Differentiation of rhesus adipose stem cells into dopaminergic neurons

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Abstract
LIM homeobox transcription factor 1a (Lmx1a) has the capacity to initiate the development program of neuronal cells and promote the differentiation of embryonic stem cells into dopaminergic neurons. In this study, rhesus adipose stem cells were infected with recombinant adenovirus carrying the Lmx1a gene and co-cultured with embryonic rat neural stem cells. Cell differentiation was induced using sonic hedgehog and fibroblast growth factor-8. Immunofluorescence staining showed that cells were positive for neuron-specific enolase and β-tubulin III. Reverse transcription-PCR results demonstrated that rhesus adipose stem cells were not only positive for neuron-specific enolase and β-tubulin III, but also positive for the dopaminergic neuron marker, tyrosine hydroxylase, neurofilament, glial cell line-derived neurotrophic factor family receptor α2 and nuclear receptor related factor 1. The number of Lmx1a gene-infected cells expressing the dopaminergic neuron marker was substantially greater than the number of cells not infected with Lmx1α gene. These results suggest that Lmx1a-mediated regulation combined with the strategy of co-culture with neural stem cells can robustly promote the differentiation of rhesus adipose stem cells into dopaminergic neurons.

Key Words
rhesus; adipose stem cells; differentiation; LIM homeobox transcription factor 1a; dopaminergic neurons; co-culture; neural stem cells; Parkinson’s disease; neurodegenerative disease; neural regeneration

Research Highlights
(1) LIM homebox transcription factor 1a gene was infected into rhesus adipose stem cells via adenovirus, and co-cultured with neural stem cells. This combination of endogenous and exogenous induction factors overcomes the problems encountered with the use of chemical agents for the differentiation of mesenchymal stem cells into neural cells.
(2) The primate rhesus monkey was selected for collection of adipose stem cells because of its similarity to humans.
(3) LIM homebox transcription factor 1a combined with neural stem cell co-culture can induce the differentiation of rhesus adipose stem cells into dopaminergic neurons. The induction efficiency using this combination approach was greater than that using neural stem cells alone.

Abbreviation
Lmx1a, LIM homebox transcription factor 1a
INTRODUCTION

Parkinson’s disease is the second most common progressive neurodegenerative disorder in the elderly, and is caused primarily by the degeneration of dopaminergic neurons in the substantia nigra pars compacta, one of the three main cell groups of the mesodiencephalic dopaminergic system. Numerous attempts have been made to reconstruct the nigrostriatal pathway by replacing the lost dopaminergic neurons in the substantia nigra pars compacta[1-3]. The first step of successful cell-replacement therapy in Parkinson’s disease is the production of mesodiencephalic dopaminergic neurons that truly represent substantia nigra pars compacta neurons, with the capacity to acquire appropriate connectivity and substitute for the lost substantia nigra pars compacta dopaminergic neuronal population. The discovery of mesenchymal stem cells has raised great hope for cell-replacement therapies in neurological disorders[4-6].

Adipose stem cells are present in the stromal vascular fraction of adipose tissues and display similar characteristics to bone marrow mesenchymal stem cells. They have high self-renewal capacity and can differentiate along several mesenchymal tissue lineages to give rise to adipocytes, osteoblasts, myocytes, chondrocytes, endothelial cells and cardiomyocytes[7-8]. However, unlike bone marrow mesenchyme stem cells, adipose stem cells can be obtained in large quantities with low risk. Furthermore, adipose tissue has the capacity to generate a greater number of stem cells than bone marrow or umbilical cord on a per gram basis[9-10]. Therefore, adipose stem cells may become the mesenchymal stem cell population of choice in future clinical strategies for replacing dopaminergic neurons[11-14]. The most important factor for successful cellular therapy using adipose stem cells in Parkinson’s disease is the induction of dopaminergic neurons that truly represent substantia nigra pars compacta neurons. There are numerous challenges to overcome for stem cell differentiation, such as inappropriate differentiation and low efficiency, associated with the use of chemical reagents and signaling molecules.

During development, lineage commitment is a multistep process requiring the activation and repression of genes at various stages[15]. It is very important to ensure that the pathways of genomic regulation are appropriately activated during the differentiation of stem cells into progenitor cells. Recent studies have provided important insight into the homeoprotein LIM homeobox transcription factor 1a (Lmx1a), showing that it plays critical roles in the recruitment of cells into a midbrain dopaminergic fate in developing mouse and sonic hedgehog-treated mouse embryonic stem cells[16]. Differentiation toward a midbrain dopaminergic phenotype is promoted by the upregulation of Lmx1a, which directly leads to an increase in tyrosine hydroxylase-positive neurons for cell replacement in Parkinson’s disease[17-18]. Neural stem cells are a self-renewing and multipotent population in the central nervous system, and are active during development and help to maintain homeostasis and tissue integrity throughout life by secreting neurotrophic factors[19]. These neurotrophic factors could provide an optimal environment for surrounding cells.

In summary, we hypothesized that the expression of an endogenous transcription factor Lmx1a plays a pivotal role in neuronal differentiation of rhesus adipose stem cells under specific culture conditions as defined in our study.

RESULTS

Morphology and pluripotency of cultured rhesus adipose stem cells

Rhesus adipose stem cells exhibited a fibroblast-like shape (similar to that of bone marrow-derived mesenchyme stem cells), and were maintained for 10 passages and reached 90% confluence every 3 days. The pluripotency of these stem cells was identified by their ability to undergo osteogenic and adipogenic differentiation[21-22]. Therefore, adipose stem cells may become the mesenchymal stem cell population of choice in future clinical strategies for replacing dopaminergic neurons[11-14]. Adipogenic differentiation was detected using oil red O and hematoxylin staining. Oil red O staining of prominent intracellular lipids on day 16 is shown in Figures 1A, B. Osteogenic differentiation was detected using 2% alizarin red staining and alkaline phosphatase activity staining. Rhesus adipose stem cells grew as clusters and prominent staining was observed on day 21 (Figures 1C, D).

Identification of rat neural stem cells

Neural stem cells isolated from the mesencephalic tissue of Sprague-Dawley rat embryos at embryonic day 14.5 were found to survive well. Three days after isolation, cell division could be observed. The cells started to
proliferate rapidly and form spherical clonal clusters 1 week later. Thereafter, cell volume and number continued to rise, and some cells began to adhere to the substrate. Significant nerve fiber growth was observed around the spherical neural clusters. Immunofluorescence labeling revealed nestin expression, confirming the presence of neural stem cells (Figures 2A, C). These neural stem cells could differentiate spontaneously into neurons \textit{in vitro}, as indicated by tyrosine hydroxylase-positive labeling, when cultured in medium containing fetal bovine serum (Figures 2B, D).

![Image](image1.png)

**Figure 1** Differentiation of rhesus adipose stem cells into adipocytes and osteoblasts (scale bars: 100 µm).

(A, B) \textit{In vitro} differentiation of rhesus adipose stem cells into adipocytes using dexamethasone, insulin, indomethacin, 3-isobutyl-1-methylxanthine, β-glycerophosphate disodium salt hydrate and L-ascorbic acid-2-phosphate.

(A) The induced adipocytes under the inverted microscope. Prominent intracellular lipids in induced adipose stem cells.

(B) Oil red O staining of the lipid droplet of cells cultured in adipogenic medium for 16 days.

(C, D) \textit{In vitro} differentiation of rhesus adipose stem cells into osteoblasts shown by alkaline phosphatase staining (C) and alizarin red staining (D) of calcified extracellular matrix of cells cultured in osteogenic medium for 21 days.

**Induction of neuron-like cells by adenovirus (Ad)-Lmx1a in co-culture**

In the induction experiment, rhesus adipose stem cells were infected with Ad-Lmx1a at a multiplicity of infection of 6; this resulted in a suitable compromise between infection efficiency and cell survival (about 70% infected cells). Ad-LacZ-infected rhesus adipose stem cells were cultured in inducing medium, whereas uninfected rhesus adipose stem cells were concomitantly cultured in growth medium and inducing medium as controls I and II, respectively.

Lmx1a protein was detected in cell nuclei after the Lmx1a-transduced cells were incubated in induction medium and co-cultured with neural stem cells. Our results showed that 80.0 ± 2.5% (mean ± SD) of rhesus adipose stem cells expressed Lmx1a on day 1 after co-culture and retained a fibroblastic-like morphology (Figure 3A). On day 10, a clear neuronal morphology was observed in cultured rhesus adipose stem cells (Figure 3B), and at week 3, a great number of these cells exhibited a neuronal morphology (Figure 3C).

![Image](image2.png)

**Figure 2** Identification of rat neural stem cells and neurons induced from neural stem cells (scale bars: 100 µm).

(A, B) Seven days after isolation, neural stem cells formed clonal clusters (balls) (A) and ventral mesencephalic neurons differentiated spontaneously into neurons 14 days later (B) under the inverted microscope.

(C) Neural stem cells were stained with nestin antibody (rabbit anti-rat, secondary antibody conjugated to FITC).

(D) About one half of neurons induced from neural stem cells were positive for tyrosine hydroxylase (rabbit anti-rat, secondary antibody conjugated to TRITC).

![Image](image3.png)

**Figure 3** Morphology of progressively induced rhesus adipose stem cells under the inverted microscope (scale bars: 100 µm).

(A) LIM homeobox transcription factor 1a-transduced rhesus adipose stem cells were incubated in our induction medium in co-culture with neural stem cells, and there were no obvious changes at day 1.

(B) Cultured rhesus adipose stem cells were subsequently incubated in a combination of media supplements at day 10. The cultured fibroblastic rhesus adipose stem cells first condense, and then extend and form process-like projections of membrane.

(C) When co-cultured with neural stem cells for 21 days, differentiated rhesus adipose stem cells displayed neuronal-like structures with a network appearance.
Neuron-specific enolase and β-tubulin III expression in neuron-like cells induced by Ad-Lmx1a in co-culture

Neural markers, such as neuron-specific enolase and β-tubulin III, were almost negative at day 1. The percentage of cells positive for neuron-specific enolase and β-tubulin III increased to 52.0 ± 1.9% and 40.0 ± 2.1%, respectively, at day 10. At week 3, a great number of rhesus adipose stem cells were positive for neuron-specific enolase, β-tubulin III and tyrosine hydroxylase (Figure 4).

Reverse transcription-PCR amplification of cultured rhesus adipose stem cells confirmed the immunocytochemistry results. Nestin mRNA was detected 1 week after co-culture, and other neuron-specific mRNAs (β-tubulin III, neurofilament, glial cell line-derived neurotrophic factor family receptor alpha 2 and tyrosine hydroxylase) were present at day 10. In contrast, nuclear receptor related factor 1 was also detected in rhesus adipose stem cells not transfected with Lmx1a (but co-cultured with neural stem cells), β-tubulin III was only weakly expressed; neuron-specific proteins failed to express in these control stem cells (Figure 4).

DISCUSSION

Mesenchymal stem cells are pluripotent stem cells that have been used in therapies for human diseases. In this study, adipose stem cells differentiated readily into adipocytes and osteoblasts, in agreement with previous data obtained with adipose stem cells from humans and rats[23-24]. These findings suggest that pluripotent stem cells reside in adult adipose tissue in a standby state, with their differentiation potential restricted by their environment. Key genetic and environmental changes allow pluripotent stem cells to differentiate into specific cell types. Non-human primates are ideal candidate models for Parkinson’s disease research because of their physiological and clinical similarities with humans. The present findings on adipose mesenchymal stem cells provide experimental support for their use in future autologous transplantation studies.
The discovery of active adult neurogenesis in the human brain has contributed to great hopes for replacement therapies for neurological disorders\cite{25-27}. As a result of the potential problems encountered with the use of small-molecule chemicals for in vitro neural differentiation of mesenchyme stem cells, a number of studies have focused on mesenchymal stem cells co-cultured with neural stem cells or neural cells\cite{28-30}. The use of stem cell-derived dopaminergic neurons for transplantation in Parkinson’s disease requires the understanding of the normal pathway of dopaminergic neuronal development. Recent studies have provided critical insight into the important role of Lmx1a in the midbrain dopamine differentiation process in embryonic stem cells and mesenchymal stem cells.

In this study, rhesus adipose stem cells were used because they easily differentiate into adipocytes and osteoblasts, and have identical characteristics to adipose stem cells isolated from humans and rats. These cells are a potential source of cells for Parkinson’s disease therapy as they can be easily isolated and have the ability to expand rapidly while maintaining pluripotency. Without Lmx1a expression, the co-culture of rhesus adipose stem cells with neural stem cells triggered their proliferation while simultaneously inducing them to differentiate. In the presence of rhesus adipose stem cells, neuronal stem cells were found to increase in number and vice versa (data not shown). These results suggest that mesenchymal stem cells could be a supporter of neural stem cell survival, consistent with previous studies\cite{31-32}. Neural stem cells could secrete numerous neurotrophic factors that have the capacity to promote the proliferation of rhesus adipose stem cells. Transwell culture dishes are frequently used to produce a cell culture environment that closely mimics the in vivo state. Direct cell contact does not occur in this system. Consequently, transdifferentiation is likely mediated by indirect communication between rhesus adipose stem cells and neural stem cells (i.e., by secreted factors). Following adenovirus vector-mediated transfection of Lmx1a into rhesus adipose stem cells, we observed a phenotypic profile of differentiated rhesus adipose stem cells similar to that of neurons. This reveals that Lmx1a plays a critical role in the direction of rhesus adipose stem cell transdifferentiation in a co-culture environment.

In conclusion, Lmx1a may play a critical role in midbrain dopamine differentiation of rhesus adipose stem cells in co-culture with rat neural stem cells. When combined with neural stem cell co-culture, Lmx1a converted the majority of rhesus adipose stem cells into neurons. These neurons displayed typical neuronal morphologies and expressed multiple neuronal markers. Our approach may facilitate the generation of autologous (patient-specific) human dopaminergic neurons derived from adipose stem cells in the future. This study sheds light on this potential autologous cell source for therapeutic strategies for Parkinson’s disease.

MATERIALS AND METHODS

Design
A neural induction study combining genetic engineering and cellular biology.

Time and setting
The experiment was performed in the Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College in China between 2010 and 2012.

Materials
A 6-year-old male rhesus monkey, weighing 6 kg, provided by the Institute of Medical Biology, Chinese Academy of Medical Science & Peking Union Medical College in China, was used for the isolation of rhesus adipose stem cells. Embryonic day 14.5 Sprague-Dawley rats purchased from Kunming Medical College were used for the isolation of rhesus adipose stem cells. Every effort was made to minimize the number of animals used and their suffering. All experimental procedures were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China\cite{33}.

Methods
Isolation and characterization of rhesus adipose stem cells
Rhesus adipose stem cells were isolated from 3 to 5 g of adipose tissue obtained from rhesus omentum under aseptic conditions. Rhesus adipose stem cells were cultured as previously described\cite{34}. To induce adipogenic differentiation, rhesus adipose stem cells at passage 3 were plated in Corning 6-well plates (1 × 10^5 cells per well) containing Dulbecco’s modified Eagle’s medium (high glucose), 10% fetal bovine serum, 1 μM dexamethasone, 10 μg/mL insulin, 200 μM indomethacin (Sigma, St. Louis, MO, USA) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). The medium was discarded every 3 days. After 2 weeks of adipogenic stimulation, cells were stained with oil red O to detect lipid vacuoles\cite{34}. To induce osteogenic differentiation, rhesus...
adipose stem cells were plated (1 × 10^5 cells per well) in Corning 6-well plates containing Dulbecco’s modified Eagle’s medium (high glucose), 10% fetal bovine serum, 10 nM dexamethasone, 10 mM β-glycerophosphate disodium salt hydrate and 150 μM L-ascorbic acid-2-phosphate (Sigma). Medium was changed every 3 days. After 3 weeks of osteogenic stimulation, cells were stained with alizarin red[24]. Alkaline phosphatase activity was assayed using an alkaline phosphatase activity staining kit (Genmed Scientifics, Inc., Washington DC, USA)[21].

**Isolation and characterization of neural stem cells**

Under aseptic conditions, neural stem cells were harvested from the mesencephalic tissue of Sprague-Dawley rat embryos at embryonic day 14.5 as described previously[35]. The cells were grown in suspension in Dulbecco’s modified Eagle’s medium/F12 medium with B27 supplement (Gibco, Carlsbad, CA, USA) and 20 ng/mL human basic fibroblast growth factor (Millipore, Billerica, MA, USA). Cells were allowed to proliferate and generate neurospheres, and were passaged every 3 days to maintain appropriate density. Seven days later, adherent cells were purified by adding arabinoside cytosine (Sigma). The medium was changed to neural basal medium (Gibco) containing 2% B27. Neural stem cells were identified by labeling for nestin (rabbit anti-rat; Millipore) and neurons derived from neural stem cells were detected by labeling for tyrosine hydroxylase (rabbit anti-rat; R&D Systems, Inc., Minneapolis, MN, USA) using immunofluorescence staining.

**Treatment with Lmx1a and co-culture with neural stem cells**

We constructed an E1/E3-deleted, replication-deficient recombinant human adenovirus serotype 5 containing Lmx1a gene using the AdEasy™ Adenoviral Vector System (Stratagene, Stevens Creek Blvd Santa Clara, CA, USA). To differentiate rhesus adipose stem cells into dopaminergic cells in vitro, rhesus adipose stem cells were trypsinized and then plated at a final density of 1 × 10^5 cells/cm² on poly-D-lysine-coated culture wells with coverslips. Rhesus adipose stem cells were infected with Ad-Lmx1a at a multiplicity of infection of 6 in 1 mL of culture medium containing 5% fetal bovine serum for 6 hours at 37°C, followed by fresh medium replacement containing 10% fetal bovine serum.

For co-culture of rhesus adipose stem cells and neural stem cells, 2.5 × 10^4 fetal neural stem cells derived from Sprague-Dawley rat embryos at embryonic day 14.5 were seeded onto poly-D-lysine-coated hanging cell culture inserts with a 0.4 μm poly(ethylene terephthalate) membrane (Millipore, Billerica, MA, USA) that is sufficient to allow for protein transfer. One day later, the membrane insert was immersed in a well containing Ad-Lmx1a infected rhesus adipose stem cells. The medium was replaced with 50 ng/mL sonic hedgehog (R&D Systems, Inc.) plus 50 ng/mL fibroblast growth factor-8 (R&D Systems, Inc.) medium (Figure 5).

**Reverse transcription-PCR analysis**

To determine whether co-culture with neural stem cells combined with Lmx1a transfection could induce neuron-like cells from rhesus adipose stem cells, reverse transcription-PCR was performed using rhesus-specific primers for neuron-specific genes and β-actin. Total RNA was isolated from induced rhesus adipose stem cells using an RNA extraction kit (Omega, Germany) and cDNA was synthesized using M-MULV reverse transcriptase (Fermentas International Inc., Burlington, Canada). DNA was amplified with rhesus-specific primers (Table 1) according to the following conditions: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; and 72°C for 10 minutes. The samples were stored at 4°C.

![Figure 5  Process of progressively induced rhesus adipose stem cells (rASCs).](image)

Half of the medium was replaced every 3 days with fresh inducing medium. In the following 3 weeks, morphology of the cultured rASCs was observed under an inverted microscope at different time points (days 1, 10 and 21); the coverslips were processed for reverse transcription-PCR amplification or immunofluorescence staining for dopaminergic neuron-related gene expression. SHH: Sonic hedgehog; FGF-8: fibroblast growth factor-8; PET: poly(ethylene terephthalate); NSCs: neural stem cells; Ad-Lmx1a: adenovirus-LIM homeobox transcription factor 1 α.
Immunofluorescence staining
To examine the expression of Lmx1a and neuron-specific genes in rhesus adipose stem cells after infection and induction, cells were fixed with 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes at 4°C, then fixed with pre-cooled 98% methanol for 10 minutes at 4°C. After washing with ice-cold PBS, samples were blocked with 2% bovine serum albumin (Sigma) in PBS for 1 hour at 37°C. The slides containing cells were incubated with antibodies, such as Lmx1a (0.1 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-tubulin III (0.1 μg/mL; Millipore), neuron-specific enolase (0.1 μg/mL; Thermo Fisher Scientific, Barrington, IL, USA) and tyrosine hydroxylase (0.1 μg/mL, Millipore) for 1.5 hours at 37°C. After incubation, cells were washed with PBS and incubated with goat anti-rabbit IgG-DyLight™ 549 (0.5 μg/mL, Rockland Immunochemicals Inc., Gilbertsville, PA, USA) or mouse anti-rabbit IgG-FITC (0.5 μg/mL; Chemicon, Santa Cruz, CA, USA) for 1 hour at 37°C. The slides were then washed three times with PBS and covered with a cover slip, followed by staining with 4, 6-diamidino-2- phenylindole dihydrochloride (Sigma). The negative controls were performed in parallel. Samples were analyzed using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis
SPSS 15.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± SD. A two-tailed Student’s t-test was used to determine the statistical significance. A value of P < 0.05 was considered statistically significant.

Acknowledgments: We are grateful to Mr. Huasheng Shi from Central Laboratory, Institute of Medical Biology, Chinese Academy of Medical Science & Peking Union Medical College for assistance with image capture during the experiments.

Funding: This research was financially supported by the Research Fund for Doctor Innovation of Peking Union Medical College and Science and Technology Project of Yunnan Province, No. 2012AE001.

Author contributions: Yan Zhou conducted the research, integrated the data and wrote the manuscript. Hongjun Li conducted the study and revised the manuscript. Min Yan conducted the experiments and analyzed the data. Maosheng Sun and Tianhong Xie revised the manuscript. All authors approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: Animal studies were conducted according to the animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC), as well as guidelines approved by the Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Edited by Tu QY, Bai H/Qiu Y/Song LP)