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A New Method for Differentiating Mesenchymal Stem Cells toward Neural Lineage

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Abstract – AIM: To investigate and find an efficient method for inducing mesenchymal stem cells (MSCs) into neural differentiation. METHODS: MSCs from rat bone marrow were purified using adhesiveness property of stem cells, then characterized with flowcytometer. The isolated cells seeded in neural induction medium for 4 days, Morphology and immunocytochemistry were used to identify the neural differentiation grade. RESULTS: MSCs exhibited neural network after differentiation, instead of homogenous fibroblast-like morphology before differentiation. Flowcytometric analysis confirmed the identity of mesenchymal stem cells. More than 80 percent of all population were stained positive for Tau protein.

CONCLUSION: Bone marrow MSCs have ectodermal differentiation capabilities. MSCs can differentiate into functional neuron-like cells, and may be used as a treatment for neurodegenerative diseases.

Keywords – Stem Cell, Neuron, Differentiation, Tau Protein, Neurodegenerative Disease.

I. INTRODUCTION

Surgical and medical therapy for central nervous system diseases, including degenerative, traumatic, and ischemic damage, are very limited, and it is too hard to expect recovery from nervous system damage. To regenerate the damaged nervous tissues, a neuron transplantation is a raised possibility. A study that transplanted fetal dopaminergic neurons to an adult who was suffering from Parkinson's disease showed positive results.[1] However, its use is controversial because there are still many ethical issues with the application of fetal or embryo stem cells to patient treatment. Also, techniques for obtaining a sufficient quantity of adult neuronal stem cells are still limited.([2],[3]) Therefore, this cannot be considered a proper method for application in clinical cases.[4]

Since the last century, many scientists have shown the existence of non-hematopoietic stem cells in bone marrow. In 1968, Friedenstein et al shown that these cells can be differentiated into bones. Later, Owen1 reported that these cells have a self-renewal and multi-lineage differentiation capacity. These population have been called marrow stromal stem cells or, bone marrow mesenchymal stem cells (MSCs), and there are several researchers who focused their work on these population. They are isolated from adipose tissue,

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umbilical cord blood, and Amniotic fluid as well as from bone marrow [5-13]. Also these cells are capable of differentiating into mesenchymal lineages such as osteoblasts, adipose cells, and chondrocytes [14]. MSCs have the ability to differentiate into stromal cells, tendinocytes, and myocytes, which are normally derived from mesenchymal stem cells. [5-14] they are also capable of differentiating into endodermal origin hepatocytes and ectodermal origin under specific conditions, in vitro. These differentiation capacity of MSC used for research into possible clinical utilization in regenerative medicine.

Generally, the number of MSCs in the bone marrow is small. There are about 2 to 5 cells present in every 1×10^6 mononuclear cells. However, They have many advantages including relatively easily separated and grown in culture, strong proliferation capacity capability of more than a billion-fold increase in number *in vitro* without losing their stemness properties, being non-immunogenic, and there are no ethical or religious issues concerning its use [15]. As a result MSCs originating from bone marrow are suggested as the best candidates for cell transplantation, repair and regeneration of damaged tissues.

Several neurodegenerative disorders have been shown to be associated with the damage of specific types of neurons accompanied by functional loss. stem cells are usually considered as candidate cells for cell transplantation to treat these diseases in clinical trials ([16], [17]), Many agents such as cytokines, growth factors, neurotrophins, and retinoic acid have been used to induce neural cell differentiation both in vivo and in vitro, and they have been used for inducing MSCs to differentiate into neural cells ([18], [19]). But all these approaches were not at satisfactory efficiencies and yields, and experimental results often fluctuated by batch. In addition, there are some limitations in the clinical setting. For example, immunological rejection, insufficient tissue supply, treatment scale, and ethical issues are common limitations.

The goal of this study is to determine an effective method of differentiating BMSCs into neural like cells. We used a cocktail of growth factors and hormones to accomplish a high percent differentiation of isolated cells. This method may provide new insights into the clinical treatment of neurodegenerative diseases and disorders.

II. Materials And Methods

2.1. Animals

Female Sprague-Dawley rats at 8-12 weeks of age were obtained from medical Experimental research center. All animal experiments were performed in accordance with the animal guidelines of Mansoura University.

2.2. Isolation and culture of MSCs from rat bone marrow

Sprage dewaly rats was obtained from, about 2 mL of marrow were extracted from both femurs and tibias of each rat. 10 mL of phosphate buffered saline (PBS) was added to marrow sample. Then, the mixture was slowly layered on Ficoll-Hipaque solution. Then centrifuged for 35 minutes at 450 xg. Next, the mononuclear layer present in the interface were retrieved and washed twice with PBS. The separated cells were placed in Dulbecco's Modified Eagle's Medium (DMEM)-Low glucose, 10 % FBS, 1% penicillin-streptomycin. Then, the cells were seeded at a density of 1.5 x ^{106} / cm^2 into a culture dish (T-75), and cultured in an CO2 incubator.

After 24 hrs the medium was replaced. When MSCs reach a conflency of about 80% of the culture flaske, it was subcultured . The medium was replaced every three days.

2.3. Cell characterization

The cells from passage 3 and later were resuspended in flowcytometry washing buffer (10 mL/L bovine serum, 2 g/L sodium azide in PBS), and stained on ice according to the manufacturer's recommendations with the monoclonal antibodies as follows: PE conjugated mouse anti-rat CD 29, FITC conjugated mouse anti-rat-CD90 and PCy5 conjugated mouse anti-rat-CD34. After washed three times with washing buffer, the stained cells were resuspended in 300 µL fixation buffer (20 g/L formaldehyde in PBS) and run on a flow cytometer (FACS Calibur, BectonDi-ckinson, USA). The results were analyzed by CellQuest software (BD, USA).

2.4. Differentiation protocols

MSCs from passage 3 were seeded at 5×10^3/cm^2 on 50 µg/mL poly-L-Ornithine (Sigma-Aldrich, USA), and then with 1 µg/mL human Fibronectin (R&D System, Cat. # FN:1918.). Cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, HyClone), supplemented with 1% N2 supplement, 20 ng/ml bFGF (basic recombinant human Fibroblast Growth Factor, 1 µg/mLTetracycline (Sigma, T7660), 2 ng/mL human recombinant GDNF and 1 mM cAMP at 37 °C in a humidified atmosphere of 5% CO2 incubator.

2.5. Immunocytochemistry

Cell cultures were washed with PBS twice and fixed with 95% alcohol/acetic acid (99:1) for 10 min at room temperature, then permeabilized with 2 g/L Triton X-100 for 10 min. After washed, cells were blocked with 5% BSA for 2

h at room temperature. Subsequently, the cells were washed with PBS three times and incubated with Primary antibodies, anti-TH antibody (1:200 dilution) and purified anti-Tau phospho (Thr181) antibody. Then cells were visualized and photomicrographed by a microscope (Olympus, Japan).

III. Results

3.1. Cell morphology

3.1.1. Cell yield and shape of MSC before and after neural differentiation

After passage 3 and before differentiation, MSCs exhibited a homogenous view and fibroblastic morphology with spindle shape cell bodies (Figure 1A), while after differentiation, the cells formed obviously neural network in shape (Figure 1B). This change occurred early on d 4 after MSC differentiation protocol.

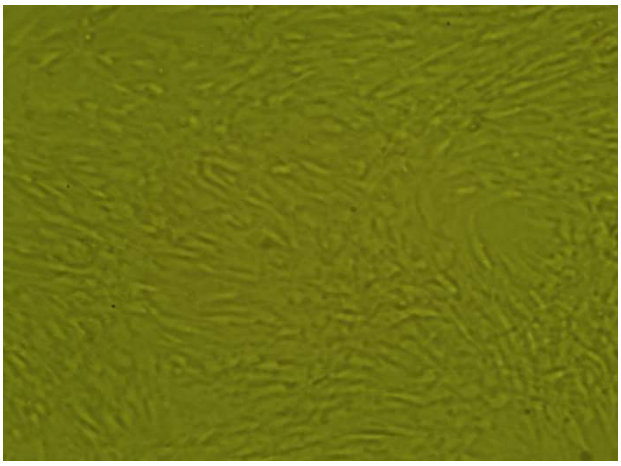

Figure (1A): morphology of stem cells at passage 3

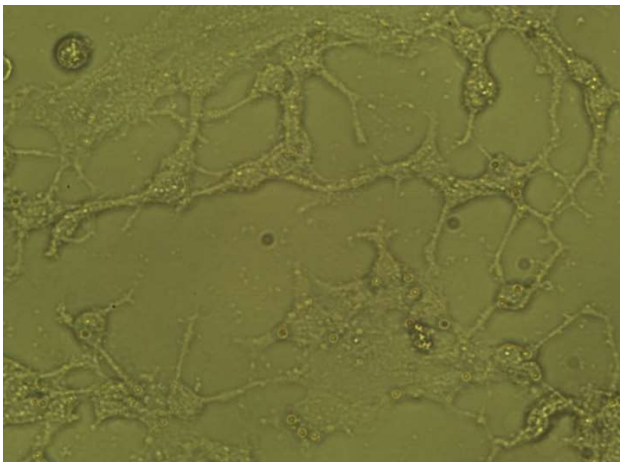

Figure (1B): after differentiation protocol, cells show clear neural network.

3.2. Flow cytometric analysis of cell-surface markers

Cells were negative for CD34 which is a hematopoietic marker and were positive for both CD90 and CD29 which are mesenchymal stem cell markers. (Figure 2).

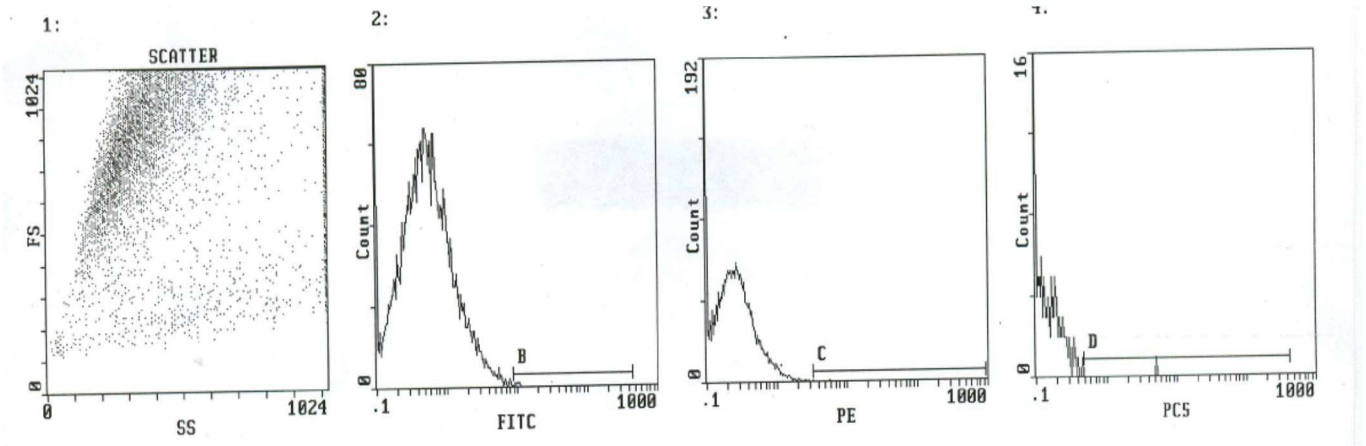

Figure 2: Flowcytometric analysis of stem cells after passage 3, PE conjugated mouse anti-rat CD 29, FITC conjugated mouse anti-rat-CD90 and PCy5 conjugated mouse anti-rat-CD34.

3.3. Immunocytochemical analysis

Differentiated MSCs were positively stained for phosphorylated Tau keeping their sharp morphology as a neural network (Figure 4).

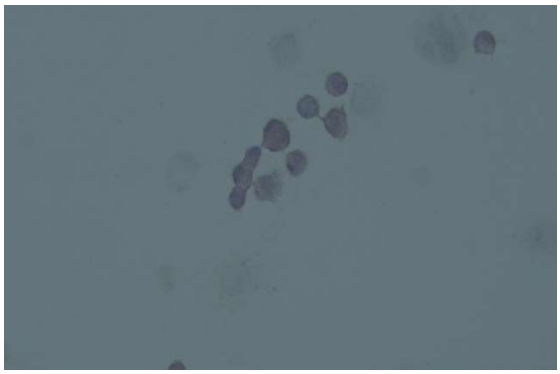

Figure 4: cells showed positive staining toward Tau antibody as well as keeping their neural network

IV. Discussion

Bone marrow Mesenchymal stem cells have clearly shown to have the ability to differentiate into a multiple types of MSCs including all mesodermal origin, such as adipocytes, osteocytes, chondrocytes and myocytes [20-23]. Besides these, MSCs are also have the ability of "transdifferentiation" into non-mesenchymal ectodermal lineage ([5], [6] ,[24]) such as neural cells. So MSCs are multipotential adult stem cells.

The traits of multipotent progenitor populations make them an attractive target for use in both therapeutic and bioengineering applications ([25] ,[26]). The differentiating capability of MSCs towards a neural lineage allows potential therapeutic applications for the treatment of neurological disorders and CNS trauma.

In previous studies, Neural Stem Cells were used as the source to induce such cell differentiation. However, due to their limited and dormant populations in adults, difficult acquisition, and secondary damage to the body, Neural Stem Cells are considered not suitable for the treatment of some central nervous system, degenerative diseases and CNS traumatic injuries, which are caused by the loss of neural cells. Therefore, it is very advantageous to use non-neural lineages as cell sources, which are easy to obtain and expand, while causing minimal damage to the body ([27] ,[28]).

BMSCs can be easily obtained and expanded. Although they originate from the mesoderm, they can differentiate into neurons. Until recently, inducing BMSCs to differentiate into ectodermal neural lineage cells was confronted with many problems that included complex induction approaches, low differentiation rates, poor functionality and poor stability [29-32].

In our study, we tried to induce standard MSCs into neural cells in an effective way that did not introduce before. After induction, MSCs showed a clear morphology that define and characterize neurons, it appeared early on d 4 after differentiation, coinciding with the change of tau protein expression.

Immunocytochemical staining confirmed the results of cell culture and differentiation at protein-level. Morphologic change of MSCs was apparent. Cell shape changed from spindle and fibroblast-like to neural network.

A Previous studies have explored the ability of neural differentiation of different subsets from bone marrow. However, Our study suggest a new approach for differentiation, and investigated their different abilities of neural differentiation simultaneously.

In this study, BMSCs were the cell source and were differentiated into neural lineage cells induced by bFGF, Tetracycline and GDNF. The MSCs used in this study were positive for CD 29, CD 90, and negative for CD 34, indicating that MSCs retain the capacity to differentiate into mesenchymal and ectodermal derivatives, and these adult cells are capable of self-renewing and exhibit multipotency, thereby fulfilling the criteria for a stem cell population.

Through the results of neuron-like cell differentiation from BMSCs using growth factors such as bFGF, Tetracycline and GDNF we can presume the following three possibilities. First, BMSCs can be differentiated using a method such as mesenchymal epithelial transformation

(MET). Second, as we already know from the results of the immunostaining, under particular conditions of differentiation, it is possible to cells to differentiate into neuron-like cells.

It is our understanding that bFGF is a type of cellular factor with wide applications. Under normal physiological conditions, bFGF can facilitate cell proliferation and division and is thus involved in neuronal generation and tissue repair for brain and spinal cord injuries ([33], [34]). The combination of bFGF and the GDNF may activate several signaling cascades, including phosphoinositide 3-kinase, phospholipase, and MAPK signaling pathways [35]. Under physiological conditions, however, bFGF has a short half-life (only 17 hours) [36] and can hardly pass through the blood-brain barrier, which results in quite low efficacy in a clinical setting. It has been reported that soluble bFGF could induce mesenchymal stem cells to differentiate into NF+ neural cells with electrophysiological characteristics in vitro, but bFGF had to be administered throughout the entire process [37], which was very expensive.The results suggest that bFGF might combine with The MAPK signaling pathway, one of the most fundamental signaling pathways in vivo, plays a significant role in cell growth, division, proliferation, and differentiation as well as in various physiological and pathological processes. It can be activated by a series of extracellular signals or stimulating factors. The ERK, JNK/SAPK, P38MAPK, and ERK5/BMK1 signaling pathways are four prominent members of the MAPK family. They are regulated by extracellular signals and activated by different stimulating factors to form different transduction pathways, which subsequently activate different transcription factors to achieve different biological effects [38].

Also, GDNF stimulates differentiation into neuron-like cells. GDNF along with bFGF is used as a factor to grow and proliferate neural stem cells or neural precursor cells derived from mouse or human brain tissue. Because previous reports already discovered that GDNF improves the survivability of neurons in the hippocampus and midbrain, and induces the growth of neurite in neocortical explants. In the peripheral nervous system, GDNF functions as a survival factor of motor neurons. Particularly, in the development process, it functions as a axonal chemoattractant to spinal motor neuron, and is also associated with the growth and survival of sensory and parasympathetic neurons [39].

In addition, Jiang, et al. [40] reported that selectively proliferated cells can be differentiated into multipotent precursor cells with the characteristic shapes of neurocytes, astrocytes, and oligodendrocytes. Chamberlain, et al. [41] also proved the possible trans-differentiation of mesenchymal stem cells. Woodbury et al. reported that MSCs undergo rapid transformation into cells with neuron-like phenotypes following treatment with a chemical induction medium containing DMSO ([42], [43]). However, Neuhuber et al. reported the formation of neurite-like processes following chemical induction, as a result of cytoplasm retraction caused by b-actin depolymerization and not by microtubule-mediated [44].

In this study, BMSCs' ability to proliferate and differentiate into neuron-like cells by has indicated a possibility for application to cell therapy for a wide range of diseases such as neurodegenerative disease and cerebral infarct. Previously known methods of inducing neuron-like cell differentiation using several chemicals have limits because the toxicity of these chemicals makes them too risky to be applied in humans.

Our results indicated that stem cells, especially bone marrow-derived neural stem cells may be therapeutically useful for treating a variety of neurodegenerative diseases that affect the brain and the behavior as a result.

Compared with other neural-related stem cells, such as embryonic stem cells, pancreatic stem cells, and liver stem cells, bone marrow-derived stem cells provide several advantages: first; Bone marrow can be obtained from living animals or recipients themselves with a moderately invasive techniques. There is no problem of limited volunteers, which restrict other organs transplantation. Second; Based on its ability of self-renewal, the amplification of bone marrow stem cells could be obtained. And the final; Utilizing patients' own bone marrow or repopulation of bone marrow system from the same donor could avoid or reduce immunogenicity, which usually affects recipients for a life-long time.

However, MSCs have amazing proliferation capabilities that can overcome the adult neuronal stem cells' limitation of quantity. It has also been confirmed that MSCs are capable of differentiating into cells that have similar Traits to neurogenic cells, and it is possible for these cells to be used in the recovery of damaged tissues.

In the upcoming, it will be of a great value to compare and define the traits of neuron-like cells originating from MSCs with other neurons in molecular biological and physiological aspects. Further researches are required to study the possibilities of transplanting them into humans and differentiating the cells within animal.

V. Conclusions

We proposed a new method that can dramatically reduce the time and efforts for the differentiation of BMSCs into functional neural cells.

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