

Research Article

# Trichostatin A Induces Both Cell Division Arrest and Neural-lineage Differentiation of the Mesenchymal Stem Cells Possibly by Triggering Wnt/ $\beta$ -Catenin Signaling

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## Abstract

**Introduction:** The bone marrow-derived mesenchymal stem cells (BM-MSC), with active proliferation and pluripotent differentiation abilities, present an ideal source of cell transplant therapy. It remains obscure, but, how to promote their neural-lineage differentiation efficiently and controlling moderate proliferation for clinical application in neurological diseases.

**Methods:** By *in vitro* culture of rat BM-MSC, regulation of a histone deacetylase inhibitor trichostatin A (TSA) in both cell proliferation and neural-lineage cell commitment, and possible involvement of Wnt/ $\beta$ -catenin signaling pathway in TSA-inducing biological effect were examined in this study.

**Results:** By cell count and flowcytometry, TSA exhibited cell division or cycle arrest of BM-MSC at doses above 10ng/ml by increased percentage of G1/S phase and apoptotic cells. Immunocytochemistry and immunoblot revealed promotion effect of TSA on neuronal cell differentiation with appearance of neuron-like ones and increased nestin and Tuj-1 expression in BM-MSC treated with TSA at dose of 100 and 500ng/ml. Enhanced glial cell differentiation was also observed by upregulation of NG2, glial fibrillary acidic protein and CNPase expression in BM-MSC with TSA treatment dose-dependently. Furthermore, significant activation of Wnt/ $\beta$ -catenin signaling was detected in TSA-treated BM-MSC, while Wnt signaling inhibitor IWR1 impeded above TSA-induced effects on BM-MSC.

**Conclusion:** This study has provided new evidence that TSA could induce neural-lineage differentiation and cell division arrest of BM-MSC dose-dependently by triggering Wnt/ $\beta$ -catenin signaling activation, suggesting that TSA may be applied as a candidate drug in manipulation of MSC therapy for the treatment of various neurological disorders.

**Keywords:** Bone marrow-derived mesenchymal stem cells; Trichostatin A; Histone deacetylase; Wnt/ $\beta$ -catenin signaling; Cell transplant therapy

## Abbreviations

BM-MSC: Bone Marrow-derived Mesenchymal Stem Cells;  
GFAP: Glial Fibrillary Acidic Protein; TSA: Trichostatin A

## Introduction

Growing studies have shown that the bone marrow-derived mesenchymal stem cells or mesenchymal stromal cells (BM-MSC) may present an ideal source for tissue engineering or cell transplant therapies in human beings [1,2]. While wide heterogeneity was recognized in BM-MSC depending on their origins, the International Society for Cellular Therapy in 2006 proposed three minimal criteria for cultured human MSC definition [3]: i) when maintained in standard culture conditions, MSC must be plastic-adherent. ii) MSC must express CD73, CD90 and CD105, and lack expression of CD34, CD45, CD14 or CD11b, CD79 or CD19 and HLA-DR surface molecules, and iii) MSC must *in vitro* differentiate to osteoblasts, adipocytes and chondroblasts. Besides, BM-MSC can also be committed into cell lineages such as neural precursors, cardiomyocytes, liver cells, and

possible other cell types. In fact, fast increasing of MSC knowledge and techniques has already led to some clinical trials for therapeutic manipulation of bone and cartilage diseases [4]. However, neural cell fate commitment or induced-differentiation mechanism of BM-MSC still remains a big clinical obstacle regarding their translational purpose for neurological disorders. Inspiringly, it is expected that effective production of MSC-derived functional neural-lineage cells shall further extend their therapeutic application in various neurodegenerative diseases and nerve injury events [4].

Obviously, it is critical that efficiency of neural cell inducement and moderate control of cell proliferation are secured for establishment of a practical and safe MSC therapy [2,4]. Though a number of substances, neurotrophic or growth factors were found to exhibit certain promoting effect on neural cell differentiation, tumorigenicity of pluripotent stem cells still remains one most concern issue in cell transplantation [4]. Indeed, currently, there are still lacked of well-defined factors, drugs or chemicals with such both natural properties, i.e. neural cell fate commitment-

promoting benefit and tumorigenicity-blocking effects in BM-MSC [4]. Recently, evidences indicated that certain epigenetic factors were actively involved in regulating differentiation of embryonic or adult stem cells [5-8]. For instance, Trichostatin A (TSA), a histone deacetylation inhibitor previously used for an anti-cancer agent, could sufficiently induce neuronal cell fate from the embryonic neural stem cells [9]. Moreover, only these embryonic neural stem cells with TSA treatment could definitely differentiate into functional neurons that were characterized with active potential and induced spikes, and underlying mystery or inducing mechanism attracts a great attention [9]. In this study, interestingly, we demonstrated that TSA could effectively induce both neural-lineage cell commitment and cell division or cycle arrest of rat BM-MSC, possibly through triggering Wnt/ $\beta$ -catenin signaling activation by using cell culture, flowcytometry, immunocytochemistry and western blot methods.

## Materials and Methods

A total of twenty adult female Sprague-Dawley (SD) rats were used in the present study and supplied from the Animal Center of the Fourth Military Medical University of China. All animal experiments followed were carried out in according with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23) revised 1996, approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University, and all efforts were made to minimize animal suffering and reduce the number of animals used.

For cell culture preparation of BM-MSC, bone marrow mesenchymal cells were isolated from the femur bone of rats, which were sacrificed by animal decapitation. The bone marrow were washed out in PBS at room temperature, collected in glass tube and allowed to precipitation for a while. The cell suspension was seeded in dish in 10ml of MEM- $\alpha$  medium (Invitrogen) supplemented with 15% fetal bovine serum (Biosera, UK) and allowed to grow for 4-6 days until cell clones appeared. Cell pellets were collected, re-suspended and seeded in density  $0.5-1 \times 10^6$  in T25 flasks (Corning) in MEM growth medium. After culture in a humidified 5% CO<sub>2</sub>/95% air incubator at 37°C about 5-7 days and cells grew fast in attached growth mode. They were dissociated by using accutase digestion and subjected to 5-6 passages each in about 3-5 days. After immunostaining confirmation of specific biomarkers (CD90<sup>+</sup>/CD45<sup>-</sup>), BM-MSC cultured in 5-6 passages were used for following TSA experiments.

TSA treatment was performed to detect its influence on cell proliferation and cell differentiation of BM-MSC *in vitro*. Cell cultures of MSC were prepared in these groups, i.e. pre-treat, control, TSA-1ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml and 2000ng/ml working dilutions. TSA (Sigma-aldrich, St Louis, MO, 78552), which was dissolved in DMSO and diluted with PBS, was added in cell culture medium, incubated with DMEM/F12 (Gibco) medium supplemented with 2% B27 (Invitrogen) without bovine serum and continued for 3 days and 7 days. Cell survival and cell growth were observed firstly under phase-contrast microscopy. MSC cell samples of control, TSA-10ng/ml, 100ng/ml and 500ng/ml group of 3d or 7d time-points were freshly collected for flowcytometry and western blot, or the cultured cells were terminated by fixation with 4% paraformaldehyde for 10 min for immunocytochemical detection of morphology and growth state of differentiated MSC.

## Flowcytometry

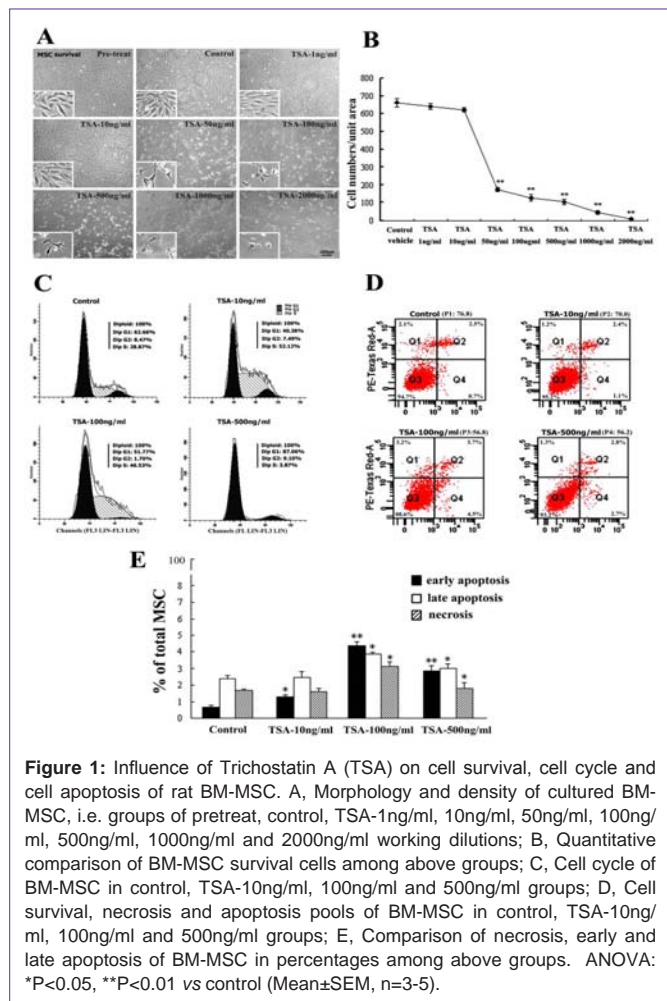
Flowcytometry was performed to detect cell cycle, cell viability and apoptosis of cultured BM-MSC in a standard protocol. Fresh cell samples were collected and centrifuged for 5min and supernatants were discarded. Cell pellets were suspended and incubated with 1-2ml accutase for 10 min at 37°C. After gentle repeated beating, cell suspension was re-centrifuged for 5min at 1000r, and supernatant was abandoned. The cells were re-suspended in 1ml PBS supplemented with 2ml dehydrated alcohol, shaken for a moment, sealed and kept at 4°C overnight. The prepared cell samples of BM-MSC were measured under a flowcytometer for cell cycle, cell survival, necrosis and apoptosis. For data presentation, percentages of defined cells like G1, S, and G2 phase, survival, necrosis, early apoptosis or late apoptosis were quantified among control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups.

## Immunocytochemistry

Immunofluorescent staining was performed to demonstrate neural cell markers and Wnt/ $\beta$ -catenin signaling molecules in cultured BM-MSC. After culture for 3d and 7d in 24-well plates, cells were processed for immunocytochemical staining protocol. Briefly, cells were incubated with 10% donkey serum-containing blocking solution for 30min at room temperature, and followed by incubation of primary antibody solution containing 10% donkey serum, 0.3% triton X-100 in PBS at 4°C for 24h, i.e. rabbit anti-nestin (Sigma-aldrich, 1:1000), rabbit anti- $\beta$ -tubulin III (Tuj-1, Sigma-aldrich, 1:500), rabbit anti-NG2 (Sigma-aldrich, 1:800), mouse anti-gial fibrillary acidic protein (GFAP, Dark, 1:4000) and rabbit anti-CNase (Sigma-aldrich, 1:500), respectively. After three washes with PBS, cells were followed with incubation of Alexa Fluor-488, or Alexa Fluor-594 conjugated donkey anti-mouse or rabbit IgG (1:500, Molecular Probes) for 4h at room temperature. Finally, nuclear counterstaining with DAPI (Sigma-aldrich, D9564) for 10min was performed to visualize total cultured MSC. After PBS wash, cell samples were mounted with Fluorescence-preserving VECTASHIELD Mounting medium (Vector, H-1000) and examined under epifluorescent microscope and laser scanning confocal microscope (LSCM, Olympus, FV-1000). For control staining experiment, the primary antibody was substituted with normal mouse or rabbit serum. Immunoreactive cells were not detected in controls (data not shown), and specificity of antibodies used was also provided by manufacture' data sheet.

## Western blot

Western blot was performed to quantify expression of several neural cell markers and Wnt/ $\beta$ -catenin signaling molecules in cell culture in a standard protocol. Briefly, protein extracts were prepared from cultured MSC. Fresh cell samples were collected and homogenized at 4°C in 5 volumes of ice-cold lysis buffer [50mM Tris (pH 7.4), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Complete, Roche Diagnostics)], and centrifugation of homogenates for 10 min (12000g) was then performed. After protein concentration was determined by the BCA assay (Beyotime, China), supernatant was mixed with four volumes of protein loading buffer, boiled for 5min at 99°C and stored at 4°C. Total protein (20-30  $\mu$ g per loading passage) was loaded using loading buffer commercial for electrophoresis on 10-12% SDS-PAGE gels and transferred to the nitrocellulose membranes (Bio-



**Figure 1:** Influence of Trichostatin A (TSA) on cell survival, cell cycle and cell apoptosis of rat BM-MSC. A, Morphology and density of cultured BM-MSC, i.e. groups of pretreat, control, TSA-1ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml and 2000ng/ml working dilutions; B, Quantitative comparison of BM-MSC survival cells among above groups; C, Cell cycle of BM-MSC in control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups; D, Cell survival, necrosis and apoptosis pools of BM-MSC in control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups; E, Comparison of necrosis, early and late apoptosis of BM-MSC in percentages among above groups. ANOVA: \*P<0.05, \*\*P<0.01 vs control (Mean±SEM, n=3-5).

Rad). After these membranes were blocked with 5% skimmed milk (Wandashan, China) in Tris-buffered saline containing 0.05% Tween 20, immunoblotting was performed with primary antibody to nestin, Tuj-1, NG2, CNPase, GFAP, Wnt1, Wnt3a, Wnt5a,  $\beta$ -catenin or phosphor- $\beta$ -catenin respectively, and followed by secondary antibody incubation. Mouse anti-Wnt1 (Sigma-aldrich, 1:1000), rabbit anti-Wnt3a (abcam, 1:500), mouse anti-Wnt5a (Sigma-aldrich,1:1000), rabbit anti- $\beta$ -catenin (Sigma-aldrich, 1:1000), rabbit anti-phosphor- $\beta$ -catenin (Sigma-aldrich, 1:1000), and rabbit anti- $\beta$ -actin (Sigma-aldrich, 1:800) were used. Visualization and detection of immunoblot bands was performed by an enhanced chemiluminescence (ECL) detection system (CWBio, China), and images were digitally acquired using ChemiScope System (Clinx, China). By using  $\beta$ -actin as internal control, quantitative analysis was carried out and data were presented in ratios.

**Wnt signaling inhibition experiment**

To confirm role of Wnt/ $\beta$ -catenin signaling pathway in TSA-induced cell differentiation of MSC, a blocking experiment was carried out in cell culture by administration of a specific inhibitor IWR-1, which can induce stabilization of APC/Axin2/GSK3 $\beta$  complex and decrease of cytoplasmic free  $\beta$ -catenin level through a direct interaction and works as Wnt signaling inhibitor [10,11]. In TSA+IWR1 group, IWR-1 (Sigma-aldrich, I0161) was dissolved in

saline and added in culture medium in 10  $\mu$ M at 10min before TSA treatment (TSA-100ng/ml plus IWR1), and saline group was used as controls. After maintaining culture for 3d and 7d, differentiation cell markers and changes were examined in BM-MSC of control, TSA-10, 100, 500ng/ml, and TSA-100ng/ml+IWR1-10  $\mu$ M group by western blot and immunocytochemistry.

**Statistic analysis**

For data analysis of flowcytometry, immunocytochemistry and western blot, total MSC cells, immunopositive cells and immunoblot density of nestin, Tuj-1, NG2, GFAP, CNPase, Wnt1, Wnt3a, Wnt5a, phosphor- $\beta$ -catenin and  $\beta$ -catenin were counted or measured and given as mean  $\pm$  S.E.M. (n=3-5, independent experiments). Differences between means were analyzed by one-way ANOVA (SPSS 18.0) with different markers or signaling molecules as independent factors. When ANOVA showed significant difference among means, the pair-wise comparisons between means were also performed by post hoc testing, and the significance level was set at a P value of 0.05 for all analyses.

**Results**

**Cell division arrest and cell apoptosis of BM-MSC induced by TSA treatment**

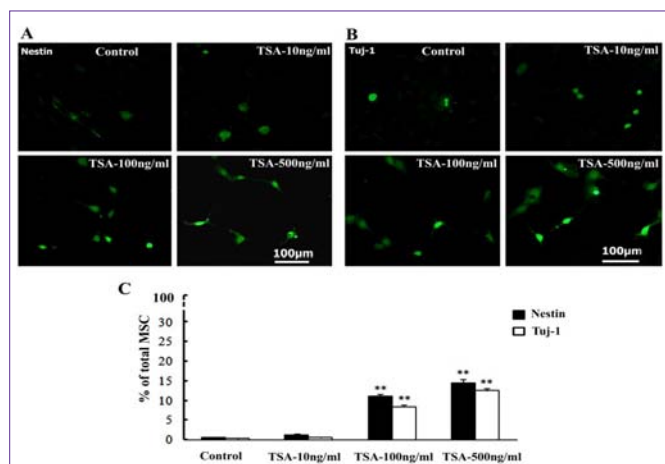
Influence of TSA treatment on cell survival, apoptosis and cell cycle were first observed in cell culture after BM-MSC in high-purity and uniform were established through 5-6 passages and characterized with specific BM-MSC biomarkers as reported previously [3,4]. Cell morphology and cell density of BM-MSC were observed and compared among these groups, i.e. pre-treat, control, TSA-1ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml and 2000ng/ml (Figure 1A). Obvious changes in morphology and cell numbers of BM-MSC dose-dependently occurred at doses of above TSA-50ng/ml working dilution. Cell count data revealed that BM-MSC in single unit field decreased after treatment of TSA dose-dependently and significantly in comparison with that of control (Figure 1B).

Flowcytometry analysis was performed to quantify cell survival, cell division and cell apoptosis of BM-MSC in control, TSA-10ng/ml, 100ng/ml and 500ng/ml group. Cell cycle analysis indicated cell division or cycle arrest occurred after treatment of TSA, and increased percentages of cells at G1 and S phase were detected in BM-MSC treated with TSA-100ng/ml and 500ng/ml (Figure 1C). The percentage of apoptotic cells also increased in BM-MSC with treatment of TSA-100ng/ml and 500ng/ml (Figure 1D). The percentages of necrosis, early and later apoptotic cells were quantified among control and TSA groups (Figure 1E). Quantitative data analysis indicated that TSA-induced decreasing of BM-MSC might be resulted from both cell cycle arrest and cell apoptosis.

**Increased neuronal-like cell differentiation of BM-MSC induced by TSA treatment**

Interestingly, promoting effects of TSA treatment on neuronal cell differentiation were observed and indicated by enhancing expression levels of neuronal cell markers and appearance of neuron-like cells in the cultured BM-MSC. Percentages of nestin (neural stem cell marker) and Tuj-1 (neuronal cell marker)-immunopositive ones of total MSC cells increased at dose of TSA-100ng/ml and 500ng/ml groups. Besides, a number of neuron-like cells appeared and grew





**Figure 2:** Increasing neuronal cell differentiation of cultured BM-MSC induced by TSA treatment. A-B, Nestin and Tuj-1-immunopositive cells in BM-MSC of control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups at 3d culture; C, Comparison of nestin and Tuj-1-immunopositive cells among above groups in the percentage (%) of total DAPI-counterstained (not shown) BM-MSC. ANOVA: \*\* $P < 0.01$  vs control (Mean $\pm$ SEM,  $n=5$ ).

thin processes in TSA-100ng/ml and 500ng/ml groups (Figure 2A). Quantitative data analysis showed increased percentages of nestin and Tuj-1-immunopositive ones constituting total MSC significantly at TSA-100ng/ml and 500ng/ml in comparison with that of control (Figure 2B).

### Enhanced glial cell differentiation of BM-MSC induced by TSA treatment

The stimulating effect of TSA treatment on glial cell differentiation were also observed in cell culture of BM-MSC, which was indicated by increasing expression of glial cell marker NG2, GFAP and CNPase. The NG2, GFAP and CNPase-immunopositive cells were observed in all groups, i.e. control, TSA-10ng/ml, 100ng/ml and 500ng/ml. However, stronger immunoreactivity to NG2, GFAP or CNPase was detected after TSA treatment and obviously at TSA-100ng/ml and 500ng/ml groups (Figure 3A). Cell count showed that increased percentages of NG2, GFAP and CNPase-immunopositive ones constituting total MSC occurred at TSA-100ng/ml and 500ng/ml group in comparison with that of control (Figure 3B).

### Activation of Wnt/beta-catenin signaling pathway in BM-MSC with TSA treatment

Western blot was applied to examine possible role of Wnt/ $\beta$ -catenin signaling pathway in TSA-induced differentiation of BM-MSC at 3d and 7d culture. It revealed increase of total  $\beta$ -catenin and decrease of phosphor- $\beta$ -catenin, the key molecules of Wnt/ $\beta$ -catenin signaling pathway (Figure 4A and 4E). Western blot also showed that expression level of total  $\beta$ -catenin molecule at 3d culture was more obvious in comparison with that of  $\beta$ -catenin at 7d culture. Quantification showed up-regulation of  $\beta$ -catenin proteins and down-regulation of phosphor- $\beta$ -catenin significantly at dose of TSA-100ng/ml and 500ng/ml in comparison with control, indicating that TSA could induce upregulation of active form of  $\beta$ -catenin molecule (Figure 4B and 4F). Besides, expression of Wnt1, Wnt3a and Wnt5a was also observed in cultured BM-MSC with TSA treatment, and data indicated increasing levels of Wnt1 and Wnt5a but not Wnt3a in cultured MSC after TSA treatment (Figure 4C and 4D).

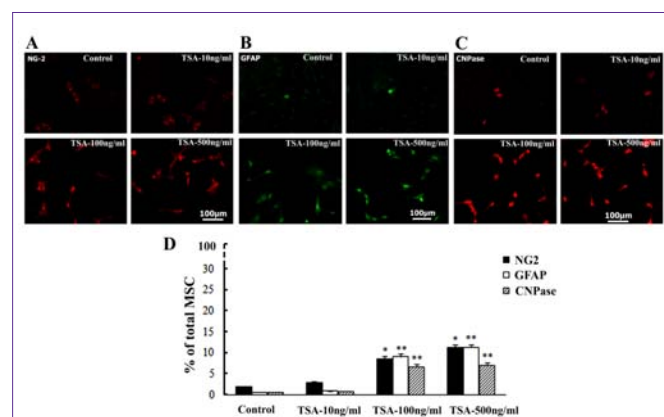
Wnt signaling inhibition experiment was performed to confirm involvement of Wnt/ $\beta$ -catenin signaling activation in TSA-inducing MSC differentiation by administration of a specific Wnt signaling blocker IWR1 [10, 11]. Western blot quantified relative levels of nestin, Tuj-1, NG2, GFAP and CNPase of cultured MSC among distinct groups of control, TSA-10ng/ml, 100ng/ml, 500ng/ml, TSA-100ng/ml+IWR1-10 $\mu$ M. Increasing of nestin, Tuj-1, NG2, GFAP and CNPase-immunoblot bands were observed in TSA-treated MSC groups (Figure 5A and 5C). Quantitative analysis confirmed that significant upregulation of nestin, Tuj-1, NG2, GFAP and CNPase expression occurred in cultured MSC in TSA groups in comparison with that of control in ratio to internal control  $\beta$ -actin (Figure 5B and 5D). On the other hand, expression of nestin, Tuj-1, NG2, GFAP and CNPase decreased significantly in TSA+IWR1 group, and these neuron-like or glia-like cells also went down obviously or almost disappeared in this TSA+IWR1 group (data not shown).

### Appearance of TSA-induced differentiated cells in prolonged BM-MSC culture

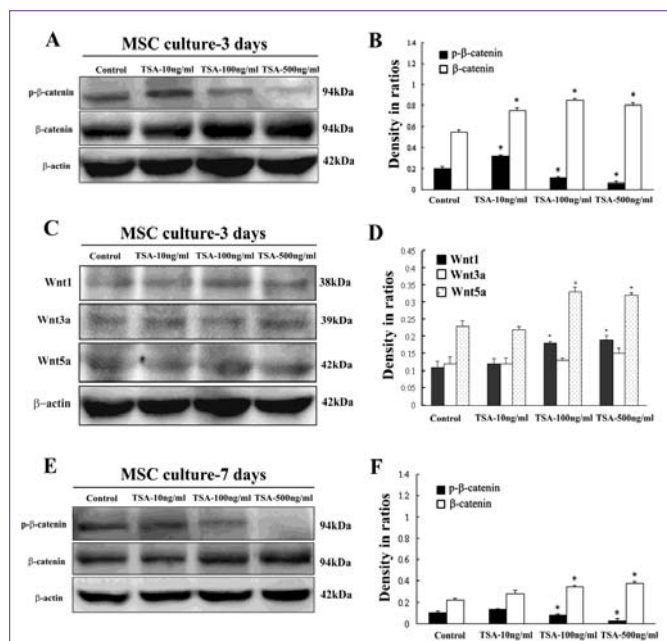
Finally, TSA-induced differentiated cells were observed at 3d and 7d MSC culture. Although Tuj-1, GFAP, and CNPase-positive cells seemed similar appearance at 3d culture, these differentiated MSC ones showed much different morphology at prolonged 7d culture. These cells with immunoreactivity to Tuj-1 (neuronal cell marker), GFAP (astrocytic cell marker), and CNPase (oligodendrocytic cell marker) are shown (Figure 6A). Besides, percentages of Tuj-1, GFAP, or CNPase-immunopositive ones constituting total MSC cells (DAPI counterstained) were counted and shown (Figure 6B). Data indicated that a small population of MSC was induced into Tuj-1, GFAP, or CNPase-immunopositive cell commitment in both 3d and 7d culture of MSC of TSA-500ng/ml groups.

## Discussion

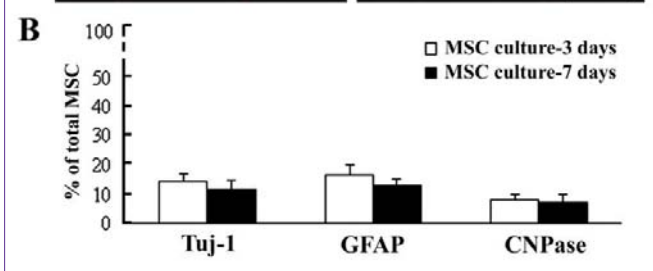
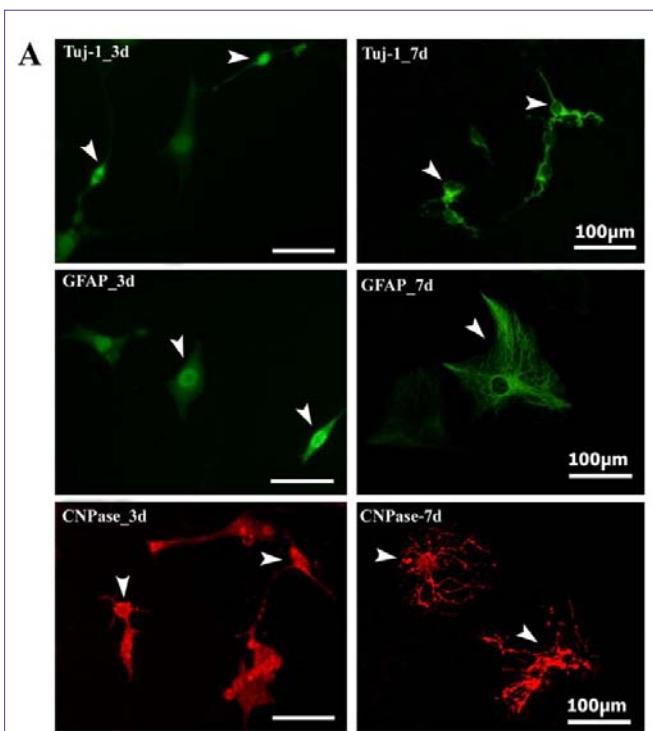
This study revealed that TSA promoted neural-lineage cell differentiation of rat BM-MSC and property of differentiated cells was indicated by morphological evaluation and differentiation marker analysis. On the other hand, TSA also induced cell division or cell cycle arrest of BM-MSC dose-dependently. More interestingly,



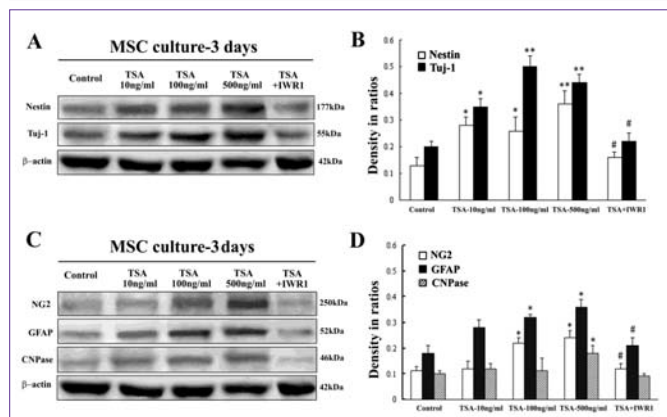
**Figure 3:** Enhancing glial cell differentiation of cultured BM-MSC induced by TSA treatment. A-C, NG2, GFAP and CNPase-immunopositive cells in BM-MSC of control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups at 3d culture; D, Comparison of NG2, GFAP and CNPase-immunopositive cells among above groups in the percentages (%) of total DAPI-counterstained (not shown) BM-MSC. ANOVA: \*  $P < 0.05$ , \*\*  $P < 0.01$  vs control (Mean $\pm$ SEM,  $n=5$ ).



**Figure 4:** Expression levels of Wnt/ $\beta$ -catenin signaling molecules in cultured BM-MSC with or without TSA treatment. A, Expression of phosphor- $\beta$ -catenin and total  $\beta$ -catenin of MSC of control, TSA-10 ng/ml, 100ng/ml and 500ng/ml groups at 3d culture; B, Comparison of phosphor- $\beta$ -catenin and total  $\beta$ -catenin among above groups in ratios to  $\beta$ -actin; C, Expression of Wnt1, 3a and 5a of MSC of control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups; D, Comparison of Wnt1, 3a and 5a among above groups; E, Expression of phosphor- $\beta$ -catenin and total  $\beta$ -catenin of MSC of control, TSA-10ng/ml, 100ng/ml and 500ng/ml groups at 7d culture; F, Comparison of phosphor- $\beta$ -catenin and  $\beta$ -catenin among above groups in ratios to  $\beta$ -actin. ANOVA: \*  $P < 0.05$ , \*\* $P < 0.01$  vs control (Mean $\pm$ SEM, n=3).



**Figure 6:** The prolonged neural-lineage cell differentiation and maturation of cultured BM-MSC induced by TSA treatment. A, Morphological appearance of Tuj-1, GFAP, CNPase-immunopositive differentiated cells in BM-MSC with treatment of TSA-500ng/ml at both 3d and 7d culture; B, Comparison of Tuj-1, GFAP, CNPase-immunopositive cells in percentages (%) of total BM-MSC between 3d and 7d.



**Figure 5:** Expression of neural-lineage cell markers, i.e. nestin, Tuj-1, NG2, GFAP and CNPase in cultured BM-MSC with or without TSA treatment. A, Expression of nestin and Tuj-1 in BM-MSC of control, TSA-10ng/ml, 100ng/ml, 500ng/ml, and TSA-100ng/ml+IWR1 group at 3d culture; B, Comparison of nestin and Tuj-1 expression levels among above groups in density ratios to  $\beta$ -actin; C, Expression of NG2, GFAP and CNPase in BM-MSC of above groups; D, Comparison of NG2, GFAP and CNPase expression levels among above groups in ratios to  $\beta$ -actin. ANOVA: \*  $P < 0.05$ , \*\* $P < 0.01$  vs control; #  $P < 0.05$  vs TSA-100ng/ml group (Mean $\pm$ SEM, n=3).

this study demonstrated potential involvement of Wnt/ $\beta$ -catenin signaling pathway in TSA-induced neural cell differentiation of BM-MSC. Further extensive studies should be needed to show functional property or maturation of the MSC-derived neuronal and glial cells. Nevertheless, this study has provided new evidence that TSA could

effectively induce neural-lineage commitment of BM-MSC possibly by triggering Wnt/ $\beta$ -catenin signaling pathway, suggesting that TSA may be applied as one candidate for MSC transplant therapy for various neurological diseases and nerve injury [2,4].

*Neural cell fate decision of BM-MSC might be induced by TSA, a histone deacetylation inhibitor* Growing studies have focused on epigenetic modulation of stem cell proliferation and differentiation, in which DNA histone acetylation, demethylation, microRNA regulation modes might be actively involved the forebrain neurogenesis [12], hippocampal neurogenesis [13-16], and cell differentiation of oligodendrocyte progenitors [17-20]. Potential interaction of epigenetic factors with growth factor or retinoic acid might occur in the neuronal differentiation [21,22]. Consistently, Balasubramanian et al reported interesting result of TSA-inducing neuronal differentiation from neural precursor cells, and only these progenitors with pretreatment of TSA could differentiate into neuronal cells [9]. It showed that TSA not only induced neuronal cell differentiation, but also fostered neuronal maturation, dendrite growth

and spikes of new-born or differentiated neurons [9]. Although it still remains unclear how TSA could improve differentiation or trans-differentiation of MSC, previous studies identified that several types of agents could increase efficiency of cell reprogramming induced by defined factors and nuclear transfer. These agents include histone deacetylase inhibitor TSA and valproic acid, and other modulators of cell specific signaling, which might confer enhancing expression of several genes, cell plasticity and pluripotency [23,24].

On other hand, it was noticed that chemically-induced MSC cells might adopt some molecular properties of neuronal-like cells but did not have basic neuronal functional activities [25]. While these MSC differentiated using chemical inducers such as  $\beta$ -mercaptoethanol showed increases in expression of some neuronal markers, they did not show electrophysiological properties of neurons [25]. Liu et al also reported that morphological changes and increases in immunolabelling for certain cellular markers might be likely a result of cytotoxicity, cell shrinkage and changes in cytoskeleton [26]. Other authors suspected that neural trans-differentiation of bone marrow stromal cells induced by chemical agents might also be a short-time reversible phenomenon [27]. In our study, DMSO was also used and remained same low concentrations in both control and all TSA groups, and data indicated that increase of differentiated MSC might be a result from specific TSA intervention effect instead of DMSO toxicity. However, we can still not completely exclude the possibility that TSA-induced MSC differentiation might result in abnormal epigenetic changes as compared to normal phenotype.

*Low tumorigenicity of BM-MSc treated with TSA may be beneficial for clinical therapy* In the cell therapy for clinical application, tumorigenicity of transplanted MSC or pluripotent stem cells raises great concern and present one big hurdle in safety [28]. Presently, no a practical method can completely eliminate tumorigenicity of stem cells including BM-MSc, and an alternative may be to use differentiated neurons [29,30]. It is well known that tumorigenicity of transplanted stem cells was greatly influenced by recipients' immune condition or response [31]. Studies have also shown that MSC exhibit immunosuppressive capacity and modulate immune function of cell populations including antigen presenting cells, T cells, B cells and natural killer cells [32,33]. Although underlying mechanisms of immunosuppressive effects of MSC or how MSC inhibit the proliferation of a variety of immune cells are still obscure, recent more evidences have suggested that MSC might down-regulate T and B lymphocytes, natural killer cells and antigen presenting cells through cell-cell interactions and soluble factor production [33-36].

A sharp decrease of MSC numbers was observed between TSA-10ng/ml and 50ng/ml treatments in this study, suggesting that effective inhibition of TSA on MSC survival or growth occurred at dose above 10ng/ml. Flowcytometry analysis showed obvious cell division or cycle arrest of BM-MSc after treatment of TSA at 100ng/ml and 500ng/ml working dilution. Besides, apparent cytotoxicity of TSA was observed at doses of 1000ng/ml and 2000ng/ml. Data of this study suggested that doses of 50-500ng/ml might be suitable for TSA intervention for purpose of promoting neural-lineage differentiation and controlling moderate proliferation of BM-MSc. While the immunosuppressive effect of MSC may foster tumorigenicity of transplanted stem cells, BM-MSc pretreated with TSA and characterized with controlling cell

proliferation and promoting cell differentiation may be alternative choice in practical transplantation therapy [32].

*Wnt/ $\beta$ -catenin signaling might be involved in regulating both neural-lineage cell commitment and cell proliferation of BM-MSc* Although TSA is clearly identified as a histone deacetylase inhibitor functionally, detailed down-stream signaling underlying MSC differentiation remains obscure [4]. The Wnt signaling functions as a key regulating signal for stem cell proliferation and cell differentiation [37,38]. In this study, western blot analysis indicated treatment of TSA at 100ng/ml and 500ng/ml decreased p- $\beta$ -catenin expression levels of MSC. By comparison with total  $\beta$ -catenin level, it indicated activation of wnt/ $\beta$ -catenin signaling in MSC after treatment of TSA above dose of 100ng/ml. Several studies showed that Wnt signaling influenced proliferation and differentiation of human MSC and osteogenic process [39-42]. Regard et al reported that Wnt/ $\beta$ -catenin signaling was required for differentiation of skeletal progenitors into osteoblastic lineage cells and also involved in the fibrous dysplasia, a disease that exhibits abnormal differentiation of skeletal progenitor cells. While Wnt/ $\beta$ -catenin signaling in osteoblast progenitors resulted in a fibrous dysplasia-like phenotype and reduction of  $\beta$ -catenin rescued differentiation defects of stromal cells. Activated G $\alpha$  proteins, which act at  $\beta$ -catenin destruction complex assembly by binding Axin, played a physiological role during skeletal development and disease by modulating Wnt/ $\beta$ -catenin signaling strength [43]. Besides, Wnt/ $\beta$ -catenin signaling actively functioned in neurogenesis of hippocampus and midbrain [44,45], and Wnts like Wnt1 and Wnt3a promoted dopamine differentiation of neural progenitors and transplant of Wnts-primed neural stem cells showed improvements in model of Parkinson disease [46-49].

In this study, our data indicated that suitable working doses of TSA might be 50-500ng/ml for promotion of neural-lineage differentiation of BM-MSc *in vitro*, while TSA doses less than 10ng/ml showed no significant effect, and doses higher than 500ng/ml showed more MSC toxicity. TSA is known as an anti-cancer agent for clinical usage, Wu et al found apoptosis-inducing effect by TSA at 50ng/ml in A549 cells, a non-small-cell lung cancer cell line. They demonstrated TSA resulted in apoptosis of A549 cells by increasing expression of TNFR and activation of caspase signaling pathway [50]. Besides, Liu et al also reported apoptosis of prostate cancer cells induced by treatment of TSA at concentration of 1mM [51]. Whether or not TSA exhibits similar effects on MSC *in situ* or transplanted MSC *in vivo* should need further investigation by animal experiment.

Finally, we especially focused on possible role of Wnt/ $\beta$ -catenin signaling in TSA effect on MSC in this study, and found that TSA-induced neural-lineage commitment from BM-MSc might be possibly attained by triggering activation of Wnt/ $\beta$ -catenin signaling and modulation of down-stream gene expression regulation cell differentiation and proliferation. Western blot confirmed IWR1 blocking effect on Tuj-1, NG2, GFAP and CNPase expression of cultured MSC induced by TSA treatment. Data of present study supported our point that Wnt signaling might be possibly required for neural-lineage cell differentiation of MSC. It is documented that Wnt/ $\beta$ -catenin signaling regulates cell adhesion, cell morphology, and gene expression controlling cell differentiation and proliferation [37,38], while HDAC inhibitor is also involved in gene transcription,



cell proliferation, differentiation and apoptosis by modulating DNA activity. Result of this study further indicated that activation of Wnt/ $\beta$ -catenin signaling induced by TSA treatment might be involved in regulation of cell division and neural-lineage differentiation of BM-MSC [39,40]. Besides, the enhanced TNF receptor, increased somatostatin receptor, cyclooxygenase-2 activity, ERK signaling inhibition, BCL-2 down-regulation, increased in cell cycle G1 arrest, decreased in cyclin D1, D2 and cyclin dependent kinases, and activation of down-stream caspase might be involved in mediation of TSA effect [50-55]. In addition, HDAC inhibitor induced E-cadherin expression and membrane localization of E-cadherin/ $\beta$ -catenin complex, leading to reduced cancer cell migration and invasion [56]. HDAC inhibitor TSA was thus applied as an effective DNA damage strategy in human neuroblastoma or effective anti-cancer agent [50-57]. It is hopefully that TSA-induced generation of neuronal cells may result in establishing new intervention for MSC transplant therapy that characterizes with lower tumorigenicity or higher safety.

In conclusion, TSA could induce both cell division arrest and neural-lineage cell differentiation of cultured BM-MSC dose-dependently most possibly by triggering activation of Wnt/ $\beta$ -catenin signaling. These biological effects of TSA intervention indicated its beneficial roles in lowering tumorigenicity of transplanted MSC and increasing MSC differentiation efficiency into neurons or glial cells, strongly suggesting that TSA may be utilized as a potential candidate drug in manipulation of MSC therapy for treatment of various neurological disorders in human beings.

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