatase assay (Sigma-Aldrich, Germany) and Oil Red O (Sigma-Aldrich, Germany) staining were performed to confirm the differentiation of hADSCs toward osteogenic and adipogenic lineages, respectively.

Construction of Vectors

The coding sequence of human PAX6 (+5a) gene was synthesized and cloned into pUC57 cloning vector. Vector was digested by BamHI and XhoI restriction enzymes and subcloned into the BamHI/XhoI site of pLEX- MCS- Pur lentiviral expression vector and was designated as pLEX-Pax6-Pur construct. The recombinant construct was confirmed by PCR ampilification, digestion and finally DNA sequencing.

Virus particles production.

Lentiviral vectors were produced by calcium-phosphate transient transfection using a vector expression system in HEK 293T cells. Briefly, HEK 293T cells were plated in 6-cm plates with 7×105 cells in 4 ml of Dulbecco's modified Eagle's medium (DMEM, high-glucose) supplemented with 10% FBS. Then pLEX-Pax6-Pur vector, as transfer vector (11 µg), envelope encoding plasmid (4 µg) and packaging lentivirus vector (7 µg) were added and transfection by CaCl2 was carried out. Fourteen hours after transfection, medium was replaced, and viral supernatant was collected 24 and 48 hours post transfection. Finally, assembled lentiviral particles were filtered, purified and concentrated by PEG 6000 (Sigma, Germany).

Transduction of hADSCs.

HADSCs were transduced by 8 hour exposure to the viral supernatant in the presence of 8 µg/ml polybrene at the 37 °C and 5% CO2 without FBS. After 72 hours post transduction, selection medium containing DMEM-F12 with 20% FBS and 1 µg/ml puromycin was added to the transduced-hADSCs and incubated. Every 3 days the selective medium was changed with the same medium. Transduced-hADSCs from passages 2 post transduction were analyzed for eGFP expression, cell proliferation and cell death using ELISA kits (Roche, Germany) according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR assay

Total RNA extraction and cDNA synthesis were performed using RNeasy kit (Qiagen, Germany), and Quantiscript® reverse transcriptase (Qiagen, Germany), respectively. Quantitative real-time PCR was performed with the Corbet Real-Time PCR system (Applied Biosystems, USA). Specific primers were used from the Quantitect primer assay (Qiagen, Germany) (Table 1). Data were normalized to the expression of GAPDH, a housekeeping gene, which has shown to have stable expression under different experimental conditions in similar studies. Each reaction contained 5 µl of Quantifast syber green master mix, 1 µl of forward and reverse mix primer (10 pm), 3 µl of RNase free water and 1 µl of cDNA. The reactions were conducted with initial enzyme activation at 95 °C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds and anneal at 60 °C for 30s. Relative gene expression was calculated using Bio-Rad software (RelQuant UpDate- for relative quantification) according to the 2-ΔΔCt method based on the threshold cycle (Ct) values (Schmittgen and Livak, 2008). All experiments were repeated duplicate and their values were presented as mean±SD. Student's t-test was used to evaluate the statistical significance of the data; $p < 0.05$ was considered statistically significant.

Table. 1 primers list which used for qPCR from quantitect primer assay (Qiagen, Germany)

	Official symbol	Amplicon length (bp)	Cat. No.
1	PAX6	113	QT00071169
2	FGF2	109	QT00047579
3	DVL3	69	QT00999810
4	SHH	136	QT00205625
5	SOX1	96	QT00215299

Immunocytochemistry

Immunocytochemical analysis was carried out for the detection of cell specific markers according to the Santa Cruz protocol with some modifications. Briefly, transduced hADSCs were cultured on FBS pre-coated glass cover slips in a 24-well microplate at a density of 6×104 cells per well and washed with PBS. Paraformaldehyde fixed cells were permeablized with chilled methanol (Merck, Germany) and then blocked in 1% BSA (Merck) in PBST (1% Triton X-100 in PBS) (Sigma) for 45 min at room temperature. Then followed by 1 h incubation in primary antibodies at room temperature (All antibodies were obtained from Santa Cruz, USA). Antibodies for retinal progenitor and retinal ganglion and biopolar cell markers were included the goat polyclonal anti-human PAX6, Thy1 and PKC. A negative secondary antibody-only control was also included. Nuclei were counter-stained with DAPI (1 mg/ml, Santa Cruz, USA) to assess the total number of cells in each field. Cover slips were then mounted onto slides using an anti-fading mounting medium (90% glycerol, 10% PBS and 10% (w/v) phenylene-diamine). Samples were observed under the Axiophot Zeiss fluorescence microscope (Germany) with a 460 nm filter for DAPI and a 520 nm filter for FITC-conjugated antibodies, and digital pictures were taken.

Results

High output hADSCs

The hADSCs were isolated successfully from human adipose tissue. Approximately 5×106 hADSCs obtained from one gram of adipose tissue. They cultured in expansion medium and passaged every 3-4 days. Human ADSCs were large, spindle-shaped cells with fibroblastic features (Figure 1A, B). Thin cell body of cells contained a large and round nucleus (Figure 1C, D). In early passages, cells displayed clonogenic properties, the ability of a single cell to proliferate independently to form a colony (Figure 1E, F). This indicated the renewing capacity of isolated stem cells. Cells kept their morphological features, without major alteration, for a maximum of 11 passages.

Analysis of multipotent differentiation capacity

Osteogenic and adipogenic differentiation capacity of hADSCs were examined. Osteogenic differentiation confirmed by mineralization of cells in osteogenic medium at week 4 that could be observed by alizarin red staining (Figure 1 G, H). Long spindle-shape morphology of the hADSCs changed into a polygonal shape 4 days after incubation in adipogenic induction medium. By the day 9, small droplets of oil lipid appeared in some of the cells. After three weeks, most of the differentiated cells showed red lipid droplet throughout the cytoplasm, which is confirmed by oil red O staining (Figure 1 I, J).

Surface marker profile expression analysis

To characterize surface markers of isolated cells, flow cytometry was performed. The flow cytometric analysis demonstrated that approximately 99% of hADSCs expressed the surface markers CD44, CD73 and CD105 (Figure 2A-C, G). The hADSCs lacked the expression of the hematopoietic markers CD34 and CD45 (Figure 2E, F). Results verified the mesenchymal origin of the hADSCs and the lack of hematopoietic markers. Each value represents the mean of two independent experiments in at least duplicate.

Successfully gene transduction of hADSCs by Lentiviral vectors

Two lentiviral vectors pLEX-eGFP-pur and

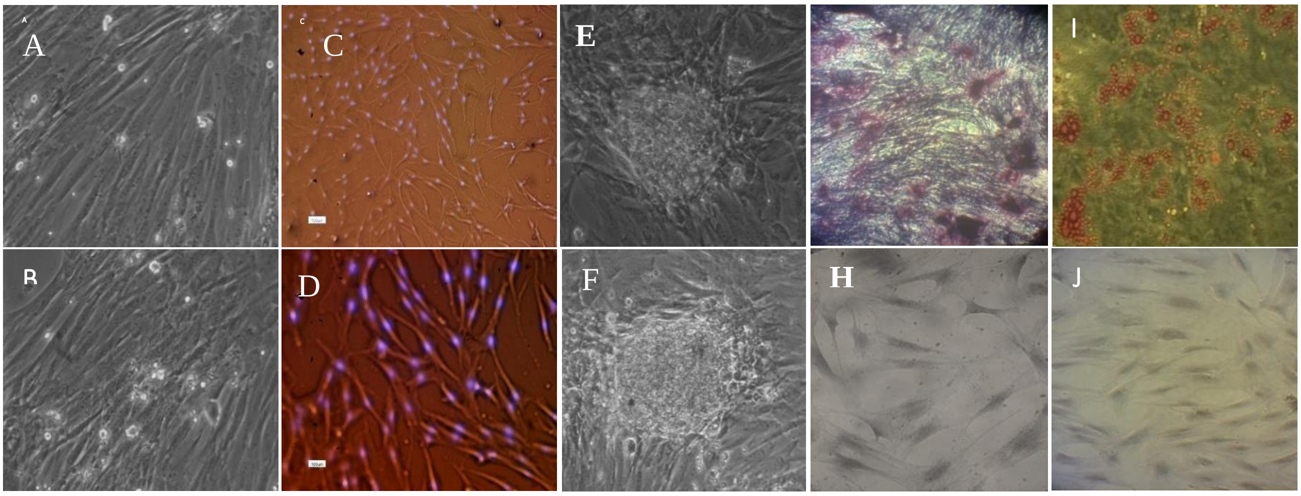

Figure 1. Characteristics of isolated hADSCs. Morphological features of cells (A-D); the hADSCs were typical fibroblast-like cells with fusiform shape from the 2nd passage and preserved their shape after expansion in vitro (A, B), Nuclei were stained with DAPI (blue) the cell body contained a large and round nucleus (C, D). Clonogenic capacity of hADSCs (E, F). Multipotential differentiation assays of hADSCs (G-J); differentiation potential of hADSCs towards the osteogenic lineages was assessed through alkaline phosphatase activity assay (G, H), hADSCs differentiated towards the adipogenic lineage and formed lipid vesicles, which were stained using oil red-O (I, J). (C 100X – all other 200 X)

pLEX-Pax6-pur, were constructed and transfered into HEK293T and hADSCs. Transduction efficiency of hADSCs was examined by expressing GFP. Lentiviral vector, pLEX-eGFP-pur, was used to transduce proliferating hADCs at a MOI of 100. One day after transduction, 90% and 75% of HEK293T and hADSCs expressed GFP, respectively. Although, the expression of GFP observed only 24 hour post transduction, it took about 3 days to observe GFP in hADSC cells (Figure 3 A, B).

Cell morphology characteristics after transduction

Three days post transduction, cells showed the characteristic morphology of neuronal cells and little axon-like processes emerged gradually. Four days post transduction, they gradually extended axon-like processes that finally led to the formation of neural-network-like structures. Cells had multiple dendrites with a long axon and a fat cell body resembling reliable RGCs (Figure 3 C-F).

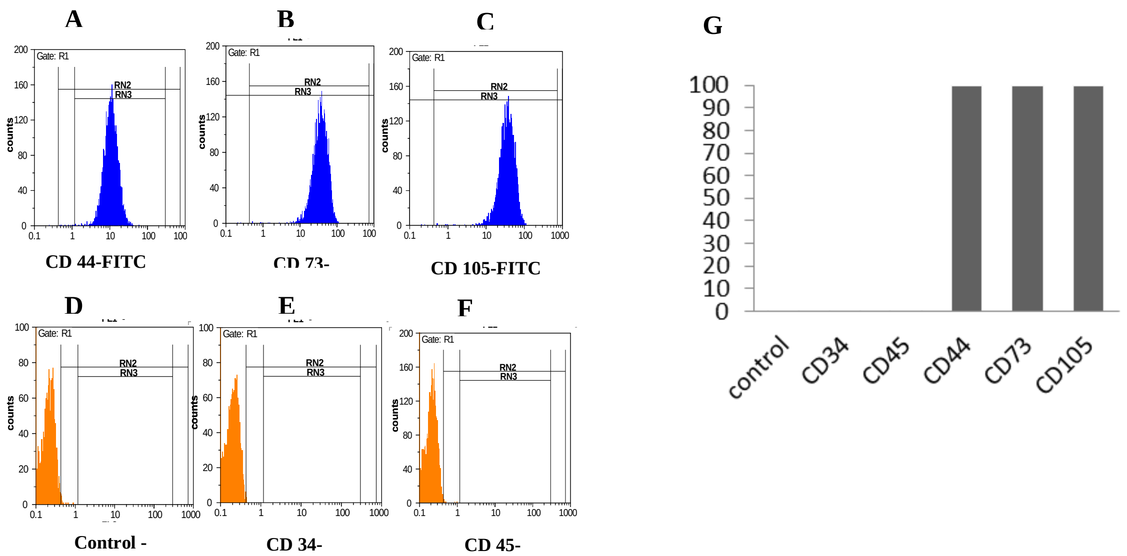

Figure 2. Flow cytometry analysis of hADSCs from fat tissue cultured in vitro. Immunophenotypic characterization of hADSCs (A-F), with cells positively expressing the antigens CD44 (A), CD73 (B) and CD105 (C), while negatively expressing the antigens CD34 (E) and CD45 (F), 99% of isolated hADSCs expressed the surface markers: CD44, CD73 and CD105 (G).

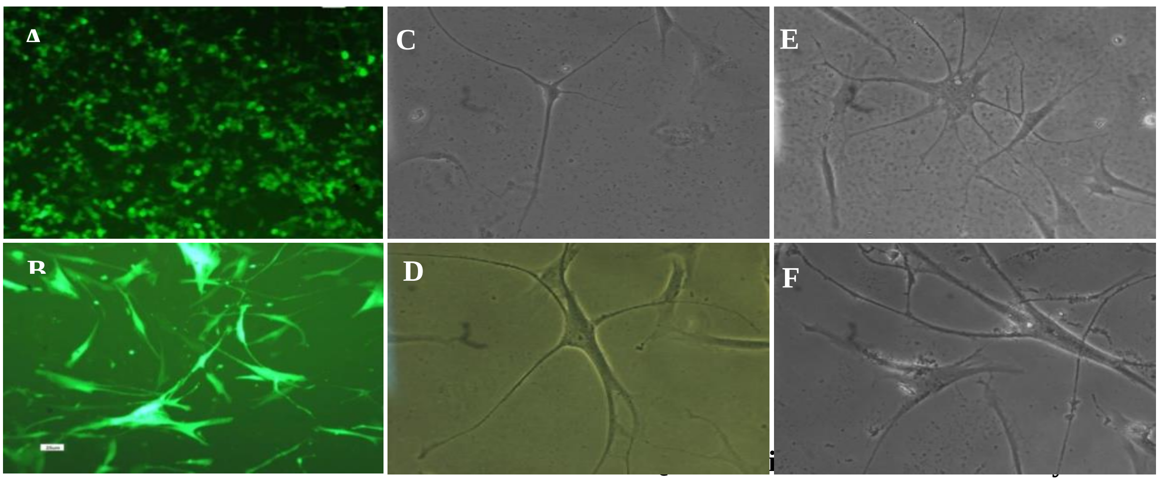

Figure 3. Transduction of hADSCs with lentiviral vectors. Efficient eGFP transduction of cells (A, B); HEK293T (A) and hADSCs (B) transduced with pLEX-eGFP-pur vector were expressed GFP. Cell morphology of transduced cell at day 4 under inverted microscopic view (C-F) (A 100X, all others 300X).

To test the expression of retinal neuron and RGC-associated mRNA, including SOX1, PAX6, Thy1 and PKC, qRT-PCR was performed. According to the qRT-PCR data, PAX6 and SOX1 expression levels increased substantially after transduction compared to cells transduced by empty vector or without transduction. Thy1, a marker of RGC, plays an important role in the formation of the visual system. Expression of FGF2 and sonic hedgehog (Shh) as molecules involved in different signaling pathways in differentiation of retinal neural cells was also examined. Results showed the increased expression of FGF2 and Shh (Figure 4). At the mRNA level, expression of PKC, a bipolar cell marker, was increased. Taken together, these results indicated that PAX6 (+5a) induction of hADSCs can cause differentiation into mostly RGCs and marginally bipolar cells.

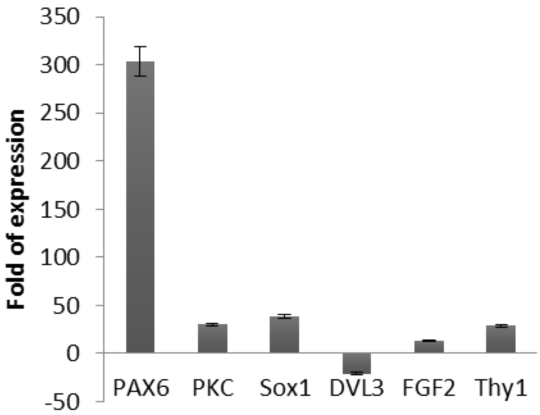

Figure 4: Relative retinal gene expression in PAX6 (5a)-transduced hADSC after 6 days in comparison to the cells transduced by pLEX-MCS-pur (empty vector). The results represent the mean of 3 independent experiments in at least triplicate.

ICC confirmed the expression of some key markers of RGCs.

To determine whether PAX (+5a) transcription factor and fibronectin were able to induce hADSCs into retinal cells, we examined the expression of PAX6, Thy1 and PKC markers in PAX6 (+5a)-transduced cells. Using ICC method, cells were positive for PAX6 (64± 1.3%), Thy1 (32± 1.1%) and PKC (29± 1.4%) (Figure 5 A-F). Cells transfected with empty vector did not show any of these markers.

Discussion

Glaucoma is the commonest cause of irreversible blindness in the world. Despite the advances in the currently available treatments, many patients experience significant visual loss due to degeneration of RGC (Kerrigan–Baumrind et al., 2000; Kuehn et al., 2005; Quigley and Broman, 2006). Right now, there is no therapeutic strategy for functional recovery of these cells. Cell therapy offers an alternative treatment for restoring the damaged cells in neurodegenerative diseases (Buchholz et al., 2013; Cao et al., 2002; Haddad-Mashadrizeh et al., 2013; Huang et al., 2013; Huang et al., 2011; John et al., 2013). It is acknowledged that damage to the neural retina during glaucoma is restricted to the degeneration of RGCs (Kerrigan–Baumrind et al., 2000); therefore, replacement of these cells might be possible and, if so, might restore the optic nerve.

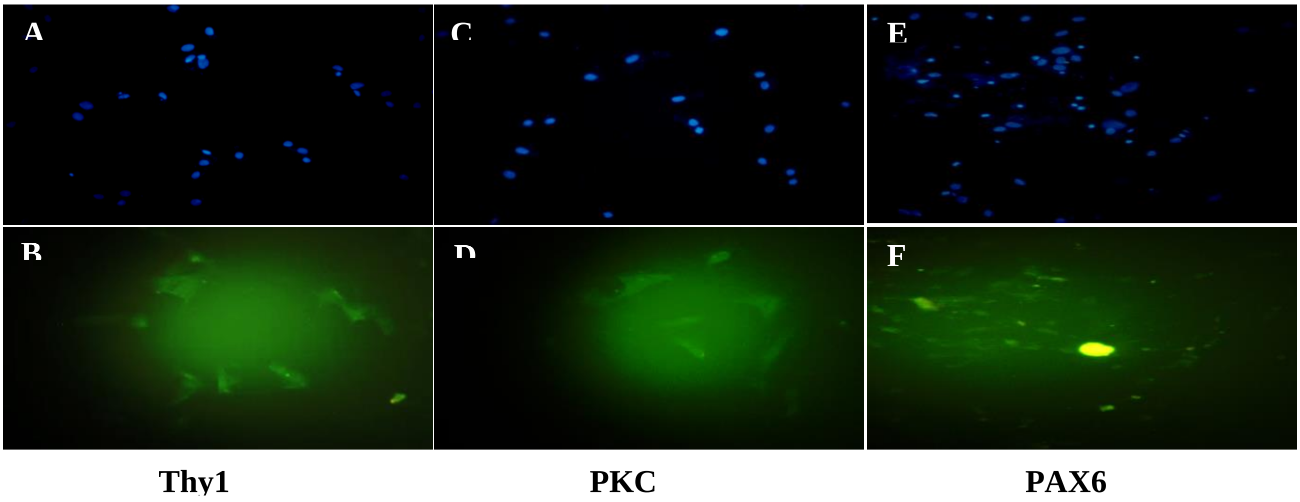

Figure 5. Fluorescence microscopy of Thy1, PKC, PAX6 in Pax6 (+5a)- transduced cells after 6 days. These Pax6 (+5a)-transduced cells were analyzed by in situ immunostaining with antibodies raised against different RGC markers. A,B) ICC for Thy1 C,D) Antibody against PKC E,F) antibody against Pax6. Each value represents the mean of 3 independent experiments in at least triplicate.

Several groups have focused on the differentiation of stem cells from different sources with different methods (Fraichard et al., 1995; Jagatha et al., 2009; Jin et al., 2009; Osakada et al., 2009; Singhal et al., 2012; Wong et al., 2011). Most of these sources have limitations for human RGC replacement in clinics (Jayaram et al., 2011). Consequently, identifying alternative sources of cells that replace these cells in the glaucomatous eye without ethical and practical restrictions is necessary. With this target in mind, this study aimed to address some of these limitations.

This study established an effective and easy way to obtain high-yield hADSCs with high purity, 99% positive staining rate observed from results of multiple surface markers, using collagenase digestion and adherence screening. No significant difference was seen among different passages in the phenotypes of hADSCs, indicating that the cells can be stably amplified in vitro for several passages. High proliferation (data not shown) and differentiation (Figure 1G-J) capacity of isolated hADSCs is consistent with stem cell characteristics.

It is reported that fibronectin may be important for differentiating ESCs into retinal neuron precursors, including RGC-like cells (Kayama et al., 2010). Based on these findings, culture medium was supplemented with fibronectin for neural cell induction. Results showed that PAX6 (+5a) expression and fibronectin supplemented medium are sufficient to induce the differentiation of retinal precursor cells from hADSCs.

Inverted microscopic examination disclosed the appearance of generally long cells that exhibited multidendrites and one axon per cell, suggesting their neuronal differentiation (Figure 3C-F). Several genes expressed in differentiating RGCs such as SOX1, PAX6, and Thy1 were tested by qPCR. The results revealed that upon differentiation, the cells had up-regulated expression of early neural markers, SOX1 and PAX6. SOX1 is one of the earliest transcription factors that is expressed in cells committed to the neural fate (Pevny et al., 1998). PAX6 is a neural/retinal progenitor marker and acts as a master switch for activation of RGC regulator, thereby supposed to initiate the RGC differentiation cascade (Jagatha et al., 2009). Thy1, a surface glycoprotein, is uniquely expressed in RGCs in retina (Huang et al., 2006).

Different signaling molecules such as FGF2 and Shh have been shown to be involved in differentiation of RGCs. It has been shown that FGF2 is a potent stimulator of axon growth during RGC development (Sapieha et al., 2003). Sonic hedgehog (Shh) has been shown to play an important role in the development of the retina in a number of different model organisms (Spence et al., 2004). Recent studies have demonstrated that the signaling molecule Shh secreted by differentiated RGCs is required to promote the progression of ganglion cell differentiation. Shh plays dual roles to orchestrate the progression of retinal neurogenic wave (Zhang and Yang, 2001) and also plays a major role in RGC axon projection inside the retina (Kolpak et al., 2005). FGF2 and SHh are known to activate PAX6 (Jagatha et al., 2009). Increased levels of FGF2 and SHh and PAX6 in this study were in line with these findings.

Since RGCs collect the messages from bipolar cells and represent the ultimate signals to the vision center in the brain, mRNA expression of PKC (protein kinase C), a bipolar cell marker, was also examined. Expression of SOX1, PAX6, FGF2, Thy1, Shh along with ICC results of PAX6, Thy1 and PKC were confirmed that the differentiated cells belonged to RGCs and bipolar cells (Figure 4 and 5).

Major disadvantages associated with the use of different sources of stem cells are their ethical concerns, shortage of donor cells, limited availability, inflammation, immunoreaction as well as their safety issues regarding teratoma formation; therefore, their potential in cell therapy may be problematic (Wong et al., 2011). Human ADSCs can overcome some of these problems.

Aside from developing a reliable source of retinal cells for transplantation, there are several additional obstacles that have to be considered. For instance, limitation of integration of graft cells into host tissue (Wong et al., 2011), ongoing disease in the host environment that may present a problem for cell transplantation (Wallace, 2007), tumorigenicity, specially, when cell cultivation period has prolonged (Wong et al., 2011). Moreover, after the transplantation of RGCs, it would need the additional challenge of regrowth of axons through the optic nerve to targets in the brain (Wallace, 2007).

Taken together, for successful retinal regeneration, improved methods for purifying donor retinal cells, optimizing host conditions, as well as using animal models of human diseases, to determine the efficacy and safety of treatments, will be crucial. Furthermore, in order to optimize the best minimal cocktail requested to achieve more authentic-differentiated neurons, more growth factors, cytokines, mRNA, microRNA and small molecules deserve to be investigated.

Future studies to determine the markers of differentiated cells and also time course expression of aforementioned genes are under investigating. Using current method, bipolar cells and RGCs beside different retinal cells including photoreceptors (our previous work, in press) were successfully differentiated from hADSCs. For an efficient differentiation of hADSC to disease-relevant cell types, novel strategies need to be developed. The current investigation demonstrates the feasibility of the differentiation of RGCs and bipolar cells from hADSCs using expression of PAX6 (+5a) in the medium supplemented by fibronectin that can be used in stem cell therapy.

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