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Genetic modification of H2AX renders mesenchymal stem cell derived dopamine neurons more resistant to DNA damage and subsequent apoptosis

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Abstract

Background—Aberrant production of reactive oxygen species (ROS) and its impact on the integrity of genomic DNA have been considered as one of the major risk factors for the loss of dopaminergic neurons in Parkinson's disease (PD). Stem cell transplantation as a strategy to replenish new functional neurons has great potential for PD treatment. However, limited survival of stem cells post-transplantation has always been an obstacle which has been ascribed to the existence of neurotoxic environment in PD patients.

Methods—In order to improve the survival of transplanted stem cells for PD treatment, we hereby explored a new strategy based on the function of H2AX gene (H2A histone family, member X) in determination of DNA repair and cell apoptosis. We introduced a mutant form Y142F of H2AX into dopamine neurons differentiated from bone marrow-derived mesenchymal stem cells (BMSC).

Results—Expression of H2AX(Y142F) renders neurons more resistant to DNA damage and subsequent cell death induced by ultraviolet irradiation and 1-methyl-4-phenylpyridinium (MPP+) treatment.

Discussion—This is a meaningful attempt to improve the sustainability of BMSCs-derived dopamine neurons under brain neurotoxic environment. Further studies will be needed to evaluate the implication of our findings in stem cell therapy for PD and related diseases.

Keywords

Parkinson's disease; DNA damage; H2AX; mesenchymal stem cells; lentivirus infection

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Disclosure statement

All authors have no actual or potential conflicts of interest.

Introduction

Aberrant production of reactive oxygen species (ROS) and their detrimental effects on integrity of genomic DNA have been considered as major risk factors for loss of dopaminergic neurons in Parkinson's disease (PD) (1–3). Evidence suggests that *substantia nigra* in PD has significantly increased ROS (4), and also higher frequency of cellular DNA damage compared to age-matched normal controls (2). Oxidative DNA damage is a major threat to viability of neurons, and effective response to this threat is critical for maintaining neuronal homeostasis and survival (5). Neurons exposed to oxidative DNA damage have fundamental options concerning the balance between DNA repair and an apoptotic response (6). Successful execution of DNA repair protects the damaged cell, allowing for recovery and resumption of cell function. Failure to repair damaged DNA gives rise to mutations and chromosomal abnormalities, eventually leading to cell death when accumulation of such damage reaches a threshold (7). The latter might contribute to irreversible neuron loss in PD because neurotoxic environment (abnormally high ROS from dopamine and high levels of iron, etc.) (8) are present in PD brains and DNA repair capacity decreases with age (9, 10). Despite the availability of effective symptomatic drugs, there is currently no cure for PD and almost every attempt to slow neuronal loss or stop disease progression has failed (11).

Stem cell transplantation as a strategy to replenish functional neurons has great potential for treatment of PD. On the other hand, limited survival of stem cells post-transplantation has been a main obstacle that has been attributed to a neurotoxic environment in PD brains (12–18). It has been suggested that a vicious cycle is initiated in the *substantia nigra* with onset of PD, that combined with deficiency in trophic factors, leads to neuronal damage (12, 19, 20). Under such conditions, implanted neurons would be predicted to suffer the same fate as innate neurons (18). Although strategies, such as ischemic preconditioning or erythropoietin exposure, have been explored to improve survival of transplanted cells (18, 21), whether such single treatments before transplantation can render transplanted cell resistant to ongoing neurotoxic factors throughout the individual's life span is questionable. It might be preferable to choose stem cells that have enhanced viability, by genetic engineering to make them resistant to the hostile microenvironment. In this regard, histone 2A family, member X (H2AX) is of interest. H2AX is a key factor involved in cellular responses to DNA damage and it is theoretically a good candidate for genetic engineering of stem cells based on its recently discovered functions (6).

H2AX is a histone that plays a crucial role in maintaining nuclear DNA integrity. Double-strand DNA breaks are known to initiate a rapid and highly coordinated series of molecular events to trigger DNA repair (22, 23). One of the earliest of such events includes the phosphorylation of H2AX at the serine residue 139, referred to as γ -H2AX, to generate a chromosomal microenvironment that facilitates recruitment of DNA repair enzymes (24, 25). Immunolabeling of γ -H2AX has been used to demonstrate DNA damage (24, 25). Importantly, it has been shown that the decision a cell makes to undergo repair of damaged DNA (i.e. survival) or turn to apoptosis is modulated by the state of H2AX phosphorylation at tyrosine 142 (Y142) (6). When phosphorylated at Y142, γ -H2AX binding of DNA repair factors is greatly reduced. Instead, the pro-apoptotic factor c-Jun N-terminal kinase (JNK1) is recruited to drive the cell to apoptosis (6). Elimination of Y142 phosphorylation by

replacing it with phenylalanine (Y142F) abolishes the interaction between H2AX and JNK1 (6), favoring a DNA repair/survival response. Compared to cells expressing wild-type (WT) H2AX, cells expressing H2AX (Y142F) are more resistant to cell death induced by high-dose ionizing radiation (6). Therefore, we chose H2AX as a target for genetic modification of stem cells.

For a stem cell model, we used bone marrow-derived mesenchymal stem cells (BMSC) because 1) they are safe for transplantation without risk of initiating immunogenic effects or forming teratomas, which happens frequently with other types of stem cells (12, 26); 2) they have been shown to have neuroprotective effects in combating oxidative stress and providing neurotrophic factors (14, 27–32); and 3) they can be induced to have a dopamine (DA) neuronal phenotype (17, 33, 34).

We used H2AX (Y142F) to modify BMSC-derived DA neurons to make them resistant to DNA damage, which lays a foundation for improving success of stem cell transplantation therapy for PD.

Material and Methods

Cell culture

The use of human BMSC followed a protocol approved by the Institutional Review Board of Mayo Clinic. BMSC were obtained from a healthy donor using Histopaque-1077 (Sigma) following density gradient protocols. The BMSC were generated after culture in a chemically defined culture medium (MEMa[®], Invitrogen) supplemented with 16.5% fetal bovine serum (FBS) (designated as complete culture medium, CCM) as described previously (35–37).

Flow Cytometry

BMSC were detached and harvested by centrifugation at $200 \times g$ for 5 min, then fixed with fresh prepared 1% paraformaldehyde in $1 \times$ phosphate buffer-saline (PBS) followed by wash in ice-cold PBS supplemented with 10% fetal bovine serum, centrifugation and resuspension for cell counting. About 10^5 cells were prepared for each antibody test. Fluorochrome-conjugated antibodies to CD34, CD45, CD73, CD90 and CD105 were added into each cell suspension, respectively. Negative controls were performed using fluorochrome-conjugated IgG1 with the respective immunoglobulin isotype. After incubation for 20 minutes at room temperature in the dark, 400 μ l of PBS supplemented with 10% FBS was added, and samples were then analyzed in the BD AccuriC6 flow cytometer.

Osteogenic and adipogenic differentiation assay

BMSC of passage 1 were plated in 6-well plate at the density of 1×10^5 cells per well and maintained until 100% confluence, then subjected to differentiation under either bone differentiation medium (CCM supplemented with 1 nmol/L dexamethasone, 20 mmol/L β -glycerolphosphate, and 50 mg/mL L-ascorbic acid 2-phosphate) or fat differentiation medium (CCM supplemented with 0.5 mmol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine, and 50 mmol/L indomethacin) for 21 days, followed by Alizarin red

S staining and Oil-Red-O staining to respectively evaluate the effect of osteogenic and fat tissue differentiation. All differentiating media supplements and stains were obtained from Sigma.

Differentiation of human BMSC into DA neurons

The differentiation of BMSC into DA neurons was based on the method described previously (17). Briefly, BMSC were seeded in 6 or 24 well plates with or without glass coverslips at the density of 2×10^5 or 5×10^4 cells per well. After incubation overnight, CCM was replaced with Neurobasal medium with 0.5% B27 supplement (Invitrogen). The neuronal differentiation of BMSC were induced with a cocktail of 250 ng/ml SHH, 100 ng/ml FGF8, and 50 ng/ml bFGF (R&D Systems) for 12 days in incubator at 37°C with 5% CO₂. Medium replacement was not needed. For non-differentiated control, BMSC maintained in CCM was used.

Lentiviral plasmids and virus preparation

Lentiviral plasmids carrying Flag-tagged H2AX (WT) and H2AX (Y142F) were purchased from Genecopoeia. The preparation of Lentivirus was the same as described previously (38). Virus infection was performed after 12 days of differentiation. After 3 days, cells were subjected to short-wavelength ultraviolet light (UVC) irradiation.

SDS-PAGE and Western blotting

Cell pellets were resuspended in Lysis Buffer (Promega) followed by freeze/thaw in dry ice/ethanol bath to obtain the whole lysate. The cell lysates were mixed with SDS sample buffer (Invitrogen) and 2% β-mercaptoethanol, boiled for 5 min and resolved by SDS-PAGE using 10–20% Tris/HCl precast gel (Bio-Rad). Precision plus protein standards (Bio-Rad) were included as references. After gel electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane for further probing with antibodies against proteins of interest. Western Lightning Plus ECL (PerkinElmer) or ECLTM Prime Western Blotting Detection Reagent (Fisher Scientific) were used for visualization of protein immunoreactivities. Immunoreactivity of β-actin was used as loading controls.

Cell growth assay

The cell growth was determined using NucleoCounter (LI-COR Biosciences), using previously published methods (39). Briefly, differentiated and non-differentiated BMSC were trypsinized and replated at the density of 5×10^5 per well in two 6 well plates, differentiation and control media were respectively used to maintain the two types of cells. After overnight incubation for adherence and 3 days of culture, the cells from each well were harvested independently by trypsinization to derive cell suspensions for cell counting. All measurements were performed in triplicate from 3 experiments.

Immunocytochemistry

Cells grown on cover slips were rinsed with phosphate buffered saline, fixed in 4% paraformaldehyde, and permeabilized with 0.1 M TBS; pH 7.6 containing 0.5% Triton X-100 for 5 minutes. They were subsequently blocked with 3% goat serum in TBS,

incubated with primary antibodies in TBS containing 1% goat serum overnight at 4°C then incubated for 1 hour with secondary antibodies. Immunolabeled cells were stained with nuclei stain DAPI (Invitrogen) for 10 minutes and observed by confocal fluorescence microscopy (Zeiss LSM 510, Carl Zeiss MicroImaging).

UV irradiation and neurotoxin treatment

BMSC-derived DA neurons, with or without lentiviral infection, were exposed to UVC irradiation (240–290 nm) for 20 J/m² at room temperature in 6 or 24 well plates using Stratalinker UV Crosslinker (Model 2400) followed by 2 days of maintenance, or exposed to 200 μM 1-methyl-4-phenylpyridinium (MPP⁺) for 2 days. After that, the neurons in 6-well plates of each group were harvested and evenly divided into 3 parts, the first and second part were respectively used for DNA and protein extraction. The third part was used for caspase 3 activity assay. Those grown in 24-well plates were only used for cell viability assays. All experiments were performed three times independently.

DNA extraction and fragmentation assay

Genomic DNA was extracted from each sample using the FlexiGene DNA Kit (QIAGEN) according to manufacturer instructions. Agarose gel (0.7%) electrophoresis of the same amount of DNA from different groups was used to assess DNA fragmentation.

Caspase 3 activity assay

Caspase 3 activity was measured using SensoLyte[®] Homogeneous AMC Caspase 3 Assay Kit. Briefly, cell pellets were lysed with 1× lysis buffer. The lysed cell suspensions were then collected and incubated at 4°C for 30 minutes followed by centrifugation at 2500 × g for 10 minutes. The supernatants from different groups with the same amount of protein were incubated with a specific caspase-3 substrate (Ac-DEVD-AMC) in a 96 well plate on a plate shaker at 100–200 rpm for 1 hour at room temperature. Caspase-3 activity was measured at 354 nm (excitation)/442 nm (emission) using a Spectra Max M4 and Soft Max Protein 4.6 software (Molecular Devices, Sunnyvale, CA).

Cell viability assay

Cell viability was assessed using Calcein AM (Invitrogen). Cells in 24-well plates were incubated with 2 μM Calcein AM in balanced salt solution for 30 minutes at room temperature in the dark. Fluorescence signals emitted from live cells by esterase-metabolized calcein were measured at 495 nm (excitation)/530 nm (emission) using a Spectra Max M4 and Soft Max Protein 4.6 software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data from at least 3 sets of independent experiments were analyzed by one-way analysis of variance with Dunnett's *post hoc* test to determine statistical significance.

Results

Isolation and characterization of BMSC

BMSC were isolated and cultured from human bone marrow aspirates as described previously (35–37), then subjected to positive and negative characterization by flow cytometry. Figure 1 is a representative example of the cell surface antigens expressed on isolated cells, showing that the isolated cells were strongly positive for three known BMSC markers, CD73, CD90 and CD105 and negative for hematopoietic stem cell marker (CD34) and pan-leukocyte marker (CD45) (Fig. 1E). Moreover, the isolated BMSC could differentiate into bone since they were positively stained with Alizarin Red S upon osteogenic differentiation (Fig. 1A), and with Oil-Red-O upon fat tissue differentiation (Fig. 1C). Such staining is not observed in non-differentiated cells (Fig. 1B and 1D). The results strongly suggested that the isolated cells were pure BMSC.

Differentiation of BMSC into dopaminergic neuronal cells

The isolated BMSC were subjected to a previous established protocol for differentiation into DA neurons (17). As shown in Fig. 2A and 2B, BMSC cells stopped growing after 12 days of differentiation, and markedly changed their morphology. Cell bodies of differentiated cells became more refractile, and they had long thin processes (Fig. 2A), which are similar to neurites of neurons. In contrast, non-differentiated BMSC remained flat without refractility. Western blotting of cell lysates and immunocytochemical staining of intact cells demonstrated that differentiated BMSC expressed not only neuronal markers NeuN (Fig. 2C) and MAP2 (Fig. 2D), but also markers of dopaminergic cells, tyrosine hydroxylase and dopamine transporter (Fig. 2C and 2D), which were not detected in undifferentiated cells. These results suggested that the isolated BMSC cells were differentiated into DA neurons.

Expression of WT and Y142F H2AX in BMSC-derived DA cells via lentiviral infection

For gene delivery, BMSC-derived DA neurons were infected with comparable titers of lentivirus carrying Flag-tagged H2AX (WT) or H2AX (Y142F), referred to as WT and Y142F. Their null differentiated counterparts without lentivirus infection were used as control, referred to as Null. Three days after infection, a fraction of cells was harvested for evaluation of expression of delivered genes. Western blotting of cell lysates showed that lentiviral delivery of genes led to robust expression of both H2AX (WT) and H2AX (Y142F) in differentiated DA neurons, as indicated by immunoreactive signals to the Flag-tag (Fig. 3). Densitometric analysis of immunoreactivities showed that both exogenous Flag-tagged and endogenous non-tagged H2AX in WT cells had comparable expression to those in Y142F cells (data not shown), whereas only endogenous H2AX, but not exogenous Flag tagged H2AX, was expressed in null counterparts (Fig. 3)

Enhanced resistance to DNA damage induced by direct UVC exposure in neurons bearing H2AX (Y142F)

To determine if expression of H2AX (Y142F) is protective to DNA damage, BMSC-derived DA neurons of WT, Y142F and Null were treated with and without UVC irradiation, then maintained for another 2 days, followed by analyses. The sibling cultures without UVC

exposure were used as controls. All three groups showed evidence of genomic DNA damage, increased activities of caspase 3, and reduced cell viability upon UVC exposure; however, Y142F was less impaired than WT and Null (Fig. 4). This indicated that Y142F expression renders BMSC-derived DA neurons partially resistant to UVC-induced DNA damage and subsequent apoptosis.

Enhanced resistance to DNA damage induced by oxidative neurotoxicity in neurons bearing H2AX (Y142F)

To further test if BMSC-derived DA neurons bearing H2AX (Y142F) are more resistant to DNA damage after exposure to oxidative damage, WT, Y142F and Null were treated with or without MPP⁺ for 2 days as MPP⁺ is a parkinsonian neurotoxin well-known to induce oxidative DNA damage and subsequent apoptosis (40–42). After exposure to MPP⁺, cells were subjected to different assays. Similar to UVC exposure, MPP⁺ treatment induced DNA damage, caspase 3 activation, and cell death in all three groups, but Y142F had less fragmented genomic DNA, lower caspase 3 activity, and increased cell survival compared with the other groups (Fig. 5). Therefore, Y142F expression can enhance the viability of BMSC-derived DA neurons to oxidative neurotoxicity.

Discussion

The capacity of transplanted stem cells to survive in the environment of a diseased brain is critical to success of stem cell therapy. In PD this may be compromised by high oxidative stress in PD (8). In order to increase the survival of transplanted stem cells, we explored a novel genetic engineering strategy. We genetically modified stem cell-derived neurons to carry protective factors for DNA damage. This strategy has advantages over previous ones in that: 1) genetic modification can endow cells with long lived favorable phenotypic properties, and 2) resistance to DNA damage can improve survival of transplanted stem cells.

We tested the effect of expressing H2AX (Y142F) on DNA damage resistance in BMSC-derived DA neurons. Genetic modification of H2AX was chosen because expression of the Y142F mutant has been previously shown to have a propensity of DNA repair/survival response after exposure to DNA damage that leads to enhanced resistance to apoptosis (6). BMSC were used as a stem cell model because they possess numerous protective features as well as the capability to differentiate into DA neurons.

We isolated and purified BMSC, then successfully differentiated them into DA neurons. The protective factor, H2AX (Y142F), was conferred by lentivirus and compared to cells expressing WT H2AX. Cells were subsequently exposed to UVC irradiation or MPP⁺ treatment. Cell damage was measured by assays of DNA fragmentation, caspase 3 activity, and cell viability. As hoped for, the BMSC-derived DA neurons expressing H2AX (Y142F) were more resistant to DNA damage and apoptosis induced by UVC exposure or MPP⁺ treatment, compared to the Null or WT H2AX cells. Therefore, this new strategy may be applicable to BMSC-derived DA neurons. It remains to be determined if DA neurons bearing H2AX (Y142F) are also more resistant to a hostile brain environment, which needs to be addressed in future transplantation studies in animal models of PD.

Since the present exploratory study was aimed at determining if a novel strategy for improving cell survival of DA neurons may be useful to treat PD, we use lentivirus infection for genetic modification as it is simple, fast and highly efficient method for gene delivery. Due to potential biohazardous risk, lentivirus infection might not be ideal for genetic modification of stem cells in future clinical use. Instead, a safer and alternative genetic engineering method such as zinc finger nuclease knock-in technology (43, 44) should be considered, which needs to be a focus of future studies.

Conclusion

In summary, our study has shown that expression of H2AX (Y142F) renders BMSC-derived DA neurons more resistant to DNA damage and subsequent cell death. This may be a method to improve survival of BMSC-derived DA neurons under the neurotoxic environment in the patient's brain. Since MPP⁺ exposure is normally used to mimic neurotoxic environment in PD brain, the promising results from our cell-based study not only strongly support the feasibility of applying this new strategy to animal models, but also may have potential implications in stem cell transplantation therapy for PD and related diseases.

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Abbreviations

Ac-DEVD-AMC	Ac-Asp-Glu-Val-Asp-AMC (AMC = 7-Amino-4-methylcoumarin)
bFGF	basic fibroblast growth factor
BMSC	bone marrow-derived mesenchymal stem cell
CCM	complete culture medium
DA	dopamine
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
FGF8	fibroblast growth factor 8
H2AX	histone 2A, family member X
JNK1	c-Jun N-terminal kinase 1
MPP⁺	1-methyl-4-phenylpyridinium
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffer-saline
PD	Parkinson's disease
ROS	reactive oxygen species
S139	serine 139
SDS	sodium dodecyl sulfate
SHH	sonic hedgehog
TBS	Tris-buffered saline
UV	ultraviolet light
UVC	short wavelength UV (100 nanometers (nm) to 280 nm)
WT	wild type
Y142	tyrosine 142
γ-H2AX	phosphorylation of H2AX at serine residue 139

References

1. Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. *J Neurochem*. 1997e; 69:1196–203. [PubMed: 9282943]
2. Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U, Subba Rao K, Jagannatha Rao KS. Studies on genomic DNA topology and stability in brain regions of Parkinson's disease. *Arch Biochem Biophys*. 2006e; 449:143–56. [PubMed: 16600170]
3. Yoshimura D, Sakumi K, Ohno M, Sakai Y, Furuichi M, Iwai S, Nakabeppu Y. An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. *J Biol Chem*. 2003e; 278:37965–73. [PubMed: 12857738]
4. Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol*. 2003e; 53(Suppl 3):S26–36. discussion S-8. [PubMed: 12666096]
5. De Zio D, Bordini M, Cecconi F. Oxidative DNA damage in neurons: implication of ku in neuronal homeostasis and survival. *Int J Cell Biol*. 2012e; 2012:752420. [PubMed: 22737170]
6. Cook PJ, Ju BG, Telese F, Wang X, Glass CK, Rosenfeld MG. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature*. 2009e; 458:591–6. [PubMed: 19234442]
7. Borges HL, Linden R, Wang JY. DNA damage-induced cell death: lessons from the central nervous system. *Cell Res*. 2008e; 18:17–26. [PubMed: 18087290]
8. Tsang AH, Chung KK. Oxidative and nitrosative stress in Parkinson's disease. *Biochim Biophys Acta*. 2009e; 1792:643–50. [PubMed: 19162179]
9. Gensler HL, Bernstein H. DNA damage as the primary cause of aging. *Q Rev Biol*. 1981e; 56:279–303. [PubMed: 7031747]
10. Hart RW, Setlow RB. Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. *Proc Natl Acad Sci U S A*. 1974e; 71:2169–73. [PubMed: 4526202]
11. Yokochi M. Reevaluation of levodopa therapy for the treatment of advanced Parkinson's disease. *Parkinsonism Relat Disord*. 2009e; 15(Suppl 1):S25–30. [PubMed: 19131038]
12. Datta I, Bhande R. Can mesenchymal stem cells reduce vulnerability of dopaminergic neurons in the substantia nigra to oxidative insult in individuals at risk to Parkinson's disease? *Cell Biol Int*. 2012e; 36:617–24. [PubMed: 22417707]

13. Shytle RD, Ehrhart J, Tan J, Vila J, Cole M, Sanberg CD, Sanberg PR, Bickford PC. Oxidative stress of neural, hematopoietic, and stem cells: protection by natural compounds. *Rejuvenation Res.* 2007e; 10:173–8. [PubMed: 17518694]
14. Brundin P, Karlsson J, Emgard M, Schierle GS, Hansson O, Petersen A, Castilho RF. Improving the survival of grafted dopaminergic neurons: a review over current approaches. *Cell Transplant.* 2000e; 9:179–95. [PubMed: 10811392]
15. Fawcett, J. Death and survival in CNS grafting. In: Dunnett, SBBA., Baker, GB., editors. *Neural Transplantation Methods*. Vol. 2000. Totowa: Humana Press; 2000. p. 441–60.
16. Gao JTJ, Dunn T. Lipoic acid enhances survival of transplanted neural stem cells by reducing transplantation-associated injury. *Journal of Neurorestoratology.* 2013e; 1:1.
17. Trzaska KA, Kuzhikandathil EV, Rameshwar P. Specification of a dopaminergic phenotype from adult human mesenchymal stem cells. *Stem Cells.* 2007e; 25:2797–808. [PubMed: 17656644]
18. Danielyan L, Schafer R, Schulz A, Ladewig T, Lourhmati A, Buadze M, Schmitt AL, Verleysdonk S, Kabisch D, Koeppen K, Siegel G, Proksch B, Kluba T, Eckert A, Kohle C, Schoneberg T, Northoff H, Schwab M, Gleiter CH. Survival, neuron-like differentiation and functionality of mesenchymal stem cells in neurotoxic environment: the critical role of erythropoietin. *Cell Death Differ.* 2009e; 16:1599–614. [PubMed: 19609278]
19. Mizuno Y, Yoshino H, Ikebe S, Hattori N, Kobayashi T, Shimoda-Matsubayashi S, Matsumine H, Kondo T. Mitochondrial dysfunction in Parkinson's disease. *Ann Neurol.* 1998e; 44:S99–109. [PubMed: 9749580]
20. Hwang O. Role of oxidative stress in Parkinson's disease. *Exp Neurobiol.* 2013e; 22:11–7. [PubMed: 23585717]
21. Theus MH, Wei L, Cui L, Francis K, Hu X, Keogh C, Yu SP. In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Exp Neurol.* 2008e; 210:656–70. [PubMed: 18279854]
22. Painter RB. DNA damage and repair in eukaryotic cells. *Genetics.* 1974e; 78:139–48. [PubMed: 4442699]
23. Jackson SP. Sensing and repairing DNA double-strand breaks. *Carcinogenesis.* 2002e; 23:687–96. [PubMed: 12016139]
24. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998e; 273:5858–68. [PubMed: 9488723]
25. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol.* 2000e; 10:886–95. [PubMed: 10959836]
26. Ganz J, Lev N, Melamed E, Offen D. Cell replacement therapy for Parkinson's disease: how close are we to the clinic? *Expert Rev Neurother.* 2011e; 11:1325–39. [PubMed: 21864078]
27. Chen MF, Lin CT, Chen WC, Yang CT, Chen CC, Liao SK, Liu JM, Lu CH, Lee KD. The sensitivity of human mesenchymal stem cells to ionizing radiation. *Int J Radiat Oncol Biol Phys.* 2006e; 66:244–53. [PubMed: 16839703]
28. Valle-Prieto A, Conget PA. Human mesenchymal stem cells efficiently manage oxidative stress. *Stem Cells Dev.* 2010e; 19:1885–93. [PubMed: 20380515]
29. Zhuo W, Liao L, Xu T, Wu W, Yang S, Tan J. Mesenchymal stem cells ameliorate ischemia-reperfusion-induced renal dysfunction by improving the antioxidant/oxidant balance in the ischemic kidney. *Urol Int.* 2011e; 86:191–6. [PubMed: 20881358]
30. Lanza C, Morando S, Voci A, Canesi L, Principato MC, Serpero LD, Mancardi G, Uccelli A, Vergani L. Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in vivo. *J Neurochem.* 2009e; 110:1674–84. [PubMed: 19619133]
31. Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinney DG. Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neurogenesis. *Exp Neurol.* 2006e; 198:54–64. [PubMed: 16336965]
32. Cova L, Armentero MT, Zennaro E, Calzarossa C, Bossolasco P, Busca G, Lambertenghi Delilieri G, Polli E, Nappi G, Silani V, Blandini F. Multiple neurogenic and neurorescue effects of human

mesenchymal stem cell after transplantation in an experimental model of Parkinson's disease. *Brain Res.* 2010e; 1311:12–27. [PubMed: 19945443]

33. Hayashi T, Wakao S, Kitada M, Ose T, Watabe H, Kuroda Y, Mitsunaga K, Matsuse D, Shigemoto T, Ito A, Ikeda H, Fukuyama H, Onoe H, Tabata Y, Dezawa M. Autologous mesenchymal stem cell-derived dopaminergic neurons function in parkinsonian macaques. *J Clin Invest.* 2013e; 123:272–84. [PubMed: 23202734]
34. Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest.* 2004e; 113:1701–10. [PubMed: 15199405]
35. Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci U S A.* 2000e; 97:3213–8. [PubMed: 10725391]
36. Grassel S, Stockl S, Jenei-Lanzl Z. Isolation, culture, and osteogenic/chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. *Methods Mol Biol.* 2012e; 879:203–67. [PubMed: 22610563]
37. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells.* 2002e; 20:530–41. [PubMed: 12456961]
38. Jiang P, Gan M, Ebrahim AS, Castanedes-Casey M, Dickson DW, Yen SH. Adenosine monophosphate-activated protein kinase overactivation leads to accumulation of alpha-synuclein oligomers and decrease of neurites. *Neurobiol Aging.* 2013e; 34:1504–15. [PubMed: 23200460]
39. Ko LW, Ko HH, Lin WL, Kulathingal JG, Yen SH. Aggregates assembled from overexpression of wild-type alpha-synuclein are not toxic to human neuronal cells. *J Neuropathol Exp Neurol.* 2008e; 67:1084–96. [PubMed: 18957893]
40. Liu HM, Yang SZ, Sun FY. 1-Methyl-4-phenyl-pyridinium time-dependently alters expressions of oxoguanine glycosylase 1 and xeroderma pigmentosum group F protein in PC12 cells. *Neurosci Bull.* 2010e; 26:1–7. [PubMed: 20101267]
41. Pettifer KM, Jiang S, Bau C, Ballerini P, D'Alimonte I, Werstiuk ES, Rathbone MP. MPP(+)-induced cytotoxicity in neuroblastoma cells: Antagonism and reversal by guanosine. *Purinergic Signal.* 2007e; 3:399–409. [PubMed: 18404453]
42. Chen LJ, Gao YQ, Li XJ, Shen DH, Sun FY. Melatonin protects against MPTP/MPP+ -induced mitochondrial DNA oxidative damage in vivo and in vitro. *J Pineal Res.* 2005e; 39:34–42. [PubMed: 15978055]
43. Le Provost F, Lillico S, Passet B, Young R, Whitelaw B, Vilotte JL. Zinc finger nuclease technology heralds a new era in mammalian transgenesis. *Trends Biotechnol.* 2010e; 28:134–41. [PubMed: 20015561]
44. Hauschild-Quintern J, Petersen B, Cost GJ, Niemann H. Gene knockout and knockin by zinc-finger nucleases: current status and perspectives. *Cell Mol Life Sci.* 2013e; 70:2969–83. [PubMed: 23161061]

Fig. 1. Characterization of human BMSC

(A and B) Isolated human BMSC with or without osteogenic differentiation were subjected to Alizarin red S staining. (C and D) Isolated human BMSC with or without fat tissue differentiation were subjected to Oil-Red-O staining. (E) Flow cytometric analysis confirmed the BMSC phenotypic characteristics of isolated cells: CD73+, CD90+, and CD105+; CD34– and CD45–. Scale bar: 200 μ m.

Fig. 2. Morphology and phenotype of BMSC-induced DA neurons

(A) Bright-field images demonstrate that the morphology of BMSCs drastically changes under differentiation, resembling native neurons. (B) Cell growth assay show that BMSC with 12 days of differentiation do not significantly proliferate after 3 days of culture, while non-differentiated counterparts do. Bar graphs summarized cell count results from all groups. [ND: Non-Differentiation; D- Differentiation. The average number of ND-0d was set as 100%. Error bars represent standard error of the mean (SEM). ** $p < 0.01$ comparing to subsets linked by bracket. N.S.: No significant difference.]; (C) Western blots of cell lysates show neuronal and dopaminergic neuronal markers – NeuN and tyrosine hydroxylase (TH) – are expressed in differentiated BMSC (D-BMSC), but not in undifferentiated BMSC. For positive control, cell lysates of human *substantia nigra* (SN) are used. (D) Immunocytochemistry of BMSC with and without differentiation show a neuronal cell marker (MAP2), dopaminergic neuron specific markers (dopamine transporter (DAT) and TH) are expressed in differentiated cells.

Fig. 3. Expression of endogenous and exogenous H2AX in BMSC-derived DA neurons
 BMSC-derived DA neurons were infected with lentivirus carrying H2AX (WT) or H2AX (Y142F) and then harvested for evaluation of H2AX expression. Non-infected null counterparts were used as controls (Null). Western blotting of cell lysates show that both endogenous (H2AX positive bands with lower molecular weight, denoted by arrowhead) and exogenous Flag-tagged H2AX (H2AX positive bands with higher molecular weight, also positive to Flag, denoted by arrow) are expressed in BMSC-derived DA neurons with lentivirus infection. In contrast, null counterparts only have endogenous expression (denoted by arrowhead).

Fig. 4. Differential sensitivity to UV radiation between Y142F, WT and null cells

BMSC-derived DA neurons of Y142F, WT or null, with or without UV irradiation and followed by 2 days of maintenance were subjected to (A) DNA fragmentation assay, (B) cell viability assay, and (C) caspase 3 activity assay. Results from (B) and (C) are summarized in bar graphs. The Y142 without UV irradiation (Y142F/UV-) was set as 100%. Error bars represent SEM (* $p < 0.05$ and ** $p < 0.01$, comparing subsets linked by line, $n=3$). The results are representative of 3 independent experiments.

Fig. 5. Differential sensitivity to MPP+ treatment between Y142F, WT and null cells
 BMSC-derived DA neurons of Y142F, WT or null, with or without MPP+ treatment for 2 days, were subjected to (A) DNA fragmentation assay, (B) cell viability assay and (C) caspase 3 activity assay. Results from (B) and (C) are summarized in bar graphs. Y142 without treatment (Y142F/MPP-) was set as 100%. Error bars represent SEM (* p < 0.05 and ** p < 0.01, comparing subsets linked by line, n=3). The results are representative of 3 independent experiments.