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Transplantation of human neuro-epithelial-like stem cells derived from induced pluripotent stem cells improves neurological function in rats with experimental intracerebral hemorrhage

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HIGHLIGHTS

- We reprogrammed ICH patient's skin cells into iPSCs.
- The iPSCs can differentiate into NES cells and neural cells *in vitro*.
- Therapeutic effects of iPSC-derived NES cells in rat ICH model were observed.
- The grafted NES cells differentiated into neural cells in the brain of ICH rats.
- Functional improvement of ICH rat is partially due to neuronal replacement.

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ABSTRACT

Specific targeted therapy for intracerebral hemorrhage (ICH), which has high disability and case-fatality rate, is currently not available. Induced pluripotent stem cells (iPSCs) generated from somatic cells of ICH patients have therapeutic potential for individualized cerebral protection. While, whether ICH patient-originated iPSCs could differentiate into neuro-epithelial-like stem (NES) cells and whether such NES cells could improve functional recovery in the hemorrhage-injured brain are unclear. Here, we showed that fibroblasts from an ICH patient can be efficiently reprogrammed to iPSCs by lentiviral vectors carrying defined transcription factors (OCT4, SOX2, KLF4, and c-MYC). These iPSCs have the typical morphology, surface antigens, capability of self-renewal and differentiating into cell types of all three embryonic germ layers that are similar to human embryonic stem cells (hESCs). Using defined serum-free neural differentiation medium, we induced the iPSCs differentiate into NES cells. Subsequently, the NES cells from ICH patient-originated iPSCs were transplanted into the perihematoma of rats with experimental ICH injury. Intriguingly, recovery of neurological dysfunction in experimental ICH rats was observed post-NES cells graftage. Transplanted NES cells migrated to the surrounding area of hematoma, survived and differentiated into neuron-like cells. Our study demonstrates that the transplantation of human iPS-originated NES cells is an effective approach of treating ICH injury and the improvement of neural function is partially due to neuronal replacement and regeneration.

1. Introduction

Intracerebral hemorrhage (ICH) is the second most common cause of stroke with high morbidity, mortality and poor functional outcome throughout the world [7]. The current treatments to ICH have little effect on the improvement of neurological function, because there are few effective interventions to activate ICH patient's endogenous neural stem cells to repopulate at stroke lesion for self-renew [12]. In such situations, it is useful to supply exogenous neural stem cells to rebuild damaged nerve network in ICH. It has been shown that transplantation of exogenous neural stem cells (NSCs) can reduce the brain edema and promote the recovery of neurological dysfunction in a rat model of ICH [9]. Embryonic stem (ES) cells have been demonstrated to have more

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significant survival rate than adult stem cells after transplantation [19]. However, the ethical issues and the possibility of immune rejection after transplantation limit the usability of ES cells and allogeneic NSCs.

Recently, induced pluripotent stem cells (iPSCs), which are extraordinarily similar to ES cells, were generated from adult somatic cells by introducing definite transcription factors [18,21]. These iPSCs may provide an adequate source for cell therapies by individualized cell transplantation, which circumvent problems associated with ethics and immunogenicity. Recent studies have been successful in deriving patient-specific iPSCs from ischemic stroke [13] and other neurological diseases [3,16]. In rat models of focal cerebral ischemia injury, iPSCs transplantation can improve behavior functions and reduce the infarct volume [13]. In addition, they were found to be able to migrate to injured brain areas and differentiate into neuron-like cells [2].

Whether ICH patient-originated iPSCs could differentiate into neuro-epithelial-like stem (NES) cells, and whether such NES cells could promote functional recovery in the hemorrhage-injured brain are unknown. In this research, we generated human iPSCs from an ICH patient by reprogramming skin fibroblasts via lentiviral transfection system and differentiate them into NES cells *in vitro*. The therapeutic effect of implanting these iPSC-derived NES cells was tested in ICH rat model, and differentiation of NES cells *in vivo* and recovery of neurological dysfunction were observed concurrently.

2. Materials and methods

2.1. iPSCs generation

A diameter (d) of 4 mm skin punch biopsy was obtained from a 53 years old patient with ICH following informed consent. This procedure that carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans was approved by the Ethics Committee of Zhengzhou University, Zhengzhou, P. R. China. Dermal tissue was digested with 0.25% trypsin/EDTA (Invitrogen) and cultured in fibroblast medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAAs), and 100 IU/ml penicillin/streptomycin) (all from Invitrogen). After 12 days fibroblasts achieved 80% confluence and were digested with trypsin and then subculture for 2–3 passages.

The fibroblasts were seeded at a density of 5×10^4 cells per well of 6-well plates 1 day before transfection. Equal amounts of lentiviruses (1.5×10^6) that carry the cDNAs of human OCT4, SOX2, KLF4, and c-Myc (SiDanSai) respectively, were mixed. Two rounds of viral transfections were performed consecutively in the presence of 10 μg/ml of polybrene (Sigma–Aldrich) over a period of 12 h. After transfection, fibroblasts were replated onto a layer of mouse embryonic fibroblasts cells (MEF) feeders. Medium was replaced with hESC medium (DMEM/F12 supplemented with 20% KnockOut serum replacement, 1 mM glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF), all from Invitrogen) the following day. 25–30 days after transfection, colonies were picked manually for expanded.

2.2. Alkaline phosphatase (AP) staining and immunocytochemistry

AP detection kit (Millipore) was utilized for AP staining. For immunocytochemistry, following fixation and permeabilization, cells were treated with blocking buffer for 1 h at room temperature. Cells were incubated with primary antibodies overnight at 4 °C and then with the appropriate secondary antibodies. 4′, 6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Images were obtained using a fluorescent microscope (Leica, DM IL LED). Primary antibodies included the hES-cell specific proteins Oct4, SOX2, SSEA4, TRA-1-60 and Nanog (1:400, Abcam). Secondary antibodies were goat anti-rabbit IgG-PE and goat anti-mouse IgG-PE (1:1000, Santa Cruz).

2.3. Pluripotent differentiation potential of iPSCs

iPSCs colonies were pipetted to small clumps and transferred to bacterial culture dish in hESC medium without bFGF to form Embryoid body (EB). After 8 days, EBs were seeded on plates for another 8 days. Immunostaining was performed using markers for endoderm (alpha-feto-protein (AFP)), mesoderm (α-smooth muscle actin (α-SMA) (Chemicon), Desmin) or ectoderm (β-III Tubulin, glial fibrillary acidic protein (GFAP)) (1:150, all from Santa Cruz except α-SMA).

iPSCs (2×10^6) were suspended in the medium less than 400 μl and injected into the hind limb muscle of 6-week-old male mice with severe combined immunodeficiency (SCID) ($n = 6$, Vital River Laboratories (VRL)). 2 months later, tumors were removed and paraffin sections were stained with hematoxylin and eosin.

2.4. Neural differentiation in vitro

The iPSCs derived from the patient with ICH were induced to differentiate as described [8]. In brief, iPSCs were cultured to forming EBs and the medium were changed to neural induction medium (DMEM/F12, 2 mM L-glutamine, N2 supplement (1:100, Invitrogen) and 10 ng/ml bFGF) on the 4th day. Day 9, clusters were plated uniformly. After seeding 7–9 days, neural tube-like rosettes in the colonies were detached by blowing gently. NSCs medium ((equal Neurobasal media (Invitrogen) and DMEM/F12, containing 1% NEAA, 2 mM-glutamine, B27 (1:100, Invitrogen)) which was added bFGF 10 ng/ml and endothelial growth factor (EGF) (10 ng/ml, Peprotech) were used for cell clumps floating cultivation to form neurospheres. Neurospheres were trypsinized into single cells and plated onto plates for continuing growth. To facilitate neuronal and astrocyte differentiations, neurospheres were plated for 2 weeks in the NSCs medium supplemented with different growth factors, BDNF (Peprotech) for neuronal and bFGF and EGF for astrocyte differentiation respectively. Neural stem cell-specific markers of Nestin (1:100, Santa Cruz), β-III Tubulin (specific marker protein of neuron) and GFAP (specific marker protein of astrocyte) were detected by immunocytochemistry.

2.5. Establishment of animal model

All animal experimental procedures that carried out in accordance with EC Directive 86/609/EEC for animal experiments were done under the Institutional Animal Care and Use approved by the Ethics Committee of Zhengzhou University, Zhengzhou, China. Twenty adult male Sprague-Dawley (SD) rats (Animal Center of Henan) weighing between 270 and 300 g were used to establish ICH model. Rats were localized in a stereotactic frame (Narishige SN-3, Tokyo, Japan) after anesthetized with 10% chloral hydrate (intraperitoneal injection). A 1-mm craniectomy was performed and a stereotactically guided needle was placed into the left striatum (3.5 mm lateral to midline, 0.5 mm anterior to bregma, 5.5 mm depth below the surface to midline). 10 μl Collagenase VII (Sigma–Aldrich, 0.25 U/μl) was injected by using a 26-gauge needle with a steady infusion rate of 1 μl/min [4].