Regulated Expression of Lentivirus-Mediated GDNF in Human Bone Marrow-Derived Mesenchymal Stem Cells and Its Neuroprotection on Dopaminergic Cells In Vitro

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Abstract

Gene regulation remains one of the major challenges for gene therapy in clinical trials. In the present study, we first generated a binary tetracycline-on (Tet-On) system based on two lentivirus vectors, one expressing both human glial cell line-derived neurotrophic factor (hGDNF) and humanized recombinant green fluorescent protein (hrGFP) genes under second-generation tetracycline-controlled transactivator - rtTA2-S-M2 under a human minimal cytomegalovirus immediate early (CMV-IE) promoter. This system allows simultaneous expression of hGDNF and hrGFP genes in the presence of doxycycline (Dox). Human bone marrow-derived mesenchymal stem cells (hMSCs) were transduced with the binary Tet-On lentivirus vectors and characterized in vitro in the presence (On) or absence (Off) of Dox. The expression of hGDNF and hrGFP transgenes in transduced hMSCs was tightly regulated as determined by flow cytometry (FCM), GDNF enzyme-linked immunosorbent assay (ELISA) and quantitative real time-polymerase chain reaction (qRT-PCR). There was a dose-dependent regulation for hrGFP transgene expression. The levels of hGDNF protein in culture medium were correlated with the mean fluorescence intensity (MFI) units of hrGFP. The levels of transgene background expression were very low in the absence of Dox. The treatment of the conditioned medium from cultures of transduced hMSCs in the presence of Dox protected SH-SY5Y cells against 6-hydroxydopamine (6-OHDA) toxicity as determined by cell viability using 3, [4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) assay. The treatment of the conditioned medium was also found to improve the survival of dopaminergic (DA) neurons of ventral mesencephalic (VM) tissue in serum-free culture conditions as assessed by cell body area, the number of neurites and dendrite branching points, and proportion of tyrosine hydroxylase (TH)-immunoreactive (IR) cells. Our inducible lentivirus-mediated hGDNF gene delivery system may provide useful tools for basic research on gene therapy for chronic neurological disorders such as Parkinson’s disease (PD).

Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder resulting in the loss of dopaminergic (DA) neurons and the impairment of motor function. Currently there is no known cure for PD. The mainstay of therapy for PD is still the oral administration of levodopa which is effective at early stage of treatment and eventually becomes ineffective with side effects associated with a long-term administration. There is an imperative need to develop new therapeutic approaches. Alternative therapeutic approaches have been developed in the use of dopamine agonists, neurosurgical treatment and neural transplantation of embryonic tissue. However, all current therapeutic approaches for PD do not arrest or reverse the fundamental neurodegenerative processes of the disease. Substantial evidence shows that neurotrophic factors can prevent nigral DA neurons from dying and improve the cell functions. Among the neurotrophic factors, glial cell line-derived neurotrophic factor (GDNF) has proven to be a potent neurotrophic factor for protection of nigral DA neurons against toxin-induced degeneration in vitro and in vivo [1–4]. As GDNF being a macromolecule with the molecular weight of 32–34 kDa does not pass through the blood-brain barrier, direct infusion of GDNF into the brain is required to achieve a therapeutic purpose. GDNF has been infused into the brain tissue [5] and the ventricles [6] in clinical trials to treat patients with PD. However, protein infusion into the brain is difficult to develop into a long-term therapeutic approach. To obtain sustained delivery, virus vectors-mediated GDNF gene delivery has been developed. The GDNF gene can be delivered into the brain using in vivo and ex vivo gene delivery approaches. For the in vivo approaches, recombinant adeno, adeno-associated, or lentivirus vectors harboring the GDNF gene are directly injected into the brain and

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gene delivery effects have been evaluated in intact and lesioned rodents [7–10] and primates [11,12]. It has been demonstrated that neuronal cells in the brain can be efficiently transduced, resulting in long-term transgene expression. However, there are concerns about live virus administration and genetically modified cells. For the ex vivo approaches, live viruses carrying the GDNF gene are used to transduce cells in vitro and then transduced cells are transplanted into the brain. For this purpose, neural stem cells [13,14], an immortalized neural stem cell line [15], primary astrocytes [16,17], and mesenchymal stem cells [18–20] have been used to serve as gene delivery vehicles. Human bone marrow-derived mesenchymal stem cells (hMSCs) are very easily accessible, prepared and cultured. The use of hMSCs can provide unlimited cell sources for gene delivery vehicles. Moreover, the use of adult hMSCs enables to do autologous transplantation and can avoid immune responses. There is accumulating evidence that MSCs can be genetically modified ex vivo by standard retroviral techniques and can stably express transgenes robustly in vivo following transplantation [18–20].

Lentivirus vectors are considered one of the most promising vehicles to efficiently deliver a gene for basic research and gene therapy, due to the ability to transduce non-dividing and dividing cells, stable transgene expression, minimal toxicity and immunity, and a large cloning capacity of 9 kb [21–23]. Numerous studies have demonstrated that lentivirus vectors can efficiently deliver the GDNF gene into the brain in animal models of PD, and that GDNF transduction protects dopaminergic neurons from neurotoxin-induced cell death [12,17,24,25]. Nevertheless, potential insertional mutagenesis of lentivirus vectors should not be ignored because lentivirus vectors transduce target cells by randomly integrating into the host chromosomes.

Considering the delivery of a therapeutic gene into the brain, one of the major challenges for clinical application remains the necessity of gene regulation in order to achieve the expected therapeutic outcome while avoiding potential limiting side effects related to the over-expression of the transgene. It is generally agreed that the introduction into the brain of a constitutively produced compound that is not normally present in the brain would not be completely harmless. Indeed, several lines of evidence show that continuous overexpression of the GDNF gene at high doses leads to adverse side effects, such as aberrant sprouting in areas outside the striatum and down-regulation of tyrosine hydroxylase (TH) in the preserved DA terminals [26]. In addition, long-term over-expression of GDNF can induce down-regulation of TH in the intact striatum [8,27]. It is therefore imperative to introduce an inducible vector system. Several systems have been generated to control gene expression by using an exogenous drug either at the transcriptional or translational level. Inducible gene expression systems are potent research tools, and are constantly developed for their use in basic research and clinical application. Among the existing inducible transcriptional gene regulatory systems, the reverse tetracycline (Tet)-controlled transactivator (rtTA)-regulated system is the most widely exploited tool for inducible gene expression. Gossen and Bujard [28] first described this system which was based on a chimeric transcription factor (the rtTA transactivator), resulting from the fusion of the bacterial Tet repressor (TetR) with the activating domain of the herpes simplex virus immediate early (IE) promoter (Fig. 1B). The TATA box of the minimal cytomegalovirus (CMV) promoter was placed 10 bp (1 helical turn) downstream of the first tetO element. The second vector encodes tet-controlled the improved reverse tetracycline-controlled transactivator (rTA) 2S-M2 [30], in which the herpes simplex virus (HSV) VP16 transactivation domain of the earlier transactivator is replaced with a human E2F4 domain, and it is controlled by the constitutive human minimal CMV-IE promoter [31]. In the present study, we modified the transgene vector, and replaced the expression cassette of hGDNF by hGDNF. The virus vectors were produced by three-plasmid transient transfection of 293T cells [40], and the titer of the virus vector was around 10^7 TU/ml.

Results

Design and production of improved binary Tet-on lentivirus vectors

To induce transgene expression in the presence of Dox and reduce background expression in the absence of Dox, Pluta et al. generated a binary Tet-On lentivirus vector system [29]. The improved binary vector system is composed of a self-inactivating (sin) transgene vector bearing a 400-bp deletion of U3 sequences in the virus’ long terminal repeats (LTRs). The transgene vector harbors second-generation tetracycline response elements (TREs) which contain repositioned tetO sequences including TRE/Pitt [39] (Fig. 1A). TRE/Pitt employs 36 bp (3.5 helical turns) between the central bases of two consecutive tetO sequences, and the TATA box of the minimal cytomegalovirus (CMV) promoter was placed 10 bp (1 helical turn) downstream of the first tetO element. The second vector encodes tet-controlled the improved reverse tetracycline-controlled transactivator (rTA) 2S-M2 [30], in which the herpes simplex virus (HSV) VP16 transactivation domain of the earlier transactivator is replaced with a human E2F4 domain, and it is controlled by the constitutive human minimal CMV-IE promoter [31]. In the present study, we modified the transgene vector, and replaced the expression cassette of enhanced GFP (EGFP) with hGDNF/hrGFP. The virus vectors were produced by three-plasmid transient transfection of 293T cells [40], and the titer of the virus vector was around 10^7 TU/ml.

Figure 1. Tet-On inducible lentivirus vector system. (A) Structure of a Tet-regulated transgene vector, pNL-TRE/Pitt-CMV-hGDNF/hrGFP-ΔU3, which carries a TRE/Pitt-hGDNF/hrGFP expression cassette linked to a 5′ V40 poly A signal in reverse orientation with respect to the 3′ LTR. The chHS4 insulator is inserted into the 3′ LTR in the forward (+) orientation. (B) Structure of a transactivator-encoding vector, pNL-CMV-IE-rta252S-M2-ΔU3. chHS4, chicken β-globin DNaseI hypersensitive site 4; cPPT, central polyuridine tract; gag, 5′ portion of the gag coding region; hGDNF, human glial cell line-derived neurotrophic factor; hrGFP, humanized recombinant green fluorescent protein; IRES, internal ribosome entry site; LTRs, long terminal repeats; RRE, Rev-response element; SA, splice acceptor site; SD, splice donor site; TRE, tetracycline response element; ΔU3, 400-bp deletion of U3 sequences present in the 3′ LTR.

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Regulated transgene expression with the binary Tet-On lentivirus vector system

We first examined the inducibility of transgene expression using HeLa cells and hMSCs. The cells were transduced with the binary Tet-On lentivirus vectors, and incubated in the presence of Dox at serial doses ranging from $10^{-4}$ – $10^{4}$ ng/ml. Flow cytometry (FCM) analysis showed that mean fluorescence intensity (MFI) units for hrGFP were increased in a Dox dose-dependent manner for both transduced HeLa cells (Fig. 2A) and hMSCs (Fig. 2B). It appeared that hrGFP transgene expression started at a very low dose (0.1 ng/ml) of Dox. We used a dose of 100 ng/ml Dox for the following experiments in the study. We next examined efficiency of regulated transgene expression and the background expression of transgenes in transduced hMSCs in the presence or absence of Dox over time (at 4, 7, 10, and 14 days). No hrGFP

expressions were observed in the cultures of transduced hMSCs at the beginning of the experiment (data not shown). For the “On – Off” treated group, numerous hrGFP

expressions were observed in the cultures 4 days in the presence (On) of Dox (Fig. 3A). Numerous hrGFP

expressions still existed in the cultures 3 days after the removal (Off) of Dox in culture medium (Fig. 3B). However, only a few hrGFP

expressions were observed in the cultures 6 days after the removal of Dox (Off) of Dox in culture medium (Fig. 3C). For the “Off – On” treated group, the pattern of transgene expression was just contrary to that of the “On – Off” treated group. No hrGFP

expressions were observed 4 days in vitro in the absence (Off) of Dox (Fig. 3D). Numerous hrGFP

expressions were observed in the cultures 3 days after the addition (On) of Dox in culture medium (Fig. 3E). The number of hrGFP

expressions remained the same level in the cultures 6 days in the presence (On) of Dox (Fig. 3F). FCM analysis of MFI units for hrGFP confirmed these dynamic changes in hrGFP expression for transduced hMSCs (Fig. 3G). No detectable MFI units for hrGFP were observed in transduced hMSCs at the beginning of the experiment (0±0). For the “On – Off” treated group, MFI units for hrGFP were increased in transduced hMSCs 4 days in vitro in the presence (On) of Dox were 2495.56±1563.53 at 4 days, MFI units were reduced to 6.41 at 7 days (Fig. 3H). For the “Off – On” group, detectable levels of hGDNF were not observed in cultures 4 days (0±0) in vitro in the absence (Off) of Dox. After the addition (On) of Dox, the levels of hGDNF were increased to 31.1±5.81 at 7 days (3 days after the addition of Dox) in culture medium, and maintained the same levels at 10 days (45.1±2.06, 6 days after the addition of Dox) and 14 days (50.7±6.41, 10 days after the addition of Dox) in vitro. These results suggest Dox can tightly regulate transgene expression in transduced hMSCs, and background expression of transgenes was very low in the absence of Dox.

The above FCM and GDNF ELISA results were confirmed and extended by quantitative real-time-polymerase chain reaction (qRT-PCR) using probes specific for hrGFP and hGDNF transcripts. Copy numbers of hGDNF and hrGFP mRNA were examined in the RNA samples isolated from the same cells used for FCM analysis (Fig. 4). The presence of Dox resulted in an average 103-fold increase in copy number of hGDNF mRNA in transduced hMSCs (5.17±0.28)×10^7 when compared to that in transduced hMSCs in the absence of Dox (0.05±0.02)×10^7 at 7 days (one-factor analysis of variance, ANOVA, followed by Fisher’s post hoc test, F(3, 20) = 347.98, p<0.05). The similar pattern was observed for copy number of hrGFP mRNA. The presence of Dox led to an average 120-fold increase in copy number of hrGFP mRNA in transduced hMSCs (4.78±0.18)×10^7 when compared to that in the absence of Dox (0.04±0.02)×10^7 at 7 days (# p<0.05).

Differentiation of transduced hMSCs

It is well known that hMSCs can be induced to differentiate into adipogenic and osteogenic cells due to their stem cell properties. In the presence of Dox, the majority of transduced hMSCs expressed...
the hrGFP transgene (Fig. 5A and B, a phase contrast image). By incubating in adipogenic or osteogenic differentiation medium, transduced hMSCs were induced to differentiate into adipogenic (Fig. 5C) and osteogenic (Fig. 5E) cells in the presence of Dox 3 weeks after induction. Transduced hMSCs in cultures did not spontaneously differentiate into adipogenic (Fig. 5D) and osteogenic (Fig. 5F) cells. These data suggest that viral transduction does not alter cell properties of hMSCs.

hMSCs G/G + Dox conditioned medium protects SH-SY5Y cells against 6-OHDA-induced cell death

To examine effects of hGDNF released by transduced hMSCs, conditioned medium was collected from cultures of transduced hMSCs in the presence or absence of Dox at 7 days. At this time point, numerous hMSCs (Fig. 6A) in the total cells (Fig. 6C) in culture expressed hrGFP transgene in the presence of Dox. In contrast, only a few hMSCs (Fig. 6B) in the total cells (Fig. 6D) in the culture expressed hrGFP transgene in the absence of Dox. FCM analysis showed that the percentage of hrGFP-positive cells was much greater in hMSCs in the presence of Dox (50.07 ± 2.76%) than in hMSCs in the absence of Dox (0.33 ± 0.03%) (Fig. 6E) (one-factor ANOVA followed by Fisher’s post hoc test, F (1, 4) = 325.78, * p < 0.05). Table 1 summarizes the data of hGDNF ELISA. Undiluted hMSCs G/G + Dox conditioned medium contained significant levels of hGDNF (93.45 ± 2.83 ng/ml). In addition, changes in the concentrations of hGDNF in conditioned medium were correlated with serial dilutions of conditioned medium. Detectable levels of hGDNF were not observed in undiluted hMSCs conditioned medium, and only very low levels of hGDNF (0.13 ± 0.00 ng/ml) were detected in undiluted hMSCs G/G – Dox conditioned medium.

As shown in Fig. 7A, the treatment with 6-OHDA for 16 h resulted in a dose-dependent decrease of SH-SY5Y cell viability (one-factor ANOVA followed by Fisher’s post hoc test, F (8, 36) = 259.70, * p < 0.05). At a concentration of 100 μM, 6-OHDA...
reduced the cell viability by about 50%, when compared to the vehicle control. We therefore used the concentration of 100 μM of 6-OHDA in the following experiments. To examine the effect of hMSCs G/G + Dox conditioned medium from transduced hMSCs in the presence of Dox alone on SH-SY5Y cell viability, SH-SY5Y cells were treated with the conditioned medium at serial concentrations ranging from 1:1 to 1:128 for 16 h (Fig. 7B). Interestingly, the treatment with hMSCs G/G + Dox conditioned medium at concentrations of both 1:1 (36.61 ± 5.23%) and 1:2 (72.65 ± 1.61%) reduced cell viability of SH-SY5Y cells when compared to the control (one-factor ANOVA followed by Fisher’s post hoc test) (* p < 0.05). The treatment with hMSCs G/G + Dox conditioned medium against 6-OHDA-induced cell death. The data suggest that hMSCs G/G + Dox conditioned medium protected SH-SY5Y cells (* p < 0.05) from 6-OHDA-induced cytotoxicity in SH-SY5Y cells. The treatment with hMSCs G/G + Dox conditioned medium protected dopaminergic cells in cultures.

It is known that the withdrawal of serum from the cultures provokes a relatively rapid degeneration of DA neurons over a few days [41,42]. We used this serum-free culture system to examine potential neuroprotective effects of hMSCs G/G + Dox conditioned medium on DA neurons. The results of TH immunocytochemistry showed that the treatment of hMSCs G/G + Dox conditioned medium improved the survival of DA neurons (Fig. 7D) (one-factor ANOVA followed by Fisher’s post hoc test) (* p < 0.05). We therefore used hMSCs G/G + Dox conditioned medium at the concentration of 1:8 in the following experiments. Similar to the treatment with GDNF protein in the culture medium (67.60 ± 2.47%), the co-treatment of hMSCs G/G + Dox conditioned medium increased cell viability of SH-SY5Y in the presence of 6-OHDA (66.07 ± 2.89%) (Fig. 7D) (one-factor ANOVA followed by Fisher’s post hoc test) (* p < 0.05). In contrast, the treatment with Dox, hMSCs and hMSCs G/G + Dox conditioned medium failed to protect SH-SY5Y cells against 6-OHDA-induced cell death. The data suggest that hMSCs G/G + Dox conditioned medium increases cell viability of SH-SY5Y cells, and prevents from 6-OHDA-induced cytotoxicity in SH-SY5Y cells.

| Conditioned hGDNF (ng/ml) | Medium | Undiluted | 1:80 | 1:160 | 1:320 |
|---------------------------|--------|----------|------|-------|-------|
| hMSCs                     | 0      | -        | -    | -     | -     |
| hMSCs G/G + Dox           | 0.13 ± 0.03 | -        | -    | -     | -     |
| hMSCs G/G + Dox           | 93.45 ± 2.83 | 1.05 ± 0.03 | 0.59 ± 0.01 | 0.27 ± 0.01 |

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Figure 4. The copy numbers of hGDNF and hrGFP mRNA as determined by quantitative real-time PCR. Transduced hMSCs were incubated in the presence (grey bar, On hGDNF and black bar, On hrGFP) or absence (grey bar, Off hGDNF and black bar, Off hrGFP) of Dox (100 ng/ml) for 7 days. Total RNA was extracted from the cells using the Qiagen Rneasy kit. Human β-actin was used as an internal control to normalize the results of hGDNF and hrGFP mRNAs in each sample. Values represent the mean ± standard error of the mean (SEM).

Figure 5. Differentiation of transduced hMSCs. Transduced hMSCs were incubated in either adipogenic or osteogenic differentiation medium in the presence of doxycycline. The scale bar = 50 μm. Values represent the mean ± standard error of the mean (SEM).
cells in the cultures when compared to those in the cultures incubated in the Control medium \( (p > 0.05) \).

**Discussion**

In the present study, we generated a binary Tet-On lentivirus vector system based on two lentivirus vectors: a bicistronic vector expressing both hGDNF and hrGFP genes under a TRE-minimal CMV promoter, and a vector expressing rtTA2S-M2 under a minimal CMV promoter. We further characterized this system in hMSCs as examined inducibility of transgene expression, background expression, and function of transgene product, hGDNF. The results show that hMSCs transduced with inducible lentivirus vectors efficiently express both the hGDNF and hrGFP transgenes in the presence of Dox. The background expression of the two genes in transduced hMSCs is very low in the absence of Dox. Conditioned medium from cultures of transduced hMSCs in the presence of Dox protects SH-SY5Y cells from 6-OHDA-induced toxicity, and improves the survival of dopaminergic neurons from ventral mesencephalic (VM) tissue in vitro.

GDNF has proved to be a potent neurotrophic factor for DA neurons [1–4]. However, several studies have also demonstrated that continuous overexpression of GDNF mediated by virus vectors results in adverse side effects for DA system in the brain [8,26,27]. It has been shown that lentivirus-mediated long-lasting overexpression of the GDNF gene in the striatum results in aberrant sprouting and downregulation of TH activity in lesioned nigrostriatal dopamine neurons [26]. For the intact striatum, long-term over-expression of the GDNF gene can lead to downregulation of TH activity [8,27]. In addition, Winkler et al. showed that continuous exposure to GDNF for nigral grafts in the striatum impaired graft-mediated function recovery [43]. A gene regulation system is therefore required to control GDNF levels for GDNF therapy. Although much effort has been made to improve inducible lentivirus vectors, lentivirus-mediated gene regulation is still unsatisfactory. By injecting inducible lentivirus vectors harboring the GDNF gene into the striatum, Georgievska et al., reported that GDNF gene expression was regulated with the tetracycline-regulated lentivirus vector system associated with a significant background leakage [38]. In an ex vivo gene delivery study, Behrstock et al. showed that transplantation of human neural progenitor cells transduced with Tet-Off lentivirus vectors carrying the GDNF gene did not lead to down-regulation of GDNF in response to doxycycline in the striatum of a rat model of PD [13]. Inability of gene regulation may represent a big obstacle for lentivirus vectors to be viable virus vectors in clinical application.

In the present study, we confirmed and extended the previous finding and showed that the modified binary Tet-On lentivirus vector system tightly regulated hGDNF and hrGFP transgene expression with a little leakage in the absence of Dox. The modifications to the binary Tet-On lentivirus vector system may attribute to the efficient inducibility of transgene expression and low background expression [29]. As the intact U3 region in the viral LTR of lentivirus vectors has promoter or enhancer elements which may attribute to background expression of transgenes, the inducible lentivirus vectors were made SIN by deleting 400 bp from the U3 region of the 3′ LTR [44]. The 1.2-kb chicken β-globin insulator element cHS4 was incorporated into the inducible lentivirus vectors at the site of the U3 deletion. Previous studies have demonstrated that cHS4 element reduces background expression in inducible adenovirus and adenov-associated virus vectors [45,46]. In addition, the second-generation TREs (TRE/Pitt) were incorporated into the transgene expression vector. Agha-
Mohammadi et al. showed that the minimal CMV promoter and tetO sequences were positioned in an optimized manner relative to the start of the transcript [39]. Indeed, by dose-dependent study, a very low dose of Dox (0.1 ng/ml) induced the hrGFP gene to express in both HeLa cells and hMSC transduced with binary Tet-On lentivirus vectors. The data from FCM, GDNF ELISA, and qRT-PCR confirmed that transduction of hMSCs with the binary Tet-On lentivirus vector system resulted in tightly regulation of hGDNF and hrGFP transgene expression. The expression of hGDNF and hrGFP transgenes in transduced hMSCs was efficiently switched “On” and “Off” by the addition or removal of Dox. With support to the previous study, our binary Tet-On lentivirus vector system induced hGDNF transgene expression in transduced hMSCs in the presence of Dox up to 719-fold above background levels as measured the levels of hGDNF protein in conditioned medium. In the “On – Off” experiment, we noticed that a certain period of time was required to wash out transgene expression after the removal (Off) of Dox. The changes in reduction of hGDNF transgene expression seemed to be slower than those in reduction of hrGFP transgene expression. The difference between GFP and GDNF gene regulation was also observed in a previous study in which a Tet-Off lentivirus vector system was used to transduce human neural progenitor cells [13]. In their system, gene regulation was very efficient for the marker gene, GFP expression, but GDNF transgene expression was only partially switched off in response to doxycycline in vitro. The properties of transgene products may contribute to this difference. GDNF has a long half-life and GFP is an unstable protein. In the present study, transduced hMSCs were able to be induced to differentiate into other cell types, adipogenic and osteogenic cells.

Figure 7. Effects of conditioned medium on SH-SYSY cells against 6-hydroxydopamine (6-OHDA)-induced toxicity. Cell viability was measured by MTT assay and presented as percentage of the control. (A) Cells were treated with 6-OHDA at serial concentrations ranging from 20 to 160 μM for 16 hours. Cell viability was reduced in a dose-dependent manner. (B) Effects of conditioned medium from transduced hMSC cultures in the presence of Dox (hMSC G/G+Dox) on cell viability of SH-SYSY cells. (C) Concentration-dependent effects of conditioned medium from hMSC G/G + Dox on 6-OHDA-induced toxicity for SH-SYSY cells. (D) Effects of various conditioned medium on 6-hydroxydopamine (6-OHDA)-induced toxicity for SH-SYSY cells. A one-factor analysis of variance (ANOVA) followed by Fisher’s post hoc test was applied to make group comparisons. * p<0.05 versus the control group. Dox, Dox (12.50 ng/ml, equal to the levels of Dox in diluted hMSC G/G + Dox medium used in the experiment) in DMEM/F12; hMSCs, medium from untransduced hMSCs cultures; hMSCs G/G – Dox, medium from transduced hMSCs cultures in the absence of Dox; hMSCs G/G + Dox, medium from transduced hMSCs cultures in the presence of Dox; GDNF, GDNF (11.68 ng/ml, equal to the levels of GDNF in diluted hMSCs G/G + Dox medium, 1:8, used in the experiment) in DMEM/F12.

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added conditioned medium. The cultures were immunostained for PLOS ONE | www.plosone.org 8 May 2013 | Volume 8 | Issue 5 | e64389
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switched from DMEM embryos of Sprague-Dawley rats. After 2 days in culture, medium was added conditioned medium. The cultures were immunostained for PLOS ONE | www.plosone.org 8 May 2013 | Volume 8 | Issue 5 | e64389
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DMEM/F12 diluted hMSCs G/G medium containing Dox (12.50 ng/ml, equal to the levels of Dox in the experiment) in DMEM/F12+N2. The scale bar = 50 μm.
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sugesting that transduction of our binary Tet-On lentivirus vectors carrying both the hGDNF and hrGFP genes did not alter the cell properties of hMSCs. The current study showed that transduced hMSCs were stable over multiple passages, and consistently released the great levels of hGDNF into the medium. This makes it possible to bank and characterize the cells prior to transplantation. It is also worthy of note that the bicistronic lentivirus vector allows the simultaneous expression of the hGDNF and hrGFP genes. Expression of the marker gene, hrGFP is significant for basic research because it facilitates the purification of transduced cells to get a large quantity of the cells in vitro by cell sorting prior to transplantation, and tracing implanted cells in the brain.

The function of hGDNF in conditioned medium was examined in two cell culture model systems: SH-SY5Y cells exposed to 6-OHDA and nigral DA neurons in serum-free culture conditions. GDNF has been well documented to protect DA cells against 6-

OHDA-induced toxicity, possibly through the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) signaling pathways [47–49]. The withdrawal of serum from the VM tissue cultures can provoke rapid degeneration of DA neurons [41,42]. Under serum-free conditions, glia-mediated trophic support to DA neurons is virtually lost as the majority of glial cells are selectively removed from total surviving cells in cultures. In addition, DA neurons are believed to be dependent on the antioxidant glutathione system within glial cells. The loss of glial cells under serum-free conditions may accelerate the degeneration of DA neurons. Accumulation of free radicals in DA neurons leads to the gradual consumption of endogenous antioxidants, leading to an excess of reactive species to damage the cells. Numerous studies have shown that GDNF promotes the survival, cell density and differentiation of cultured VM neurons [50–52]. Sawada et al. showed that GDNF exerted neuroprotection against apoptosis through the PI3K signaling pathway and the subsequent up-regulation of Bcl-2 and Bcl-x [53]. Here we demonstrated that significant levels of hGDNF protein were detected in the hMSCs G/G + Dox conditioned medium. The treatment of the hMSCs G/G + Dox conditioned medium protected SH-SY5Y cells against 6-OHDA-induced cell loss as measured by MTT assay, and promoted the survival of cultured nigral DA neurons as examined by cell body area, number of neuritis and branching points, and proportion of TH-IR cells. The neuroprotection of the hMSCs G/G + Dox conditioned medium was similar to that in the GDNF control, suggesting that hGDNF in the hMSCs G/G + Dox conditioned medium plays a critical role in neuroprotection. The treatment of the hMSCs G/G – Dox conditioned medium failed to protect both SH-SY5Y cells and cultured nigral DA neurons, further confirming that the background expression of hGDNF gene was very low, if there was any. It is noted that hMSCs without virus transduction do not have neuroprotective effects. Although several studies have reported that hMSCs can produce a number of cytokines and growth factors [54,55], we speculate that hMSCs-produced growth factors may not be sufficient enough to protect cells in our cell model system.

In summary, we developed an inducible cellular hGDNF gene delivery system which can tightly regulate hGDNF and hrGFP transgene expression with a little leakage of the system in the absence of Dox. hGDNF released from transduced hMSCs is functional although further studies are warranted to examine the mechanisms underlying the neuroprotection of hGDNF. It is imperative to examine if the system works in vivo. Our inducible cellular hGDNF gene delivery system may provide useful tools for basic research on gene therapy for chronic neurological disorders such as PD.

Materials and Methods

Plasmids and virus vector production

In a previous study, Pluta et al. developed an improved binary Tet-On system based on two lentivirus vectors, transfer vectors harboring the EGFP gene under second-generation TRE and rtTA2S-M2 vectors carrying a gene encoding advanced rtTA - rtTA2S-M2 under a human minimal CMV-IE promoter with efficient regulation of transgene expression [29]. In the present study, we modified this system and generated a binary Tet-On lentivirus vector system to deliver both the hGDNF and hrGFP genes (Fig. 1). Transfer plasmid, pNL-TRE/Pit-hGDNF-IRES-hrGFP-U3 was constructed by removing a BamHI/BstGI fragment containing the EGFP sequence and replacing it with a BamHI/Xhol fragment containing hGDNF-coding sequence derived from pLVT-hGDNF-rTRKRA2SM2, provided by Dr.
Aebischer (Lausanne, Switzerland) [34], and a XhoI/SmaI fragment containing an internal ribosome entry site (IRES)-hrGFP sequence derived from pIRES-hrGFP (Stratagene, USA), as shown in Figure 1. This bicistronic lentivirus vector allows the simultaneous expression of the hGDNF and hrGFP genes. The key element of these vectors is an optimized IRES which permits two genes of interest to be co-expressed as separate proteins from a single mRNA transcript. rtTA2S-M2 plasmid, pNL-CMV-IE-rtTA2S-M2-DU3 was also prepared. Three plasmids, the transfer or rtTA2S-M2 plasmid, pCMVdR8.74 and pMD2VSV.G plasmids (provided by Dr. Didier Trono, Lausanne, Switzerland) were used for packaging lentivirus vectors. The lentivirus vectors were prepared by the transient transfection of 293T cells using the calcium phosphate precipitation method [40].

Cell culturing and in vitro transduction
HeLa cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and hMSCs [56] [kindly provided by Dr. Darwin J. Prockop at Center for Gene Therapy, Tulane University Health Sciences Center, New Orleans, USA] were maintained in DMEM supplemented with 16.5% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin at 37 °C under an atmosphere of 5% CO₂ and 95% air. Tet-free FBS (Clontech, USA) was used in the experiments to reduce the background leak of transgene expression. We transduced HeLa cells and hMSCs with both pNL-TRE/Ptt-hGDNF-IRES-hrGFP-AU3 vectors and pNL-CMV-IE-rtTA2S-M2-AU3 lentivirus vectors at a ratio of 1:2. The concentration of pNL-TRE/Ptt-hGDNF-IRES-hrGFP-AU3 vectors was a multiplicity of infection (MOI) of 5 and pNL-CMV-IE-rtTA2S-M2-AU3 vectors 10. Virus particles were washed away with a replacement of new culture medium after 8 h transduction. To examine dose-dependent expression of hrGFP transgene in HeLa cells and hMSCs, Dox in serial concentrations (ranging from 10⁻⁴ to 10⁻⁵ ng/ml) was added to cultures 8 h post-transduction. hrGFP expressing cells were examined 4 days after Dox treatment using FCM. To examine the efficiency of regulated transgene expression by a binary Tet-On lentivirus vectors, “On-Off” and “Off-On” treatment groups were designed. In the “On-Off” group, transduced hMSCs were first incubated in the presence (On) of Dox (100 ng/ml) for 4 days, and then in the absence (Off) of Dox with a replacement of culture medium for additional 3, 6 and 10 days. In the “Off-On” treatment group, transduced hMSCs were first incubated in the presence (On) of Dox (100 ng/ml) for 4 days, and then in the absence (Off) of Dox with a replacement of culture medium for additional 3, 6 and 10 days. At desired time points (0, 4, 7, 10, and 14 days after the first treatment with or without Dox), transduced hMSCs were prepared for FCM analysis to determine the MFI units of hrGFP. Transduced hMSCs were also prepared for qRT-PCR to determine mRNA copy numbers for hGDNF and hrGFP at 4 days. In addition, conditioned medium was collected from transduced hMSCs in the presence or absence of Dox for hGDNF ELISA (Promega, USA) to determine protein levels of hGDNF in culture medium at 0, 4, 7, 10, and 14 days. After collecting culture medium at each time point, fresh culture medium was added to cultures.
FCM analysis
Transduced HeLa cells and hMSCs in cultures were digested with 0.25% trypsin/EDTA (Sigma, USA) and centrifuged at 1000 rpm for 5 min, the supernatant was then removed. The cells were washed twice with phosphate buffered saline (PBS) and adjusted to a concentration of 1×10^6 cells/ml for FCM analysis to determine the MFI units for hrGFP or hrGFP' cell counts.

qRT-PCR
The copy number of hGDNF mRNA was determined by qRT-PCR with the RNA samples isolated from transduced hMSCs. Total RNA was extracted using the Qiagen Rneasy kit (P/N 74104), then treated twice with DNase TURBO DNA-free (Ambion, Austin, TX). 2 µg of total RNA was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). TaqMan PCR was conducted with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA) in a total volume of 50 µl. The PCR mix contained 25 µl of 2× TaqMan Universal PCR Master Mix, 10 pmol each of the forward and reverse primers and probe. The sequences of primers and probe specific for hGDNF were as follows: forward primer, 5'-CTGACTTGAGTGTGGCTATG-3'; reverse primer, 5'-TTGTCACTCAACCGGTCTATTT-3'; probe, 5'-TGCGATGCTGAGACACGTACG-3'. Human β-actin was used as an internal control to normalize the results of hGDNF and hrGFP mRNAs in each sample (forward primer: 5'-ACCTGATGAGGAGATTGCTTCG-3'; reverse primer: 5'-AGGCGGGTGATGCTCTACTT-3'; probe: 5'-CAAAGGGCCGAACCTTCACCATGAC-3'). The protein levels of hGDNF in conditioned media (hMSCs G/G + Dox, hMSCs G/G + Dox, and hMSCs) were determined using hGDNF ELISA (Promega, USA) according to the manufacturer’s instructions. Briefly, a ninety-six well ELISA plate was coated with 100 µl of buffer containing 0.1 µl anti-GDNF monoclonal antibody for overnight at 4°C. The wells were then blocked with 200 µl of 1 × block & sample buffer for 1 hour at room temperature. 100 µl of diluted GDNF standard protein and conditioned medium (1:80, 1:160, 1:320 of hMSCs G/G + Dox, and no dilution of the other groups) were added to wells with shaking for 6 hours at room temperature. The wells were washed and incubated with 100 µl of anti-GDNF polyclonal antibody (1:500) overnight at 4°C. The wells were washed and incubated with 100 µl of HRP-conjugated anti-chicken IgY for 2 hours at room temperature, then washed and developed with TMB micropate plate reader. The levels of hGDNF protein were presented as the optical density (OD) value.

SH-SY5Y cell culture and 6-hydroxydopamine (6-OHDA) treatment
SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS at 37°C under an atmosphere of 5% CO2 and 95% air. Culture medium was changed twice a week. To insult SH-SY5Y cells, stock solution of 6-OHDA (5 mM) were prepared in cold saline containing 0.15% ascorbic acid and used for all experiments. To determine an appropriate dose of 6-OHDA, we first performed dose-dependent cell viability assays. SH-SY5Y cells were treated with 6-OHDA at different concentrations (from 0.1 µM to 160 µM) for 16 hours. To examine effects of hMSCs G/G + Dox conditioned medium on cell viability of SH-SY5Y cells, the cells were treated with hMSCs G/G + Dox conditioned medium with serial dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) for 16 hours. To examine which concentrations of hMSCs G/G + Dox conditioned medium protected SH-SY5Y cells against 6-OHDA-induced toxicity, SH-SY5Y cells were co-incubated with 6-OHDA (100 µM) and hMSCs G/G + Dox conditioned medium at serial concentrations for 16 hours. To examine specificity of protective effects for hMSCs G/G + Dox conditioned medium, SH-SY5Y cells were co-treated with 6-OHDA (100 µM) and various conditioned media (hMSCs, hMSCs G/G + Dox, hMSCs G/G + Dox) for 16 hours. In addition, DMEM/F12 containing GDNF (GDNF, 11.68 ng/ml, equal to the levels of GDNF in diluted hMSCs G/G + Dox medium, 1:8, used in the experiments), DMEM/F12 containing Dox (Dox, 12.50 ng/ml, equal to the levels of Dox in diluted hMSCs G/G + Dox medium used in the experiment) and DMEM/F12 were also used as controls. Cells viability of SH-SY5Y cells was determined by the conventional 3, [4,5-
dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) assay (Sigma, USA).

Determination of cell viability

SH-SY5Y cells viability was determined by MTT using a MTT assay kit according to the manufacturer’s instructions. Briefly, SH-SY5Y cells were plated at a density of 1.0 × 10^5 cells/well in a 96-well plate, pre-coated with poly-D-lysine (Trevigen, USA). Cells were treated with the MTT solution (final concentration, 0.5 mg/ml) for 4 hours at 37°C. The medium was replaced with 100 μl of DMSO for each well. The formazan dye crystals were solubilized for 30 min, and absorbance at 570 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

Preparation of ventral mesencephalic tissue cultures

All animal procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Use and Care Committee of Capital Medical University, Beijing, China. The number of animals used was the minimum required for statistical analysis, and all precautions were taken to minimize animal suffering. All surgery was performed under sodium pentobarbital anesthesia (100 mg/kg body weight, i.p.), and animals were sacrificed with an overdose of sodium pentobarbital (200 mg/kg body weight, i.p.) in the end of experiments. Pregnant Sprague-Dawley (SD) rats at gestation day 14 were obtained (Vital River Laboratory Animal Technology, China). VM tissue was dissected from the brain of embryos on ice and trypsinized into single-cell suspension with 0.25% trypsin/EDTA for 10 minutes at 37°C. The cells were re-suspended in DMEM using sterilized micropipette tips, supplemented with 10% FBS and plated at a final density of 2 × 10^5 viable cells/well in four-well chamber slides (Thermo Scientific Nunc Lab-Tek II Chamber Slide, USA). The chamber slides were pre-coated with 500 μl of poly-D-lysine (20 μg/ml) for 2 hours at 37°C. The single cells were incubated at 37°C under atmosphere of 5% CO_2 and 95% air for 2 days. To examine the functional effects of hGDNF released by transduced hMSCs in the presence of Dox, the medium was switched to new serum-free DMEM/F12 supplemented with N_2 medium. At this time point, hMSCs G/G + Dox, hMSCs G/G – Dox, hMSCs, GDNF, Dox, or DMEM/F12+N2 conditioned medium was added to VM tissue cultures for additional 7 days in vitro.

Immunocytochemistry

VM tissue cultures were fixed at day 7 by 4% paraformaldehyde in PBS for 20 minutes at room temperature. The avidin-biotin complex immunoperoxidase technique was used to visualize TH immunoreactivity. The primary antibody used was against TH (rabbit polyclonal antibody, 1:300, Santa Cruz Biotechnology). The secondary antibody was biotinylated goat anti-rabbit (1:200, Vector Laboratories, Burlingame, CA, USA). Cells were incubated in ABC solution (Vectastain ABC Elite kit, Vector Laboratories, USA) followed by development with 3,3'-diaminobenzidine solution (Vectastain DAB kit, Vector Laboratories, USA) to visualize immunoreactivity. To evaluate the specificity of immunostaining, normal goat serum was used instead of a primary antibody, or primary antibody was omitted during the immunostaining as a negative control.

Morphological assessment

TH-immunostained cell cultures were examined at 200× and 400× magnification using light microscope (Nikon, Japan) with bright field illumination in a blinded manner. The original codes of the slides, which indicated treatment groups, were covered by opaque tape and the slides were re-numbered. After evaluation, the original codes were revealed. TH-IR cells in cultures were examined in a 400 μm² ocular grid which was randomly placed at 4 predetermined sites of each well. TH-IR cells were only evaluated in an ocular grid which covers a field containing at least 4 TH-IR cells. TH-IR cells in about 36 fields were examined for each treatment group. By 200× magnification, the number of neurites and branching points for dendrites were counted, and cell body area of TH-IR cells were quantified using HCImage software (HCImage, 3.0, Hamamatsu, Japan). By 400× magnification, the number of TH-IR cells and total cells in a field was counted, and the proportion of TH-IR cells in the total cells was calculated and presented as a percentage of the control without conditioned medium.

Statistical analysis

For all in vitro experiments, at least three replicas per group were used in each experiment. Data were collected from at least three independent experiments, and were presented as the mean ± standard error of the mean (SEM). All data were subjected to statistical analysis using StatView software. A one-factor ANOVA followed by Fisher’s post-hoc test was used for group comparisons. Statistical significance was defined as P<0.05.

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Author Contributions

Conceived and designed the experiments: WMD. Performed the experiments: WHY CY YQX TL WMD. Analyzed the data: WHY CY YQX JR LRZ WMD. Wrote the paper: WMD.

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