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Tea Polyphenol-Induced Neuron-Like Differentiation of Mouse Mesenchymal Stem Cells

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Abstract

Bone marrow mesenchymal stem cells (BMSCs) can be induced to differentiate into neuron-like cells under appropriate conditions often involving toxic reagents that are not applicable for clinical transplantation. The present study investigated whether tea polyphenol (TP), a native nontoxic antioxidant, could induce mouse neuron-like cell differentiation of BMSCs *in vitro*. BMSCs, dissected from mouse femur bone marrow, were amplified in culture and treated with TP or β-mercaptoethanol (BME, control). Morphological changes were observed under light microscopy. After 12 h treatment with 50 μg/ml TP or 5 mM BME, most cells differentiated into neuron-like cells exhibiting neuronal morphological characteristics, cellular shrinkage and neurite growth. Immunocytochemistry and reverse transcription (RT)-PCR results demonstrated neuronal marker expression in the induced cells with no glial fibrillary acidic protein expression. Taken together, TP induced mouse BMSCs to differentiate into neuron-like cells *in vitro*. These findings provide a potential source for the treatment of various neurological diseases.

Key Words: bone marrow mesenchymal stem cells, tea polyphenol, neuron-like cells, differentiation

Introduction

Bone marrow mesenchymal stem cells (BMSCs), which are multipotent somatic stem cells, can differentiate into several types of mesenchymal cells including osteocytes, chondrocytes, adipocytes and muscle cells (5, 13, 14, 18). Under appropriate *in vivo* and *in vitro* experimental conditions, BMSCs also differentiate into non-mesenchymal cells such as neuron-like cells (3, 6, 8, 9, 12, 17, 20). Many previously reported chemical inducers, such as β-mercaptoethanol (BME), dimethyl sulfoxide (DMSO) and butylated hydroxyanisole (BHA), all of which have antioxidant functions, have been shown to promote neural cell induction and differentiation both *in vivo* and *in vitro*. These compounds have also been used to induce neuronal differentiation of BMSCs (7, 16, 17, 20). These observations have raised interests in the possible use of BMSCs for cell therapy strategies of various neurological disorders. However, because

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these compounds are toxic reagents resulting in a large amount of cell death, they are not suitable for *in vivo* clinical transplantations (15, 21). Therefore, it is of importance to find a nontoxic inducer of neuron-like cell differentiation.

Tea polyphenols (TP) are native, nontoxic complexes of catechin, brass, flavonol, anthocyan and phenolic acid polyphenolic substances that usually include the four major polyphenols: epicatechin, epigallocatechin, epcatechin-3-gallate and epigallocatechin-3-gallate (EGCG) (1). Previous studies have shown that TP accounts for 30% of green tea moisture-free weight, and possesses strong anti-oxidative characteristics (2). TP has also been shown to induce differentiation in epidermal keratinocytes (1, 4). The aim of the present study was to investigate whether TP could induce neuron-like cell differentiation in mouse BMSCs. This method could provide a unique and nontoxic experimental model for studying early steps of neuronal differentiation *in vitro*. In addition, this method, if successful, could serve as a potential source for treating a variety of neurological diseases.

Materials and Methods

Isolation and Culture of Mouse BMSCs

Adult (8-12 weeks old) mice (Balb/C) were provided by the Medical Experimental Animal Center of Shaanxi Province, PRC. The experimental protocol was approved by the Institutional Animal Care Committee of Xi'an Jiaotong University and was performed in accordance with the European Animal Care and Use Guidelines. The mice were sacrificed, and the femurs were subsequently dissected. Delbucco's modified Eagle's medium-low glucose (DMEM-LG; Hyclone, Logan, UT, USA) cell culture medium (5 ml) was injected into the bone central canal to extrude the marrow. Whole marrow cells ($10\text{-}20 \times 10^6$) were plated in a 75 cm^2 plastic flask. The cells were incubated in DMEM medium, supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C, 5% CO_2 and 95% humidity. After 24 h, non-adherent cells were removed by changing the medium. The remaining cells were incubated in basic medium, and the medium was changed every other day until the culture was confluent. The cells were split at a 1:3 dilution, and after 3 passages were utilized for subsequent experiments. Prior to immunocytochemistry analysis, the cells (after 3 passages) were trypsinized into a single-cell suspension and transferred to coverslips placed in 24-well plates with basic medium. The cultures were grown until the cells were confluent.

Characterization of Culture-Expanded BMSCs

BMSC adherent cells were phenotypically characterized by immunocytochemistry. Coverslips containing the adherent cells were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. Subsequently, the coverslips were washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and incubated in blocking buffer (PBS/10% goat serum/0.2% Triton X-100) for 1 h at room temperature. The cells were then incubated overnight at 4°C in the following primary antibodies diluted with blocking buffer: rabbit anti-CD34 (1:200), CD44 (1:200), CD105 (1:200) and CD45 (1:200) (Chemicon, Temecula, CA, USA). Specific labeling was revealed with 3, 3-diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan) colorization subsequent to incubation with a goat anti-rabbit secondary antibody (1:200, Vector, Burlingame, CA, USA) for 1 h and ABC complex (1:100, Vector) for 30 min. The coverslips were then dehydrated in alcohol, cleared in xylene, and coverslipped with DPX. Stainings were observed under a light microscope (BX-60; Olympus, Tokyo, Japan), and microphotographs were obtained with a digital camera (DP-70; Olympus) attached to the microscope.

Induced Differentiation of Mouse BMSCs

Cells were cultured as near-confluent monolayers in 24-well plates or 25 cm^2 plastic flasks. The cells were then preinduced with DMEM-LG plus 10% FBS, supplemented with either 10 μg/ml TP or 1 mM BME; the control cells were incubated with DMEM-LG plus 10% FBS. After 24 h, the medium was replaced with new serum-free induction medium that consisted of DMEM-LG containing either 50 μg/ml TP or 5 mM BME. The control cells were changed to serum-free DMEM-LG medium. After 1, 3, 6 and 12 h, cellular morphology was observed by light microscopy. Coverslips containing cells were fixed in 24-well culture plates for subsequent immunocytochemistry, and cells from the plastic flasks were harvested for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Immunocytochemistry Analysis of Differentiated BMSCs

Upon removal of culture medium, the cells were rinsed twice with 0.01 M PBS (pH 7.4). The cells were fixed with 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, and subsequently washed three times with PBS. The cells were then incubated in blocking buffer for 45 min at room temperature, followed by overnight incubation at 4°C in the following primary antibodies diluted in blocking buffer: rabbit anti-mouse nestin (1:200, Chemicon), mouse anti-neuron-specific nu-

Table 1. RT-PCR primers

Gene	Sense sequence	Anti-sense sequence	Annealing temperature (°C)	Size of product (bp)
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA	56.0	200
Nestin	TATGTCTGAGGCTCCCTATCC	CTGTGGCTGCTTCTTTCTTTA	55.5	335
NSE	TCCAGATAGTGGGCGATGA	CACAGTCCGACGACAAGA	53.5	227
GFAP	CAAGGAGCCCACCAAAC	GCAAACTTAGACCGATACCA	55.0	468

clear protein (NeuN; 1:1000, Chemicon) and mouse anti-glial fibrillary acidic protein (GFAP; 1:500, Chemicon). Cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:200; Chemicon) for 4 h at room temperature, followed by counter-staining with 4', 6-diamidino-2-phenylindole (DAPI, Pierce Bio, Pittsburgh, PA, USA) for 10 min. For negative controls, the primary antibodies were omitted or replaced with normal pre-immune mouse serum, and no specific positive staining was detected. Cells were examined by fluorescence (Olympus) and laser scanning confocal microscopy (LSCM) (LSM, FV1000; Olympus). The digital images were captured with an attached digital camera and modulated for better light/contrast with the Adobe Photoshop software (Version 7.0). The analysis was performed on three independent experiments.

RT-PCR Analysis

RT-PCR was performed to assess expression of selected neuron-like related mRNAs. Total RNA was isolated from undifferentiated cells at various stages of induction (described above) using Tri-Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma), random primers and RevertAid™ First Strand cDNA Synthesis Kit (K1621, Fermentas) according to the manufacturer's instructions. To minimize variation, all RNA samples from a single experimental setup were reverse transcribed simultaneously.

The primer sequences of the neuronal-specific genes, as well as the annealing temperatures, are described in Table 1. cycle conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 30 s at annealing temperature, and 30 s at 72°C. A final elongation step was performed for 5 min at 72°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel. The gels were stained with ethidium bromide (10 μg/ml) and photographed using an UV transilluminator (Uvidoc, UK). Gel images were analyzed using the UVIbandmap program (Uvitec, Cambridge, UK). β-actin served as an endogenous control, and expression levels were found to be similar among all samples.

Results

Isolation and Culture of Mouse BMSCs

Cells were dispersively attached and grew like fibroblastic cells, and developed into visible symmetric colonies approximately 5-7 d after initial plating (Fig. 1A). However, a number of cell colonies did not grow like fibroblast-like cells (Fig. 1B). Non-adherent cells were removed by medium changes at 24-48 h. Colonies were readily expanded for 15 passages *in vitro* by successive cycles of trypsinization, seeding and culture every 3 d without visible morphological changes.

Immuno-Phenotypic Characterization of BMSCs

BMSCs were analyzed for expression of various antigens. The culture-expanded, adherent cells were uniformly positive for CD44 (Fig. 1C) and CD105 (Fig. 1D). In contrast, these cells were negative for other markers of the hematopoietic lineage CD34, such as the leukocyte common antigen CD45. All BMSC-derived isolated colonies maintained a similar phenotype, even at passage 15. In addition, cryopreservation did not alter these immunophenotypic features.

Morphological Changes Induced by Mouse BMSCs Differentiation

As visualized by inversed microscopy, undifferentiated BMSCs exhibited a typical, adherent spindle and were fibrocyte-like. Upon exposure to induction medium containing either 10 μg/ml TP or 1 mM BME for 24 h, a small portion of BMSCs with flat cytoplasm began to retract towards the nucleus. Subsequently, the adherent cells were exposed to induction medium containing either 50 μg/ml TP or 5 mM BME. In the TP group, some cells began to retract towards the nucleus by 6 h, forming a contracted multi-polar body. At 12 h, most cells exhibited

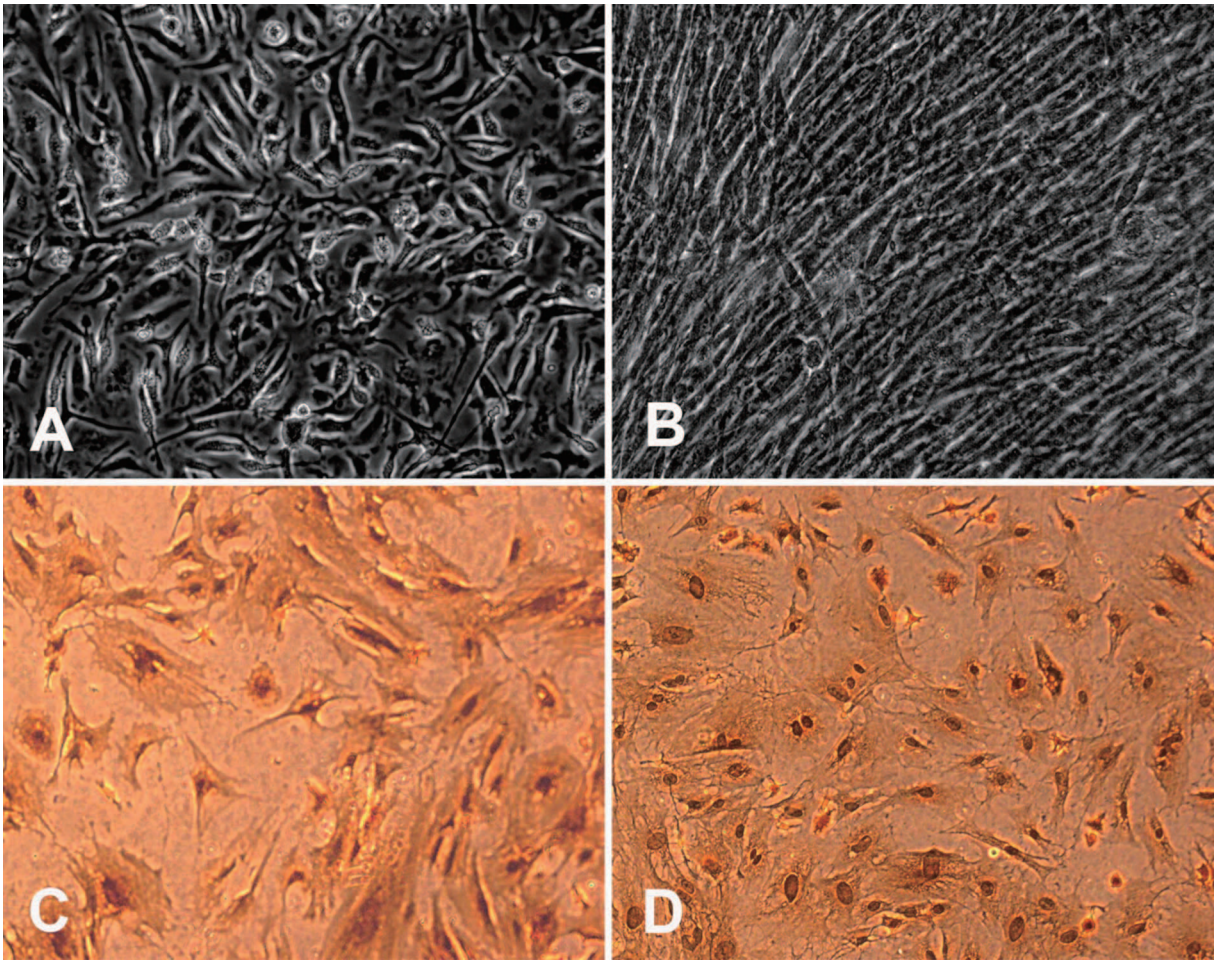

Fig. 1. Primary mouse BMSC cultures at day 5 (A). Mouse BMSC fibroblast-like colonies (B). Culture-expanded, adherent cells were uniformly positive for CD44 (C) and CD105 (D).

spherical cell bodies with bipolar or multi-polar processes, and the cells appeared to be connected to each other. At 24 h, the cells began to take on a neuronal morphology. By contrast, at 3 h, a large portion of cells from the BME group began to retract towards the nucleus, with several axon or dendrite-like processes reaching out from cell body. By 12 h, most cells also exhibited typical neuronal features. Nevertheless, after 12 h, some BMSCs began to detach from the coverslip. The untreated cells did not show any morphological changes.

Protein Expression of Bone Marrow-Derived Neuron-Like Cells

We evaluated the effect of "chemical induction" on protein expression of nestin, NeuN and GFAP in bone marrow-derived neuron-like cells by immunocytochemistry. Upon exposure to 50 μg/ml TP for 12 h, 76% of BMSCs was positive for nestin (Fig. 2A), a marker of neural precursors, and 88% was positive for NeuN (Fig. 2B), a mature neuronal marker. Comparatively, 5 mM BME resulted in expression of nestin and NeuN in 64% and 80% of the cells, respectively. GFAP immunoreactivity was not detected in either the TP (Fig. 2C) or BME group. In contrast, only 8% of the untreated BMSCs was nestin-positive, and no NeuN or GFAP expression was detected. Taken together, "chemical induction" amplified nestin immunoreactivity and induced NeuN expression.

Gene Expression of Bone Marrow-Derived Neuron-Like Cells

To determine whether BMSCs underwent neuronal differentiation, gene expression profiles for neuronal cell differentiation markers were assessed using RT-PCR. β-actin expression served as the normalization control and was similarly assayed in this experimental setup. As illustrated in Fig. 3, undifferentiated BMSCs did not express neuron-specific enolase (NSE) or GFAP. However, nestin

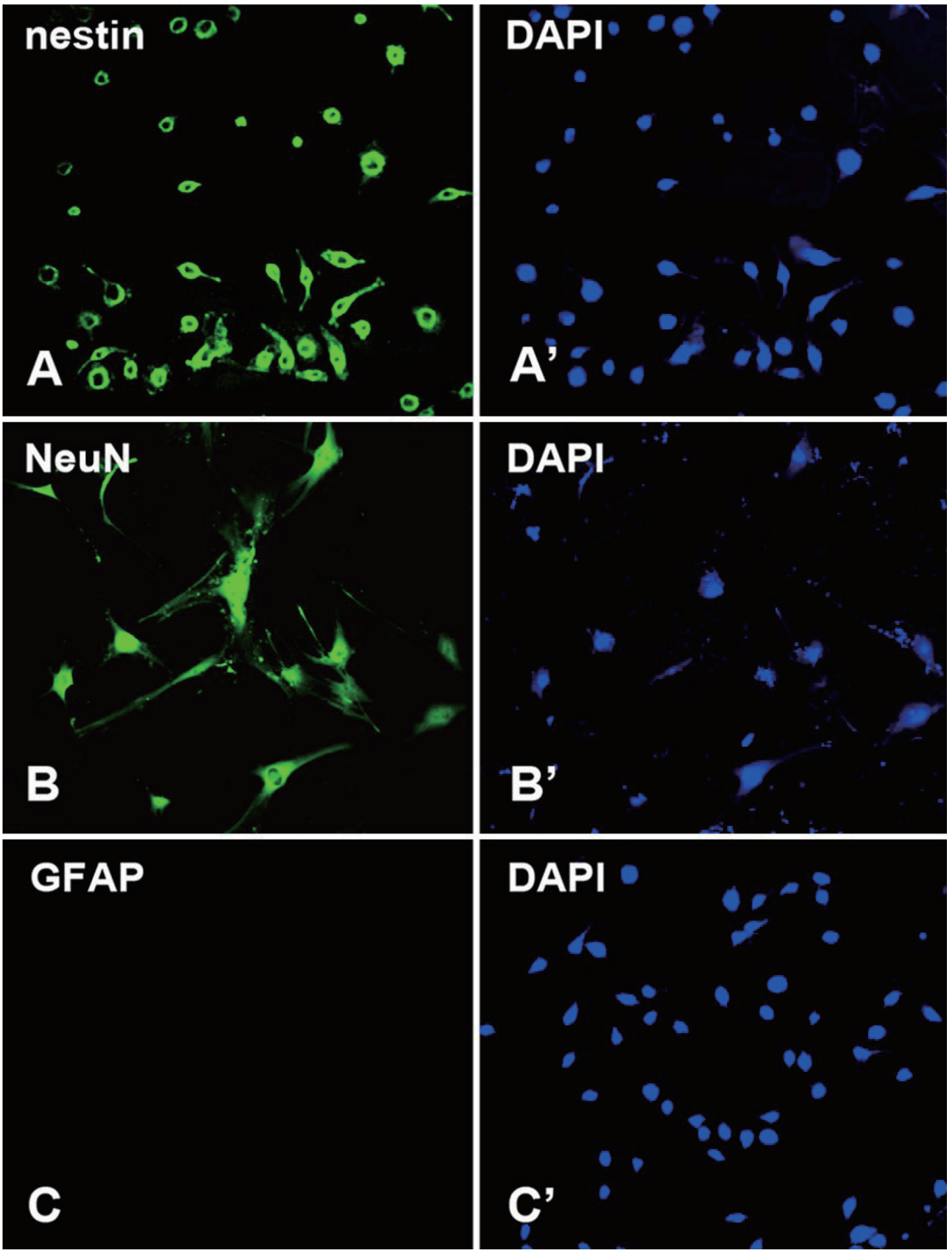

Fig. 2. Immunocytochemical characterization of differentiated cells in the TP group. At 12 h after plating, cells were positive for NeuN (A) and nestin (B) expression, but not GFAP (C). NeuN-, nestin- and GFAP-positive cells were stained with FITC; the nuclei were labeled with DAPI (A'-C').

expression was detected in the undifferentiated BMSCs. When the cells were induced for 12 h with 50 μg/ml TP or 5 mM BME, high levels of NSE and nestin expression were observed. By contrast, GFAP expression was not detectable in the induced cells.

Discussion

The present study demonstrated that TP was capable of inducing neuron-like cellular differentiation in mouse BMSCs. These results are consistent

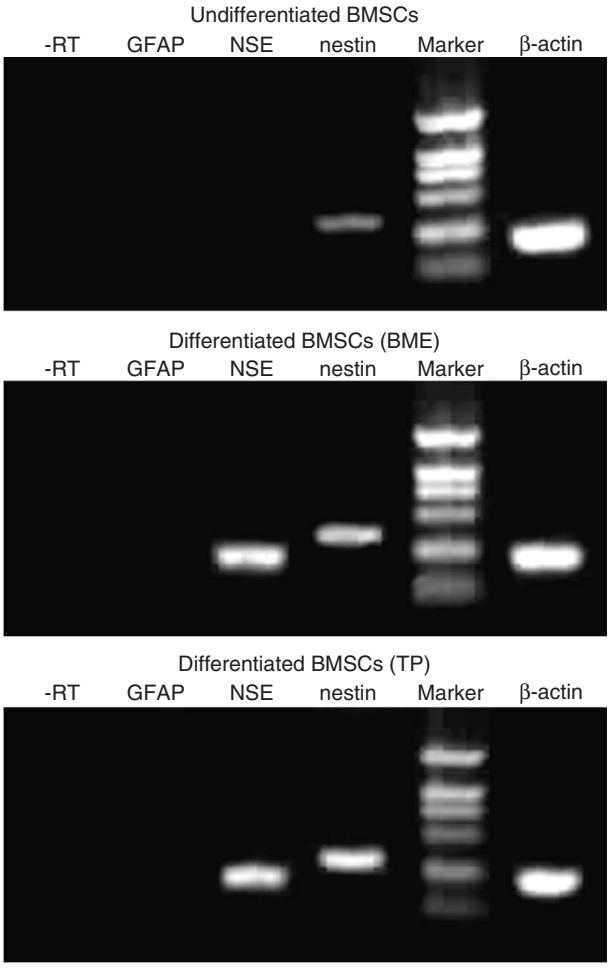

Fig. 3. RT-PCR gene expression analysis of differentiated cells induced for 12 h by 50 μg/mL TP or 5 mM BME, compared with undifferentiated cells.

with previous reports showing that a minor proportion of mouse and human BMSCs differentiates into neuron-like cells and expresses NeuN in the presence of epidermal growth factor or brain-derived neurotrophic factor (17). Woodbury *et al*. (20) reported that *in vitro* neuronal differentiation (70%) could be rapidly induced (within minutes to a few hours) in rodent and human BMSCs by simple chemical inducers such as DMSO, BME or BHA. The majority (80%) of induced mesenchymal stem cells not only exhibits a neuronal morphology, but also has been shown to express several neuronal markers such as nestin, NSE, NeuN, neurofilament-M, tau and trkA. These studies clearly demonstrated that BMSC *in vitro* neuronal differentiation could be induced by exposure to these chemical agents (20). Nevertheless, these chemical inducers are toxic reagents that cause a high percentage of cell death and are, therefore, not suitable for clinical use (16, 21). In the present study, BMSCs were cultured in a medium containing TP. Morphological and phenotypical changes were evaluated and compared with cells grown in BMC-containing medium. A larger number of cells remained viable in the TP group, compared with the BME group, which may be related to BME toxicity. These findings suggest that TP could serve as a neuron-like cellular inducer of BMSCs which may be applicable for the clinical treatment of neurologic diseases. This report presents only preliminary results, further confirmation is needed using other undifferentiated and neuronal markers with neurotransmitters in addition to nestin and NeuN as well as electrophysiological analysis The induced cells will also be transplanted into the central nervous system to further demonstrate that the neuronal phenotype can also be expressed *in vivo*. If successful, the induced differentiated cells may be used as a potential source for the treatment of various relevant clinical neurological diseases.

Previous studies have demonstrated that TP could induce differentiation and proliferation in epidermal keratinocytes with associated induction of p57/KIP2, a cyclin-dependent kinase inhibitor that conferred growth arrest and differentiation (1, 4). TP-induced neuron-like cellular differentiation of BMSCs maybe have the same mechanism as TP-induced epidermal keratinocytes differentiation. In addition, several reports have demonstrated neuroprotective effects of TP. For instance, as free radical scavengers and conjugates of metals (iron and copper) during transient states, TP can ameliorate dysgnosia in elderly patients and delay the degenerative cerebral processes in Alzheimer's disease (11, 19). Lee *et al*. (10) reported that epigallo catechin gallate, one of the main catechins in TP, significantly inhibited glutamic acid-induced excitotoxicity, decreased the generation of malondialdehyde, reduced encephaledema and neuronal injuries, and minimized the amount of infarcted brain tissue. However, the precise mechanisms of TP-induced neuron-like cellular differentiation of BMSCs remain to be explored. In the future, we plan to investigate the molecular mechanisms and downstream signals underlying neuronal differentiation induction, the importance of fate determination of BMSCs, and the therapeutic potential of these observations.

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