Neurospheres Induced from Human Adipose-Derived Stem Cells as a New Source of Neural Progenitor Cells

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Abstract

Human adipose-derived stem cells are used in regenerative medicine for treating various diseases including osteoarthritis, degenerative arthritis, cartilage or tendon injury, etc. However, their use in neurological disorders is limited, probably due to the lack of a quick and efficient induction method of transforming these cells into neural stem or progenitor cells. In this study, we reported a highly efficient and simple method to induce adipose-derived stem cells into neural progenitor cells within 12 hours, using serum-free culture combined with a well-defined induction medium (epidermal growth factor 20 ng/ml and basic fibroblast growth factor, both at 20 ng/ml, with N2 and B27 supplements). These adipose-derived stem cell-derived neural progenitor cells grow as neurospheres, can self-renew to form secondary neurospheres, and can be induced to become neurons and glial cells. Real-time polymerase chain reaction showed significantly upregulated expression of neurogenic genes Sox2 and Nestin with a moderate increase in stemness gene expression. Raybio human growth factor analysis showed a significantly upregulated expression of multiple neurogenic and angiogenic cytokines such as brain-derived neurotrophic factor, glial cell line-derived neurotrophic growth factor, nerve growth factor, basic fibroblast growth factor and vascular endothelial growth factor etc. Therefore, adipose-derived stem cell-derived neurospheres can be a new source of neural progenitor cells and hold great potential for future cell replacement therapy for treatment of various refractory neurological diseases.

Keywords

Human adipose-derived stem cells, neural progenitor cells, trans-differentiation

Introduction

Human adipose-derived stem cells (ADSCs) are a common type of mesenchymal stem cell (MSC) and have been mainly used in regenerative medicine for treating various diseases including osteoarthritis, degenerative arthritis, cartilage or tendon injury, graft-versus-host diseases, chronic kidney diseases, etc. Currently, ADSCs are among the mostly used stem cell sources in the cell transplantation field. As of 17 September 2019, there were 994 MSC clinical trials registered at clinicaltrials.org (either completed or recruiting), of which 176 (17.7% of all MSC trials) were using human adipose-derived MSCs. The unique advantage of using ADSCs in regenerative medicine is that patients usually have excess fatty tissue and these cells can be easily obtained using minimally invasive procedures such as conventional or hydrodynamic liposuction, thus enabling autologous cell replacement therapy.

The use of ADSCs in treating neurological disorders is relatively limited. Less than 10% of current ADSC clinical trials are targeting refractory neurological disorders such as spinal cord injury, stroke, dementia, and various neurodegenerative disorders. Ideally, these diseases could be treated using human neural stem cells (NSCs). However,
as we all know, the unfavorable accessibility combined with ethical issues of human NSCs have long restricted their wide clinical use. This problem could be potentially solved if we could develop an easy way to obtain NSC/neural progenitor cells (NPCs) from more accessible stem cell sources19–22, such as ADSCs.

MSCs have been reported to be able to generate NSCs/NPCs23–25. Most studies used bone marrow-derived MSCs. Hermann et al. first reported human bone marrow stromal cells can be induced to produce NSC-like cells23. Fu and colleagues24 repeated the Hermann induction method, but only induced 8% of bone marrow MSCs into neurospheres. These MSC-derived neurospheres not only express the NSC antigens Nestin and Musashi-1, but can also self-renew and differentiate into neurons, astrocytes, and oligodendrocytes. Furthermore, these differentiated neurons are capable of producing tetrodotoxin-sensitive action potential. Ma et al.25 reported using NSC-conditioned medium to induce 80% of bone-marrow MSCs into NSC-like cells, but the induction was a lengthy process with total induction time of 3 weeks.

Several studies have demonstrated trans-differentiation of ADSCs into NSCs/NPCs. For instance, Feng and colleagues26 reported using a three-step induction method to induce high-purity NSCs from ADSCs. The neurospheres derived from ADSCs were found to express Sox1, Pax6, Nestin, and Vimentin by flow cytometry. Qin et al.27 reported similar findings by transfecting Sox2 gene with a retrovirus to induce ADSCs into NSC-like cells. Others have reported functional neural differentiation using basic fibroblast growth factor (bFGF) and forskolin28, or an epidermal growth factor (EGF) and bFGF combination29. Overall, these studies reported a varied induction protocol with varied neural differentiation efficiency. But so far, a fast and efficient induction method of trans-differentiating these cells into NSCs/NPCs is lacking.

We are interested to see whether highly efficient neural induction can be achieved in ADSCs using a well-defined induction medium, without retro-lentiviral mediated gene intervention. In this study, we used serum-free culture conditions and a well-defined medium mainly comprised of EGF and bFGF to induce ADSCs into neurospheres. We carried out functional experiments to analyze the self-renewal and multi-potency capacity of these neurospheres. We further analyzed the change in gene expression and cytokine profile after induction.

Materials and Methods
Study Samples and Ethics Statement
Human adipose tissues were obtained from patients who underwent hydrodynamic liposuction procedures after obtaining written informed consent and approval by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China.

Isolation and Culture of ADSCs
ADSCs were isolated and amplified as described elsewhere30,31. Briefly, adipose tissue was dissociated into small pieces of about 25–50mm3 and digested with 0.1% collagenase type I (Gibco™, Thermo Fisher, Waltham, MA, USA) at 37°C for 60 minutes. The single-cell suspension was obtained by filtering the digested material through a 100μm mesh filter. ADSCs were cultured in Dulbecco’s modified eagle medium: nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (HyClone™, GE Healthcare, Chicago, IL, USA) and 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2. ADSCs were continuously passaged until passage 30 (P30). ADSCs at P5, P15, and P30 were subjected to karyotyping and neurosphere formation assay.

Adipogenic, osteogenic, neurogenic differentiation and immunophenotyping experiments were performed as described elsewhere32.

ADSC-Derived Neurosphere Generation
In total, 2–4 x 10⁶ ADSCs (P5, 15, 30) cultured in T75 cell culture flasks were dissociated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C, washed twice with phosphate buffered saline (PBS) and plated on T25 cell culture flasks (Nalge Nunc International, Rochester, NY, USA) at a concentration of 2 x 10⁵ cells/cm² in medium containing DMEM/F12 with 20 ng/mL of both EGF (R&D Systems, Inc., Minneapolis, MN, USA) and bFGF (R&D Systems, Inc., Minneapolis, MN, USA), N2, and B27 supplements (Invitrogen, Carlsbad, CA, USA). Then 12 hours later, after ADSC-derived neurospheres were generated, neurospheres were enumerated under a 100x microscope by adding the total neurosphere numbers from six random fields. Neurospheres were maintained by replenishing the medium containing DMEM/F12 with 20 ng/mL of both EGF and bFGF every 3 days. At day 5, primary neurospheres were dissociated into single cells by digesting them with 0.25% trypsin-EDTA for 5 minutes at 37°C and continuous pipetting using a fire-polished Pasteur pipette and 100 cells were re-plated in each well of 96-well plates for secondary neurosphere formation. Cultures were replenished with fresh growth factors (EGF and bFGF) every 3 days and assayed for neurosphere formation after 10–14 days in vitro. Secondary neurosphere formation capabilities were measured by the percentage of positive neurosphere formation after 14 days.

Apoptosis Assay
ADSC-derived neurospheres were digested with 0.25% trypsin/0.04% EDTA for 5 minutes at 37°C and continuous pipetting using 200 ul pipetting tips to ensure a single-cell suspension was achieved under a microscope. After spinning down, dissociated neurospheres (105 cells) were resuspended...
in a 500 μl binding buffer. Then they were incubated for 5 minutes at room temperature in the presence of 0.5 μg/mL Annexin V-FITC (R&D Systems, Minneapolis, MN, USA) and propidium iodide (PI) in binding buffer as described by the manufacturer. The percentage of apoptosis was determined by fluorescent-activated cell sorting.

**Multi-Differentiation Assay**

On the third day after ADSC-derived neurosphere formation, these neurosphere-like structures were plated on six-well plates with coverslips (coated with laminin from Sigma-Aldrich, St. Louis, MO, USA) and cultured in DMEM supplemented with 2% fetal bovine serum. Then 8–10 days after induction, neurogenic differentiation was assayed by immunofluorescence staining for neural and glial specific protein expression. Cells were washed with PBS, fixed with 4% para-formaldehyde, blocked with 1% bovine serum albumin for 30 minutes, and then incubated for 1 hour at room temperature with the following antibodies: rabbit anti-Tuj1 mAb (Abcam, Cambridge, MA, USA at final concentrations of 1/250), mouse anti-microtubule associated protein 2 (Map2) mAb (Abcam, Cambridge, MA, USA, at final concentrations of 1/200) and mouse anti-glial fibrillary acidic protein (GFAP) mAb (Abcam, Cambridge, MA, USA, at final concentrations of 1/200). Primary antibodies were developed with secondary Dylight 488-goat anti-rabbit IgG and Dylight 546-rat anti-mouse IgG, both at final concentrations of 1/500. Secondary antibodies were incubated for 45 minutes at room temperature in the dark. After labeling, nuclei were stained with Dapi for 5 minutes and then the slides were immediately examined on a three-color immunofluorescence microscope (Nikon Instruments Inc., Tokyo, Japan).

**Quantitative Real-Time Polymerase Chain Reaction Assay**

RNA was isolated using Trizol reagent (InVitrogen, Carlsbad, CA, USA). cDNA was transcribed using Superscript III First Strand cDNA Synthesis kit following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR reagents on an ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA, USA). Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The normalized fold expression was obtained using the 2^-ΔΔCT method. Primers used for real-time PCR are shown in Table 1.

**Raybio Cytokine Microarray Analysis**

ADSCs at P5 were grown to sub-confluency and the medium was switched to DMEM/F12 only. ADSCs were continued to culture for 48 hours. After filtration 1 ml of the supernatant was collected and assayed by RayBio® Biotin Label-based Human Growth Factor Cytokine Array I (Cat#: QA-GF-1-1 Human Growth Factor Array, Norcross, GA, USA). Then ADSCs were induced to form neurospheres as described previously. Then 72 hours after neurosphere formation, 1 ml of supernatant was collected and was assayed by the same Raybio Cytokine Array. Commercially available human induced pluripotent stem cell-derived NSCs (Nouv-Neu hNSC, Catalogue No. NC0001, iRegene, Wuhan, Hubei, China) were used as a positive control.

**Statistical Analysis**

All values were expressed as mean ± SD. The comparison between two groups was checked by a t-test, and one-way analysis of variance followed by either a Dunnett or a Tukey post hoc test was used for multiple sample means. A p value < 0.05 was considered to indicate statistical significance. A p value < 0.01 was considered statistically very significant. All analyses were performed with GraphPad Prism 8.

**Results**

**Characterization of ADSCs**

Human ADSCs were isolated and characterized by flow cytometry, multi-differentiation assay as reported elsewhere30,31. ADSCs can be differentiated into osteocytes, adipocytes, and neurons. They are positive for CD13, CD71, CD44, CD90, and CD105, and negative for CD14, CD45, CD34, and human leukocyte antigen-antigen D related (HLA-DR) expression, as shown in Supplementary Figure 1.

**Generation of ADSC-Derived Neurospheres**

We cultured ADSCs (P5–30) under serum-free induction medium (DMEM/F12, EGF, bFGF 20 mg/ml with N2, B27 supplements). ADSCs can be efficiently induced to form neurosphere-like structures under this culture
condition within 12 hours. As early as 4–6 hours after converting the culture medium into a neurosphere medium, quick clustering of ADSCs into sphere-like structures were seen. Within 24 hours of converting the culture medium into a neurosphere medium, almost all ADSCs formed neurosphere-like structures, as shown in Figure 1(B). Neurospheres usually have a round shape, a clear outline, and a dense core. Apart from the ADSC-derived neurosphere-like structures, there were some irregular-shaped cell clusters formed at the same time. These cell clusters underwent apoptosis soon after. The apoptosis rate is around 18%, as assayed by Annexin V-PI flow cytometric assay, as shown in Figure 1(I).

We next wanted to know more about the neurosphere formation process. We tested several different conditions: (a) complete induction medium, which is comprised of DMEM/F12, EGF 20 ng/ml, bFGF 20 ng/ml, and N2 and B27 supplements; (b) DMEM/F12, EGF 20 ng/ml, no
bFGF, with N2 and B27 supplements; (c) DMEM/F12, bFGF 20 ng/ml, no EGF, with N2 and B27 supplements; (d) DMEM/F12 with N2 supplement only, no EGF nor bFGF; (e) DMEM/F12 with B27 supplement only, no EGF nor bFGF.

For the ADSC-derived neurosphere assay, we found the complete induction medium led to most prominent neurosphere-like structure production. Interestingly, EGF and bFGF are not indispensable. DMEM/F12 and N2 and B27 supplements are already enough to induce neurosphere formation. But N2 supplement alone is not enough to induce neurosphere formation. The result is shown in Figure 1(B)–(G).

ADSC-Derived Neurospheres can Self-Renew and can be Induced to form Neurons and Glial Cells

To confirm these ADSC-derived neurospheres are indeed NPCs rather than culture artifacts, ADSC-derived neurospheres were subjected to self-renewal and multipotency tests. For the self-renewal assay, ADSC-derived neurospheres were dissociated into single cells and re-plated for secondary neurosphere formation. Within 3 days after re-plating, secondary neurospheres were formed, as shown in Figure 2(A). Secondary neurospheres can be dissociated again into single cells and then re-plated for tertiary neurosphere formation. Figure 2(B)–(D) show the formation of...
tertiary neurospheres. Nascent tertiary neurospheres are usually smaller than secondary neurospheres but they grow bigger over time, and around 7 days they mature. Nascent neurospheres usually have very clear borders with a dense core, but when they mature, the border becomes darker (Figure 2(D)) and therefore they are easily distinguishable from nascent neurospheres. At this point, they need to be passaged. Secondary neurospheres express Nestin, an NSC-specific marker, as shown in Figure 2(H).

For the multipotency assay, neurosphere-like structures were plated on six-well plates with coverslips and cultured in DMEM supplemented with 2% fetal bovine serum. Then 6 days after induction, neurogenic differentiation was assayed by immunofluorescent staining for Tuj1 and GFAP expression. Then, 10–12 days after induction, neurogenic differentiation was assayed by immunofluorescent staining for mature neuron specific marker Map2 expression. As shown in Figure 2(I)–(O), ADSC-derived neurospheres can be induced to form neurons and glial cells.

**Real-Time PCR Analysis**

Real-time PCR showed expression of multiple genes important for NSC self-renewal and multipotency such as Sox2, Olig2, Nestin, and Bmi1 in ADSCs in a standard MSC culture condition. When cultured under complete induction medium conditions (DMEM/F12, EGF, bFGF, N2, and B27), the expression of these genes is further upregulated. We found the expression levels of pluripotent genes Olig2, Oct4, and Bmi1 were modestly increased (5–10 fold) from as early as 24 hours post-induction compared to pre-induction. The expression of Sox2 increased steadily from 10–35 fold after the induction. The expression level of Nestin was about 8 times higher just 24 hours after the induction and increased to 15 times higher at day 3 post-induction, as shown in Figure 3(A).

**ADSCs in Early Passages Possess Higher Neurosphere Potentiality than Late-Passage ADSCs**

We compared neurospheres formed by different passage ADSCs: P5 (early passage), P15 (moderate passage), and P30 (extensive passage). The primary neurosphere formation efficiency does not change much when comparing early-passage ADSCs with moderate-passage ADSCs; however, there is a significant decrease in primary neurosphere formation efficiency when comparing moderate-passage ADSCs with extensive-passage ADSCs, despite the fact that ADSCs as late as P30 can readily form neurospheres within 24 hours, just like as early-passage ADSCs do (Figure 3(B)). Moreover, when we performed a secondary neurosphere formation assay, we found significantly less secondary neurosphere formation in late-passage ADSCs in comparison with early-passage ADSCs, indicating decreased self-renewal capacity with extensive passage numbers, as shown in Figure 3(C). It is noteworthy that ADSCs at P30 still maintain a normal karyotype, as shown in Figure 3(D)–(E).

**Raybio Cytokine Array**

We are interested to see whether this fast induction protocol also led to an increase in neurogenic cytokine expression. Using RayBio® Biotin Label-based Human Growth Factor Cytokine Array 1, we detected a significant increase in expression levels for two-thirds of the human growth factors, especially for neurogenic growth factors such as brain-derived neurotrophic growth factor (BDNF), bFGF, glial cell line-derived neurotrophin growth factor (GDNF) etc. The expression of BDNF increased 13.31 fold only 72 hours after the induction. The expression of GDNF increased to 3.8 fold and bFGF level increased to 26.41 fold. The hepatocyte growth factor level increased to 21.77 fold. Overall, the cytokine secretion pattern shifted significantly towards the NSC profile after induction, as is shown in Figure 4(A). In comparison with the human NSC secretome, ADSCs exhibit even higher neurogenic growth factor levels. At the same time, some angiogenic growth factors also significantly increased after induction, for example, ADSCs expressed an extremely high level of vascular endothelial growth factor (VEGF) before the induction and the VEGF level further increased to 3.90 fold after the induction, as shown in Figure 4(B).

**Discussion**

Neurological disorders such as Parkinson’s disease, stroke and multiple sclerosis have varied causes, but in many cases the symptoms of neurological deficit are caused by the loss of neurons and glial cells. Therefore, neural replacement therapy based on NSCs/NPCs has become a new direction in the treatment of neurological diseases21. NSCs/NPCs have a clear ability to differentiate into all types of nerve cells, such as neurons, astrocytes, and oligodendrocytes. But due to the limited source of NSCs and the immune response to transplantation, their clinical applications are greatly limited20.

In this study we showed human ADSCs can be efficiently induced to form neurospheres under serum-free culture conditions using well-defined medium (mainly composed of EGF and bFGF) within 12 hours. There are several important features for this simple induction method, which distinguish our study from other studies previously reported: (a) the induction is immediate and highly efficient; (b) the induction method is simple and well defined; there is no need to use retro-lentiviral vectors or other conventional methods to overexpress or downregulate certain genes specifically and there is no need to use low-attachment plastic cell culture flasks for neurosphere formation; and (c) the induction is real; the ADSC-derived neurospheres are not spheres merely growing as neurospheres (culturing artifacts), but they can readily form secondary and tertiary neurospheres and be
induced to further differentiate into neurons and glial cells; and (d) this induction method is scalable and can easily meet Good Manufacturing Practice standards for large-scale clinical application.

To understand why this trans-differentiation happened so quickly, we checked the expression of several major neurogenic and stemness genes by real-time PCR. We were interested to see the change in gene-expression levels after the induction. We found the expression levels of stemness genes such as Bmi1, Oct4, and Olig2 increased, whereas neurogenic genes such as Nestin and Sox2 increased much more after the induction. The increase in stemness genes was around 5–10 fold, whereas the increase in Sox2 expression was about 35 fold, and the increase in Nestin expression was around 9–14 fold. Real-time PCR assay showed significant overexpression of Sox2 after induction, starting as early as 24 hours after the induction with a near 10-fold increase in expression level and near 35-fold increase at day 7 of the induction. Sox2 is a very important transcription factor that is essential for the self-renewal and multipotency of

Figure 3. Quantitative real-time polymerase chain reaction (PCR) analysis and comparison of neurosphere formation capabilities of adipose-derived stem cells (ADSCs) with different passage numbers. (A) Quantitative real-time PCR of Sox2, Oct4, Nestin, Nanog, Olig2, and Bmi1 after complete medium (epidermal growth factor (EGF) 20 ng/ml, basic fibroblast growth factor (bFGF) 20 ng/ml and N2 and B27 supplements) induction. The induction medium led to significant overexpression of Sox2 and Nestin within 72 hours. The expression of genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Triplicate PCR amplifications were performed for each sample, and the results were represented as mean values ± SD of three triplicate samples. (B) and (C) Comparison of neurosphere formation capabilities of ADSCs with different passage numbers. (B) ADSCs as late as passage 30 can readily form neurospheres, but the efficiency is significantly lower than early-passage ADSCs. Overall the neurosphere formation capacity decreased with extensive passages. **p<0.01. (C) Significantly less secondary neurosphere formation in late-passage ADSCs in comparison to early passage ADSCs. ***p<0.01. (D) Representative karyotyping analysis of ADSCs at passage 5. (E) Representative karyotyping analysis of ADSCs at passage 30.
embryonic stem cells and NSCs\textsuperscript{32,33}. In contrast, the expression level of Nestin was 8 times higher just 24 hours after the induction and was even higher at 72 hours post-induction. Significant overexpression of neurogenic genes, possibly triggered by the induction medium we used, led to the highly efficient trans-differentiation process, as we demonstrated in this study.

We wanted to know more about the neurosphere formation process. We tested several different induction conditions. Overall, adding N2 and B27 supplements to the EGF and bFGF induction regimen produced by far the best performance in terms of neurosphere formation efficiency. But we found the minimum requirement is either DMEM/F12 medium supplemented with EGF and bFGF (both at 20 ng/ml) or B27 only. EGF and bFGF are mitogen-specific growth factors widely regarded as key factors for the self-renewal, proliferation, and maintenance of NSCs\textsuperscript{34–37}. Our previous assumption is that these two factors should be indispensable for ADSC-derived neurosphere formation. But in contrast, we found N2 and B27 supplements are already enough to induce neurosphere formation. It is interesting that N2 alone cannot induce neurosphere formation. We suspect some ingredients within the B27 supplement might play an important role in this conversion.

The paracrine effects of MSCs have been shown to play an important role in a large number of preclinical and clinical trials\textsuperscript{38–40}. We therefore used Raybio Cytokine Array analysis to analyze the expression levels of 41 human growth factor cytokines secreted by ADSCs, and compared the expression of these cytokines before and after the induction of neurosphere formation, using human NSCs as a positive control. Human NSCs have some characteristic growth factor expression levels. For example, they express high levels of BDNF, bFGF, insulin growth factor (IGF), insulin, platelet-derived growth factor, NT-3, NT-4, VEGF, etc. Most of these cytokines are neurogenic (BDNF, IGF, EGF, bFGF, insulin, etc.) or angiogenic (VEGF). We found ADSCs already express these cytokines before induction, but what is most striking is the amplitude and the relative values of increase in the expression levels of these cytokines after induction. For example, the increase in BDNF expression is 13.31 fold, whereas the increase in insulin expression

Figure 4. Raybio human growth factor analysis. (A) Heatmap. The adipose-derived stem cell (ADSC)-derived neurosphere secretome profile is significantly skewed towards human neural stem cells after induction. NouvNeu human neural stem cells were used as a positive control. (B) The growth factors that showed significant increase after the induction. Signal strength is expressed as mean ± SD. **p<0.01.
level is 74 fold. It must be noted that ADSCs express very low levels of insulin, whereas human NSCs express high levels. After induction, the ADSC-derived neurospheres have an insulin expression level well over that of human NSCs. The whole secretome shifted significantly to the NSC secretome. After the induction, 30/41 (73.2%) of the human growth factor cytokines we analyzed exhibited larger than 1.5-fold increases in expression levels, 8/41 (19.5%) exhibited larger than 5-fold increase in expression levels, ranging from 13 fold to 74 fold increase in expression levels. Overall, we see a dramatic shift toward that NSC secretome after induction. In fact, ADSC-derived neurospheres are in many aspects superior to NSCs in neurogenic cytokine secretion. Therefore, our short neural induction protocol not only produced cells phenotypically resembling neurospheres but also led to a quick upregulation and expression of neurogenic cytokines, skewing strongly towards the NSC secretome.

In summary, we reported human ADSCs can be efficiently induced to form neurospheres using serum-free culture with EGF and bFGF within 12 hours. These ADSC-derived neurospheres display self-renewal and multi-potentialities comparable to NSCs/NPCs while they maintain the favorable characteristics of MSCs with scarce HLA-DR expression. The advantage of ADSCs is that they can be safely obtained during a conventional liposuction procedure. Using this easy one-step induction method, patient-specific autologous neurospheres can be obtained within 12 hours. Therefore, ADSC-derived neurospheres can serve as an autologous source of NSCs/NPCs for patients with spinal cord injuries, who have suffered a stroke and in various neurodegenerative disorders. We believe further exploration of this unique cell source can bring hope to those patients with intractable neurological disorders.

Ethical Approval
The procedures had been approved by the ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China.

Statement of Human and Animal Rights
All procedures in this study were conducted in accordance with the protocol approved by the Ethical Committee Board of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology and complied with the recommendations of the Declaration of Helsinki.

Statement of Informed Consent
All the patients involved in the study had been given written informed consent.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References
1. Mazini L, Rochette L, Amine M, Malka G. Regenerative capacity of adipose derived stem cells (ADSCs), comparison with mesenchymal stem cells (MSCs). Int J Mol Sci. 2019;20(10):E2523.
2. Rodeo SA. Cell therapy in orthopaedics: where are we in 2019? Bone Joint J. 2019;101-B(4):361–364.
3. Si Z, Wang X, Sun C, Kang Y, Xu J, Wang X, Hui Y. Adipose-derived stem cells: sources, potency, and implications for regenerative therapies. Biomed Pharmacother. 2019;114:108765.
4. Rivera-Izquierdo M, Cabeza L, Láinez-Ramos-Bossini A, Quesada R, Perazzoli G, Alvarez P, Prados J, Melguizo C. An updated review of adipose derived-mesenchymal stem cells and their applications in musculoskeletal disorders. Expert Opin Biol Ther. 2019;19(3):233–248.
5. Nahar S, Nakashima Y, Miyagi-Shiohira C, Kinjo T, Toyoda Z, Kobayashi N, Saitoh I, Watanabe M, Noguchi H, Fujita J. Cytokines in adipose-derived mesenchymal stem cells promote the healing of liver disease. World J Stem Cells. 2018;10(11):146–159.
6. Kucharzewski M, Rojczyk E, Wilemska-Kucharzewska K, Wilk R, Hudecki J, Los MJ. Novel trends in application of stem cells in skin wound healing. Eur J Pharmacol. 2019;843:307–315.
7. Argentati C, Morena F, Bazzucchi M, Armentano I, Emiliani C, Martino S. Adipose stem cell translational applications: from bench-to-bedside. Int J Mol Sci. 2018;19(11):E3475.
8. Trávníčková M, Bačáková L. Application of adult mesenchymal stem cells in bone and vascular tissue engineering. Physiol Res. 2018;67(6):831–850.
9. Gaur M, Dobke M, Lunyak VV. Mesenchymal stem cells from adipose tissue in clinical applications for dermatological indications and skin aging. Int J Mol Sci. 2017;18(44):843:307–315.
10. Jayaram P, Ikpeama U, Rothenberg JB, Malanga GA. Bone marrow-derived and adipose-derived mesenchymal stem cell therapy in primary knee osteoarthritis: a narrative review. PM R. 2019;11(2):177–191.
11. Gutiérrez-Fernández M, Otero-Ortega L, Ramos-Cejudo J, Rodriguez-Frutos B, Fuentes B, Diez-Tejedor E. Adipose tissue-derived mesenchymal stem cells as a strategy to improve recovery after stroke. Expert Opin Biol Ther. 2015;15(6):873–881.
12. Palumbo P, Lombardi F, Siragusa G, Cifone MG, Cinque B, Giuliani M. Methods of isolation, characterization and expansion of human adipose-derived stem cells (ASCs): an overview. Int J Mol Sci. 2018;19(17):E1897.
13. Schneider S, Unger M, van Griensven M, Balmayor ER. Adipose-derived mesenchymal stem cells from liposuction and resected fat are feasible sources for regenerative medicine. Eur J Med Res. 2017;22(1):17.

14. Konno M, Hamabe A, Hasegawa S, Ogawa H, Fukusumi T, Nishikawa S, Ohita K, Kano Y, Ozaki M, Noguchi Y, Sakai D, et al. Adipose-derived mesenchymal stem cells and regenerative medicine. Dev Growth Differ. 2013;55(3):309–318.

15. Damia E, Chicharro D, Lopez S, Cuervo B, Rubio M, Sopena JJ, Vilar JM, Carrillo JM. Adipose-derived mesenchymal stem cells: are they a good therapeutic strategy for osteoarthritis?. Int J Mol Sci. 2018;19(7):E1926.

16. Hur JW, Cho TH, Park DH, Lee JB, Park JY, Chung YG. Intrathecal transplantation of autologous adipose-derived mesenchymal stem cells for treating spinal cord injury: a human trial. J Spinal Cord Med. 2016;39(6):655–664.

17. Tsai YA, Liu RS, Lirng JF, Yang BH, Chang CH, Wang YC. Treatment of spinocerebellar ataxia with mesenchymal stem cells: a phase I/IIa clinical study. Cell Transplant. 2017;26(3):503–512.

18. Fernández O, Izquierdo G, Fernández V, Leyva L, Reyes V, Guerrero M, León A, Arnaiz C, Navarro G, Páramo MD, Cuesta A, et al. Adipose-derived mesenchymal stem cells (AdMSC) for the treatment of secondary-progressive multiple sclerosis: a triple blinded, placebo controlled, randomized phase I/II safety and feasibility study. PLoS One. 2018;13(5):e0195891.

19. Petersen ED, Zenchak JR, Lossia OV, Hochgeschwender U. Neural stem cells derived directly from adipose tissue. Stem Cells Dev. 2018;27(9):637–647.

20. Casarosa S, Bozzi Y, Conti L. Neural stem cells: ready for therapeutic applications? Mol Cell Ther. 2014;2:31.

21. Tang Y, Yu P, Cheng L. Current progress in the derivation and therapeutic application of neural stem cells. Cell Death Dis. 2017;8(10):e3108.

22. Zhang SC, Werning M, Duncan ID, Brüistle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001;19(12):1129–1133.

23. Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO,Maisel M, Lerche H, Schwarz J, Brenner R, Storch A. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci. 2004;117(Pt 19):4411–4422.

24. Fu L, Zhu L, Huang Y, Lee TD, Forman SJ, Shih CC. Derivation of neural stem cells from mesenchymal stem cells: evidence for a bipotential stem cell population. Stem Cell Dev. 2008;17(6):1109–1121.

25. Ma K, Fox L, Shi G, Shen J, Liu Q, Pappas JD, Cheng J, Qu T. Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells. Neurol Res. 2011;33(10):1083–1093.

26. Feng N, Han Q, Li J, Wang S, Li H, Yao X, Zhao RC. Generation of highly purified neural stem cells from human adipose-derived mesenchymal stem cells by Sox1 activation. Stem Cells. 2014;23(5):515–529.

27. Qin Y, Zhou C, Wang N, Yang H, Gao WQ. Conversion of adipose tissue-derived mesenchymal stem cells to neural stem cell-like cells by a single transcription factor, Sox2. Cell Reprogram. 2015;17(3):221–226.

28. Jang S, Cho HH, Cho YB, Park JS, Jeong HS. Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC Cell Biol. 2010;11:25.

29. Yang E, Liu N, Tang Y, Hu Y, Zhang P, Pan C, Dong S, Zhang Y, Tang Z. Generation of neurospheres from human adipose-derived stem cells. Biomed Res Int. 2015;2015:743714.

30. Hu L, Hu J, Zhao J, Liu J, Ouyang W, Yang C, Gong N, Du L, Khanal A, Chen L. Side-by-side comparison of the biological characteristics of human umbilical cord and adipose tissue-derived mesenchymal stem cells. Biomed Res Int. 2013;2013:438243.

31. Yang C, Lei D, Ouyang W, Ren J, Hu J, Huang S. Conditioned media from human adipose tissue-derived mesenchymal stem cells and umbilical cord-derived mesenchymal stem cells efficiently induced the apoptosis and differentiation in human glioma cell lines in vitro. Biomed Res Int. 2014;2014:109389.

32. Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M, Pevny L. SOX2, a persistent marker for multipotent neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev Neurosci. 2004;26(2–4):148–165.

33. Feng R, Wen J. Overview of the roles of Sox2 in stem cell and development. Biol Chem. 2015;396(8):883–891.

34. Phinney DG. Advancing mesenchymal stem/stromal cells-based therapies for neurologic diseases. Neural Regen Res. 2017;12(1):60–61.

35. Wu Y, Chen X, Xi G, Zhou X, Pan S, Ying QL. Long-term self-renewal of naïve neural stem cells in a defined condition. Biochim Biophys Acta Mol Cell Res. 2019;1866(6):971–977.

36. Darvishi M, Tiraihi T, Mesbah-Namin SA, Delshad A, Taheri T. Motor neuron transdifferentiation of neural stem cell from adipose-derived stem cell characterized by differential gene expression. Cell Mol Neurobiol. 2015;37(2):275–289.

37. Boregowda SV, Booker CN, Phinney DG. Mesenchymal stem cells: the moniker fits the science. Stem Cells. 2018;36(1):7–10.

38. Baez-Jurado E, Hidalgo-Lamussa O, Barrera-Bailón B, Sahebkar A, Ashraf GM, Echeverria V, Barreto GE. Secretome of mesenchymal stem cells and its potential protective effects on brain pathologies. Mol Neurobiol. 2019;56(10):6902–6927.

39. Eleuteri S, Fierabracci A. Insights into the secretome of mesenchymal stem cells and its potential applications. Int J Mol Sci. 2019;20(18):e4597.

40. Harrell CR, Fellabaum C, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Molecular mechanisms responsible for therapeutic potential of mesenchymal stem cell-derived secretome. Cells. 2019;8(5):e467.