Differentiation of Mesenchymal Stem Cells Into Dopaminergic Neuron-like Cells *in vitro*[1]

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Objective   To explore the way to induce mesenchymal stem cells (MSCs) to differentiate into dopaminergic neurons *in vitro*.   **Methods**   MSCs were obtained from rat bone marrow, cultured and passaged. MSCs used in this experiment had multipotency, which was indirectly proved by being induced to differentiate into chondrocytes and adipocytes. MSCs were cultured in medium containing 0.5 mmol/L IBMX for 2 days. Then the medium was replaced with induction medium, which contained GDNF, IL-1β, mesencephalic glial-cell-conditioned medium and flash-frozen mesencephalic membrane fragments. The surface markers of the differentiated neurons, such as NSE, nestin, MAP-2a, b and TH were detected by immunocytochemistry and Western blot after MSCs were cultured in induction medium for 7 days and 15 days.   **Results**   MSCs differentiated into neural progenitors and expressed nestin after MSCs were incubated with medium containing IBMX for 2 d. After the medium was replaced with induction medium containing many inducing agents, MSCs differentiated into neuron-like cells and dopaminergic neuron-like cells and expressed NSE, MAP-2a, b and TH. The percentage of   NSE-positive cells, MAP-2a, b-positive cells and TH-positive cells was $30.032 \pm 2.489\%$, $41.580 \pm 5.101\%$ and $34.958 \pm 5.534\%$, respectively after MSCs were induced in medium containing GDNF, IL-1β, mesencephalic glial-cell-conditioned medium and flash-frozen mesencephalic membrane fragments for 15 days.   **Conclusion**   MSCs can differentiate into dopaminergic neuron-like cells and are a new cell source for the treatment of neurodegeneration diseases and have a great potential for wide application

Key words: Mesenchymal stem cells (MSCs); Dopaminergic neuron-like cells (DA neuron-like cells); Differentiate

INTRODUCTION

The therapy of Parkinson's disease is difficult, so people have been exploring more effective methods. Drugs can only treat symptoms of the diseases, and brain tissue transplantation has been the most prospective way, but the sources of transplantation cells need to be explored. The people have a new viewpoint of the central nervous system (CNS) regeneration and the therapy of CNS diseases[1,2] because of the recent discovery of stem cell populations in CNS. But adult neural stem/progenitor cells can not be obtained easily, and it is dangerous to get neural stem cells from the living body; at the same time rejections

often occurr, so a new cell resource will have to be explored. MSCs in bone marrow derived from mesoderm. MSCs can not only differentiate into osteoblasts, chondrocytes and adipocytes and so on, but also generate skeletal muscle, cardiac muscle and hepatocytes, as well as glia and neurons[3-7]. This supplies a new way for therapy of neurodegenerative diseases like Parkinson's disease. MSCs may be useful in the treatment of a wide variety of neurologic diseases, and possess significant advantages over other stem cells. They are easily available and can overcome the risks of obtaining neural stem cells from the brain. They are also immunologically inert and their autologous transplantation can overcome

the ethical and immunologic concerns associated with the use of fetal tissues.

But the researches of differentiation of MSCs into nerve cells *in vitro* have just begun. It is very important to induce MSCs to differentiate into specific neuronal cell types with function for therapy of CNS diseases like Parkinson's disease. Therefore, we explored the way to induce MSCs to differentiate into dopaminergic neuron *in vitro* for the purpose of supplying a continuous cell source for the treatment of CNS neurodegenerative diseases like Parkinson's disease.

METHODS

Main Reagents and Animals

DMEM medium was purchased from GibcoBRL, mouse monoclonal NSE and MAP-2a, b antibody were purchased from Neomarkers, rabbit polyclonal TH antibody (gift from Dr. Yu Shun, Beijing Institute of Geriatrics, Xuanwu Hospital of Capital University of Medical Sciences), mouse monoclonal nestin antibody was purchased from Chemicon (gift from Prof. CHEN Dong at Department of Histology and Embryology of School of Basic Medical Sciences, Jilin University). SP and DAB kit were purchased from Fuzhou Maixin Company. Recombinant human GDNF and recombinant rat interleukin-1 β were purchased from Strathmann Biotec AG. Peroxidase-conjugated goat anti-mouse IgG was purchased from Beijng Zhongshan Biotechnology Company. Western blot kit was purchased from Wuhan Boshide Company. Six-week-old Wistar rats and newborn Wistar rats were purchased from the Center of Experimental Animals of Jilin University.

Rat Bone Marrow MSCs Culture

Rat bone marrow cells were collected, after sacrifice of 6-week-old rats from femurs and tibias by flushing the shaft with DMEM using a syringe. Cells were disaggregated by gentle pipetting several times and centrifuged for 10 min at 1 000 r/min and supernatant was removed, then resuspended in DMEM supplemented 10% FCS, and plated in a 75 mL flask at 4×10^6 cells/mL. Medium consisted of DMEM supplemented with 10% FCS was added and replaced every other 3 or 4 days. When cells grew to 70%-90% confluences, they were passaged with 0.25% trypsin.

Identification of MSCs Multipotency

Passaged MSCs were divided into two groups and plated onto Costar 24 well plates where poly-L-lysine-coated coverglasses were placed. (1) MSCs differentiated into chondrocytes: MSCs in experimental groups were cultured in induction medium containing IMDM, 10 ng/mL TGF-β1, 10^{-7} mol/L desamethasone, 50 mg/L Vitamin C, 10% FCS. MSCs in control groups were cultured in IMDM containing 10% FCS. After 7 days, the coverglasses were stained by toluidine blue and immunocytochemistry for collagen Ⅱ. (2) MSCs were differentiated into adipocytes: MSCs in experimental groups were cultured in induction medium containing DMEM, 10 mg/L insulin, 10^{-6} mol/L desamethasone, 10% FCS. MSCs in control groups were cultured in DMEM containing 10% FCS. After 10 days, the coverglasses were stained by Sudan IV.

Differentiation of Bone Marrow MSCs into Dopaminergic Neuron-like Cells

Mesencephalic neuronal cell culture and membrane fragments preparation　The heads of new-born Wistar rats were broken and meninges were removed as described previouslyby Ling Z. P.[8]. The mesencephalon tissue was dissected out, and treated with 0.25% trypsin. Cells were passed through a 30-μm nylon mesh. Then cells were plated onto Costar 6 well plates at 2×10^6 cells/mL. After 3 days, the medium was removed, and 2 mL complete medium containing DMEM, 10% FCS, 2.2 g/L NaHCO₃, 100U/mL benzylpenicillin, 100 U/mL streptomycin,10 mmol/L HEPEs and 100 U/L insulin was added. The cultures were then placed in a -80℃ freezer for 1 h and thawed at room temperature three successive times. Using a Pasteur pipette, 2mL complete medium in each well was drawn and expelled from the pipette several times to place the membrane fragments in suspension. The membrane fragments were then collected and the fragments from one primary culture were added to nine MSCs cultures (10%v/v).

Mesencephalic glia cultures and conditioned medium　The mesencephalons of 3 to 4 days old new-born Wistar rats were dissected out. Cells were plated at 1×10^6 cells/mL and passaged using 0.25% trypsin. Three days later, the glia-conditioned medium was collected, and added into MSCs cultures where the glia-conditioned medium accounted for 15%.

Differentiation of bone marrow MSCs into dopaminergic neuron-like cells　Two to four passaged MSCs were plated onto Costar 24 wells plates where poly-L-lysine-coated coverglasses and 75 mL flasks were placed. MSCs in the control groups were not treated. MSCs in the experimental groups were cultured in the medium containing 0.5 mmol/L IBMX for 2 days. Then medium was replaced with

induction medium containing the following agents GDNF (10 ng/mL), IL-1β (100 pg/mL), GDNF (10 ng/mL)+IL-1β (100 pg/mL) abbreviated to "G+I", GDNF (10 ng/mL)+IL-1β (100 pg/mL)+mesencephalic glial-cell-conditioned medium abbreviated to "G+I+conditioned medium", GDNF (10 ng/mL) +IL-1β (100 pg/mL) + mesencephalic glial-cell-conditioned medium+flash-frozen mesenceph- alic cellular fragments abbreviated to "G+I+conditioned medium+ fragments". After MSCs were treated by IBMX for 2, 7, and 15 days by induction medium, the coverglasses were taken out and the surface markers of neurons, such as NSE, nestin, MAP-2a, b and TH were detected by immunocytochemistry. MSCs in 75 mL flasks were collected, and the surface markers of the differentiated cells, such as NSE and MAP-2a, b, were detected by Western blot after MSCs were cultured in induction medium for 15days.

Immunocytochemistry   In all studies the cultures were fixed for 20 min with 95% ethanol and immunocytochemistry was performed. The cultrues were incubated for 10 min with a solution of 0.2% Triton X-100, blocked for 30 min with an endogenous peroxidase blocking solution and normal nonimmune serum, and then incubated with a primary antibody overnight at 4℃. Sequential 30-min incubation with biotin-conjugated second antibody and streptavidin-peroxidase was performed. The immunoreactivity was developed for approximately 1 min incubation using 3-3′diaminobenzidine. The primary antibodies used were nestin (1:800), NSE and MAP-2a, b (1:100), TH(1:500). The number of immunoreactive cells was counted. Primary antibody replaced with PBS was used as the negative control, and cultured neurons as the positive control.

Western blot   Cultures were washed in 800 μL TEN (40 mmol/L Tris·Cl pH7.5, 1 mmol/L EDTA, 150 mmol/L NaCl), the samples were centrifuged at 3 000 r/m for 5min, and supernatants were removed. Cell pellet was lysed in an ice-cold lysis buffer containing 10 mmol/L Tris·Cl pH 7.4, 1 mmol/L $MgCl_2$, 0.5%NP40, 20 μg/mL DNase I. After incubation on ice for 10 min, the samples were centrifuged at 5000 r/min for 5 min and supernatants were collected. An aliquot was separated by SDS-PAGE (10% for NSE antibody, 6% for MAP-2a, b antibody), and transferred electrophoretically to nitrocellulose filters. Nonspecific binding of antibody was blocked with blocking solution for 2 h at room temperature. Immunoblotting was carried out with NSE (1:100) or MAP-2a, b (1:100) for 2 h at 37℃, followed by peroxidase-conjugated secondary anti-immunoglobulin antibodies. The membranes were processed using DAB test kit.

Statistical Treatment

t test was performed on immunocytochemical cell counts using SPSS software.

RESULTS

Microscopy

Most of the cells adhered to the flask after they were plated for 72 h, and took on fibroblast-like shape. The medium was changed 4-5 d later. The cell clones formed 5-6 d and cells grew to confluence over 15 d.

Identification of MSCs Mulipotency

Chondrocyte   In vitro marrow MSCs differentiated into chondrocytes. In the control groups, cells adhered to the flask fast. MSCs cultured in induction medium proliferated slowly and showed cellular rounding with cell cluster aggregation. Cellular form changed into triangle and polygon. Toluidine blue metachromatic staining of differentiated chondrocytes was positive (Fig. 1). Additionally, many differentiated chondrocytes in the experimental groups expressed collagen type II, whereas only several cells were positive in the control groups (Fig. 2).
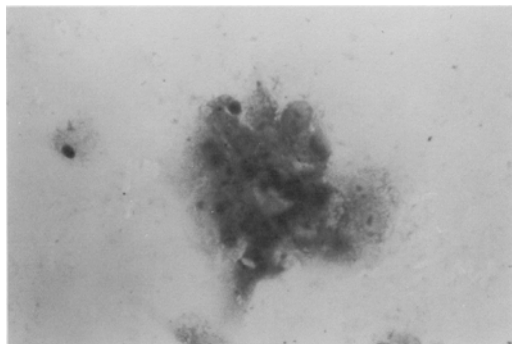

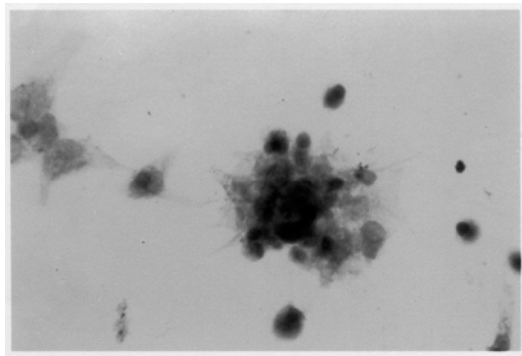FIG. 1. MSCs differentiated into chondrocytes (toluidine blue stain, 400×).

FIG. 2. MSCs differentiated into chondrocytes (immunocytochemistry, 400×).

Adipocyte *In vitro* marrow MSCs differentiated into adipocytes. Multiple induction treatments resulted in lipid-rich vacuoles accumulated within experimental cells, lipid vacuoles continued to develop with time, coalesced in the cell. MSCs changed into round and triangle shapes. After Sudan staining, lipid droplet was scarlet red, nuclei were blue and there were many different size lipids in the cells (Fig. 3). In the control groups, there was no lipid in cells or there were lipids in several cells.
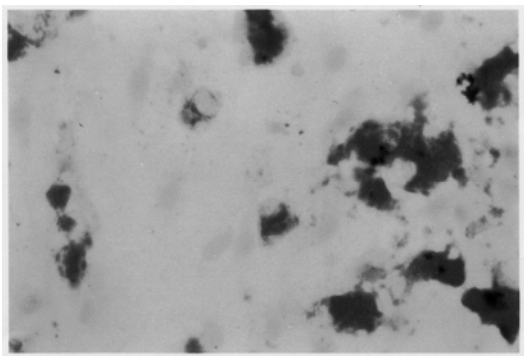

FIG. 3. MSCs differentiated into adipocytes (sudan IV stain, 400×).

Bone marrow MSCs differentiated into neural progenitors and early neuron-like cells MSCs were induced to differentiate into neural progenitors and early neuron-like cells in culture by incubation with 0.5 mmol/L IBMX for 2 d. The results of immunocytochemistry showed that the percentage of nestin-positive cells in the control groups (25.206± 3.391)% was significantly less than that in the experimental groups induced for 2 days ((56.904± 4.684)%, *P*<0.01, *t*=12.257). It showed that MSCs differentiated into neural progenitors. In the control groups, the NSE expression of MSCs was very low. The expression of NSE was intense after being induced for 2 days. There were no MAP-2a, b-immunoreactive cells and TH-immunoreactive cells in the control and experimental groups during experiment.

Differentiation of Bone Marrow MSCs Into Dopaminergic Neuron-like Cells

Morphology The morphology of differentiated cells changed after induction agents, especially mesencephalic glial-cell-conditioned medium+flash-frozen mesencephalic cellular fragments, were added for 30 min. Two to three days later, in the experimental groups, many cells exhibited contracted cell bodies, and some of them transformed into spindle-shaped cells with short processes. Some smaller round cells have no processes, some others have bipolar proce-sses. In the control groups the form of untreated MSCs

had no changes.

Immunocytochemistry In this experiment, the morphology of MSCs did not change and MSCs did not express MAP-2a, b and TH after being induced by IL-1β or GDNF, so the results about the two experimental groups were not discussed. The analysis of immunocytochemistry showed that there were various numbers of NSE, MAP-2a, b and TH-positive cells in the other experimental groups. The bodies of round or oval- shape positive cells were smaller than those of negative cells.

A small part of MSCs expressed NSE before induction by immunocytochemistry. The NSE⁻ positive cells increased after induction (Fig. 4). There were no MAP-2a, b-positive cells in the control groups, whereas MAP-2a, b-positive cells appeared in the experimental groups (Fig. 5). There were no TH-positive cells in the control group. Only few of TH-positive cells began to appear after having been cultured in G+I+conditioned medium for 15 d and TH-positive cells significantly increased after incubation in the G+I+conditioned medium+fragments group for 7 d and 15 d (Fig. 6), and there were no TH-positive cells in the other experimental groups. The results are summarized in Tables 1 and 2.
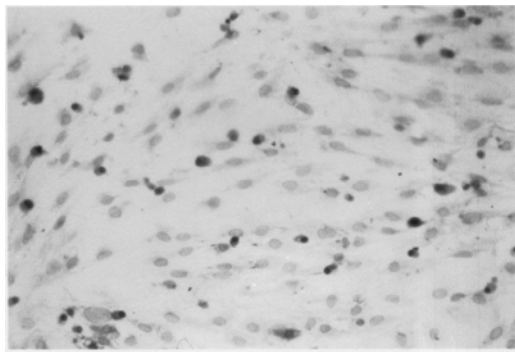

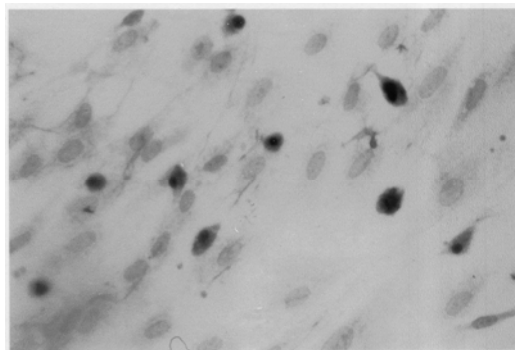FIG. 4. NSE-positive cells increased after MSCs were induced for 15 d (immunocytochemistry, 200×).

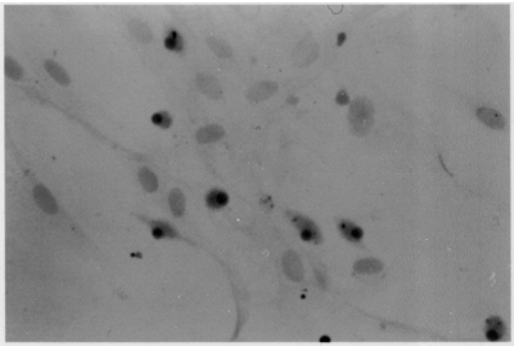FIG. 5. MAP-2a,b-positive cells increased after MSCs were induced for 15 d (immuno- cytoche-mistry, 400×).

FIG. 6. TH-positive cells increased after MSCs were induced for 15 d (immunocytochemistry, 400×).

Western blot   Western blot of proteins in cytoplasm of the untreated control group, G+I+ conditioned medium group and G+I+conditioned medium+ fragments group demonstrated the presence of proteins NSE (Fig. 7). There was no protein of MAP- 2a, b in the control medium group (Fig. 8).

DISCUSSION

The bone marrow MSCs used in this experiment were cultured and passaged. MSCs used in this experiment had multipotency, which was indirectly proved by the induction to differentiate into three kinds of cells, such as adipocytes, chondrocytes and neuron-like cells and resolved the question that MSCs can only be identified by surface markers.

The specific markers were different at different stages of CNS development. In this experiment, several specific markers at several neuron developmental stages were selected to explore differentiation of marrow MSCs into neurons, such as nestin, neural stem/ progenitor cell characteristic marker, NSE, (an early marker for neuron), MAP-2a, b (a marker for mature neurons), and TH (a marker for DA neurons).

In this experiment, MSCs differentiated into progenitors of neural cells after being induced for 2 d by IBMX. Then the medium was replaced with an induction medium containing GDNF, IL-1β, mesencephalic glial-cell-conditioned medium and flash-frozen mesencephalic cellular fragments. The results showed: (1) There were no MAP-2a,b-positive and TH-positive cells when MSCs were incubated in the induction medium only containing IL-1 β or GDNF. (2) MSCs began to express MAP-2a,b when MSCs were incubated in the induction medium containing both IL-1β

TABLE 1

Differentiated Neurons Expressed NSE After MSCs Were Induced for Different Time in Different Induction Medium ($\bar{x} \pm s$, %)

Day	Control	G+I	G+I+Conditioned Medium	G+I+Conditioned Medium+Fragments
7 d	7.904±0.396	15.658±0.682 [a]	22.142±2.506 [a, b]	25.608±1.193 [a, b]
15 d	8.676±0.684	20.060±0.790 [a, d]	27.744±1.450 [a, c, d]	30.032±2.489 [a, d]

Note. [a]$P<0.01$ *vs* control group. [b]$P<0.05$. [c]$P<0.01$ comparison of neighbor two groups. [d]$P<0.01$ compared with groups of 7 d and 15 d.

TABLE 2

Differentiated Neurons Expressed MAP-2a,b and TH After MSCs Were Induced for Different Time in Different Induction Medium ($x \pm s$, %)

Day	MAP-2a, b			TH
	G+I	G+I+Conditioned Medium	G+I+Conditioned Medium+Fragments	G+I+Conditioned Medium+Fragments
7 d	15.786±1.265	28.616±2.364[a]	32.660±2.749[a, b]	6.626±0.754
15 d	17.364±5.738	34.602±2.252[a,c]	41.580±5.101[a, b, c]	34.958±5.534 [c]

Note. [a]$P<0.01$ *vs.* G+I group. [b]$P<0.05$ compared with groups of G+I+conditioned Medium group and G+I+conditioned Medium+ fragments group. [c]$P<0.01$ compared with groups of 7 d and 15 d.
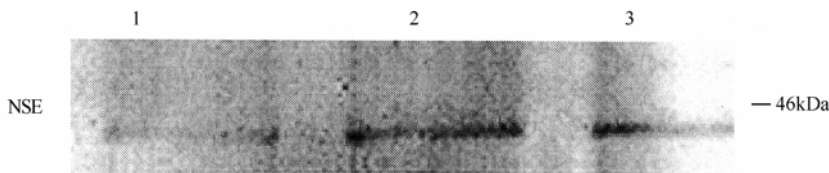

FIG. 7.   Western blot NSE:1. Control group, uninduced MSCs; 2. Induction medium containing GDNF (10 ng/mL), IL-1β (100 pg/mL), mesencephalic glial-cell-conditioned medium; 3. Induction medium containing GDNF (10 ng/mL), IL-1β (100 pg/mL), mesencephalic glial-cell-conditioned medium, flash-frozen mesencephalic cellular fragments.
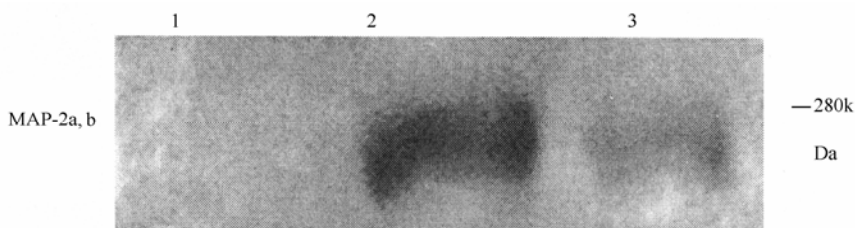
FIG. 8. Western blot MAP-2a, b:1. Control group, uninduced MSCs; 2. Induction medium containing GDNF (10 ng/mL), IL-1β (100 pg/mL), mesencephalic glial-cell-conditioned medium, flash-frozen mesencephalic cellular fragments; 3. Induction medium containing GDNF (10 ng/mL), IL-1β (100 pg/mL), mesencephalic glial-cell-conditioned medium.

and GDNF. The number of MAP-2a, b-positive cells increased with the time. But there were no TH-positive cells. At the same time, NSE-positive cells significantly increased. (3) Few of TH-positive cells began to appear after being cultured in the medium containing IL-1, GDNF and mesencephalic glial-cell-conditioned medium for 15 days. MAP-2a.b-positive cells and NSE-positive cells significantly increased. (4) The percentage of TH-positive cells significantly increased after MSCs were cultured in the induction medium containing GDNF, IL-1β, mesencephalic glial-cell-conditioned medium and flash-frozen mesencephalic cellular fragments. TH-positive cells significantly increased over the time. TH-positive cell counts for 15 days were approximately 84% of the MAP-2a, b-positive cell counts.

Mesencephalic glial-cell-conditioned medium contained many cytokines from glia, such as IL-1, IL-2, IL-3, IL-6, IFN-α, IFN-γ, TNF-α, TNF-β, transforming growth factor-β, colony-stimulating factors, and etc.[9]. Traditionally, cytokines played an important role in the initiation, regulation and suppression of immune and inflammatory responses occurring in peripheral tissues[10]. Now it has been found that these proteins are also synthesized in the central nervous system (CNS) and have direct or indirect actions on various neural cells, and affect the neuronal development and maturation[11]. During normal brain development, cytokines such as IL-6, IL-1, and TNF could express and take part in the regulation of brain development[12-15]. Furthermore, some researches revealed that IL-1 could prolong the survival of DA neurons, increase the DA activity and induce DA neuron phenotype development, etc.[13]. GDNF has been shown to prolong the survival and morphological differentiation of DA neurons, and to increase primary DA neuron number and their high-affinity dopamine uptake[16]. It was estimated that various cytokines and the unknown trophic activity found in mesencephalic glial-cell-conditioned medium could interact each other, initiate signal path of DA neuron differentiation, synthesize and express MAP-2a, b and TH. MAP-2a, b is a marker for

mature neuron. Low-molecular weight MAP-2c expressed at the early development stage, but high-molecular weight MAP-2a, b expressed at the late developmental stage[17]. MAP-2a, b could play an important role in neuronal development, differentiation and plastic[18].

TH-positive cells increased in G+I+conditioned Medium+fragments group after flash-frozen mesencephalic cellular fragments were added for 7 days (6.626%) and 15 days (34.958%), indicating that flash-frozen mesencephalic cellular fragments played a key role in differentiation process. It could be presumed that cellular fragments provided extracellular matrix proteins and environment agent for MSCs differentiating into DA neurons. It is now firmly established that ECM profoundly influences the major cellular programmes of growth, differentiation and apoptosis and regulates the transcription of genes associated with specialized differentiating functions. The question which of these programmes a cell would select could be ultimately determined by the composition of the surrounding ECM[19]. In this experiment, differentiated DA-like cells had no long process, because the time was too short, and /or the additional cytokine increasing process should be extended.

It is still unclear by what mechanism MSCs differentiate into neurons. We think the autocrine cytokines of MSCs, the added cytokines, the cytokines produced from glia provided by mesencephalic glial-cell-conditioned medium would interact each other, which ultimately results in genetic reprogramming of the MSCs nuclei and activates the initiation of nerve genetic reprogramming of ectoderm, and as a consequence MSCs can differentiate into neuron-like cells and dopaminergic neuron-like cells, but such hypothesis needs to be explored further.

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