J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-010-0113-5 30(1):75-80,2010

Neurogenic Differentiation of Murine Adipose Derived Stem Cells Transfected with EGFP *in vitro**

Zhong FANG (方 忠)[1], Qin YANG (杨 琴)[2], Wei XIONG (熊 伟)[1], Guanghui LI (李光辉)[1], Jun XIAO (肖 骏)[1], Fengjing GUO (郭风劲)[1], Feng LI (李 锋)[1], Anmin CHEN (陈安民)[1#]
[1]*Department of Orthopedics,* [2]*Department of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

©Huazhong University of Science and Technology and Springer-Verlag Berlin Heidelberg 2010

Summary: Some studies indicate that adipose derived stem cells (ADSCs) can differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells *in vitro*. However, whether ADSCs can be induced to differentiate into neural cells *in vitro* has not been clearly demonstrated. In this study, the ADSCs isolated from the murine adipose tissue were cultured and transfected with the EGFP gene, and then the cells were induced for neural differentiation. The morphology of those ADSCs began to change within two days which developed into characteristics of round cell bodies with several branching extensions, concomitantly expressing EGFP fluorescence. Approximately 60% of the total cell populations were bipolar or multipolar in shape. Some of them appeared to make contact with their neighboring cells. RT-PCR, Western blot and Immunocytochemistry revealed that the expression levels of the markers of neurons and oligodendrocytes such as MAP2, NF-70, Neu N and RIP upon neural induction were increased, but the expression of the special marker of astrocytes, GFAP, was undetectable until 96 h after induction when a small signal was observed. It was concluded that the ADSCs transfected with EGFP possessed the ability to undergo morphologic and phenotypic changes consistent with neural differentiation *in vitro*. It suggests that these cells might provide an ideal source for further stem cell research with possible therapeutic application for spinal cord injury.
Key words: adipose derived stem cells; enhanced green fluorescent protein; neural induction; spinal cord injury

Spinal cord injury (SCI) is a severe neurological disorder which results from damage or severance of axons, loss of neurons and glia, and demyelination. Due to its complicated pathophysiology of SCI, various combination strategies, such as the regeneration of neuron, neuroprotection from second injury, and enhancement of axonal regrowth, are required for SCI repair.

Cell transplantation provides great promise for treating SCI and has achieved great success in experimental investigations[1], but this technique is hindered by the choice of donors. Embryonic stem cells, bone marrow stromal cells (BMSCs), and neural stem cells should be confronted by the insufficient cell population[2], problems of histocompatibility, inadequate tissue supply, and ethical concerns exist[3, 4], and so on.

Isolation of adipose derived stem cells (ADSCs) from adipose tissue was reported firstly by Zuk[5]. These cells show many advantages as a donor as they can be easily obtained in ubiquitously distributed adipose tissue with little harm to patient and are expandable *in vitro*[5-9]. Some studies indicate that ADSCs can differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells *in vitro* under specific culture conditions[10-12]. However, whether ADSCs can be induced to differentiate into neural cells *in vitro* and have positive effects on the SCI when they are transplanted has not been clearly demonstrated[13-15]. This study aimed to shed some light on the application of ADSCs to neural differentiation.

1 MATERIALS AND METHODS

1.1 Experimental Procedures

1.1.1 Animals All animal procedures were approved by Animal Use and Care Committee of HUST (China). Healthy Sprague-Dawley rats (10 to 12 weeks old) of both sexes were used for these studies. Activities related to animal care were performed according to standard operating procedures accepted by Chinese National Animal Research Center.

1.1.2 Preparation of Recombinant Adenoviral Constructs The AdEasy Vector System was used for construction of the EGFP adenoviral vector. The transfer vector pShuttle-EGFP was constructed using standard method. This vector contained EGFP reporter gene derived from pEGFP-C. pShuttle-EGFP was linearized with *Pme* Ⅰ and separately co-transformed into the competent *E. coli* strain BJ5183 together with pAdeasy-1, the viral DNA plasmid. Briefly, 1 μg of linearized recombinant transfer vector

Zhong FANG, E-mail: hbycfz@yahoo.com.cn
#Corresponding author, E-mail: hbwhlf@yahoo.com.cn
*This project was supported by the "Eleventh Five-Year" plan to support the National Science and Technology key project(No. 2007BAI04B07) and a grant from National Natural Sciences Foundation of China (No. 30772206)

pShuttle-EGFP (5 μL) and 1.0 μL of pAdEasy-1 vector (100 μg/μL) were added to 200 μL competent-BJ5183 cells in a 14-mL culture tube. These components were mixed gently and incubated on ice for 1 h, heat-shocked at 42°C for 1 min and then immediately returned to ice for 5 min. Subsequently, 1000 μL LB media were added and the cells were shaken (280 r/min) and incubated for 1 h at 37°C. Cells were plated onto 100 mm Petri dishes and incubated overnight at 37°C. The recombinant clones (pAd5-EGFP) were identified by restriction enzyme analysis.

The pAdeasy-1 is E1 and E3 deleted, and its E1 function can be complemented in 293A cells. The recombinant adenoviral construct, pAd5-EGFP, was then cleaved with *Pac* Ⅰ to expose their inverted terminal repeats and transfected into 293A cells to produce viral particles. The recombinant viruses were identified with PCR, RT-PCR, immunocytochemical staining and Western blot. The Ad5-EGFP construct was purified through two cesium chloride gradients, and the purified virus was desalted by dialysis at 4°C against 10 mmol/L Tris-HCl buffer with 4% sucrose. Virus was stored in aliquots in liquid nitrogen. The titer of the viral preparations was determined using Adeno-X^{TM} Rapid Titer Kits.

1.1.3 Isolation and Culture of Rat ADSCs Rat adipose tissue from the inguinal region was obtained under local anesthesia. The raw adipose tissue was processed according to established method to obtain a stromal vascular fraction[7]. To isolate stromal cells, samples were washed extensively with equal volumes of phosphate-buffered saline (PBS), and digested at 37°C for 30 min with 0.075% collagenase Ⅰ (Sigma, USA). Enzyme activity was neutralized with α-modified eagle's medium (DMEM) (Invitrogen, USA) containing 10% FBS and centrifuged at 1200 g for 10 min to obtain a high-density cell pellet. The pellet was resuspended in red blood cell (RBC) lysis buffer (Biowhittaker, USA) and incubated at room temperature for 10 min to lyse contaminating RBCs. The stromal cell pellet was collected by centrifugation, as described above, and incubated overnight at 37°C in 5% CO_2 in DMEM containing 10% FBS.

1.1.4 Adenoviral Transfection of Rat ADSCs Third passages ADSC cultures were prepared for transfection. In brief, trypsin dissociated cells were resuspended in DMEM containing 10% PDHS and plated onto poly-*L*-lysine-precoated culture dishes at a density of 3×10^6/35-mm dish or 24-well plates at a density of 1.5×10^6/well. Cells were incubated at 37°C in a humidified incubator with 5% CO_2/95% air. On the day 3, cells were treated with 10 μmol/L β-cytosine arabinoside in DMEM containing 10% PDHS. After 2 days of treatment with ARC, the culture media were replaced with DMEM containing 10% PDHS and cultures were grown for an additional 7–9 days before being used in experiments. At this time, Ad5-EGFP was added directly to the growth media (5×10^7 infectious units per well or 1×10^8 infectious units per dish) and 3 days later the expression of EGFP was analyzed.

1.1.5 Generation of Neurospheres From ADSCs Undifferentiated ADSCs cultured at high densities spontaneously formed spherical clumps of cells that were isolated in 0.25% trypsin (Invitrogen, USA). We also collected free floating neurospheres that were released from the cell culture surface into the culture media. The spheres of cells were transferred to a Petri dish and cultured in Neurobasal medium (Invitrogen, USA) supplemented with B27 (Invitrogen, USA), 20 ng/mL bFGF, and 20 ng/mL EGF (Sigma, USA) for 4–7 days. The culture density of the spheroid bodies was maintained at 10–20 cells/cm^2 to prevent self aggregation.

1.1.6 *In vitro* Differentiation of ADSCs to Neural Cells For neural lineage differentiation, neurospheres derived from ADSCs were layered on PDL-laminin double-coated chamber slide. Spheres were cultured and maintained for 10 days in NB media containing only the B27 supplement. 70% of the media was replaced every 4 days. These cells were examined 10 days after differentiation by reverse transcription polymerase chain reaction (RT-PCR), immunocytochemistry and Western blot. All data to be shown were representative of at least three different experiments.

1.1.7 RT-PCR Analysis of Differentiated Neural Cells To assess the efficiency of neural differentiation of ADSCs and compare the levels of expression of nestin, microtubule associated protein-2 (MAP2), neurofilament-70 (NF-70), neuron-specific nuclear protein (Neu N), receptor interacting protein (RIP), and glial fibrillary acid protein (GFAP) after neural differentiation, quantification was performed using RT-PCR. Total cellular RNA was isolated with Trizol (Invitrogen, USA) and reversely transcribed into first strand cDNA using oligo-dT primer and amplified by 35 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) of PCR using 20 μmol/L of specific primers. PCR amplification was performed using the primer sets. All primer sequences (table 1) were determined using established human GenBank sequences. Duplicate PCR were amplified using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for assessing PCR efficiency and for subsequent analysis by 1.5% agarose gel electrophoresis.

Table 1 Primer sequences and cycling conditions in RT-PCR

Genes	Length of amplified genes (bp)	Primer sequences (forward/reverse)	Cycles
Nestin	220	GCCCTGACCACTCCAGTTTA/GGAGTCCTGGATTTCCTTCC	33 cycles: 30 s, 94°C 30 s, 55°C 30 s, 72°C
MAP2	774	TCAGACTTCCACCGAGCAG/AGGGGAAAGATCATGGCCC	33 cycles: 30 s, 94°C 30 s, 55°C 30 s, 72°C
NF-70	333	TGGGAAATGGCTCGTCATTT/CTTCATGGAAGCGGCCACTT	30 cycles: 30 s, 94°C 60 s, 55°C 90 s, 72°C
Neu N	329	TGAACACAGACGCTATGCGCTCAG/CACCTTTATGTGAGTGGACACAGA	30 cycles: 60 s, 94°C 60 s, 60°C 120 s, 72°C
IP	349	ACACGACACCGGATAAACCAA/CGAGTGCGCACCTCGGTA	25 cycles: 30 s, 94°C 30 s, 60°C 30 s, 72°C
GFAP	200	ACCAGGACCTGCTCAATGTC/ATCTCCACGGTCTTCACCAC	30 cycles: 30 s, 94°C 30 s, 55°C 30 s, 72°C
GAPDH	347	GCCAAAAGGGTCATCATCTCTG/CATGCCAGTGAGCTTCCCGT	25 cycles: 30 s, 96°C 60 s, 58°C 30 s, 74°C

1.1.8 Immunocytochemistry of Neural Differentiated Cells For analysis of neural differentiation of ADSC neurospheres, differentiated cells were fixed with 4% paraformaldehyde, and incubated with 10% goat serum to prevent nonspecific antibody binding. The cells were incubated overnight at 4°C with several species-specific monoclonal

antibodies against neuronal or glial cell proteins. After extensively washing in PBS, the cells were incubated for 30 min with FITC conjugated secondary antibodies (1:250, Molecular Probe, USA). Controls in which primary antibodies were omitted or replaced with irrelevant IgG resulted in no detectable staining. Specimens were examined using a Leica TCS SP2 laser scanning microscope equipped with three lasers (Leica Microsystems, USA). Immunocytochemical studies were repeated at least three times.

1.1.9 Western Blot Analysis of Differentiated Cells Protein extracts were prepared from undifferentiated or differentiated ADSCs by the treatment of lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin and 1 mmol/L sodium orthovanadate. Total protein (30–40 μg/mL) was resolved on 12.5% acrylamide gel and electroblotted onto polyvinyldiethylfluoride (PVDF) membrane. The blot was probed with rat anti-nestin (1:500), anti-MAP2 (1:500), anti-Neu N (1:500), anti-NF-70 (1:500), anti-RIP (1:500), and anti-GFAP (1:1000) antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated anti-rat IgG antibodies (Amersham, USA) and visualized by enhanced chemiluminescence (Amersham, USA).

1.2 Statistical Analysis

Data were presented as $\bar{x}\pm s$. Statistical analyses were performed using one way repeated measure ANOVA (using SigmaStat 3.1, SigmaPlot 9.0). Student's *t*-test was used to compare the results between two groups at each time point. $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Isolation and Morphology Characterization of ADSCs after Transfected with EGFP Gene

The inguinal fat pads from each rat yielded approximately 5×10^6 nucleated cells. When plated, the primary ADSCs from these rats exhibited a heterogeneous population of spindle-shaped cells morphologically, among which a few lipid droplets precipitated (fig. 1A). Within two passages after the initial plating of the primary culture, ADSCs appeared as a monolayer of flat fibroblast-like cells (20–30 μm in diameter) (fig. 1B), which was consistent with the results of previous reports. These EGFP-transfected ADSCs exhibited bright green fluorescence under fluorescent microscope (fig. 1C).

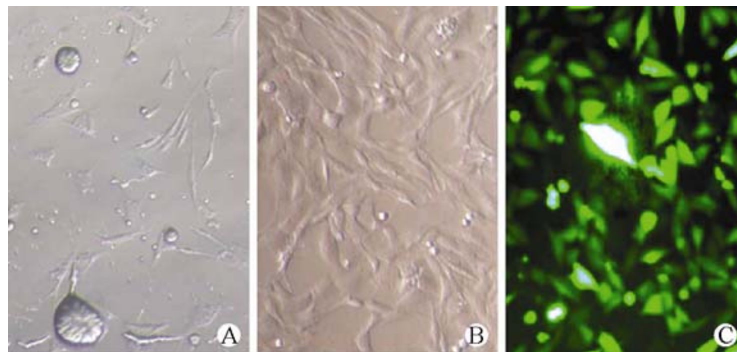

Fig. 1 (A) The primary ADSCs exhibited a heterogeneous population of spindle-shaped cells morphologically; (B) ADSCs appeared as a monolayer of flat fibroblast-like cells; (C) ADSCs transfected with EGFP reporter gene showed an elongated fibroblastic appearance with concomitant EGFP fluorescence under fluorescent microscopy (×400)

2.2 Morphological Changes of ADSCs upon Neural Induction

Following the neural induction, the morphology of ADSCs began to change within two days. The cells developed into characteristics of round cell bodies with several branching extensions (fig. 2A), concomitantly expressing GFP fluorescence (fig. 2B). Approximately 60% of the total cell populations were bipolar or multipolar in shape. Some of them appeared to make contact with their neighboring cells (fig. 2C).

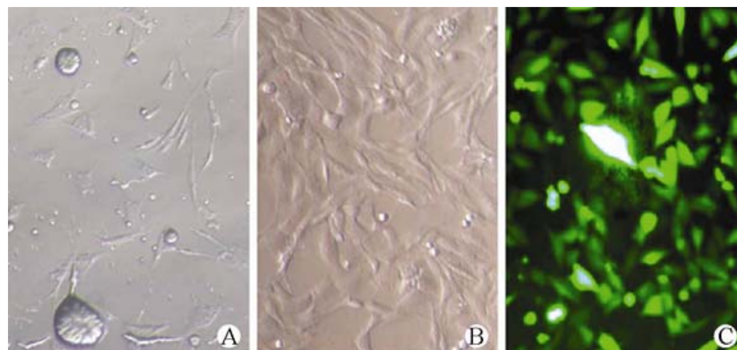

Fig. 2 (A) A light microscopic image shows that ADSCs developed characteristics of round cell bodies with several branching extensions 48 h after neural induction; (B) A fluorescence microscopic image of the cells with branching extensions retaining EGFP fluorescence; (C) Some of induced ADSCs appeared to make contact with their neighboring cells under fluorescence microscope (×400)

2.3 Expression of Neural Markers in Differentiated ADSCs

To fully characterize the differentiated ADSCs after the neural induction, RT-PCR, Western blot and immunocytochemistry for specific antigens indicative of neural cell lineages from 0 to 4 days were performed. The data from RT-PCR (fig. 3A) analyses indicated the expression of nestin was at a high degree early after induction, but decreased remarkably after 96 h of induction. Furthermore, the expression of nestin in undifferentiated ADSCs was also confirmed. The increased expression of the markers of neurons and oligodendrocytes such as MAP2, NF-70, Neu N and RIP upon neural induction was confirmed; at the 96th h, their expression all had shown a high level. However, the expression of the special marker of astrocytes, GFAP, was undetectable until 96 h after induction when a small signal was observed. Western blot analysis (fig. 3B) revealed the same results as the RT-PCR. Immunofluorescence was used to detect changes of phenotypes after the differentiation of ADSCs(fig. 4A-H). After the neural induction, most of the ADSCs with bipolar processes expressed nestin (fig. 4F), while those with multipolar processes tended to be stained with more MAP2 (fig. 4C), but less nestin. RIP-positive cells could be easily seen under a microscope (fig. 4G). However, only a few GFAP-positive cells were observed (fig. 4B and D).

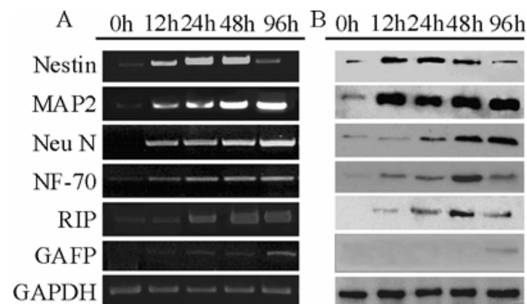

Fig. 3 (A) Expression pattern of various neuronal genes in differentiated ADSCs. RT-PCR showed an increasing trend in levels of MAP2, NF-70, NeuN, and RIP mRNA at different days of cells induction. The nestin expression was apparently down-regulated. GFAP was weakly expressed only at the 96th h. (B) Western blot analysis of the expression of various special markers in differentiated ADSCs. The protein levels of MAP2, NF-70, NeuN, and RIP were up-regulated at the whole phases of neuronal commitment. The expression of Nestin apparently showed a down-regulated profile. Low levels of GFAP protein were detected after 4 days of cell induction

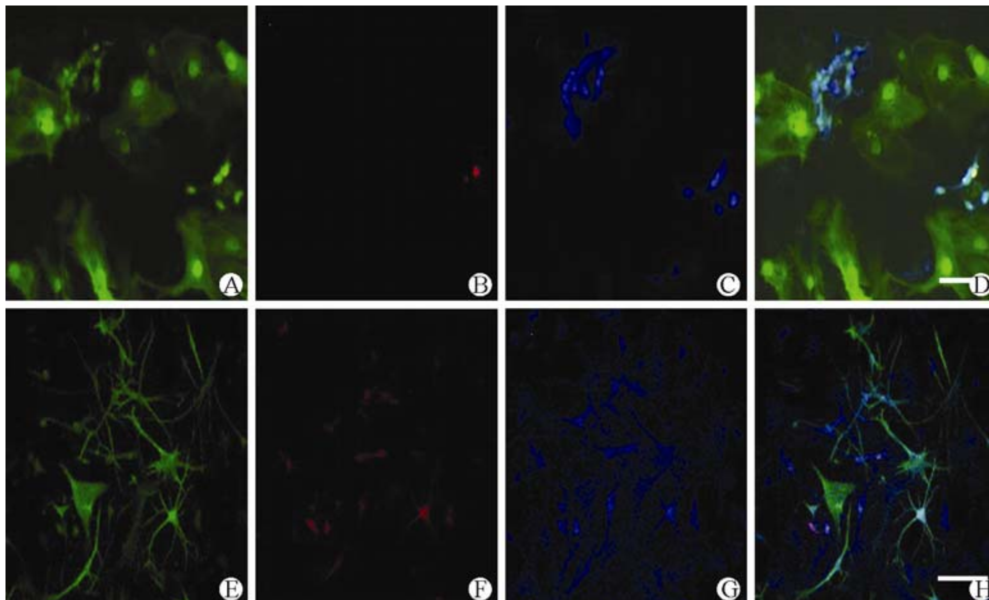

Fig. 4 Immunocytochemical staining for special phenotypic markers after neuronal induction
(A) and (E) EGFP (green); (B) Anti-rat GFAP (red); (C) MAP2 (blue); (D) Photomicrographs of ADSCs after differentiation *in vitro* immunostained with selective phenotypic markers: triple-stained with EGFP (green), GFAP (red), and MAP2 (blue); (F) nestin (red); (G) RIP (blue); (H) Photomicrographs of ADSCs after differentiation *in vitro* immunostained with selective phenotypic markers: triple-stained with EGFP (green), nestin (red), and RIP (blue). Scale bars=100 μm

3 DISCUSSION

Our findings have demonstrated that rat ADSCs transfected with EGFP possessed the ability to undergo morphologic and phenotypic changes consistent with neural differentiation. At first, the low level of nestin expression in undifferentiated ADSCs was in line with the previous reports on stromal cells of bone marrow origin[12, 16], suggesting that ADSCs may retain a native potential for neural differentiation. Secondly, within a few days of neural induction, the ADSCs changed shape from a fibroblast-like appearance to bipolar and multipolar one. And immunostaining revealed that the bipolar cells expressed nestin and the multipolar ones were positive for one or several of the markers: MAP2, NF-70, Neu N and RIP. Usually, nestin is thought to be expressed at high levels in neural stem cells[17], whereas MAP2, NF-70 and Neu N are used as markers for mature neurons[12, 18, 19]; And RIP is considered as the special marker for oligodendrocytes[20]. In addition, co-expression of MAP-2 and Neu N is thought to coincide with terminal differentiation of ADSCs[14], and co-expression of Neu N, MAP-2, and nestin may indicate potential neurogenic capacity in ADSCs. Consequently these morphological and phenotypic changes may represent neuronal maturation and oligodendrocyte generation. Moreover, immunochemical studies using GFAP as a marker for astrocytes[21] demonstrated that there were far fewer glial cells than neurons and oligodendrocytes among the induced cells. Therefore, ADSCs tended to differentiate into neurocytes[22-24] rather than astrocytes under the condition provided in this study.

Furthermore, we found that EGFP-transfected ADSCs can keep stable expression of EGFP for a long-term. And it provided an easy method for tracing and observing of ADSCs *in vitro* or *in vivo*[25]. Moreover, owing to the EGFP gene tranfected facilely, it can be inferred that ADSCs transfected with EGFP gene from rats may be an ideal source for further experiments on neural differentiation and transplantation in animal models of neurological diseases and also maybe is a good gene vector and a better seed cells for the tissue engineering.

In conclusion, we were able to induce EGFP-transfected ADSCs into neurogenesis *in vitro* giving rise to neurons and oligodendrocytes as shown both morphologically and phenotypically. It suggests that these cells might provide an ideal source for further stem cell research with possible therapeutic application for SCI.

REFERENCES

1 Iwanami A, Kaneko S, Nakamura M, *et al*. Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res, 2005,80(2):182-190
2 Schwartz PH, Bryant PJ, Fuja TJ, *et al*. Isolation and characterization of neural progenitor cells from post-mortem human cortex. J Neurosci Res, 2003,74(6):838-851
3 Gottlieb DI. Large-scale sources of neural stem cells. Annu Rev Neurosci, 2002,25:381-407
4 Lenoir N. Europe confronts the embryonic stem cell research challenge. Science, 2000,287(5457):1425-1427
5 Zuk PA, Zhu M, Mizuno H, *et al*. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng, 2001,7(2):211-218
6 Safford KM, Hicok KC, Safford SD, *et al*. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun, 2003,294(2):371-379
7 Zuk PA, Zhu M, Ashjian P, *et al*. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell, 2002, 13(12):4279-4295
8 Wang KH, Kao AP, Wangchen H, *et al*. Optimizing proliferation and characterization of multipotent stem cells from porcine adipose tissue. Biotechnol Appl Biochem, 2008,51(4):159-166
9 Kim JH, Lee MR, Kim JH, *et al*. IFATS collection: Selenium induces improvement of stem cell behaviors in human adipose-tissue stromal cells via SAPK/JNK and stemness acting signals. Stem Cells, 2008,26(10):2724-2734
10 Lee DH, Kang SK, Lee RH, *et al*. Effects of peripheral benzodiazepine receptor ligands on proliferation and differentiation of human mesenchymal stem cells. J Cell Physiol, 2004,198(1):91-99
11 Lee DH, Kim B, Choi I, *et al*. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem, 2004,14(4-6):311-324
12 Kang SK, Lee DH, Bae YC, *et al*. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol, 2003,183(2):355-366
13 Fujimura J, Ogawa R, Mizuno H, *et al*. Neural differentiation of adipose-derived stem cells isolated from GFP transgenic mice. Biochem Biophys Res Commun, 2005,333(1):116-121
14 Kang SK, Putnam LA, Ylostalo J, *et al*. Neurogenesis of rhesus adipose stromal cells. J Cell Sci, 2004,117(18):4289-4299
15 Bunnell BA, lostalo JY, Kang SK. Common transcriptional gene profile in neurospheres-derived from pATSCs, pBMSCs, and pNSCs. Biochem Biophys Res Commun, 2006,343(3):762-771
16 Dhar S, Yoon ES, Kachgal S, *et al*. Long-term maintenance of neuronally differentiated human adipose tissue-derived stem cells. Tissue Eng, 2007,13(11):2625-2632
17 Franco Lambert AP, Fraqa Zandonai A, Bonatto D, *et al*. Differentiation of human adipose-derived adult stem cells into neuronal tissue: does it work? Differentiation, 2009,77(3):221-228
18 Safford KM, Safford SD, Gimble JM, *et al*. Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. Exp Neurol, 2004,187(2):319-328
19 Ashjian PH, Elbarbary AS, Edmonds B, *et al*. *In vitro* differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg, 2003,111(6):1922-1931
20 Keirstead HS, Nistor G, Bernal G, *et al*. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion. after spinal cord injury. J Neurosci, 2005,25(19):4694-4705
21 Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). Neurochem Res, 2000,25(9-10):1439-1451
22 Xu Y, Liu Z, Liu L, *et al*. Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells *in vitro*. BMC Neurosci,

2008,9:21
23 Radtke C, Schmitz B, Spies M, *et al*. Peripheral glial cell differentiation from neurospheres derived from adipose mesenchymal stem cells. Int J Dev Neurosci, 2009,27(8):817-823
24 Ning H, Huang YC, Banie L, *et al*. MicroRNA regulation of neuron-like differentiation of adipose tissue-derived stem cells. Differentiation, 2009,78(5):253-259
25 Bierhujzen MF, Westermany Y, Visser TP. Enhanced green fluorescent protein as selectable marker of retroviral mediated gene transfer in immature hematopoietic bone marrow cells. Blood, 1997,90(9):3304-3315

(Received Sep. 18, 2009)