Combination of Temperature-Sensitive Stem Cells and Mild Hypothermia: A New Potential Therapy for Severe Traumatic Brain Injury

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Abstract

Stem cell transplantation holds great potential for the treatment of traumatic brain injury (TBI). However, the micro-environment of reduced oxygen and accumulated toxins leads to low survival rates of grafted cells, which dramatically limits their clinical application. Mild hypothermia has been demonstrated to improve the micro-environment after severe TBI. Thus, we speculate that combinational therapy of mild hypothermia may promote survival of grafted cells, especially temperature-sensitive stem cells, which show the most activity in mild temperatures. In this study, we first isolated mesenchymal stem cells from umbilical cord (UCSMCs) and generated the temperature-sensitive UCSMCs (tsUCSMCs) by infection with a retrovirus carrying the temperature-sensitive tsA58 SV40 LT antigen gene. We demonstrated that tsUCSMCs grew and proliferated with more activity at 33°C than at 37°C by counting cell numbers with a hematocytometer, measuring the cell cycle with flow cytometry, and detecting proliferating cell nuclear antigen (PCNA) with immunofluorescence staining. Thereafter, we established the rat severe TBI model by fluid percussion, and injected PBS, UCSMCs, or tsUCSMCs into the injured region, and subject the animals to normothermia or mild hypothermia (33°C). We found that, compared with UCSMC or tsUCSMC treatment alone, their combination with hypothermia could significantly improve motor and cognitive function with more survival of the grafted cells. Furthermore, we observed that combined therapy with hypothermia and tsUCSMCs exerted the most protective effect on the recovery of neurological function of all the tested treatments, with the highest survival and proliferation rates, and the lowest apoptosis rate. Thus this may represent a new therapeutic strategy for the treatment of severe TBI.

Key words: mild hypothermia; stem cell; temperature-sensitive; transplantation; traumatic brain injury

Introduction

Stem cell transplantation provides new hope for the treatment of traumatic brain injury (TBI; Gao et al., 2006; Riess et al., 2007). However, the micro-environment of reduced oxygen and accumulated toxins leads to low survival rates of grafted cells (Karlsson et al., 2005), which dramatically limits the clinical application of cell transplantation in TBI, and underlies the need to focus on strategies to solve this problem.

TBI is the leading cause of mortality and disability among male adolescents and young adults and constitutes a major health and socioeconomic problem throughout the world (Lingsma et al., 2010). The primary injury and secondary events initiated minutes after TBI lead to cellular dysfunction and death and determine the extent of brain damage. The extended nature of these events and the multiplicity of targets offer opportunities for therapeutic interventions. So far, no single-agent treatment has been successfully used in the clinical setting (Loane and Faden, 2010; Maas et al., 2008), emphasizing the need to focus on strategies that simultaneously affect multiple injury mechanisms. Cell transplantation has garnered much attention for the treatment of TBI (Zanier et al., 2011). Most severe TBI patients receive craniotomies. In situ transplantation during craniotomy post-TBI may be a better way to perform cell grafting than lumbar puncture, ventricular puncture, or intravenous infusion. However, TBI-induced free-radical generation, mitochondrial
dysfunction, and the inflammatory response, create a devastating micro-environment for the survival of grafted cells, which further limits their effects in injured brain tissue (Hernandez, 2006). Therefore, improving the micro-environment is an important issue impeding cell transplantation.

Therapeutic hypothermia has been reported to improve functional outcomes and limit secondary damage in models of focal and diffuse TBI (Marion and Bullock, 2009). After TBI, brain tissues undergo edema and release catecholamines and free radicals that lead to degeneration of brain tissue (Busto et al., 1987; Globus et al., 1987). Accumulating evidence demonstrates that hypothermia has neuroprotective effects on TBI, especially severe TBI, involving attenuation of intracranial pressure (Kawai et al., 2000), cerebral perfusion pressure (Yan et al., 2010), brain metabolism (Bacher et al., 1998), free radical production (Globus et al., 1995), brain edema, neurotransmitter release, energy depletion, and production of reactive oxygen species, and reduces neuronal cell death and apoptosis (Brinkkoetter et al., 2008; Maier et al., 2002; Zhang et al., 2009), all of which play beneficial roles in the micro-environment post-TBI.

Increasing evidence demonstrates the multipotency of mesenchymal stem cells (MSCs) and their capability to exert a protective effect after injury in organs through paracrine production of mitogenic, antiapoptotic, and trophic factors through their immunomodulatory action (Salem and Thiemermann, 2010), and by their ability to efficiently scavenge reactive oxygen species (Valle-Prieto and Conget, 2010). Furthermore, MSCs have been shown to express neuronal and glial markers in vitro and in vivo (Munoz-Elias et al., 2003; Yano et al., 2005), thus possessing reparative potential after brain injury. Some researchers have cultured MSCs from the Wharton’s jelly (WJ) of the human umbilical cord (UCSMCs) and differentiated them in vitro into several tissue types (Weiss et al., 2006). Mesenchymal cells from the WJ of the umbilical cord possess stem cell properties (Fu et al., 2004; Mitchell et al., 2003; Wang et al., 2004). Studies have demonstrated that UCSMSCs can be induced to differentiate into neuron-like cells (Fu et al., 2004), and are also capable of differentiating into osteogenic, chondrogenic, adipogenic, and myogenic cells in vitro (Wang et al., 2004). These cells have the advantage of ready availability, do not require invasive bone marrow biopsies, and are more plentiful than umbilical cord blood–derived MSCs.

Immunolocalization techniques have previously been used to produce large numbers of cells to overcome the limitations of donor cell supplies. However, their tumorigenic potential has been an obstacle to the transplantation of immortalized cells. Consequently, conditionally immortalized cells have been generated by transduction by retroviruses (Jat et al., 1986), or with transgenic mice (Kohno et al., 2011; Oostendorp et al., 2002) harboring the temperature-sensitive tsA58 simian virus 40 large T (SV40LT) gene (Tegtmeyer, 1975). The immortalized cells proliferate at 33°C because the large T-antigen of SV40 binds to the tumor suppressor gene product p53, and/or the retinoblastoma gene Rb, which regulate cell proliferation (Obinata, 1997). At temperatures over 37°C, which are non-permissive, the large T gene becomes unstable and is degraded, which stops the growth of the cells (Kanehira et al., 2006). Thus, cell lines carrying the conditionally-immortalizing gene grow continuously at permissive temperatures, whereas growth arrest, apoptosis, and/or cell differentiation are induced at nonpermissive temperatures, which could successfully overcome the problem of tumorigenicity. In light of the fact that mild hypothermia (33°C) is suitable for the conditionally-immortalized stem cells, we speculate that mild hypothermia may not only provide a better micro-environment for the grafted cells, but it may induce these temperature-sensitive stem cells to grow and proliferate. In this study, we applied a combination of hypothermia and temperature-sensitive UCSMCs to treat experimental TBI.

**Methods**

**Antibodies and chemicals**

Mouse monoclonal antibody (mAb) BrdU was from Santa Cruz Biotechnology (Santa Cruz, CA). mAb DM1A against α-tubulin was from Sigma-Aldrich (St. Louis, MO). mAb PCNA were from Millipore (Billerica, MA). Rabbit polyclonal antibody (pAb) neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP), FITC-conjugated mAb CD29, CD44, CD90, CD34, CD45, CD14, and CD31 were from Abcam (Cambridge, U.K.). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Geneticin-418®, and trypsin were from Gibco (Grand Island, NY). BrdU, collagenase, and propidium iodide were from Sigma-Aldrich. The TUNEL apoptosis detection kit was from Millipore, and the BCA kit was from Pierce (Rockford, IL). Peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were from Pierce. Rhodamine red-X or Oregon Green® 488-conjugated secondary antibodies were from Invitrogen (Sydney, N.S.W., Australia).

**UCSMC isolation, culture, and immunophenotype analysis with FACS**

Human UCMSCs were isolated from WJ of the umbilical cord by methods previously described. Briefly, the cords were dissected and the blood vessels removed. The remaining WJ was cut into small pieces (1–2 cm²), treated with collagenase at 37°C for 18 h, and further digested with 0.25% trypsin. The digested mixture was then passed through a 100-μm filter to obtain cell suspensions. Cell suspension was seeded in the presence of DMEM supplemented with 20% FBS, 2 mM L-glutamine, and antibiotics (100 U penicillin and 100 μg/mL streptomycin). Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C. After 3 days of culture, the medium was replaced and non-adherent cells were removed. The medium was then changed twice weekly thereafter.

UCMSCs were characterized by flow cytometry during culture. Cells (10⁶) were washed in phosphate-buffered saline (PBS) for 20 min at room temperature, and incubated in the dark with FITC-conjugated antibodies against CD29, CD44, CD90, CD34, CD45, CD14, and CD31. Mouse isotypic antibodies served as the control. At least 50,000 events were acquired with a Cytometers FC500 flow cytometer (BD Biosciences, San Jose, CA).

**Generation of an immortalized UCSMC line (tsUCSMCs)**

An immortalized cell line was generated according to a previously described procedure (Hasan et al., 2010). UCSMC cultures at passage 4 were infected at 60% confluency for 8 h with fresh medium containing a retrovirus carrying the
temperature-sensitive SV40 LT antigen gene (PsC10-SV40-tssA58, a kind gift from Dr. Klucher of California University) in the presence of 4 μg/mL polybrene. Three successive 8-h infections were carried out, followed by antibody selection (150 μg/mL Geneticin-418). One of the clones was selected for this study. The tsUCSMCs were cultured at the permissive temperature of 33°C. The cell medium was replaced twice weekly, and the cells were seeded at a density of approximately 10^5 cells/cm² at 33°C, or at the nonpermissive temperature of 37°C to facilitate cell differentiation.

Assay of cell growth and cell cycle

The UCSMCs or tsUCSMCs were cultured in a 24-well plates at 37°C for 24 h, followed by culturing at 33°C or 37°C for 0–72 h. The culture medium was changed every day. After washing once with PBS, the cell number was counted using a hemocytometer. To examine the cell cycle, the cells were permeabilized with 70% alcohol for 2 h at 4°C. Then the fixed cells were treated with 10 μg/mL RNase A, followed by incubation with 50 μg/mL propidium iodide. DNA content was analyzed by a FACS flow cytometer.

Establishment of the TBI model

Sprague-Dawley rats were purchased from Chinese Military Academy of Medical Sciences and maintained in the animal center of the Institute of TBI and Neurology of the Chinese People’s Armed Police Forces (CPAPF). All rats were kept under standard laboratory conditions: a 12-h light/dark cycle with lights on at 6:00 am, kept under standard laboratory conditions: a 12-h light/dark cycle with lights on at 6:00 am, temperature 22±2°C, and water and food ad libitum. All animal experiments were performed according to the “Policies on the Use of Animals and Humans in Neuroscience Research,” revised and approved by the Society for Neuroscience in 1995.

The rats were randomized into the different treatment groups. The rats were anesthetized with 6% intraperitoneal chloral hydrate (6 mL/kg), and then surgically prepared for lateral fluid percussion brain injury or sham surgery as previously described (McIntosh et al., 1989). In brief, a 4-mm craniotomy was performed overlying the right parietal cortex (3.8 mm posterior to the bregma and 2.5 mm lateral to the midline). A plastic injury tube was then placed over the exposed dura, and was bonded by adhesive and dental acrylic. An endotracheal tube was inserted orally, and the rats were mechanically ventilated and maintained on a mixture of 70% nitrous oxide and 0.5–1.0% isoflurane, with the balance of oxygen. The rats were attached to the fluid percussion device (a saline-filled cylinder) via a female Luer-Lok fitting. Severe (2.5 atm) brain injury was then induced by the rapid injection of a pressure pulse of saline into the closed cranial cavity. Sham-operated animals were anesthetized and surgically prepared, but were not subjected to brain injury. After brain injury or sham surgery, the Luer-Lok and the dental cement were removed, and the skin was sutured. Animals recovered after injury or sham surgery on heating pads to maintain the body temperature at 37°C.

Mild hypothermia therapy and cell transplantation

Rats in the hypothermic group were cooled by an ice blanket. Body and brain temperatures were measured using a rectal thermometer and a thermistor in the temporalis muscle, respectively. The target rectal temperature of 33°C was achieved within 15 min. A constant rectal temperature of 33–33.5°C was maintained for 24 h using an ice blanket and an automated feedback heat lamp system, then the animal was rewarmed naturally. Normothermic rats were kept at a constant temperature (37°C) with a heating pad connected to a rectal temperature probe.

When the temperature reached 33°C, transplantation was performed in the hypothermic rats. Surviving TBI animals were randomized to receive either transplantation of PBS, UCSMCs, or tsUCSMCs. Prior to transplantation, the cells were incubated with 1 μg/mL BrdU for 24 h. Immediately after injury, 1×10⁶ cells in a 3- to 5-μL PBS suspension were injected through a 26-gauge syringe into areas adjacent to the injured region, 3.0–5.0 mm below the dura. The needle remained in place for 5 min after each injection, and a piece of bone wax was applied to the skull defect to prevent leakage of the injected solution.

Evaluation of the neurological impairment score and the Morris water maze test

Neurologic motor function was evaluated 24 h before injury, and 7 days, 14 days, 21 days, and 28 days after transplantation. Evaluation of motor function was performed by blinded, trained observers, using standard, well-established tests of balance, vestibulomotor function, and coordination. The composite neuroscore paradigm employed a previously described battery of behavioral tests (McIntosh et al., 1989). Performance on this battery of neurological tests has been shown to correlate with injury severity. The animals were graded using a scale from 4 (normal) to 0 (severely impaired) for each of the following seven indices: (1) left and (2) right forelimb contraflexion upon suspension by the tail, (3) left and (4) right hindlimb flexion when the forelimbs remain on a surface and the hindlimbs are lifted up and back by the tail, and (5) left and (6) right resistance to lateral pulsing. A composite neuroscore (maximum score of 24) was generated by combining the scores for each of these tests.

Morris water maze testing was performed according to a previously described procedure (Li et al., 2012). Briefly, the rats were trained to find a submerged platform by using a stationary array of cues outside the pool. The water was made opaque by milk powder. Acquisition training consisted of a total of 24 trials, given as four spaced trials per day from 21 to 26 days after transplantation. The probe tests were performed with the platform on the 26th day, and by removing the platform on the 28th day. Swimming paths in the probe test were monitored using an automatic tracking system. This system was used to record the swimming trace and the time spent in each quadrant.

Immunofluorescence and immunohistochemistry

For cell studies, the cells were cultured on cover-slips and fixed with 4% paraformaldehyde. Rhodamine red-X or Oregon Green 488-conjugated secondary antibodies were used for immunofluorescence. The images were visualized using confocal microscope.

For animal studies, the rats were sacrificed by an overdose of chloral hydrate and transcardially perfused with 50 mL normal saline, and then with 100 mL 4% cooled paraformaldehyde solution. The brains were post-fixed in the same
fixation of hippocampal tissue were cut at 25 μm using a vibratome. For immunohistochemistry, the immunoreaction was detected using horseradish peroxidase-labeled antibodies, and visualized with a diaminobenzidine tetrachloride system. For immunofluorescence, the immunoreaction was examined with rhodamine red-X- or Oregon Green 488-conjugated secondary antibodies. The images were visualized using a confocal microscope.

The quantitative data of the immunostaining intensity from fields around transplanted sites in 3 to 5 successive sections of 3 rats each were analyzed. The quantitative analysis was carried out by using the “measure IOD” function of Image Pro Plus according to the manufacturer’s instructions as reported previously (Chen et al., 2012).

**Stereological assessment**

To quantify the number of BrdU-positive cells at the implanted site at 7 days, 2 mm away from the implanted site at 7 days, and 2 mm away from the implanted site at 28 days, sections were examined using an Olympus Image System (Olympus, Tokyo, Japan), using an unbiased stereological method described previously (Tran et al., 2006; Sun et al., 2009). A total of 45 sections (25 μm; 15 sections at 0 mm/7 days, 2 mm/7 days, and 2 mm/28 days, respectively) spaced 105 μm apart from −0.8 mm to −6.8 mm from the bregma (n = 4 rats per group) were examined by a blinded observer. The region of interest was outlined using a 4× objective, and a 60× objective was used for cell counting. The area (A) of the counting frame was known relative to the stage-stepping intervals over the section, to calculate the sampling fraction (asf) = A (frame)/a (x,y step). The dissector height (h) was known relative to the section thickness (t). With these parameters, the number of total cell counts (N) was estimated as N = (∑Q)/(t/h)/(1/asf)(1ssf), where ssf was the section-sampling fraction, and ∑Q was the number of cells counted.

**TUNEL staining**

Apoptosis analysis was performed using a fluorescence TUNEL apoptosis detection kit (Millipore) according to the manufacturer’s instructions. The field was chosen randomly around the injured sites at 3 days after transplantation in 3 to 5 successive sections of 3 rats. The quantitative data of the immunostaining intensity were analyzed with Image Pro Plus.

**Reverse transcription PCR**

Total ribonucleic acid (RNA) was isolated using Trizol reagents according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA), and reverse transcribed to produce cDNA using the TaqMan reverse transcription reagent kit. The cDNA produced (50 ng) was used to detect the transcripts. The primers used were tsSV40LT: 5'-TGA GGC TAC TGC TGA CTC TCA ACA-3' (forward), and 5'-GCA TGA CTC CAA AAC TTA GCAATT CTG-3' (reverse). For polymerase chain reaction (PCR) amplification, 30 cycles were performed, and each cycle was programmed as follows: denaturing at 94°C for 30 sec, annealing at 64°C for 60 sec, and chain extension at 72°C for 90 sec. The PCR products were separated on 1.5% agarose gels.

**Statistical analysis**

Data were analyzed using SPSS 11.0 statistical software (SPSS Inc., Chicago, IL). Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Fisher’s LSD post-hoc test with 95% confidence, and the Student’s two-tailed t-test.

**Result**

**UCSMC culture and identification**

At approximately day 30, we observed stable fibroblast-like UCSMCs in culture using phase-contrast microscopy (Fig. 1A). To characterize the MSCs, we performed fluorescence-activated cell sorting (FACS) analysis using a battery of markers for characterizing MSCs. We found that MSCs were positive for the typical mesenchymal pattern of markers CD29 (99.83%; Fig. 1B), CD44 (99.77%; Fig. 1C), and CD90 (99.90%; Fig. 1D), whereas they were negative for the hematopoietic markers CD34 (0.73%; Fig. 1E), CD45 (1.09%; Fig. 1F), and CD14 (1.51%; Fig. 1G), and negative for the endothelial marker CD31 (3.21%; Fig. 1H). These data suggested that the UCSMCs were isolated and identified successfully.

**Generation and growth characterization of tsUCSMCs**

To harvest more stem cells, we conditionally immortalized UCSMCs by transfection of plasmid carrying the mutant SV40LT antigen gene (tsA58). The generation of tsUCSMCs was identified by examination of tsSV40LT mRNA expression. We observed that, compared with UCSMCs without transfection, tsSV40LT mRNA (Fig. 2A) was positively expressed in UCSMCs with transfection of tsSV40LT, suggesting the successful establishment of tsUCSMCs. To analyze the characterization of tsUCSMCs, we cultured tsUCSMCs at 33°C or 37°C. We first counted the cell numbers at passage 4 with a hematocytometer, and found that the cell numbers cultured at 33°C were significantly higher than those seen at 37°C, at 24, 48, 72, 96, 120, and 144 h (Fig. 2B). Then we detected the cell cycle at passage 4 with flow cytometry, and found that the time spent in S phase was longer at 33°C than at 37°C. These data indicated that tsUCSMCs could proliferate dramatically at 33°C, but nearly completely stopped growing at 37°C (Fig. 2C). We next examined the expression of proliferating marker PCNA in tsUCSMCs at 24, 48, and 72 h with immunofluorescence staining, and observed that the level of PCNA at all time points was higher at 33°C than at 37°C (Fig. 2D and E).

**Alteration of neurological motor function after TBI**

Injured animals showed slightly less weight loss than sham-operated animals. There were no significant differences in body weight among the different treated TBI groups (data not shown). The temporalis muscle temperature was 0.5–1.0°C lower than the rectal temperature. In the hypothermic groups, the rats were cooled to a brain temperature of 33°C within 20 min. No significant differences were found in the intervals required to reach this temperature between hypothermia groups. Rats were maintained at this level for 24 h, and were then rewarmed naturally. There were no significant differences in rewarming rates between hypothermia groups (data not shown).
FIG. 1. Human mesenchymal stem cells from umbilical cord (UCSMCs) culture and identification. Human UCSMCs were harvested as described in the text. (A) Cell morphology of UCSMCs at passage 4 was examined by phase-contrast microscopy. (B–H) Surface markers of cells at passage 4 were analyzed by flow cytometry with the FITC-conjugated monoclonal antibodies (mAbs) CD29 (B), CD44 (C), CD90 (D), CD34 (E), CD45 (F), CD14 (G), and CD31 (H; scale bar in A = 50 μm). Color image is available online at www.liebertonline.com/neu

FIG. 2. Growth characterization of temperature-sensitive mesenchymal stem cells from umbilical cord (tsUCSMCs). ts-UCSMCs were established by transfection of plasmid carrying the temperature-sensitive SV40 LT antigen gene (PrC10-SV40-tsA58). (A) mRNA extract of ts-UCSMCs or UCSMCs were examined with reverse-transcriptase polymerase chain reaction (positive: positive control, namely the tsSV40LT gene; tsUC, tsUCSMCs; UC, UCSMCs). (B) Cell numbers of tsUCSMCs at passage 4 cultured at 33°C or 37°C were counted at 0, 24, 48, 72, 96, 120, and 144 h (*p < 0.05, **p < 0.01 versus the 37°C group). (C) Cell cycle of tsUCSMCs at passage 4 cultured at 33°C or 37°C was detected with flow cytometry (**p < 0.01 versus S phase of 37°C group; #p < 0.01 versus G1 phase of the 37°C group). (D and E) The proliferation of tsUCSMCs cultured at 33°C or 37°C was examined by immunofluorescence staining with PCNA antibody at 24, 48, and 72 h. E shows the quantitative analysis of D (*p < 0.05, **p < 0.01 versus the 37°C group; scale bar in D = 50 μm). Color image is available online at www.liebertonline.com/neu
A group of rats \( (n=20) \) underwent only the initial surgical procedure without any injury and served as sham controls, and other rat groups received a severe brain injury. Immediately after brain injury, PBS, UCSMCs (UC), or tsUCSMCs (tsUC) were implanted around the injured region \( (n=40) \). Meanwhile, half of the rats in each group received hypothermia therapy \( (n=20) \). A total of 13 rats subjected to brain injury died within the first 2 h. Within the first 28 days following TBI, 3 rats died in each group. The investigators were blinded to the experimental groups. The individuals that conducted the TBI and transplant studies were different from those that conducted the neurological impairment tests and Morris water maze testing.

To evaluate the effects of different treatments on neurological motor functions, neurological impairment scores were calculated at 2 h before sham surgery, or at 3, 7, 14, and 28 days after treatment. At 3 days post-injury, all injured rats revealed a significant impairment in motor function compared to sham-operated rats (Fig. 3A and B). A distinct improvement in motor function was observed from 7 to 28 days with hypothermia therapy in rats injected with either PBS (PBS+hypo) or UCSMCs (UC+hypo) (Fig. 3A), indicating that hypothermia therapy contributed to the amelioration of neurological impairment after TBI. However, the impairment of motor function seen after the injection of UCSMCs (UC) had no obvious difference compared with the injection of PBS (PBS). Meanwhile, compared to the hypothermia and PBS group (PBS+hypo), the scores for combined therapy with hypothermia and UCSMCs (UC+hypo) had no obvious difference (Fig. 3A), suggesting that under hypothermia therapy, the implantation of UCSMCs did not improve neurological motor function. Compared to UCSMCs, the implantation of tsUCSMCs (tsUC) also did not alter the neurological impairments induced by TBI (Fig. 3B). Nevertheless, with hypothermia, implantation of tsUCSMCs (tsUC+hypo) led to a significant elevation of scores (Fig. 3B), suggesting that...
combined therapy with hypothermia and tsUCSMC transplantation could facilitate the recovery of neurological motor function after TBI.

**Alteration of cognitive function after TBI**

To explore the effects of hypothermia and tsUCSMCs on cognitive function of rats, we used Morris water maze testing. The rats were trained to find the hidden platform from 21 to 25 days post-transplantation. The latency or time spent in the target quadrant was recorded on day 26 or day 28, respectively. We found that hypothermia significantly restored the increase of latency (Fig. 3C and D), and decreased the time spent in target quadrant (Fig. 3E), compared with the use of stem cells alone. The combination of hypothermia and tsUCSMCs was most effective at increasing latency (Fig. 3C and D), and the time spent in the target quadrant (Fig. 3E), of all groups (data for the sham, sham+hypo, PBS, and PBS+hypo groups are not shown). This in vivo evidence suggests that combined therapy of hypothermia and tsUCSMCs could reverse the cognitive impairments seen after TBI.

**Survival, migration, and differentiation of implanted cells**

To trace the implanted cells in vivo, UCSMCs or tsUCSMCs were incubated with BrdU for 24 h prior to transplantation. With double-labeled immunofluorescence staining, we found that 98.4% of tsUCSMCs were marked by BrdU (Fig. 4). Then we transplanted BrdU-labeled UCSMCs or tsUCSMCs into the injured region. To examine the survival and migration of the implanted cells, slices were taken of the injured region at 7 days (0 mm/7 days), 2 mm away from the injured region at 7 days (2 mm/7 days), 2 mm away from the injured region at 28 days (2 mm/28 days), and 8 mm away from the injured region at 28 days (8 mm/28 days), and were evaluated by quantitative analysis of BrdU-positive cells at 0 mm/7 d (B), 2 mm/7 d (C), and 2 mm/28 d (D), after treatment with UCSMCs (UC), UCSMCs+hypothermia (UC+hypo), tsUCSMCs (tsUC), and tsUCSMCs+hypothermia (tsUC+hypo; \( p < 0.05, \quad **p < 0.01 \); scale bars in A = 50 \( \mu \)m). Color image is available online at www.liebertonline.com/neu

**FIG. 4.** Temperature-sensitive mesenchymal stem cells from umbilical cord (tsUCSMCs) were labeled with BrdU before transplantation. UCSMCs and tsUCSMCs were incubated with BrdU for 24 h prior to transplantation. Double-labeled immunofluorescence staining was done with BrdU antibody and Hoechst (scale bar = 20 \( \mu \)m). Color image is available online at www.liebertonline.com/neu

**FIG. 5.** The combination of hypothermia and temperature-sensitive mesenchymal stem cells from umbilical cord (tsUCSMCs) promoted survival and migration of implanted cells. BrdU-labeled UCSMCs or tsUCSMCs were transplanted into the injured region. (A) Injured region at 7 days (0 mm/7 d), 2 mm away from the injured region at 7 days (2 mm/7 d), 2 mm away at 28 days (2 mm/28 d), and 8 mm away at 28 days (8 mm/28 d), after transplantation of tsUCSMCs under hypothermia were examined by immunofluorescence staining with antibody against BrdU and Hoechst. The lower panels show higher-magnification images of the boxed areas in the upper panels (arrowheads indicate BrdU-positive cells). (B–D) Quantitative analysis of BrdU-positive cells at 0 mm/7 d (B), 2 mm/7 d (C), and 2 mm/28 d (D), after treatment with UCSMCs (UC), UCSMCs+hypothermia (UC+hypo), tsUCSMCs (tsUC), and tsUCSMCs+hypothermia (tsUC+hypo; \( p < 0.05, \quad **p < 0.01 \); scale bars in A = 50 \( \mu \)m). Color image is available online at www.liebertonline.com/neu
immunohistochemical analysis using BrdU antibody. The total number of BrdU-positive cells was counted using a non-biased stereological method. In tsUC+hypo group, we found that there was high expression of BrdU-positive cells at 0 mm/7 days (510,500 – 85,400), low expression of BrdU-positive cells at 2 mm/7 days (20,350 – 3680), moderate expression of BrdU-positive cells at 2 mm/28 days (163,500 – 29,860), and no expression at 8 mm/28 days (Fig. 5A), suggesting that implanted cells could survive at least for 28 days in vivo, and could migrate 2 mm, but not 8 mm. We also observed that hypothermia therapy contributed to improved survival and migration at 0 mm/7 days (Fig. 5B), and 2 mm/28 days (Fig. 5D). The combination of hypothermia and tsUCMSMs (tsUC+hypo group) led to improved survival and migration at 2 mm/7 days (Fig. 5B), 2 mm/7 days (Fig. 5C), and 2 mm/28 days (Fig. 5D), compared to the UC+hypo group.

To determine whether grafted stem cells differentiate into neural cells, at 7 days after transplantation, brain coronal sections were co-stained with BrdU antibody, neuronal nuclear antigen (NeuN), or the astrocyte-specific marker GFAP antibody. We found that few grafted stem cells differentiated into neurons or astrocytes in animals with UCSMCs alone, UCSMCs combined with hypothermia, and tsUCSMCs alone. In the tsUCSMCs+hypothermia group, we notes that a few grafted cells differentiated into neurons (Fig. 6A), and even fewer cells differentiated into astrocytes (Fig. 6B).

To investigate the proliferation of implanted UCSMCs or tsUCSMCs under hypothermia therapy 3 days post-transplantation, the levels of proliferating marker PCNA were evaluated by immunofluorescence analysis using antibody against PCNA. We found that the PCNA expression of the tsUC+hypo group was much higher than of the UC+hypo group (Fig. 7A and B), suggesting that the combination of hypothermia therapy and tsUCSMCs induced more proliferation of the implanted cells. To evaluate the apoptosis of implanted UCSMCs or tsUCSMCs under hypothermia therapy 3 days post-transplantation, the apoptosis of the implanted cells was examined with in situ apoptosis kits (Fig. 7C and D). We observed that the tsUC+hypo group harbored less apoptosis than the UC+hypo group (Fig. 7C and D), indicating that the combination of hypothermia therapy and tsUCSMCs could decrease apoptosis of the implanted cells.

**Discussion**

In the present study, we first generated the temperature-sensitive UCSMCs (tsUCSMCs), and investigated the growth characterization of tsUCSMCs at 33°C and 37°C. Then we...
established the rat severe TBI model by fluid percussion, and injected PBS, UCSMCs, or tsUCSMCs into the injured region, and implemented normothermia or mild hypothermia therapy (33°C) within 30 min. We found that, compared with UCSMCs or tsUCSMCs treatment alone, their combination with hypothermia therapy could improve neurological motor and cognitive function. Furthermore, we observed that combined therapy of hypothermia with tsUCSMCs exerted the most protective effect on the recovery of neurological function among all of the tested treatments, and had the highest rates of survival and proliferation and the lowest apoptosis rate.

TBI remains a major health care problem with devastating social and health care costs. Despite the significant progress made in our understanding of TBI, effective management has remained elusive (Dietrich et al., 2009; Marklund et al., 2006). To date, many therapeutic approaches have been tested in clinical trials, but they have not proven to be effective (Roberts et al., 2004). Mild hypothermia has been used in numerous studies of TBI (McIntyre et al., 2003). The neuroprotective effect afforded by mild hypothermia in the current study strongly emphasizes the importance of the period immediately after grafting for transplant survival. In contrast to pretreatment of donor tissue, few attempts have been made to improve transplant survival by treating the graft recipient for the first few days after implantation. Karlsson and colleagues recently showed a significant increase in graft survival when mesencephalic tissue was implanted into rats kept hypothermic (32–33°C) during surgery (Karlsson et al., 2000). In our study, we observed that the combination of hypothermia exerted more protective effects on recovery of neurological impairment after TBI than monotherapy with PBS injection or UCSMC or tsUCSMC implantation, a finding consistent with those of previous researchers.

Cell-replacement therapies, which have potential as effective treatments for TBI, have recently attracted considerable attention. In particular, the utility of stem and progenitor cells to reconstitute function post-TBI has been increasingly investigated. A major practical shortcoming of this technique is the low survival rate of the grafted cells (Karlsson et al., 2005). The lack of survival/integration of transplanted cells may be related to the severity of injury. Recent reports have described differences in neuronal stem cell survival, neuronal differentiation, and neurological improvement seen after transplantation of neural stem cells, between mild and severe experimental TBI in rats. Neuronspheres transplanted following mild TBI survived well in the hippocampus, but barely survived following severe TBI (Shindo et al., 2006). We used an injury that was defined as a moderate to severe injury. However, due to the relatively high number of animals that did not survive the injury, the induced injuries have to be redefined as severe. Therefore, enhancing the survival rate of grafted cells has become a key issue to be resolved in TBI, especially in severe TBI.

The tsSV40LT antigen confers temperature-dependent conformational changes on the large T-antigen, which permit cell growth at permissive temperatures (33°C), but arrests growth in the G1 (Larsson et al., 2004) or G2/M phase (Koizumi et al., 2004) at nonpermissive temperatures. At a permissive temperature (33°C), the tsSV40LT-antigen induces immortalization by inactivating the functions of several tumor suppressor molecules, such as p53 and pRB, but at a nonpermissive temperature it is rapidly inactivated and degraded, and releases pRB or p53 from the complexes (DeCaprio et al., 1988; Hsieh et al., 2000; Jat and Sharp, 1989; Yanai and Obinata, 1994). Previous findings suggest that the tsSV40LT antigen gene is useful for establishing conditionally-immortalized cell lines that have proved difficult to culture in vitro (Ebihara et al., 2004; Tabuchi et al., 2005). However, in this study, the use of temperature-sensitive UCSMCs was mainly an attempt to improve the cell survival and differentiation rate after hypothermic transplantation. In our study, we generated the tsUCSMCs by transfecting UCSMCs with a retrovirus carrying the A58 SV40 LT gene, and identified them with RT-PCR. In accordance with previous studies, tsUCSMCs here showed high growth at 33°C, and low growth at 37°C.

The most successful strategy to improve survival of grafted neurons has focused on treating donor tissue with single pharmacological agents, such as growth factors, antioxidants, or inhibitors of apoptotic pathways. However, whether combined approaches have additive beneficial effects is unclear. The temperature of mild hypothermia used in our study was 33°C, which corresponds to the permissive temperature (33°C) of tsUCSMCs. Hence, we speculated that the combined use of hypothermia and tsUCSMCs to treat TBI may have more beneficial effects on neurological motor and cognitive impairments. By implanting tsUCSMCs into the injured region and adopting mild hypothermia therapy, we observed that this combined therapy not only significantly attenuated the impairment of motor and cognitive function compared with PBS injection, tsUCSMCs implantation, or hypothermia therapy alone, but it was also more effective than therapy with hypothermia and normal UCSMCs. Under hypothermia, compared with normal UCSMCs, the implantation of tsUCSMCs induced better survival and migration with more proliferation and less apoptosis, all of which are consistent with improvements in neurological function. This phenomenon may partially be attributable to the improved microenvironment afforded by mild hypothermia, which induced tsUCSMCs to grow more actively at the permissive temperature used.

Although mesenchymal stem cells have the potential to become neurons, previous studies found only low percentages of neurons after grafting stem cells into TBI patients, a finding in agreement with ours. Though a few neurons differentiated from grafted cells were found by co-staining with BrdU and NeuN antibody, it is unlikely that this contributed to the motor and cognitive functional improvements seen at 4 weeks in injured rats following transplantation. Any functional contributions of neurons derived from stem cells would occur later, after the newly differentiated neurons had matured and integrated into the host circuitry. Thus the differentiation of grafted tsUCSMCs into neurons requires further experimentation.

In summary, here we demonstrated that combined therapy with temperature-sensitive UCSMCs and mild hypothermia had beneficial effects on the impairments suffered post-TBI, and may represent a new therapeutic strategy for severe TBI patients.

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