# Surface marker changes during induced differentiation of bone marrow mesenchymal stem cells into neuronal-like cells★

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## Abstract

**BACKGROUND:** Adult central nervous system lacks the ability to regenerate, so it is of great significance to find a new source of neural stem cells.

**OBJECTIVE:** To investigate the differences between basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) in inducing bone marrow mesenchymal stem cells (MSCs) to differentiate into neurons *in vitro*.

**METHODS:** MSCs were isolated from normal human bone marrow using density gradient centrifugation and cell attachment method. MSCs were plated in 96-well culture plates at a density of $0.25 \times 10^8$/L and cultured with 200 μL DMEM/F12 for 0, 1, 2, 3, 4, 5, 6, and 7 days, with 20 μL MTT (5 mg/mL) in 5 wells at each time point. The supernatant was removed and 100 μL dimethyl sulphoxide was added to each well for 4 additional hours of incubation. In addition, some cells were exposed to bFGF and EGF. Growth curve was determined with MTT method. Telomerase activity were examined by TRAP(PCR)-ELISA. Additionally, the functional differences of the two cytokines were checked by RT-PCR.

**RESULTS AND CONCLUSION:** RT-PCR revealed that nestin, glial fibrillary acidic protein (GFAP) and neurofilament subunit M (NF-M) mRNA were expressed in un-induced MSCs of passage 4. Nestin expression reduced at 7 days. The expression of micro-tubule-associated protein-2 (MAP$_2$) mRNA was not detected until the induction, and increased thereafter. The expression of MAP$_2$ mRNA was greater in bFGF+EGF and bFGF alone groups compared with EGF alone group, and the expression of GFAP in EGF alone group was greater than other groups. Results showed that MSCs can be cultivated, proliferated and differentiated into neural stem cells *in vitro*. The differentiated neural stem cells have the activity of proliferation, but not have the ability of infinite proliferation as tumor cells.

## INTRODUCTION

Adult central nervous system lacks the ability to regenerate, so they will be replaced by glial cells when injured or degenerated. Recent studies have shown that neural stem cells (NSCs) have distributed in embryonic and adult brain tissues and peripheral nervous system[1-2], and can be induced into neurons, astrocyte and oligodendrocytes by cytokines to compensate for the damaged nervous tissue[3-5]. However, endogenous NSCs lack necessary stimulating signals so that they cannot differentiate into neurons automatically when damage occurs. In addition, the utility of NSCs from embryonic brain is restricted methodically and ethically[6-7]. Therefore, it is of great significance to find a new source of NSCs for clinical use in neuroscience field[8].

The existence of non-hematopoietic stem cell in bone marrow was first proposed by Cohnheim in 1876. In 1976, Friedenstein *et al*[9-10] isolated mesenchymal stem cells (MSCs) roughly from bone marrow through cell adhering method. Thereafter more and more cultures of bone marrow-derived MSC were established. MSCs are the portion of adherent cells that are different from the hematopoietic stem cells in bone marrow. They can differentiate into multiple cell lineages *in vitro* and can be cultured and proliferated easily. These characteristics of MSCs allow for application in cell treatment and gene therapy. Recent studies have demonstrated that MSCs can differentiate into neuronal-like cells *in vitro*[11-13]. The present study established a bone marrow-derived MSCs culture system to induce MSCs to differentiate into neural-like cells under basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF)[8, 14].

## MATERIALS AND METHODS

### Design

Grouping and comparative experiment of MSCs.

### Time and setting

This study was performed at the Stem Cells Central Laboratory of Zhengzhou University from March to December 2004.

### Materials

Human bone marrow mesenchymal stem cells (hMSCs) were provided by healthy donator with informed consent. Reagents and instruments are listed as follows:

| Reagent and instrument | Source |
|---|---|
| DMEM/F12 culture solution | Gbico, USA |
| Fetal bovine serum, MTT, EGF, bFGF | Sigma, USA |
| CO2 incubator, refrigerated centrifuge | Jouan, France |
| Enzyme immunoassay analyzer | TECAN, USA |
| Inverted phase contrast microscope | OLYMPUS, Japan |
| RevertAid™ First Strand cDNA Synthesis Kit | MBI Fermentas, USA |
| Telo TAGGG Telomerase PCR ELISA Kit | Roche, USA |

### Methods

#### Cell isolation

Bone marrow mononuclear cells were isolated from sterile PBS (pH7.4) and heparinized bone marrow at a ratio of 1: 1 using density gradient centrifugation method. Cell viability was observed following trypan blue staining.

### Cell cultures

Cells at a density of $1.0 \times 10^9$/L were seeded in DMEM/F12 solution (T-50 culture flask) containing 15% fetal bovine serum and incubated in 5% $CO_2$, saturated humidity at 37 ℃. After 3 days, the solution was discarded, and the medium was exchanged every 3–4 days thereafter. Cultured cell appearance was observed by microscopy. Cells were passaged at 90% confluency.

### MTT assay

Single cell suspension were plated in 96-well culture plates at a density of $3 \times 10^7$/L well and cultured with 200 μL DMEM/F12 for 0, 1, 2, 3, 4, 5, 6 and 7 days. At each time point, 20 μL MTT (5 mg/mL) were added in 5 wells. The supernatant was discarded and 100 μL dimethyl sulphoxide was added to each well after incubating for another 4 hours. The absorbance ($A$) of each well at 450 nm was read with an ELISA reader.

### Induction and differentiation of hMSCs into neural-like cells

After four passages, the adherent cells were dissociated, and the density was adjusted to $1 \times 10^5$/mL. The cells were seeded onto 8-well chamber slides. When the cells reached 30%–40% confluency, cells were exposed to neural induction medium (DMEM/F12 supplemented with 10% fetal bovine serum, 10 ng/mL EGF and 10 μg/L bFGF). To determine the functional differences of two cytokines (EGF and bFGF), three different culture conditions were used.

Neural inducing conditions are as follows:

| Group | Induced differentiation conditions |
|---|---|
| 1 | 10 μg/L bFGF |
| 2 | 10 μg/L EGF |
| 3 | 10 μg/L bFGF+10 μg/L EGF |

### RT-PCR analysis of nestin, micro-tubule-associated protein-2 (MAP2), glial fibrillary acidic protein (GFAP), neurofilament-M (NF-M) expression in induced hMSCs

Total RNA was isolated from adherent cells using Trizon Total RNA Isolation System according to the manufacture's instructions. cDNA were synthesized from total RNA using the AMV First Strand cDNA Synthesis Kit under condition recommended by the manufacture. Nestin, NF-M, GFAP, MAP2 and β-actin gene were examined by PCR using a LA-PCRTM kit. PCR was performed according to the manufacture's instructions. Primers used in the experiments are listed in Table 1.

Table 1    Primers

| Target gene | Primer sequence | Size of product (bp) |
|---|---|---|
| Nestin | 5'- AGG ATG TGG AGG TAG TGA GA-3'<br>5'-TGG AGA TCT CAG TGG CTC TT-3' | 251 |
| NF-M | 5'-GAG CGC AAA GAC TAC CTG AAG A-3'<br>5'-CGA CTC TAG CTC GAT GCT CTT G -3' | 231 |
| MAP2 | 5'-TCA GAG GCA ATG ACC TTA CC-3'<br>5'-GTG GTA GGC TCT TGG TCT TT -3' | 321 |
| GFAP | 5'-CAA GCA CGA AGC CAA CGA CT-3'<br>5'-GTT CTC CTC GCC CTC TAG CAG-3' | 292 |
| β-actin | 5'-ATG GGT CAG AAG GAT TCC-3'<br>5'-ATG TCA CGC ACG ATT TCC CGC-3' | 500 |

For verification, the PCR products were electrophoresed on 2% agarose gel, and the bands were identified by fluorescence.

### PCR-ELISA analysis

Telomerase activity for un-induced MSCs and induced MSCs for 7 days were detected using the Telo TAGGG Telomerase PCR ELISA kit according to the manufacturer's instructions.

### Main outcome measures

Growth curve was observed by MTT; differences in cells induced by two cytokines were compared by RT-PCR; telomerase activity was determined by TRAP(PCR)-ELISA.

### Design, enforcement and evaluation

The experiment was designed, performed and evaluated by all authors using a blind method.

### Statistical analysis

Data were processed using SPSS 11.0. Data were expressed as Mean±SD. Paired comparison of mean was performed by $t$-test. $P < 0.05$ was considered statistically significant.

## RESULTS

### MTT assay of cell proliferation

The 1, 5 and 9 passages of MSCs were used in the MTT assay; the results expressed as absorbance were normalized and growth curve was drawn with growth time as X-axis and A value as Y-axis (Figure 1).
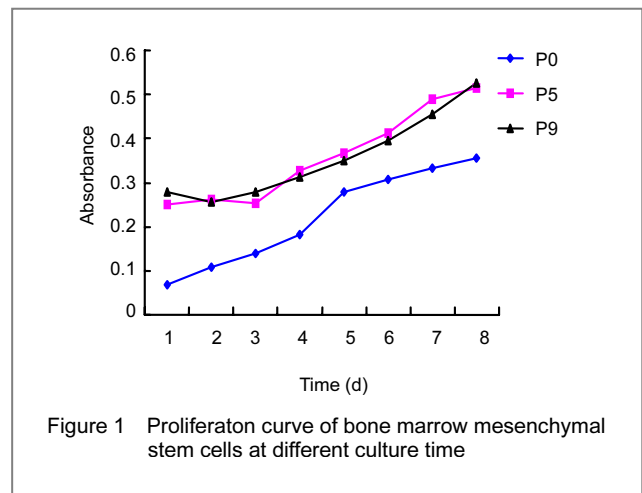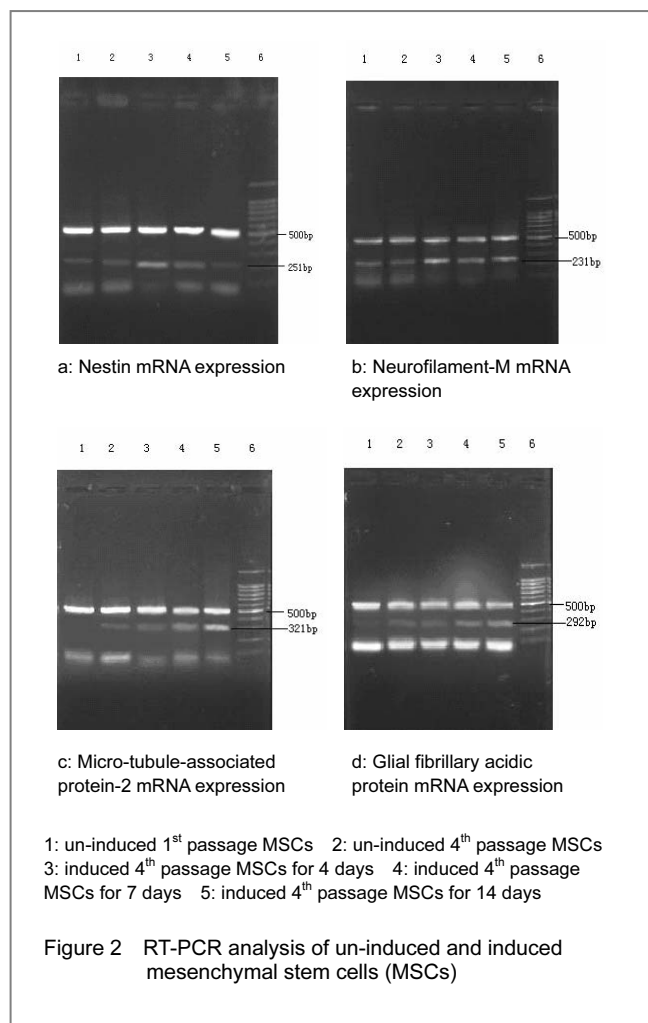


Figure 1    Proliferaton curve of bone marrow mesenchymal stem cells at different culture time

Cell proliferation ability at different passages was compared. Results showed that the first 3 days was growth latent period for attachment. The passage cells grew rapidly, and attached in 24 hours. The growth curve of primary, 5 and 9 passages of cells showed that 24–48 hours was a latent period; 3–6 days was exponential growth phase; cells grew slowly since the 6th day, and entered the platform phase. The proliferation of the 5th and 9th passages was similar ($P = 0.190$).
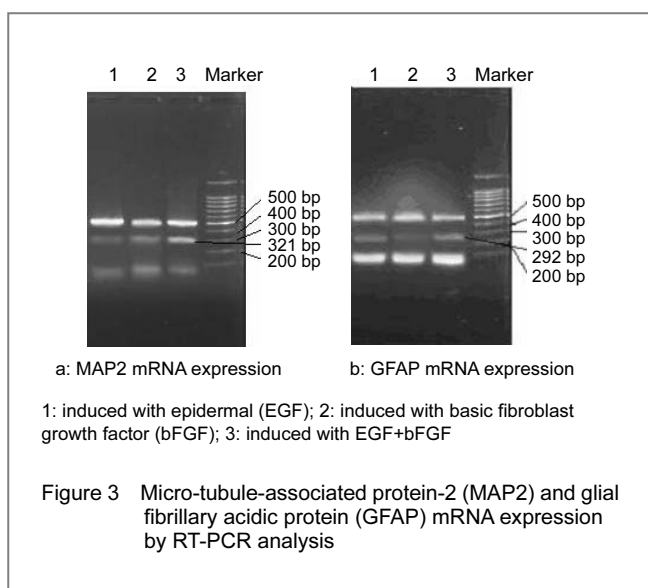
### RT-PCR results

The fourth passage un-induced cells expressed nestin, NF-M and GFAP mRNA, and after induction, the expression was increased. But the expression of nestin mRNA reduced gradually after induction for 7 days. There was no expression of MAP2 mRNA in un-induced fourth passage cells until the

induction (Figure 2).



a: Nestin mRNA expression

b: Neurofilament-M mRNA expression

c: Micro-tubule-associated protein-2 mRNA expression

d: Glial fibrillary acidic protein mRNA expression

1: un-induced 1st passage MSCs   2: un-induced 4th passage MSCs
3: induced 4th passage MSCs for 4 days   4: induced 4th passage MSCs for 7 days   5: induced 4th passage MSCs for 14 days

Figure 2   RT-PCR analysis of un-induced and induced mesenchymal stem cells (MSCs)

The expression of MAP2 mRNA was greater in bFGF+EGF and bFGF alone groups compared with EGF alone group, and the expression of GFAP in EGF alone group was greater than other groups (Figure 3).



a: MAP2 mRNA expression

b: GFAP mRNA expression

1: induced with epidermal (EGF); 2: induced with basic fibroblast growth factor (bFGF); 3: induced with EGF+bFGF

Figure 3   Micro-tubule-associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP) mRNA expression by RT-PCR analysis

## PCR-ELISA results

With the induced MSCs, low levels of telomerase activity were detected. But the result of un-induced MSCs was negative (Figure 4). The telomerase activity of the induced MSCs was 0.379, exceeding the positive standard 0.2, but much lower than that of the tumor cells 2.502, suggesting that telomerase activity of the induced MSCs was weakly positive.

## DISCUSSION

Stem cells obtained from bone marrow can differentiate into cells with morphological and phenotypic characteristics of neurons, and these cells express several neural proteins[15-19]. We observed an increase in the expression of more mature neuronal/glial proteins after exposure to neural induction medium, which confirms the differentiation of MSCs into neurons and astrocytes. The constitutive expression of nestin by MSCs suggests that these cells are "multidifferentiated" cells and could retain the ability for neuronal differentiation, enhancing their potentiality to be employed in the treatment of neurological diseases[20].

EGF and bFGF are cytokines that can promote proliferation and differentiation of MSCs into neurons through receptor of plasma membrane[21-22]. bFGF is the mitogen of the cells which can promote the proliferation of the neuron precursor cells, while recent study has indicated that bFGF also can determine the differentiation directions. bFGF has been shown to exhibit strong ability of stimulating proliferation of stem cells and to induce stem cell differentiation into neural cells[23]. EGF is a mitogen of glial cells, which is of vital importance in maintaining the survival of MSCs. EGF and bFGF play different roles in inducing MSCs into neurons. bFGF produces a mitogenic effect in the early period of differentiation of MSCs and make the MSCs to obtain the reaction of another mitogenic factor EGF. EGF plays an important role in the anaphase of the proliferation of MSCs[24]. The present study demonstrated that bFGF had stronger effects on stimulating proliferation of MSCs than EGF, and can induce them to express the specific markers of neurons. bFGF promotes the proliferation of MSCs and induces them to express the specific markers of neurons while EGF accelerates the MSCs turning to glial cell direction. Possible reasons are as follows: (1) MSCs may have the different mitogen-growth factor reaction subgroups which may have the different differentiation potentials. MSCs that can react with bFGF may differentiate more to neuronal cells. (2) bFGF can enhance the survival of neural progenitors or promote differentiation of MSCs to neural progenitors, so as to induce MSCs differentiation into neurons[25].

Telomerase activity of natural body cells is negative except germ cells, stem cells and tumor cells, and the telomerase activity of the stem cell is correlated with the cell period[26-27]. Telomerase activity is negative when stem cells are in the G0 stage. But under the function of cytokines and the change of microenvironment, the cells of G0 stage are induced into multiplication period, and telomerase activity also increases[28-30]. Results of examination of telomerase activity of induced MSCs showed that telomerase activity were enhanced in MSCs by EGF in combination with bFGF, suggesting that cytokines can induce $G_0$ stage cells to enter generation cycle but not have the ability of infinite proliferation as tumor cells[31].

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# 间充质干细胞向神经细胞诱导过程中表面标记的变化★

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## 摘要

**背景：**成熟的中枢神经系统缺乏再生能力。因此，找寻新的神经干细胞来源就显得尤为重要。

**目的：**观察碱性成纤维细胞生长因子和表皮生长因子对间充质干细胞诱导分化为神经元样细胞的差异。

**方法：**从正常人骨髓中分离获取间充质干细胞，体外培养扩增、纯化后种植于 96 孔板中，调整细胞密度为 $0.25×10^8$ $L^{-1}$，每孔 200 μL DMEM/F12 培养液，从第 0~7 天，每天于固定时间在 5 个孔里加入 20 μL MTT，放入培养箱继续培养 4 h 后吸净孔内的培养液，加入二甲基亚砜 100 μL/孔。另外，一部分细胞用碱性成纤维细胞生长因子和表皮生长因子单独或联合对其进行诱导分化。MTT 法绘制细胞的生长曲线，RT-PCR 比较两种细胞因子诱导细胞的差异，TRAP(PCR)-ELISA 检测诱导后细胞的端粒酶活性。

**结果与结论：**未诱导的第 4 代间充质干细胞有 nestin，GFAP 和 NF-M mRNA 的表达，随着诱导时间延长表达逐渐加强，但是诱导 7 d 后 nestin 的表达量逐渐下降。MAP2 mRNA 的表达在诱导后出现，随着诱导，表达逐渐加强。碱性成纤维细胞生长因子+表皮生长因子诱导组和碱性成纤维细胞生长因子诱导组 MAP2 mRNA 的表达量明显多于表皮生长因子诱导组，而表皮生长因子组可见较多胶质纤维酸性蛋白的表达。提示间充质干细胞可以在体外培养，扩增以及向神经干细胞分化。分化后的神经干细胞具有增殖的活性，但是不具备肿瘤细胞的无限增殖性。

**关键词：**骨髓；间充质干细胞；碱性成纤维细胞生长因子；表皮生长因子；神经元；诱导分化