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Short report

Lmx1a enhances the effect of iNSCs in a PD model

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Abstract Lmx1a plays a central role in the specification of dopaminergic (DA) neurons, which potentially could be employed as a key factor for trans-differentiation to DA neurons. In our previous study, we have converted somatic cells directly into neural stem cell–like cells, namely induced neural stem cells (iNSCs), which further can be differentiated into subtypes of neurons and glia in vitro. In the present study, we continued to test whether these iNSCs have therapeutic effects when transplanted into a mouse model of Parkinson's disease (PD), especially when Lmx1a was introduced into these iNSCs under a Nestin enhancer. iNSCs that over-expressed Lmx1a (iNSC-Lmx1a) gave rise to an increased yield of dopaminergic neurons and secreted a higher level of dopamine in vitro. When transplanted into mouse models of PD, both groups of mice showed decreased ipsilateral rotations; yet mice that received iNSC-Lmx1a vs. iNSC-GFP exhibited better recovery. Although few iNSCs survived 11 weeks after transplantation, the improved motor performance in iNSC-Lmx1a group did correlate with a greater tyrosine hydroxylase (TH) signal abundance in the lesioned area of striatum, suggesting that iNSCs may have worked through a non-autonomous manner to enhance the functions of remaining endogenous dopaminergic neurons in brain.

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Introduction

Induced neural stem cells (iNSCs) are reprogrammed cells that possess features like bona fide neural stem cells (NSCs). In 2012, we have reported that mouse somatic cells—sertoli cells, can be directly converted to iNSCs by introduction of 8 transcription factors (Sheng et al., 2012a), which provides further evidence that somatic cells can be induced to not only pluripotent stem cells (PSCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007), mature neurons (Vierbuchen et al., 2010; Pfisterer et al., 2011; Caiazzo et al., 2011; Kim et al., 2011), but also to adult stem cells. The above findings support the concept that the fates of any two different lineages are interchangeable, given sufficient and necessary conditions.

In our previous work, we have shown that iNSCs converted from sertoli cells demonstrate the characteristics of NSCs in vitro, such as self-renewal, differentiation to neurons and glia, and similar gene expression profiles, etc. After transplantation into the native area of neurogenesis—dentate gyrus in adult mouse brain, iNSCs can survive and further mature to neurons. However, whether iNSCs have therapeutic efficacy in neurodegenerative disease models, for example, Parkinson's disease (PD), has not been tested yet.

Lmx1a is a key determinant in the specification of dopaminergic (DA) neurons (Deng et al., 2011; Andersson et al., 2006). When exogenously expressed in embryonic stem (ES) cells, LMX1a markedly enhances the efficiency for DA neuron production (Friling et al., 2009). We thus speculate that expression of Lmx1a in iNSCs may further increase their capacity to differentiate to DA neurons. In this study, we aim to investigate whether iNSCs are safe and/or efficacious in a mouse PD model, and whether expression of Lmx1a in iNSCs can further enhance the therapeutic efficacy.

Materials and methods

Cell culture

iNSC 1-9, an induced neural stem cell line, trans-differentiated from sertoli cells from neonatal C57BL/6 mice, has been described in our previous work (Sheng et al., 2012a). In this study, iNSC 1-9 was firstly labeled by lentiviral vectors expressing GFP – FUGW (Addgene, Cambridge, MA), and then infected with lentiviral vectors pNestin-Lmx1a-2A-BlastR (pNestin-GFP-2A-BlastR as control) (Fisher Scientific, Pittsburgh, PA). After 1 week of selection with 6 μg/ml blasticidin (Invitrogen, Carlsbad, CA), the surviving cells were replated on PDL (Sigma-Aldrich, St Louis, MO)/Laminin (Roche, Mannheim, Germany)-coated plates in N2B27 medium plus 20 ng/ml bFGF and EGF (both from Peprotech, Rocky Hill, NJ) (Ying et al., 2003). iNSC 1-9 FUGW pNestin-Lmx1a (iNSC-Lmx1a) and iNSC 1-9 FUGW pNestin-GFP (iNSC-GFP) at passage 27 were used for karyotype analysis as previously described (Sheng et al., 2012a).

PCR analysis

To confirm the integration of exogenous LMX1A, PCR-amplification of genomic DNA extracted from iNSC-Lmx1a was performed using primers specific for the transgenic Lmx1a sequences (Forward 5′- CCTCAGTGGATGTTGCCTTTA-3′; Reverse 5′- GCTCCCGTTCTTTCTCATAG-3′). Genomic DNA extracted from iNSC-GFP was used as a negative control.

In vitro differentiation

2500 iNSC-Lmx1a or iNSC-GFP cells were plated on PDL/Laminin-coated 24-well plates. After exposure to 100 nM SAG (Enzo, Farmingdale, NY), 100 ng/ml FGF-8 (Peprotech) and 10 ng/ml bFGF (Peprotech) for 2 days, the culture medium was switched to N2B27 medium with 20 ng/ml BDNF (Peprotech) and 200 nM ascorbic acid (AA, Sigma-Aldrich).

Immunofluorescence

On the 6th or 14th day of differentiation, iNSC-Lmx1a and iNSC-GFP cells were washed and fixed by 4% paraformaldehyde (Sigma-Aldrich). For immunofluorescence staining, cells were washed and blocked by 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) in 0.3% Triton-TBS for 1 h, and incubated with primary antibodies overnight at 4 °C followed by secondary antibody staining for 2 h at room temperature. The primary antibodies used were rabbit anti-tyrosine hydroxylase (TH) (1:1000, Millipore, Billerica, MA), mouse anti-β III tubulin (Tuj-1) (1:1000, Millipore), mouse anti-NeuN (1:500, Millipore). The secondary antibodies were cy3-conjugated donkey anti-rabbit and cy5-conjugated donkey anti-mouse (both 1:400, Jackson ImmunoResearch). After nuclear staining with DAPI (Sigma-Aldrich), pictures were captured by using Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany).

To compare the capacity to differentiate to DA neurons, 3 or 9 areas on each coverslip were randomly selected for imaging using the same confocal settings. The proportions of TH-positive cells were calculated by using Image J software (National institutes of health, USA).

Animal experiments

All the animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals established by Beijing Association for Laboratory Animal Science. Unilateral PD mouse model was performed as previously described (Tabar et al., 2008): eight week old male C57BL/6 mice were used to establish PD models by 6-hydroxydopamine (6-OHDA, Sigma-Aldrich) administration. Mice were injected intraperitoneally (i.p.) with 25 mg/kg desipramine (Sigma-Aldrich) 20 min before anesthetization with ketamine and Xylazine (Sigma-Aldrich), followed by injection of 8 μg 6-OHDA in 2 μl saline into the right striatum (A/P 0.5 mm, M/L −2.3 mm, D/V −3.2 mm). Four weeks after 6-OHDA injection, the mice were i.p. injected with 5 mg/kg amphetamine (Sigma-Aldrich), and the number of ipsilateral turns per min (every 5th min in 1 h) was scored. Mice with stable lesions (>8 turns/min) were selected for transplantation studies.

Five weeks after 6-OHDA injection, stable PD mice were randomly divided into 4 groups for engraftment experiments, iNSC-Lmx1a (n = 9), iNSC-GFP (n = 9), sertoli cells (n = 8), and sham control (n = 7) groups. After being cultured in N2B27 medium with 100 nM SAG (ENZO),

100 ng/ml FGF-8 (Peprotech) and 10 ng/ml bFGF (Peprotech), 8×10^5 iNSC-Lmx1a or iNSC-GFP cells, suspended in 4 μl transplantation buffer (5 g/l glucose (Sigma-Aldrich) and 100 ng/ml bFGF (Peprotech) in HBSS (Life technologies, Grand Island, NY)), were injected into the right side of striatum (A/P 0.5 mm, M/L −2.1 mm, D/V −3.4 mm). Sertoli cells isolated from newborn C57BL/6 mice were labeled with FUGW virus and engrafted into striatum. In sham control group, only buffer was injected.

Behavioral tests were performed in the 2nd, 4th, 6th and 10th week after transplantation. The results were expressed as ipsilateral rotations minus contralateral rotations per min. Videos were recorded by EOS 550D (Canon, Tokyo, Japan) or DSC-V1 (Sony, Tokyo, Japan) digital camera, and edited by Final Cut Pro 10.9 (Apple, Cupertino, CA).

Histological analysis

Eleven weeks after transplantation, all mice were perfused with 4% paraformaldehyde and brains were taken out, followed by dehydration in 30% sucrose (Sigma-Aldrich) and sliced coronally at 40 μm thickness. The slices were stained by using the following primary and secondary antibodies: rabbit anti-TH (1:500, Millipore), sheep anti-TH (1:500, Millipore), rat anti-dopamine transporter (DAT) (1:5000, Millipore), Cy3-conjugated donkey anti-sheep, Cy3-conjugated donkey anti-rat and Cy5-conjugated donkey anti-rabbit (all 1:200, Jackson ImmunoResearch).

To evaluate the effect of engrafted cells on the remaining endogenous DA neurons, we measured the signal intensity of TH in the right striatum (lesioned and engrafted) relative to that of the left striatum (no lesion or graft) using the same confocal settings.

HPLC

In vitro HPLC was performed as previously described (Roy et al., 2006; Ren et al., 2013). On the 7th and 14th day of differentiation, 500 μl supernatant from iNSC-Lmx1a, iNSC-GFP and mouse embryonic fibroblast (MEF) cultures was collected. After addition of 7 μl perchloric acid (7 N, Sigma-Aldrich), the supernatant was stored at −80 °C for future use. For HPLC assay, the supernatant was centrifuged at 12,000 *g*, 4 °C for 15 min, and 50 μl supernatant was used for examining levels of dopamine and DOPAC. Each sample was injected using a 542 automatic injector (Thermo Scientific, Chelmsford, MA), and separation was achieved on Zorbax ODS (C-18) reverse-phase column (150 mm × 4.6 mm, 5 μm) (Agilent, Santa Clara, CA). Mobile phase containing 90% of 70 mM sodium acetate, 50 mM citric acid, 0.1 mM EDTA, 0.2 mM 1-octanesulfonic acid sodium salt, and 10% methanol (adjusted to pH 4.1 with NaOH 10 M) (all from Sigma-Aldrich), was pumped at 0.1 ml/min through the C-18 column connected to the electrochemical detectors (5600A, Thermo Scientific). There were 3 biological replicates for each group at every time point and the experiments were repeated twice.

Statistical analysis

The results were expressed as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 5 (Graphpad software, La Jolla, CA) or PASW Statistics 18 software (IBM, Armonk, NY). Three or more groups were compared by using ANOVA. Pair wise comparisons were made by using Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Generation of iNSC-Lmx1a

iNSCs 1-9, a cell line that shows similar in vitro and in vivo functions as naturally existing NSCs, were generated from our previous work (Sheng et al., 2012a,b). To explore whether *Lmx1a* can promote the differentiation of iNSCs into DA neurons, and enhance its therapeutic efficacy in mouse PD models, iNSC 1-9 were first labeled with FUGW, a GFP-expressing lentivirus, followed by infection with pNestin-Lmx1a lentivirus (or its control virus), which encodes *lmx1a* (*GFP* in control vector) and a blasticidin-resistant gene under Nestin enhancer (Fig. S1A). To examine whether pNestin-GFP virus works, normal NSCs were infected with the virus and stained for nestin and GFP. About 21% of nestin-positive cells were also GFP-positive (Fig. S1B). After 6 days of selection with 6 μg/ml blasticidin, many surviving cells were found in pNestin-Lmx1a and pNestin-GFP groups (Fig. S2A). Surviving cells showed normal morphology and karyotype, and could proliferate as monolayer or spheres (Fig. 1A–C). More than 95% of these cells were GFP-positive (Fig. 1D). Exogenous lmx1a integration was confirmed by genomic PCR analysis (Fig. S2B). The established cell lines were herein named iNSC-Lmx1a and iNSC-GFP.

Lmx1a enhances DA neuron production from iNSCs in vitro

To test the efficiency of DA neuron production, equal numbers of iNSC-Lmx1a or iNSC-GFP cells were treated for 2 days with SAG and FGF8, followed by differentiation for 6 or 14 days with BDNF and AA. TH-positive cells differentiated from iNSC-Lmx1a and iNSC-GFP cells expressed Tuj-1, and DA neurons from iNSC-Lmx1a were also positive for NeuN (Fig. S3). On the 6th or 14th day of differentiation, the proportion of TH-positive cells was measured. More TH-positive cells were found in iNSC-Lmx1a group (8.686 ± 1.391% on the 6th day, 15.99 ± 2.104% on the 14th day) vs. iNSC-GFP group (4.877 ± 0.7151% on the 6th day, 5.271 ± 0.8127% on the 14th day, $p < 0.05$ on the 6th day, $p < 0.001$ on the 14th day, Fig. 2A).

We also examined the concentrations of dopamine and its metabolite DOPAC in culture supernatant from iNSC-Lmx1a, iNSC-GFP and MEF cells (Fig. 2B). On the 7th, and 14th day of differentiation, the concentrations of dopamine and DOPAC in iNSC-Lmx1a group were higher than those in iNSC-GFP group, and dopamine and DOPAC were hardly detectable in MEF supernatant (Fig. 2C).

iNSC-Lmx1a graft improves the behavioral performance of PD mice

To test the therapeutic effect, iNSC-Lmx1a, iNSC-GFP, sertoli cells or buffer were introduced into the right side of striatum

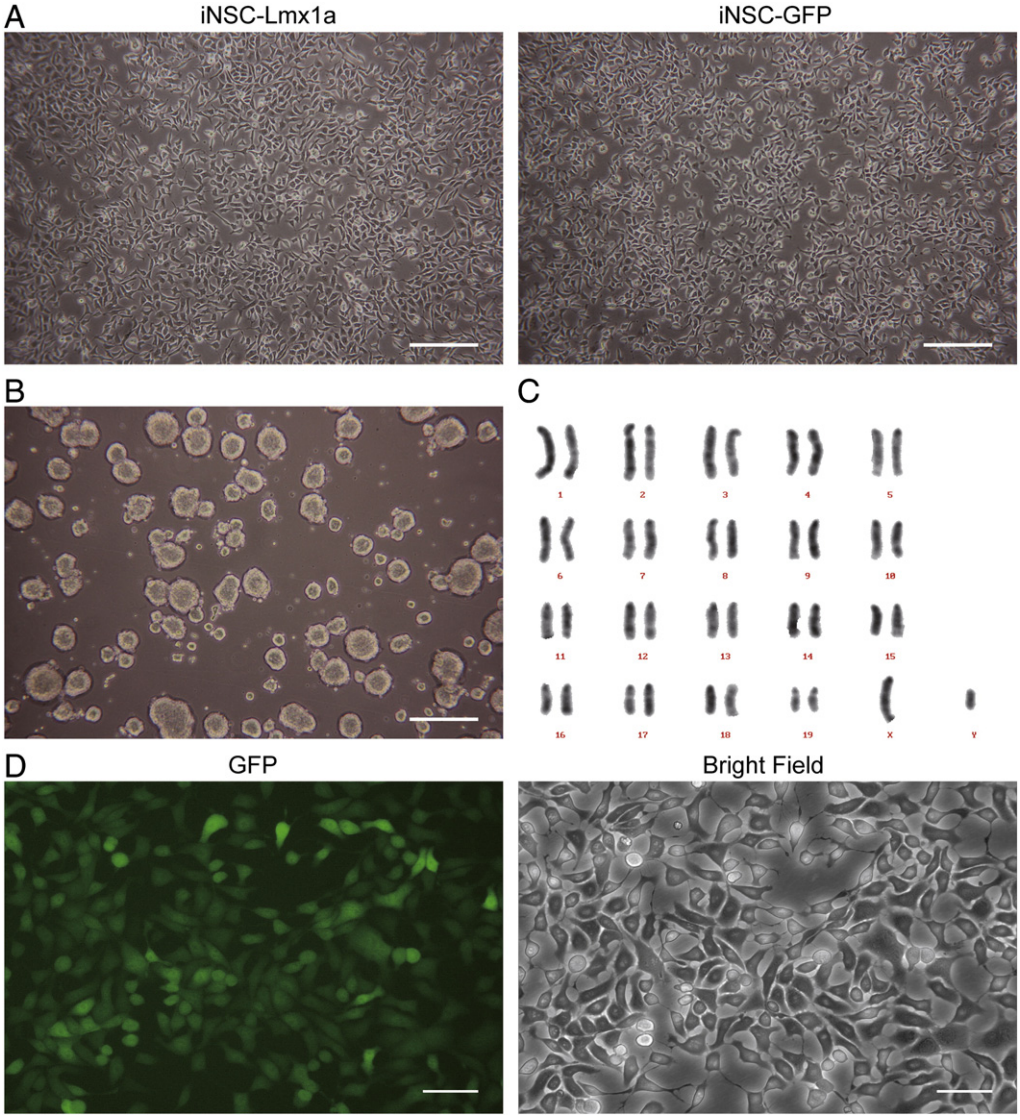

Figure 1 Morphology of iNSC-Lmx1a and iNSC-GFP cells. A: Monolayer culture of iNSC-Lmx1a and iNSC-GFP at passage 28. B: Suspension culture of iNSC-Lmx1a at passage 26. C: iNSC-Lmx1a cells (passage 27) show normal male mouse karyotype. D: iNSC-Lmx1a cells under fluorescent and bright field. More than 95% (261 of 273) of iNSC-Lmx1a cells are GFP-positive. Scale bars: 200 μm (A and B), and 50 μm (D).

(the same side of 6-OHDA injection) of the PD mice (Fig. 3A). All mice survived transplantation and behavioral tests were performed at 2, 4, 6, and 10 weeks thereafter. At 2 weeks post-transplantation, the numbers of ipsilateral turns were significantly reduced in iNSC-Lmx1a and iNSC-GFP groups, but not in sertoli or buffer groups (Fig. 3B and Table 1). At 4 weeks, the effects from iNSC-Lmx1a and iNSC-GFP started to differentiate, and both iNSC groups still showed improved performance compared to that of control groups (Fig. 3B and Table 1). At 10 weeks, similar trends existed as at 4 weeks, with iNSC-GFP groups performing better than buffer group, and iNSC-Lmx1a better than iNSC-GFP group, suggesting that iNSCs may have enhanced the nigro-striatum functions by an autonomous and/or non-autonomous manner (Videos 1 and 2). Interestingly, the numbers of ipsilateral turns showed a trend of decline even in buffer group (Fig. 3B), probably reflecting an adaptation to amphetamine treatment and/or ageing-associated behavioral change.

We next tried to investigate the mechanisms underlying the behavioral improvement. Eleven weeks after engraftment, the mice were sacrificed and brain slices examined. No overgrowth of graft was found in any mice; rather, very few engrafted cells had survived. On each of the 6th consecutively sliced section, only one or two TH-positive cells were found in the striatum receiving graft (Fig. 3C), and these TH-positive cells were positive for GFP and DAT (Fig. S4). Given the small number of surviving cells, the chance was slim that the behavioral changes were caused by the differentiation and integration of incoming iNSCs exerting functions by participating in the wiring circuits in charge of motor behavior.

The other possibility was that the transplanted cells may have produced neurotrophic or other soluble factors that

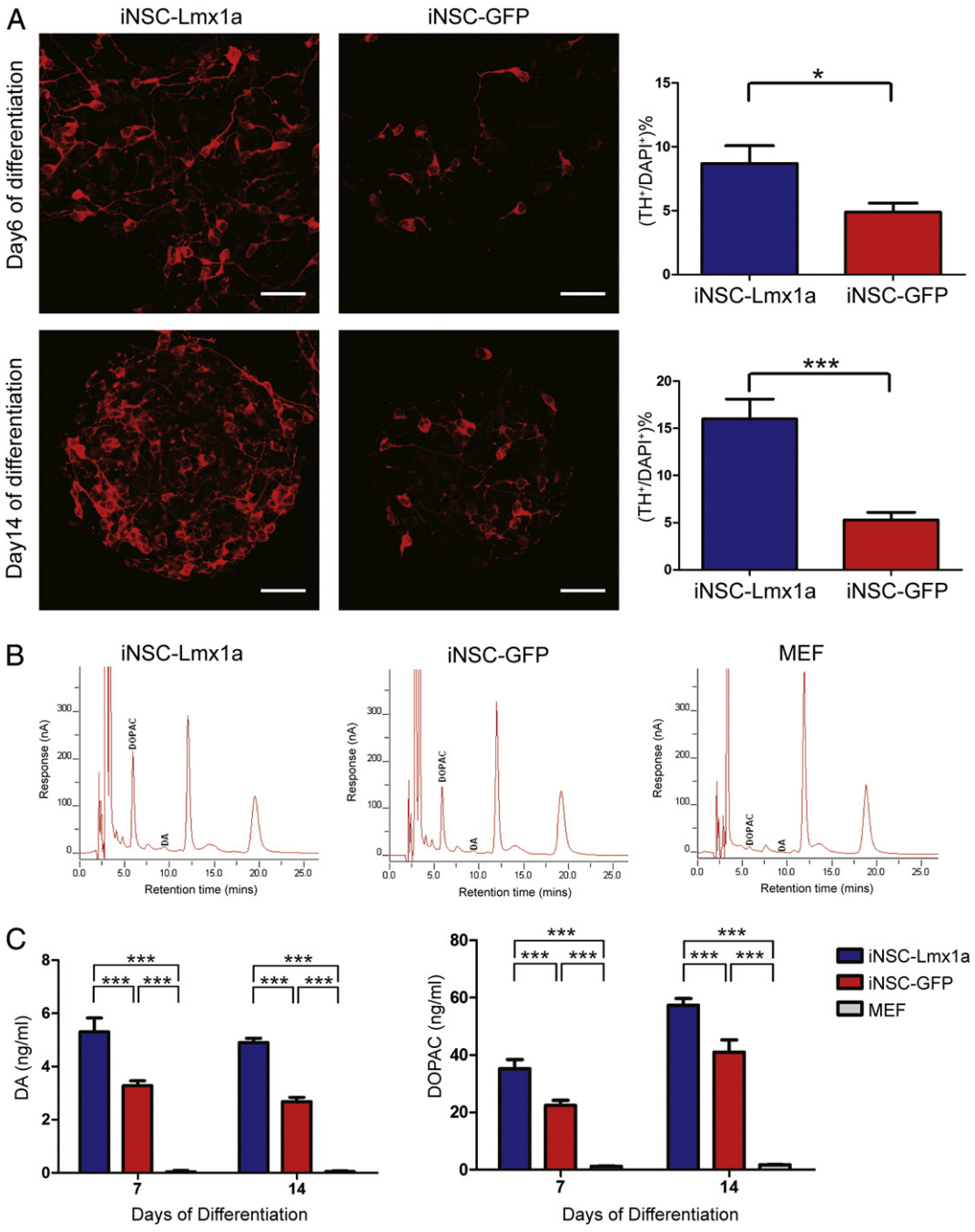

Figure 2 Lmx1a promotes differentiation of iNSCs into DA neurons in vitro. A: The proportion of TH-positive cells in iNSC-Lmx1a group was significantly higher than that in iNSC-GFP group on the 6th day and 14th day of differentiation. Data were represented as means ± SEM, $n = 18$ (6th day of differentiation) and 9 (14th day of differentiation). *, $p < 0.05$; ***, $p < 0.001$. B: HPLC chromatograms of supernatant from MEF, iNSC-GFP or iNSC-Lmx1a culture (24 h incubation) on the 14th day of differentiation. C: DA and DOPAC concentrations in MEF (gray), iNSC-GFP (red) or iNSC-Lmx1a (blue) culture supernatants (24 h incubation) on the 7th or 14th day of differentiation. Data were represented as means ± SEM in all the figures of this study unless specifically noted, $n = 6$; ***, $p < 0.001$. Scale bars: 40 μm (A).

could enhance the functions of the endogenous DA neurons. Following 6-OHDA injection, the TH signal abundance was markedly reduced in the dorsal lateral quadrant of the striatum where lesion was made (Fig. 3D), which may correlate with the decreased capacity to produce dopamine and subsequently the compromised motor performance. We hypothesized that if the transplants worked by augmenting the nigro-striatum circuit, the TH signals would have been changed accordingly. Indeed, the relative TH signal abundance in the lesioned quadrant normalized to its counterpart in the left striatum was significantly higher in iNSC-GFP group, and was even higher in iNSC-Lmx1a group (Fig. 3E). We plotted

the TH signal abundance against the behavioral results for each mouse and found a negative correlation between the ipsilateral turns and TH signals ($p = 0.05$, $R^2 = 0.122$, Fig. 3F), indicating that the restored capacity to produce dopamine, probably resulted from engraftment, may account for the improved motor behavior.

Discussion

In this study, we continued our previous work and examined the safety and therapeutic efficacy of iNSCs in a mouse PD model. We found that iNSCs grafts are safe and can improve the motor performance of PD mice, and expression of Lmx1a

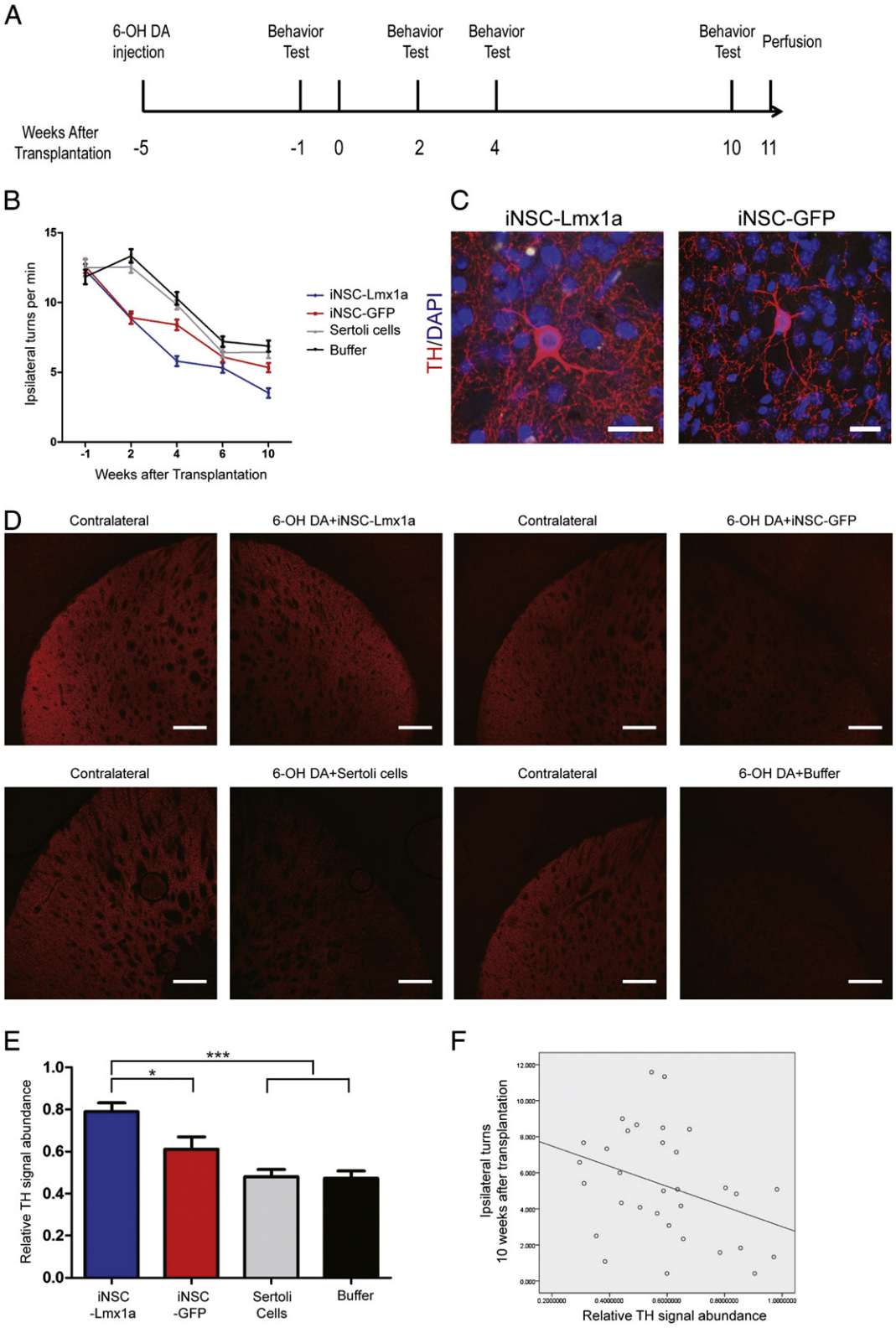

Table 1 Statistical analysis of behavioral tests.
Results of behavioral tests for PD model mice transplanted with iNSC-Lmx1a, iNSC-GFP, sertoli cells or buffer at different time points were analyzed by two-way ANOVA. P values were showed. NA, non-significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Weeks after transplantation	Groups					
	iNSC-Lmx1a VS Buffer	iNSC-Lmx1a VS Sertoli cells	iNSC-Lmx1a VS iNSC-GFP	iNSC-GFP VS Buffer	iNSC-GFP VS Sertoli cells	Sertoli cells VS Buffer
−1	NS	NS	NS	NS	NS	NS
2	***	***	NS	***	***	NS
4	***	***	***	**	NS	NS
6	**	NS	NS	NS	NS	NS
10	***	***	**	*	NS	NS

NS, non-significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

at neural stem cell stage can further enhance the therapeutic efficacy. iNSCs have worked probably by restoring the functions of the endogenous DA neural circuits.

Since the report of iNSCs in 2012 (Sheng et al., 2012a,b), several other groups have also published studies on iNSCs, using different starter cells and reprogramming factors (Kim et al., 2014; Cheng et al., 2014a; Cheng et al., 2014b; Cheng, 2014; Maucksch et al., 2012; Mitchell et al., 2014; Zou et al., 2014; Han et al., 2012; Lujan et al., 2012). Nevertheless, none of those studies had tested the efficacy of iNSCs in a PD model. To our knowledge, the current study is the first to provide proof-of-principle that iNSCs exert therapeutic effects in a PD model. We understand that sertoli cells which iNSCs were derived from in this study are not readily accessible in clinics and the number of exogenous factors may be further reduced using different starter cells; yet the present study could be a good beginning on the long way towards clinical applications of iNSCs. Future work is needed to test if more clinically relevant cells, for example, iNSCs derived from human fibroblasts or blood cells converted with fewer factors in a non-integrative manner, would work in small and large PD animal models.

The iNSCs used in this study had the same genetic background as the recipient PD mice, and should not elicit adaptive immune recognition in host (Chen et al., 2011; Chen and Palmer, 2008). The low survival rate of grafts may reflect a relatively vulnerable feature of this cell line, compared to iPSC-derived DA precursor cells (Wernig et al., 2008), which, however, have an inherent problem of tumorigenicity. In our previous work (Sheng et al., 2012a; Sheng et al., 2012b), iNSCs without patterning by SAG and FGF8 were deposited at the native neurogenic area—dentate gyrus of naïve mice, and showed a decent survival rate. The poor survival observed in the current study may be due to a lack of supportive niche in striatum, and/or the two-day patterning period decreasing the capacity of the iNSCs to survive transplantation.

As expected, mice receiving iNSC-Lmx1a demonstrated better behavioral results than did mice receiving iNSC-GFP. In vitro data showed that iNSC-Lmx1a yielded a higher percentage of DA neurons and a higher level of dopamine secretion. One of the direct causes of the motor symptoms in PD patients is the progressive depletion of dopamine supply in striatum (Buttery and Barker, 2014). Dopamine replenishment has been shown to alleviate the motor problems. For example, levodopa—the drug that has been used in clinics for years and shown good therapeutic effects in the first few years of treatment, is the precursor to dopamine and can be converted to dopamine once it enters the central nervous system (Marsden and Parkes, 1976). Transplantation of fetal ventral mesencephalon into the striatum of PD patients can enhance dopamine levels and improve motor functions in some patients (Olanow et al., 2003; Freed et al., 2001; Lindvall et al., 1990). In the current study, although only few TH-positive cells had survived by the end of the experiment (11 weeks post-transplantation), it is possible that more cells were surviving at earlier time points and the replenishment of dopamine by the engrafted cells may have accounted for the improved motor behavior. Another possibility is that the engrafted cells had directly or indirectly produced neurotrophic factors that could nourish the endogenous DA neurons and enhance their functions. GDNF is a well-studied factor that has a robust neurotrophic effect on DA neurons (Lin et al., 1993). Astrocytes express dopamine receptor D1 and upon activation by dopamine, astrocytes may produce GDNF (Kuric et al., 2013) that is

Figure 3 Lmx1a enhances the beneficial effects of iNSC in improving motor behavior recovery in PD mice and in restoring TH signal abundance in the engrafted striatum. A: Schematic representation of in vivo study. B: Ipsilateral rotations of PD mice transplanted with iNSC-Lmx1a (blue), iNSC-GFP (red), sertoli cells (grey) or buffer (black) 4 weeks after 6-OH DA injection (one week before cell transplantation), and 2, 4, 6 and 10 weeks after transplantation. The numbers represented ipsilateral minus contralateral rotations. $n = 9$ in iNSC-Lmx1a group; $n = 9$ in iNSC-GFP group; $n = 8$ in sertoli cell group; and $n = 7$ in buffer group. C: TH-positive neurons differentiated from iNSC-Lmx1a or iNSC-GFP cells were found in the transplantation area in striatum. Bar = 20 μm. D: Representative pictures showing the TH signal abundance in the dorsal lateral quadrant of striatum relative to its contralateral counterpart. E: Relative TH signal abundance in the dorsal lateral quadrant of striatum relative to its contralateral counterpart. $n = 9$ in iNSC-Lmx1a group; $n = 9$ in iNSC-GFP group; $n = 8$ in sertoli cell group; and $n = 7$ in buffer group. *, $p < 0.05$; ***, $p < 0.001$. F: Scatter plots showing the correlation between relative TH signal abundance and the ipsilateral turns at 10 weeks post-transplantation. After one outlier was removed, ipsilateral turns negatively correlated with the relative TH signal abundance (simple regression, $p = 0.05$). Scale bars: 20 μm (C) and 250 μm (D).

beneficial to the endogenous DA neurons. Another possible effector is BDNF, which also has a protective role on DA neurons (Spina et al., 1992; Hyman et al., 1991). Both NSCs and mature neurons can synthesize BDNF (Blurton-Jones et al., 2009; Conner et al., 1997; Legutko et al., 2001). In our present work, the greater abundance of TH signals in striatum at the axonal terminals of DA neurons may have possibly resulted from these non-autonomous effects from engrafted iNSCs.

In summary, this study for the first time showed that induced neural stem cells directly converted from somatic cells exerted therapeutic effect when transplanted into a mouse PD model, and this effect may have possibly resulted from iNSCs through a non-autonomous manner.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.10.004.

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