FULL PAPER *Laboratory Animal Science*

Intracerebellar Transplantation of Neural Stem Cells into Mice with Neurodegeneration Improves Neuronal Networks with Functional Synaptic Transmission

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ABSTRACT. Recent studies have shown that many kinds of stem cells are beneficial for patients suffering with neurodegenerative diseases. We investigated the effects of neural stem cell (NSC), Maudsley hippocampal clone 36 (MHP36) in the Niemann-Pick disease type C (NP-C) model mice. Herein, we demonstrate that MHP36 transplantation improves the neuropathological features without acute immune response and promotes neuronal networks with functional synaptic transmission. The number of surviving Purkinje neurons substantially increased in MHP36 transplanted NP-C mice compared with sham-transplanted NP-C mice. MHP36 significantly reduced both of astrocytic and microglial activations. We also found that these surviving Purkinje neurons have normal functional synapses with parallel fibers that have normal glutamate release probability in MHP36 transplanted NP-C mice. Furthermore, real-time PCR analysis revealed up-regulation of genes involved in both excitatory and inhibitory neurotransmission encoding subunits of the ionotropic glutamate receptors GluR2, 3 and $GABA_A$ receptor $\beta 2$. These findings suggest that NSC, MHP36 transplantation may have therapeutic effects in the treatment of NP-C and other neurodegenerative diseases.

KEY WORDS: neural stem cell, neuronal networks, Niemann-Pick disease type C.

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Many Studies demonstrating the influence of transplantation of stem cell derived from embryonic and adult tissues of various species into degenerative disorders of the central nervous system has made that be thought a promising therapeutic strategy [1–3, 10, 18, 21, 22, 27, 29]. Although these many kinds of stem cells have therapeutic effects, the selection of stem cells that have a specific therapeutic effect for the certain disease may be the key for the treatment.

In most neurodegenerative diseases characterized by inflammation and another neurodegenerative disease, Niemann-pick disease type C (NP-C), chronic inflammatory reaction has been considered to be a key process leading to neuronal degeneration [1, 4, 20]. NP-C is an inherited lysosomal storage disorders resulting from a mutation of the *NPC* gene, and characterized by progressive ataxia, cerebellar atrophy, psychomotor deterioration, extrapyramidal deficits, and dementia. Pathological characteristics are progressive Purkinje neuron loss, intracellular accumulation of sphingomyelin, cholesterol and neuroglial cell (astrocyte, microglial cell) infiltration in the cerebellum [6, 11]. Our studies have shown that transplantation of bone marrow-derived mesenchymal stem cells (BM-MSC) results in alleviation of pathology associated with murine NP-C cerebellum through fusion of BM-MSC and Purkinje neuron and promotion of neuronal networks with functional synaptic transmission [1–3]. Herein, we assessed the therapeutic effects of conditionally immortalized multipotential neural stem cells (NSCs), Maudsley hippocampal clone 36 (MHP36), in a NP-C model mice.

MHP36, conditionally immortalized NSCs, originated from the hippocampus of the H-2K^b-tsA58 immortomouse that express the temperature-sensitive oncoprotein, thermolabile form of simian virus 40 (SV40) large tumor (T) antigens (Tag), under the control of the MHC I antigen promoter [9]. MHP36 proliferates at a permissive temperature of 33°C and stops a proliferation and differentiates at the non-permissive temperature of 37°C, so, can be controlled whose differentiation [15]. These characteristics of MHP36 are considered to induce a successful engraftment. Because to yield the large number of engrafted cells typically observed, NSCs should be in an undifferentiated state and still capable of mitosis [10].

Transplantation of NSCs, however, evokes immunological responses in the host. These immune rejection responses against the transplanted cells are crucially dependent on host's immune system that is mediated by recognition of cell surface proteins known as major histocompatibility complex (MHC) antigens [17]. Incompatibility between graft donor and recipient host MHC molecules is considered as a main source of the sensitization of the host immune system, so, subsequently immune rejection response is induced [16]. Although transplantation of stem cells induces immune responses, McLaren *et al.* observed that MHC antigens on the surface of NSCs were down-regulated as cells

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differentiate [14]. Differentiation of MHP36 cells leads to the down-regulation of MHC class I and II molecule expression [17]. Therefore, we expect MHP36 would not induce an acute immunological response and may have protective effects in neurodegenerative environment of NP-C model mice's cerebellum. Furthermore, we demonstrate elevation of genes encoding neuronal transmission following transplantation of MHP36 in NP-C mice.

MATERIALS AND METHODS

Animals: A colony of BALB/c npc^{nih} (NP-C) mice has been maintained and genotyped by polymerase chain reaction (PCR) as described [11]. The NP-C *Gsbs*$^{GFP+}$ mice were created by crossing NP-C mice with *Gsbs*$^{GFP+}$ mice, which carry a null allele of *Gsbs* (GeneBank accession No. 1333876) on BALB/c back ground and concomitantly have the green fluorescent protein (GFP) reporter gene inserted into the *Gsbs* locus [2, 6]. Animals were housed on a 12 hr light/dark cycle and given water and food *ad libitum*. All procedures were in accordance with an animal protocol approved by the Kyungpook National University Institutional Animal Care and Use Committee (IACUC).

Culture of MHP36: Frozen MHP36 (gift of Dr. J. Price) were thawed and cultured [15]. MHP36 were cultured in fibronectin-coated (Sigma-Aldrich, St. Louis, MO, U.S.A.) Flask at 33°C with Dulbecco's modified Eagle medium/F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with bovine serum albumin (MP Biomedicals, Aurora, OH), apo transferrin (Sigma-Aldrich) 94 μg/m*l*, putrescine dihydrochloride (Sigma-Aldrich) 15.3 μg/m*l*, insulin (Sigma-Aldrich) 4.7 μg/m*l*, L-thyroxine (Sigma-Aldrich) 380 *n*g/m*l*, tri-iodo-L-thyronine (Sigma-Aldrich) 317 *n*g/m*l*, L-glutamine (Invitrogen) 1.9 mM, progesterone (Sigma-Aldrich) 58 *n*g/m*l*, sodium selenite (Sigma-Aldrich) 38 *n*g/m*l*, heparin sodium salt (Sigma-Aldrich) 9.4 unit/m*l*, basic fibroblast growth factor (FGF) (Peprotech, Rocky Hill, NJ, U.S.A.) 10 *n*g/m*l*, interferon (Peprotech) 1.2 *n*g/m*l*. The culture condition was maintained in an undifferentiated state at 33°C and 5% CO_2 [19, 20]. For transplantation, the cells were liberated from culture flasks using versene (Invitrogen) rather than a trypsin-based technique to preserve protein expression, and were pelleted from Hank's Balanced Salt Solution (HBSS). MHP36 were resuspended at HBSS.

Cell transplantation: NP-C and NP-C *Gsbs*$^{GFP+}$ mice (approximately 4 weeks of age, *n*=4 each group) were anesthetized with a combination of 100 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal injection. Skin of the head was incised along the midline, and the skull was exposed. Hole at the appropriate site of the skull and then, mouse were placed on the Styrofoam platform and were transplanted cells using stereotaxic frame (Stoelting Co., Wood Dale, IL, U.S.A.) as described [2]. U373, glioma cell line, was used as a non-stem cell control condition. U373 was cultured with Dulbecco's modified Eagle medium (Invitrogen) supplemented with fetal bovine serum (Invitrogen), penicillin and streptomycin (Invitrogen). MHP36 and U373 were transplanted into the cerebellum using a glass capillary (1.2 × 0.6 mm) [2]. Using bregma as a reference point, a Hamilton syringe was moved to the following coordinates. The injection coordinates were 5.52 mm posterior to bregma, and injection depth was 2.50 mm from the surface of the brain. Each recipient received approximately 1×10^6 cells in 3 μl of cell suspension at a rate of 0.15 μl/min. Sham-transplanted mice received injections of equal volume of Hanks' balanced salt solution. After transplantation, sutured and closed the scalp, and the animals recovered from the anesthesia before they were returned to their cage. Age matched normal littermates in the NP-C colony were used as controls.

Histological analysis: Mice were sacrificed at 2 and 4 weeks after transplantation. The mice were anesthetized with 2.5% avertin in phosphate buffered solution (PBS). Animals were immediately cardiac perfused with 4% paraformaldehyde in PBS. After perfusion, decapitated and brain tissue were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.2 for 2 hr. And then they cryoprotected in a 20% sucrose in PBS overnight after wash the tissue with cold 0.2 M PBS, pH 7.4 for 1 hr. Then transfer the tissue to cold 30% sucrose in 0.1 M PBS at 4°C overnight. Thin sections for immunofluorescence were made on a cryostat (CM3050S; Leica, Heidelberg, Germany) at 8 μm and mounted on superfrost/plus glass slides (Fisher Scientific, Morris Plains, NJ, U.S.A.). For immunofluorescence, we used primary antibodies, anti-calbindin-D 28K (rabbit, diluted 1:1,000; Chemicon, Temecula, CA, U.S.A.) which recognizes Purkinje neuron, anti-GFAP (rabbit, diluted 1:1,000; Dakocytomation, Carpinteria, CA, U.S.A.) which recognizes astrocyte, anti-F4/80 (rat, diluted 1:10; Serotec, Oxford, U.K.) which recognizes microglia, anti-MHC class II and anti-CD45. They were applied in a humidified chamber for 24 hr at 4°C to the sections after pre-incubation in blocking solution for 1 hr. Either Alexa Fluor 488 or Alexa Fluor 568 (diluted 1:1,000; Molecular Probes, Carlsbad, CA, U.S.A.) were used as a secondary antibodies.

For sphingomyelin stain, we used lysenin which specifically binds to the sphingomyelin, anti-lysenin antiserum, Alexa Fluor 568 (diluted 1:1,000; Molecular Probes) were used [25, 30]. For filipin stain, the sections were incubated with 50 μg/m*l* filipin in 1% BSA in PBS for 2 hr at room temperature after blocking with 1% BSA in PBS for 1 hr. Fluorescence microscopy was performed using an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan).

Electrophysiology: Wild type or NP-C *Gsbs*$^{GFP/+}$ mice transplanted with MHP36 were anesthetized using urethane (1.5 gm/kg, i.p.) and decapitated. A rapid craniotomy was performed in ice-cold Krebs' solution (composition in 1 mM: NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11; pre-oxygenated with 95% O_2/5% CO_2 at pH 7.4) to remove the occipital bone and mastoid process, which allowed the cerebellum to be detached and removed. The removed cerebellum was glued with cyanoacylate to the wall of agar block on the stage of a vibratome, and then 250 μm parasagittal slices were cut and

recovered in the oxygenated Krebs' solution. The slices were placed in a recording chamber of the microscope stage which was continuously superfused with the oxygenated Krebs' solution (flow rate: 3 m*l*/min) at room temperature.

Whole-cell recordings were performed under the microscope (BX51WI, Olympus) equipped with BX-FLA attachment, the appropriate filter cube and the stage chamber. Fluorescent cells expressing GFP in the parasagittal slices were visually identified beneath a long-working distance (3.3 mm) 40 × water-immersion objective (NA 0.9, Olympus) with differential interference contrast (DIC) optics. Whole-cell recordings were then obtained from the identified fluorescent cells under infrared (IR)-DIC system, and made using patch pipettes (TW150F-4, WPI) with the resistance of 8–10 Ω when filled with internal solution (composition in 1 mM: 135 K gluconate, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 KCl, 5 EGTA, 5 HEPES, 5 Na_2ATP). Cells were voltage-clamped at holding potential of –70 mV to record spontaneous excitatory postsynaptic currents (sEPSCs), parallel fiber stimulation-evoked EPSCs, and current-clamped to record membrane potentials by various steps of intracellular current injections (–0.5~1.1 nA, 300 msec). The molecular layer of cerebellar slices was stimulated using bipolar concentric electrode to record parallel fiber stimulation-evoked EPSCs. The responses were amplified by Multiclamp 700A (Axon Instruments, Sunnyvale, CA, U.S.A.), sampled at 10 kHz (Digidata 1320, Axon Instruments) and low-pass filtered at 2 kHz. After finishing each recording, the recorded cell was photographed using charge-coupled device (CCD) camera ($CoolSNAP_{HQ}$, Photometrics) under each filter cube for GFP excitation and emission wavelengths. sEPSCs were manually picked, and analyzed using pClamp software (version 9.2, Axon Instruments) for frequency and amplitude. Amplitude, 20–80% rise time and decay time constant (τ) of evoked EPSCs were measured.

Real-time PCR: To validate the promotion of neuronal networks, several genes involving synaptic transmission were chosen for real-time PCR. Particularly, we focused on the GABA receptor and AMPA receptors because inhibitory synapse in the cerebellum is related with GABA and excitatory synapse is related with glutamate receptor, especially Non N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) receptor, ionotropic glutamate receptors (GluR). The RNA samples from total of three individual animals per group in the different experiment were used to prepare cDNA for PCR using the Superscript III RT (Invitrogen). The cDNA was quantified using the SensiMixPlus SYBR Green PCR Kit (Quantace, London, U.K.). The PCR primers used were as follows: GluR2 forward, 5'-AAATTGCCAAACATTGTGGA-3'; GluR2 reverse, 5'-ATGGAGCCATGGCAATATCA-3'; GluR3 forward, 5'-ACACCATCAGCATAGGTGGA-3'; GluR3 reverse, 5'-TCAGTGGTGTTCTGGTT-GGT-3'; $GABA_A$ $\beta 2$ forward, 5'-AAAGCAGACGGAAATTGCTT-3'; $GABA_A$ $\beta 2$ reverse, 5'-CGGTTCTGCAGACTTCATGT-3'. For each transcript investigated, a mixture of the following reaction components was prepared to the indicated end concentration: 10 pM forward primer, 10 pM reverse primer, and SensiMixPlus SYBR Green PCR Master mix. The 10 μl of master mix was fill in the 0.1 m*l* tubes, and a 5 μl volume, containing 125 *n*g of reverse-transcribed total RNA, was added as PCR template. The 0.1 m*l* tubes were closed, and placed into the Corbett research RG-6000. The real time PCR primers and experimental protocol for each gene was designed according to previous report [2].

Immunoblotting: Brain samples were homogenized in Laemmli lysis buffer plus protease inhibitors. Aliquots containing 30 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis. Protein bands were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, U.S.A.) and probed by incubating with the primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). We used the primary antibodies raised against GluR2/3 (1:500; Upstate Biotechnology, Charlottesville, VA, U.S.A.), and $GABA_A$ receptor $\beta 2$ (1:1,000; Chemicon, Temecula, CA, U.S.A.). To determine the specificity of the primary antibodies, we used antibodies preabsorbed with blocking peptides instead of primary antibodies. Blots were visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's direction and exposed to x-ray film. Equal protein loading was confirmed by measuring β-actin (1:5,000; anti-β-actin; Sigma-Aldrich). Densitometric measurements were made from the film using an imaging densitometer (Bio-Rad, Hercules, CA, U.S.A.) and then quantified using Bio-Rad analysis software. For quantification of relative protein expression, the optical density of the protein band of interest was normalized to the optical density of β-actin on the same gel.

Accelerating Rota-Rod analysis: The Rota-Rod apparatus is used to measure an animal's balance and coordination by recording the amount of time the animal is able to remain on a rotating rod [5]. The Rota-Rod apparatus (accelerating model 47600; Ugo Basile, Comerio, Italy) has a 3 cm diameter, soft rubber covering rod, suitably machined to provide grips. The animals put on the rod that is rotating at a selected speed via the gear belt. When the mouse falls off from its cylinder section, the plate below trips, and the corresponding counter is disconnected, thereby recording the animal's endurance time can be recorded in seconds. The screening results are more correctly assessed by using an accelerating model. The machine was set to an initial speed of 32 rpm, and the acceleration was increased by 32 rpm every 25–30 sec. Transplanted NP-C mice were analyzed along with sham-transplanted NP-C and normal control mice. Scores were registered once per week (at least three independent tests were performed for each time point) beginning the day of transplantation. Uniform conditions were carefully maintained for each test, and there was a rest time of 30 min between each trials. A maximum time limit of 400 sec per test was established.

Statistical analysis: The Student's *t*-test was used to com-

pare two groups, whereas the Tukey's HSD test and Repeated Measures Analysis of Variance test was used for multi group comparisons according to the SAS statistical package (release 9.1; SAS Institute Inc., Cary, NC, U.S.A.). $P<0.05$ was considered to be significant.

RESULTS

MHP36 transplantation promotes Purkinje neuron survival: NP-C is characterized by progressive Purkinje neuron loss in the cerebellum. We investigated whether MHP36 is contributed to Purkinje neurons survival. Purkinje neurons in the cerebellum were visualized by immunofluorescence assay with anti-calbindin-D28K at 2 and 4 weeks following transplantation. Total cell numbers of surviving Purkinje neurons were significantly increased in MHP36 transplanted NP-C group compared with sham-transplanted NP-C group especially 2 weeks after transplantation (Fig. 1a). The greatest effect on the survival of Purkinje neurons was evident in lobe I, II, III (Fig. 1b). Cerebellum from sham-transplanted NP-C group showed severe depletion of calbindin-positive Purkinje neurons. In U373 transplanted NP-C group and sham-transplanted NP-C group, there was an almost complete disappearance of Purkinje neurons, and there was no differences between the two groups. However, the protective effects of MHP36 were decreased at 4 weeks after transplantation. Our results suggest that MHP36 transplantation promotes Purkinje neuron survival in NP-C mice.

MHP36 transplantation attenuates astrocytic and microglial infiltration: Glial activation has been considered to be a key process leading to neuronal degeneration in NP-C mice [4]. Thus, we examined the inflammatory responses in

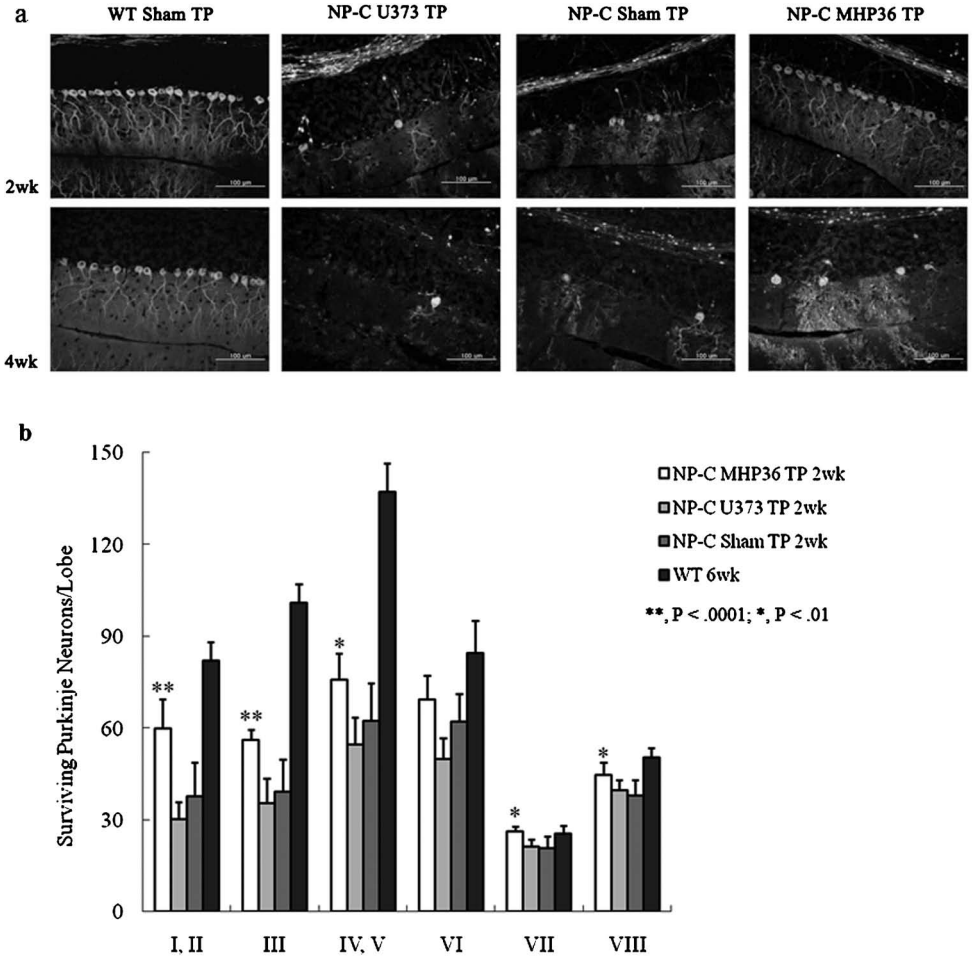

Fig. 1. Effects of neural stem cell, MHP36 transplantation on Purkinje neurons in the cerebellum. (a) Immunofluorescence of the cerebellum for calbindin-D28K demonstrates that MHP36 transplantation promotes survival of Purkinje neurons in NP-C mice at 2 weeks after transplantation. Scale bar: 100 μm. (b) Quantification of Purkinje neurons in cerebellar vermis in transplanted and sham-transplanted NP-C mice. Data are mean SEM (n=4 brains and n=32 sections in each group). The number of Purkinje neurons was significantly increased in MHP36 transplanted NP-C mice especially at 2 weeks after transplantation. **, $P<0.0001$; *, $P<0.01$ compared with U373 and sham-transplanted mice.

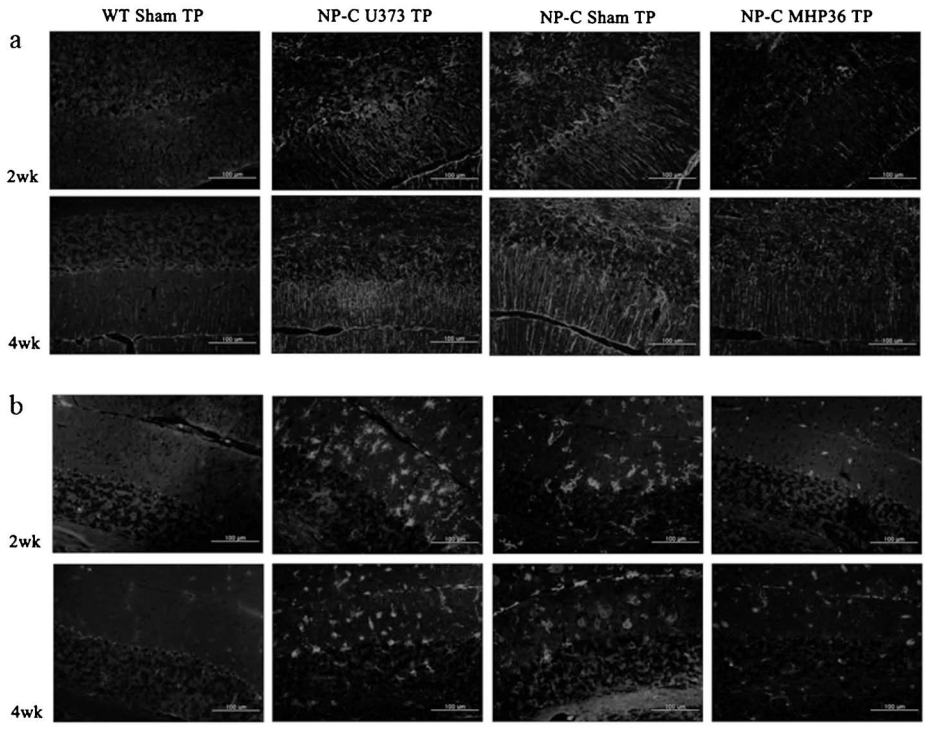

Fig. 2. Inflammatory reaction in the cerebellum of NP-C mice. (a) Pattern of astrocytic activation in transplanted and sham-transplanted group was assessed using GFAP. GFAP immunoreactivity was markedly reduced in the MHP36 transplanted NP-C group compared with sham-transplanted NP-C group. (b) Immunofluorescence of F4/80-positive microglial cells were decreased in MHP36 transplanted NP-C group as compared to U373 and sham-transplanted NP-C group. Scale bar: 100 μm.

the MHP36 transplanted NP-C group, i.e., changes in infiltration of activated microglia and astrocyte in the cerebellar tissue. At 2 weeks after MHP36 transplantation, there was a significant difference between MHP36 transplanted NP-C group and sham-transplanted NP-C group. From the staining of GFAP-positive cells, astrocytic activity was markedly reduced in MHP36 transplanted NP-C group compared with sham-transplanted NP-C group (Fig. 2a). The presence of microglia/macrophage was determined by F4/80. Low level of microglial/macrophage activations was presented in MHP36 transplanted NP-C group compared with sham-transplanted NP-C group (Fig. 2b). These phenomena were continued till 4 weeks after transplantation. In addition, the stained cells with GFAP or F4/80 in U373 transplanted NP-C group were widely distributed in the cerebellum. This reveals that MHP36 transplantation markedly reduced inflammatory reactions in the cerebellum of NP-C mice.

MHP3 reduces sphingomyelin and cholesterol accumulation: Progressive lipid accumulation is one of the pathological characteristics of NP-C. The most pronounced cellular phenotypes in NP-C are an accumulation of unesterified cholesterol in the perinuclear lysosomal compartments and the increasing level of sphingomyelin [11]. We investigated the effect of MHP36 transplantation on the changes of intracellular lipid storage assessed using specific probes, lysenin and filipin. Lysenin, a sphingomyelin-specific binding protein, was applied as a probe to assess sphingomyelin storage. U373 and sham-transplanted NP-C group showed very high levels of sphingomyelin accumulation in the cerebellum. There was a marked reduction in lysenin staining in the MHP36 transplanted NP-C group (Fig. 3a). As shown in Fig. 3B, cholesterol accumulation is also greatly reduced by transplantation of MHP36. Cholesterol accumulation was prominent in the U373 and sham-transplanted NP-C groups. Thus, MHP36 transplantation in NP-C mice has the effects of marked decrease in intracellular lysosomal storage and cholesterol accumulation pathology. These effects were maintained till 4 weeks post transplantation.

Transplanted MHP36 reduces acute host immune rejection responses: Next, we sought to correlate the transplantation of MHP36 and host immunological response because immune rejection response to the transplanted stem cells might limit the duration of their action. There is protective system which recognizes or eliminates foreign material when the foreign material comes in or transplanted on the body. This protective mechanism is celled immune rejection response and is mediated MHC molecule. Thus it is important to evaluate the immune rejection response that may determine integration and acceptance, or rejection of transplanted cells. So, we studied the degrees of host immu-

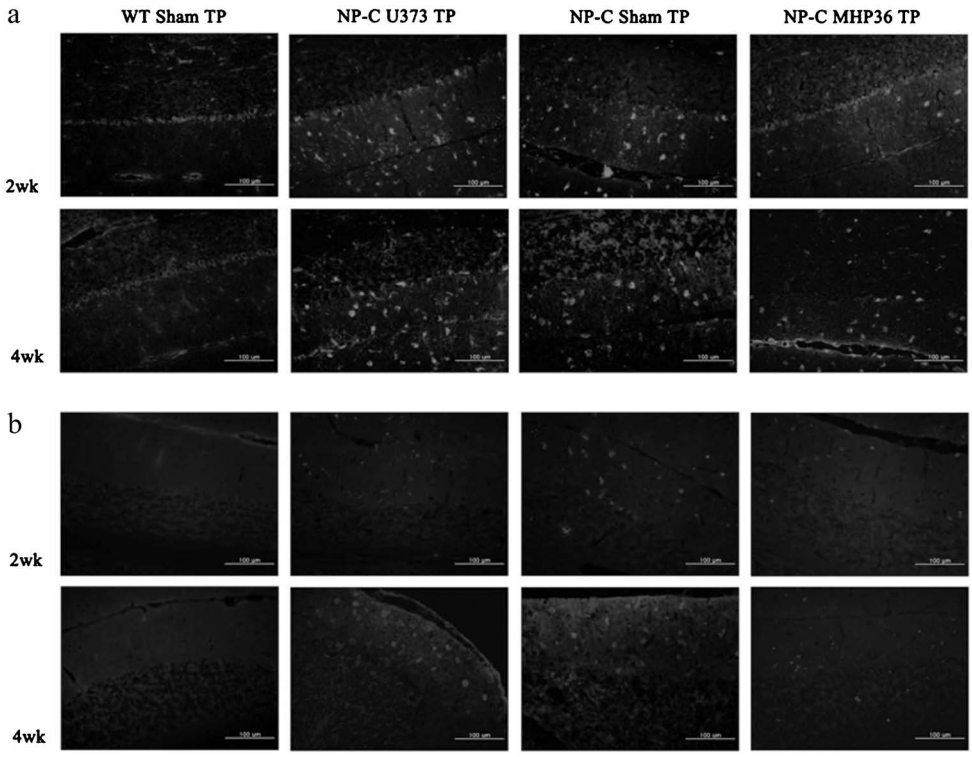

Fig. 3. Abnormality of cholesterol and sphingomyelin storage were improved in the cerebellum of MHP36 transplanted NP-C group. (a) Lysenin and its polyclonal antibody were used to visualize sphingomyelin in cerebellar sections from transplanted and sham-transplanted NP-C group. The lysenin reaction was decreased in the MHP36 transplanted NP-C group compared with sham-transplanted NP-C group. (b) Filipin was used to visualize cholesterol in cerebellar sections of transplanted and sham-transplanted groups. Note that signals seen clearly in U373 and sham-transplanted NP-C group were markedly reduced in MHP36 transplanted NP-C cerebellum. Scale bar: 100 μm.

nological response by estimating the MHC class II and CD45 (leukocyte common antigen) expression at 2 and 4 weeks following transplantation of MHP36. At 2 weeks after transplantation, the patterns of immunological response such as MHC class II and CD45 expression were similar to animals receiving a MHP36 and sham-transplanted NP-C group. But, U373 transplanted NP-C group showed that greatly increase of immunological response (Fig. 4). Nonetheless, at 4 weeks post transplantation, MHP36 induced strong immunological response as shown in U373 transplanted NP-C group. There was no increase of immunological response in sham-transplanted group at 4 weeks after transplantation. These results suggest that MHP36 was not recognized as a foreign material and did not also induce acute immunological response in NP-C mice at 2 weeks after transplantation, even though no immunosuppressive agents were used. At 4 weeks after transplantation, host immunological responses were increased. Maybe, it seemed to be as a result of that transplanted MHP36 died or immune response was turned on at 4 weeks after transplantation by becoming much more responsive to inflammatory cytokine released by host as stem cell differentiate [14].

Purkinje neurons in MHP36 transplanted NP-C mice have normal synaptic function: In the previous result, we demonstrated protective effect of MHP36 transplantation by assessing the number and morphology of Purkinje neurons. Therefore we investigated functional improvement by MHP36 transplantation. To investigate any possible change in synaptic function and in glutamate release from parallel fibers, we recorded parallel fiber stimulation-evoked EPSCs in Purkinje neurons in wild type mice and the surviving Purkinje neurons in mice transplanted with MHP36. As summarized in Table 1, the parallel fiber stimulation-evoked EPSCs were not significantly different in amplitudes, 20–80% rise time and decay τ between MHP36 transplanted NP-C group and wild type group. In addition, paired-pulse ratios of EPSCs evoked by two successive pulses of stimuli spaced at 50 msec are not significantly different between these groups (Fig. 5). These results together indicate that the survival Purkinje neurons in MHP36 transplanted NP-C group form normal functional synapses with parallel fibers that have normal glutamate release probability as wild type group.

MHP36 transplantation improves neuronal networks:

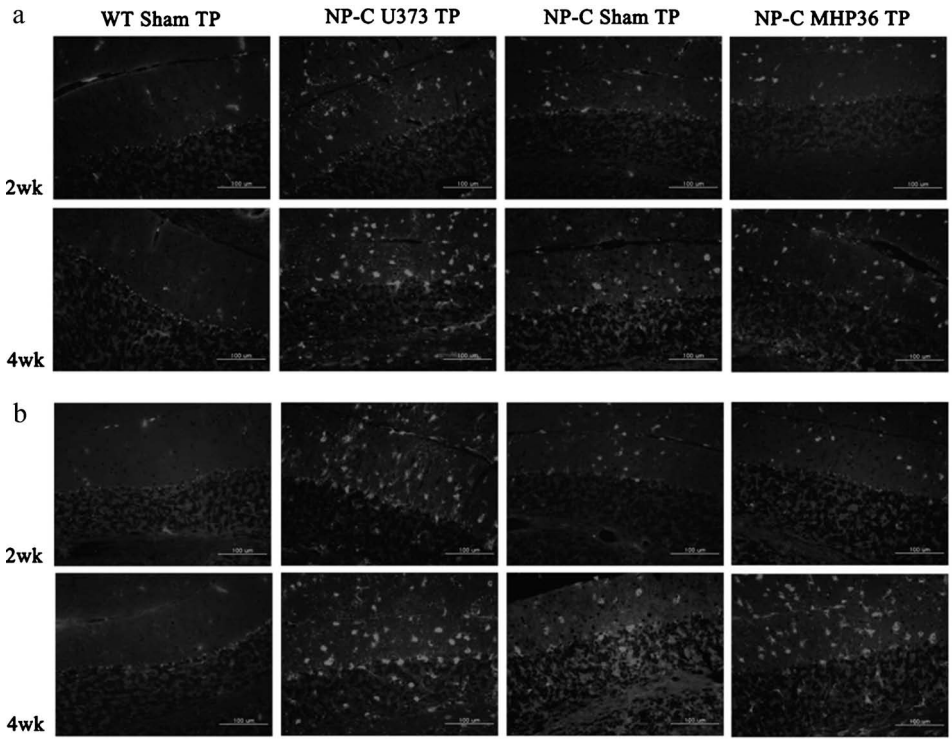

Fig. 4. MHP36 did not induce acute immunological response in NP-C mice. (a) There was no up-regulation of MHC class II by transplantation of MHP36 compared with sham- transplanted NP-C group at 2 weeks post transplantation. On the other hand, U373 transplantation greatly up-regulated the expression of MHC class II. (b) Likewise, patterns of CD45 expression were similar to those of MHC class II. CD45 expression was not increased in MHP36 transplanted NP-C group compared with sham-transplanted NP-C group. Scale bar: 100 μm.

Table 1. EPSCs recorded in Purkinje neurons by stimulation of parallel fibers

	NP-C MHP36 TP (n=6)	WT (n=8)
Stimulus Intensity (μA)	400.0 ± 45.6	364.5 ±116.2
EPSC (amplitude)	103.3 ± 33.4	95.0 ± 11.7
EPSC (20–80% rise time)	1.8 ± 0.2	2.2 ± 0.2
EPSC (decay τ)	19.4 ± 3.5	21.3 ± 5.5
Paired-pulse ratio	1.7 ± 0.3	1.4 ± 0.3

Numbers represent number of neurons recorded. Stimulus duration was fixed to 0.1 msec.

Purkinje neurons in MHP36 transplanted NP-C mice have synaptic function, thus we further examined to understand how MHP36 improves neuronal function and environment. We focused on the neurotransmitter related genes which are responsible synaptic transmission and function. In the cerebellum, inhibitory synaptic transmission is mediated by GABA and excitatory synaptic transmission is mediated by AMPA receptor. We analyzed expression of genes encoding neurotransmitters of the brain using real-time PCR. MHP36 transplantation caused increase of genes related synaptic transmission in NP-C mouse. Especially, genes encoding subunits of the ionotropic glutamate receptors, GluR2 and 3, and ionotropic GABA receptors ($GABA_A$ β2Rs) were up-regulated. All of these genes have been implicated in neurotransmission in the cerebellum (Fig. 6). To validate whether the gene expression profile in this experiment is stem cell specific, we examined non-stem cell line, U373 transplanted mouse brain. As shown in Fig. 6, the expression profiles of genes involved in synaptic transmission were not elevated in U373 transplanted NP-C group. Especially, up-regulation of GluR2, 3 is elucidated in western blot analysis (Fig. 6d). Our result demonstrated that MHP36 transplantation have the specific effects that promote neuronal network with increased synaptic transmitters.

MHP36 transplantation improves motor function: Motor dysfunction and ataxia was evident in the course of degeneration of Purkinje neuron. But, as mentioned above, we demonstrated that total cell counts of surviving Purkinje neurons were significantly increased in MHP36 transplanted NP-C group. Therefore, to further understand the contributions of MHP36 transplantation to the improvement of pathology in NP-C mice, we studied the motor functional changes. Motor function was evaluated by counting the mean time to fall off a rotating Rota-Rod. This frame can assess an ani-

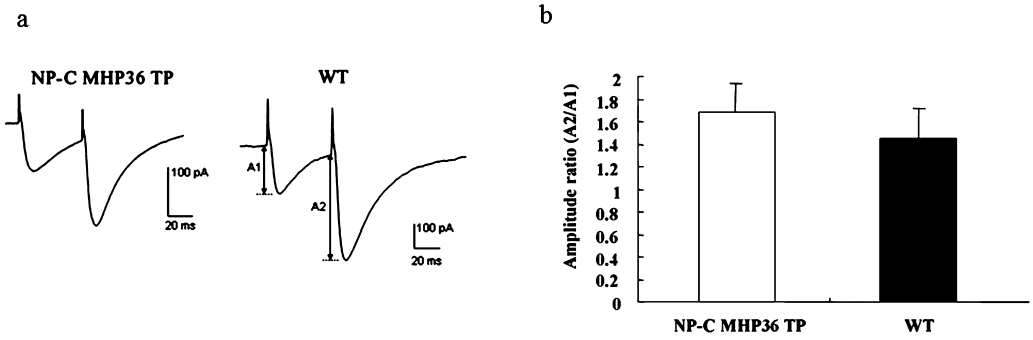

Fig. 5. (a) Paired-pulse ratio of EPSCs recorded in Purkinje neurons by stimulating parallel fibers in wild-type, and NP-C mice transplanted with MHP36. A1 and A2 respectively indicate amplitudes of the first and the second EPSCs, evoked by two successive pulses of stimuli spaced at 50 msec, recorded in a wild type Purkinje neuron. (b) The paired-pulse ratios (A2/A1) of EPSCs from two groups are summarized. White bars, MHP36 transplantation; Black bars, sham-transplanted wild type mice.

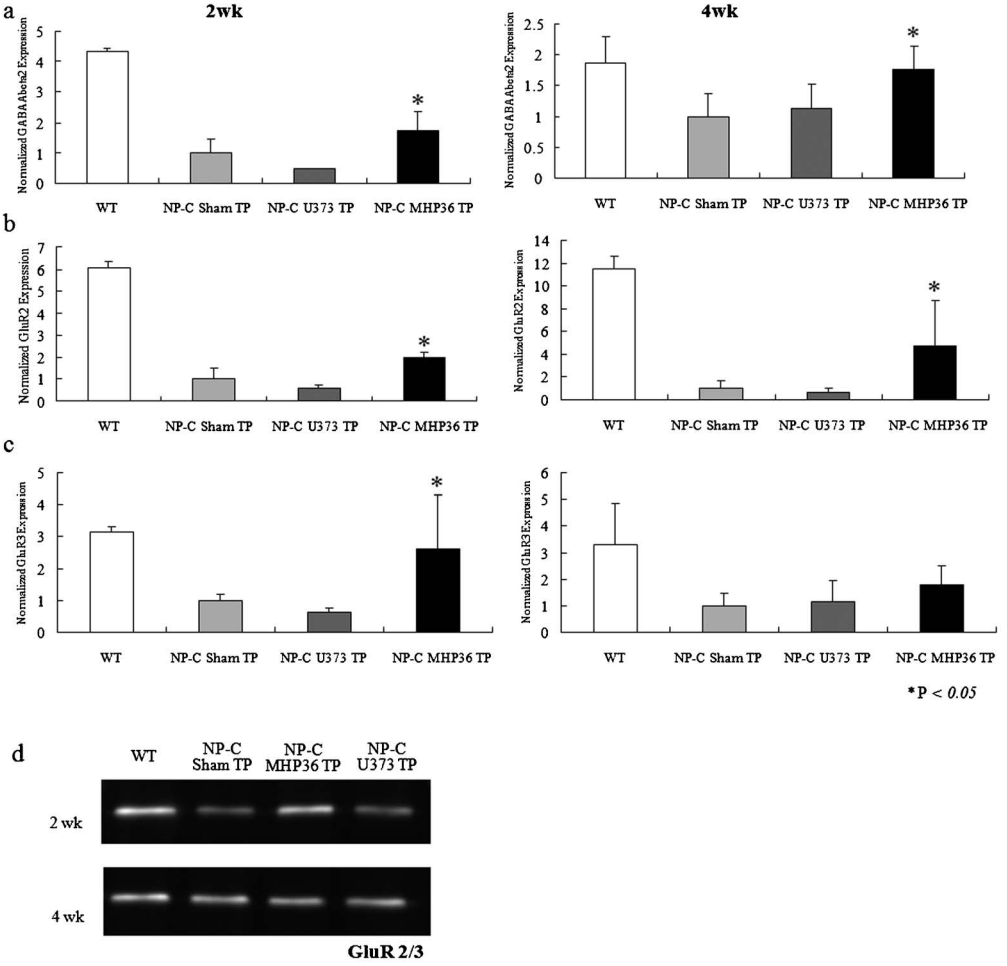

Fig. 6. Gene expressions of GluR2, 3 and $GABA_A$ receptor $\beta 2$ were analyzed using real time PCR. Increase in GluR2/3 protein was also detected using western blot analysis. MHP36 transplantation results in increase of synaptic transmission related gene expressions in NP-C mouse. Black bars, MHP36 transplantation; white bars, sham transplanted wild type mouse; light gray bars, sham transplanted NP-C mouse; dark gray bars, U373 transplanted NP-C mouse. Gene expression of (a), $GABA_A$ receptor $\beta 2$; (b), GluR2; (c), GluR3 using real-time PCR. All of the genes are elevated in MHP36 transplanted NP-C group compared with sham transplanted and U373 transplanted NP-C group. *, $P<0.05$ compared with U373 and sham transplanted mice. (d), Up-regulation of protein expression of GluR2/3 also confirmed using western blot analysis.

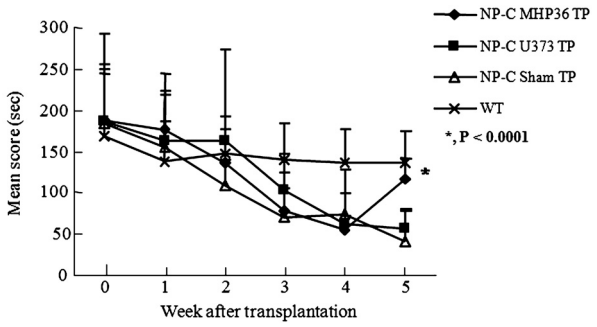

Fig. 7. Rota-Rod scores for transplanted and sham transplanted NP-C mice. Rota-Rod scores of MHP36 TP (filled diamond), U373 TP (filled square), sham TP (open triangle), and WT (× mark) were averaged and plotted beginning the day of transplantation (4 weeks of age). All of the NP-C mice died by 9 weeks of age (n=8 in each group). MHP36 transplanted NP-C group showed an improved performance in Rota Rod test. *, $P<0.0001$.

mal's balance and coordination. Mice were tested three trials, and Rota-Rod testing was conducted once a week until mice would die of natural causes. The result of the test is summarized in Fig. 7. Although the motor function was deteriorated, a more gradual decline and improved performance was evident in MHP36 transplanted NP-C group. In contrast, U373 transplanted NP-C group presented a significantly reduced motor function. The results suggest that MHP36 transplantation not only altered cerebellar environment, but also improved the cerebellar motor function.

DISCUSSION

Among the many kinds of stem cells, NSCs have emerged some important considerations. Despite their origins from one of the most quiescent tissue in the body, NSCs can undergo effective long-term culturing, proliferation and expansion while retaining stable functional characteristics. These cells have been widely used *in vivo* without causing tumor formation or other side effects to date [7]. Immortalized cell lines of embryonic brain cells has been obtained by reversible oncogene transfection allowing the replicative signal to be turn off. As an example, v-*myc*-immortalized cerebellar mouse C17.2-CD cells have been shown to efficiently ameliorate clinicopathological feature of neurodegenerative disease model mice [13, 27]. MHP36, conditionally immortalized neural stem cells, is also has the capacity to repair and restore cognitive functions in stroke which is one of the diseases characterized neuronal loss and behavioral disorder [18, 29]. MHP36 is derived from a transgenic mouse strain carrying the temperature-sensitive (ts) allele of the SV40 large T-oncogene [9]. At the permissive temperature (33°C), the oncoprotein is active and the cells proliferate continuously. But at the non-permissive temperature (37°C), the oncoprotein is inactive, the cell division is stopped and differentiation is started. Thus, MHP36 is accurately controlled its differentiation, and these characteristics of MHP36 are considered to induce a successful engraftment.

Moreover, the effects of transplanted MHP36 only have been recognized if these cells survive in a host environment and integrate into the brain parenchyma. For improvement of the effects of transplanted MHP36, immune rejection should be avoided. The survival of transplanted cells is dependent on the host immune system recognizing and destroying foreign cells [17]. These identifications of foreign cells are commonly mediated by major histocompatibility complex (MHC) molecule, which is involved cellular rejection. Litchfield *et al.* observed that when transplanted of astrocyte conditionally immortalized with the SV40 large T gene, they did not express MHC molecule and did not evoke the immunological rejection response [12]. Although transplantation of NSCs induces the host immunological response, MHC antigens on NSCs were down-regulated upon to differentiation. MHP36 may also prove less likely to evoke an immunogenic response in the host compared to other kinds of cell, i.e., fetal graft [16]. M. Modo *et al.* reported the down-regulation of MHC antigens upon differentiation following cell transplantation [17]. Furthermore, MHP36 is derived from the E14 mouse. MHC expression is reduced during embryonic development, so, MHC expression in E14 mouse is lesser than earlier embryonic stage. Moreover the decreased expression of MHC antigens and the inability of transplanted stem cells to present antigen are important determinants of the survival or rejection of stem cell transplants [16]. In our experiment, transplantation of MHP36 did not evoke immune response. This means that not only MHP36 is not recognized as a foreign material in the host environment but also additional immunosuppression treatment for MHP36 transplantation is not necessary.

In the present study, we evaluated the suitability of MHP36 immortalized multipotent neural stem cell in the cerebellum of NP-C mice. The result demonstrated that the transplanted MHP36 had a protective effect on Purkinje neurons in NP-C mice. In NP-C mice transplanted with MHP36, the number of Purkinje neurons was increased compared with sham-transplanted NP-C group, and these surviving Purkinje neurons form normal functional synapses. However, normal synaptic function of the surviving Purkinje neuron did not support sufficiently improvement of cerebellar environment and did not validate the precise mechanism by stem cell transplantation. In real-time PCR analysis, we demonstrated the clues that prove the underlying mechanism improve the cerebellar environment. That is up-regulation of genes encoding GluR2, 3 and $GABA_A$ receptor $\beta 2$ which are involved in synaptic transmission. Purkinje neurons express mainly GluR as a glutamate receptor. Especially GluR2, 3 are abundant in Purkinje neuron [8]. Moreover GluR2, 3 are main mediators involving synaptic transmission in not only interaction of Basket cells and Purkinje neurons but also that of climbing fibers and Purkinje neurons [24]. $GABA_A$ receptor $\beta 2$ is also one of the principal synaptic transmitter receptor components.

When we examined that the changes in lipid storage deposits in the areas around the cerebellum, one of the pathological characteristics in NP-C disease, sphingomyelin and cholesterol accumulation were also ameliorated in MHP36 transplanted NP-C mouse. Furthermore, we observed that neuroglial activation was down regulated by transplantation of MHP36. Inflammatory reactions such as astrogliosis, microgliosis are typical responses in neurodegenerative diseases such as NP-C [1, 4]. Glial cells have the cytotoxic effects and glial activation has been considered to be a key process leading to neuronal degeneration in NP-C mice [10]. In this study, we demonstrated that the astrogliosis and microgliosis were reduced in MHP36 transplanted NP-C group compared with sham-transplanted NP-C group, suggesting these means that transplanted MHP36 regulated the inflammatory reaction.

To further investigate this point, we used anti-MHC antibodies to assess the degree of immunological rejection in the cerebellum. As a result, the immunoreactivity of MHC class II was similar between MHP36 transplanted and sham transplanted NP-C group. These support that transplanted MHP36 did not induce significant immunological rejection in host environment. Nonetheless, the immune response was significantly increased at 4 weeks in MHP36 transplanted NP-C group as U373 transplanted NP-C group. These are related to the decrease of Purkinje neuron number. It seems to be that MHP36 did not survive for a long time and eventually die, so, they did not rescue host Purkinje neurons destined to die on the basis of certain genetic deletion. Otherwise, the susceptibility to the inflammatory cytokine released by host was increased as NSCs differentiate. NSCs become low expressing MHC but high susceptible to the inflammatory cytokine like interferon as differentiate [18]. Although the loss of Purkinje neurons were still presented at 4 weeks post transplantation, the other pathological symptoms that lipid storage and inflammatory response were down-regulated and motor function were improved.

In this study, we have demonstrated MHP36 transplantation could be an effective therapy to protect the Purkinje neurons without significant immunological response. Further studies will be aimed at elucidating the molecular mechanism(s) of protective effects and inducing long term survival of transplanted stem cell for investigation of more specific therapeutic approaches in various neurodegenerative diseases.

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