(Research Article)

X-linked severe combined immunodeficiency (X-SCID) rats for xeno-transplantation and behavioral evaluation

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ABSTRACT

Background

To evaluate the *in vivo* function of human dopaminergic (DA) neurons, Parkinson's disease (PD) model rats made by the hemi-lateral injection of 6-hydroxydopamine (6-OHDA) are widely used as host animals. In the case of such xeno-transplantation, however, immunosuppression is needed for good survival of the grafted cells.

New Methods

In order to determine whether human mature neurons can survive in X-linked severe combined immunodeficiency (X-SCID) rats without immunosuppression, we grafted human embryonic stem cell (ESC)-derived DA neurons into the striatum of X-SCID rats. We next treated the X-SCID rats with 6-OHDA and grafted mouse fetal DA neurons **or human induced pluripotent stem cell (iPSC)-derived DA neurons** to examine whether these rats can be used as PD model rats.

Results

X-SCID rats did not elicit immune responses against human ESC-derived DA neurons and consequently resulted in good survival of the cells without immunosuppression. Furthermore, 6-OHDA-lesioned X-SCID rats exhibited rotational behavior, which was recovered by grafting mouse fetal DA neurons or human iPSC-derived DA neurons.

Comparison with Existing Methods

Immunosuppression by drugs such as Cyclosporine A requires daily injection, which is stressful for rats and moreover may cause renal or hepatic failure. Furthermore, blood levels of the drug may not be stable, which weakens the reliability of the data.

Conclusions

Our results provide a more accessible and reliable method to evaluate the *in vivo* function of human DA neurons, potentially offering a pre-clinical study for the application of pluripotent stem cells.

Keywords

severe combined immunodeficiency rats; xeno-transplantation; Parkinson's disease;
dopaminergic neurons; 6-hydroxydopamine; immune response

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by a loss of midbrain dopaminergic (DA) neurons (Marsden et al., 1994) and is a major target disease for cell replacement therapy using fetal cells or stem cells (Bjorklund and Kordower et al., 2013). To evaluate the *in vivo* function of DA neurons derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), PD model rats made by hemi-lateral injection of 6-hydroxydopamine (6-OHDA) has been widely used (Ungerstedt U et al., 1970). This model animal shows abnormal rotations in response to methamphetamine because of an imbalance between right and left extremities, which are improved when the grafted DA neurons function in the brain (Sundberg et al., 2013; Kriks et al., 2011; Brundin et al., 1986). However, the xeno-transplantation causes an intense immune response in rat brain (Jensen et al., 2012). Furthermore, cell injection through a needle disrupts the blood-brain barrier and evokes inflammation. Therefore, immune-suppression of the model rats is required.

Cyclosporine A (CsA) is a widely used immunosuppressive drug that suppresses the activation and proliferation of lymphocytes by blocking interleukin (IL)-2 synthesis through inhibition of the calcineurin pathway (Clipstone et al., 1992). Consequently, it

reduces the acquired immune response and promotes survival of grafted neural progenitor cells (NPCs) in the brain (Hovakimyan et al., 2012). CsA, however, inhibits the maturation of NPCs (Guo et al., 2007) and its long-term administration can cause nephrotoxicity, liver dysfunction and a compromised host (Burdmann et al., 2003; Grub et al., 2000). Therefore, immune-deficient rats are preferred recipients for such xeno-transplantation.

Recently, X-linked severe combined immunodeficiency (X-SCID) rats were established by gene knockout of IL-2 receptor gamma (Il2rg) using Zinc-finger nucleases (ZFNs) (Mashimo et al. 2010). Il2rg is essential for signaling by several interleukins such as IL-2, IL-4, IL-7 and IL-15. In addition, the gene is involved in X-SCID, one of the most common forms of human SCID (Noguchi et al., 1993; Leonard et al., 2001). The thymus of X-SCID rats is extremely hypoplastic, and the immunodeficient phenotypes have been characterized as a nearly complete lack of T-cells, B-cells and NK-cells. When human ovarian cancer cells were subcutaneously injected into X-SCID rats, these cells formed tumors within 14 days, while control F344 rats showed no tumor growth.

These characteristics may make X-SCID rats a preferred recipient of human stem cell-derived DA neurons. It is not known, however, whether human mature neurons can survive in these rats without immunosuppression. It is also unknown whether

6-OHDA-**lesioned** X-SCID rats can be used as a PD model for evaluating the DA function of the grafted cells. In order to answer these questions, we induced DA neurons from human ESCs and grafted them into the striatum of X-SCID rats. We next treated the X-SCID rats with 6-OHDA and grafted mouse fetal DA neurons **or human iPSC-derived** DA neurons into their striatum to examine whether these rats can be used as PD model rats.

2. Materials and methods

2.1. Human **ESC/iPSC culture.**

Undifferentiated human ESCs (KhES-1; Suemori et al., 2006) were maintained on a SNL feeder in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1% MEM non-essential amino acids solution (NEAA; Gibco), 1% sodium pyruvate solution (Pyruvate; Sigma), 25% knockout serum replacement (KSR; Gibco) and 0.1 mM 2-mercaptoethanol (2-ME; Wako).

DA neurons were differentiated from human ESCs by using a serum-free floating culture of embryoid bodies-like quick-aggregation (SFEBq) culture, which is a modification of a previously described method (Watanabe et al., 2005). For differentiation, human ESCs were dissociated into single cells using Accumax (Innovative Cell

Technologies) and seeded into 96-well low cell-adhesion plates (Nunc) at a density of 9000 cells/well to induce sphere formation on day 0 in differentiation medium containing Glasgow minimum essential medium (GMEM; Wako) supplemented with 1% NEAA, pyruvate, L-glutamine (L-Glu; Sigma), 8% KSR and 0.1 mM 2-ME. We added 500 nM A83-01 (Wako) and 100 nM LDN193189 (Stemgent) to induce neuronal differentiation. The next day, we also added 100 ng/ml FGF8b (Peprotech) and 2 μ M purmorphamine (Wako) until day 7, and 3 μ M CHIR99021 (Wako) from day 3 to day 12 to induce floor plate cells (Kriks et al., 2011). On day 7, A83-01, FGF8b and purmorphamine were removed from the differentiation medium. On day 12, we changed GMEM for neurobasal medium (Gibco) supplemented with 2 mM L-Glu, 1 $\times$ B-27 supplement (Gibco), 20 ng/ml BDNF (R&D), 10 ng/ml GDNF (R&D), 200 nM ascorbic acid (Nacalai tesque) and 400 mM dbcAMP (Sigma). We added 30 μ M of Y-27632 (Wako) in the first medium to avoid apoptosis and changed the medium every three days.

Human iPSC lines, 1039A1 (N=2) and 1231A3 (N=2), were generated from peripheral blood mononuclear cells (PMNCs) by transfection of episomal plasmid vectors (OCT3/4, SOX2, KLF4, L-MYC, LIN28, shRNA for TP53 and EBNA1) and then plated on rLN511E8-coated cell culture plates, as described previously (Okita et al., 2013; Nakagawa

et al., 2014).

Undifferentiated human iPSCs were maintained by the same method as human ESCs. DA neurons were differentiated from human iPSCs by using LM511-E8-coated dishes, as described previously (Doi et al., 2014). We added A83-01, LDN193189, FGF8b, purmorphamine and CHIR99021 in the same manner as used for human ESCs (Figure 1A). On day 12, we performed cell sorting using anti-CORIN antibody (1:200, a kind gift from Dr. Ono Y, KAN Research Institute, Japan) and then incubated the CORIN⁺ cells in neurobasal medium supplemented with 2 mM L-Glu, 1×B-27 supplement, 20 ng/ml BDNF, 10 ng/ml GDNF, 200 nM ascorbic acid, 400 mM dbcAMP and 1% penicillin/Streptomycin (P/S; Gibco) for 16 days to be used for transplantation. We added 30 μM of Y-27632 in the first medium after sorting and changed the medium every three days.

2.2. Quantitative RT-PCR.

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN), and cDNA was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR reactions were performed with SYBR Premix Ex Taq (Takara) and the Thermal Cycler Dice Real Time System (Takara). The data were assessed using a delta-delta Ct method and

normalized by GAPDH levels. The primer sequences are shown in Table 1.

2.3. Immunofluorescence study.

For *in vitro* experiments, the spheres were fixed with 4% paraformaldehyde for 15 min then cut at 20- μ m thickness using a cryostat (Leica). Following *in vivo* experiments, the brains were fixed with 4% paraformaldehyde overnight and then in PBS containing 30% sucrose for 2 days. Brains were sliced coronally at 30- μ m thickness using a cryostat. Frozen sections were permeabilized with 2% Triton X-100 in PBS for 30 min and blocked with PBS containing 4% Block Ace (Megmilk Snow Brand Co., Ltd) and 0.1% Triton X-100 (PBST-BA) for 10 min. The primary antibodies used overnight were mouse anti-HNA (1:1000, Millipore), anti-NESTIN (1:500, Millipore), anti-TUJ1 (1:2000, Covance), anti-MHC-II (1:200, Abcam), anti-CD4 (1:500, Millipore), anti-CD8 (1:500, LifeSpan), anti-CD45 (1:500, BD), rabbit anti-CD3 (1:500, Abcam), anti-IBA1 (1:500, Millipore), anti-MPO (1:50, Millipore), anti-F4/80 (1:100, Abcam), anti-TH (1:1000, Millipore), goat anti-FOXA2 (1:1000, Santa cruz biotechnology), hamster anti-LMX1A and rat anti-NURR1 (1:200 and 1:1000, a kind gift from Dr. Ono Y, KAN Research Institute, Japan). After rinsing with 0.1% PBST, the samples were stained with appropriate secondary

antibodies conjugated with Alexa 488, Alexa 594, Alexa 647 (1:400, Invitrogen) or Dylight 594 (1:200, Thermo Scientific) for 30 min at room temperature. Following additional rinsing with 0.1% PBST, the samples were stained with 200 ng/ml of 4', 6'-diamidino-2-phenylindole (DAPI).

The procedure of 3,3'-diaminobenzidine (DAB) staining was carried out as previously described (Morizane et al., 2013). We performed antigen retrieval in 10 mM citrate buffer at 70 °C for 40 min and then incubated with 0.1% PBST containing 30% H₂O₂ for 15 min before primary antibody reaction. The primary antibodies used overnight were mouse anti-CD45 (1:1000, BD), anti-CD45RA (1:1000, Abcam), anti-MHC class II (1:200, Abcam), rabbit anti-CD3 (1:1000, Abcam) and anti-TH (1:1000, Millipore). After rinsing with 0.1% PBST, the samples were stained with biotinylated secondary antibody reactions for 2 hours. Finally, we carried out chromogenic reactions using the ABC Elite Kit (Vector Labs). In the case of IgG deposition, we started with a secondary antibody reaction without antigen retrieval.

Images were visualized using a fluorescence microscope (BZ-9000; Keyence) and a confocal laser microscope (Fluoview FV1000D; Olympus). The number of immunoreactive cells (cell diameter = 10-15 µm) was counted in every eighth (1 week

post-transplantation) or sixth (12 weeks post-transplantation) section throughout the graft and corrected using the Abercrombie formula (Abercrombie et al., 1946).

2.4. Flow Cytometry.

Flow cytometric analysis was carried out by FACS AriaII (BD Biosciences), and the data were analyzed by FACSDiva software (BD Biosciences) and Flowjo flow cytometry analysis software (Tree Star, Inc). The differentiated cells on day 12 were dissociated into a single-cell suspension and resuspended in PBS with 2% fetal bovine serum (FBS; Cell Culture Bioscience), 20 mM D-glucose (Nacalai tesque) and 1% P/S. Subsequently, the cells were incubated with anti-mouse PSA-NCAM antibody (1:200, Millipore). After primary antibody reaction, the cells were incubated with Alexa 488 donkey anti-mouse IgG (1:400, Invitrogen). Dead cells were distinguished by 7-amino-actinomycin D (7AAD; BD).

2.5. Animals and Transplantation.

All animal experiments were performed according to the Guidelines for Animal Experiment of Kyoto University and the Guide for the Care and Use of Laboratory Animals of the

Institute of Laboratory Animal Resources (ILAR; Washington, DC, USA). Adult female Fischer 344 (F344), F344/NJcl-*rnu/rnu* (CLEA Japan, Inc., Japan), F344-*Il2rg*^{em2Kyo} X-SCID rats (NBRP-Rat No.0586, Kyoto University, Japan; <http://www.anim.med.kyoto-u.ac.jp>) and C57BL/6CrSlc pregnant mice at gestation day 11 (Shimizu Laboratory Supplies, Co., Ltd., Japan) were used. Athymic nude (Nude) and X-SCID rats were also used for the 6-OHDA-lesioned model. We injected 6-OHDA into the medial forebrain bundle (MFB) in the right side of the rat brain. The coordinates were calculated with reference to the bregma: anterior (A), -5.3; lateral (L), -1.2; ventral (V), -7.0; and tooth bar (TB), +1.5. A total of 16 µg of 6-OHDA was injected per rat in 2.5 µl of saline with 0.02% ascorbic acid. Cell transplantation was performed with stereotactic injection of 4×10^5 cells in 2 µl (2×10^5 cells/µl, 0.16 µl/s) through a 22-gauge needle into the right and left side of the striatum (from the bregma: A, +1.0; L, ± 3.0 ; V, -5.0 and -4.0, TB, 0). At 1, 4, 12 or 16 weeks after transplantation, the animals were euthanized with pentobarbital and perfused with paraformaldehyde. F344 rats received intraperitoneal injections of the immunosuppressant CsA (Wako) every day starting 3 days before transplantation until the day of sacrifice. We determined the average blood level of CsA as 1550 ± 354 ng/ml by taking samples from 10 rats after injections for 10 days (Supplementary Table 1).

2.6. Behavioral analysis.

The methamphetamine-induced rotation assay was carried out before surgery and 2, 4, 8 or 16 weeks after surgery. Rotational behavior in rats was recorded for 90 min after intraperitoneal injection of methamphetamine (2.5 mg/kg, Dainippon Sumitomo Pharma) and automatically assessed by video-monitored rotational bowls. The cylinder test was carried out by placing rats in a glass cylinder and counting the independent use of the right and left forelimb. The number of ipsilateral versus contralateral paw touches (out of 20 touches) was analyzed at 4 weeks after 6-OHDA injection.

2.7. Statistical analysis.

Statistical analysis was carried out using a software package (GraphPad Prism 5; GraphPad). Data from the *in vitro* and *in vivo* experiments were analyzed by a one-way ANOVA and Bonferroni's post-hoc analysis (Figures 2J, 2K, 3I, 3J, 4E, 4L, 5E and 5O-Q), two-way ANOVA and Bonferroni's post-hoc analysis (Figure 6K and Supplementary figure 2A) or a paired t-test (Figure 6G and H). The data were considered statistically significant for $P < 0.05$ and are shown as the mean $\pm$ standard error (s.e.m.). All data were acquired from at least three independent experiments

3. Results

3.1. Human ESCs differentiate into DA neurons by SFEBq method

As donor cells for transplantation, we induced midbrain DA neurons from human ESCs (KhES-1; Suemori et al., 2006) based on a previous protocol (Doi et al., 2014) with modification for the initial neural induction by using the SFEBq method (Figure 1A). Quantitative PCR analysis revealed that the expression of pluripotent markers (*OCT3/4* and *NANOG*) gradually decreased from day 0. With regard to differentiation to three germ layers, the expression of ectodermal markers (*SOX1* and *MAP2ab*) gradually increased until day 35, while that of mesodermal markers (*GSC* and *BRACHYURY*) and an endodermal marker (*SOX17*) showed only slight changes. On the other hand, DA neuron markers (*NURR1* and *TH*) and ventral markers (*FOXA2* and *CORIN*) increased with neuronal differentiation (Figure 1B).

Flow cytometry on day 12 revealed that 98.4% of the cells expressed PSA-NCAM, a cell adhesion molecule on neural cells (Figure 1C). An immunofluorescence study showed that most of the cells expressed a NPC marker, NESTIN (Figure 1D). On day 24, about half of the cells expressed a midbrain marker, LMX1A, and a floor plate marker, FOXA2 (Figure 1E). On day 35, all the cells expressed a post-mitotic neuronal marker,

TUJ1, and some of them co-expressed a DA neuron marker, TH (Figure 1F). Furthermore, most TH⁺ cells also expressed midbrain DA neuron markers, FOXA2 and NURR1 (Figure 1G, H). These results suggested that the human ESCs committed into neural lineage including DA neurons on day 35.

3.2. Immune responses were reduced in the brain of immunodeficient rats

In order to investigate the immune response by the host brain, we injected human ESC-derived neural cells (day 35) into the striatum of wild type rats with (CsA) or without CsA (Wild) and immunodeficient rats, namely Nude and X-SCID rats, without CsA. Human ESC/iPSCs express only a low level of major histocompatibility complex class-I (MHC-I) even after they differentiate into neural cells (Morizane et al., 2013). Consistently, we could not find MHC-I⁺ cells in any of the grafts in all conditions by immunofluorescence. Next, we examined the expression of MHC-II, because once the host microglia cells are activated, they develop antigen-presenting activity and express MHC-II. Immunofluorescence at 1 and 12 weeks post-transplantation showed that MHC-II⁺ cells were most frequently found in Wild rats at 1 week (Figure 2A-D). The expression of MHC-II⁺ generally overlapped with that of IBA1 (Figure 2I), indicating that MHC-II was

expressed by host-derived microglia. The total numbers of MHC-II⁺ cells in the grafts at 1 week were 39,235 $\pm$ 25,801 (Wild), 15,593 $\pm$ 14293 (CsA), 4,694 $\pm$ 2,795 (Nude) and 2,647 $\pm$ 2,155 (X-SCID). There were statistically significant differences between the Wild rats and other rats (Figure 2J). In contrast, we found fewer MHC-II⁺ cells at 12 weeks (Figure 2E-H): none (Wild), 1,603 $\pm$ 882 (CsA), 1,162 $\pm$ 1,175 (Nude) and 376 $\pm$ 362 (X-SCID). There were no statistically significant differences in MHC-II⁺ cells among the rat types (Figure 2K).

Because the expression of MHC-II might induce the recruitment of circulating immune cells, including T cells and B cells, we examined the accumulation of CD45⁺ cells (a marker for pan-leukocytes) in the grafts at 1 and 12 weeks. Immunofluorescence revealed that the accumulated CD45⁺ cells were most frequently found in Wild rats at 1 week (Figure 3A-D). The total numbers of CD45⁺ cells in the grafts at 1 week were 62,542 $\pm$ 25,575 (Wild), 17,215 $\pm$ 15,799 (CsA), 619 $\pm$ 472 (Nude) and 1,172 $\pm$ 1,036 (X-SCID). There were statistically significant differences between the Wild rats and other rats (Figure 3I). In contrast and like above, the numbers of CD45⁺ cells were fewer at 12 weeks (Figure 3E-H): none (Wild), 4,716 $\pm$ 6,214 (CsA), 1,694 $\pm$ 1,492 (Nude) and 336 $\pm$ 336 (X-SCID). There were no statistically significant differences among rat types

(Figure 3J). Further specification revealed that a small number of CD45⁺ cells co-expressed a macrophage marker, F4/80 (<5%), or a neutrophil marker, MPO (<5%), at 1 week post-transplantation (Figure 3K and L).

3.3. Very few T- and B-cells accumulated in immunodeficient rat brain

In order to investigate the immune response in more detail, we performed immunofluorescence at 1 week post-transplantation to detect T- and B-cells using antibodies for CD3 and CD45RA, respectively. Although a large number of CD3⁺ T-cells accumulated in the grafts of Wild rats (Figure 4A), the numbers were much fewer in the CsA, Nude and X-SCID rats (19,038 $\pm$ 14,096 vs. 1235 $\pm$ 1899, 653 $\pm$ 909 cells and 1296 $\pm$ 1385, respectively; Figure 4B-E). Furthermore, 67% of CD3⁺ T-cells expressed a killer T-cell marker, CD8⁺ (Figure 4F), while 17% of CD3⁺ T-cells expressed a helper T-cell marker, CD4⁺ (Figure 4G), in the graft at 1 week post-transplantation. On the other hand, a large number of CD45RA⁺ B-cells accumulated in Wild rats (Figure 4H), but the numbers were much smaller in CsA, Nude, and X-SCID rats (22,289 $\pm$ 16,854 vs. 1,412 $\pm$ 3,359, 87 $\pm$ 115 cells and 287 $\pm$ 547 cells, respectively; Figure 4I-L).

We also performed immunostaining for immunoglobulin (Ig) to detect the

accumulation of antibodies by the host. Immunostaining against anti-rat IgG at 1 week post-transplantation revealed an intense accumulation of IgG in the grafts of Wild rats, but very little in Nude or X-SCID rats (Supplementary Figure 1). Taken together, these results suggested that immunodeficient rats exhibited only a minimum immune response in the brain even against xeno-grafts.

3.4. Human ESC-derived DA neurons survived in immunodeficient rat brain

In order to evaluate the survival of human ESC-derived DA neurons, we performed immunofluorescence on the same brain slices used for the immune response analysis. Immunostaining for HNA at 12 weeks demonstrated that all grafted cells were rejected in Wild rats (Figure 5A). In contrast, the grafted cells survived well in 4 of the 10 CsA rats, all 10 nude rats and 7 of the 8 X-SCID rats (Figure 5B-D). The total numbers of HNA⁺ cells were $51,033 \pm 25,630$ (CsA), $33,837 \pm 23,771$ (Nude) and $21,019 \pm 15,583$ (X-SCID). There were no statistically significant differences among these numbers (Figure 5E).

Among the HNA⁺ cells, 2-3% expressed a DA neuron marker, TH, in CsA and immunodeficient rats (Figure 5F-H, O). To further investigate the ventral and midbrain

identity of the survived cells, we performed double-labeled immunostaining with TH and FOXA2 (a ventral midbrain marker; Figure 5I-K) or NURR1 (a midbrain marker; Figure 5L-N). Most of the TH⁺ cells simultaneously expressed these markers, and there were no statistically significant differences in the number of double positive cells (Figure 5P, Q). These results suggested that an equivalent number of human ESC-derived midbrain DA neurons can survive in the brains of X-SCID rats as well as in CsA or Nude rats, indicating that X-SCID rats can be used as host animals for xeno-transplantation.

3.5. X-SCID rats can be used as Parkinson's disease models

A next question was whether X-SCID rats can be used as PD models. Unilateral injection of 6-OHDA into the MFB is an established method to make a rat PD model. This model rat exhibits methamphetamine-induced rotations caused by an imbalance of dopamine concentration between the right and left striatum (Ungerstedt et al., 1970). Therefore, we injected 6-OHDA into the MFB of X-SCID rats and performed immunostaining for TH at four weeks. TH⁺ DA neurons were almost entirely absent in the substantia nigra pars compacta (SNpc), and TH⁺ fibers extending into the striatum were also undetectable in the lesion side (Figure 6A-F). To evaluate motor functions, we next performed cylinder and

rotation tests. The cylinder test revealed that the X-SCID rats with 6-OHDA injection showed preferential use of the ipsilateral paws (>80%) in contrast to those without 6-OHDA (Figure 6G). The rotation test revealed that the 6-OHDA-lesioned X-SCID rats showed methamphetamine-induced ipsilateral rotations (>6 rotations/minute) (Figure 6H). The number of rotations increased between 4 and 16 weeks post-injection in the case of X-SCID rats (Figure 6I). In contrast, Nude rats showed variable rotation numbers and some (4 of 10) showed spontaneous recovery (Figure 6J), suggesting these rats may not be suitable as a PD model.

Finally, to examine the functional recovery of the PD models after the transplantation of DA neurons, we grafted ventral midbrain (VM) cells of E11.5 mice into the striatum of X-SCID PD model rats. These fetal cells are known to cause functional recovery of PD models made from Wild rats (Brundin et al., 1986). Similarly, X-SCID PD model rats showed a decrease in the number of methamphetamine-induced rotations four weeks after transplantation (Supplementary Figure 2A), and according to immunostaining, showed good survival of DA neurons that extended TH⁺ fibers into the striatum (Supplementary Figure 2B-C). Next, we grafted human iPSC-derived CORIN⁺ DA neurons (Doi et al., 2014) into the striatum of 6-OHDA-lesioned X-SCID rats. These rats also

exhibited fewer rotations at 16 weeks post-transplantation (Figure 6K), and an immunofluorescence study showed good survival of human iPSC-derived TH⁺ neurons (Figure 6L) that extended TH⁺ fibers into the host striatum (Figure 6M). Furthermore, these TH⁺ cells were co-localized with HNA and most of them expressed NURR1 (Figure 6N and O), suggesting that the survived DA neurons were human-derived and contributed to the functional recovery.

4. Discussion

In this study, we demonstrated that X-SCID rats exhibited minimal immune response against the xeno-transplantation of human ESC-derived NPCs into the striatum. This absence of immune response associated with good survival of the human ESC-derived DA neurons even without immunosuppression. Furthermore, 6-OHDA-lesioned X-SCID rats showed behavioral recovery by transplantation of ventral midbrain cells from mouse fetuses or of human iPSC-derived DA neurons. This is the first report suggesting that X-SCID rats can be used to assess *in vivo* function of human ESC/iPSC-derived cells.

Usually, SD rats are used as a recipient of cell transplantation, especially in the case of the 6-OHDA-lesioned PD model (Kriks et al., 2011; Kirkeby et al., 2012; Doi et al.,

2014). This model is a reliable method to evaluate the DA function of grafted cells. For xeno-grafts, however, an immunosuppressive drug such as CsA is necessary for good survival of the cells. Yet CsA can cause nephrotoxicity and liver dysfunction, which may lead to weakness or death of the animal (Burdmann et al., 2003; Grub et al., 2000). Furthermore, blood levels of the drug may not be stable, which weakens the reliability of the data. In this study, we found that the blood levels of CsA ranged from 1,100 to 2,100 ng/ml after 10 days of administration (Supplementary Table 1) and that only 4 of 10 CsA rats showed good survival of the graft. Another report found that neural precursor cells derived from a human fetus survived in the brains of 6 of 8 CsA-treated rats, but that all rats had to be sacrificed at 12 weeks after transplantation because of poor healthy (Kelly et al., 2009). Thus, there is a legitimate concern about the quality and stability of immunosuppression by CsA.

Activated T-cells contribute to the immunological rejection of intracerebral neural xenografts (Pakzaban and Isacson., 1994; Wood et al., 1996). To become activated, a naïve T-cell must first receive the target's information via antigen presenting cells. Activated T-cells or T-cell related factors, conversely, play a critical role in the activation of macrophages and the antigen presentation (Van Luyn et al., 1998). CsA, however,

suppresses the activation and proliferation of lymphocytes by inhibiting the calcineurin pathway to block IL-2 synthesis (Clipstone et al., 1992). Nude rats lack mature T-cells (Festing et al., 1978), and X-SCID rats have very few T-, B- or NK-cells (Mashimo et al., 2010). These findings may support our results that not only T-cells, but also B-cells and MHC-II⁺ cells less frequently accumulate in the grafts of immunosuppressed rats, including CsA and Nude rats. After the transplantation of human ESC-derived DA neurons, we found a negligible number of T- or B-cells in the grafts of Nude and X-SCID rats at 1 week post-transplantation, and these rats showed good survival of the grafts for 12 weeks. These results suggest that Nude and X-SCID rats can be used as a good host animal of neural xeno-transplantation even without immunosuppression.

However, there are only few papers that use 6-OHDA-lesioned PD models made from Nude rats (Kuan et al., 2006), and the behavioral assessment of these rats has not been well established (Nelander et al., 2013). In this study, we could reproducibly make X-SCID PD models in terms of histology and behavior. Furthermore, the motor dysfunction of X-SCID PD models was ameliorated by the transplantation of ventral midbrain cells of mouse embryos or human iPSC-derived DA neurons. These results suggest that X-SCID rats can be used for assessing *in vivo* function of human DA neurons. In the case of Nude

rats, however, 4 of 10 rats showed spontaneous recovery of rotational behavior, which makes them seem less suitable as a 6-OHDA lesion model.

5. Conclusion

In conclusion, we show that X-SCID rats exhibited minimal immune response against xeno-transplantation and have promise as a PD model in preclinical studies of cell transplantation.

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Figure Legends

Figure 1. Induction of DA neurons from human ESCs by the SFEB method. (A) An overview of the culture protocol. (B) Gene expression analyses for pluripotent (*OCT3/4* and *NANOG*), mesodermal (*GSC* and *BRACHYURY*), endodermal (*SOX17*), ectodermal (*SOX1* and *MAP2ab*), DA neuron (*NURR1* and *TH*) and ventral (*FOXA2* and *CORIN*) markers by quantitative RT-PCR. The expression level of the undifferentiated hESCs (day 0) was set to 1, and the relative expression levels are presented as the mean $\pm$ s.e.m. (n=3 independent experiments). (C) Flow cytometry analysis for the expression of PSA-NCAM on day 12 (red). Data from a cell sample stained with anti-PSA-NCAM antibody and secondary antibody. Blue indicates data from a cell sample stained only by a secondary antibody (negative control). (D-H) Immunofluorescence images of hESC-derived cells on day 12 (D), 24 (E) and 35 (F-H). Scale bar, 50 μ m.

Figure 2. MHC-II expression by host-derived microglia. Representative images of DAB staining for the expression of MHC-II at 1 week (A-D) and 12 weeks (E-H) post-transplantation. Scale bar, 200 μ m. (I) Representative immunohistochemical image of the grafts for MHC-II and IBA1 in wild type rats without CsA (Wild) at 1 week

post-transplantation. Scale bar, 20 μm . (J) Comparison of total numbers of MHC-II⁺ cells in the graft at 1 week revealed a significant main effect of each rat type ($F_{(3,40)}=13.99$; $p=2.20\text{E-}06$). N=10, 12, 10 and 12 for Wild, CsA, Nude and X-SCID rats, respectively. Values are mean $\pm$ s.e.m. ** $p < 0.01$ and *** $p < 0.001$ by a one-way ANOVA with Bonferroni's all pairs-comparison tests. (K) Comparison of total numbers of MHC-II⁺ cells in the graft at 12 weeks revealed no statistically significant differences between each rat type ($F_{(2,18)}=2.57$; $p=0.10$). N=0, 4, 10 and 7 for Wild, CsA, Nude and X-SCID rats, respectively.

Figure 3. CD45 expression by host-derived leukocytes. Representative images of DAB staining for the expression of CD45 at 1 week (A-D) and 12 weeks (E-H) post-transplantation. Scale bar, 200 μm . (I) Comparison of total numbers of CD45⁺ cells in the graft at 1 week revealed a significant main effect of each rat type ($F_{(3,40)}=40.38$; $p=3.54\text{E-}12$). N=10, 12, 10 and 12 for wild type rat without CsA (Wild), wild type rat with CsA (CsA), Nude rat and X-SCID rat, respectively. Values are mean $\pm$ s.e.m. *** $p < 0.001$ by a one-way ANOVA with Bonferroni's all pairs-comparison tests. (J) Comparison of total numbers of CD45⁺ cells in the graft at 12 weeks revealed no statistically significant

differences between each rat type ($F_{(2,18)}=2.81$; $p=0.09$). N=0, 4, 10 and 7 for Wild, CsA, Nude and X-SCID rats, respectively. (K and L) Double-labeled immunohistochemical images of the graft for F4/80 and CD45 or MPO and CD45 of Wild rats at 1 week post-transplantation. Scale bar, 50 μ m.

Figure 4. CD3 and CD45RA expression by host-derived lymphocytes. Representative images of DAB staining for the expression of CD3 at 1 week post-transplantation (A-D). (E) Comparison of total numbers of CD3⁺ T-cells in the graft at 1 week revealed a significant main effect of each rat type ($F_{(3,40)}=17.90$; $p=1.60E-07$). N=10, 12, 10 and 12 for wild type rat without CsA (Wild), wild type rat with CsA (CsA), Nude rat and X-SCID rat, respectively. (F and G) Double-labeled immunohistochemical images of the graft for CD8 and CD3 or CD4 and CD3 of Wild rats at 1 week post-transplantation. Scale bar, 50 μ m. (H-K) Representative images of DAB staining for the expression of CD45RA at 1 week post-transplantation. (L) Comparison of total numbers of CD45RA⁺ cells in the graft at 1 week revealed a significant main effect of each rat type ($F_{(3,40)}=18.07$; $p=1.43E-07$). N=10, 12, 10 and 12 for Wild, CsA, Nude and X-SCID rats, respectively. Values are mean $\pm$ s.e.m.

*** $p < 0.001$ by a one-way ANOVA with Bonferroni's all pairs-comparison tests.

Figure 5. Survival of grafted hESC-derived DA neurons. (A-D) Immunohistochemical images of the grafts for HNA at 12 weeks post-transplantation. Scale bar, 200 μ m. (E) Comparison of total numbers of HNA⁺ cells in the graft at 12 weeks revealed no statistically significant differences between each rat type ($F_{(2,19)}=2.65$; $p=0.10$). N=0, 4, 10 and 8 for wild type rat without CsA (Wild), wild type rat with CsA (CsA), Nude rat and X-SCID rat, respectively. Values are mean $\pm$ s.e.m. (F-N) Double-labeled immunohistochemical images of the grafts for HNA, FOXA2, NURR1 and TH at 12 weeks post-transplantation. Scale bar, 50 μ m. (O-Q) Comparison of percentages of TH/HNA and total numbers of TH⁺FOXA2⁺ and TH⁺NURR1⁺ cells in the graft at 12 weeks revealed no statistically significant differences between each rat type ($F_{(2,18)}=0.27$; $p=0.77$), ($F_{(2,18)}=0.79$; $p=0.47$) and ($F_{(2,18)}=0.45$; $p=0.65$). N=4, 10 and 7 for CsA, Nude and X-SCID rats, respectively. Values are mean $\pm$ s.e.m.

Figure 6. Histological and behavioral analysis of 6-OHDA-lesioned X-SCID rats. DAB staining of the midbrain (A) and striatum (F) of a 6-OHDA-lesioned X-SCID rat stained for

TH. Scale bars, 1 mm. (B-E) Magnified images of the frames indicated in (A). The images show host DA neurons of the intact side (B, C) and lesioned side (D, E). Scale bar, 50 μ m.

(G) Results of the cylinder test measuring ipsi- versus contra-lateral paw touches by X-SCID rats with or without 6-OHDA injection ($p=0.0001$). Values are mean $\pm$ s.e.m. *** $p < 0.001$ by the unpaired t test. N=8 for unlesioned and 6 for lesioned X-SCID rats. (H) Methamphetamine-induced rotations by X-SCID rats with or without 6-OHDA injection ($p=0.0008$). Values are mean $\pm$ s.e.m. *** $p < 0.001$ by the unpaired t test. N=5 for unlesioned and 6 for lesioned X-SCID rats. (I, J) Time course analysis of the methamphetamine-induced rotation at 4 and 16 weeks after 6-OHDA injection. N=5 for X-SCID rats and 10 for Nude rats. (K) Methamphetamine-induced rotations of 6-OHDA-lesioned X-SCID rats after transplantation of human iPSC-derived CORIN+ DA neurons. Values are mean $\pm$ s.e.m. *** $p < 0.001$ by a two-way ANOVA with Bonferroni's post-test. N=4 for culture medium and 4 for human iPSC-derived cells. (L) DAB staining of a representative graft derived from human iPSCs stained for TH. Scale bar, 1 mm. (M) Magnified images of the frame indicated in (L). Scale bar, 50 μ m. (N and O) Double-labeled immunofluorescent images of the grafts for HNA, NURR1 and TH at 16 weeks post-transplantation. Scale bar, 50 μ m.

Table.1 Summary of primers for quantitative RT-PCR

Gene name	Forward primer	Reverse primer
<i>Gapdh</i>	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
<i>Nanog</i>	GGCTCTGTTTTGCTATATCCCCTAA	CATTACGATGCAGCAAATACGAGA
<i>Oct3/4</i>	AGACCATCTGCCGCTTTGAG	GCAAGGGCCGCAGCTT
<i>Sox1</i>	GCGGAGCTCGTCGCATT	GCGGTAACAACACTACAAAAAACTTGTA
<i>Map2</i>	GGATCAACGGAGAGCTGAC	TCAGGACTGCTACAGCCTCA
<i>Gsc</i>	GAGGAGAAAGTGGAGGTCTGGTT	CTCTGATGAGGACCGCTTCTG
<i>Brachyury</i>	ATGGAGGAACCCGGAGACA	TGAGGATTTGCAGGTGGACA
<i>Th</i>	GCAGTTCTCGCAGGACATTG	CGGCACCATAGGCCTTCA
<i>Nurr1</i>	CGAAACCGAAGAGCCACAGGA	GGTCATAGCCGGGTGGAGTCG
<i>Sox17</i>	CGCTTTCATGGTGTGGGCTAAGGACG	TAGTTGGGGTGGTCCTGCATGTGCTG
<i>Foxa2</i>	TTCAGGCCCCGGCTAACTCT	AGTCTCGACCCCCACTTGCT
<i>Corin</i>	CACAGCCAGGGTCTGGTGGAATGCAG	GAGAGCTACCACCACATGAATCAAGG

Figure 1

Figure 1.

Figure 2.

Figure 3

Figure 3.

Figure 4.

Figure 5.

Figure 6.