Therapeutic effect of autologous compact bone-derived mesenchymal stem cell transplantation on prion disease

Running title: Mesenchymal stem cell therapy for prion disease

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Abbreviations

AD, Alzheimer's disease; ANOVA, analyses of variance; BDNF, brain-derived neurotrophic factor; BM-MSCs, bone marrow-derived MSCs; CB-MSCs, compact bone-derived MSCs; DMEM, Dulbecco's Modified Eagle Medium; Dpi, days post-inoculation; GFAP, glial fibrillary acidic protein; HBSS, Hanks' Balanced Salt Solution; HE, haematoxylin-eosin; Iba-1, ionized calcium binding adaptor molecule 1; IHC, immunohistochemistry; MACS, Magnetic Activated Cell Sorting; MSCs, mesenchymal stem cells; NT, neurotrophin; PK, proteinase K; PrP^{C}, cellular form of prion protein; PrP^{Sc}, disease-specific prion protein; qRT-PCR, quantitative RT-PCR; VEGF, vascular endothelial growth factor

Abstract

Prion diseases are fatal neurodegenerative disorders of humans and animals and no effective treatments are available to date. Allogenic transplantation of immortalized human mesenchymal stem cells (MSCs) can prolong the survival of mice infected with prions. However, autologous transplantation is an appropriate model for evaluating the effects of MSCs on prion diseases. Therefore, we isolated and purified MSCs from the femur and tibia of mice as compact bone-derived MSCs (CB-MSCs). Flow cytometric analysis showed that CB-MSCs were negative for myeloid stem cell-derived cell markers CD11b and CD45, but positive for molecules such as Sca-1, CD105, and CD90.2, which are reported to be expressed on MSCs. The ability of CB-MSCs to migrate to brain extracts from prion-infected mice was confirmed by an *in vitro* migration assay. Intra-hippocampus transplantation of CB-MSCs at 120 days post inoculation marginally but significantly prolonged the survival of mice infected with the Chandler prion strain. The transplantation of CB-MSCs did not influence the accumulation of disease-specific prion protein. However, the CB-MSCs transplantation enhanced microglial activation, which appeared to be polarized to the M2-type activation state. These results suggest that autologous MSC transplantation is a possible treatment for prion diseases and the modification of microglial activation may be a therapeutic target for neurodegenerative diseases.

Introduction

Prion diseases are neurodegenerative disorders, which include bovine spongiform encephalopathy, scrapie in sheep and goats, chronic wasting disease in deer and Creutzfeldt-Jakob disease in humans. The diseases are characterized by vacuolation of neurons and neuropil, astrocytosis, microglial activation, and the deposition of disease-specific prion protein (PrP^{Sc}) in the central nervous system. Prion diseases have a long incubation period; however, after the clinical onset, the diseases are subacutely progressive and inevitably fatal. No therapeutics are available at present. The process of the conversion of the cellular form of prion protein (PrP^{C}) into PrP^{Sc} is believed to be associated with the propagation of prions, the causative agent of the disease, and neurodegeneration [1].

Stem cells privde a new approach for the treatment of degenerative diseases because of their potential applications in regenerative medicine. Mesenchymal stem cells (MSCs) are pluripotent cells that can be isolated from adult tissues of mesodermal origin, such as bone marrow, adipose tissue, compact bones, and umbilical cord blood [2]. MSCs can be differentiated into various cell types, not only mesodamal lineages (osteocytes, chondrocytes, adipocytes and skeletal muscle cells), but also ectodermal lineages (neuronal cells, glial cells, and schwann cells) and endodermal lineages (hepatocytes, insulin-producing pancreatic cells) [3, 4]. MSCs have some advantages for the use in cell therapy, such as the minimal ethical problem and extensive sources. Safety of MSCs is also empirically recognized because little adverse effects were reported in clinical trail of MSCs to neurological diseases such as stroke, spinal cord injury, and amyotrophic lateral sclerosis [5, 6].

MSCs have been reported to have beneficial effects as cell therapies in animal models of

stroke [7, 8], spinal cord injury [9], brain tumor [10], and neurodegenerative diseases such as Alzheimer's disease (AD) [11, 12], Parkinson's disease [13-15], and amyotrophic lateral sclerosis [16, 17]. In clinical trials, MSCs transplantation for neurological disorders also tended to achieve functional recovery or partial improvement in patients with stroke [18], spinal cord injury [19], and multiple sclerosis [20]. In neurodegenerative and neurological disorders, MSCs transplanted via the intracerebral or intravenous route migrate to brain lesions and ameliorate functional deficits or exhibit neuroprotective potential. This may possibly be attributable to immunomodulatory and anti-inflammatory effects by producing cytokines and chemokines or modulating microglial activation [11, 21]; neuroprotection by producing neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor [22, 23]; neuronal differentiation or stimulation of differentiation of endogenous neural stem cells [24-26]; or neurovascularization by producing vascular endothelial growth factor (VEGF) [27] or decreasing oxidative stress [28]. These findings demonstrate that MSCs are promising tools for cell therapy in neurodegenerative disorders. However, the detailed mechanisms that allow MSCs to relieve detrimental conditions and restore functional deficits still remain to be elucidated.

Previously, we reported that intracerebral or intravenous transplantation of immortalized human bone marrow-derived MSCs (BM-MSCs) prolonged the survival of mice infected with prions. The transplanted MSCs migrated to brain lesions and produced neurotrophic factors such as BDNF, VEGF, and neurotrophin (NT) 3 and 4/5 [29]. These results suggest that human BM-MSCs have neuroprotective effects in prion disease. However, our experiments involved allogenic transplantation and thus instead of the human MSCs themselves, human PrP^{C} expressed in human BM-MSCs might have impeded prion propagation in mouse brains

by interfering with the intermolecular interaction between mouse PrP^{C} and PrP^{Sc} [30-32]. Alternatively, immortalized human BM-MSCs that can proliferate more effectively than primary MSCs are expected to exist for a longer period in the brain lesions, which might have resulted in an overestimation of the effect.

Therefore, in this study, to evaluate the effect of autologous MSC transplantation on prion disease, we isolated MSCs from compact bone from the femur and tibia of mice, and analyzed the therapeutic potential of compact-bone derived MSCs (CB-MSCs) in prion disease.

Result

Expression of cell surface markers on CB-MSCs

After removing CD11b- and CD 45-positive cells by a Magnetic Activated Cell Sorting (MACS), CB-MSCs were serially passaged to expand cell numbers. At the 4^{th} passage, the expression of surface markers was analyzed by flow cytometry (Fig. 1). The cells were confirmed as negative for myeloid-derived markers CD11b and CD45, but molecules that are reportedly expressed on the surface of MSCs, i.e., CD29, CD44, CD73, CD90.2, CD105, and Sca-1 [33, 34], and CD106 [35, 36], were expressed on the surface of the CB-MSCs. However, the histograms for CD73, CD90.2, CD105, and CD106 showed that the CB-MSCs included cells that were both positive and negative for these molecules, thereby indicating that CB-MSCs comprise heterologous cell populations.

Migration of CB-MSCs to brain extracts from prion-infected mice

MSCs are known to migrate to brain lesions in animal models of neurodegenerative disorders, such as Parkinson's disease [13], ischemia [7], glioma [10], and brain tumor [37]. Previously, we reported that when immortalized human BM-MSCs are transplanted intracerebrally or intravenously, they migrate to brain lesions in prion disease [29]. Human BM-MSCs migrate to brain extracts from prion-infected mice according to an *in vitro* migration assay, so the capability of BM-MSCs to migrate to lesions in prion disease *in vivo* can be assessed using an *in vitro* migration assay [38]. Thus, we assessed the ability of CB-MSCs to migrate to brain extracts from Chandler strain-infected mice using the *in vitro* migration assay (Fig. 2). Figure 2(a) shows that the CB-MSCs migrated to the bottom side of the insert wells. Quantitative analysis of the migrated CB-MSCs showed that 3.0 and 2.1 times as many CB-MSCs migrated to the 1% and 0.1% brain extracts from prion-infected mice, respectively, compared with those from the corresponding uninfected mice (Fig. 2b).

Effect of CB-MSCs on survival of prion-infected mice

The results of the migration assay suggested that CB-MSCs are capable of migration into lesions of prion disease. Thus, we examined the therapeutic potential of CB-MSCs in prion disease. Under the experimental condition used in the current study, early clinical signs, such as ataxia of hind limbs and changes in pelage and posture, appear at around 120 days post-inoculation (dpi). The decrease in body weight also becomes apparent at around 110 - 120 dpi. Thus, CB-MSCs were transplanted into the left hippocampus of Chandler strain-infected mice at 120 dpi in order to evaluate the therapeutic potential of CB-MCSs in clinical phase. The intra-hippocampal transplantation of CB-MSCs significantly prolonged the survival of mice infected with the Chandler strain (163.8 ± 6.2 days, n = 9) compared with that of PBS-injected group (155.0 ± 2.4 days, n = 7) (Fig. 3a, $p < 0.01$).

Loss of body weight with disease progression is one of the prominent features in Chandler strain-infected mice [39, 40]. To evaluate the effects of CB-MSCs more objectively, we weighed the mice every week from 120 dpi. At 141 dpi, the decrease in body weight appeared to slow down in the CB-MSC transplanted group compared with the PBS-injected group (Fig. 3b, $p < 0.05$), and the differences in body weight were more obvious at 148 and 155 dpi (Fig. 3b, $p < 0.01$).

We also transplanted CB-MSCs to mice infected with the Obihiro strain, as another scrapie strain (Supplementary figure 1). Although the effect of CB-MSCs on prolongation of the survival of mice infected with the Obihiro strain was not statistically significant (154.4 ± 7.5 days, n = 7) compared with that of PBS-injected group (151.5 ± 1.9 days, n = 4). We reported that intreventricular administration of anti-PrP mAbs mitigated the disease progression of mice infected with the Chandler strain even when the administration started at 120 dpi. However, no protective effect was observed in mice infected with the Obihiro strain under the same condition [39]. This fact suggests that the Obihiro strain is more resistant to the treatment against prion diseases than the Chandler strain. Nonetheless, 3 out of 7 Obihiro strain-infected mice transplanted CB-MSCs apparently survived longer than those with sham operation. This suggests that the therapeutic potential of CB-MSCs is not limited to the Chandler strain.

Effects of CB-MSCs on PrP^{Sc} accumulation and neuropathology

To analyze whether CB-MSCs influence the accumulation of PrP^{Sc}, we transplanted CB-MSCs into the hippocampus of Chandler-infected mice at 120 dpi and the kinetics of the total PrP and proteinase K-resistant PrP^{Sc} levels were analyzed by immunoblotting (Fig. 4).

When we initiated intravenous administration of anti-PrP mAb to the Chandler strain-infected mice at 120 dpi, the decrease in body weight became significantly slower compared to control mice at around 140 dpi [40]. Similar tendency was observed on the decrease in the body weight in the Chandler strain-infected mice transplanted with CB-MSCs (Fig 3b). Thus we set 145 dpi as an examination point in the following experiments. At 145 dpi, the mean PrP^{Sc} level in the PBS-injected group increased nearly 2-fold compared with the level at 120 dpi; however, no difference was observed in the PrP^{Sc} levels in the CB-MSC-transplanted and PBS-injected groups. The PrP^{Sc} levels increased further at the terminal stage of the disease, however, no difference was observed in the PrP^{Sc} levels between the CB-MSC-transplanted and PBS-injected groups (Figs. 4a & 4c). The mean total PrP levels of the CB-MSC-transplanted group increased 1.3- and 1.8-fold at 145 dpi and at the terminal stage, respectively, compared with the levels at 120 dpi; however, there were no differences in the total PrP levels were observed between CB-MSC-transplanted group and PBS-injected groups (Figs. 4b & 4d). These results indicate that CB-MSCs did not influence the accumulation of PrP^{Sc} in brains.

Consistent with the results of PrP^{Sc} accumulation obtained by immunoblotting, there were no obvious differences in the accumulation of PrP^{Sc} in the hippocampus of CB-MSC-transplanted and PBS-injected groups by immunohistochemistry (IHC) for PrP^{Sc} at 145 dpi and at the terminal stage (Fig. 5a, PrP^{Sc}). No difference was observed in the astrogliosis in the hippocampus of CB-MSC-transplanted and PBS-injected groups according to IHC for glial fibrillary acidic protein (GFAP) (Fig. 5a, GFAP). By contrast, there were differences in microglial activation, which was assessed by the ionized calcium binding adaptor molecule 1 (Iba-1) (Fig. 5a, Iba-1). The quantitative analysis detected more

Iba-1-positive cells in the CB-MSC-transplanted group than in PBS-injected group in hippocampus at 145 dpi (Fig. 5b, $p < 0.05$). However, there were no differences in the number of microglia at the terminal stage of the disease (Fig. 5b). No difference was observed in vacuolation in the hippocampus of CB-MSC-transplanted and PBS-injected groups (Fig. 5a, HE & Fig. 5c).

In the HE staining section, large cells with abundant pale cytoplasm were observed in the MSC-transplanted side of the hippocampi of Chandler strain-infected mice (Supplementary figure 2a, at 145 dpi, 25 days after MSC-transplantation). Similar cells were observed in the ipsilateral side of the hippocampus of mock-infected mice transplanted with CB-MSCs. However, such cells were not observed in the Chandler-infected or mock-infected mice with sham operation. Thus, the large cells with pale cytoplasm are likely to be transplanted CM-MSCs. At the terminal stage of the disease, such cells were not observed but instead, aggregates of necrotic cells that were not observed in sham-operated mice, were observed in the hippocampi of the Chandler strain-infected mice transplanted with CM-MSCs (Supplementary figure 2b). These results suggest that the transplanted CB-MSCs survived at least by 25 days after transplantation.

Effects of CB-MSCs on gene expression in the brains of prion-infected mice

To analyze the activation state of microglia, quantitative RT-PCR (qRT-PCR) was carried out (Fig. 6). The expression of the *Aif1* gene, which encodes Iba-1, was up-regulated in prion-infected mice; however, greater up-regulation of *Aif1* gene was observed in prion-infected mice transplanted with CB-MSCs compared with those with sham operation. No difference was observed in *Aif1* gene expression between mock-infected mice transplanted

with CB-MSCs and those with sham operation. The expression of *CD68*, which is used as a marker for activated microglia, was also up-regulated more significantly in prion-infected mice transplanted with CB-MSCs than those with sham operation. The expression of *IL-1β*, a representative pro-inflammatory cytokine, was up-regulated upon prion infection (compare Mock PBS vs Chandler PBS) as reported. Transplantation of CB-MSCs itself up-regulated the expression of *IL-1β* in mock-infected mice: the expression level was 3.1 times higher in mock-infected mice with CB-MSC transplantation than those with sham-operation. However, interestingly, compared to prion-infected mice with sham-operation, 5.3 times higher up-regulation was observed in prion-infected mice transplanted with CB-MSCs. The expression of another pro-inflammatory cytokine gene, *TNF-α*, exhibited a similar trend. The transplantation of CB-MSCs did not influence the gene expression of some marker genes for M2-type activation in macrophages, i.e., *Chil3, Retnla and Mrc1*, in mock-infected mice. However, the gene expression levels of *Chil3* and *Retnla* were up-regulated remarkably in prion-infected mice transplanted with CB-MSCs. A marginal but significant up-regulation of Mrc1 expression was also observed. These results suggest that the transplantation of CB-MSCs influenced the activation state of microglia in prion-infected mice. The gene expression level of *IL-10*, an anti-inflammatory cytokine that stimulates microglia to shift into the M2-type activation state, was up-regulated markedly by CB-MSCs transplantation in prion-infected mice. The expression level of *IL-10* also appeared to be up-regulated by CB-MSCs transplantation in mock-infected mice, although the degree of up-regulation was smaller than that observed in prion-infected mice. No difference was observed in the gene expression levels of *NGF* and *BDNF*.

Discussion

The primary aim of this study was to analyze the efficacy of autologous MSCs transplantation in the treatment of prion diseases. Previously, we reported that the transplantation of immortalized human MSCs prolonged the survival of mice infected with prions [29]. That experiment involved the transplantation of heterologous MSCs, and thus human PrP^{C} from human MSCs was present. It is well known that heterologous PrP^{C} interferes with PrP^{Sc} formation of homologous PrP^{C} and PrP^{Sc} combination [30, 31]. Thus, it is possible that the extended survival might not have been caused by any direct or indirect neuroprotective effects of human MSCs, but instead it may have been attributable to the inhibition of PrP^{Sc} formation by human PrP^{C} produced by human MSCs. In addition, the use of immortalized MSCs had an apparent protective effect due to their higher proliferation capacity [29]. However, in the present study, we showed that autologous and un-immortalized MCSs transplantation prolonged the survival of mice infected with prions even when they were transplanted in the early clinical phase of Chandler strain-infected mice (at 120 dpi). The kinetics of PrP^{Sc} accumulation indicated that there were no differences in the PrP^{Sc} levels between MSC-transplanted and sham operation groups (Fig. 4), suggesting that mouse CB-MSCs exerted their neuroprotective potential without inhibiting PrP^{Sc} formation.

The primary CB-MSCs used here comprised heterologous cell populations (Fig. 1), but were effective in mitigating the disease progression. Thus it is possible that certain sub-population of CB-MSCs have a higher potential to exert neuroprotective effects. It is reported recently that cells that are positive for platelet-derived growth factor receptor α and Sca-1 are more potent population of MSCs [41]. Cells that are positive for stage-specific

embryonic antigen-3 and CD105, distinct stem cells in mesenchymal cell population, show triploblastic differentiation to all three germ layers [42]. Thus, identification of MSC sub-populations that have a higher neuroprotective potency will be of interest to enhance the protective effects against prion diseases.

In prion-infected mice, microglial activation is often associated spatially with the brain regions where PrP^{Sc} accumulates before clinical onset [43-45]. However, it is controversial whether activated microglia have neuroprotective or neurotoxic functions. The blockade of colony-stimulation factor receptor 1 signaling prolonged the survival of prion-infected mice, which was accompanied by reduced microglial activation [46]. However, knockout of the CD40 ligand reduced the survival of mice infected with prions with enhanced microglial activation [47]. These findings suggest a detrimental effect of activated microglia in prion diseases. By contrast, the depletion of microglia in cerebellar slice culture increased the accumulation of PrP^{Sc} and severe neuronal loss occurred as a consequence [48, 49]. Recently, we reported that prion-infected CD14-deficient mice exhibited prolonged survival with increased activation of microglia compared with wild-type mice [50]. These findings suggest neuroprotective roles of microglia. In the present study, prion-infected mice transplanted with CB-MSCs survived longer than mice in the sham-operated group and they exhibited increased microglial activation without any reduction in the PrP^{Sc} level (Figs. 4 & 5). These results suggest that the enhancement of microglia activation by CB-MSCs partly mitigated the disease progression without influencing the clearance of PrP^{Sc}. In AD model mice, microglial activation in the early stage of the disease is considered to be neuroprotective due to the Aβ clearance functions by phagocytosis and the production of Aβ-degrading enzyme [51, 52]. However, the phagocytic activity is attenuated by the stimulation of pro-inflammatory cytokine [53]. It is well known that the up-regulation of pro-inflammatory cytokines such as *IL-1β, TNFα, and IL-6* occurs *w*ith the disease progression in prion diseases [54-56]. In the

present study, we detected greater up-regulation of *IL-1β* gene expression in CB-MSC-transplanted prion-infected mice (Fig. 6). Thus, it is conceivable that the microglia activated further by CB-MSC-transplantation had little effect on the clearance of PrP^{Sc}. The intracerebral injection of lipopolysaccharide induced microglial activation with marked production of IL-1β but little clearance of PrP^{Sc} [57], which supports our hypothesis.

MSCs produce various cytokines, ckemokines and inflammation mediators depending on microenvironments and pleiotropically modulate activities of immune cells such as T-cell, NK cell, monocyte/macrophages and microglia [58, 59]. The immunomodulation is believed to be one of the mechanisms that mediate the neuroprotective effects of MSCs in neurodegenerative diseases and traumatic injuries. [21, 60]. The enhancement of microglial activation in CB-MSC-transplanted prion-infected mice appeared to be an immunomodulatory effect of CB-MSCs. In addition to greater microglial activation, the expression of pro-inflammatory cytokine genes, i.e., *IL-1β* and *TNF-α*, was up-regulated significantly in CB-MSC-transplanted prion-infected mice than sham-operated prion-infected mice. Interestingly, the gene expression level of *IL-10*, an anti-inflammatory cytokine that stimulates microglia to shift into the M2-type activation state, and the gene expression levels of *Chil3* and *Retnla*, well-known M2-type microglia activation marker genes, were also up-regulated in CB-MSC-transplanted prion-infected mice (Fig. 6). These results suggest that CB-MSCs influenced the microglial activation state and the microglia polarized more into the M2-type activation state. The enhancement of microglial activation in response to BM-MSC transplantation as well as improvements in cognitive decline were also reported in AD model mice [11], where the expression levels of the *Chil3* and *Retnla* genes and anti-inflammatory cytokine *IL-4* gene were up-regulated, thereby suggesting the M2-type activation of microglia. However, unlike our results, the expression levels of pro-inflammatory cytokines genes, *IL-1β* and *TNF-α*, were down-regulated [11, 61, 62]. It is unknown whether the differences in the

pro-inflammatory cytokine gene expressions may be attributed to differences in the origin of the MSCs, *i.e.*, isolated from compact bone in the current study and from bone marrow in the previous studies, or differences in pathobiology of prion diseases and AD. Interestingly, the transplantation of BM-MSCs into a rat model of traumatic brain injury reduced the number of microglia as well as down-regulating the expression levels of some pro-inflammatory cytokine genes and up-regulating those of some anti-inflammatory cytokine genes [63]. This also suggests a shift of microglia into the M2-type activation state in the presence of MSCs in response to acute traumatic injury. It is expected that the neuro-pathobiology of acute traumatic injury and slow progressive encephalopathies such as AD and prion diseases will differ from each other; however, these findings suggest that MSCs could modulate microglial activation state into the M2-type by responding to each disease condition in a different manner to exert neuroprotective functions. The M2-type of microglia can produce anti-inflammatory cytokines and neurotrophic factors [64], so it is possible that alternatively activated microglia may facilitate neuroprotection and regeneration.

Our previous study showed that the immortalized human BM-MSCs transplanted to prion-infected mice produced various neurotrophic factors, such as NGF, BDNF, NT3/4 and VEGF (Song *et al*., 2009). However, in the current study, we used primary CB-MSCs without any genetic modification or labeling in order to exclude any clonal or gene modification effects. Thus, we could not assess other neuroprotective mechanisms of MSCs, such as neuronal differentiation, stimulation of differentiation of endogenous neural stem cells, neurovascularization, or decrease of oxidative stress, or assess the distribution of the transplanted CB-MSCs.

In this study, we showed that the autologous transplantation of CB-MSCs mitigated the disease progression of prion-infected mice. The CB-MSCs transplantation did not influence the accumulation of PrP^{Sc} but it enhanced microglial activation, which appeared to be

polarized into the M2-type activation state. It remains to be elucidated whether the M2-type polarized microglia have neuroprotective roles against prion propagation. However, the fact that CB-MSC transplantation partly prevents disease progression even after the clinical onset will encourage further studies for the application of regenerative medicine in the treatment of prion diseases.

Materials & Methods

Animals and prion inoculation

All procedures for animal experiments were conducted according to protocols approved by the Institutional Committee for Animal Experiments at Hokkaido University. The mouse-adopted scrapie Chandler strain was used in this study. Four-week-old female ICR mice (CLEA, Japan) were inoculated intracerebrally (into the left hemisphere) with 20 μl of 2.5 % brain homogenate from the Chandler strain-infected, Obihiro strain-infected, or mock-infected mice.

Preparation of CB-MSCs from mouse compact bone by MACS

Six-week-old Jcl:ICR female mice were euthanized under anesthesia with Sevofrane (Maruishi Pharmaceutical Co. Ltd., Japan). The femur and tibia were obtained to isolate CB-MSCs [65]. Both ends of the femur and tibia were cut by scissors and the bone marrow was washed out with Hanks' Balanced Salt Solution (HBSS, Sigma, USA) by inserting a 27 G needle (Terumo, Japan) into the cavity. The femur and tibia were then cut into small pieces

using bone scissors. After washing with HBSS, the bone fragments were digested with 1 mg collagenase II ml^{-1} (Sigma) in HBSS with constant shaking at 220 rpm for 2 h at 37°C. The digest was filtered through a 100-µm cell strainer (BD Falcon, USA) and the bone fragments remaining on the cell strainer were washed three times with HBSS. The bone fragments were cultured with Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 10% FBS (Gibco, USA), 10% horse serum (Gibco), 2 mM L-glutamine (Wako, Japan), 10 mM HEPES (Gibco) and 100 U Penicillin-Streptomycin ml^{-1} (Gibco) (FBS-HS-DMEM) in 10 cm plates. The filtered cells were also cultured with FBS-HS-DMEM in 10 cm plates at 37 °C under 5% CO_2 and 5% O_2. The cells were freshly fed every day for the first 3 days and passaged every 3 to 4 days.

Compact bone-derived cells that adhered to plastic plate were harvested with 0.1% collagenase I (Wako, Japan) in PBS (pH 7.2) when the cells were reached about 70% confluence. The cells were collected by centrifugation and incubated for 15 min on ice with 200 µl CD11b microbeads (Miltenyi Biotec, USA) diluted at 1:10 with HBSS containing 0.5% FBS (0.5% FBS-HBSS). The CD11b-positive cells were then removed by passing through an MS column set on MACS separators (Miltenyi Biotec), and the pass through fraction was collected. The collected cells were subsequently incubated for 15 min with 200 µl CD 45 microbeads (Miltenyi Biotec) diluted at 1:10 with 0.5% FBS-HBSS. CD45-positive cells were removed by the MS column set on MACS separators. Cells in the pass through fraction were cultured as CB-MSCs.

Flow cytometric analysis

The CB-MSCs were harvested by collagenase treatment and suspended with 0.5% FBS-HBSS. The CB-MSCs were added to 96-well plates (1×10^5 cells per well). After centrifugation, cells were incubated for 30 min on ice with 100 µl primary rat antibodies against mouse CD11b, CD45, CD29, CD44, CD73, CD90.2, CD105, CD106, and Sca-1 at a 1:200 dilution. Except for anti-CD73 antibody, all of the antibodies were purchased from Biolegend (USA). Anti-CD73 antibody was purchased from BD Bioscience (USA). Rat IgG2a kappa and IgG2b kappa, both from Biolegend, were used as isotype controls. The cells were washed three times with 0.5% FBS-HBSS and incubated for 30 min on ice with anti-rat Alexa Fluor 488 (Molecular Probes, USA) at a dilution of 1:1,000. After washing three times with 0.5% FBS-HBSS, the cells were stained with 5 µg propidium iodide ml^{-1} (Molecular Probes) in HBSS for 5 min. The cells were analyzed using FACSVerse flow cytometer (BD Biosciences).

Transplantation of CB-MSCs

To transplant CB-MSCs into the hippocampus, mice were anesthetized by intramuscular injection with xylazine (10 mg kg^{-1}) and ketamine (50 mg kg^{-1}) and placed on a stereotaxic apparatus (Narishige, Japan). After making a linear scalp incision, burr holes were drilled to accommodate stereotaxic placement into the left hippocampus (2.0 mm caudal; 2.0 mm lateral to bregma; depth, 2 mm). CB-MSCs (1×10^5 cells in 2 µl PBS) were transplanted over a period of 15 min using a Hamilton syringe with a 31-gauge needle set in a micromanipulator. In the sham-operation, 2 µl of PBS was injected into the same position.

Immunoblotting

Brains were hemi-sectioned sagittally and homogenized in 20% (w/v) TMS buffer [10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5% glucose]. The 20% brain homogenate (250 μl) was mixed with an equal volume of a detergent buffer [8% Zwittergent 3-14, 1% Sarcosyl, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5)] and digested with collagenase I at 0.5 mg ml-1 for 15 min at 37°C in a water bath. To detect proteinase K (PK)-resistant PrP^{Sc}, the samples were digested with PK (Roche Diagnostics, Germany) at 20 μg ml^{-1} for 30 min at 37°C in a water bath. After stopping PK digestion by adding Pefabloc (Roche Diagnostics) to 2 mM, DNase I was added to the samples at 40 μg ml^{-1} and incubated for 5 min at room temperature. A half volume of butanol-methanol solution (2-butanol : methanol = 5 : 1) was added and PK-resistant PrP^{Sc} was recovered by centrifugation at 20,000 × g for 10 min at 20°C. The resulting pellet was dissolved in 1× SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 5% glycerol, 3 mM EDTA, 5% SDS, 4 M urea, 0.04% bromophenol blue, 4% β-mercaptoethanol] by boiling for 5 min. To detect the total PrP, PK digestion and Pefabloc treatment were omitted. SDS polyacrylamide gel electrophoresis and immunoblotting for the detection of PrP were performed as described previously [66]. The specific bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare, UK) and a LAS-3000 chemiluminescence image analyzer (Fujifilm, Japan). Quantitative analysis was performed with Image Reader LAS-3000 version 1.11 (Fujifilm).

Histopathology and IHC

Mice were dissected under anesthesia and brains were fixed with 10% formalin (Wako), soaked in 60% formic acid (Wako) for 1 h and then kept in 70% ethanol (Wako). After embedding in paraffin, the samples were sectioned coronally at a thickness of 3 μm. The

sections were deparaffinized, rehydrated and subjected to haematoxylin-eosin (HE) staining or IHC. For HE staining, sections were stained in haematoxylin (Wako) for 3 min and washed with tap-water for 5 min and then washed again with de-ionized water. After pre-treatment with 95% ethanol, the sections were stained with 0.5% eosin (Wako) for 2 min and dehydrated using an ethanol series. The sections were permeabilized against xylene (Wako) and enclosed with cover glass using Mount-Quick (Daido Sangyo, Japan).

To detect PrP^{Sc} by immunohistochemistry, sections were autoclaved for 20 min at 135°C [67]. The sections were treated with 3% H_2O_2 in methanol for 15 min, blocked with 5% FBS in PBS for 30 min and then incubated with mAb 31C6 [68] (0.5 μg ml^{-1}) for 1 h at 37°C. After washing with PBS containing 0.1% Tween 20 (PBST), the sections were incubated with Envision+ system-HRP labeled polymer conjugated to goat anti-mouse immunoglobulins (Dako, Denmark) for 1 h at 37°C. The sections were washed with PBST and developed with DAB peroxidase (Vector Laboratories, USA), followed by counterstaining with Mayer's haematoxylin.

To detect GFAP and Iba-1, markers for activated astrocytes and microglia, respectively, sections were treated twice in 500W microwave with citric acid buffer [0.01M citric acid and 0.01M sodium citrate] for 5 min for antigen retrieval [67]. Next, the sections were treated with 0.3% H_2O_2 and blocked with FBS as described above. After blocking, the sections were incubated with anti-GFAP antibodies (Dako) at 1:2,000 or anti-Iba-1 antibodies (Wako) at 1:200 dilution for 1 h at 37°C. After washing with PBST, the sections were incubated with EnVision+ system-HRP labeled polymer conjugated to goat anti-rabbit immunoglobulins (Dako) for 1 h at 37 °C. The sections were then developed and counterstained as described above.

In vitro migration assay

Brains from Chandler strain-infected mice at 120 dpi, or age-matched mock-infected mice, were homogenized to 20% (w/w) in DMEM. Two brains were pooled for each homogenate. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C, and the resulting supernatants were passed through a 0.22-μm pore size filter. Aliquots of the brain extracts were stored at –80°C until use. CB-MSCs starved in serum-free DMEM for 24 h were harvested with 0.1% collagenase I. Wells in the 24-well plate were supplied with serum-free DMEM containing brain extracts, and CB-MSC suspensions (5×10^4 cells with 400 μl of DMEM) were then added to the insert well with a polycarbonate membrane (24-well Millicell Hanging Cell Culture Inserts, pore size, 8.0 μm, Millipore, USA). The 24-well plate containing with the insert wells was incubated for 16 h at 37°C. The CB-MSCs on the polycarbonate membrane were stained for 1 h on ice with 1% crystal violet in methanol. After washing with de-ionized water, non-migratory cells that stayed on the upper side of the polycarbonate membrane were removed using a cotton swab. The migrated CB-MSCs that passed through the pores and clung to the underside of the membrane were observed using a BIOREVO BZ-9000 microscope (Keyence, Japan), and the cell numbers were counted using the NIH Image J Program.

qRT-PCR

After collecting the mouse brains, the hippocampi were isolated under the microscope and total RNA was extracted using TRIzol Reagent (Life Technologies, USA). First-strand cDNA was synthesized from 1 μg of the total RNA using a First Strand cDNA Synthesis Kit

(GE Healthcare). The qRT-PCR reaction mixtures contained the diluted cDNA synthesis reaction mixture (2 µl, diluted at 1:8 with de-ionized water unless stated otherwise), predesigned TaqMan Gene Expression Assays (0.5 µl), and 2× TaqMan Fast Universal PCR Master Mix (5 µl) in a final reaction volume of 10 µl. The TaqMan Gene Expression Assays (Applied Biosystems, USA) used were as follows: mouse Iba-1 (*Aif1,* Mm 00479862-g1), *CD68* (Mm 00839636), *Chil3* (Mm 00657889-mH), *Retnla* (Mm00445109-m1), *Mrc1* (Mm 00485148-m1), *IL-10* (Mm 99999062-m1), *TNF-α* (Mm 00443258-m1), *IL-1β* (Mm 01336189-m1), *BDNF* (Mm 04230607-s1), and *NGF* (Mm 00443039-m1). The expression of mouse β-actin gene was analyzed using Mouse ACTB (4352933E, Applied Biosystems) and used for normalization. The TaqMan assays were performed using a 7900HT Fast Real-Time PCR system (Life Technologies). The results were analyzed using the comparative cycle threshold (2-ΔΔct) method to calculate fold changes.

Statistical analysis

Statistical analyses were done with JMP Pro 12.2.0 statistical software (SAS Institute).

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Conflicts of interest

The authors have no conflict of interest to declare

Ethical statement

All procedures for animal experiments were conducted according to protocols approved by the Institutional Committee for Animal Experiments at Hokkaido University (protocol no. 13-0143, 03-0144).

Reference

1. **Prusiner SB**. Prions. *Proc Natl Acad Sci U S A* 1998;95(23):13363-13383.
2. **Nombela-Arrieta C, Ritz J, Silberstein LE**. The elusive nature and function of mesenchymal stem cells. *Nat Rev Mol Cell Biol* 2011;12(2):126-131.
3. **Kuroda Y, Kitada M, Wakao S, Dezawa M**. Bone marrow mesenchymal cells: how do they contribute to tissue repair and are they really stem cells? *Arch Immunol Ther Exp (Warsz)* 2011;59(5):369-378.
4. **Snykers S, De Kock J, Rogiers V, Vanhaecke T**. In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem cells (Dayton, Ohio)* 2009;27(3):577-605.
5. **Qayyum AA, Haack-Sorensen M, Mathiasen AB, Jorgensen E, Ekblond A et al.** Adipose-derived mesenchymal stromal cells for chronic myocardial ischemia (MyStromalCell Trial): study design. *Regen Med* 2012;7(3):421-428.
6. **Qi Y, Feng G, Yan W**. Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. *Mol Biol Rep* 2012;39(5):5683-5689.
7. **Chen J, Li Y, Wang L, Lu M, Zhang X et al.** Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci* 2001;189(1-2):49-57.
8. **Li Y, Chen J, Zhang CL, Wang L, Lu D et al.** Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells. *Glia* 2005;49(3):407-417.
9. **Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A et al.** Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci U S A* 2002;99(4):2199-2204.
10. **Nakamura K, Ito Y, Kawano Y, Kurozumi K, Kobune M et al.** Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004;11(14):1155-1164.
11. **Lee JK, Jin HK, Endo S, Schuchman EH, Carter JE et al.** Intracerebral transplantation of bone marrow-derived mesenchymal stem cells reduces amyloid-beta deposition and rescues memory deficits in Alzheimer's disease mice by modulation of immune responses. *Stem cells (Dayton, Ohio)* 2010;28(2):329-343.
12. **Kim S, Chang KA, Kim J, Park HG, Ra JC et al.** The preventive and therapeutic effects of intravenous human adipose-derived stem cells in Alzheimer's disease mice. *PLoS One* 2012;7(9):e45757.
13. **Hellmann MA, Panet H, Barhum Y, Melamed E, Offen D**. Increased survival and migration of engrafted mesenchymal bone marrow stem cells in 6-hydroxydopamine-lesioned rodents. *Neurosci Lett* 2006;395(2):124-128.
14. **Blandini F, Cova L, Armentero MT, Zennaro E, Levandis G et al.** Transplantation of undifferentiated human mesenchymal stem cells protects against 6-hydroxydopamine neurotoxicity in the rat. *Cell Transplant* 2010;19(2):203-217.
15. **Park HJ, Shin JY, Lee BR, Kim HO, Lee PH**. Mesenchymal stem cells augment neurogenesis in the subventricular zone and enhance differentiation of neural precursor cells into dopaminergic neurons in the substantia nigra of a parkinsonian model. *Cell Transplant* 2012;21(8):1629-1640.
16. **Vercelli A, Mereuta OM, Garbossa D, Muraca G, Mareschi K et al.** Human mesenchymal stem cell transplantation extends survival, improves motor performance and decreases neuroinflammation in mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* 2008;31(3):395-405.
17. **Zhou C, Zhang C, Zhao R, Chi S, Ge P et al.** Human marrow stromal cells reduce microglial activation to protect motor neurons in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neuroinflammation* 2013;10:52.
18. **Honmou O, Houkin K, Matsunaga T, Niitsu Y, Ishiai S et al.** Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 2011;134(Pt 6):1790-1807.

19. **Yoon SH, Shim YS, Park YH, Chung JK, Nam JH et al.** Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: Phase I/II clinical trial. *Stem cells (Dayton, Ohio)* 2007;25(8):2066-2073.
20. **Karussis D, Karageorgiou C, Vaknin-Dembinsky A, Gowda-Kurkalli B, Gomori JM et al.** Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. *Arch Neurol* 2010;67(10):1187-1194.
21. **Ohtaki H, Ylostalo JH, Foraker JE, Robinson AP, Reger RL et al.** Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. *Proc Natl Acad Sci U S A* 2008;105(38):14638-14643.
22. **Chopp M, Li Y**. Treatment of neural injury with marrow stromal cells. *Lancet Neurol* 2002;1(2):92-100.
23. **Bai L, Lennon DP, Caplan AI, DeChant A, Hecker J et al.** Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nat Neurosci* 2012;15(6):862-870.
24. **Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ**. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *Proc Natl Acad Sci U S A* 2005;102(50):18171-18176.
25. **Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N et al.** Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 2004;113(12):1701-1710.
26. **Woodbury D, Schwarz EJ, Prockop DJ, Black IB**. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000;61(4):364-370.
27. **Toyama K, Honmou O, Harada K, Suzuki J, Houkin K et al.** Therapeutic benefits of angiogenetic gene-modified human mesenchymal stem cells after cerebral ischemia. *Exp Neurol* 2009;216(1):47-55.
28. **Calio ML, Marinho DS, Ko GM, Ribeiro RR, Carbonel AF et al.** Transplantation of bone marrow mesenchymal stem cells decreases oxidative stress, apoptosis, and hippocampal damage in brain of a spontaneous stroke model. *Free Radic Biol Med* 2014;70:141-154.
29. **Song CH, Honmou O, Ohsawa N, Nakamura K, Hamada H et al.** Effect of transplantation of bone marrow-derived mesenchymal stem cells on mice infected with prions. *J Virol* 2009;83(11):5918-5927.
30. **Horiuchi M, Priola SA, Chabry J, Caughey B**. Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97(11):5836-5841.
31. **Prusiner SB, Scott M, Foster D, Pan KM, Groth D et al.** Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* 1990;63(4):673-686.
32. **Priola SA, Caughey B, Race RE, Chesebro B**. Heterologous PrP molecules interfere with accumulation of protease-resistant PrP in scrapie-infected murine neuroblastoma cells. *J Virol* 1994;68(8):4873-4878.
33. **Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R et al.** Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284(5411):143-147.
34. **Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F et al.** Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8(4):315-317.
35. **Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A et al.** Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *J Immunol* 2010;184(5):2321-2328.
36. **Yang ZX, Han ZB, Ji YR, Wang YW, Liang L et al.** CD106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. *PLoS One* 2013;8(3):e59354.

37. **Kim TE, Kim CG, Kim JS, Jin S, Yoon S et al.** Three-dimensional culture and interaction of cancer cells and dendritic cells in an electrospun nano-submicron hybrid fibrous scaffold. *International journal of nanomedicine* 2016;11:823-835.

38. **Song CH, Honmou O, Furuoka H, Horiuchi M**. Identification of chemoattractive factors involved in the migration of bone marrow-derived mesenchymal stem cells to brain lesions caused by prions. *Journal of virology* 2011;85(21):11069-11078.

39. **Song CH, Furuoka H, Kim CL, Ogino M, Suzuki A et al.** Effect of intraventricular infusion of anti-prion protein monoclonal antibodies on disease progression in prion-infected mice. *The Journal of general virology* 2008;89(Pt 6):1533-1544.

40. **Ohsawa N, Song CH, Suzuki A, Furuoka H, Hasebe R et al.** Therapeutic effect of peripheral administration of an anti-prion protein antibody on mice infected with prions. *Microbiology and immunology* 2013;57(4):288-297.

41. **Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M et al.** Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. *Nature protocols* 2013;8(7):1391-1415.

42. **Houlihan DD, Mabuchi Y, Morikawa S, Niibe K, Araki D et al.** Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-alpha. *Nature protocols* 2012;7(12):2103-2111.

43. **Sasaki A, Hirato J, Nakazato Y**. Immunohistochemical study of microglia in the Creutzfeldt-Jakob diseased brain. *Acta Neuropathol* 1993;86(4):337-344.

44. **Williams AE, Lawson LJ, Perry VH, Fraser H**. Characterization of the microglial response in murine scrapie. *Neuropathol Appl Neurobiol* 1994;20(1):47-55.

45. **Williams A, Lucassen PJ, Ritchie D, Bruce M**. PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Experimental neurology* 1997;144(2):433-438.

46. **Gomez-Nicola D, Fransen NL, Suzzi S, Perry VH**. Regulation of microglial proliferation during chronic neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2013;33(6):2481-2493.

47. **Burwinkel M, Schwarz A, Riemer C, Schultz J, van Landeghem F et al.** Rapid disease development in scrapie-infected mice deficient for CD40 ligand. *EMBO reports* 2004;5(5):527-531.

48. **Falsig J, Julius C, Margalith I, Schwarz P, Heppner FL et al.** A versatile prion replication assay in organotypic brain slices. *Nature neuroscience* 2008;11(1):109-117.

49. **Zhu C, Herrmann US, Falsig J, Abakumova I, Nuvolone M et al.** A neuroprotective role for microglia in prion diseases. *The Journal of experimental medicine* 2016;213(6):1047-1059.

50. **Sakai K, Hasebe R, Takahashi Y, Song CH, Suzuki A et al.** Absence of CD14 delays progression of prion diseases accompanied by increased microglial activation. *J Virol* 2013;87(24):13433-13445.

51. **El Khoury J, Toft M, Hickman SE, Means TK, Terada K et al.** Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nature medicine* 2007;13(4):432-438.

52. **Hickman SE, Allison EK, El Khoury J**. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008;28(33):8354-8360.

53. **Koenigsknecht-Talboo J, Landreth GE**. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2005;25(36):8240-8249.

54. **Campbell IL, Eddleston M, Kemper P, Oldstone MB, Hobbs MV**. Activation of cerebral cytokine gene expression and its correlation with onset of reactive astrocyte and acute-phase response gene expression in scrapie. *Journal of virology* 1994;68(4):2383-2387.

55. **Kordek R, Nerurkar VR, Liberski PP, Isaacson S, Yanagihara R et al.** Heightened expression of tumor necrosis factor alpha, interleukin 1 alpha, and glial fibrillary acidic protein in experimental Creutzfeldt-Jakob disease in mice. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93(18):9754-9758.

56. **Hwang D, Lee IY, Yoo H, Gehlenborg N, Cho JH et al.** A systems approach to prion disease. *Molecular systems biology* 2009;5:252.

57. **Hughes MM, Field RH, Perry VH, Murray CL, Cunningham C**. Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrPSc, even upon LPS stimulation. *Glia* 2010;58(16):2017-2030.

58. **Konala VB, Mamidi MK, Bhonde R, Das AK, Pochampally R et al.** The current landscape of the mesenchymal stromal cell secretome: A new paradigm for cell-free regeneration. *Cytotherapy* 2016;18(1):13-24.

59. **Salem HK, Thiemermann C**. Mesenchymal stromal cells: current understanding and clinical status. *Stem cells (Dayton, Ohio)* 2010;28(3):585-596.

60. **Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T et al.** Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World journal of stem cells* 2014;6(5):552-570.

61. **Kim KS, Kim HS, Park JM, Kim HW, Park MK et al.** Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging* 2013;34(10):2408-2420.

62. **Naaldijk Y, Jager C, Fabian C, Leovsky C, Bluher A et al.** Effect of systemic transplantation of bone marrow-derived mesenchymal stem cells on neuropathology markers in APP/PS1 Alzheimer mice. *Neuropathol Appl Neurobiol* 2016.

63. **Zhang YK, Liu JT, Peng ZW, Fan H, Yao AH et al.** Different TLR4 expression and microglia/macrophage activation induced by hemorrhage in the rat spinal cord after compressive injury. *Journal of neuroinflammation* 2013;10:112.

64. **Chio CC, Lin MT, Chang CP**. Microglial activation as a compelling target for treating acute traumatic brain injury. *Current medicinal chemistry* 2015;22(6):759-770.

65. **Zhu H, Guo ZK, Jiang XX, Li H, Wang XY et al.** A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nature protocols* 2010;5(3):550-560.

66. **Shindoh R, Kim CL, Song CH, Hasebe R, Horiuchi M**. The region approximately between amino acids 81 and 137 of proteinase K-resistant PrPSc is critical for the infectivity of the Chandler prion strain. *J Virol* 2009;83(8):3852-3860.

67. **Furuoka H, Yabuzoe A, Horiuchi M, Tagawa Y, Yokoyama T et al.** Effective antigen-retrieval method for immunohistochemical detection of abnormal isoform of prion proteins in animals. *Acta neuropathologica* 2005;109(3):263-271.

68. **Kim CL, Umetani A, Matsui T, Ishiguro N, Shinagawa M et al.** Antigenic characterization of an abnormal isoform of prion protein using a new diverse panel of monoclonal antibodies. *Virology* 2004;320(1):40-51.

Figure Legends

Figure 1. Expression of surface markers on CB-MSCs cells

Compact bone derived cells that adhered to plastic plate were purified by MACS to remove CD11b- and CD45-positive cells and analyzed by flow cytometry. The expression of surface markers on CB-MSCs was analyzed at the 4th passage (purple lines) and the black lines show the histograms for the corresponding isotype control antibody.

Figure 2. Migration of CB-MSCs to brain extracts from prion-infected mice

Insert wells containing the pre-starved CB-MSC suspension (5×10^4 cells) were placed in the wells of a 24-well plate, which contained serum-free DMEM with 1%, 0.1%, or 0.01% brain extracts from the Chandler strain- or mock-infected mice, and they were incubated for 16 h. The membranes of the insert wells were stained with crystal violet. (a) Migrated CB-MSCs clung to the underside of the membrane. Bars = 100 μm. (b) Numbers of CB-MSCs that migrated to brain extracts from Chandler strain or mock-infected mice. Stains areas greater larger than 2×10^2 μm^2 were counted as cells. Means and SD from a total of 10 areas (1.20×10^6 μm^2 per area) in two wells (five areas per well) are shown. The graphs show the cell counts per 1.20×10^6 μm^2 per area. *, $p < 0.05$; **, $p < 0.01$ (Student's *t*-test).

Figure 3. Effect of CB-MSCs on the survival of mice infected with the Chandler prion strain

(a) Survival curves and survival periods for individual mice. CB-MSCs (1×10^5 cells in 2 μl PBS) were transplanted into Chandler strain-infected mice at 120 dpi (n = 9, closed square). As a sham-operation group, the same volume of PBS was injected into Chandler strain-infected mice at 120 dpi (n = 7, open square). The X-axis indicates the survival time

after prion inoculation, and the Y-axis indicates the survival rate (%). The table on the right shows the survival times for individual mice in each group. **, $p < 0.01$ (Kaplan-Meier survival estimate, Generalized Wilcoxon test) (b) Changes in body weight. After the transplantation of CB-MSCs, mice were weighed weekly up to the terminal stage of the disease. The graph shows mean weight ± SD. *, $p < 0.05$; **, $p < 0.01$, [one way analysis of variance (ANOVA) with *post hoc* Boniferrori test].

Figure 4. Detection of PrP^{Sc} and total PrP in brain homogenates

Ten mice infected with the Chandler strain were used in each experiment. Two of the mice were sacrificed at 120 dpi. The remaining mice were divided into two groups; four mice were transplanted with CB-MSCs and the other four were injected with PBS as the sham operation. At 145 dpi (25 days after transplantation), two mice each from the CB-MSC-transplanted group and PBS-injected group were sacrificed, and the remaining two mice in each group were sacrificed at the terminal stage of the disease. The left halves of the brains were collected and used for the detection of PrP^{Sc} and total PrP by immunoblotting, whereas the right hemispheres were used for histopathological examinations (Fig. 5). (a and b) samples (10 μl, 100 μg brain equivalent) were loaded onto each well to detect PrP^{Sc} and total PrP. To avoid sampling bias that often occurs if small brain regions were collected for making brain homogenates, whole brain hemispheres were used for making 20% brain homogenates. In addition, we did sample preparation at the same time in each experiment to minimize the variation of PK treatment. PrP^{Sc} (a) and total PrP (b) at 120 dpi, 145 dpi and at the terminal stage (CB-MSC-transplanted group, 161 and 168 dpi; PBS-injected group, 156 and 158 dpi). PrP^{Sc} and total PrP were detected using anti-PrP mAb 31C6. Molecular mass markers are shown on the left. (c and d) Relative levels of total PrP^{Sc} (c) and total PrP (d). The same experiment was repeated independently and the results were quantified at each time point

based on a total of four mice. The mean levels ± SD PrP^{Sc} and total PrP relative to those at 120 dpi are indicated.

Figure 5. Effects of intra-hippocampus transplantation of CB-MSCs on neurohistopathological changes

The right hemispheres of mice (as described in Fig. 4) were fixed with 10% formalin for histopathological examinations. Paraffin-embedded sections were subjected to immunohistochemical analysis for PrP^{Sc} using mAb 31C6, astrocytes using anti-GFAP antibody, microglia using anti-Iba-1 antibody and HE staining. (a) Immunohistochemistry and HE staining of hippocampus at 145 dpi and the terminal stage of the disease. The microglia in the region of interest (blue squares) in Iba-1-stained sections were magnified and they were shown under each original section. Bars = 20 μm. (b) Numbers of microglia in the hippocampus. Iba-1-positive microglia in sections were counted by Image J. Stained areas larger than 10 μm^2 were counted as cells. Means and SD from a total of 15 areas (3.4×10^{-2} mm^2 per area, five areas per one section from each mouse, three mice) are shown. (c) Quantitative analysis of vacuolar regions in the hippocampus. Vacuolar regions in HE-stained sections were quantified by Image J. Stained areas of 20 μm^2 to 30 μm^2 were measured as vacuolar regions. The means and SD from three areas (2.6×10^{-2} mm^2 per area, one area per one section from each mouse, three mice) are shown. Gray and black bars indicate CB-MSC-transplanted and PBS-injected groups of mice, respectively (*$p < 0.05$, Student's *t*-test).

Figure 6. Effect of CB-MSCs on gene expression in brains of prion-infected mice

CB-MSCs (1×10^5 cells in 2 μl PBS) were transplanted into Chandler strain-infected mice

762 (n = 3, black bars “Chandler MSCs”) and mock-infected mice (n = 3, white bars “Mock
763 MSCs”) at 120 dpi. The same volume of PBS was injected into Chandler strain-infected mice
764 (n = 3, gray bars “Chandler PBS”) and mock-infected mice (n = 3, red bars “Mock PBS”) as a
765 sham-operation group. At 145 dpi, 21 days after transplantation, mice were sacrificed and
766 hippocampi were collected for total RNA isolation. qRT-PCR was performed as described in
767 the Methods. Gene expression levels relative to mock-infected/sham-operated group (Mock
768 PBS group) are shown (mean ± SD). Only significant differences between the
769 CB-MSC-transplanted group and PBS-injected group are indicated by asterisks. *, $p < 0.05$;
770 **, $p < 0.01$, one-way ANOVA with Tukey *post hoc* tests.