

## Transplantation of Adipose-Derived Stem Cells is Associated with Neural Differentiation and Functional Improvement in a Rat Model of Intracerebral Hemorrhage

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### Keywords

Apoptosis; Hemorrhagic stroke; Progenitor cells; Rat; Transplantation.

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Received 1 June 2012; revision 27 June 2012;

accepted 5 July 2012.

### SUMMARY

**Aims:** To examine whether transplantation of adipose-derived stem cells (ADSCs) induces neural differentiation and improves neural function in a rat intracerebral hemorrhage (ICH) model. **Methods:** Adipose-derived stem cells were isolated from inguinal fat pad of rat. ICH was induced by injection of collagenase type IV into the right basal ganglia of rat. Forty-eight hours after ICH, ADSCs cells ( $10 \mu\text{L}$  of  $2\text{--}4 \times 10^7$  cells/mL) were injected into the right lateral cerebral ventricle. The differentiation of ADSCs was detected *in vitro* and *in vivo*. The neural function was evaluated with Zea Longa 5-grade scale at day 1, 3, 7, 14, or 28. **Results:** Our data demonstrated that ADSCs differentiated into cells that shared the similarities of neurons or astrocytes *in vitro*. Transplantation of ADSCs decreased cell apoptosis and the transplanted ADSCs were able to differentiate into neuron-like and astrocyte-like cells around the hematoma, accompanied with upregulation of vascular endothelial growth factor expression and improvement of neural function. **Conclusions:** Our data suggest that transplantation of ADSCs could be a therapeutic approach for ICH stroke.

doi: 10.1111/j.1755-5949.2012.00382.x

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### Introduction

Intracerebral hemorrhage (ICH) is a major cause of mortality and disability. Currently, there is no effective treatment for ICH. Recent studies suggest that transplantation of neural stem cells holds great promise for treatment of ICH [1,2]. However, it is a difficult and injurious procedure to obtain neural stem cells from the brain. An alternative source of stem cells is in need.

Adipose-derived stem cells (ADSCs) have multilineage differentiation potential and have been shown to differentiate into adipogenic, chondrogenic, myogenic, osteogenic, and hepatic cells

in the presence of lineage-specific induction factors *in vitro* [3–6]. More recently, ADSCs have been shown to be able to be induced to undergo morphologic and phenotypic changes consisting with neuronal differentiation [7,8]. Following neuronal induction, murine and human ADSCs display immunocytochemical staining for glial fibrillary acidic protein (GFAP), nestin, and neuronal nuclei (NeuN). They are emerging as an alternative source of stem cells. However, *in vivo* neuronal differentiation of ADSCs and their therapeutic effects on ICH have not been explored.

In this study, we have investigated whether ADSCs can be induced to neuronal differentiation and whether transplanta-

tion of ADSCs can improve the neural function of rats with ICH.

## Materials and Methods

### Animals

Male Sprague-Dawley (SD) rats (body weight, 120–150 g) were provided by the Animal Center of Huazhong University of Science and Technology (Wuhan, China) and housed under controlled temperature (23–25°C) and lighting (8:00–20:00 light, 20:00–8:00 dark) conditions with free access to standard rat chow and drinking water. The experimental procedures regarding the use of animals were approved by the Institutional Review Board (IRB) of Huazhong University of Science and Technology China and performed in accordance with the guidelines of the Ministry of Science and Technology of the People's Republic of China.

### Isolation and Culture of ADSCs

The adipose tissue was aseptically collected from the inguinal fat pads of the rats under anesthesia (6% chloral hydrate, 1 mL/200 g, and intraperitoneal injection). Fat pad tissues were excised, finely minced, and digested with 0.1% collagenase type I (Gibco, Los Angeles, CA, USA) at 37°C for 40 min. An equal volume of basic medium (Dulbecco's Modified Eagle Medium [DMEM; Thermo Scientific Hyclone, Waltham, MA, USA]) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific Hyclone) was added to neutralize the collagenase. Then, samples were centrifuged at  $367 \times g$  for 10 min at room temperature to obtain pellets. The pellets were washed with basic medium once, resuspended in basic medium, and filtered through a 0.075-mm cell strainer to remove undigested debris. The resulting cells were planted in a 25-cm<sup>2</sup> culture flask for growth. Medium was changed every 3 days. Cells were passaged until reaching approximately 70–80% confluence.

### Adipogenic and Osteogenic Differentiation of ADSCs

The second passage of cultured ADSCs was used in these experiments. To evaluate ADSCs for adipogenic differentiation capacity, cells were planted on cover slips and cultured in basic medium for 3 days and replaced with adipogenic medium (DMEM medium containing 0.5 mM 3-Isobutyl-1-Methylxanthine [IBMX; Sigma-Aldrich, Louis, MO, USA], 1  $\mu$ M dexamethasone [Biosharp, Seoul, Korea], 10  $\mu$ M insulin [Sigma-Aldrich], and 200  $\mu$ M indomethacin [Aladdin Chemistry Co., Ltd, Xi'an, China]) for 14 days. The cells were visualized with an inverted phase-contrast microscope. Adipogenic differentiation was determined by staining with Oil-Red O (Amresco, Solon, OH, USA). To evaluate ADSCs for osteogenic differentiation capacity, basic medium was replaced with osteogenic medium (DMEM medium supplemented with 0.1  $\mu$ M dexamethasone, 10 nM VitD<sub>3</sub> [Biosharp], 50  $\mu$ M VitC [Biosharp], and 10 mM beta-2-glycerophosphate [Biosharp]) for 14 days. Osteogenic differentiation was analyzed by alkaline phosphatase (AP) staining.

### In vitro Analysis of Neural Differentiation from ADSCs

The third passage of cultured ADSCs was evaluated for neural differentiation. ADSCs were cultured in induction medium (neurobasal medium [Gibco] supplemented with 20 ng/mL basic fibroblast growth factor [bFGF; Sigma-Aldrich], 20 ng/mL epidermal growth factor [EGF; Sigma-Aldrich], and 2% B27 supplement [Gibco]) for 7 days. Medium was changed every 3 days. The cells were visualized with an inverted phase-contrast microscope. When the number of neurosphere-like cell masses reached 15–20, cells were resuspended in neurobasal medium and passaged (1:3).

Fluorescent immunostaining analyses of neural differentiation of ADSCs were performed as previously described [7,9]. Five days after culture, the suspension of neurosphere-like cell masses was centrifuged at  $500 \times g$  for 2 min to obtain cell pellets. The cell pellets were resuspended in 2 mL neurobasal medium and planted on coverslips coated with polylysine in six-well plates. Cells were cultured in neurobasal medium (1 mL/well) for 24 h to allow cell adherence. For fluorescence immunostaining, cells were fixed with 4% paraformaldehyde for 30 min and blocked with 5% bovine serum albumin (BSA; Thermo Scientific Hyclone) for 1 h at room temperature. After blocking, the cells were incubated with rabbit monoclonal antibodies of nestin (1:150, Sigma-Aldrich) overnight at 4°C followed by incubation with a FITC-conjugated goat anti-rabbit secondary antibody (1:50) (Jackson ImmunoResearch Laboratories, Suffolk, UK) at room temperature for 1 h and 50  $\mu$ g/mL DAPI (Sigma-Aldrich) for 5 min. Between each step, cells were washed with phosphate buffer solution (PBS; Sigma-Aldrich). Reaction products were visualized with a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Neurosphere-like cell masses were seeded into six-well plates and cultured in the neurobasal medium supplemented with B27 for 10 days. 70% of the medium was changed every 4 days. Cells were fixed with 4% paraformaldehyde for 30 min and blocked with 5% BSA for 1 h at room temperature. After blocking, cells were incubated with mouse monoclonal antibodies of NeuN (1:100; Sigma-Aldrich) or mouse monoclonal antibodies of GFAP (1:100; Millipore, Billerica, MA, USA) overnight at 4°C followed by incubation with a Cy3-conjugated goat anti-mouse secondary antibody (1:50; Jackson ImmunoResearch Laboratories) for 1 h and 50  $\mu$ g/mL DAPI for 5 min. Between each step, cells were washed with PBS. Reaction products were visualized with a fluorescence microscope (BX51; Olympus).

### Preparation of Bromodeoxyuridine (BrdU)-conjugated ADSCs

Cultured ADSCs of the third passage were incubated with 20  $\mu$ mol/L BrdU (Sigma-Aldrich) at 37°C and 5% CO<sub>2</sub> for 48 h. BrdU-conjugated ADSCs were centrifuged at  $367 \times g$  for 1 min and washed with 0.01 mM PBS. The cells were counted under a microscope. The viability of the cells was assessed by trypan blue exclusion. Cells ( $2\text{--}4 \times 10^7$ /mL) with 90–95% viability were used for transplantation.

## Animal Model of ICH

The ICH model was induced to SD rats by injecting collagenase type IV according to previous report [10]. Rats were anesthetized with 6% chloral hydrate (1 mL/200 g, intraperitoneal injection) and positioned in a stereotactic frame (Jangwan I; Zhenghua Bioinstrumentation Co., Ltd., An'Hui, China). A cranial burr hole (1 mm) was drilled on the right coronal suture 3.2 mm lateral to the midline. Collagenase IV (0.5 u/1.5  $\mu$ L) was injected into the right basal ganglia (coordinates: 1.4 mm posterior, 5.6 mm ventral, 3.2 mm lateral to the bregma) by using a 26-gauge needle connected to a microinfusion pump (0.3  $\mu$ L/min). After injection, the needle was removed and the burr hole was filled with bone wax. The skin incision was closed with sutures.

## Transplantation of ADSCs

Rats were anesthetized with 6% chloral hydrate (1 mL/200 g, intraperitoneal injection) 48 h after ICH induced by injection of collagenase type IV. Rats were randomly divided into two groups ( $n = 40$ /group): ADSCs and control. In the ADSCs group, rats were injected with BrdU-conjugated ADSCs (10  $\mu$ L,  $2-4 \times 10^4$ / $\mu$ L) [11–13] into the right lateral cerebral ventricle (coordinates: 0.2 mm posterior, 4.5 mm ventral, 1.5 mm lateral to the bregma) by using a 26-gauge needle connected to a microinfusion pump at a rate of 1  $\mu$ L/min. Rats in the control group were injected with an equal volume of normal saline solution (NSS) as previously reported [14,15]. After injection, the burr hole was filled with bone wax and the skin incision was closed with sutures. Some rats died of ICH during the experiment were excluded from analysis. There were 15 rats per group that died during the period of ICH induction for 48 h by injecting of collagenase type IV and were excluded from analysis. No rats died after ADSCs or NSS treatment.

## Behavioral Tests

The neural function was evaluated with Zea Longa 5-grade scale at day 1, 3, 7, 14, or 28 after ADSCs or NSS transplantation according to previous report [16]. The neurologic findings were scored on a five-point scale: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) and 4 (no spontaneous walk) indicates severe focal deficits [16]. Behavioral assessments were performed by investigators unaware of treatment applied to the animals.

## In vivo Analysis of Neural-like cells Differentiation from ADSCs

At day 7 after ADSCs or NSS transplantation ( $n = 5$ /group), brains were immediately snapped frozen in OCT blocks. The brain tissues surrounding the injection needle were sectioned at a thickness of 10  $\mu$ m in the coronal plane, mounted on slides coated with polylysine, and fixed with 4% paraformaldehyde (15 min). After permeated with 0.5% Triton X-100 (15 min), the sections were acidified with 2 N hydrochloric acid (30 min) and neutralized with boric acid (10 min). The sections were then blocked with 10% fetal calf serum (1 h) and incubated with primary antibodies

(4°C, overnight). After washing three times, the sections were incubated with secondary antibodies (room temperature, 30 min). Antibodies used in this study included mouse monoclonal antibodies of NeuN (Sigma-Aldrich) at a dilution of 1:100, mouse monoclonal antibodies of GFAP (Millipore) at a dilution of 1:100, rabbit anti-BrdU polyclonal antibody (Millipore) at a dilution of 1:200, Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:250, and FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:100. The ADSC-differentiated neuron-like (BrdU+NeuN+) and glia-like cells (BrdU+GFAP+) in the areas surrounding hematoma and the corresponding regions of contralateral side were analyzed under the fluorescence microscopy. The stained cells in five random fields (200 $\times$ ) were counted and averaged for each section and the average of five sections represented the data for each side of the brain. The cells were counted by an investigator who was unaware of animal group information.

## Measurement of VEGF expression and Cell Apoptosis

Rat brains were randomly harvested at day 1, 3, 7, 14, or 28 after ADSCs or NSS transplantation ( $n = 5$ /group/time point). The frozen rat brain sections (10  $\mu$ m) were stained using an anti-vascular endothelial growth factor (VEGF) kit (Zhongshan Co., Ltd., Beijing, China) according to the manufacturer's instructions. VEGF intensity was quantified using an image analysis system linked to an Olympus camera (Olympus BX51). Five randomly chosen fields (400 $\times$  magnifications) were examined. Data were analyzed and quantified using stereological image processing software Image-Pro Plus (Version 6.0 for windows). Investigators were blinded to the treatment.

For analysis of cell apoptosis, the adjacent sections (10  $\mu$ m) of the brains harvested in each group and time point were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an *in situ* cell death detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. TUNEL-positive cells were counted in five randomly chosen fields (400 $\times$  magnifications). The apoptotic rate (%) was calculated as the TUNEL-positive cells divided by the total number of cells. Investigators who measured apoptotic cells were blinded to the treatment.

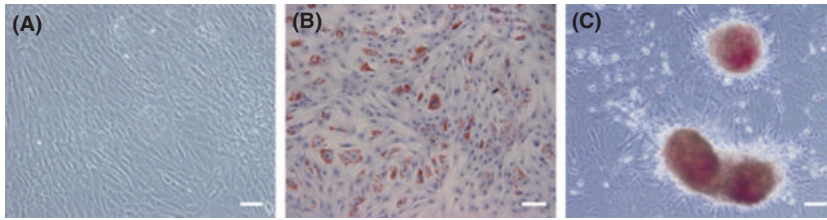
## Statistical Analysis

Data are presented as mean  $\pm$  SEM. Continuous variances were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni–Dunn *post hoc* test for comparison of differences between groups were determined. For comparison of the neurological deficit scores among different groups, we used significant difference, the Mann–Whitney *U*-test was applied. A *P*-value < 0.05 was considered significant.

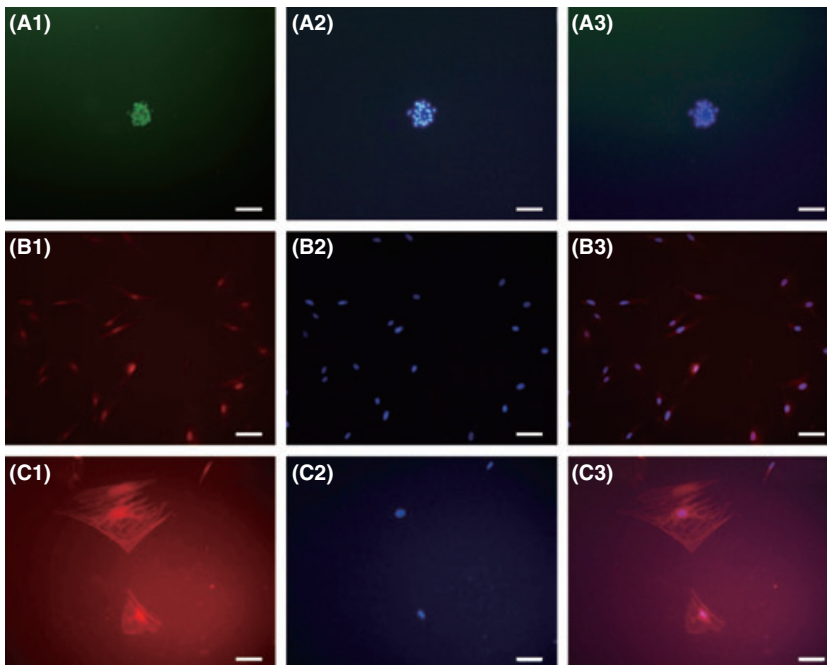
## Results

### ADSCs Were Able to Differentiate into Adipogenic and Osteogenic Cells *in vitro*

Cultured ADSCs grew as a monolayer of spindle-shaped cells with large nuclei morphologically (Figure 1A). At day 12 after



**Figure 1** Morphological characteristic of adipogenic and osteogenic differentiation from adipose-derived stem cells (ADSCs). **(A)** Morphology of ADSCs. **(B)** Adipogenic differentiation (Oil-Red O staining). **(C)** Osteogenic differentiation (alkaline phosphatase staining). Scale bar: 100  $\mu\text{m}$ .



**Figure 2** Neural-like cells differentiations of adipose-derived stem cells (ADSCs) *in vitro*. **(A)** Immunofluorescent staining showed that the neurosphere-like cells were nestin positive (green: Nestin, blue: cell nuclear). **(B)** Neurosphere-like cells were NeuN positive (red: NeuN, blue: cell nuclear). **(C)** Glia-like cells were glial fibrillary acidic protein (GFAP) positive (red: GFAP, blue: cell nuclear). Scale bar: 100  $\mu\text{m}$ .

adipogenic induction, lipid droplets accumulated within the cells were revealed by Oil-Red O staining (Figure 1B). At day 14 after osteogenic induction, mineralized bone nodules were detected within the cells by AP staining (Figure 1C). Meanwhile, the cell morphology changed to a polygonal or cubic shape.

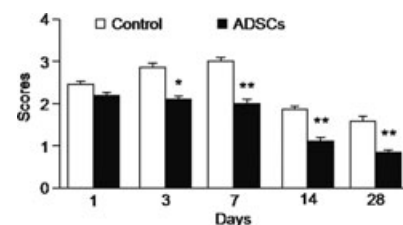
### ADSCs Were Able to Differentiate into Neural-like and Glia-like Cells *in vitro*

Five to 7 days after culturing ADSCs in induction medium, neurosphere-like cells were detected showing nestin expression (Figure 2A). By day 10, these neurosphere-like cells started to express neural markers, NeuN (Figure 2B) or GFAP (Figure 2C).

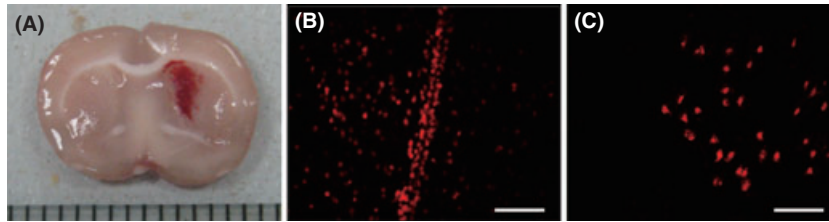
### Transplantation of BrdU-conjugated ADSCs Decreased ICH-induced Neural Dysfunction

Neural function was evaluated by Zea Longa 5-grade scale. There was no significant difference in neurological deficit score at

day 1 between ADSC transplantation group and control group (vs. control,  $P > 0.05$ ). However, at day 3, 7, 14, and 28, the scores were significantly lower in ADSC transplantation than those in control group (vs. control,  $P < 0.05$  or 0.01, Figure 3).



**Figure 3** Effect of adipose-derived stem cells (ADSC) transplantation on neurologic deficit score in intracerebral hemorrhage rats. ADSC transplantation improved neural function at different time points determined by Zea Longa 5-grade scale. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group,  $n = 5/\text{group}/\text{time point}$ .

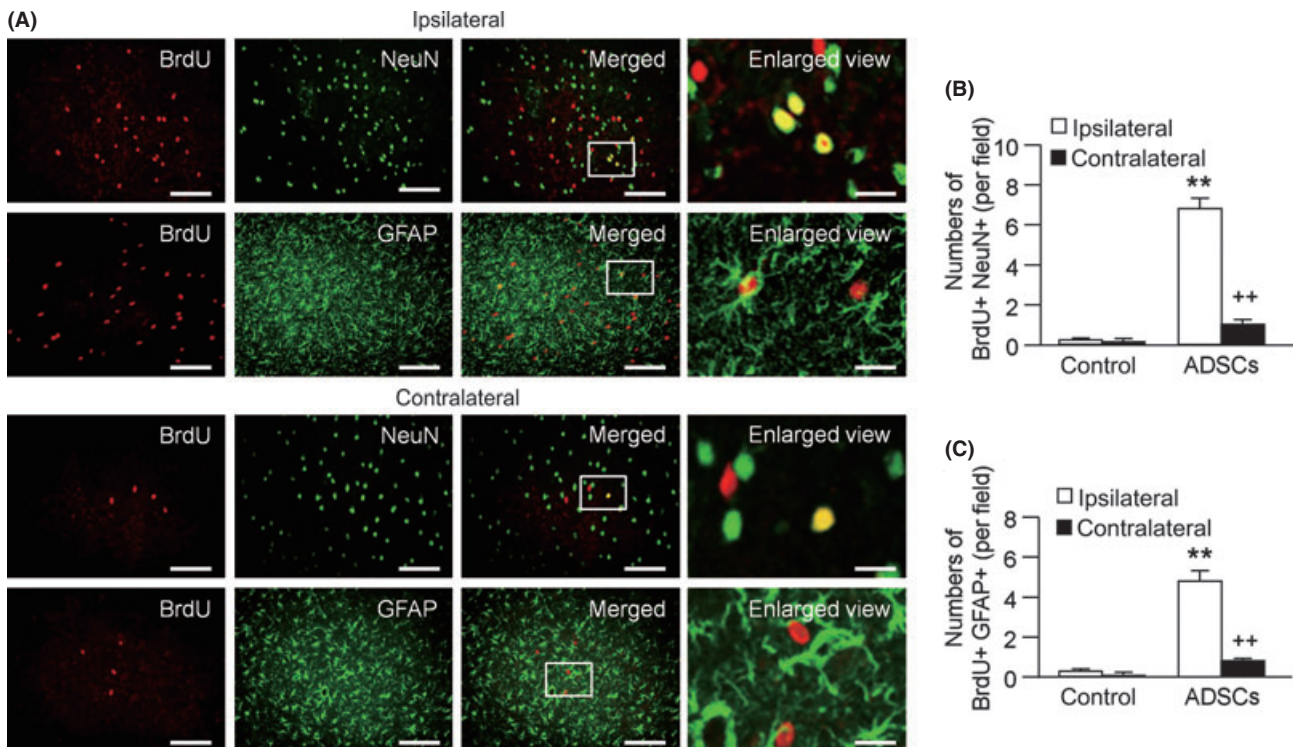


**Figure 4** A rat model of intracerebral hemorrhage (ICH) and migration of BrdU-conjugated adipose-derived stem cells (ADSCs) after transplantation. **(A)** Representative coronal brain section showing the success of ICH model in rat. **(B)** On day 1 after transplantation, BrdU-conjugated ADSCs concentrated on the tunnel made by injection. **(C)** On day 7 after transplantation, BrdU-conjugated ADSCs migrated to the surrounding area of hematoma. Scale bar: 100  $\mu$ m.

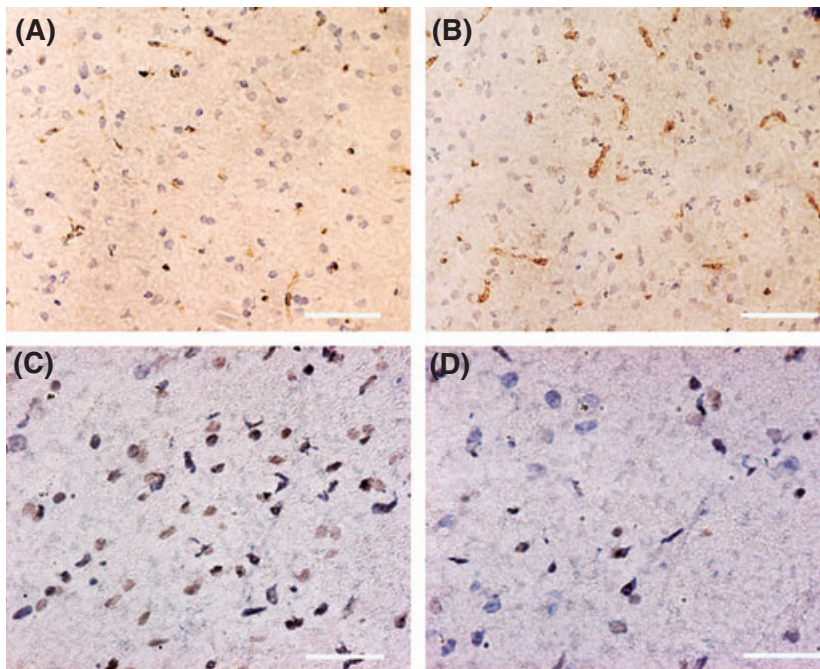
**BrdU-conjugated ADSCs Differentiated into Neural-like and Glia-like Cells *in vivo* after Transplantation**

In the coronal section, hematoma appeared in the basal ganglia area of all the rats 24 h after collagenase injection suggesting the success of ICH induction (Figure 4A). Fluorescent immunostaining and immunohistochemical staining showed that BrdU-conjugated ADSCs concentrated around the tunnel made by injection in day 1 (Figure 4B), and some of them were able to

migrate to the area surrounding the hematoma after 7 days (Figure 4C). In the corresponding brain region of the contralateral side, few labeled BrdU-ADSCs were found. Double-fluorescence labeling for BrdU and NeuN or GFAP revealed that ADSCs were able to differentiate into neuron-like (BrdU+/NeuN+) and glia-like cells (BrdU+/GFAP+), which were predominated in the surrounding area of hematoma (Figure 5A–C). The data suggested that the transplanted ADSCs were able to migrate to the ICH location, and to differentiate into neuron-like and glia-like cells.



**Figure 5** Neural-like cells differentiations of adipose-derived stem cells (ADSCs) *in vivo*. **(A)** Representative images of neuron-like cells (red: BrdU, green: NeuN, yellow: merged) or glia-like cells (red: BrdU, green: glial fibrillary acidic protein, yellow: merged) differentiation on day 7 after BrdU-conjugated ADSC transplantation. Scale bar: 100  $\mu$ m. The images in the last column are the enlarged views of the squared areas. Scale bar: 20  $\mu$ m. **(B)** Summarized data of neuron-like cell differentiation. **(C)** Summarized data of glia-like cell differentiation. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group; \* $P < 0.05$ , \*\* $P < 0.01$ , compared with ipsilateral side,  $n = 5$ /group.



**Figure 6** Representative images of vascular endothelial growth factor (VEGF) expression and cell apoptosis in the hematoma surrounding area on day 3 after adipose-derived stem cells (ADSC) transplantation. **(A)** VEGF in control group. **(B)** VEGF in ADSC group. **(C)** Apoptosis in control group. **(D)** Apoptosis in ADSC group. Scale bar: 100  $\mu\text{m}$ .

### Transplantation of BrdU-conjugated ADSCs upregulated VEGF expression and decreased ICH-induced cell apoptosis

The levels of VEGF expression were significantly higher in the ADSC transplantation group than in the control group at day 3, 7, 14, and 28 (vs. control,  $P < 0.05$ , Figure 6A and B, Table 1). TUNEL staining showed that most of the apoptotic cells were around the hematoma. Apoptotic rate was significantly lower at day 3 after ADSC transplantation (vs. control,  $P < 0.05$ , Figure 6C and D, Table 1). The apoptotic rates were slightly lower in the ADSC transplantation group than those in the control group at day 7, 14, and 28 ( $P > 0.05$  for all, Table 1).

### Discussion and Conclusion

Intracerebral hemorrhage causes severe neurological deficits and extensive death rate in patients. Medical therapies against ICH such as mechanical removal of hematoma, prevention of edema

formation by drugs, or reduction of intracranial pressure show only limited effectiveness. Alternative approach is required by NINDS ICH Workshop Participants [17,18]. Recent progress in stem cell biology has opened up a new way of therapeutic strategies in brain injury and disease by replacing lost neural cells with transplanted neural stem cells [19–21]. However, obtaining neural stem cells from the brain is a difficult, injurious procedure and painful to patients. A source for easier and higher yield of adult stem cell is needed.

Adipose tissue is an alternative source of pluripotent mesenchymal stromal cells [22]. Cells isolated from adipose tissue are self-renewing and can be induced to differentiate along several mesenchymal tissue lineages, such as adipocytes, osteoblasts, myocytes, and chondrocytes. Our study showed that ADSCs can be induced into neuron-like cells *in vitro*, which is consistent with previous reports [7,23,24]. However, whether these cells can actually differentiate into neurons remains controversial [25–27]. In addition, the functional importance of these cells is poorly understood.

**Table 1** The effects of ADSCs transplantation on VEGF expression and cell apoptosis following intracerebral hemorrhage

Group	Variants	Days after transplantation				
		Day 1	Day 3	Day 7	Day 14	Day 28
Control	VEGF (density)	0.21 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.01	0.18 $\pm$ 0.01
	Apoptosis (%)	0.64 $\pm$ 0.12	0.57 $\pm$ 0.11	0.51 $\pm$ 0.09	0.45 $\pm$ 0.15	0.33 $\pm$ 0.08
ADSCs	VEGF (density)	0.23 $\pm$ 0.01	0.24 $\pm$ 0.03*	0.23 $\pm$ 0.03*	0.21 $\pm$ 0.01*	0.20 $\pm$ 0.01*
	Apoptosis (%)	0.64 $\pm$ 0.18	0.36 $\pm$ 0.04*	0.42 $\pm$ 0.12	0.34 $\pm$ 0.04	0.28 $\pm$ 0.07

ADSC, adipose-derived stem cells; VEGF, vascular endothelial growth factor. Data are means  $\pm$  SD. n = 5/group, \* $P < 0.05$  compared to control.

Studies of ADSC transplantation in several disease models start to shed light on their function roles. In an ICH model, transplanted ADSCs are found to densely populate in perihematomal areas at week 6 and significantly reduce both acute cerebral inflammation and chronic brain degeneration promoting long-term functional recovery [11]. However, these cells express endothelial markers (von Willebrand factor and endothelial barrier antigen) rather than neuronal or glial markers arguing against neuronal differentiation of these cells [11]. Intracerebral grafting of BDNF-transduced human adipose tissue stromal cells (hATSCs) significantly improves motor recovery of functional deficits in rats following middle cerebral artery occlusion [28]. Some of the engrafted cells express neural markers such as MAP2 and GFAP. However, the functional improvement might be owing to increased neurotrophic factors other than differentiation into neurons because the number of neural marker-positive cells is extremely low [4].

In our study, we found that ADSC transplantation significantly decreased the neurologic deficit scores at day 3, 7, 14, and 28 after ICH, demonstrating that transplantation of ADSCs can improve neuronal function. There are three mechanisms that might be involved in the beneficial effect of ADSC treatment for ICH. Firstly, the ability of transplanted ADSCs to differentiate into neuron-like and glia-like cells that compensate for ICH-induced loss of the nearby neuronal cells can account for the improved functional outcomes. This is supported by the evidence that ADSCs injected into the lateral cerebral ventricle were able to migrate to the surrounding area of hematoma at day 7. Some of these cells were differentiated into neuron-like cells expressing NeuN or glia-like cells expressing GFAP. Secondly, VEGF might be another possible mechanism because recent *in vivo* and *in vitro* studies showed that VEGF protects neurons from cell death [29–31]. In our study, we found the expression of VEGF in the surrounding area of hematoma was significantly upregulated in the ADSC transplantation group when compared with the control group. Increased VEGF expression was detected as early as on day 3 and remained higher till day 28 after ADSC transplantation. This may be attributed to ADSC transplantation, because ADSCs have been reported to stimulate the release of multiple growth factors including VEGF [32,33]. It should be noted that the data from others [34,35] showed that ICH itself could induce the upregulation of VEGF in the brain as early as on day 3. These help to interpret the functional improvement in the control group and persistent functional improvement after ADSC transplantation. Finally, the reduction of cell apoptosis in the surrounding area of hematoma could be

also involved in the beneficial effects of ADSC transplantation. Our data showed that the number of apoptotic cells around the hematoma was significantly decreased in the transplantation group on day 3.

Our results are not totally agreed with a previous report by Kim et al. [11], which showed that transplanted ADSCs differentiated into endothelial cells rather than neuronal or glial markers [11]. We believe that the disagreement could come from the difference of the medium used for culturing ADSCs. In Kim's study, ADSCs were cultured with endothelial growth medium supplemented with 2–5% FBS, which contains various growth factors, including VEGF, fibroblast growth factor, EGF, and insulin-like growth factor, whereas we cultured ADSCs in DMEM supplemented with 10% FBS. Nevertheless, our *in vitro* and *in vivo* findings showing the ability of ADSCs in differentiating into neuron-like and glial-like cells were supported by previous studies in ischemic stroke models [4,28] and in a spinal cord injury model [27].

In summary, our study demonstrated that isolated ADSCs from inguinal fat pads of rats could be induced to neuronal differentiation both *in vivo* and *in vitro*. The results suggest that improvements of neurological function after transplantation of ADSCs to rats with ICH might be owing to neuronal differentiation of implanted stem cells, which protect cells against ICH-induced apoptosis. Taking together, these data reveal that ADSCs could be an alternative source of stem cells for treatment of patients with ICH. However, the safety, time window, and proper dose of ADSC transplantation as well as the functional and characterizing studies on the neurons derived from ADSCs need further investigation.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (30770751 & 81171089 to Z.T., 81070878 to B.Z.), Health Commission of Hubei Province of China (JX4A03 to Z.T.), and the National Heart, Lung, and Blood Institute (HL-098637 to Y.C.).

## Disclosures

None.

## Conflicts of Interest

The authors declare no conflict of interest.

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