Transdifferentiation of human synovium-derived mesenchymal stem cell into neuronal-like cells in vitro

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Abstract:

Introduction: Synovial mesenchymal stem cells (SMSCs) could be expanded extensively in monolayer with limited senescence, maintaining their multilineage differentiation potential in vitro. We characterized the multi-potent ability of human synovial membrane derived stem cells (SMSCs) and investigated the neural differentiation potential of these cells.

Materials and Methods:

SMSCs were isolated from the knee joint of ACL (Anterior cruciate ligament) and baker cyst patient. These cells were proliferated and amplified with the indicated concentration of FBS (Fetal Bovin Serum) and DMEM in vitro. Alizarin red and oil red staining was done to investigate stemness property of the cells. Then, they were induced in vitro by β-mercaptoethanol (BME), and Retinoic acid (RA). Reverse transcription polymerase chain reaction (RT-PCR) analysis was done to prove the neural gene and osteocyte and adipocyte cells specific genes.

Results:

RT-PCR analysis showed the expression of osteogenic and adipogenic genes. Following neural induction, SMSCs were differentiated into various types of neural cells in vitro. RT-PCR analysis also demonstrated that the mRNA levels encoding for neurofilament medium (NFM), neuron specific enolase (NSE) were also highly increased in induced SMSCs.

Conclusion:

These results suggest that synovium tissue, which is discarded in most of the knee operations, can be used for cell therapy and tissue engineering protocols as an enrichment source of potent mesenchymal stem cells. Induction of SMSCs by inducers such as BME-RA could highly transdifferentiate SMSCs into neuronal-like cells.

Keywords: Stem Cells, Cell Differentiation, Synovium

Introduction

Stem cells are undifferentiated somatic cells that can divide into adult or fully differentiated cells under specific circumstances (1). According to their origin, stem cells are categorized as either embryonic or adult. Adult stem cells are found in various specialized tissues including the brain, bone marrow, liver, pancreas, gastrointestinal tract, skin, cornea, retina, skeletal muscles, blood vessels, and even dental pulp. Although they possess different properties depending on their origin, their major role...
is to produce adult specialized cells in their residing tissues. In other words, these cells are responsible for the protection and maintenance of cells in all tissues and organs during an individual’s life (2). Adult stem cells are generally multipotent cells whose division results in semi-differentiated cells known as progenitor cells. Frequent divisions reduce the differentiation potential of the cells and lead them toward adult cells with definitive fate.

Mesenchymal stem cells have attracted researchers’ attention as one the most important groups of stem cells. They have high self-renewal and differentiation potential and have even been shown to differentiate into ectoderm and endoderm. Besides, their fairly simple isolation and proliferation along with their autologous transplantation make them good candidates for stem cell therapy. While bone marrow is considered as the major source of mesenchymal stem cells, they have also been isolated from other sources such as adipose tissue, placenta and umbilical cord tissue, and peripheral blood (3-5).

Synovium is a tissue containing stem cells. Research has proven synovium-derived mesenchymal stem cells not only to have a differentiation potential a hundred times higher than that of bone marrow-, periosteum-, adipose tissue-, and muscle-derived mesenchymal stem cells, but also to possess long-term self-renewal capacity and not to undergo aging during frequent passages and stages of freezing (6-7). Moreover, as these cells have immunosuppressive properties similar to those of bone marrow-derived mesenchymal stem cells, they can evade the immune system and suppress the immune response (8). Extracting synovium tissue and isolating mesenchymal stem cells occurs through arthroscopy, a minimally invasive procedure that incurs no pain to the patient. The procedure is simple and complication-free and a small amount of tissue suffices for successful isolation of stem cells (9). Previous studies have reported efficient isolation and proliferation of mesenchymal cells from both healthy synovium tissue and damaged tissue of patients with rheumatoid arthritis and osteoarthritis (10). Therefore, the unique characteristics of synovium-derived stem cells necessitate research on their use in clinical applications of cell differentiation.

Diseases affecting central nervous system are among the most harmful and painful human diseases. Degenerative nerve disorders comprise a wide range of diseases from Alzheimer’s disease to stroke. Since embryonic and adult stem cells can differentiate into various cell lines including nerve cells, they can contribute to a better understanding of the brain and nervous system and causes of disease progression and thus be valuable in the development of possible treatments and novel medicines for degenerative nerve disorders. Recent research has suggested that human brain-derived neural progenitor cell transplantation into an animal model with spinal cord injury caused remyelination similar to the process in Schwann cells (11). Considering the significance of synovium-derived mesenchymal stem cells, the present study assessed the potential of these cells to differentiate into nerve cells. In case of successful differentiation of synovium-derived mesenchymal stem cells into nerve cells, the differentiated cells can be utilized in treatment protocols for neurological damages.

Materials and Methods

Isolation and Culture of Synovium-Derived Mesenchymal Stem Cells

This empirical study was conducted in 2010 after obtaining necessary permissions from Jahrom University of Medical Sciences (Iran) and informed consent from the participants. Synovium tissue was taken from 12 male patients (age: 21-50 years) who had undergone anterior cruciate ligament reconstruction surgery.
Transdifferentiation of human synovium-derived mesenchymal cells was studied by Kashafi et al. (Journal of Jahrom University of Medical Sciences, Vol. 11, No. 2, Summer 2013).

The study involved four men (age: 37-50 years) who had undergone a Baker’s cyst removal. The tissues were transferred to the research laboratory of the mentioned university under sterile conditions. For enzymatic tissue digestion, the samples were cut and kept in culture media containing 0.25 mg/cc collagenase D (Roche Applied Science, USA) for three hours. The digested tissues were then filtered through a nylon filter (pore size: 0.70 µm) and washed with phosphate buffered saline (PBS) (Gibco-BRL, USA).

After centrifuging the obtained suspension at 1500 rounds per minute (rpm) for five minutes, the resulting sediment was cultured in cell culture flasks containing Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco-BRL, USA) with 15% embryonic bovine serum (FBS) (Gibco-BRL, USA), 100 U/ml penicillin (Sigma, USA), and 100 µg/ml streptomycin (Sigma, USA) at 37°C and carbon dioxide content of 5%. Twenty-four hours later, cell media were removed and non-adherent cells were discarded. Fresh medium was then added to adherent cells and this process was repeated every three days.

After 70%-80% confluency, the cells were passaged using 0.25% trypsin (Gibco-BRL, USA) and 0.04% ethylenediaminetetraacetic acid (EDTA) (Sigma, USA). The procedure was repeated for four passages to purify mesenchymal cells.

To prove that the cultured and passaged cells were true stem cells, differentiation tests and specific staining techniques were applied after replacing culture media with osteogenic and adipogenic differentiation media. Media was regularly replaced at three-day intervals for three weeks. Negative control cells were also simultaneously cultured in DMEM and 10% serum. Histochemical staining of cells differentiated to osteocytes and adipocytes was performed using alizarin red and oil red, respectively. Reverse transcriptase polymerase chain reaction (RT-PCR) was also carried out to confirm the formation of the mentioned cells (7).

**Pre-Induction and Induction of Synovium-Derived Mesenchymal Stem Cells**

Following the trypsinization, synovium-derived mesenchymal stem cells from the fourth passage were differentiated into nerve cells through pre-induction and induction. During the pre-induction stage, beta-mercaptoethanol (1 mM) was used in DMEM culture medium without serum. Twenty-four hours after pre-induction, a culture medium containing 15% serum and retinoic acid (1 mM) was added to PBS-washed cells. The flasks were then incubated under appropriate conditions for three days and the cells were prepared for ribonucleic acid (RNA) extraction and RT-PCR (12,16).

**RT-PCR**

Differentiated and undifferentiated cells were first trypsinized and centrifuged. The supernatant was then discarded and the sediment was prepared for RNA extraction by utilizing a Qiagen kit (#74134) and according to the manufacturer’s instructions. A one-step RT-PCR kit (Cat No. K-2055, Bioneer) was used to perform reverse transcription reaction at a final volume of 20 µl which was achieved by adding sterile distilled water and upstream and downstream primers to mastermix tubes (Cat No. K-2016, Bioneer). The primer used in the current research had been employed by various earlier studies and confirmed by the Basic Local Alignment Search Tool (BLAST).

After reaching the determined volume, RT-PCR was conducted at 42°C for 70 minutes. The obtained cDNA was added to a 35-cycle PCR for NFM (neurofilament medium, a neurofilament intermediate filament protein gene). The PCR included an initial denaturation at 95°C for five minutes, denaturation at 92°C for 45 seconds, primer binding at 54°C for one minute, and primer extension with DNA polymerase at 72°C and final extension
time of 10 minutes. Similarly, 35 cycles of PCR were repeated for neuron-specific enolase (NSE), lipoprotein lipase (LPL), alkaline phosphatase (ALP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes with primer binding temperatures equal to 55.1°C, 54°C, 54.5°C, and 58°C, respectively. All PCR tests were performed with a thermal cycler. The GAPDH gene was considered as a housekeeping gene for internal control. All primers and genes used in this study are presented in Table 1.

**Table 1. Primer sequences used in the study**

<table>
<thead>
<tr>
<th>Gene name and Reference Sequence</th>
<th>Primer sequence</th>
<th>Amplified fragment length (base pairs)</th>
<th>Primer binding temperature (°C)</th>
</tr>
</thead>
</table>
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | F: GTCAACGGATTGGTCGTATTG  
R: CATGGGTTGAATCATATTGGAA | 139 | 58 |
| Lipoprotein lipase (LPL) | F: GTCGTCGACCTGCTAGCTTACC  
R: AGCCCTTTCTCAGGCTCTC | 717 | 55 |
| Alkaline phosphatase (ALP) | F: CTCTCTCAGTCTAGCTTAC  
R: AGGGTCAAGGAGATGAGACTGG | 300 | 54.5 |
| Neurofilament intermediate filament protein (NFM) | F: GCAATATGAGGGGACTGCAT  
R: ACTGCTGTGACGTTAAACATCG | 336-337 | 54 |
| Neuron-specific enolase (NSE) | F: GGGACTGAGAACAAAATCCTAAGG  
R: CTCCAAAGCTTTACTGTTCTC | 572 | 54.1 |

**Results**

**Morphological Observations before Induction**

Cells were generally available in two forms during the first day, i.e. a number of them were dividing and thus looked round, spherical, and small while others, which were greater in number, were spindle-shaped and fibroblastoid. There were also some larger flat cells adherent to the flask. Three days after the initial culture, a few elongated, fibroblastoid cells were observed (Figure 1-A). On day seven, the number of spindle-shaped, fibroblastoid cells increased. The cells were more purified after the initial culture phase and entering the passage (Figure 1-B). Over increased passages, their division occurred at higher speed. At passage 4, synovium-derived mesenchymal stem cells had roughly uniform morphology.

![Figure 1-A](image1.png)  
Figure 1-A. Cells derived from synovium of a patient with cruciate ligament rupture three days after initial culture (20x magnification);  
Figure 1-B. Cells derived from synovium of a patient with Baker’s cyst at passage 2 (20x magnification)
**Morphological Observations after Induction**

Morphological assessment of synovium-derived mesenchymal stem cells after pre-induction and induction stages revealed that cell bodies swelled and developed elongations, sometimes even larger than the cell body itself, that were in contact with adjacent cells (Figures 2-A and 2-B).

![Figure 2-A. Cells during pre-induction with beta-mercaptoethanol (20x magnification)](image1)

![Figure 2-B. Cells pre-induced with beta-mercaptoethanol and induced with retinoic acid (20x magnification)](image2)

**Testing If the Purified Cells Are Stem Cells**

**Differentiation Test with Oil Red and Alizarin Red Staining**

Negative control cells kept in non-inducing media for 21 days were not stained with either oil red or alizarin red staining. However, 21-day culture of cells in adipogenic media and the consequent formation of fat vacuoles caused cells to be stained with oil red. Osteogenic differentiation of the cells was also evaluated to determine calcium contents. Differentiated cells with bone trabeculae turned red using alizarin red staining (Figure 3).

![Figure 3. Alizarin red and oil red staining. (A) Negative control cells kept in non-inducing media for 21 days (20x magnification); (B) Cells kept in adipogenic differentiation media for 21 days and stained with oil red (100x magnification); (C) Cells kept in osteogenic differentiation media for 21 days and stained with alizarin red (100x magnification)](image3)
Assessment of Cell Differentiation through RT-PCR

RT-PCR was used to examine lineage-specific gene expression in differentiated cells (Figure 4). Adipocyte-specific and osteocyte-specific gene expression was assessed using LPL and ALP, respectively. GAPDH gene with 139 base pairs was considered as control. The fragment lengths of ALP and LPL were 300 and 717 base pairs, respectively.

![Figure 4](image-url)

**Figure 4.** Assessment of cell differentiation into adipocytes and osteocytes. (1) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (139 base pairs) expression as the control; (2) Lipoprotein lipase (LPL) gene (717 base pairs) expression in cells induced into adipocytes; (3) Alkaline phosphatase (ALP) gene (300 base pairs) expression in cells induced into osteocytes

RT-PCR was employed to prove the differentiation of mesenchymal cells into nerve cells. The results suggested that GAPDH gene (the control gene which is expressed in all cells) was expressed in both induced and undifferentiated groups of cells. Amplified fragment length in the GAPDH primer was 139 base pairs. Besides, NSE (572 base pairs) and NFM (337 base pairs) were specifically expressed in neural progenitors and adult neural cells, respectively. Figure 5 demonstrates the electropherogram of RT-PCR products following GAPDH, NSE, and NFM gene mRNA extraction in synovium-derived mesenchymal stem cells before and after induction (the right column shows DNA ladder).

Discussion

Repair of the central nervous system and neural regeneration are among the major challenges for neurologists. Recent research has shown embryonic, neural, and mesenchymal stem cells to have the potential of differentiation into neurons. These stem cells have in fact demonstrated neural function after transplantation in animal models (13). Due to the ethical and legal barriers to extraction and differentiation of embryonic stem cells and their limited resources, mesenchymal stem cells are widely used since they raise no ethical concerns and possess high self-renewal potential, simple accessibility, differentiation potential into various...
lineages, and low immunity. The present study was the first Iranian research to evaluate the in vitro differentiation of synovium-derived stem cells into nerve cells.

Figure 5-A. Electropherogram of reverse transcriptase polymerase chain reaction (RT-PCR) following glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (139 base pairs) mRNA extraction in synovium-derived mesenchymal stem cells before and after induction. (1) Molecular weight marker; (2) GAPDH gene expression in non-induced synovium-derived mesenchymal stem cells; (3) GAPDH gene expression (139 base pairs) in synovium-derived mesenchymal stem cells induced into nerve cells using beta-mercaptoethanol.

Figure 5-B. Electropherogram of reverse transcriptase polymerase chain reaction (RT-PCR) following neuron-specific enolase (NSE) gene (572 base pairs) mRNA extraction in synovium-derived mesenchymal stem cells induced with beta-mercaptoethanol. (1) Molecular weight marker; (2) NSE gene expression in synovium-derived mesenchymal stem cells induced into nerve cells using beta-mercaptopoethanol.
Many researchers have used mesenchymal stem cells to induce glial and neural cells. Woodbury et al. were the first to report the ability of bone marrow-derived stem cells to differentiate into not only mesenchymal cell line but also neurons. In order to make mesenchymal cells differentiate into neurons, they were induced with beta-mercaptopethanol, dimethyl sulfoxide (DMSO), and butylated-hydroxyanisole (BHA). In the pre-induction stage, the cells were cultured in the DMEM with 20% serum and 1 µM beta-mercaptopethanol. The induction was performed in the DMEM with 1-10 µM beta-mercaptopethanol. The next experimental groups were exposed to DMEM with 2% DMSO and 200 µM BHA. Using this treatment protocol, 80% of cells expressed NSE and NFM genes (14). Sanchez-Ramos et al. suggested rat and human bone marrow-derived mesenchymal stem cells to have the potential of differentiation into neurons and glia cells in presence of 0.5 µM retinoic acid and brain-derived neurotrophic factors (i.e. epidermal growth factor). They found bone marrow-derived mesenchymal stem cells induced with DMEM and the mentioned factors to express neural progenitor markers such as nestin and NeuN (a neuronal specific nuclear protein) (15).

The type of serum, dissimilar races or species, and the use of differentiation inducing agents are the major causes of difference between studies on differentiating mesenchymal stem cells into glial and neuronal cells (16). In the current study, we found human synovium-derived mesenchymal stem cells to differentiate into neuronal cells in presence of beta-mercaptopethanol without serum and also retinoic acid and serum. Previous research using similar inducers has observed similar cell morphology (14,16). Although the exact mechanism through which beta-mercaptopethanol induces neural differentiation is unknown, its antioxidant properties are believed to promote neuronal survival in culture and thus be partially responsible for the induction. On the other hand, retinoic acid is derived from retinol and plays a
significant role in regulating cell proliferation, differentiation, and apoptosis. It exists in various embryonic and adult tissues and enhances neural differentiation (17,18).

Using any of the induction agents in the present study turned cells into neuron-like cells (Figure 2-A). Three days after adding retinoic acid to pre-induced cells, cell concentration increased and elongated cells with yet neuronal morphology were observed (Figure 2-B). Since a crucial characteristic of stem cells is their potential to differentiate into various adult cells, adult mesenchymal stem cells should be able to produce all types of mesenchymal lineages including fibroblasts, chondrocytes, osteocytes, and adipocytes. After 21 days of induction in osteogenic and adipogenic differentiation media, mesenchymal cells formed fat vacuoles and bone trabeculae in alizarin red and oil red staining, respectively. These results confirmed that the cultured mesenchymal cells had been stem cells. Kadivar et al. employed the same staining method and authenticated synovium-derived mesenchymal stem cells as stem cells (19).

RT-PCR is one of the most common techniques to evaluate cell differentiation. It shows the expression of any specific gene with high accuracy and sensitivity. LPL and ALP gene expression in the current study was suggestive of cell differentiation into adipocyte and osteocyte lineages, respectively. Such finding in turn proved that the cultured cells had been stem cells (Figure 4). Similarly, De Bari et al. had previously detected cell differentiation using other genes (20).

We also found NSE and NFM to be expressed in induced cells but not in non-induced cells (Figure 5-2 and 5-3). NSE is a gene expressed in neurons. Research has shown enzyme, protein, and mRNA activity of NSE in cultured mouse oligodendrocytes compared to cultured mouse neurons. NSE expression increases during the differentiation of oligodendrocyte progenitors. NSE proteins are fully suppressed in differentiated oligodendrocytes and adult cells in vivo. On the other hand, much lower enzyme, protein, and mRNA activity of NSE has been detected in type 1 astrocytes, i.e. although NSE cannot specifically prove neural differentiation, it can indicate glial differentiation (21). Hence, NSE expression in the present study can be proof of synovium-derived mesenchymal stem cell differentiation into glial cells.

NFM was another expressed gene in the current study. Neurofilaments are a large group of intermediate filaments with low, medium, and high molecular weight. They are found in the neurons of central and peripheral nervous system and ensure heteropolymerization. A number of neurofilaments including NFM are present in adult differentiated neural cells (22). Therefore, it seems that the induced synovium-derived stem cells in this research have been able to differentiate into neuron-like cells. Previous studies have also reported consistent findings. Liu et al. used basic fibroblast growth factor (bFGF, 25 ng/ml) to differentiate synovial stem cells extracted from temporomandibular joint into neural cells. They utilized fetal calf serum and DMEM to culture the cells. RT-PCR and immunocytochemistry results indicated the expression of neurofilament light chain and nestin in the induced cells (23). Likewise, utilizing bFGF (100 ng/ml) and 10 μM forskolin, Sujeong et al. differentiated adipose tissue-derived stem cells into glial cells and neurons. They also confirmed the expression of numerous neuronal markers such as NSE, NFM, neurofilament light chain, and microtubule-associated protein 2 (MAP2) (24).

Generally, the difference between various studies seems to be mainly caused by the type of induction agent and serum employed. Furthermore, different proliferation and survival ability of the
cells might be responsible for their dissimilar differentiation potential.

**Conclusion**

The proliferative potential of synovium-derived mesenchymal stem cells is a hundred times higher than that of similar cells extracted from bone marrow, periosteam, adipose tissue, and muscles. Moreover, these cells demonstrate high self-renewal ability and slow aging. Due to these properties, they are good candidate for differentiation into other cells and can thus be used in clinical studies. Apparently, synovium tissue can be utilized as a rich source of mesenchymal stem cells in cell therapy and tissue engineering.

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**Conflict of Interests**

None to declare.

**References:**
