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Original Article

Motor neurons differentiation of encapsulated human endometrial stem cells in collagen without HLA-DR expression

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Abstract:

Background: Human endometrial stem cells (hEnSCs) are attractive cells in regenerative medicine and neurons derived from EnSCs are one of the best targets for cell therapy due to the fact that they do not stimulate immune system. Small molecules as useful chemical tools can effectively change cell fate. Thus, these small molecules provide a promising strategy for differentiation of cells in regenerative medicine. Purmorphamine (PMA) is a small molecule that possesses certain differentiation effects by activating SHH signaling pathway. In this study, we used PMA as a small molecule to differentiate motor neurons from hEnSCs by using collagen polymer.

Methods and Materials: hEnSCs were cultured in differentiation medium containing 1µM PMA in 2D and 3D environments. Scanning electron microscope (SEM) was used for cell attachment and cell viability was assessed by and 3-(4,5-dimethylthiazoyl-2-yl)2,5-diphenyltetrazolium bromide (MTT). Immunocytochemistry was performed to confirm the expression of islet-1 and Acetylcholine transferase (Chat). Real-time PCR also confirmed for expression of neural markers such as NF, Chat, islet-1, HB9.

Results: Therefore, our data revealed that induced hEnSCs with PMA could significantly express motor neuron markers in RNA and protein levels. Further, flow cytometry was done for the expression of the cell surface antigen HLA-DR in endometrial stem cells and motor neurons derived from these cells. The results confirmed that hEnSCs and motor neurons derived from them do not express HLA-DR. Our motor neurons differentiation protocol provides an accessible system to provide motor neurons that do not stimulate immune system.

Conclusion: In conclusion, encapsulation of hEnSCs in collagen along with induction media containing PMA have potential for being used in neural tissue engineering through activation of the SHH signaling pathway.

Key words: Human endometrial stem cell, Purmorphamine, Collagen, Scaffold, HLA-DR

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Introduction

Neurodegenerative diseases are the most important disease in the world, which is increased because of injury to the central nervous system (CNS) and as well as the the aging of population (1). Many CNS diseases such as amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, and Huntington's diseases occur as a result of neurodegenerative processes (2,3). Axonal regeneration in CNS is restricted by a combination of different intrinsic and extrinsic factors, including inflammation, glial scar formation, absence of growth-guiding astrocytes (4,5). Motor neuron (MN) is a specialized neuron whose soma is located in different areas of the CNS, such as spinal cord, brain stem or motor cortex and whose axon projects to outside of the spinal cord is organized and discrete patterns to control the activity of muscles (6).

Stem cell derived MNs are extensively used for developing cellular strategies for replacement of neural cells lost after spinal cord injury and other diseases such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) (7). Cell replacement therapy is promising method to repair of neurodegenerative diseases (8). Mesenchymal stem cells (MSCs) are the type of adult stem cells that have been described in different tissues (3) capable of differentiating various mesenchymal tissues. Endometrium is highly dynamic tissue that during woman's productive life undergoes approximately 400 cycle of proliferation (9). The level of tissue growth in endometrium is equivalent to the cellular turnover in other organs (10). It has been shown that endometrial stem cells (EnSCs) play a role in triggering of neovascularization, increase of tissue integration and reduction of chronic inflammation (11). Many investigations have shown the potency of EnSC to differentiate into neural cells (12). EnSCs can be released neuroprotective trophic factors such as NT-3, BDNF and VEGF and also releasing factors that promoted survival of neural cells (11). It has been indicated that human EnSCs have high potential to differentiate into cholinergic and dopaminergic neurons (11).

Small molecules have defined an obtainable naive ground state of pluripotency and are facilitating the accurate and effective differentiation of stem cells into target cells (12). reprogramming of cells by using small molecules (SM) provides a promising strategy for target cell-based therapy (13). Sonic hedgehog (SHH) is a morphogen that is necessary to the developing nervous system and play an important role in adult life contributing for cell proliferation and differentiation (14). Pormurphamin (PMA) is a purine-derivative small molecules agonist of SMO receptor. PMA promoted transient upregulation of tissue-type plasminogen activator in injured neurons (15). Recent studies have indicated that it is possible to generate motor neurons in culture upon treatment with SHH and RA and transplanted into spinal cord (16). It was discovered that this process can be achieved by using small molecule PMA instead of Shh. The expression of class II genes (Nkx6.1) is increased along with combination of PMA and RA and the expression level of class one genes (Irx3 and Pax7) and Olig2 is decreased. This result suggests that PMA acts through molecular pathway similar to that of Shh during the induction of motor neuron specifications (17).

MSCs produce a variety of immunosuppressive molecules and growth factors that facilitate tissue repair and maintenance of immune homeostasis. The plasticity of immunoregulatory effects of MSCs is dependent on the inflammatory status. (18).

HLA mismatches are associated with more frequent rejection episodes that require increased

immunosuppression that, in turn, can enhance the risk of malignancy and infection (19). HLA-DR, biomarker of inflammation, is normally expressed by most immune competent cells and some non-immune epithelial cells. HLA-DR molecules are up regulated in response to activation of immune system (20,21).

We have developed a system to encapsulate adult human Endometrial stem cells (hEnSC) within spherical three-dimensional (3D) microenvironments consisting of a collagen Type I. Our goal was to produce defined 3D microenvironments for the culture and differentiation of hEnSC to motor neurons for tissue engineering applications in central nervous system.

Material and methods

Biopsy and human EnSCs isolations

Human endometrium was obtained from biopsy samples from patient referred to the hospital for infertility treatment. The biopsy tissues were washed in DPBS (Sigma,USA) and transferred to laboratory and maintained in Hanks balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) (Sigms,USA) with 10% fetal bovine serum (FBS) (Gibco,USA), and 1% (v/v) penicillin/streptomycin (Gibco,USA) and cells were isolated by previous our protocol(8). Endometrial tissue was dissociated into single-cell suspensions using collagenase type I (Sigma,USA). Cells were cultured in DMEM/F12 (Gibco,USA) medium with 20% FBS (Gibco,USA) and 1% antibiotic pen/strep and incubated at 37°C and 5% CO2. After passage three, the cells trypsinized and flow cytometry were done for detection of CD90, CD105, CD31, CD34, CD146 (22).

Collagen preparation and cell encapsulation

Rat tail tendons was used for extraction of type I collagen stock solution. Collagen gels were prepared by mixing collagen solution, distilled water, and 4× DMEM so that the final concentration was 1.0 mg/ml. After this, 500 µl of the mixture was put into each well of a 24-well culture plate. The solutions gelled in approximately 20 min at room temperature. Alternatively, cells were suspended in the collagen solution and the mixture was immediately put in the wells. After 2 to 8h, the cells attached. Gels were released from the well in which they were cast with a sterile spatula, floated in culture medium, and incubated at 37°C in a 5% CO2 atmosphere (23).

Cell attachment and morphology analysis of encapsulated cells in collagen

Following the sterilization by exposure to UV radiation for 1 h, 5×104 cells were encapsulated in collagen and incubated in DMEM/F12 containing 10% FBS at 37°C in 5% CO2 for 24 h. The cell attachment and cell morphology of seeded cells into scaffolds was visualised by SEM at the 5th day of culture period. Cell-seeded scaffolds were fixed in 2.5% glutaraldehyde for 1 h, rinsed three times in phosphate buffer salin and then dehydrated in increasing concentrations of ethanol from 30%, 50%, 70%, 80%, 90%, and 100% for 10 minutes per each. Finally, samples were sputter-coated with gold and observed using a scanning electron microscope (Philips XL-30, Netherland), operated at 15 kV.

Determination of cell viability by MTT assay

Cell viability of the hEnSCs encapsulated in the Collagen was studied by the 3-(4, 5dimethylthiazoyl-2-yl) 22, 5- diphenyltetrazolium bromide (MTT) assay on days 1, 3, 5 and7. For MTT assay, cells were seeded

at density of 1×104 cells/scaffolds in 96-well plates and maintained at 37 °C under 5 % CO2 for 1, 3, 5 and 7 days. The culture medium of each cultured specimen was removed. After incubation of cells with MTT solution for 4 h, the solution was removed and formazan crystals dissolved in 100 µl DMSO. The plate left at room temperature in the darkness for 10 min on a rotary shaker. The absorbance of plates was measured at 570 nm using an ELIZA reader (Expert 96, Asys Hitch, Ec Austria).

Chemical induction for motor neuron differentiation

To induce motor neuron differentiation, human EnSCs were encapsulated at 500,000 cells/ml in collagen as 3D culture and 24-well tissue culture polystyrene (TCP). The cells were incubated with DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, and 1 mg/ml streptomycin for 24h. Differentiation of cells was induced by exposing the cells to preinduction medium composed of DMEM/F12 (1:1), 20% FBS, 2% B27, 10 ng/ml fibroblast growth factor 2 (FGF2) (Gibco,USA), 250 µM isobutylmethylxanthin (Sigma,USA), 100µM 2-metcaptoethanol (Sigma,USA) and incubate for 24h at 37°C and 5% CO2. The treated cells were then cultured in induction media containing DMEM/F12 (1:1), 0.2% B27 (Gibco,USA), 1µM of purmorphamin (PMA) (Abcam,USA), and 0.01ng/ml retinoid acid (RA) (Sigma,USA) for 1 week. Then the induced media was replaced with medium composed of DMEM: F12 (1:1), 0.2% B27 and 200 ng/ml brain-derived neurotrophic factor (BDNF) (Sigma,USA) for other 1 week. As a control, a group of hEnSCs were cultured on the 3D cultures or TCP in the absence of differentiation factors for 15 days. The medium was changed every 3 days. This signaling protocol leads to neural differentiation.

Investigation of gene expression using real time-PCR

For the mRNA expression level of neuronal-markers in treatment groups, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used. Total RNA was extracted by using RNeasy Plus Mini kit (Qiagen, USA, 74134). Revert Aid First Strand cDNA Synthesis kit (Takara, USA, K1632) was used for cDNA synthesis from 1 µg of RNA. qRT-PCR reactions were carried out in the 48-well optical reaction plates on StepOneTM Real-Time PCR machine. In each PCR reaction, 30 ng synthesized cDNA was used to PCR by mixing with 10 µl of Power SYBER Green master mix (2X, Applied Biosystems), 0.5 µM of each primer (Table 1) in a total volume of 20 µL at the annealing temperature. The groups were compared with control group. The comparative Ct method, 2^{-DDCt}, was used for relative gene expression analysis.

Immunofluorescence analysis

After induction to motor neural cells, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and permeabilized with 0.1% TX-100 in TBS. The cells were blocked for 30 min at room temperature with 5% BSA and incubated with primary antibodies against choline acetyltransferase (Chat) (Rabbit monoclonal antihuman; Abcam, 1:200), Islet-1 (mouse monoclonal antihuman; Abcam, 1:200) diluted in 5% BSA in PBS overnight. Secondary antibodies included Alexa fluor 594 donkey anti mouse (1:500; Gibco, A-11058) and Alexa Fluor 594 donkey anti-rabbit (1:700; Gibco, A21207) and the nuclei were counterstained with DAPI (Sigma-Aldrich, D8417).

For negative controls, only the secondary antibodies were used. The nuclei were stained with DAPI.

Flow cytometry for HLA-DR expression

The expression of MHC class II (HLA-DR) was investigated in hEnSCs and hEnSCs derived motor neurons using flow cytometry technique. Cells were initially supplemented with suspensions and purified as much as possible. They were stained with a fluorescence material and labeled with a conjugate monoclonal antibody. Positive (peripheral blood mononuclear cells) and negative (human endometrial stem cells) control groups were used to determine whether hEnSCs and motor neurons derived from them express HLA-DR or not. Then 20µ anti HLA-DR (Gibco,USA) was added to differentiated cells and 10µ IgG1 (Gibco,USA) was added to isotype group. After that they were kept in darkness and 4 ˚C for 30 min and then flow cytometry was performed.

Statistical Analysis

The data were presented as means ± standard deviation of the means (n=3). Statistical analysis was carried out using one-way ANOVA and difference between groups was considered statistically significant if P >0.05.

Results

Isolation and identification of human EnSCs

Flow cytometry analysis which was published in our previous report (13) showed that CD90+ (80%), CD105+ (79%) and CD146+ (97%) were highly expressed and CD31, CD34, CD133and CD45 were extremely down regulated.

Assessment of Cell attachment, Viability and Proliferation

After hEnSCs were cultured in TCP and collagen treated with induction media containing PMA for 18days. The morphology of encapsulated cells in collagen was validated by inverter microscope and SEM. After 18 days, the cells showed neural morphological characteristics with neurite-like processes (Fig1a,b). The viability of cells cultured in collagen scaffolds was assayed by DAPI staining and MTT assay at 1, 3, 5 and 7 days. As shown in Fig. 2, until day 3, the cells cultured on TCP showed higher viability than cells cultured in the collagen scaffold, however this trend was not statistically significant. However, in days 5 and 7, the viability of cells cultured on collagen scaffold significantly enhanced relative to cells cultured on TCP (Fig.2). The results obtained from MTT assay showed that collagen was a more suitable substrate than TCP in case of cell attachment and proliferation.

Evaluation of motor neuron genes expression in hEnSCs differentiated cell after expose to PMA

After 18 days post induction, the morphological changes of cells were confirmed with the expression of specific neural markers such as NF-H, Chat, Islet-1 and HB9 by real-time PCR. qRT-PCR data showed that mRNA levels of NF-H, Chat, Islet-1 and HB9 were significantly increased in hEnSCs induced by PMA compared to the control (Fig. 3). The comparison between TCP and collagen groups showed that in the collagen group, the expression of Chat, HB9, Islet-1 and NF-H were higher than in the TCP group and were statistically significant (Fig.3).

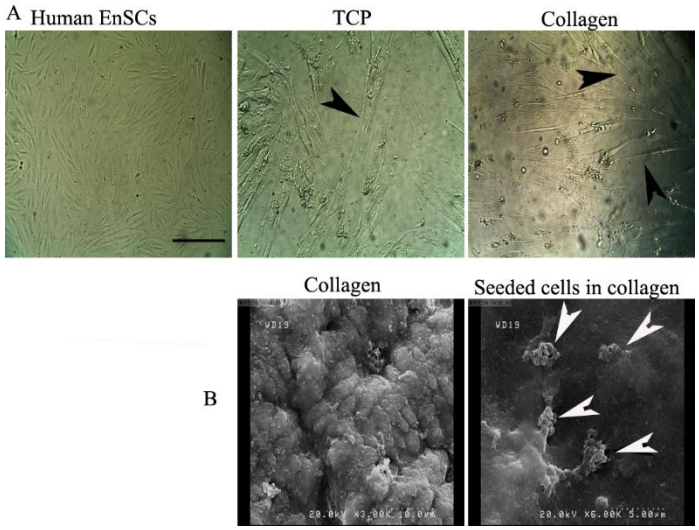

Fig1. A) Morphological images from differentiated cells in TCP and collagen. B) Scanning electron micrographs showing encapsulated cells in Collagen. The arrows show the projection of neural cells.

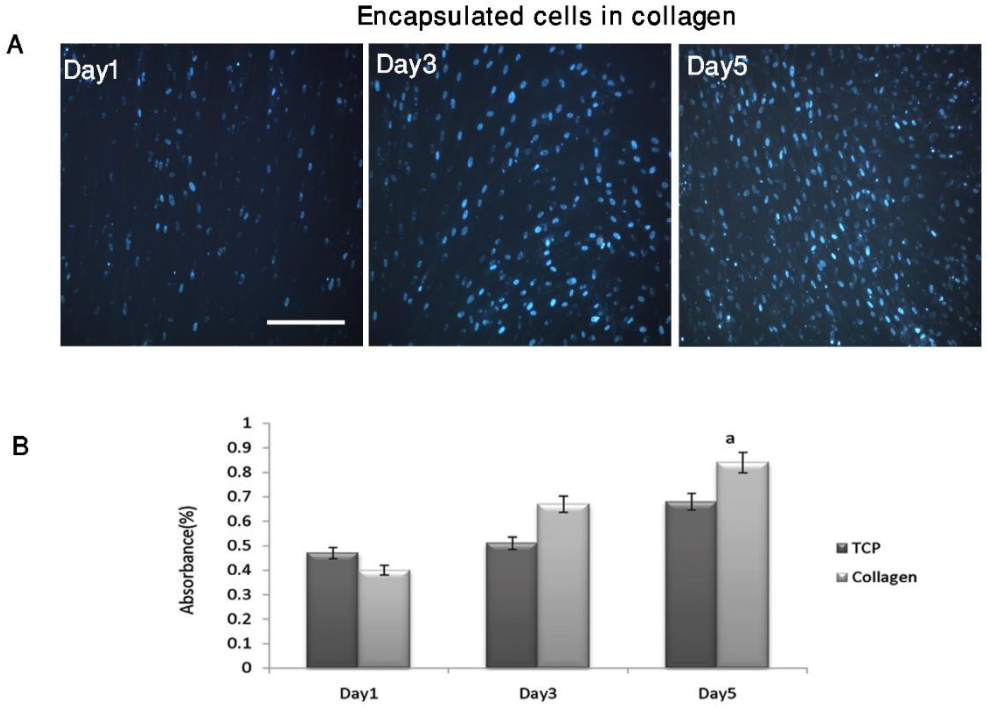

Fig 2. DAPI staining and MTT assay. Formosan absorbance expressed as a measure of cell viability from the cell cultured on TCP and Collagen. *P>0.05, **P>0.01 vs. TCP (n=3 biological samples, mean±SD)

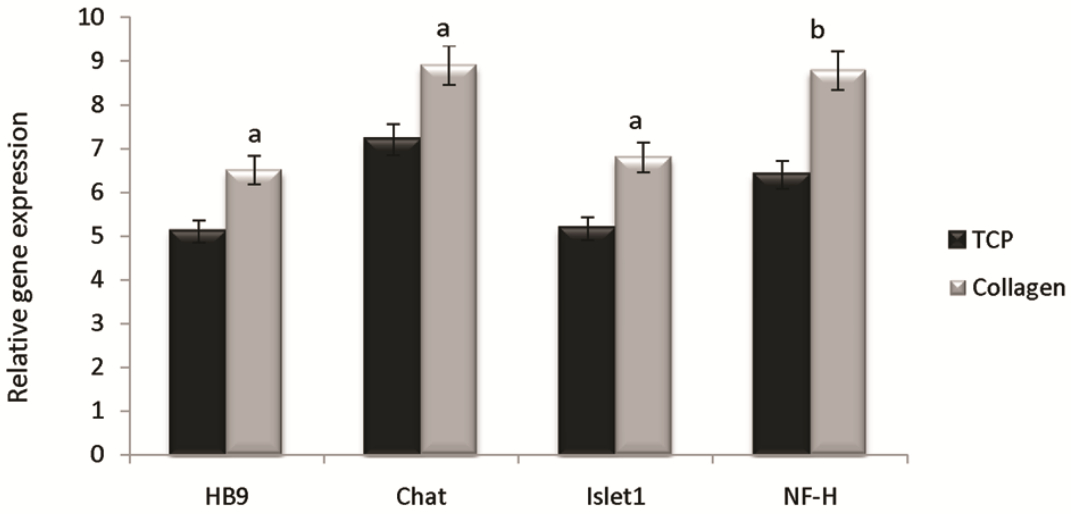

Fig3. Quantitative mRNA expression analysis of motor neuron-like cells derived from human EnSCs seeded in tissue culture plate (TCP) and Collagen after 18 days. The TCP group was compared with collagen groups. a:*P>0.05, b:**P>0.01 (n=3 biological samples, mean±SD)

Immunocytochemical evaluation of motor neuron markers expression

Cells were fixed and stained for the motor neuron markers such as Chat and Islet-1. Immunofluorescence analysis showed that Chat and Islet-1 specific motor neuron markers were widely distributed in the cell cytoplasm. The ratio of Chat and Islet-1-positive cells were also increased by PMA treatment and culturing in collagen scaffolds compared to TCP group (Fig. 4).

Evaluation of the expression of surface marker HLA-DR in hEnSCs differentiated by exposure to PMA using flow cytometry

In this study, flow cytometry was performed to investigate the cells immune reactions after transplantation and grafts. Expression of MHC II,(HLA-DR) was investigated in hEnSCs and differentiated cells treated with PMA. According to subsequent data endometrial stem cells do not express MHC II, so they do not stimulate immune system. Results demonstrated that our hEnSCs and motor neurons derived from them do not expressed HLA-DR. PBMCs, which express HLA-DR, were selected as positive control (Fig.5).

Discussion

The main goal of this study was to investigate the capability of hEnSCs encapsulated in collagen to differentiate into motor neuron-like cells in the presence of PMA. We found that activation of SHH signaling pathway by means of PMA led to enhancement of hEnSCs differentiation into motor neuron-like cells. Here we indicated that EnSCs derived motor neurons do not stimulate immune system, so they can be the best target for transplantation and grafts. RT-PCR and immunocytochemistry were used to show the expression of specific markers in mRNA levels and

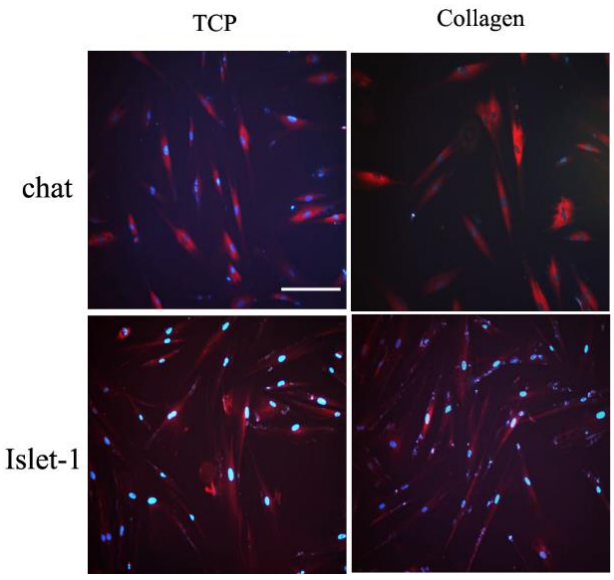

Fig4. Immunofluorescence staining of differentiated cells on TCP (a) and Collagen scaffolds
(b) After 18 days post induction for motor neuron markers including Chat, Islet-1, Tuj-1 and NF-H. Scale bar=100 µm.

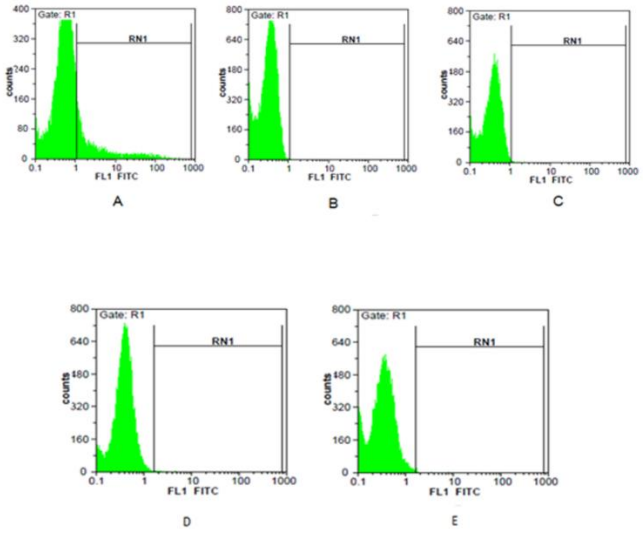

Fig5. Flow cytometry analysis was down to show the expression of HLA-DR in EnSCs and motor neurons derived from them. As we Know PBMC expresses HLA-DR (A). Negative control (undifferentiated hEnSCs) (B), isotype group(C), differentiated group in TCP (D) and differentiated group in Collagen (E) did not express this immune marker.

protein. The results of immunocytochemistry and RT-PCR Represented the higher level of hEnSCs differentiation into motor neuron-like cells in cultured cells in collagen relative to those on a TCP surface. It is well-documented that biomaterials aim to provide a suitable substrate and appropriate microenvironment to enhance cellular viability (22). Chemical factors can enhance neuronal differentiation using stimulation

signaling pathways that lead to control of the neural development (22). PMA as a synthetic small molecule improve neuronal differentiation with smoothened receptor activation, which directly acts on the same signaling pathway as Shh (23). PMA acts on the SMO receptor, binding of PMA to this receptor leads to increase of GLI1 expression, which is downstream of the Hedgehog pathway (16). Moreover, it has been demonstrated that Gli1 also has a neuroprotective effect on dopaminergic neurons in experimental models of neurodegenerative diseases. Additionally, there is also a growing body of evidence about the activation of pro-survival signaling pathways such as autophagy under control of SHH pathway (7). Autophagy pathway plays a critical role in maintenance of neural precursor cells. Thus, activation of SHH pathway by means of PMA may lead to an increase in cellular viability during differentiation of hEnSCs into motor neuron-like cells(15). Recent studies indicate that dopaminergic cell transplantation provides long-term neuromodulation. Host immune response to the transplant has been implicated as a major cause for these adverse outcomes. One major problem with cell transplantation is the issue of host immune response (24). One factor affecting long-term graft survival is the development of antibodies to mismatched HLA antigens after transplantation. It has been shown that even a single mismatched antigen can result in sensitization (25). HLA class two phenotype, HLA-DR in particular, contributes to the immunogenicity of HLA mismatch by presenting allo-HLA-derived T-cell epitopes and thus influencing CD4+ T cell and B cell interactions (26,27). Based on subsequent data, EnSCs were selected as the best available source of cells that applied the least stimulus to the immune system. According to our previous information, EnSCs do not express HLA-DR. On the basis of our finding, in the present research it was stated that motor neurons developed by small molecule mentioned, do not expressed HLA-DR. Our finding indicated that EnSCs derived motor neurons developed by inducing small molecules do not stimulate immune system. Because of this unique feature, they can be used in therapeutic cases such as transplantation and grafts.

Conclusion

Taken together, we have found that hEnSCs were encapsulated on collagen and can be induced to differentiate into motor neuron-like cells with PMA. Expression of motor neuron markers at the RNA and protein levels by RT-PCR and immunocytochemistry showed that PMA, a synthetic small molecule, promotes differentiation of cells into motor neuron–like cells. Our finding indicated that EnSCs derived motor neurons developed by inducing small molecule PMA do not stimulate immune system because these cells do not express HLA-DR after differentiation and can be useful for stem cell-based therapy and transplantation in neurological disorders in future.

Conflict of interest

None

Acknowledgments

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