

A New Multistep Induction Protocol for the Transdifferentiation of Bone marrow Stromal Stem Cells into GABAergic Neuron-Like Cells

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ABSTRACT

Background: Bone marrow stromal stem cells (BMSC) are appropriate source of multipotent stem cells that are ideally suited for use in various cell-based therapies. It can be differentiated into neuronal-like cells under appropriate conditions. This study examined the effectiveness of co-stimulation of creatine and retinoic acid in increasing the differentiation of BMSC into GABAergic neuron-like cells (GNLC). **Methods:** BMSC isolated from the femurs and tibias of adult rats were cultured in DMEM/F12 medium supplemented with 10% FBS, pre-induced using β -mercaptoethanol (β ME) and induced using retinoic acid (RA) and creatine. Immunostaining of neurofilament 200 kDa, neurofilament 160 kDa, nestin, fibronectin, Gamma-amino butyric acid (GABA) and glutamic acid decarboxylase (GAD) 65/67 were used to evaluate the transdifferentiation of BMSC into GNLC and to evaluate the effectiveness of pre-induction and induction assays. The expression of genes that encode fibronectin, octamer-binding transcription factor 4 (Oct-4), GAD 65/67 and the vesicular GABA transporter was examined in BMSC and GNLC by using RT-PCR assays during transdifferentiation of BMSC into GNLC. **Results:** Co-stimulation with RA and creatine during the induction stage doubled the rates of GABAergic differentiation compared with induction using creatine alone, resulting in a 71.6% yield for GNLC. RT-PCR showed no expression of Oct-4 and fibronectin after the induction stage. **Conclusion:** The results of this study showed that the application of β ME, RA, and creatine induced the transdifferentiation of BMSC into GNLC. *Iran. Biomed. J. 17 (1): 8-14, 2013*

Keywords: Cell therapy, GABAergic neurons, Creatine, Bone marrow stem cell

INTRODUCTION

Gamma-amino butyric acid (GABA), a major inhibitory neurotransmitter in the mammalian central nervous system, plays an important role in the positioning of neuronal precursors [1], maturation of neural circuitry during postnatal development [2] and the control of behaviorally relevant patterns and oscillations [3]. GABA also play an essential role during nervous system development including the proliferation and differentiation of nerve cells into neural circuits and networks [4]. GABA are secreted by neurons and glial cells as a neurotransmitter and act as an inducer and regulator of the nervous system [4]. GABA-secreting neurons are referred to as GABAergic neurons [4]. Dysfunction of GABAergic neurons have been strongly associated

with many neurological disorders, including Huntington's disease [5] schizophrenia, bipolar depression [6], autism, mood disorders, epilepsy and Alzheimer's disease [4-6]. Given that GABAergic neurons have been used to treat ischemic injuries [7], transplantation of GABAergic neurons may provide a feasible therapeutic approach for various diseases.

Bone marrow stromal stem cells (BMSC) are currently considered as potential agents for stem cell-based therapy. Enthusiasm about their potential is based on the capacity of BMSC to differentiate into neural cells, the ease with which BMSC can be isolated and expanded and the low frequency of immunological rejection of BMSC [8].

The *in vitro* differentiation of BMSC into chondrogenic, adipogenic, osteogenic, myogenic and other lineages have been reported using different

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induction protocols [8-10]. Gharibani *et al.* [10] showed that BMSC were transdifferentiated into GABAergic neuron-like cells (GNLC) after pre-induction in the presence of β -mercaptoethanol (β ME) and retinoic acid (RA), followed by induction in the presence of potassium chloride. Creatine, which is synthesized from the amino acid arginine, glycine and methionine, serves as a substrate for creatine kinase (CK) and regulates cellular ATP levels. Previously, creatine was shown to induce the differentiation of the striatal GABAergic neurons [11]. The present study showed an induction protocol for transdifferentiation of BMSC into GNLC, which uses RA and creatine as inducers. We improved the yield of GNLC generated from BMSC.

MATERIALS AND METHODS

Preparation of rat bone marrow cells. All animal experiments in this study were based on approved protocols that follow the guidelines of the Ethical Committee at Tarbiat Modares University (Tehran, Iran). Adult female Sprague-Dawley rats (200-250 g) were obtained from the Razi Vaccine and Serum Research Institute (Tehran, Iran). BMSC were collected by marrow aspiration of the shafts of femurs and tibias into DMEM/F12 (GIBCO, BRL, Eggenstein, Germany) supplemented with 10% FBS using a 21-G needle. Cells were separated through gentle pipetting and filtered through a 60- μ m nylon mesh. The cells were then washed in PBS and centrifuged at $400 \times g$ for 5 min. The cells were seeded onto 25-cm² flasks containing DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin/streptomycin (GIBCO, BRL, Eggenstein, Germany) and 2.5 μ g/ml fungizone (Invitrogen, Paisley, Scotland). They were then incubated at 37°C in 95% relative humidity and 5% CO₂ for 2 days. Any non-adherent cells in the flasks were discarded. Upon reaching confluence, the cells were harvested by treating with 0.25% trypsin and 1 mM EDTA at 37°C for 5 min, split at a ratio of 1:3 and propagated until the third passage. The viability of the cell cultures was 95%. Cells from the third passage were then used for RT-PCR and immunostaining analyses using the following marker fibronectin, octamer-binding transcription factor 4 (Oct-4), glutamic acid decarboxylase 1 and 2 (GAD1 and GAD2) and vesicular GABA transporter (VGAT). The experiments involved two stages of culture, namely pre-induction and induction. Pre-induction was performed by seeding the cells onto the 24-well plates at a density of 10^4 cells/cm², replacing the culture medium with serum-free DMEM-F12 (Gibco, BRL) containing 1 mM β ME and 2% B₂₇ and then incubating

the cells for 24 h [6]. To determine the optimal dose and time for induction, pre-induced cells were treated with different concentrations of creatine (0, 2.5, 5 and 10 mM), which were added to the DMEM-F12 culture medium containing 5% FBS and 2% B₂₇ on days 1, 2, 4 and 6 of the induction stage. The percentage of immunoreactive cells was used as a parameter to determine the extent of the induction. After determining the optimal exposure time and dose of creatine for induction of the cells, the cells were then co-stimulated with creatine and 10 μ M RA.

Immunocytochemistry. Pre-induced and induced third-passage BMSC were washed three times in 0.1 M PBS, fixed in 4% paraformaldehyde at room temperature for 30 min, pre-incubated in 0.1% Triton X-100 in PBS plus 10% FBS for 1 h, washed in PBS and incubated with the primary antibody at 4°C overnight, then rinsed 3 times in PBS. The cells were incubated with the secondary antibody at room temperature for 2 h. All antibodies were purchased from Millipore, Germany except the secondary antibody. The secondary antibodies used were anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC, 1:300; Chemicon, Hofheim, Germany) and anti-mouse IgG antibody conjugated with FITC (1:100; Millipore, Germany). The cells were then rinsed twice in PBS for 15 min and counterstained with ethidium bromide for 1 min to visualize the nuclei. The cells were washed again in PBS and examined using a fluorescence microscope (Olympus model, 1 \times 71, Japan). The following primary antibodies and dilutions were used: mouse anti-CD106 monoclonal antibody (1:300; Millipore), mouse anti-CD31 monoclonal antibody (1:200; Millipore), rabbit anti-CD45 polyclonal antibody (1:300; Millipore), mouse anti-CD90 monoclonal antibody (1:300; Millipore), anti-CD44 monoclonal antibody (1:200; Millipore), mouse anti-CD34 monoclonal antibody (1:300; Millipore), mouse anti-fibronectin monoclonal antibody (1:400 Millipore), mouse anti-nestin monoclonal antibody (1:300; Millipore), mouse anti-neurofilament 200 kDa (anti-NF-H) monoclonal antibody (1:400; Millipore), mouse anti-neurofilament 160 kDa (anti-NF-M) monoclonal antibody (1:300; Millipore) and mouse anti-GABA monoclonal antibody (1:500). The number of immunoreactive cells was determined to estimate the percentage of immunoreactive cells. A random table was used to select the fields and in total, 200 cells were counted [12]. Immunostaining assays were performed in triplicate for each marker.

RT-PCR. Total cellular RNA was isolated from the BMSC and the GNLC by using the High Pure RNA

Isolation Kit (Roche Biochemicals, Mannheim, Germany). This was followed by treatment with DNase I amplification grade kit (Invitrogen, Paisley, Scotland). Single-stranded cDNA was subsequently generated using a first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) and cDNA amplification was performed with the following primers: fibronectin (GenBank ID: NM01914), 5'-CTGTCCTGTGGCTGTGTCC-3' (sense) and 5'-CAGTAGTAAAGTGTGGCATGT-3' (antisense, 221-bp product); Oct4 (GenBank ID: NM001009178), 5'-GGCTGTGTCCTTCTCT-3' (sense) and 5'-TCTCTTTGTCTACCTCCCTTC-3' (antisense, 217 bp); GAD1 (GenBank ID: NM017007), 5'-AACAGTAGAGACCCCAAGAC-3' (sense) and 5'-GCAGATCTTGAGCAAACAG-3' (antisense, 336 bp); GAD2 (GenBank ID: NM012563), 5'-AGAGA GGAGGGACTGATGC-3' (sense) and 5'-TTGTGTG CTGAGGCTTCC-3' (antisense, 279 bp); VGAT (GenBank ID: NM031782), 5'-TTCCTATCTCCAT CGGCATC-3' (sense) and 5'-TCCGTGATGACTTCC TTGG-3' (antisense, 198 bp). All procedures were performed according to the manufacturer's instructions. Following 34 cycles of amplification in a thermocycler, *PCR products were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. The fluorescent bands were photographed using a gel documentation system (Uvtec D55, France).*

Statistical analysis. The results were analyzed using the SPSS software release 13 (SPSS Inc., Chicago, IL, USA) and analysis of variance (ANOVA) with Tukey's multiple test for comparison among groups.

RESULTS

Preparation of bone marrow stromal stem cells. BMSC (third passage) were immunostained with antibodies against CD90 (Fig. 1A) and CD44 (Fig. 1B), both of which are markers of mesenchymal stem cells, as well as with antibodies against CD106 (Fig. 1C), which is a marker of mesenchymal stem cells derived from BMSC. The cells were negatively immunostained with antibodies against CD34 (Fig. 1D), CD45 (a hematopoietic cell marker, Fig. 1E) and CD31 (an endothelial cell marker, Fig. 1F).

Dose response and time course. At pre-induction stage, β ME (24 h) and B₂₇ effects were done using immunoreactive cells for different antibodies. The expression of fibronectin was decreased during the pre-induction stage, whereas the expression of nestin and

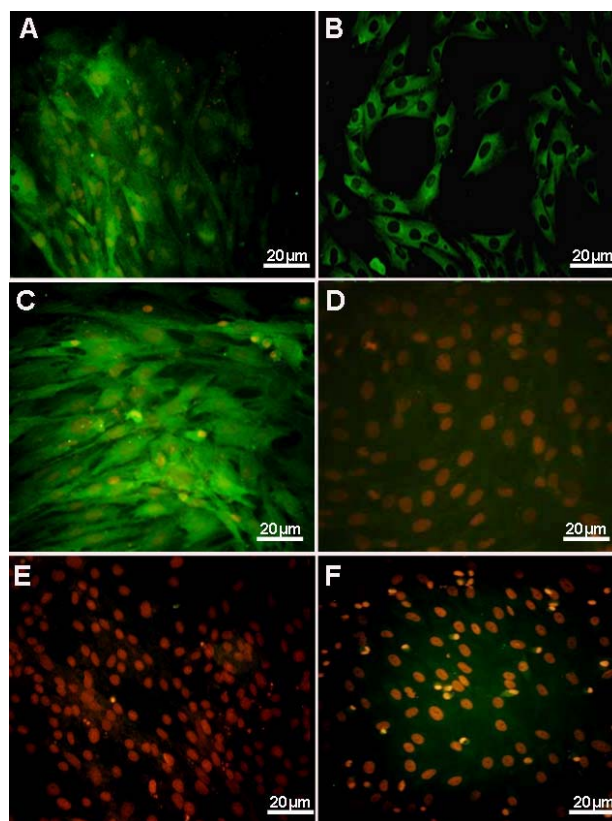


Fig. 1. Immunostaining of BMSC for different markers of cell differentiation. (A) CD90, (B) CD44, (C) CD106, (D) CD34, (E) CD45 and (F) CD31. BMSC were immunolabeled with primary antibody, incubated with FITC-conjugated secondary antibody and counter-stained using ethidium bromide.

NF-M was increased. Although the level of NF-H expression increased during the pre-induction stage, the increase in the level of GABA was not considerable. We thus performed an induction stage to increase GABA expression in pre-induced cells.

A dose-response analysis that involved immunostaining with anti-GABA antibody was conducted to determine the optimal dose of creatine for trans-differentiation of BMSC to GNLC. Figure 2 shows the means and the standard errors of the means of the data collected using the immunoreactive cells. These data revealed positive correlations between rates of transdifferentiation and both the duration of exposure to creatine and the concentration of creatine. A time-dependent increase in GABA expression was observed in all creatine doses tested.

Treatment of pre-induced cells with creatine at concentrations of 1, 2.5, 5 and 10 mM on days 1, 2, 4, and 6, respectively showed that GABA expression in GNLC was significantly higher after treatment with 5 mM creatine (without RA) for 4 days ($30.8\% \pm 1.4$) when compared with the other doses of creatine and

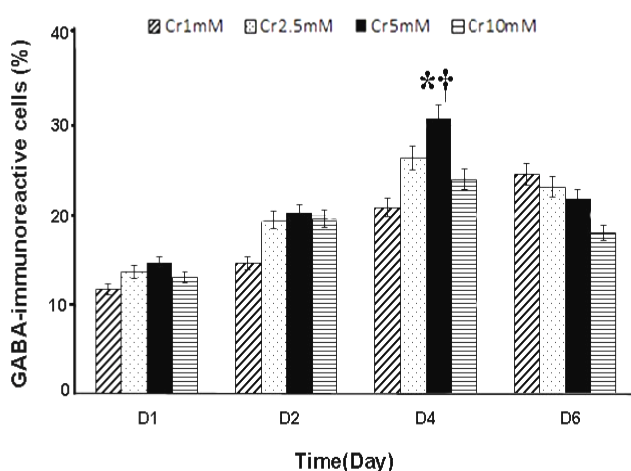


Fig. 2. Histogram of the mean and the standard errors of the mean percentage of GABA-immunoreactive cells at the induction stage in the presence of creatine alone (1, 2.5, 5 and 10 mM) at days 1 (D1), 2 (D2), 4 (D4) and 6 (D6) of induction, respectively. Values are represented as mean \pm SEM; * P <0.05, compared to other doses at the same time point; † P <0.05, compared to other days at the same concentration.

other days (P <0.05). The maximum effective dose was determined to be 5 mM (Fig. 2). The optimal creatine treatment (exposure to 5 mM creatine for 4 days) was used for costimulation with RA (10 μ M) and transdifferentiation of BMSC into GNLC. Figure 3 shows the means and the standard errors of the means of the percentages of the immunoreactive cells. The highest levels of differentiation of GNLC ($71.6\% \pm 1.36$) were observed after 4 days.

Immunocytochemistry. The anti-fibronectin antibody, which was employed as a stromal marker, was present in approximately 90% of the BMSC examined, whereas very few of the cells demonstrated expression of the transdifferentiation markers nestin, NF-M, NF-H and GABA (Fig. 3). A small (<4%), but significant, reduction in levels of fibronectin during the pre-induction stage was associated with a 21.6% increase in levels of nestin. Figure 3 shows low levels of expression of GABA (7%) and NF-H (8.4%) during the pre-induction stage. Also, Figure 4 shows images obtained using fluorescence microscopy.

RT-PCR. The levels of transcripts that encode Oct-4, fibronectin, GAD1, GAD2 and VGAT in BMSC were compared before and after transdifferentiation. The results of the RT-PCR analysis revealed that Oct-4 and fibronectin were expressed in the BMSC, whereas the GABAergic markers GAD1, GAD2 and VGAT were not expressed. On the other hand, induction with B₂₇, RA and creatine resulted in repression of Oct-4 and fibronectin expression, whereas the expression of GABAergic neuron markers was detected (Fig. 5).

DISCUSSION

Cell replacement therapy using transdifferentiated BMSC has the potential to restore impaired inhibitory transmissions and dysfunctions [13]. Studies have earlier shown that induced BMSC could transdifferentiate into ectodermal cells [14, 15]. After the induction stage, neither fibronectin nor Oct-4 were expressed, whereas nestin, NF-M and NF-H were found to have increased expression [9, 16, 17] GAD1, GAD2 and VGAT were expressed during the induction stage as reported elsewhere [10]. Nestin was also expressed after pre-induction of the cells with β ME [13, 18] *in vitro* transdifferentiation of BMSC into neuron-like cells was initially reported by Woodbury *et al.* [13] using β ME and dimethyl sulfoxide.

Since 1993, B₂₇ with defined components has been employed as a serum-free medium that eliminates the need for supplementation with serum [19]. In this study, the viability of cell cultures after pre-induction was 70% that was higher than the viabilities reported previously [10]. B₂₇ may prevent the development of side effects attributable to β ME at the pre-induction stage, thus leading to an increased cell resistance to biological oxidants. In our study, both β ME and B₂₇ were employed as pre-inducers; however, other studies have used β ME and RA for inducing the transdifferentiation of BMSC into neuronal cells [9, 20].

The number of both GABAergic phenotypic cells and nestin-positive progenitor cells was increased 4 days after induction. The failure to observe a change in

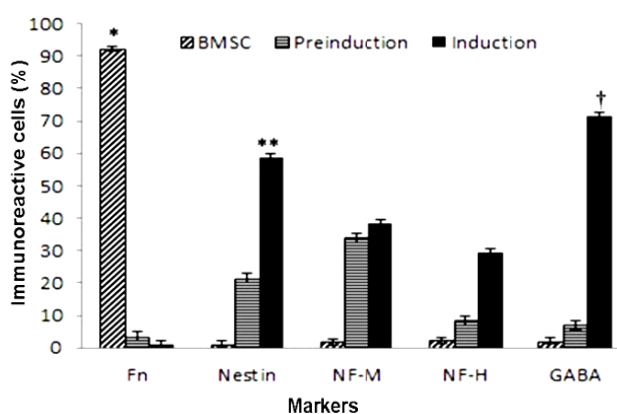


Fig. 3. The mean and the standard errors of the mean percentages of cells immunoreactive to fibronectin (BMSC marker), nestin (neuronal progenitor cells marker), neurofilament 160 and 200 (neuronal marker) and GABA (GABAergic neuron-like cells marker). Levels of these proteins were compared among BMSC, pre-induced cells and induced cells after 4 days of exposure to retinoic acid and creatine. Values are represented as mean \pm SEM; * P <0.05, BMSC compared with other groups; ** P <0.05, nestin expression compared to pre-induced cells; † P <0.05, GABA expression compared to other experimental groups.

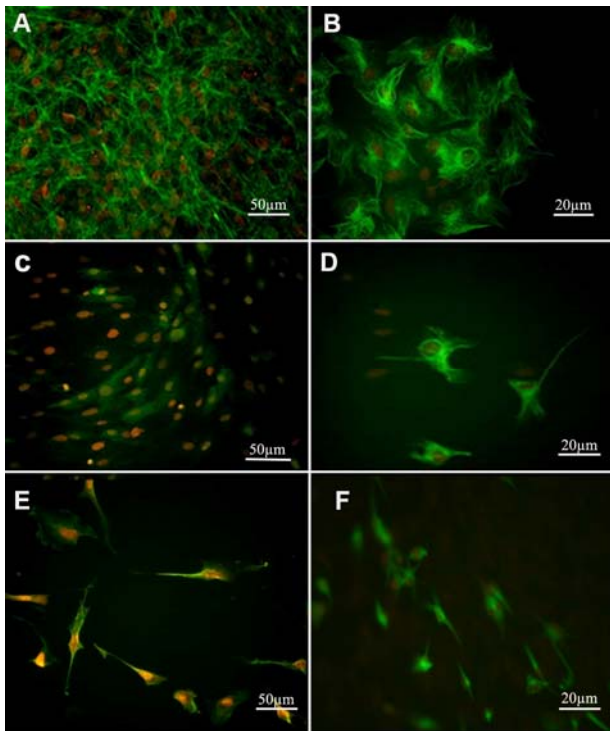


Fig. 4. Photomicrographs of bone marrow stromal stem cells (BMSC) and GNLG derived from BMSC after immunostaining. Cells were labeled with primary antibody against the following markers: fibronectin in BMSC (A) and nestin(B), neurofilament 160 (C), neurofilament 200 (D), GAD65/67 (E) and GABA (F) in GNLG, followed by incubation with secondary antibody conjugated to fluorescein isothiocyanate. Nuclei were counterstained with ethidium bromide (1 $\mu\text{g}/\text{ml}$). [Scale bars: A, C and E = 50 μm and B, D and F = 20 μm].

the number of NF-H immunoreactive neurons suggests that the majority of the dividing cells possessed an immature neuronal phenotype [9, 21]. The high percentage of cells that were immunoreactive for nestin

indicated that most of the cells in the cultures were neural progenitors. The results of this study demonstrate that BMSC can efficiently differentiate into GNLG in the presence of a combination of RA and creatine, thus providing an alternative technique for generating sufficient numbers of GNLG from BMSC. This RA-creatine treatment combination resulted significantly in a higher percentage of GNLG as compared to treatment with creatine alone. Interestingly, treatment with creatine alone caused only a slight (30%) increase in the total number of GABAergic neurons. Among reagents used to differentiate embryonic stem cells into the neural progenitors, RA remains the most effective result [22, 23]. This study demonstrates that exposure of rat BMSC to RA promotes the differentiation of neuronal precursors into GNLG (71.6%). Previous studies have shown that endogenous and exogenous RA are needed for GABAergic differentiation from embryoid bodies-derived from embryonic stem cells [23, 24].

In the central nervous system, creatine serves as a substrate for various isoforms of creatine kinase, including cytosolic brain-specific creatine kinase and ubiquitous mitochondrial creatine kinase and possibly induces more inhibitory synapses [25, 26]. The phosphocreatine *system* plays a key role in regulating ATP metabolism by promoting energy homeostasis [27]. Moreover, creatine imparted neuroprotective effects against various toxic insults in rat fetal ventral mesencephalic and striatal cultures [11, 28] and prevented complications related to traumatic brain injury in children and adolescents [29].

Andres *et al.* [11] have shown that creatine directly influences the differentiation of neural stem cells into GABAergic neurons. Chatzi *et al.* [30] have shown that 93-96% of embryonic bodies cultured in neural

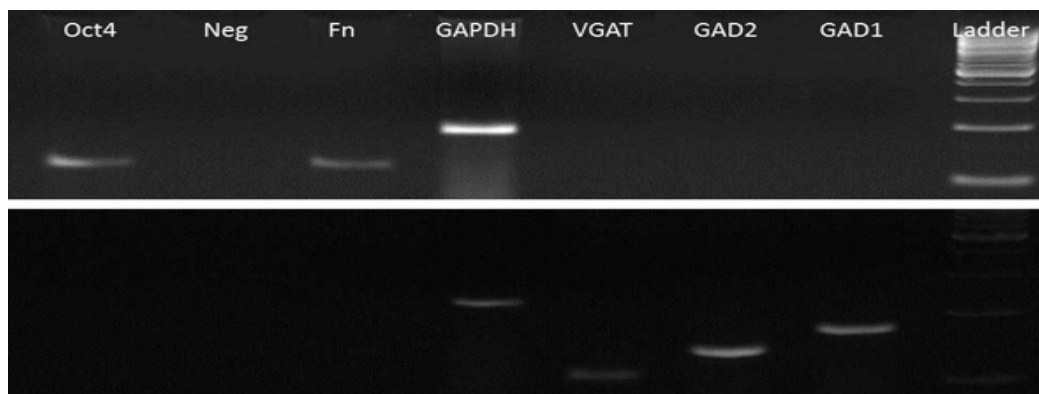


Fig. 5. RT-PCR analysis of bone marrow stromal stem cells (BMSC, upper panel) and GABAergic-like neurons transdifferentiated from BMSC (lower panel) using primers that specifically amplify transcripts that encode GAD1 (GAD67), GAD2 (GAD65), VGAT, fibronectin (Fn) and Oct-4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as an internal control housekeeping gene and negative control (Neg; RT-PCR with all of the RT-PCR components except the DNA template).

media were differentiated into GABAergic neurons. Gharibani *et al.* [10] have reported that sequential treatment of BMSC with β ME and RA followed by potassium chloride resulted in a 60% differentiation rate into GABAergic neurons. More recently, Gharibani *et al.* [31] have demonstrated the same proportion of GNLC (60%) using creatine as an inducer.

In this study, there was a significant increase in the yield of GNLC despite the use of creatine as an inducer. This issue demonstrates the importance of RA as a co-stimulator with creatine. The induction of neurotrophins may account for the action of creatine in the transdifferentiation of BMSC into cells with a GABAergic phenotype [31].

The results of this study showed that 71.6% of the BMSC were transformed into GLNC. The values obtained in this study are relatively high despite the heterogeneous nature of BMSC population when compared with broadly comparable studies that used neural stem cells and embryonic stem cells.

To understand the mechanisms that mediate creatine-induced differentiation, Andres *et al.* [28] have investigated the possible involvement of mitogen-activated protein kinase (MAPK) signal pathways. The canonical MAPK pathway has emerged as a major contributor to plasticity in vertebrates. MAPK are a family of serine/threonine kinases that include the extracellular-signal-regulated protein kinases [32]. These kinases are strongly activated by mitogens and in the central nervous system by neurotrophins and neurotransmitters. Creatine has been found to upregulate the expression of neurotrophins [31]. Neurotrophins are major effectors of signal transduction from the cell surface to the nucleus and are implicated in cell growth and differentiation [28, 32]. On the other hand, MAPK signaling is required for RA-triggered G0 cell cycle arrest and cell differentiation [33, 34].

Our findings may have significant implications for cell replacement therapies for the treatment of neurological disease. The induction of the GABAergic phenotype in BMSC by creatine and RA may increase yields of GLNC relative to the best current options. In addition, the differentiation-promoting properties of creatine demonstrated in the present study may also be relevant to both understanding and manipulating cell-fate decisions for a range of stem cells.

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