Enhancement of Polysialic Acid Expression Improves Function of Embryonic Stem-Derived Dopamine Neuron Grafts in Parkinsonian Mice

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**ABSTRACT**

There has been considerable progress in obtaining engraftable embryonic stem (ES) cell-derived midbrain dopamine neurons for cell replacement therapy in models of Parkinson’s disease; however, limited integration and striatal reinnervation of ES-derived grafts remain a major challenge for future clinical translation. In this paper, we show that enhanced expression of polysialic acid results in improved graft efficiency in correcting behavioral deficits in Parkinsonian mice. This result is accompanied by two potentially relevant cellular changes: greater survival of transplanted ES-derived dopamine neurons and robust sprouting of tyrosine hydroxylase-positive processes into host tissue. Because the procedures used to enhance polysialic acid are easily translated to other cell types and species, this approach may represent a general strategy to improve graft integration in cell-based therapies.

**INTRODUCTION**

The feasibility of experimental cell replacement therapy in Parkinson’s disease (PD) has been illustrated using human fetal tissue [1], and recent studies have greatly improved our ability to establish an unlimited source of engraftable, authentic midbrain dopamine (DA) neurons in vitro from human embryonic stem (ES) cells [2]. Although current protocols can provide significant results in small animal models, major challenges remain to improve clinical outcome. At the cellular level, these include the limited survival and fiber outgrowth of the grafted cells in host tissue. Because these challenges are likely to be more severe in the larger human striatum, their resolution is an important step toward the effective clinical application of the ES-derived DA cells. Improved fiber outgrowth and graft integration may also reduce the risks associated with multiple injections or poor distribution of DA neurons in vivo.

Regulation of cell interactions by polysialic acid (PSA) is an important factor in the promotion of cell translocation, axon outgrowth, and target innervation during vertebrate development [3]. Attached to the neural cell adhesion molecule, PSA is a large carbohydrate polymer that attenuates cell-cell interactions and thereby promotes tissue plasticity [3]. PSA remains active in only a few regions of the adult brain (e.g., the migration of neuronal precursors from the subventricular zone), but its upregulation has been shown to improve axonal growth through a glial scar [3, 4]. In view of the action of PSA in both the developing and adult central nervous systems, we reasoned that its enhanced expression on DA neurons would improve their integration after transplantation into the striatum. In this paper, we report that an engineered increase in PSA expression in purified mouse ES-derived DA neurons results in enhanced behavioral recovery in Parkinsonian mice and that this outcome is accompanied by increased graft cell survival and more extensive DA neuron fiber outgrowth into host striatum.

**RESULTS**

**Cell Transduction With the Polysialyltransferase Gene**

Expression of mammalian polysialyltransferase (PST) is known to be sufficient for PSA synthesis on a neural cell adhesion molecule [5, 6]. Green fluorescent protein (GFP) expression under control of the Nurr1 promoter in mouse ES cells allows purification of differentiated DA neurons for engraftment in the hemiparkinsonian mouse model [7]. Consequently, the mouse Nurr1 cell
line (Nurr1::GFP ES cells) was stably transduced with a lentiviral vector ubiquitously expressing PST, and blasticidin-resistant Nurr/PST subclones were selected. One line showed a dramatic increase in PST mRNA compared with controls (Fig. 1A) and was found to have an increase in PSA expression at day 14 of DA neuron differentiation (Fig. 1B). Treatment with the purified endoneuraminidase (endoN) specifically and completely cleaves PSA’s α-2,8-linked sialic acid polymers [8–10], and we found that preincubation with endoN was sufficient to abolish both the endogenous and induced PSA expression (Fig. 1B–1E). Furthermore, PST transduction did not affect expression of neuronal or midbrain markers in fluorescence-activated cell sorting-purified DA neurons (supplemental online Fig. 1), which were used for engraftment on further exclusion of any contaminating SSEA-1-positive cells (supplemental online Fig. 2).

**Increased PSA Expression Enhanced the Functional Efficacy of DA Transplant**

Previous studies in 6-hydroxydopamine-lesioned hemiparkinsonian mice (supplemental online Fig. 3) have shown that transplantation of ~100,000 ES-derived DA neuron precursors is required to produce robust functional recovery [7, 11] as measured by the amphetamine-enhanced rotation test. In the present study, we sought to graft a suboptimal number (55,000) of GFP-positive cells in order to scale for possible augmentations in motor behavior recovery on increased PSA expression. Without PST overexpression, the reduced graft size failed to produce a significant behavioral recovery (Fig. 2, black line). In contrast, with enhanced PSA expression, the same number of Nurr1/PST DA neurons resulted in a significant correction of PD behavioral impairment (p < .01; two-way analysis of variance [ANOVA]), with complete recovery ~5 weeks after surgery (Fig. 2, red line). Sustained PSA

Figure 1. PST overexpression resulted in increased levels of PSA on NCAM in differentiating mouse embryonic stem cells. (A): Quantification of PST mRNA by quantitative polymerase chain reaction in control cells (Nurr1) and in cells overexpressing PST (Nurr1/PST). Data are expressed as the fold enrichment of PST levels in Nurr1/PST versus Nurr1 cells. (B): PSA immunostaining in dopamine neuron cultures at day 14 of differentiation shows increased levels of PSA in Nurr1/PST cells (scale bar = 100 μm). (C): Western blot for NCAM in differentiated cells. Nurr1/PST cells (lane 2) show increased levels of the polysialylated form of NCAM (smear, brackets) compared with control (lane 1). PSA is removed from NCAM after endoN treatment (lane 3). (D): Quantification of the intensity of the PSA smear expressed in arbitrary units. (E): PSA-NCAM fluorescence-activated cell sorting analysis at day 14 of differentiation. Treatment of cells with 20 units of endoN, 24 hours before the end of differentiation, abolished the PST effect. Abbreviations: AI, arbitrary intensity; endoN, endoneuraminidase; NCAM, neural cell adhesion molecule; PSA, polysialic acid; α-tub, α-tubulin.

Figure 2. Nurr1/PST grafts are more effective at inducing behavioral recovery in a 6-hydroxydopamine mouse model. Nurr1::green fluorescent protein cells were differentiated and sorted at day 14 for green fluorescent protein-positive/SSEA-1-negative population. Cells treated with endoN were cultured for 12 hours before sorting with 20 units of the enzyme. There were 55,000 cells grafted in 1 μl of N2 media with BDNF and ascorbic acid. Animals scored for amphetamine-induced rotation (rotations per minute over 20 minutes) for 3 weeks prior to grafting, then for 7 weeks after. Nurr1/PST cells significantly improve the outcome compared with Nurr1 controls (two-way analysis of variance: p < .01, with Bonferroni post test; *, p < .05; **, p < .01; ***, p < .001; six animals per group). Removal of PSA by endoN abolishes the PST effect (p = .26). Abbreviations: endoN, endoneuraminidase; PST, polysialyltransferase.
removal from the injected cells, achieved by a combination of pretreatment with and coinjection of endoN, resulted in a decrease in this enhanced behavioral recovery (Fig. 2, red dotted line), indicating a PSA-specific effect following engraftment of the cells.

The PST Modified Cells Exhibit Increased Postgraft Survival Without Altering Their Phenotype

To examine the characteristics of the grafted cells, animals were processed for immunohistochemistry 2 months after transplantation. Nurr1/PST grafts displayed higher levels of PSA expression in vivo (Fig. 3A, 3B). There was also an approximately twofold increase in GFP-positive cell survival (Fig. 3C; 5,571 ± 1,008 vs. 2,480 ± 719 GFP-positive cells; p < .05, one-way ANOVA). With the endoN pretreatment and coinjection, cell counts were intermediate in average value but with greater spread than in accompanying readouts and thus were not significantly different from either the Nurr1 controls or the Nurr1/PST results (Fig. 3C). The percentages of cells expressing the midbrain DA markers tyrosine hydroxylase (TH) and Foxa2 within the graft core (supplemental online Fig. 4) were comparable for the Nurr1 and Nurr1/PST lines (Fig. 3D; TH: 62.0% ± 8.0% vs. 51.3% ± 7.0%, p = .33; Foxa2: 63.2% ± 8.6% vs. 55.4% ± 2.0%, p = .3). Similarly, the neuronal processes that emerged from the Nurr1 and Nurr1/PST cells had

Figure 3. The PSA augmentation improved graft survival without altering cell phenotype. (A): GFP, TH, and PSA immunofluorescence. Scale bars = 200 μm. (B): Ratio of PSA immunopositive cells among the GFP-positive population present at the core of the graft (**, p < .01; Student’s t test; n = 5 per graft type). (C): There were more GFP-positive cells in the Nurr1/PST graft at endpoint than in control (p < .05, one-way analysis of variance, with Dunnett’s post-test). Counts in Nurr1/PST and Nurr1/PST plus endoN samples were not statistically different. Values are mean ± SEM. (D): The percentage of midbrain dopamine neuron markers expressed in the Nurr1 and Nurr1/PST grafts were similar. Abbreviations: endoN, endoneuraminidase; GFP, green fluorescent protein; PSA, polysialic acid; TH, tyrosine hydroxylase.
comparable levels of TH, GIRK2 (formal gene name is Kcnj6) (supplemental online Fig. 4), and synapsin.

**PSA Enhancement Promoted Neurite Outgrowth From Grafted DA Neurons**

Unlike our previous studies with transplanted Schwann cells [12], enhanced PSA expression had little effect on the migration of DA neurons from the graft site; however, there were striking changes in neurite outgrowth. As shown in Figure 4A and supplemental online Figure 6, there were more DA neuron processes emerging from Nurr1/PST cells than from Nurr1 controls. To quantify this effect, the intensity of GFP and TH immunofluorescence was measured in five successive 100-μm zones away from the transplant. In order to compensate for the larger number of surviving cells in the Nurr1/PST grafts, as well as to more accurately assess the direct effect of PSA on neurites, we normalized the density of processes in each zone to that of the initial fiber segments observed in the most proximal zone to the graft core. This analysis confirmed that Nurr1/PST grafts had a much higher relative density of neurites.
of processes than the Nurr1 controls (Fig. 4B, 4C; p < .01 for both GFP and TH, two-way ANOVA). Exposure to endoN reversed the increase in neuronal process density observed in the Nurr1/PST grafts (p < .01, two-way ANOVA), demonstrating the specificity of the PSA effect (Fig. 4A, 4D).

Importantly, there was a strong correlation between graft function and the relative extent of GFP-positive fiber outgrowth, for example, into zone IV (Fig. 4E; p < .001, r² = 0.65, n = 17). This fiber outgrowth and behavioral relationship was consistent for all experimental groups (control, PSA enhanced, and endoN-treated). A synaptic marker, synapsin, consistently colocalized with axonal varicosities, which were increased in size and number by PSA enhancement, and this change correlated with functional outcome (supplemental online Fig. 5; p < .005, r² = 0.72). These correlations suggest that graft-host innervation is a key parameter for behavior recovery in this mouse Parkinsonian model.

**DISCUSSION**

The results obtained indicate three significant changes in the properties of the grafted PST-modified DA/Nurr1 neurons: (a) a more efficient amelioration of PD-associated behavior, (b) better cell survival, and (c) enhanced neurite outgrowth within the host tissue. Although these correlations do not directly prove that the cellular effects led to the altered behavior, it is reasonable to suggest that improvement of the survival and integration of the Nurr1 cells can contribute to the amelioration of the studied PD effects.

For the observed PSA-specific increase in DA fiber outgrowth, several mechanisms can be considered, including enhanced penetration of the zone of reactive glia encapsulating the graft core, increased sprouting and branching activity, more rapid and extensive process elongation, and prevention of premature connections in close proximity to the graft core. In fact, all of these possibilities are consistent with PSA’s known role in facilitating process outgrowth during normal development and in the adult nervous system (reviewed in [3]). The accompanying increase in DA neuron survival is also consistent with earlier findings on the role of PSA in the developing spinal cord [13], and it has been proposed that PSA can stimulate cell survival through a change in sensitivity to BDNF [14]. Because endoN treatment did not significantly reduce survival in Nurr1/PST grafts, it remains unclear whether the survival effects reflect a direct action of PST. In contrast, endoN did significantly affect both fiber outgrowth and behavioral outcome in the Nurr1/PST group, suggesting that those parameters are PSA dependent.

In future studies, it will be interesting to test whether endoN treatment also adversely affects behavioral outcome of grafted DA neurons that are not PSA enhanced. The current study did not address this point because we intentionally grafted suboptimal numbers [7] of DA neurons into animals with high rotational scores [15, 16] (>14 rotations per minute at grafting) and thus did not observe behavioral recovery in both the Nurr1 and Nurr1/endoN treated groups. We demonstrated that ES-derived Nurr1 neurons express low levels of PSA, which is cleaved by endoN treatment at the time of transplantation; however, the endoN effect is not permanent, and reappearance of PSA over time could complicate the interpretation of a long-term behavioral study.

The absence of an effect of PSA on cell migration is not surprising in this context. As in development, striatal tissue appears to be attractive for DA fibers rather than cell bodies. In addition, although the grafting procedure could have produced a glial scar-like structure that might inhibit migration, we have not previously observed a change in gliosis in lesioned spinal cord following PSA overexpression in astrocytes or Schwann cells [3, 4]. Similarly, we did not observe an obvious change in the glial reaction of Nurr1 versus Nurr1/PST grafts. Moreover, cells overexpressing PSA have been shown to be able to traverse a glial scar [12].

**CONCLUSION**

The present study offers a proof-of-principle demonstration of the use of engineered PSA in DA neuron grafting. Future studies will be needed to extend the results to other PD models including the nonhuman primate brain. This approach is attractive for future clinical translation because it involves a relatively straightforward manipulation of cells before transplantation. In principle, it can be combined with other methods that also provide survival and sprouting effects (e.g., viral [17–19] or polymer-encapsulated cell delivery of Gdnf [20]) for additive benefit. Because PSA is a generally permissive factor in these contexts, it is possible that, in the presence of PSA, such a powerful neurotrophic factor might cause excessive sprouting and possible physiological impairment [17–19]. For future studies, in addition to the genetic modification of cells, PST may be delivered directly to the cells by exposure to the purified enzyme and substrate [21]. Independent of the method of delivery, the cellular effects observed represent familiar axonal behaviors that are logically consistent with the behavioral improvements obtained. The implementation of the PSA strategy for human translation in PD grafting could minimize the need for multiple injections and thereby reduce the surgical risks they engender. Finally, this technology is readily adapted to other cell types and species; for example, it is under development for improving the migration of grafted Schwann cells in creating a bridge for regrowth of axons at the site of spinal cord injury [12].

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**AUTHOR CONTRIBUTIONS**

D.B.: conception and design, manuscript writing, data analysis and interpretation, collection and/or assembly of data; Y.G.: collection and/or assembly of data, conception and design, data analysis and interpretation; A.E.M.: collection and/or assembly of data, data analysis and interpretation; L.S.: conception and design, final approval of manuscript; U.R.: conception and design, financial support, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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