

Induction of Bone Marrow Stromal Cells into Cholinergic-Like Cells by Nerve Growth Factor

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ABSTRACT

Background: Bone marrow stromal cells (BMSC) are used as a source for cell therapy in different model for neurological disorder such as stroke and spinal cord injury. However, the transdifferentiation of BMSC into cholinergic phenotype requires more investigation. **Methods:** BMSC were isolated from adult rats, pre-induced with β -mercaptoethanol (BME) and followed by nerve growth factor (NGF) induction. Neurofilaments of 68 kDa, 160 kDa and 200 kDa (NF-200, NF-160 and NF-68, respectively) immunostaining were used for evaluating the transdifferentiation of BMSC into neuronal phenotype. The percentage of neurofilaments immuno-reactive cells was applied in order to evaluate the results at the pre-induction and the induction stages. Also, NeuroD and Oct-4 expressions, using RT-PCR, were used in assessing the progression of BMSC into neuronal lineage. Choline acetyltransferase immuno-reactive cells were used for estimating the percentage of cholinergic neuronal phenotype. Immuno-staining with anti-microtubule-associated protein-2 (MAP-2) and anti-synapsin-I antibodies was done in order to evaluate cell tendency for synaptogenesis. **Results:** The yield of cholinergic neurons with BME as pre-inducer and NGF as inducer was 80%. Also, NF-200, NF-160, NF-68, MAP-2 and synapsin-I were detected in the transdifferentiated cells. RT-PCR showed the expression of NeuroD, while Oct-4 was not detected. **Conclusion:** BME as pre-inducer and NGF as inducer for BMSC transdifferentiation into cholinergic phenotype are potential sources in traumatic injury therapy in the central nervous system. *Iran. Biomed. J. 13 (2): 117-123, 2009*

Keywords: Bone marrow stromal cells (BMSC), Cholinergic neuron, Nerve growth factor (NGF)

INTRODUCTION

Cholinergic neurons in the central nervous system include cells in the basal forebrain, cortex, motor nuclei and the spinal cord. Acetylcholine is a neurotransmitter released from the synapses of cholinergic neurons. Choline acetyltransferase (ChAT), which synthesizes the neurotransmitter acetylcholine (ACh), is a phenotypic marker for cholinergic neurons [1].

The cholinergic neurons in the brain are involved in motor control and they decrease ChAT activity found in several neurological disorders including strokes [2] and amyotrophic lateral sclerosis [3].

Current advances offer hopes that human embryonic stem cells [4], neural stem cells [5] and bone marrow stromal cells (BMSC) [6] can be candidates for the lost motoneurons. From these

potential cell types for clinical transplantation which can be used as a replacement of lost neurons, BMSC represent an attractive cell source for restoring these neurons. They also can be easily isolated from bone marrow aspirates and expanded efficiently in culture and reintroduced into patients as autografts [7]. However, less elucidation about the mechanisms or agents can induce BMSC differentiation into cholinergic phenotype.

It is well known that β -mercaptoethanol (BME) is an anti-oxidant and thiol-reducing agent which was used by Woodbury *et al.* [6] for transdifferentiating BMSC into neuronal phenotype and was reported as a rapid and effective inducer and a contributor for the survival and differentiation of cholinergic precursors prepared from embryonic septal nuclei and basal forebrain *in vitro* [8]. But, other investigators contradicted these findings; a study

*Corresponding Author; Fax: (+98-21) 8801 6544; E-mail: ttiraihi@yahoo.com/takialtr@modares.ac.ir; BMSC, bone marrow stromal cells; BME, β -mercaptoethanol; NGF, nerve growth factor; MAP-2, microtubule-associated protein-2; ChAT, Choline acetyltransferase; ACh, acetylcholine; RT, room temperature; NDRB, ratio of NeuroD band, IBMX, isobutyl-methylxanthine.

showed that transdifferentiation of BMSC by this agent was an artifact [9], so that the effect of BME on differentiation of BMSC needs more investigation.

On the other hand, nerve growth factor (NGF) exerts anti-apoptotic, trophic and differentiating actions on the sympathetic neurons [10] and survival of septal cholinergic neurons after axotomy [11]. However, the *in vitro* transdifferentiation of adult stem cells such as BMSC into cholinergic phenotype is not carefully studied and needs more investigation.

Thus, the aim of this study is to evaluate the transdifferentiation of BMSC into cholinergic neuron-like cells by a consecutive neuronal pre-induction (by BME) and induction (by NGF) protocol.

MATERIALS AND METHODS

The experiments were approved by the Ethical Committee of the School of Medical Sciences, Tarbiat Modares University (Tehran, Iran).

The BMSC were isolated from adult Sprague-Dawley rats (200-250 g, Razi Vaccine and Serum Research Institute, Tehran, Iran). The tibias and femurs were dissected, the epiphyses of each bone were cut under aseptic condition and the whole marrow was aspirated with 5 ml of culturing media containing 500 units of heparin, using a 10-ml syringe and an 18-gauge needle. The content of each femur and tibia was transferred to a flask and expanded for 5 times and about 20 animals were sacrificed in this study. The isolation and culture medium was alpha-MEM (Gibco, UK) supplemented with 20% heated fetal bovine serum, 100 u/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2 mM L-glutamine [12].

The harvested cells were seeded in a 75-cm² flask (Nunc, Denmark) and a 5% CO₂ incubator at 37°C. After 48 h, the non-adherent floating cells including hematopoietic cells and debris were removed by changing the medium. The remaining adherent cells were incubated with the same medium and were passaged before reaching confluence. The cells were split 1:3 and passaged up to five times for subsequent experiments. Neuronal pre-induction was done with BME. The culturing medium was changed with serum-free media containing 1 mM BME. The cells were incubated for 24 h [6], then they were incubated with 100 ng/ml NGF as cholinergic

neuron inducer for 6 days [8]; therefore, the total days for pre-induction and induction were 7.

Immuno-cytochemistry. Before the neuronal pre-induction step, fibronectin, a BMSC marker, was used in order to evaluate the purity of BMSC. The cultured cells were plated in 60-mm dishes contained cover slips coated with gelatin. The cells were washed with PBS three times, fixed with acetone for 15 min and washed with PBS again. They were treated with 0.3% Triton X-100 for 1 h, and non-specific antibody reaction was blocked with 10% normal goat serum at room temperature (RT) for 30 min. It was followed by incubation with monoclonal mouse anti-fibronectin antibody (Chemicon, UK) for 2 h (1:300 dilution), and anti-mouse FITC conjugated antibody (Chemicon, UK) for 1 h (1:100 dilution) at RT. The labeled cells were visualized using fluorescence microscope and digitally photographed (Zeiss, Axiophot, Germany).

After pre-induction for 24 h, the cells were fixed with 4% paraformaldehyde (Merck, Germany), and immuno-cytochemistry was done according to the above mentioned method with polyclonal mouse anti-NF 200 kDa antibody (Chemicon, UK, 1:400 dilution), polyclonal mouse anti-NF 160 kDa antibody (Chemicon, UK, 1:300 dilution), polyclonal mouse anti-NF 68 kDa antibody (Chemicon, UK, 1:300 dilution), and polyclonal mouse anti-ChAT antibody (Chemicon, UK, 1:300 dilution). This was followed by incubation with secondary antibody, anti-mouse FITC conjugated antibody (Chemicon, UK, 1:100 dilution; for 1 h at RT). Other markers were evaluated using polyclonal rabbit anti-microtubule-associated protein-2 (MAP-2, Chemicon, UK, 1:500 dilution) and polyclonal rabbit anti-synapsin-I (Chemicon, UK, 1:400 dilution) as primary antibodies, followed by secondary antibody: anti-rabbit FITC conjugated antibody (Chemicon, UK, 1:100 dilution; for 1 h at RT). Each experiment was replicated at least 5 times in order to ensure reproducibility.

RNA extraction. The expression of genes shown in Table 1 was evaluated using RT-PCR. The total RNA was extracted using the RNX plus™ kit (Cinnagen, Tehran, Iran) according to the manufacturer's recommendations. Briefly, 1 ml of RNX plus was added to a tube containing 2 millions homogenized cells, and the mixture was incubated at RT for 5 min. Chloroform was added to the

Table 1. The primers used in the study using RT-PCR technique.

Genes	Primer (5'→3')	bp	Accession No.	Annealing temp.
β 2 microglobulin	F: CCGTGATCTTTCTGGTGCTT R: TTTTGGGCTCCTTCAGAGTG	300	NM-012512	58°C
NeuroD	F: AAGCACCAGATGGCACTGTC R: CAGGACTTGCATTCGATACAC	215	XM-341822	56°C
Oct-4	F: AAGCTGCTGAAACAGAAGAGG R: ACACGGTTCCTCAATGCTAGTC	285	NM-001009178	57°C

solution and centrifuged at 12,000 \times g for 15 min. The upper phase was then transferred to another tube and an equal volume of isopropanol was added. The mixture was then centrifuged at 12,000 \times g for 15 min and the resulting pellet was then washed in 70% ethanol and dissolved in DEPC-treated water. The purity and integrity of the extracted RNA were evaluated by optical density measurements (260/280 nm ratios) and examined using electrophoresis on agarose gel.

RT-PCR. One microgram of the total RNA was used as a template in a 20 μ l volume cDNA synthesis reaction containing 0.5 μ g oligo dT₍₁₈₎. This solution was first denaturated at 70°C for 5 min and chilled on ice immediately. Then, the mixture of 20 U ribonuclease inhibitor, 1 mM dNTPs, the 5 \times buffer supplied by the manufacturer and deionized water (nuclease free) up to 19 μ l was added and the mixture was incubated at 37°C for 5 min. The 200 U RevertAidTM M-MuLV reverse transcriptase (Fermentas, K1622, EU) was added to the reaction and the tube was incubated in a thermocycler (BIO RAD, USA) at 42 °C for 60 min, and at 70 °C for 10 min afterwards.

PCR was performed using 2 μ l of synthesized cDNA with 1.25 U Taq polymerase (Cinnagen, Tehran, Iran), 1.5 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M of each primer, 10x buffer supplied by the company, and deionized distilled water in a 50 μ l total reaction volume. All common components were added into the master mix and then aliquoted in tubes. The cycling conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of: 94°C for 30 s, 56-58°C (depending on the primers described in Table 1) for 30 s at 72°C for 45 s, and a final extension at 72°C for 5 min.

Each experiment was repeated at least 3 times in order to ensure reproducibility. The size of the digested products was checked on 1.5% agarose gel electrophoresis. The semi-quantitative analysis was done using UVI Tech software, where the ratio of

the band density of NeuroD to the β 2-microglobulin was calculated for assessing NeuroD gene expression.

RESULTS

Figure 1 demonstrates a time course at the induction stage using the time points 1, 3, 5 and 7 days. The expression of NF-200, NF-160, NF-68, ChAT, MAP-2 and synapsin-I was evaluated following the induction with NGF of the pre-induced BMSC with BME. The histogram shows a sustained increase in the expression of neurofilaments 200 kDa and 160 kDa. Also, a sustained increase in the expression of ChAT and synapsin-I was noticed, but the level of neurofilament of 68 kDa decreased, the level of MAP-2 expression was variable.

Figures 2 and 3 represent the immuno-reactivity of the markers in BMSC treated with the pre-inducer

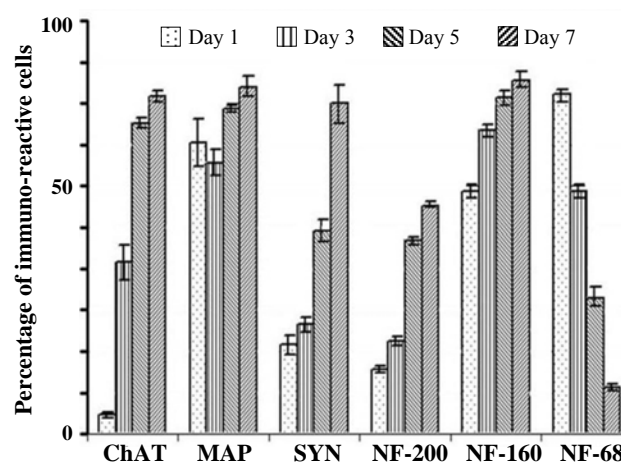


Fig. 1. A histogram represents the time course study with sampling on day 1, 3, 5 and 7 after pre-induction with BME and induction with nerve growth factor. It shows that there is a sustained increase in the expression of Nf-200 and NF-160. Similar trend is noticed in the expression in ChAT and synapsin-I, while there is a sustained decline in the expression of NF-68. The trend of MAP-2 expression is variable.

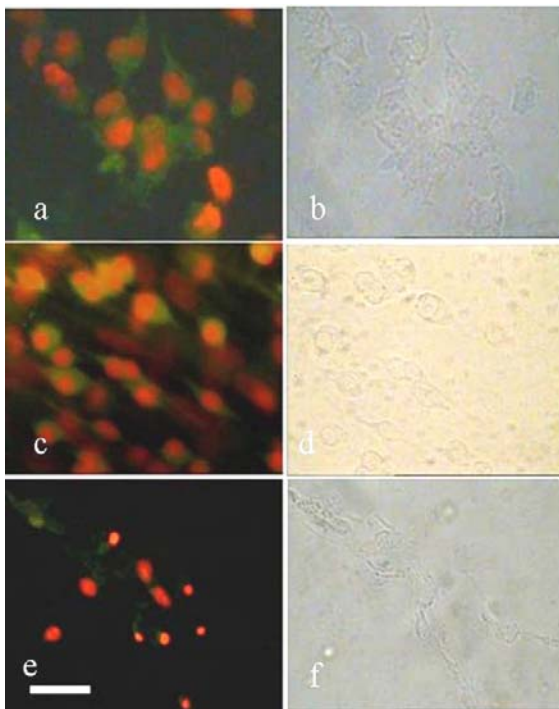


Fig. 2. Photomicrographs of immuno-histochemistry for neurofilaments used for characterizing the transdifferentiated bone marrow stromal cells. The right panels represent the phase contrast of the immuno-stained cells. All pictures were taken at day 7 of the experiment. (a) represents the immuno-stained cells with anti-neurofilament 200 kDa antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 45 μ m), (b) represents the phase contrast image. (c) represents the immuno-stained cells with anti-neurofilament 160 kDa antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 45 μ m), (d) represents the phase contrast image. (e) represents the immuno-stained cells with anti-NF-68 antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 45 μ m), (f) represents the phase contrast image.

(BME) and the inducer (NGF). Figures 4 and 5 demonstrate the electrophoresis of Oct-4 and NeuroD in the untreated, the pre-induced and the induced BMSC. The expression in the cells induced with NGF was done at 3, 5 and 7 days. The results of the semi-quantitative NeuroD expression were assessed using densitometry of electrophoresis NeuroD expression band. The mean ratio of NeuroD band density to that of β 2-microglobulin (NDRB) was done at days 3, 5, and 7. At day 1, using BME only as pre-inducer (negative control), NDRB was 1.2 ± 0.4 . At day 3 (pre-induction 1 day and induction for 2 days), NDRB was 1.1 ± 0.2 ; at day 5 (pre-induction 1 day and induction for 4 days), NDRB was 0.9 ± 0.1 ; and at day 7 (pre-induction 1 day and induction for 6 days), NDRB was 0.7 ± 0.3 . The results showed that there was a general declining trend in NeuroD expression; however,

there was no significant difference in NeuroD expression in this time course.

DISCUSSION

Results of this study offer a protocol for step by step neural differentiation. The pre-induced cells showed a high percentage of immuno-reactivity to NF-68, a marker for neuroblast [12], expressed NeuroD, a gene encoding transcription factors in the basic helix-loop-helix family and a key marker in early differentiation of neurons [13] and also NF-160, a marker for neuroblast and neuron [12]. The percentage of NF-200 immuno-reactive, a marker for differentiated neurons, was low, which is same conclusion and indicates that the majority of the cells transdifferentiated into neuroblast [12, 14].

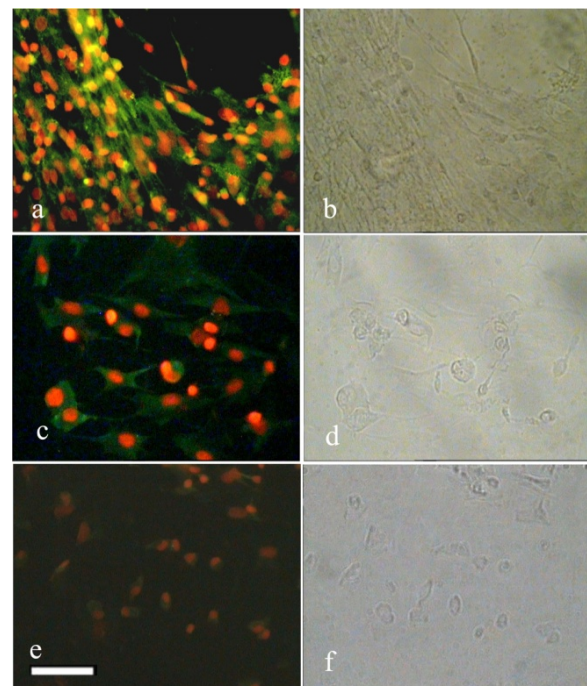


Fig. 3. Photomicrographs of immuno-histochemistry for choline acetyltransferase (ChAT), microtubule-associated protein 2 (MAP-2) and synapsin-I used for characterizing the transdifferentiated bone marrow stromal cells. All pictures were taken at day 7 of the experiment. (a) represents the immuno-stained cells with ChAT antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 90 μ m), (b) represents the phase contrast image. (c) represents the immuno-stained cells with MAP-2 antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 90 μ m), (d) represents the phase contrast image. (e) represents the immuno-stained cells with synapsin-I antibody which reacted with FITC conjugated secondary antibody (counter stained with ethidium bromide: scale bar = 90 μ m), (f) represents the phase contrast image.

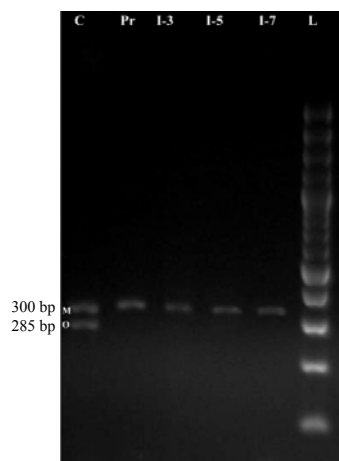


Fig. 4. An electropherogram represents the expression of Oct-4 gene in untreated BMSC "C". The BMSC were pre-induced with β -mercaptoethanol for 24 h "Pr". The pre-induced BMSC were induced with nerve growth factor for 3, 5 and 7 days "I-3", "I-5" and "I-7", respectively. L represents DNA ladder. "O" and "M" represent Oct-4 and β 2 microglobulin bands, respectively.

The use of BME as a pre-inducer for trans-differentiation of BMSC into neurons was reported by Woodbury *et al.* [6] showed anti oxidant and thiol reduction effects of BME [8], induces the BMSC and express the neuroblastic markers, like nestin and NF-160 [6] in these cells. Though the result of a study revealed the transdifferentiation of BMSC by BME is a artifact [9] we re-examined the BME as a pre-inducer and the results of this study, confirms the early differentiation of BMSC by BME into neuronal phenotype, but no artifact [9].

MAP-2 is a structural protein which is found in dendrites where its function is to modulate the stability of microtubules by direct linkage with cytoskeleton [15], where MAP-2 was reported to be localized in the presynaptic and postsynaptic side of the neuronal synapse [16], while synapsin-I is located at the presynaptic side [17]. The profile of MAP-2 and synapsin-I expression at the pre-induction stage was consistent with immaturity of the differentiating cells.

BME and other antioxidant such as N-acetylcystein inhibited neuronal apoptosis by increasing glutathione levels, the later can substantially increase the activity of ChAT and alter patterns of neurite outgrowth in cholinergic precursor's cells of basal forebrain [8].

Interestingly, in this study, the expression of ChAT occurred in the BMSC (4%), similar to the mentioned approach but very low than the result of study by Li *et al.* [8] the explanation of this, may because of the different kinds of cells were used in these two experiments.

Expression of Oct-4 gene, a stem cell marker, was reported in undifferentiated BMSC [18]. In this study, Oct-4 was detected in undifferentiated BMSC, and was not detected in BMSC after pre-induction and induction. Other investigators confirmed these finding by reporting that Oct-4 expression is suppressed in differentiating embryonic stem cells [19], neural stem cells [20] and umbilical cord blood stem cells [21]. Detectable level of NeuroD in undifferentiated BMSC was not found by Seung *et al.* [22]. This is consistent with the finding of this investigation. Expression of NeuroD gene was detected during the pre-induction and induction stages of this experiment; also Sanchez-Ramos *et al.* [23] reported the expression of this gene in the presence of RA and brain derived neurotrophic factor in BMSC. This pattern of expression was also seen when adult neural stem cells were exposed to retinoic acid [24]. The implication of the above results is that BMSC express Oct-4 which is suppressed by induction of differentiation, while NeuroD is not expressed in BMSC, then the expression is detected in BMSC differentiated into neuronal phenotype.

In order to identify factors able to lead differentiation of BMSC towards cells of different neural lineage, treatments by many inducers were examined. Expression of early neuronal markers, NF-160, nestin [6, 8, 23], and vimentin [25] were reported by two single inducers, BME, or isobutyl-methylxanthine (IBMX), respectively. IBMX increased level of the intracellular cyclic AMP, induce the BMSC and express the neuroepithelial marker, vimentin, in BMSC [25].

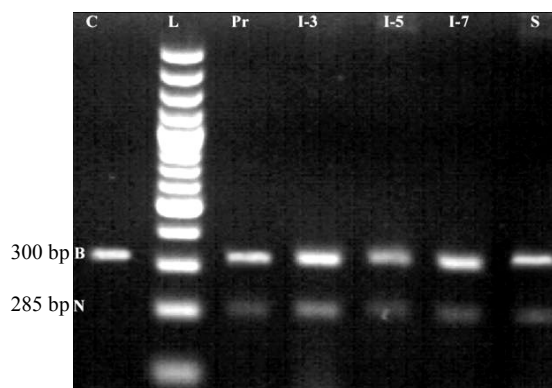


Fig. 5. An electropherogram represents the expression of NeuroD gene in untreated BMSC "C". The BMSC were pre-induced with β -mercaptoethanol for 24 h "Pr". The pre-induced BMSC were induced with nerve growth factor for 3, 5 and 7 days "I-3", "I-5" and "I-7", respectively, "S" represents the expression profile of NeuroD in the embryonic spinal cord. L represents DNA ladder. "N" and "B" represent NeuroD and β 2 microglobulin bands, respectively.

Effect of BME and RA on BMSC revealed the differentiation of these cells into Schwann cells [26] and effect of glial growth factor on BMSC leads the cells to astrocytes [27]; while induction of these cells by combination of RA, basic-fibroblast growth factor and NGF leads the cells to neuroglial differentiation [28]. The results of these studies were shown each inducer have the special effect on BMSC and may produce a specific type of neurons. NGF was reported to enhance the outgrowth of nerve fibers in cultured chicken sympathetic (a type of cholinergic neurons) and sensory [29]; exhibit an anti-apoptotic, trophic and differentiating functions on sympathetic neurons [10]; increase the activity of the expression of genes regulating the synthesis of acetylcholine [30] and facilitate the maturation and repair of basal forebrain and striatal cholinergic neurons *in vivo* [11]. Here we reported the use of NGF after pre-induction by BME leads the BMSC into cholinergic phenotype.

The pre-induction stage with BME for 24 h was followed by the induction stage using NGF for 6 days, which resulted in 80% increase in cholinergic neuron like cells, a high *in vitro* yields by this method. NGF was reported to have a direct effect on *in vivo* cholinergic precursor's differentiation [8]. Other investigators were shown an increase in *in vivo* Ach release [31]; Ach content and ChAT activity neuronal precursors cells by NGF [32]. Also, results of this study shows presence of ChAT in the BMSC by this factor.

Though cholinergic neurons are important neurons in the CNS and may damage in many CNS disorders like Alzheimer's disease, transplantation of NGF-gene-modified BMSC into model of Alzheimer was done by Li *et al.* [33] and the effect of NGF on injured cholinergic neurons, lead to improvement of functional recovery in the animals. Direct *in vitro* differentiation of cholinergic neurons in our study have a potential for cell therapy in cholinergic disease and offer hopes for more *in vivo* investigations.

According to our knowledge, there is the first report for *in vitro* transdifferentiation of BMSC into cholinergic neuron-like cells.

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