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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