Brief Communications

Reducing Glypican-4 in ES Cells Improves Recovery in a Rat Model of Parkinson's Disease by Increasing the Production of Dopaminergic Neurons and Decreasing Teratoma Formation

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The heparan sulfate proteoglycan Glypican 4 (Gpc4) is strongly expressed in mouse embryonic stem (ES) cells where it controls the maintenance of self-renewal by modulating Wnt/β-catenin signaling activities. Here we show that mouse ES cells carrying a hypomorphic Gpc4 allele, in a single-step neuronal differentiation protocol, show increased differentiation into dopaminergic neurons expressing tyrosine hydroxylase (TH) and nuclear receptor related-1 protein (Nurr1) 1. In contrast to wild-type cells, these differentiating Gpc4-mutant cells expressed high levels of DOPA decarboxylase and the dopamine transporter, two markers expressed by fully mature dopaminergic neurons. Intrastriatal transplantation of Gpc4 hypomorphic cells into a 6-OHDA rat model for Parkinson's disease improved motor behavior in the cylinder test and amphetamine-induced rotations at a higher level than transplanted wild-type cells. Importantly, Gpc4 hypomorphic cell grafts, in contrast to wild-type cells, did not generate teratomas in the host brains, leading to strongly enhanced animal survival. Therefore, control of Gpc4 activity level represents a new potential strategy to reduce ES cell tumorigenic features while at the same time increasing neuronal differentiation and integration.

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. It is characterized by a progressive loss of midbrain dopamine (DA) neurons with a subsequent reduction in striatal DA (Olanow, 1999). Pharmacological treatment with L-DOPA temporarily ameliorates clinical symptoms but induces severe complications, such as dyskinesias (Pearce et al., 2001). Deep brain stimulation, although representing an efficient alternative treatment, can also have side effects (Olanow et al., 2000). Cell-therapeutic approaches in diseased humans have so far been based on the transplantation of fetal DA neurons, and clinical studies indicate that transplantation of these cells can induce symptomatic relief (Freed et al., 2001; Mendez et al., 2005). However, immunological, logistical, technical, and ethical difficulties have limited the development and application of this approach (Lindvall, 2012).

Embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, differentiated toward the midbrain dopaminergic fate *in vitro*, represent an alternative source for cell replacement therapy in PD (Björklund and Isacson, 2002; Kim et al., 2002; Perrier et al., 2004; Arenas, 2010). However, *in vitro* dopaminergic differentiation has to be finely controlled, and current protocols involving morphogens and drugs are complex and costly (Kriks et al., 2011). Moreover, the risk associated with uncontrolled tissue growth in the brain needs to be abolished (Björklund and Isacson, 2002; Isacson, 2003). Indeed, rodent studies have shown tumor formation after ES cell transplantation (Björklund and Isacson, 2002; Barberi et al., 2003) that was insufficiently reduced by the available differentiation protocols (Barberi et al., 2003) or cell sorting (Hedlund et al., 2008). Therefore, the use of ES cells and iPS cells for therapy in PD remains currently elusive.

The cell surface protein Glypican 4 (Gpc4), one of the most abundant heparan sulfate proteoglycans expressed by mouse ES cells, controls the maintenance of stem cell self-renewal (Fico et al., 2012). Remarkably, downregulation of Gpc4 in ES cells suppressed teratoma formation after grafting into the flank of nude mice. Moreover, ES cells with reduced expression levels for Gpc4 ($Gpc4^{gt-1}$) showed increased neuronal differentiation *in vitro* (Fico et al., 2012).

Here we explore the ability of Gpc4 mutant cells to decrease teratoma formation and improve neuronal differentiation in a PD cell-based therapeutic context. We show that $Gpc4^{gt-1}$ ES cells generate increased amounts of mature DA neurons *in vitro*.

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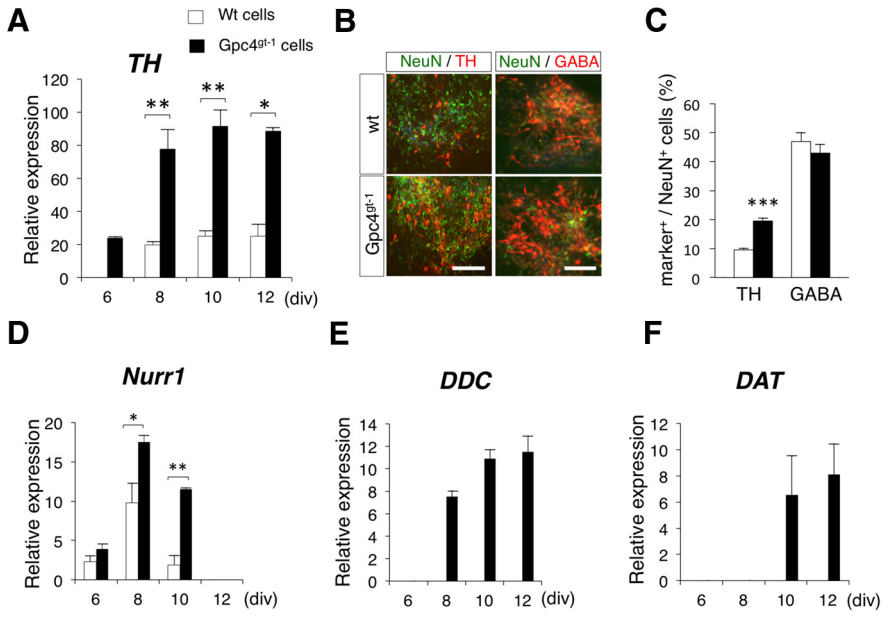

Figure 1. In vitro differentiation of *Gpc4*$^{gt-1}$ ES cells increases the number of DA neurons. ***A***, TH mRNA measured by qRT-PCR at different steps of wt and *Gpc4*$^{gt-1}$ ES cell differentiation. ***B***, NeuN and TH double immunostaining of *Gpc4*$^{gt-1}$ and wt cultures at 12 DIV. ***C***, Quantitative analysis of the proportion of TH$^+$ and GABA$^+$ cells among NeuN$^+$ neurons. Note the significant increase in TH$^+$ cells in the *Gpc4*$^{gt-1}$ condition (wt: 9.6 ± 0.6%; *Gpc4*$^{gt-1}$: 19.6 ± 1%). ***$p < 0.0001$ (Mann–Whitney test). ***D–F***, Expression of DA markers at different steps of wt and *Gpc4*$^{gt-1}$ ES cell differentiation, measured by qRT-PCR. mRNA expression was normalized to *GAPDH* expression. $n = 2$ experiments. *$p < 0.05$ (unpaired Student's *t* test). **$p < 0.01$ (unpaired Student's *t* test). Scale bars: ***B***, 100 μm (NeuN/TH); 50 μM (NeuN/GABA).

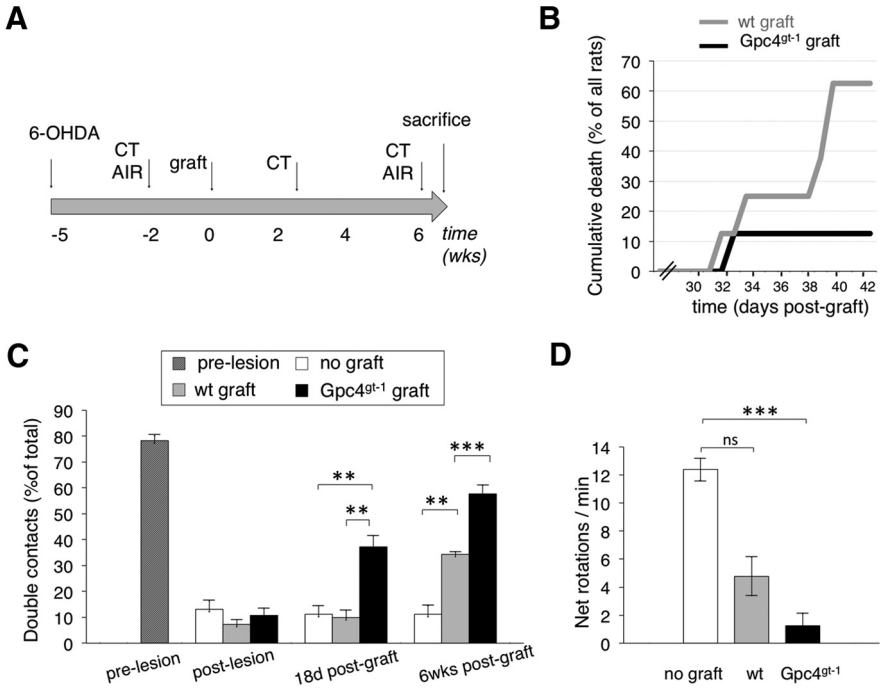

Figure 2. *Gpc4*$^{gt-1}$ ES cell grafts result in increased survival and behavioral improvements in parkinsonian rats. ***A***, Timeline of the experimental approach. CT, Cylinder test; AIR, amphetamine-induced rotations test; wks, weeks. ***B***, Graph reporting the cumulative death of rats after grafting. ***C***, Data presented in the graph are the mean ± SEM of the double contacts on the wall of the cylinder, expressed as the percentage of the total number of contacts. Note very consistent behavioral improvement in *Gpc4*$^{gt-1}$ after only 18 d after cell transplantation. Analyses were performed on $n = 7$ animals at time 0 and 18 d, and on $n = 3$ for wt graft and $n = 5$ for *Gpc4*$^{gt-1}$ graft at 6 weeks. **$p < 0.01$ (Mann–Whitney test). ***$p < 0.0001$ (Mann–Whitney test). ***D***, Amphetamine-induced rotational behavior in sham (no graft, white bar, $n = 8$), wt graft (gray bar, $n = 4$), and *Gpc4*$^{gt-1}$ graft animals (black bar, $n = 9$). ***$p < 0.0001$ (Kruskal-Wallis test). ns, not significant.

After transplantation of these cells in a rat PD model, dopaminergic differentiation *in vivo* was increased and accompanied by improved motor behavior. Moreover, *Gpc4*$^{gt-1}$ ES cells showed decreased teratoma formation, which correlated with enhanced animal survival.

Materials and Methods

In vitro *experiments*

ES cell cultures and differentiation. ES cell lines PST132 and E14Tg2a were previously described (Niwa et al., 2000; Fico et al., 2012). GFP was inserted in both ES cell lines at the Rosa26 locus by transfecting them with the R26P-SA-EGFP-puro plasmid (Addgene). Puromycin-selected ES cell clones were verified for correct self-renewal and differentiation properties and used as pools in all grafting experiments.

Cell lines were maintained on a monolayer of mitomycin-C-inactivated mouse fibroblasts in the presence of DMEM supplemented with 15% FBS, 100 μM 2-mercaptoethanol, 1 × MEM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/ml LIF ESGRO (Millipore Bioscience Research Reagents).

Neuronal differentiation used a serum-free neural induction protocol (Fico et al., 2008).

Immunofluorescence stainings and cell counts

Immunofluorescence stainings were done as described previously (Fico et al., 2012). Primary antibodies used were mouse anti-NeuN (Millipore, 1:100), rabbit anti-GABA (Sigma, 1:300), and rabbit anti-tyrosine hydroxylase (TH, Millipore, 1:300). Quantitative immunocytochemical analysis was performed on randomly selected visual fields from two independent differentiation experiments. On average, 10 visual fields, at 20× magnification were acquired and a total of 3000 NeuN$^+$ cells were counted per experiment.

RNA preparation and RT-PCR

Total RNAs were isolated using RNeasy mini kit (QIAGEN) and reverse transcribed using QuantiTect Reverse Transcription kit (QIAGEN) following the manufacturer's instructions. qRT-PCR was performed using SYBR Green PCR master mix (EuroClone). Primers: TH, sense (s) tgtcacgtccccaaggttcat, antisense (as) gggcaggccgggtctctaagt; nuclear receptor related-1 protein (Nurr1), (s) gcccgatgtgggacgat, (as) tctgctcgatcatatgcgtagtg; DOPA decarboxylase (DDC), (s) gaacagacttaacgggagccttt, (as) aatgccggtagtcagtgataagc; dopamine transporter (DAT), (s) gtgggcttcactgtcatcctca, (as) cccaggtcatcaatgccacga; GAPDH, (s) atggggaaggtgaaggtcg, (as) gggtcattgatggcaacaatatc; no. of cycles was in all cases 30.

In vivo *experiments*

6-Hydroxydopamine (6-OHDA) lesions. Male Wistar rats were lesioned by intranigral 6-OHDA injection as described previously (Oueslati et al., 2007). Lesioned animals were

selected for transplantation based on their response to amphetamine-induced rotational behavior and asymmetry score in the cylinder test to get homogeneous groups of animals.

Immunosuppression
Cyclosporine A (10 mg/kg Sandimmun, Novartis) in sunflower seed oil (Sigma, S5007) was injected daily starting 1 d before surgery.

Transplantation procedures
For transplantations, ES cells were prepared as described by Björklund et al. (2002). Briefly, the cells were seeded at 5×10^6 cells in the absence of LIF. ES cells did not adhere to the dish but instead formed embryoid bodies. Four days later, embryoid bodies were dissociated with trypsin. 6-OHDA-lesioned animals received injections of 1 μl of suspension (10,000 cells/μl) into two sites of the right striatum (bregma: anterior 1.0 mm, lateral 3.0 mm, ventral 5.0 and 4.5 mm). Two independent cell transplantation experiments were performed.

Behavioral tests
Cylinder test was performed as reported previously to assess akinesia-like deficit of the forepaw contralateral to the lesion (Oueslati et al., 2007; Jouve et al., 2010). Lesioned rats were tested for rotational behavior in response to amphetamine (4 mg/kg i.p., Sigma). Behavior was monitored by a computerized activity monitor system and analyzed as described previously (Björklund and Isacson, 2002).

Histological procedures, immunohistochemistry, and cell counting
Fifty-micrometer sections were prepared and labeled as described previously (de Chevigny et al., 2012). Antibodies included the following: anti-TH (Aves, 1:1000; or Immunostar 1:1600), anti-GFP (Aves; 1:500), anti-nestin (Aves; 1:1000), anti-DAT (Santa Cruz Biotechnology; 1:100), anti-Pitx3 (gift from Austin Smith, 1:1000), anti-FoxA2 (Santa Cruz Biotechnology; 1:100), anti-calbindin (Millipore; 1:1000), anti-GABA (Sigma; 1:300), anti-serotonin (5-HT) (Immunostar; 1:500), and anti- dopamine D2 receptor (DRD2) (Millipore/Abcam; 1:150; specificity of the antibodies was validated on sections at the level of the substantia nigra). Images were acquired with Nikon macroconfocal or with Zeiss 510 Meta/780 confocal microscopes.

Measures of the three largest graft areas for each animal were performed from Nikon macroconfocal images (5× lens, optical zoom ×3) using ImageJ. TH cell density was counted semiautomatically using ImageJ on every sixth section for each rat.

Evaluation of FoxA2, Pitx3, calbindin, and DAT expression in TH^+ cells was done on 63× Zeiss confocal microscope. Evaluation of GABA, 5-HT, and DRD2 positive cells was done on 20× Zeiss confocal microscope

Statistical analysis
Data are presented as mean ± SEM. *n* represents number of animals. Statistical tests are specified in figure legends (Instat software, Graph Pad Software). Differences were considered statistically significant when $p < 0.05$.

Results

Gpc4^{gt-1} hypomorphic ES cell cultures showed increased morphological and immunohistochemical signs of neuronal differentiation in a well-defined 12 d single-step neuronal differentiation protocol as previously reported (Fico et al., 2008). We further characterized the properties of Gpc4 loss-of-function cells under these culture conditions. TH, the rate-limiting enzyme for the synthesis of DA, was strongly induced (Fig. 1*A*). qRT-PCR showed that as early as at day 6 of *in vitro* differentiation, when TH transcripts were not detectable in wild-type (wt) cultures, mutant cells (*Gpc4*$^{gt-1}$) already expressed TH mRNA. Over the entire analyzed time window, TH expression remained approximately fourfold over controls (Fig. 1*A*).

The increased expression of TH in mutant cells could arise either from globally accelerated differentiation or from specifically enhanced DA neuron fate commitment. Quantitative analysis at 12 d *in vitro* (DIV) showed that in Gpc4 mutant cultures the proportion of TH^+ presumptive DA neurons among the NeuN-expressing neuronal population was doubled compared with wt cells (wt: 9.6 ± 0.6%; *Gpc4*$^{gt-1}$: 19.6 ± 1%; Fig. 1*B*,*C*). In contrast, the proportion of $GABA^+$ cells among the $NeuN^+$ neurons was unchanged (wt: 46.9 ± 3%; *Gpc4*$^{gt-1}$: 42.9 ± 3.%; Fig. 1*B*,*C*). We also stained for parvalbumin and Islet-1 but found too few positive cells in both conditions to allow for meaningful quantification. Together, these results indicate that Gpc4 loss-of-function promotes the specification toward the DA lineage rather than promoting differentiation globally.

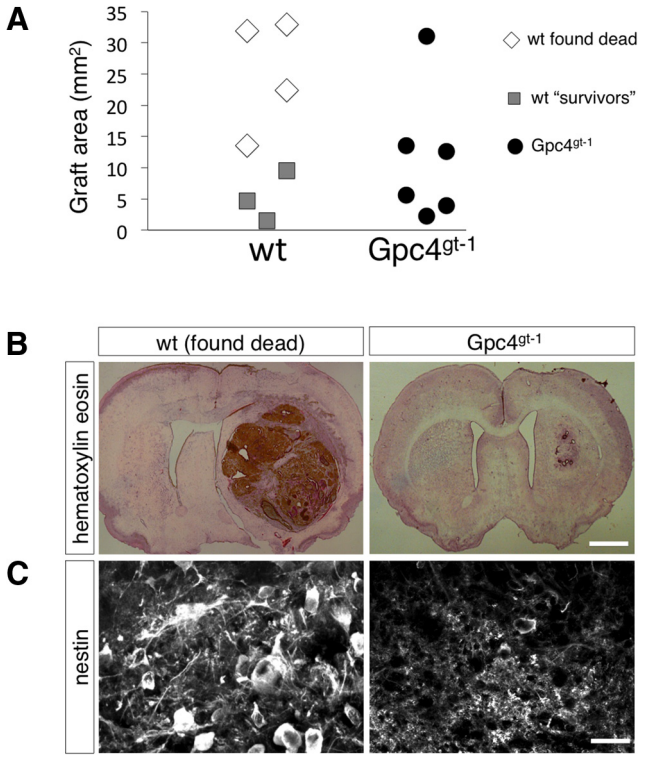

Figure 3. *Gpc4*$^{gt-1}$ ES cell grafts do not generate teratoma-like structures in parkinsonian rats. ***A***, Graph showing the mean area of wt dead before sacrifice (white), wt survivors (gray), and *Gpc4*$^{gt-1}$ (black) grafts (in square millimeters). Data were determined from the three largest graft sections. ***B***, Examples of hematoxylin/eosin staining of graft sections derived from wt and *Gpc4*$^{gt-1}$-transplanted animals. wt cells developed teratoma-like structures, in contrast to *Gpc4*$^{gt-1}$ hypomorphic cells. ***C***, Nestin expression was stronger in wt cells than in *Gpc4*$^{gt-1}$ hypomorphic cells. Scale bars: ***B***, 3 mm; ***C***, 20 μm.

We next analyzed the expression dynamics of midbrain DA neuron markers in *Gpc4*$^{gt-1}$cultures. Nurr1 displayed increased mRNA levels in *Gpc4*$^{gt-1}$ cells until 10 DIV and became undetectable at 12 DIV (Fig. 1*D*), thus showing similar dynamics as in the control situation, but at higher absolute levels. Interestingly, DDC, the last enzyme in the DA biosynthesis pathway, was strongly expressed in *Gpc4*$^{gt-1}$-derived cultures, whereas wt cells never expressed detectable mRNA levels (Fig. 1*E*). Finally, the DAT was strongly induced from 10 DIV in mutant cells but not detected in wt cultures (Fig. 1*F*). Thus, decreased expression of Gpc4 in differentiating ES cells leads to increased neuronal differentiation (Fico et al., 2012) and a strong shift toward the mature DA neurotransmitter phenotype, even in the absence of factors, such as SHH and FGF8, that are normally added to trigger DA differentiation (Ye et al., 1998).

As *Gpc4*$^{gt-1}$ ES cells (1) generate mature dopaminergic neurons *in vitro* and (2) reduce tumor formation after flank trans-

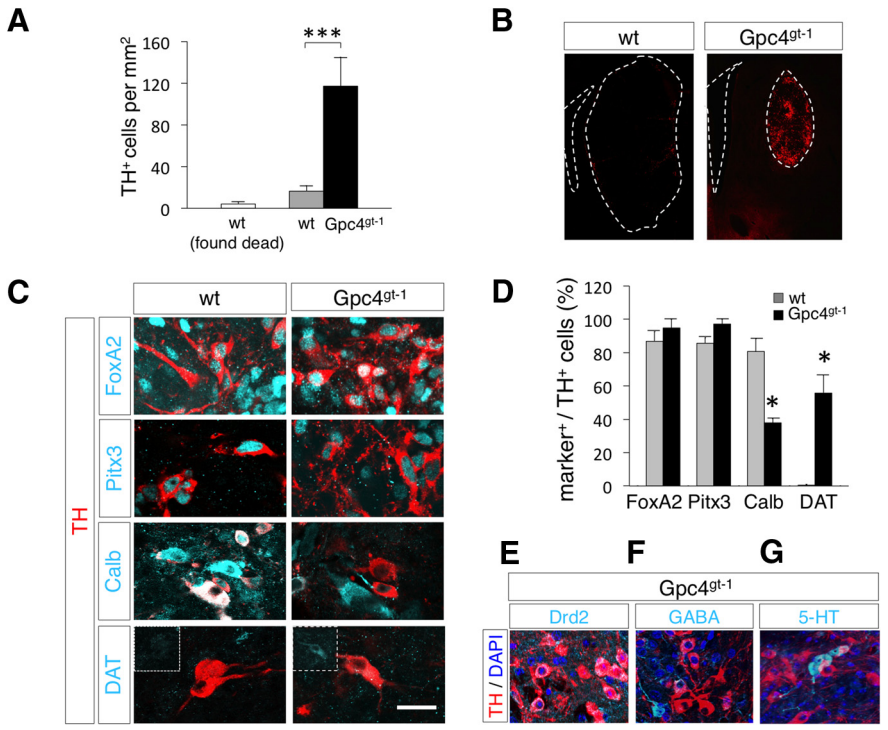

Figure 4. Grafted $Gpc4^{gt-1}$ ES cells generate large numbers of TH^+ cells with ventral midbrain DA features. ***A***, Density of TH^+ cells per mm^2 revealed that $Gpc4^{gt-1}$ grafts ($n = 11$) contained more DA neurons than wt grafts ($n = 6$). ***$p < 0.0001$ (Mann–Whitney test). wt-grafted rats that died prematurely (wt dead) showed comparably low levels of TH neurons than survivors. ***B***, Representative images of a wt (left) and a $Gpc4^{gt-1}$ (right) graft labeled with TH (red). ***C***, Immunohistochemical characterization of TH^+ cells in grafts. TH^+ cells coexpressed some typical midbrain DA neuron markers, both in wt and $Gpc4^{gt-1}$ grafts. FoxA2, forkhead box protein A2; Pitx3, pituitary homeobox 3; Calb, calbindin. ***D***, Percentage of TH^+ neurons that express also midbrain specific markers. *$p < 0.05$ (Mann–Whitney test). ***E–G***, Coexpression of DRD2 (***E***), GABA (***F***), and 5-HT (***G***) with TH in mutant grafts. Scale bar, 20 μm.

plantation in nude mice (Fico et al., 2012), we assessed $Gpc4^{gt-1}$ cell transplants in a rodent model of PD. Hemi-parkinsonian rats were generated by unilateral injection of 6-OHDA in the substantia nigra pars compacta. Five weeks later, we transplanted GFP-labeled wt versus $Gpc4^{gt-1}$ ES cells at low doses in the ipsilateral striatum using a embryonic body based protocol (Björklund et al., 2002). To challenge $Gpc4^{gt-1}$ cells, we intentionally chose this minimal protocol because it results in teratoma formation and induces only weak behavioral improvements. At the time of brain transplantation, control and mutant cells still expressed pluripotent cell markers, such as nanog, but no neural differentiation markers, such as nestin (data not shown). Four of seven rats grafted with wt ES cells died within 6 weeks after transplantation, the scheduled endpoint of this experiment. Lethality peaked between 30 and 39 d post grafting (dpg; Fig. 2*B*). In contrast, only one $Gpc4^{gt-1}$-grafted animal died before the end of the experiment (day 31; Fig. 2*B*).

We measured the motor improvements in grafted rats using the cylinder test and amphetamine induced rotation behavior (Björklund and Isacson, 2002). Tests were performed on three groups: nongrafted, wt-grafted, and $Gpc4^{gt-1}$-grafted rats (Fig. 2*C*). The cylinder test was performed before lesion, after the lesion, and at two different time points after grafting in wt- and $Gpc4^{gt-1}$-grafted rats. In the cylinder test, nongrafted hemiparkinsonian rats lost the ability to use their contralateral forepaw, inducing a dramatic decrease in double contacts on the cylinder walls (prelesioned: 78 ± 2% double contacts; postlesioned/nongrafted: 13 ± 3%). At 18 dpg, lesioned animals transplanted with wt cells were indistinguishable from nongrafted controls. However, at 6 weeks after grafting, use of the left paw improved in this group (nongrafted: 11 ± 3%; wt-grafted: 34 ± 1%). Importantly, rats grafted with $Gpc4^{gt-1}$ cells showed significant improvement already at 18 dpg (37 ± 4%) and the effect further increased, reaching 58 ± 3% of double contacts 6 weeks after transplantation (Fig. 2*C*).

Animals of two independent transplantation experiments were scored for amphetamine-induced rotations at the endpoint of the experiment (Fig. 2*D*). Analysis of the lesioned/nongrafted and of the lesioned/wt-grafted animals showed no significant change in net ipsilateral rotations, although there was a tendency toward improvement (sham: 12.4 ± 0.8 rotations/min, wt-grafted: 4.8 ± 1.4 rotations/min; Fig. 2*D*). Importantly, $Gpc4^{gt-1}$-grafted animals displayed a highly significant reduction in ipsilateral rotations compared with lesioned/nongrafted ($Gpc4^{gt-1}$-grafted: 1.3 ± 0.9 rotations/min; Fig. 2*D*). In conclusion, transplantation of $Gpc4^{gt-1}$ ES cells in a PD context was associated with strongly decreased lethality and was beneficial for motor functions.

We investigated size and cellular composition of the transplanted tissue. Generally, graft area was smaller in animals that received $Gpc4^{gt-1}$ ES-derived cells, although there was considerable heterogeneity (Fig. 3*A*). In wt cell-grafted animals, graft area was positively correlated with premature death before sacrifice (Fig. 3*A*). In contrast, only one of the $Gpc4^{gt-1}$ ES cell-grafted animals died within 6 weeks of transplantation (Fig. 2*B*). Hematoxylin/eosin staining demonstrated that all wt grafts showed the typical morphological array and dense structures indicative of teratomas (Lensch et al., 2007), whereas the $Gpc4^{gt-1}$ grafts showed rather homogeneous morphology and displayed no obvious signs of teratoma-like structures (Fig. 3*B*). Moreover, expression of nestin, a marker of immature neural cell types (Tohyama et al., 1992), was observed in all grafts, but staining was considerably stronger in wt cells (Fig. 3*C*). Thus, transplantation of Gpc4 hypomorphic cells strongly limited the occurrence of teratoma-like structures, which were likely the reason for augmented premature death in controls.

We then focused on the TH^+ cells in the grafts. Importantly, $Gpc4^{gt-1}$ grafts displayed a sevenfold increase in TH^+ cells compared with wt (wt grafts: 16.5 ± 5.1 cells/mm^2; mutant grafts: 117.2 ± 27.6 cells/mm^2; Fig. 4*A*,*B*). We immunohistochemically characterized the phenotypes of TH^+ neurons in grafts. FoxA2 and Pitx3, involved in ventral midbrain dopaminergic neuron production (Nunes et al., 2003), were both expressed in most TH^+ neurons in grafts of wt and $Gpc4^{gt-1}$ origins (Fig. 4*C*,*D*). In contrast, calbindin, a protein enriched in ventral tegmental area (Thompson et al., 2005), was decreased in dopaminergic neurons derived from $Gpc4^{gt-1}$ grafts (Fig. 4*C*,*D*), suggesting that dopaminergic neurons in $Gpc4^{gt-1}$ grafts were preferentially of the substantia nigra subtype. Importantly, DAT was exclusively expressed in TH^+ neurons in $Gpc4^{gt-1}$ grafts (Fig. 4*B*,*C*), reminis-

cent of the *in vitro* situation (Fig. 1). In addition, most TH$^+$ neurons in the *Gpc4*$^{gt-1}$ cell grafts coexpressed the DRD2 (Fig. 4*E*; DRD2/(TH + DRD2): 89 ± 2%), suggesting that they are capable of autoregulation. Indeed, activation of D2 autoreceptors is known to tune DA synthesis, release, and uptake as well as dopamine neuron firing. Finally, contribution of GABA and 5-HT-positive neurons to the *Gpc4*$^{gt-1}$ grafts was minor with respect to TH (GABA/(TH + GABA): 8.8 ± 0.4%; 5-HT/(TH + 5-HT): 11.4 ± 1.7%; Figure 4*F*,*G*).

In conclusion, decreased levels of Gpc4 increased the level of mature midbrain dopaminergic neurons after transplantation into the dopamine-depleted striatum.

Discussion

Our data suggest that interfering with Gpc4 expression or function could represent a powerful strategy to increase the efficiency and safety of cell transplantation in PD. Cultured Gpc4 hypomorphic ES cells show a strong tendency to differentiate into cells with properties of mature DA neurons. Moreover, *in vivo* grafted *Gpc4*$^{gt-1}$ cells show augmented differentiation into TH$^+$ neurons with molecular features of midbrain DA neurons and improve recovery of motor functions in a rat model of PD. Importantly, knockdown of Gpc4 decreases the formation of teratoma-like structures and increases animal survival.

Our data indicate that Gpc4 depletion enhances DA neuron fate commitment *in vitro* and *in vivo*. Further studies will clarify whether loss of Gpc4 activity impacts also on the development of other neuronal cell types and whether distinct culture conditions could promote the capability of Gpc4 mutant cells to develop into DA neurons. Mechanistically, glypicans can interact with several signaling pathways, such as Wnt, SHH, FGFs, and BMPs, and they function either as positive or negative regulators of these signaling pathways depending on the cellular context (Galli et al., 2003; Fico et al., 2011). We have recently shown that Gpc4 maintains mouse ES cell self-renewal by positively modulating Wnt/β-catenin signaling (Fico et al., 2012). Both the canonical (Wnt/β-catenin) and noncanonical (Wnt/PCP) Wnt pathways have been implicated in midbrain DA neuron generation *in vivo* (Castelo-Branco and Arenas, 2006). In particular, Wnt5a deficiency induces premature Nurr1 expression and a transitory increase in DA neurons in the ventral midbrain (Andersson et al., 2008). In ES cells, blocking the Wnt canonical signaling increases neural induction and dopaminergic differentiation (Cajánek et al., 2009). Thus, it is possible that fine-tuning of Wnt signaling by Gpc4 might be a regulatory mechanism controlling the generation of midbrain DA neurons *in vitro* and/or *in vivo*.

Independent of the mechanisms by which the loss of Gpc4 increases midbrain-like DA neurons, we show that reduction of Gpc4 signaling permits enhancement of DA neuron differentiation from ES cells without adding any exogenous factor, both *in vitro* and *in vivo* in a 6-OHDA rat model of PD. Moreover, at the functional level, Gpc4 hypomorphic cell-grafted rats showed strong relief of the lesion-induced motor dysfunction not only in the amphetamine-induced rotation test but also in the cylinder test, a nondrug induced test for akinesia-like deficits that is considered very stringent.

Finally, we find reduced cell overgrowth by Gpc4 hypomorphic grafts. Even the one animal in this group showing a large graft area (Fig. 3*A*) showed no signs of teratogenesis and survived until the end of the experiment. This is in agreement with previous work showing that Gpc4 hypomorphic ES cells do not generate teratomas after injection in the flank of nude mice (Fico et al., 2012). Thus, control of Gpc4 may represent a means to reduce ES cell tumorigenic features while at the same time increasing neuronal features. Because Gpc4 is a cell surface protein, it appears as an attractive target to design drugs that improve security and functional improvement in PD cell therapeutic approaches.

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