



# The therapeutic effect of CD133<sup>+</sup> cells derived from human umbilical cord blood on neonatal mouse hypoxic-ischemic encephalopathy model



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## ABSTRACT

**Aims:** Brain damage at birth can cause lifelong neurodevelopmental deficits. Recently, stem cell therapies have been used in several fields of medicine. We previously reported that CD133<sup>+</sup> cells, endothelial progenitor cells derived from human umbilical cord blood, induce nerve extension in an *ex vivo* hypoxic-ischemic encephalopathy model. Here, we used an *in vivo* model to examine the effect of CD133<sup>+</sup> cells in neonatal hypoxic-ischemic encephalopathy.

**Main methods:** Hypoxic-ischemic brain lesions were induced in neonatal severe combined immunodeficiency mice using the Rice–Vannucci method. CD133<sup>+</sup> cells were administered by intraperitoneal injection 24 h after injury.

**Key findings:** Immunohistochemical analysis revealed that intraperitoneally transplanted CD133<sup>+</sup> cells migrate towards the brain 48 h after injection. Moreover, in CD133<sup>+</sup> cell-treated animals, motor function improved and the brain was protected from the hypoxic-ischemic insult compared with untreated animals.

**Significance:** Our results suggest that CD133<sup>+</sup> cells derived from human umbilical cord blood have therapeutic potential in neonatal hypoxic-ischemic encephalopathy.

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## 1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a major cause of cerebral palsy (CP) in full-term infants. Although perinatal medicine has rapidly progressed, HIE occurs at a rate of about three per thousand live-born infants, even in developed countries [1]. Of these HIE patients, 15–20% die in the neonatal period and 30% suffer from neurological deficits including CP, mental retardation, learning disabilities, and epilepsy [2]. Although some new treatments have been developed (e.g., hypothermia and brain hypothermia therapy), they have limited therapeutic effect. A recent trial of hypothermia treatment reduced the risk of death or major sensorimotor disability by only 15% [3,4]. Therefore, new and more effective treatments for HIE urgently need to be developed.

Recently, stem cell therapies have been developed in several fields of medicine. Endothelial progenitor cells (EPCs) are one of the major stem/progenitor cell subsets with potential for repairing vascular injury and protecting from ischemic lesions [5,6]. EPCs derived from umbilical cord blood show higher regenerative potential than adult bone marrow-derived EPCs. Because umbilical cord blood is a readily available source of cells for autotransplantation, EPCs from umbilical cord blood

are advantageous for therapeutic use in infants [7]. We hypothesized that transplantation of CD133<sup>+</sup> cells, an EPC-containing fraction from umbilical cord blood, may be a useful therapy in neonatal hypoxia-induced brain injury.

Previously, we established an *ex vivo* hypoxic encephalopathy model comprising organ co-cultures from the cerebral cortex and spinal cord, to assess the effects of CD133<sup>+</sup> cells derived from human umbilical cord blood, and confirmed their effect on nerve extension [8]. Hypoxia markedly suppressed axonal growth in organ co-cultures, and the suppression was significantly restored by addition of CD133<sup>+</sup> cells to the culture. CD133<sup>+</sup> cells also reduced hypoxia-induced destruction of cortical blood vessels and apoptosis [8]. Here, we further investigated the therapeutic potential of CD133<sup>+</sup> cells using an *in vivo* neonatal mouse HIE model.

## 2. Materials and methods

### 2.1. Animals

This study was performed in accordance with guidelines from the Committee of Research Facilities for Laboratory Animal Science at Hiroshima University. Postnatal day 7 severe combined immunodeficiency (SCID) mice (C.B-17/lcr-scid/scidJcl; CLEA Japan Inc., Tokyo, Japan)

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**Table 1**  
Body weight of hypoxic-ischemic or control (sham-operated) mice, untreated or intraperitoneally injected with CD133<sup>+</sup> cells.

| Group              | P7        | P28                     | P35                     | P42                     | P49                     | P56                     |
|--------------------|-----------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Sham-operated      | 3.6 ± 0.1 | 15.4 ± 0.4              | 17.6 ± 0.8              | 20.1 ± 0.6              | 21.1 ± 0.6              | 22.7 ± 0.7              |
| HI                 | 3.8 ± 0.2 | 12.8 ± 0.8 <sup>a</sup> | 14.0 ± 0.4 <sup>b</sup> | 14.6 ± 0.6 <sup>c</sup> | 15.8 ± 0.6 <sup>d</sup> | 16.7 ± 0.6 <sup>e</sup> |
| CD133 <sup>+</sup> | 3.8 ± 0.2 | 12.8 ± 1.0 <sup>f</sup> | 14.9 ± 0.9 <sup>g</sup> | 16.4 ± 0.9              | 17.6 ± 1.0              | 18.5 ± 1.1              |

Body weight is in grams (mean ± SE). Postnatal day 7 (P7) = day of surgery, P28 = day of first rotarod test.

- <sup>a</sup> P = 0.015,
- <sup>b</sup> P = 0.002,
- <sup>c</sup> P < 0.001,
- <sup>d</sup> P < 0.001
- <sup>e</sup> P < 0.001
- <sup>f</sup> P = 0.014
- <sup>g</sup> P = 0.015

were used. As human cells were administered to mice, in this study, we used immunocompromised mice in order to suppress immunological reactions due to xenotransplantation.

2.2. Hypoxic-ischemic model

The Rice–Vannucci model [9,10] was applied to establish hypoxia-ischemia in neonatal SCID mice. Unilateral hypoxic-ischemic cerebral damage was induced in 7-day-old SCID mice [11] by permanent occlusion of the left common carotid artery under anesthesia. There is compelling evidence that postnatal day 7 mice have brain maturity equivalent to that of third trimester human fetuses [12]. After surgery, pups were allowed to recover from anesthesia in their home cages for 1 h. Pups with unilateral carotid artery occlusion were then exposed to 8% O<sub>2</sub>/92% N<sub>2</sub> for 30 min at 34 °C (ambient temperature) [13,14]. Twenty-four hours after the hypoxic-ischemic insult, pups received 1 × 10<sup>5</sup> human umbilical cord blood-derived CD133<sup>+</sup> cells (in a volume of 100 µL 0.9% sodium chloride) by intraperitoneal injection. Pups from five litters were randomly assigned to three experimental groups: (1) sham-operated group (sham-operated animals in which the left common carotid artery was exposed but not ligated, n = 7); (2) HI group (hypoxic-ischemic insult and 100 µL 0.9% sodium chloride injection as sham injection, n = 7); and (3) CD133<sup>+</sup> group (hypoxic-ischemic insult and CD133<sup>+</sup> cell injection, n = 8). There was no difference in body weight between the three groups on postnatal day 7, but the mice in the HI group were significantly smaller than sham-operated mice on postnatal day 28 and thereafter. The weight of animals in the CD133<sup>+</sup>

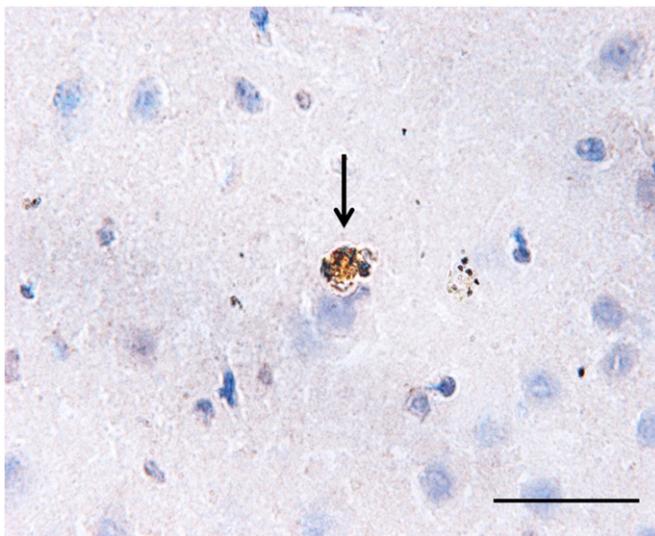
group was significantly lower than those of the sham-operated group on postnatal days 28 and 35; however, there was no significant difference between these two groups on postnatal day 42 and thereafter. There was no significant difference in body weight between the CD133<sup>+</sup> and HI groups (Table 1).

2.3. Isolation and preparation of human CD133<sup>+</sup> cells

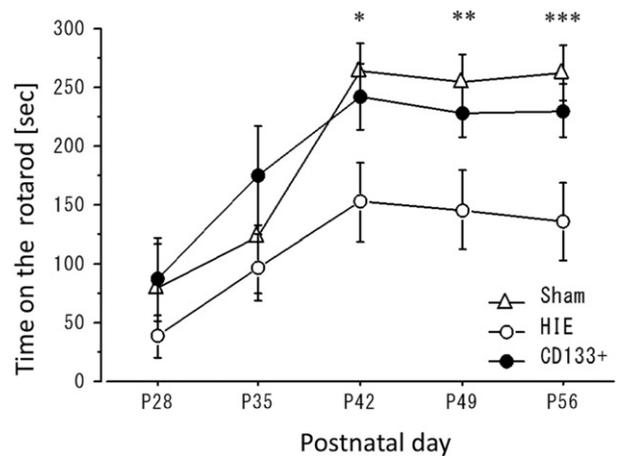
The protocol used was approved by the Hiroshima University Research Ethics Committee, and informed consent was obtained from all participants. Human CD133<sup>+</sup> cells were prepared from freshly obtained human umbilical cord blood, as described in our previous report [15]. Only full-term umbilical cord blood was used because lower numbers of endothelial colonies are generated from preterm compared with term umbilical cord blood [16]. Umbilical cord blood mononuclear cells were isolated by density-gradient centrifugation of buffy coats using histopaque1077 (Sigma-Aldrich, Oakville, Canada). CD133<sup>+</sup> cells were purified from mononuclear cells using anti-CD133 monoclonal antibody-conjugated microbeads (CD133 MicroBead Kit; Miltenyi Biotec, Bergisch-Gladbach, Germany) and a magnetically activated cell sorter (auto-MACSpro; Miltenyi Biotec), following the manufacturer's protocol. Isolated CD133<sup>+</sup> cells were suspended in freezing medium (CELLBANKER; Zenoaq, Fukushima, Japan) and cryopreserved until use.

2.4. Motor function analysis

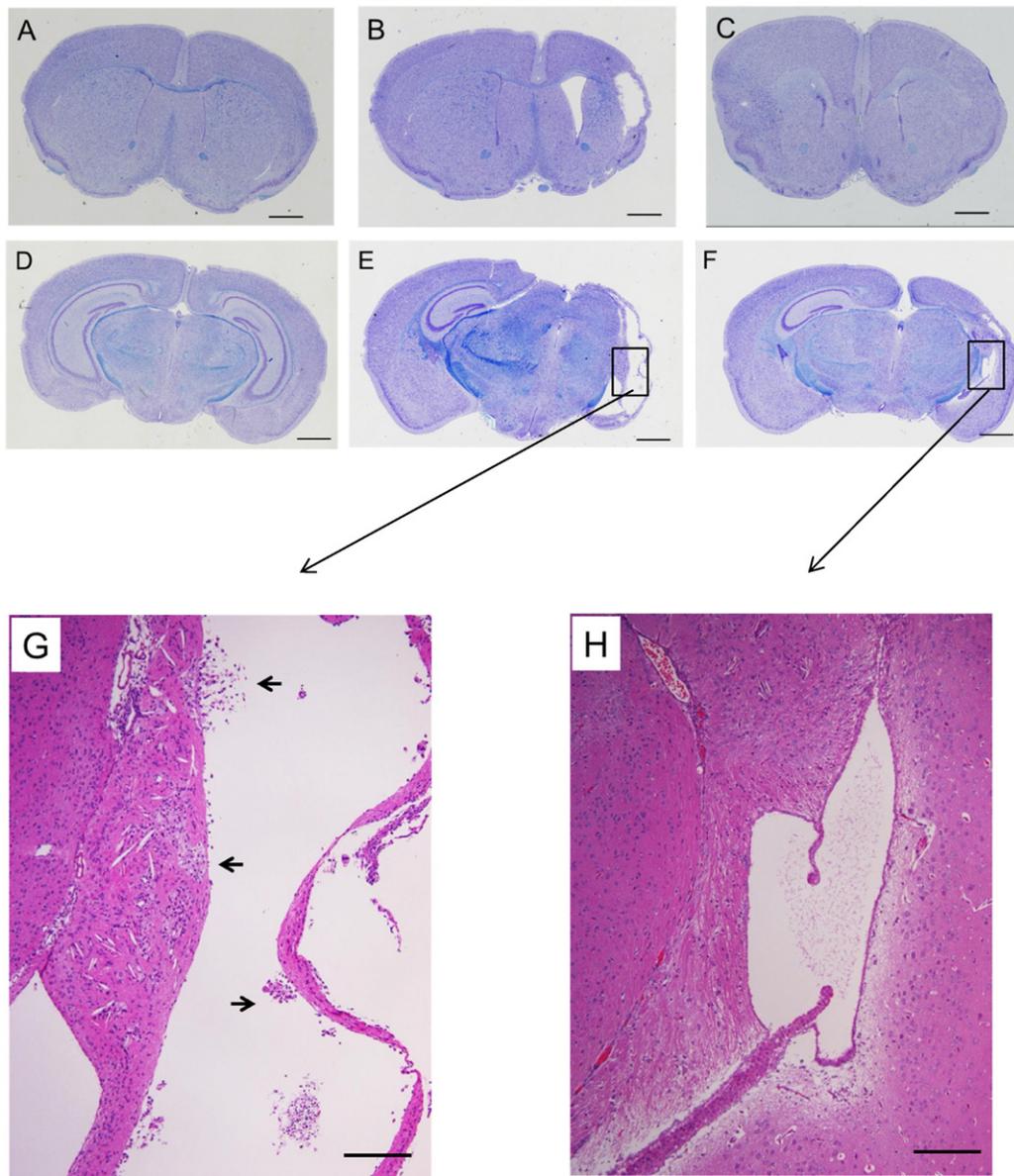
To evaluate motor function, the rotarod test was performed every 7 days between postnatal days 28 and 56. The rotarod test is commonly used for determining motor coordination in rodents [17]. When placed



**Fig. 1.** CD133<sup>+</sup> cell migration to the hypoxic-ischemic lesion site. P7 mice were exposed to hypoxia-ischemia according to the Levine method, followed by intraperitoneal injection of CD133<sup>+</sup> cells. CD133<sup>+</sup> cells were identified in the brain by immunohistochemical detection using anti- HLA-DR α-chain surface antigen. Scale bars: 50 µm. Magnification: ×400.



**Fig. 2.** Performance of hypoxic-ischemic lesioned mice on the rotarod test. P7 mice were exposed to hypoxia-ischemia according to the Levine method, followed by intraperitoneal injection of CD133<sup>+</sup> cells. The rotarod test was performed every 7 days. The time spent on the rotarod was plotted for each group. Δ: sham-operated group (n = 7); ○: HI group (n = 7); ●: CD133<sup>+</sup> group (n = 8). \*P = 0.035, \*\*P = 0.027, \*\*\*P = 0.009. Data are expressed as mean ± SE.



**Fig. 3.** Brain damage after hypoxic-ischemic insult. P7 mice were exposed to hypoxia-ischemia according to the Levine method, followed by intraperitoneal injection of CD133<sup>+</sup> cells. Cerebral hemisphere coronal sections were obtained at the level of the optic chiasm (A–C) and hippocampus (D–F) in sham-operated (A, D), HI (B, E), and CD133<sup>+</sup> groups (C, F) at postnatal day 42. (G) Higher-magnification image of the cystic lesion in (E), which shows several macrophage foci (arrows). (H) Higher-magnification image of the cystic lesion in (F) showing no macrophages. Scale bars, (A–F): 200 μm; (G, H): 100 μm.

on the rotarod, animals must continuously walk forward to remain on the rod. The rotation speed of the rod was fixed at 15 rpm. The mice rested in their cage for 20 min between each trial. The mean time (maximum 5 min) for each mouse on the rotarod was determined from five trials. After completion of the rotarod test, brains were collected on postnatal days 42 or 56 for histological analysis.

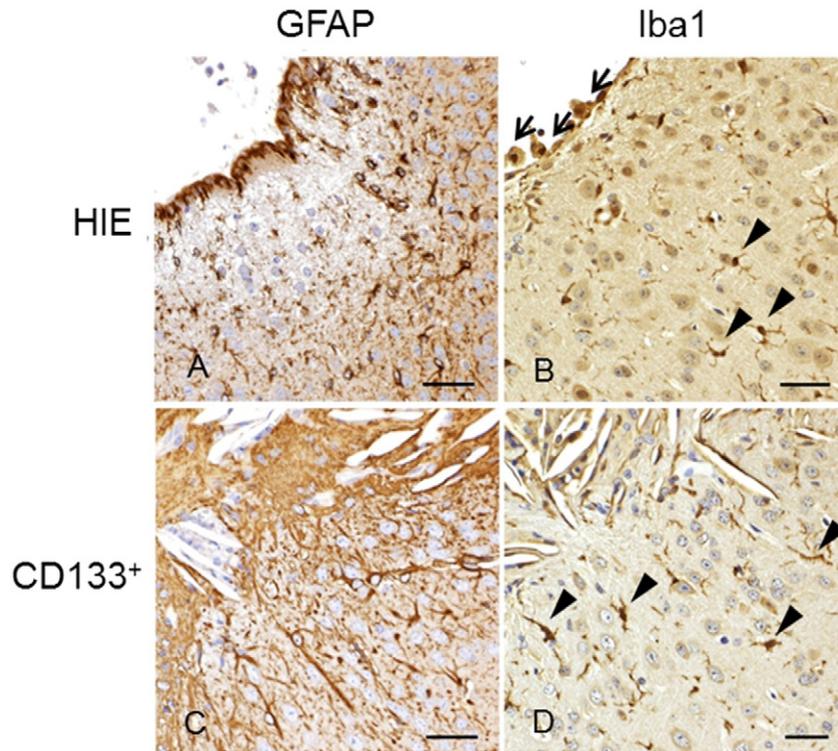
### 2.5. Histological examination

After the rotarod test on postnatal days 42 and 56, animals in each group were deeply anesthetized with diethyl ether, and the brains removed (sham-operated group,  $n = 7$ ; HI group,  $n = 7$ ; and CD133<sup>+</sup> group,  $n = 8$ ). The specimens obtained on day 42 were fixed with 4% paraformaldehyde and paraffin embedded. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin or by the Klüver–Barrera method [18]. Brains obtained on day 56 were immersed in SCEM gel (Section Lab, Hiroshima, Japan) and completely frozen with the gel in cooled hexane. Embedded brains were sectioned coronally at

8-μm thickness, according to Kawamoto's film method [19]. Lesion sizes were estimated after Klüver–Barrera staining, which shows myelinated fiber tracts in blue and nerve cell bodies in violet. Lesion size was determined by measuring the hemispheric area of the brain in sections at two defined coronal levels (the optic chiasm and hippocampus) using Image J software [20]. The ratio of left to right cerebral hemispheric area was measured to determine atrophy of the lesioned hemisphere. In addition, the sizes in hippocampus, striatum and thalamus were compared.

### 2.6. Immunohistochemistry

As previously described [21], we also examined paraffin-embedded sections by immunohistochemistry using the following primary antibodies: mouse monoclonal antibodies against human leukocyte antigen class II (HLA-DR) α-chain (Dako, Hamburg, Germany; 1:200), α-smooth muscle actin (anti-α-SMA; Funakoshi, Tokyo, Japan; 1:200), rabbit polyclonal antibodies against Iba1 (Wako, Osaka, Japan; 1:500), cleaved caspase 3 (Cell Signaling Technology, Danvers, MA; 1:200)



**Fig. 4.** Reactive astrocytosis (A, C) and infiltration of microglia (arrowheads) and macrophages (arrows) (B, D) in and around the cystic lesion in HIE (A, B) and CD133<sup>+</sup> (C, D) groups. A, C: GFAP immunostain. B, D: Iba1 immunostain. Scale bars, (A–D): 50  $\mu$ m.

and glial fibrillary acidic protein (GFAP) (Dako; 1:500). The vascular endothelium was stained by fluorescein Isolectin B4 (IB4; 1:100, Vector). The sections were subjected to immunohistochemical processing using the avidin–biotin–peroxidase complex method with diaminobenzidine. These assessments were performed on slides without knowledge of animal's identity by two neuropathologists (Yasuo Miki and Koichi Wakabayashi). Since there are no immunohistochemical markers to distinguish between activated microglia and infiltrated macrophages [22], we identified these two cell types morphologically: activated microglia were defined as small rod cells with wavy, branching processes and infiltrated macrophages were defined as round cells with abundant granular and vacuolated cytoplasm.

### 2.7. Statistical analysis

Values were expressed as mean  $\pm$  standard error. Statistical differences between two groups were assessed using the Student *t*-test. Data of between three groups were analyzed using two-way ANOVA followed by the appropriate post hoc test (Student–Newman–Keuls). *P*-values less than 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. CD133<sup>+</sup> cell migration to the brain

Intraperitoneally transplanted CD133<sup>+</sup> cells were immunohistochemically detected in the lesioned hemisphere at 48 h after transplantation (Fig. 1). The number of detectable CD133<sup>+</sup> cells peaked at 96 h after transplantation and declined thereafter. Only a few CD133<sup>+</sup> cells were detectable in the non-lesioned hemisphere.

### 3.2. Analysis of motor function

To examine motor function, the rotarod test was performed every 7 days between postnatal days 28 and 56. Fig. 2 shows the latency

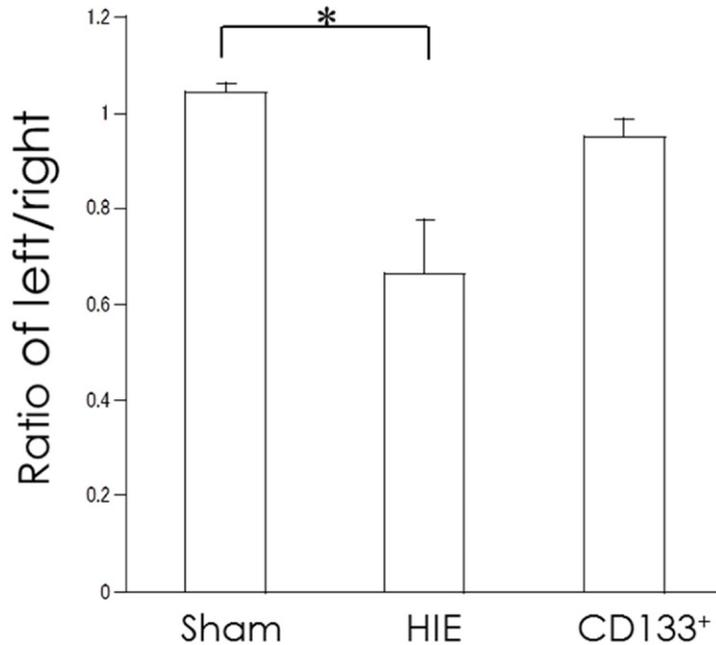
before falling from the rotarod. Motor function was significantly impaired in the HI group compared with the sham-operated group ( $P = 0.010$ ) on postnatal day 42. Moreover, motor function was significantly improved in the CD133<sup>+</sup> group compared with the HI group ( $P = 0.038$ ) on postnatal day 42. Significant differences were also observed between the HI and CD133<sup>+</sup> groups on postnatal days 49 ( $P = 0.037$ ) and 56 ( $P = 0.019$ ).

### 3.3. Histological analysis of the brain

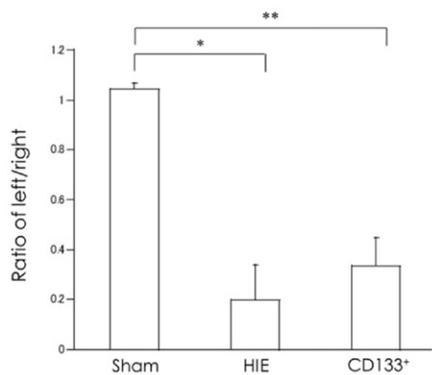
Histopathological examination revealed no obvious abnormalities in the brains of sham-operated mice on postnatal day 42 (Fig. 3A and D). After hypoxic-ischemic insult, infarction was observed in the frontoparietal region, caudate-putamen complex, and hippocampus. Large cystic lesions were found in the left hemisphere (Fig. 3B and E). These cysts contained several foci of macrophages and reactive astrocytes, and microglia were found in the surrounding area (Figs. 3G and 4A,B). These macrophages and microglia were immunopositive for Iba1 (Fig. 4B). In the CD133<sup>+</sup> group, cystic lesions with reactive astrocytes and microglia were also observed (Fig. 3H and Fig. 4C, D). No apoptotic cells were identified by cleaved caspase 3 immunohistochemistry in the surrounding area in both the HI and CD133<sup>+</sup> groups. However, the cystic lesions in the CD133<sup>+</sup> group were much smaller than those in the HI group. In addition, no or only few macrophages were found in the CD133<sup>+</sup> group (Fig. 3H and Fig. 4D).

Left (lesioned) and right (non-lesioned) hemisphere size was approximately the same in the sham-operated group at postnatal day 56. In the HI group, the lesioned hemisphere area was approximately 70% that of the non-lesioned hemisphere ( $P = 0.005$ ). In the CD133<sup>+</sup> group, the lesioned hemisphere area was approximately 90% that of the non-lesioned hemisphere. The ratio of lesioned to non-lesioned hemisphere area in the CD133<sup>+</sup> group was significantly higher than in the HI group ( $P = 0.004$ ) (Fig. 5). The hippocampus was specifically affected in the lesioned hemisphere in the HI and CD133<sup>+</sup> groups ( $P < 0.0001$ ). Damage to the striatum was detectable, but it was not

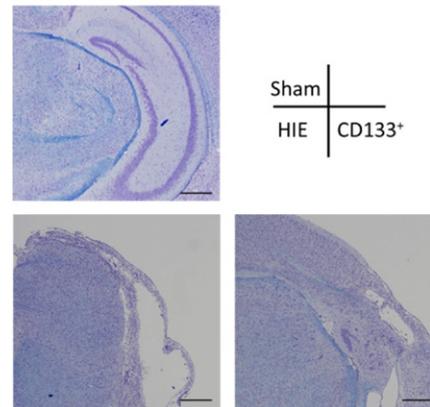
## A. Hemisphere



## B. Hippocampus



## C. Klüver-Barrera stain of hippocampus



**Fig. 5.** Ratio of right/left hemispheres. The ratios of left (lesioned) to right (non-lesioned) hemisphere were measured using ImageJ software, to evaluate brain damage on postnatal day 56. A: Comparison of total area of each hemisphere (\* $P = 0.005$ , \*\* $P = 0.004$ ), B: Comparison of each hippocampus, (\* $P = 0.001$ , \*\* $P = 0.006$ ), C: Klüver–Barrera stain of hippocampus, D: Comparison of each striatum (\* $P = 0.004$ , \*\* $P = 0.026$ ), E: Klüver–Barrera stain of striatum, F: Comparison of each thalamus (\* $P = 0.015$ ), G: Klüver–Barrera stain of thalamus. Data are expressed mean  $\pm$  SE. Scale bars, (C, E, F): 500  $\mu$ m.

stronger than the damage to the hippocampus in the HI and CD133<sup>+</sup> groups ( $P = 0.001$ ). Moderate damage was also observed in the thalamus ( $P = 0.042$ ).

To examine brain vessels, brain sections at postnatal day 56 were immunohistochemically stained with anti-isolectin B4 and anti- $\alpha$ -SMA antibodies. There were no significant differences between the three groups in number or total length of blood vessels (Table 2).

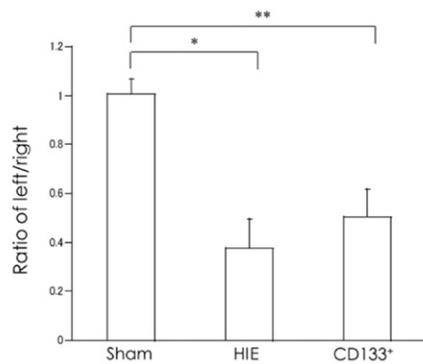
## 4. Discussion

Here, we have demonstrated that CD133<sup>+</sup> cells derived from human umbilical cord blood protect mouse brain from neonatal hypoxic-ischemic injury. Our conclusion is based on the following observations: (1) CD133<sup>+</sup> cells migrate from the abdominal cavity to damaged brain; (2) motor function is recovered in animals with transplanted CD133<sup>+</sup>

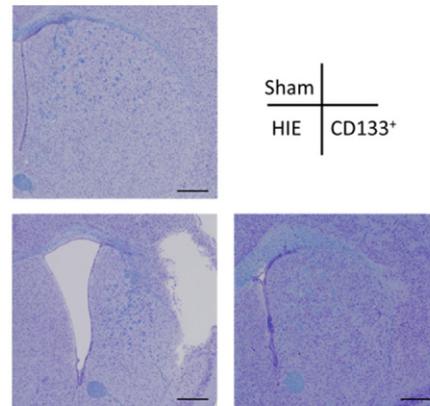
cells; and (3) lesion size in the CD133<sup>+</sup> group is smaller compared with the HI group.

Asahara et al. first described EPCs in 1997 [5]. The CD133 antigen is expressed on the surface of EPCs, hematopoietic stem cells, and very small embryonic-like stem cells [23]. The therapeutic effects of EPCs in post-ischemic myocardial regeneration and adult cerebral infarction have previously been investigated [24,25]. EPCs induce neovascularization and inhibit myocardial cell apoptosis, and these beneficial effects play an important role in improvement of myocardial function [26,27]. Additionally, EPCs accelerate functional recovery in both *in vivo* and *ex vivo* rat spinal cord injury studies [28,29]. During the regenerative period, EPCs specifically migrate to the injured endothelium and damaged vascular and ischemic organs [30], which prompted us to determine if EPCs can be a useful and safe cell therapy for neonatal HIE.

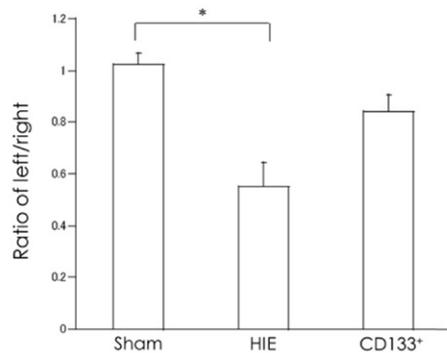
## D. Striatum



## E. Klüver-Barrera stain of striatum



## F. Thalamus



## G. Klüver-Barrera stain of thalamus

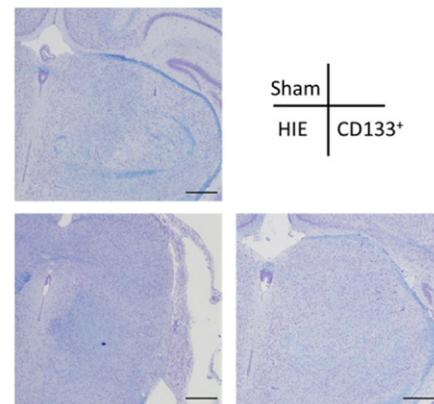


Fig. 5 (continued).

As demonstrated using our *in vivo* HIE model, hypoxic-ischemic injury characteristically leads to an extensive unilateral lesion in the cerebral cortex, basal ganglia, and hippocampus, which explains the loss of motor coordination and learning ability. HI mice had a lower body weight compared with sham-operated group. The body weight of CD133 mice were lower than sham-operated mice until postnatal day 35, however there was no difference in body weight between these two groups from postnatal day 42 and thereafter. Furthermore, using this model we show that CD133<sup>+</sup> cells improve motor function by protecting the brain from hypoxic-ischemic injury-induced damage, although the underlying mechanisms of this effect have not yet been determined. CD133<sup>+</sup> cells are able to differentiate into neural cells *in vitro* [31], but few transplanted stem cells differentiate into vascular endothelial cells and neurons in *ex vivo* [32] and *in vivo* [33] mouse brain. Using an *in vivo* neonatal hypoxic-ischemic mouse model, Van Velthoven et al. reported that cell replacement appears to be a rare or absent event [34]. Beneficial effects (such as functional recovery

Table 2

Length and number of brain blood vessels in hypoxic-ischemic or control (sham-operated) mice, untreated or intraperitoneally injected with CD133<sup>+</sup> cells.

| Group              | Length of vessels (mm/mm <sup>2</sup> ) | Number of vessels (/mm <sup>2</sup> ) |
|--------------------|---|---------------------------------------|
| Sham-operated      | 0.97 ± 0.15                             | 169.57 ± 9.66                         |
| HI                 | 1.03 ± 0.12                             | 171.14 ± 17.56                        |
| CD133 <sup>+</sup> | 0.76 ± 0.13                             | 145.50 ± 20.83                        |

Vascular endothelial cells were specifically stained with anti-isolectin B4, and vascular smooth muscle cells with anti- $\alpha$ -SMA. The length and number of blood vessels stained only with anti-isolectin B4 were measured (mean ± SE).

following cell transplantation) involve multiple mechanisms including angiogenesis, and neuroprotective and anti-inflammatory effects [35]. While we previously reported that CD133<sup>+</sup> cells reduce hypoxia-induced damage of cortical blood vessels in organ co-cultures [8] in our present *in vivo* study, the number and length of blood vessels did not change using transplanted CD133<sup>+</sup> cells. The beneficial effects of transplanted cells are suggested to be mediated by production of various neurotrophic factors [6], including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 [36]. Correspondingly, BDNF and NGF block caspase-3-mediated apoptosis and microglial activation in ischemic brain, and these neuroprotective factors may improve the microenvironment of injured brain tissue. Cell transplantation significantly reduces the number of activated microglia [37]. Microglia activation increases production of cytokines such as tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ , which appear to be involved in apoptosis of premyelinating oligodendrocytes and cell damage [38]. Microglia also produce anti-inflammatory cytokines and it is disturbances in the balance between pro- and anti-inflammatory cytokines that directs pro-oligodendrocyte precursors to differentiate into astrocytes, and not oligodendrocytes, thus affecting subsequent myelination [39,40]. Human umbilical cord blood cells secrete the interleukins, IL-6, IL-8, and IL-10, which are known to exert anti-inflammatory effects *in vivo* [41]. Thus the human umbilical cord cells contribute to restoration of the pro-/anti-inflammatory cytokine balance, and induce protection. One limitation of this study was that we were required to use immunodeficient mice for the HIE model to avoid inducing an immune response through the administration of human cells. Therefore, it is unknown whether similar results are obtained to normal mice.

Several investigators have found that transplanted stem cells migrate to the lesioned brain, and that the chemokine stromal-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) and its receptor, chemokine receptor type 4 (CXCR4), play important roles in trafficking transplanted cells [42–45]. Transplanted stem cells migrate to the brain 24 h after either intraperitoneal or intravascular administration, but appear to disappear from the brain a few weeks after administration [46,47]. In the present study, CD133<sup>+</sup> cells exhibited a similar pattern of behavior. The optimal route, timing, and cell number for transplantation must be determined before cell therapy using EPCs can be applied to neonatal HIE. For ischemic heart disease in adults, EPCs are injected directly into the myocardium during coronary artery bypass grafting [48] and their effects are dependent on cell number [49]. For treatment of neonatal HIE, we intraperitoneally transplanted cells to avoid cerebral injury caused by direct administration of EPCs to the brain. Intravenous [50], intranasal [47], and intrathecal [51] administration have also been performed, but result in cells becoming trapped in other organs [50]. It is therefore necessary to develop a safe and effective administration route for introducing cell therapies for neonatal HIE. As for the timing of administration, neonatal animal studies suggest transplantation proximal to the time of injury provides better therapeutic effects [33].

Hypoxic-ischemic encephalopathy is a leading cause of neonatal death and neurological disability. However, current treatments such as hypothermia have limited therapeutic effect. The techniques of neurotransplantation and neuroregeneration, which replace and repair injured cells, are still experimental [52]. Cell therapy using umbilical cord blood cells was reported to have a good safety profile in a clinical trial for the treatment of neonatal HIE; however, this trial is ongoing and efficacy has not yet been demonstrated [53]. The major advantages of human umbilical cord blood cells are their availability, safety, immaturity, and heterogeneous properties.

## 5. Conclusions

Although further studies are required to determine the precise mechanisms underlying the effects of CD133<sup>+</sup> cells on protection from brain damage induced by a hypoxic-ischemic insult, our results suggest the possibility of a novel treatment for neonatal HIE.

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Conflicts of interest/disclosure statement: The authors declare that there are no conflicts of interest.

## References

- [1] M.V. Johnston, A. Fatemi, M.A. Wilson, F. Northington, Treatment advances in neonatal neuroprotection and neurointensive care, *Lancet Neurol.* 10 (2011) 372–382.
- [2] M. Shinoyama, M. Ideguchi, H. Kida, K. Kajiwara, Y. Kagawa, Y. Maeda, S. Nomura, M. Suzuki, Cortical region-specific engraftment of embryonic stem cell-derived neural progenitor cells restores axonal sprouting to a subcortical target and achieves motor functional recovery in a mouse model of neonatal hypoxic-ischemic brain injury, *Front. Cell. Neurosci.* 7 (2013) 128.
- [3] S. Shankaran, A.R. Laptook, R.A. Ehrenkrantz, J.E. Tyson, S.A. McDonald, E.F. Donovan, A.A. Fanaroff, W.K. Poole, L.L. Wright, R.D. Higgins, N.N. Finer, W.A. Carlo, S. Duara, W. Oh, C.M. Cotten, D.K. Stevenson, B.J. Stoll, J.A. Lemons, R. Guillet, A.H. Jobe, H. National Institute of Child, N. Human Development Neonatal Research, Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy, *N. Engl. J. Med.* 353 (2005) 1574–1584.
- [4] S.E. Jacobs, C.J. Morley, T.E. Inder, M.J. Stewart, K.R. Smith, P.J. McNamara, I.M. Wright, H.M. Kirpalani, B.A. Darlow, L.W. Doyle, C. Infant Cooling Evaluation, Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: a randomized controlled trial, *Arch. Pediatr. Adolesc. Med.* 165 (2011) 692–700.
- [5] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzgenbichler, G. Schatteman, J.M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, *Science* 275 (1997) 964–967.
- [6] C.T. van Velthoven, A. Kavelaars, F. van Bel, C.J. Heijnen, Regeneration of the ischemic brain by engineered stem cells: fuelling endogenous repair processes, *Brain Res. Rev.* 61 (2009) 1–13.
- [7] T. Murohara, S. Kamijikkoku, T. Honda, Increased circulating soluble intercellular adhesion molecule-1 in acute myocardial infarction: a possible predictor of reperfusion ventricular arrhythmias, *Crit. Care Med.* 28 (2000) 1861–1864.
- [8] N. Tanaka, N. Kamei, T. Nakamae, R. Yamamoto, M. Ishikawa, H. Fujiwara, H. Miyoshi, T. Asahara, M. Ochi, Y. Kudo, CD133<sup>+</sup> cells from human umbilical cord blood reduce cortical damage and promote axonal growth in neonatal rat organ co-cultures exposed to hypoxia, *Int. J. Dev. Neurosci.* 28 (2010) 581–587.
- [9] J.E. Rice 3rd, R.C. Vannucci, J.B. Brierley, The influence of immaturity on hypoxic-ischemic brain damage in the rat, *Ann. Neurol.* 9 (1981) 131–141.
- [10] S. Levine, Anoxic-ischemic encephalopathy in rats, *Am. J. Pathol.* 36 (1960) 1–17.
- [11] B. Clancy, R.B. Darlington, B.L. Finlay, Translating developmental time across mammalian species, *Neuroscience* 105 (2001) 7–17.
- [12] H. Hagberg, D. Peebles, C. Mallard, Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults, *Ment. Retard. Dev. Disabil. Res. Rev.* 8 (2002) 30–38.
- [13] N. Rodriguez-Alvarez, E.M. Jimenez-Mateos, M. Dunleavy, J.L. Waddington, G.B. Boylan, D.C. Henshall, Effects of hypoxia-induced neonatal seizures on acute hippocampal injury and later-life seizure susceptibility and anxiety-related behavior in mice, *Neurobiol. Dis.* 83 (2015) 100–114.
- [14] X.B. Liu, Y. Shen, D.E. Pleasure, W. Deng, The vulnerability of thalamocortical circuitry to hypoxic-ischemic injury in a mouse model of periventricular leukomalacia, *BMC Neurosci.* 17 (2016) 2.
- [15] H. Muramatsu, F. Katsuoka, K. Toide, Y. Shimizu, S. Furusako, M. Yamamoto, Nrf2 deficiency leads to behavioral, neurochemical and transcriptional changes in mice, *Genes Cells* 18 (2013) 899–908.
- [16] M.J. Javed, L.E. Mead, D. Prater, W.K. Bessler, D. Foster, J. Case, W.S. Goebel, M.C. Yoder, L.S. Haneline, D.A. Ingram, Endothelial colony forming cells and mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood, *Pediatr. Res.* 64 (2008) 68–73.
- [17] R.M. Deacon, Measuring motor coordination in mice, *J. Vis. Exp.* (2013) e2609, <http://dx.doi.org/10.3791/2609>.
- [18] H. Kluver, E. Barrera, A method for the combined staining of cells and fibers in the nervous system, *J. Neuropathol. Exp. Neurol.* 12 (1953) 400–403.
- [19] T. Kawamoto, Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants, *Arch. Histol. Cytol.* 66 (2003) 123–143.
- [20] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH image to ImageJ: 25 years of image analysis, *Nat. Methods* 9 (2012) 671–675.
- [21] Y. Miki, K. Tanji, F. Mori, J. Utsumi, H. Sasaki, A. Kakita, H. Takahashi, K. Wakabayashi, Alteration of Upstream Autophagy-Related Proteins (ULK1, ULK2, Beclin1, VPS34 and AMBRA1) in Lewy Body Disease (Brain Pathol) 2015, <http://dx.doi.org/10.1111/bpa.12297>.
- [22] D. Hambardzumyan, D.H. Gutmann, H. Kettenmann, The role of microglia and macrophages in glioma maintenance and progression, *Nat. Neurosci.* 19 (2016) 20–27.
- [23] J. Ratajczak, M. Kucia, K. Mierzejewska, W. Marlicz, Z. Pietrzowski, W. Wojakowski, N.J. Greco, M. Tendera, M.Z. Ratajczak, Paracrine proangiopoietic effects of human umbilical cord blood-derived purified CD133<sup>+</sup> cells—implications for stem cell therapies in regenerative medicine, *Stem Cells Dev.* 22 (2013) 422–430.
- [24] M. Meregalli, A. Farini, M. Belicchi, Y. Torrente, CD133(+) cells isolated from various sources and their role in future clinical perspectives, *Expert. Opin. Biol. Ther.* 10 (2010) 1521–1528.
- [25] R. Flores-Ramirez, A. Uribe-Longoria, M.M. Rangel-Fuentes, P. Gutierrez-Fajardo, R. Salazar-Riojas, D. Cervantes-Garcia, J.H. Trevino-Ortiz, G.J. Benavides-Chereti, L.P. Espinosa-Oliveros, R.H. Limon-Rodriguez, R. Monreal-Puente, J.L. Gonzalez-Trevino, A. Rojas-Martinez, Intracoronary infusion of CD133<sup>+</sup> endothelial progenitor cells improves heart function and quality of life in patients with chronic post-infarct heart insufficiency, *Cardiovasc. Revasc. Med.* 11 (2010) 72–78.
- [26] A. Kawamoto, H.C. Gwon, H. Iwaguro, J.I. Yamaguchi, S. Uchida, H. Masuda, M. Silver, H. Ma, M. Kearney, J.M. Isner, T. Asahara, Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia, *Circulation* 110 (2001) 634–637.
- [27] A. Kawamoto, T. Tkebuchava, J. Yamaguchi, H. Nishimura, Y.S. Yoon, C. Milliken, S. Uchida, O. Masuo, H. Iwaguro, H. Ma, A. Hanley, M. Silver, M. Kearney, D.W. Losordo, J.M. Isner, T. Asahara, Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia, *Circulation* 107 (2003) 461–468.
- [28] N. Kamei, S.M. Kwon, C. Alev, K. Nakanishi, K. Yamada, H. Masuda, M. Ishikawa, A. Kawamoto, M. Ochi, T. Asahara, Ex-vivo expanded human blood-derived CD133<sup>+</sup> cells promote repair of injured spinal cord, *J. Neurol. Sci.* 328 (2013) 41–50.
- [29] H. Sasaki, M. Ishikawa, N. Tanaka, K. Nakanishi, N. Kamei, T. Asahara, M. Ochi, Administration of human peripheral blood-derived CD133<sup>+</sup> cells accelerates functional recovery in a rat spinal cord injury model, *Spine (Phila Pa 1976)* 34 (2009) 249–254.
- [30] M. Hristov, W. Erl, P.C. Weber, Endothelial progenitor cells: mobilization, differentiation, and homing, *Arterioscler. Thromb. Vasc. Biol.* 23 (2003) 1185–1189.
- [31] T. Tondreau, N. Meuleman, A. Delforge, M. Dejeneffe, R. Leroy, M. Massy, C. Mortier, D. Bron, L. Lagneaux, Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity, *Stem Cells* 23 (2005) 1105–1112.
- [32] R. Yamamoto, M. Ishikawa, N. Tanaka, N. Kamei, K. Nakanishi, H. Sasaki, T. Nakamae, Y. Mochizuki, T. Asahara, M. Ochi, CD133<sup>+</sup> cells from human peripheral blood promote corticospinal axon regeneration, *Neuroreport* 19 (2008) 799–803.

- [33] C. Meier, J. Middelalan, B. Wasielewski, S. Neuhoﬀ, A. Roth-Haerer, M. Gantert, H.R. Dinse, R. Dermietzel, A. Jensen, Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells, *Pediatr. Res.* 59 (2006) 244–249.
- [34] C.T. van Velthoven, A. Kavelaars, F. van Bel, C.J. Heijnen, Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration, *Brain Behav. Immun.* 24 (2010) 387–393.
- [35] F. Wang, N. Maeda, T. Yasuhara, M. Kameda, E. Tsuru, T. Yamashita, Y. Shen, M. Tsuda, I. Date, Y. Sagara, The therapeutic potential of human umbilical cord blood transplantation for neonatal hypoxic-ischemic brain injury and ischemic stroke, *Acta Med. Okayama* 66 (2012) 429–434.
- [36] P. Lu, L.L. Jones, E.Y. Snyder, M.H. Tuszynski, Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury, *Exp. Neurol.* 181 (2003) 115–129.
- [37] P.M. Pimentel-Coelho, E.S. Magalhaes, L.M. Lopes, L.C. de Azevedo, M.F. Santiago, R. Mendez-Otero, Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum, *Stem Cells Dev.* 19 (2010) 351–358.
- [38] C. Kaur, G. Rathnasamy, E.A. Ling, Roles of activated microglia in hypoxia induced neuroinflammation in the developing brain and the retina, *J. Neuroimmune Pharmacol.* 8 (2013) 66–78.
- [39] J.M. Bain, A. Ziegler, Z. Yang, S.W. Levison, E. Sen, TGFbeta1 stimulates the over-production of white matter astrocytes from precursors of the “brain marrow” in a rodent model of neonatal encephalopathy, *PLoS One* 5 (2010), e9567.
- [40] E. Rocha-Ferreira, M. Hristova, Antimicrobial peptides and complement in neonatal hypoxia-ischemia induced brain damage, *Front. Immunol.* 6 (2015) 56.
- [41] M.B. Newman, A.E. Willing, J.J. Manresa, C.D. Sanberg, P.R. Sanberg, Cytokines produced by cultured human umbilical cord blood (HUCB) cells: implications for brain repair, *Exp. Neurol.* 199 (2006) 201–208.
- [42] K. Rosenkranz, S. Kumbruch, K. Lebermann, K. Marschner, A. Jensen, R. Dermietzel, C. Meier, The chemokine SDF-1/CXCL12 contributes to the ‘homing’ of umbilical cord blood cells to a hypoxic-ischemic lesion in the rat brain, *J. Neurosci. Res.* 88 (2010) 1223–1233.
- [43] T. Bogoslovsky, M. Spatz, A. Chaudhry, D. Maric, M. Luby, J. Frank, S. Warach, N.N.H.o.S. Investigators, Stromal-derived factor-1[alpha] correlates with circulating endothelial progenitor cells and with acute lesion volume in stroke patients, *Stroke* 42 (2011) 618–625.
- [44] M. Song, O. Mohamad, X. Gu, L. Wei, S.P. Yu, Restoration of intracortical and thalamocortical circuits after transplantation of bone marrow mesenchymal stem cells into the ischemic brain of mice, *Cell Transplant.* 22 (2013) 2001–2015.
- [45] Y. Fan, F. Shen, T. Frenzel, W. Zhu, J. Ye, J. Liu, Y. Chen, H. Su, W.L. Young, G.Y. Yang, Endothelial progenitor cell transplantation improves long-term stroke outcome in mice, *Ann. Neurol.* 67 (2010) 488–497.
- [46] M. Tsuji, A. Taguchi, M. Ohshima, Y. Kasahara, Y. Sato, H. Tsuda, K. Otani, K. Yamahara, M. Ihara, M. Harada-Shiba, T. Ikeda, T. Matsuyama, Effects of intravenous administration of umbilical cord blood CD34(+) cells in a mouse model of neonatal stroke, *Neuroscience* 263 (2014) 148–158.
- [47] V. Donega, C.T. van Velthoven, C.H. Nijboer, F. van Bel, M.J. Kas, A. Kavelaars, C.J. Heijnen, Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement, *PLoS One* 8 (2013), e51253.
- [48] J. Forcillo, L.M. Stevens, S. Mansour, I. Prieto, R. Salem, C. Baron, D.C. Roy, E. Larose, D. Mascachaun, N. Noiseux, Implantation of CD133+ stem cells in patients undergoing coronary bypass surgery: IMPACT-CABG pilot trial, *Can J. Cardiol.* 29 (2013) 441–447.
- [49] C. Kalka, H. Masuda, T. Takahashi, W.M. Kalka-Moll, M. Silver, M. Kearney, T. Li, J.M. Isner, T. Asahara, Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 3422–3427.
- [50] T. Yasuhara, K. Hara, M. Maki, R.W. Mays, R.J. Deans, D.C. Hess, J.E. Carroll, C.V. Borlongan, Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells in neonatal hypoxic-ischemic rats, *J. Cereb. Blood Flow Metab.* 28 (2008) 1804–1810.
- [51] C. Purandare, D.G. Shitole, V. Belle, A. Kedari, N. Bora, M. Joshi, Therapeutic potential of autologous stem cell transplantation for cerebral palsy, *Case Rep. Transplant* 2012 (2012) 825289.
- [52] A.E. Willing, J. Lixian, M. Milliken, S. Poulos, T. Zigova, S. Song, C. Hart, J. Sanchez-Ramos, P.R. Sanberg, Intravenous versus intrastriatal cord blood administration in a rodent model of stroke, *J. Neurosci. Res.* 73 (2003) 296–307.
- [53] C.M. Cotten, A.P. Murtha, R.N. Goldberg, C.A. Grotegut, P.B. Smith, R.F. Goldstein, K.A. Fisher, K.E. Gustafson, B. Waters-Pick, G.K. Swamy, B. Rattray, S. Tan, J. Kurtzberg, Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy, *J. Pediatr.* 164 (2014) 973–979 (e971).