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Can manipulation of differentiation conditions eliminate proliferative cells from a population of ES cell-derived forebrain cells?

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ABSTRACT

There is preliminary evidence that implantation of primary fetal striatal cells provides functional benefit in patients with Huntington's disease, a neurodegenerative condition resulting in loss of medium-sized spiny neurons (MSN) of the striatum. Scarcity of primary fetal tissue means it is important to identify a renewable source of cells from which to derive donor MSNs. Embryonic stem (ES) cells, which predominantly default to telencephalic-like precursors in chemically defined medium (CDM), offer a potentially inexhaustible supply of cells capable of generating the desired neurons. Using an ES cell line, with the forebrain marker *FoxG1* tagged to the *LacZ* reporter, we assessed effects of known developmental factors on the yield of forebrain-like precursor cells in CDM suspension culture. Addition of FGF2, but not DKK1, increased the proportion of FoxG1-expressing cells at day 8 of neural induction. Oct4 was expressed at day 8, but was undetectable by day 16. Differentiation of day 16 precursors generated GABA-expressing neurons, with few DARPP32 positive MSNs. Transplantation of day 8 precursor cells into quinolinic acid-lesioned striata resulted in generation of teratomas. However, transplantation of day 16 precursors yielded grafts expressing neuronal markers including NeuN, calbindin and parvalbumin, but no DARPP32 6 weeks post-transplantation. Manipulation of fate of ES cells requires optimization of both concentration and timing of addition of factors to culture systems to generate the desired phenotypes. Furthermore, we highlight the value of increasing the precursor phase of ES cell suspension culture when directing differentiation toward forebrain fate, so as to dramatically reduce the risk of teratoma formation.

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Introduction

Huntington's disease (HD) is an inherited autosomal dominant disorder characterized by progressive neurodegeneration including extensive atrophy of dopamine and cyclic adenosine 3′, 5′-monophosphate-regulated phosphoprotein, 32kDa (DARPP32) positive striatal medium-sized spiny projection neurons (MSNs) which leads to motor, cognitive and psychiatric disturbances. There is currently no known cure for HD and only limited symptomatic treatments are available. Cell replacement by transplantation is one therapeutic strategy being explored. The current 'gold standard' for transplantation in HD utilizes primary fetal whole ganglionic eminence; the site of MSN genesis.[1,2] However, the use of fetal tissue brings with it logistical and ethical issues; in particular fetal tissue is a scarce resource. Thus, there is a need for a renewable source of donor cells, with pluripotent stem cells offering an attractive, potential alternative.[3]

Embryonic stem (ES) cells have the capacity for unlimited self-renewal and proliferation, and are pluripotent, with the capability to generate any cell type from the 3 germ layers.[4,5] These characteristics make ES cells an attractive source of cells for use in cell-replacement strategies. The main challenge with the use of any renewable donor cell source is to generate the specific phenotype required; for HD, striatal MSNs.[6] In addition, it may be necessary to eliminate proliferative cells from pluripotent cultures (such as

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undifferentiated ES cells or proliferative precursors) that may produce neurological damage through continued proliferation in the graft.

It is well documented that, following withdrawal of serum and leukemia inhibitory factor (LIF), mouse ES cells cultured in free-floating suspension under defined conditions without addition of exogenous factors, generate a proportion of neural precursor cells.[7,8,9] Different protocols yield varying percentages of neural precursors (ranging from 60-90%), as indicated by expression of Nestin and Sox1.[8,9,10,11,12] Neural induction of mouse ES cells is enhanced by addition of growth factors such as fibroblast growth factor-2 (FGF2), which induces an increase in Nestin,[8] Sox19 and FoxG1 expression.[10] In most reports analysis has been performed following up to 8 d in culture. Inhibition of Wnt signaling is reported to be necessary for telencephalic generation during neural development.[13] Addition of the Wnt inhibitor Dickkopf-1 (DKK1) has been shown to increase the proportion of FoxG1-expressing cells, if added to cultures between days 0–5, but not later,[11] indicating the importance of timing for addition of factors for induction of specific markers.

Neural induction protocols for ES cells have not resulted in exclusively neural differentiation and the resulting population is often heterogeneous, containing multiple cell types.[10,14,15] Specifically, derivation of striatal-like neurons, as evaluated with markers such as gamma-amino butyric acid (GABA), glutamate decarboxylase and DARPP32, has been achieved following differentiation of mouse ES cells, albeit in small numbers.[16-19] However, the continued presence of undifferentiated ES cells in the neural induction culture system makes this cell source less appealing for use in cell-replacement therapies because of the potential to form teratomas. Indeed uncontrolled proliferation has been observed in several studies following transplantation of neurally differentiated mouse and human ES cells.[19-23] In addition, it has been shown that the greater the proportion of dividing cells (determined by bromodeoxyuridine (BrdU) immuno-reactivity) and neural precursors (determined by Nestin immunoreactivity) within the transplanted population, the greater the potential to generate tumors.[20]

One strategy to reduce the risk of teratoma formation would be to prolong the length of time ES cell-derived precursors spend in neural induction culture prior to transplantation, so that they may be more completely differentiated with accompanying down-regulation of pluripotent markers. Neural induction of mouse ES cells using the suspension method has, to date, been described after short periods of culture only, and analysis of precursors beyond 8-10 d has not been reported.

In this study, which follows on from previous work from our group,[10,15] we use a mouse ES cell line where most of the *FoxG1* coding sequence was replaced with the *LacZ* reporter gene and expression of the β-galactosidase (β-Gal) enzyme is under the control of the *FoxG1* promoter.[24] *FoxG1* is the earliest and most specific determinant of telencephalic fate.[25,26] Neural induction in chemically defined

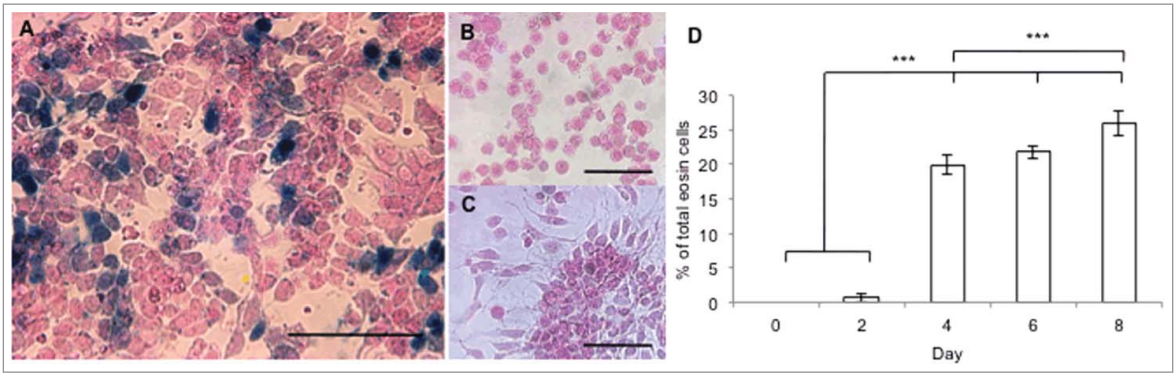

Figure 1. X-Gal expression in FoxG1Z-derived precursors. Within cultures there were cells present exhibiting no X-Gal expression (pink), interspersed with X-Gal positive cells (blue) (A). Undifferentiated FoxG1Z ES cells (B) and precursors derived from a non-*LacZ* reporter ES cell line (C) exhibited no X-Gal positive cells. X-Gal positive cells were counted at days 0, 2, 4, 6 and 8 of neural induction and are represented as a percentage of total eosin stained cells (D). Each bar on the graph represents a mean of 3 different cultures and error bars represent SEM. There were significantly more X-Gal positive cells with increasing time in culture. Significant *post-hoc* differences are indicated with brackets; $^{***}p < 0.001$. Scale bars = 50 μm

medium (CDM) suspension culture with and without the addition of growth factors was assessed at day 8 with analysis of expression of regional neural precursor markers. Cultures were compared at day 8 and day 16 for expression of markers of ES cells and neural precursor cells, and subsequently, neuronal markers, following neuronal differentiation. Further characterization of the mature differentiated phenotype from neural precursors was assessed following transplantation into the rat quinolinic acid (QA)-lesioned striatum, in particular looking for differentiation toward striatal neuronal phenotypes.

Results

Forebrain-like character of ES cell-derived precursors

The use of the FoxG1Z mouse ES cell line in this study enabled detection of FoxG1-positive cells following incubation with X-Gal, which yields a blue product. FoxG1Z cells were cultured in CDM alone and analyzed at different time points up to day 8. Within cultures there was a mix of cells that were positive or negative for X-Gal (Fig. 1A). Undifferentiated FoxG1Z ES cells were negative for X-Gal, as were precursors derived from a mouse ES cell line without the *LacZ* reporter (CGR8.8) (Figs. 1B, 1C). Counts of X-Gal positive cells revealed a significant increase in the proportion of forebrain cells with increasing time in culture ($F_{4,15} = 117.31$, $p < 0.001$) (Fig. 1D). There were no X-Gal positive cells identified at day 0 and the greatest proportion of X-Gal positive cells was seen at day 8 (25.91 ± 1.78%).

Effect of addition of FGF2 and DKK1 on FoxG1 expression

We have previously shown, and validated using multiple mouse ES cell lines (E14, CGR8.8 and IMT11), that addition of FGF2 to CDM neural induction cultures results in increased expression of FoxG1 and Nestin.[10,15] Here, we found that addition of increasing concentrations of FGF2 to CDM neural induction cultures on day 4 resulted in a significant increase in the percentage of X-Gal positive cells at day 8 ($F_{4,15} = 5.57$, $p < 0.05$) (Fig. 2A).

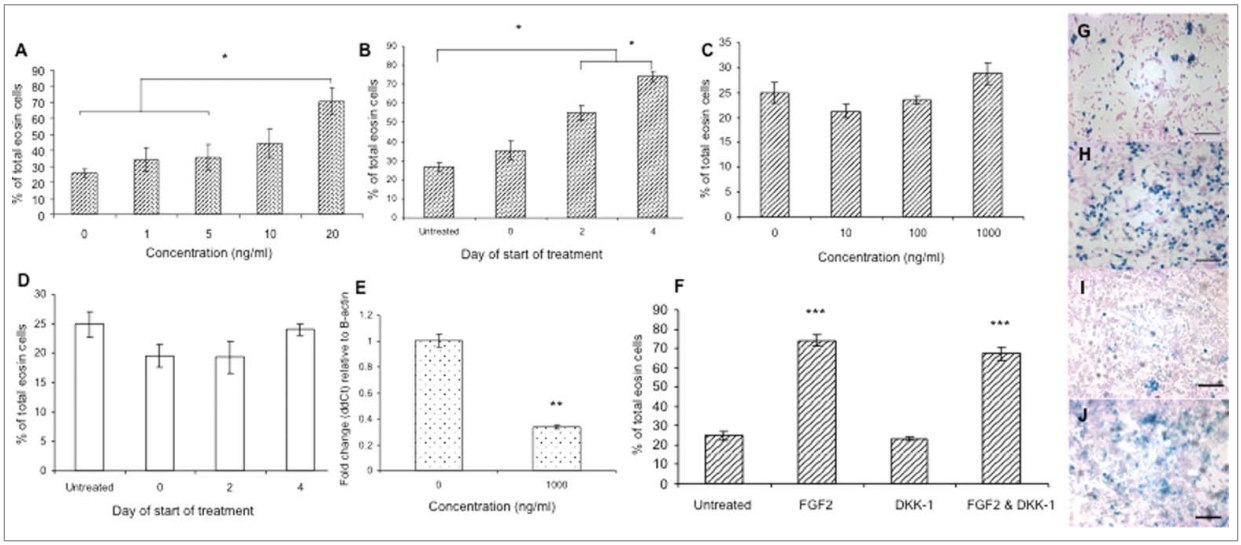

Figure 2. Effect of addition of FGF2 and DKK1 on the proportion of forebrain cells. FoxG1Z cells were cultured in neural induction medium with or without the addition of other factors. FGF2 (1, 5, 10 and 20 ng/ml) was added at day 4 and cultures were analyzed at day 8 (A). Addition of 20 ng/ml FGF2 was initiated on either day 0, 2 or 4, and maintained through to analysis at day 8 (B). DKK1 (10, 100, 1000 ng/ml) was added at day 4 and cultures were analyzed at day 8 (C). Addition of 1000 ng/ml DKK1 was initiated on either day 0, 2 or 4 and maintained through to analysis at day 8 (D). Untreated or DKK1 treated (1000 ng/ml) precursors from day 4 to day 8 were analyzed for expression of *Axin* using semi-quantitative RT-PCR (E). Cultures treated with 20 ng/ml FGF2 alone or both 20 ng/ml FGF2 and 1000 ng/ml DKK1 have significantly more X-Gal positive cells than untreated cultures and those treated with 1000 ng/ml DKK1 alone (F). X-Gal positive cells were counted and are represented as a percentage of total eosin stained cells. Photomicrographs showing X-Gal (blue) and eosin (pink) counterstaining at day 8 of neural induction, in untreated cultures (G); cultures treated with 20 ng/ml FGF2 at day 4 (H); cultures treated with 1000 ng/ml DKK1 at day 4 (I); and cultures treated with 20 ng/ml FGF2 and 1000 ng/ml DKK1 at day 4 (J). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. Significant *post-hoc* differences are indicated with brackets. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars = 100 μm

There was no significant difference between cultures receiving 1, 5 and 10 ng/ml FGF2, but those receiving 20 ng/ml FGF2 yielded a significantly higher proportion of X-Gal positive cells. When addition of 20 ng/ml FGF2 was initiated on different days (day 0, 2 or 4) and maintained through to analysis at day 8, the percentage of X-Gal positive cells was significantly increased the later the initial addition ($F_{3,12} = 33.89$, $p < 0.05$) (Fig. 2B).

Next we wanted to target a different pathway and determine the effects of Wnt inhibition on the FoxG1 population. Addition of increasing concentrations of the Wnt inhibitor DKK1 to CDM neural induction cultures on day 4 did not result in a significant difference in the percentage of X-Gal positive cells ($F_{3,12} = 3.45$, p = n.s) (Fig. 2C). The lack of effect of 1000 ng/ml DKK1 was not modified by altering the day of treatment initiation (day 0, 2 or 4; $F_{3,11} = 2.19$ p = n.s) (Fig. 2D). The integrity of the Wnt-signaling pathway was confirmed by the reduced expression of the pathway component *Axin* following addition of the top dose of DKK1, when compared to untreated cultures ($t_2 = 19.54$, $p < 0.01$) (Fig. 2E).

The initial aim here was to determine the optimum concentrations of FGF2 and DKK1 for maximum expression of FoxG1, and then to add them in combination to determine if a further increase in FoxG1 expression would ensue. The effect of DKK1 was initially analyzed in the absence of FGF2. To determine if there was an additive effect of these 2 factors, 20 ng/ml FGF2 was added in conjunction with 1000 ng/ml DKK1 at day 4 for analysis at day 8 (Figs. 2F-J). There was a highly significant overall effect of treatment ($F_{3,12}= 114.60$, $p < 0.001$) (Fig. 2F). Cultures treated with both 20 ng/ml FGF2 and 1000 ng/ml DKK1, or with 20 ng/ml FGF2 alone, generated significantly higher numbers of X-Gal cells compared to untreated cultures and cultures treated with DKK1 alone. However, there was no difference between cultures treated with FGF2 alone and those that also received DKK1.

In vitro *characterization of FoxG1Z-derived precursors*

Expression of Oct4 (Figs. 3A, 3B) and Nestin (Figs. 3C, 3D) were assessed in ES cell-derived precursors in neural induction culture at day 8 and day 16. Oct4 expression significantly decreased over time $F_{8,27} = 115.81$, $p < 0.001$) (Fig. 3E). Notably, expression

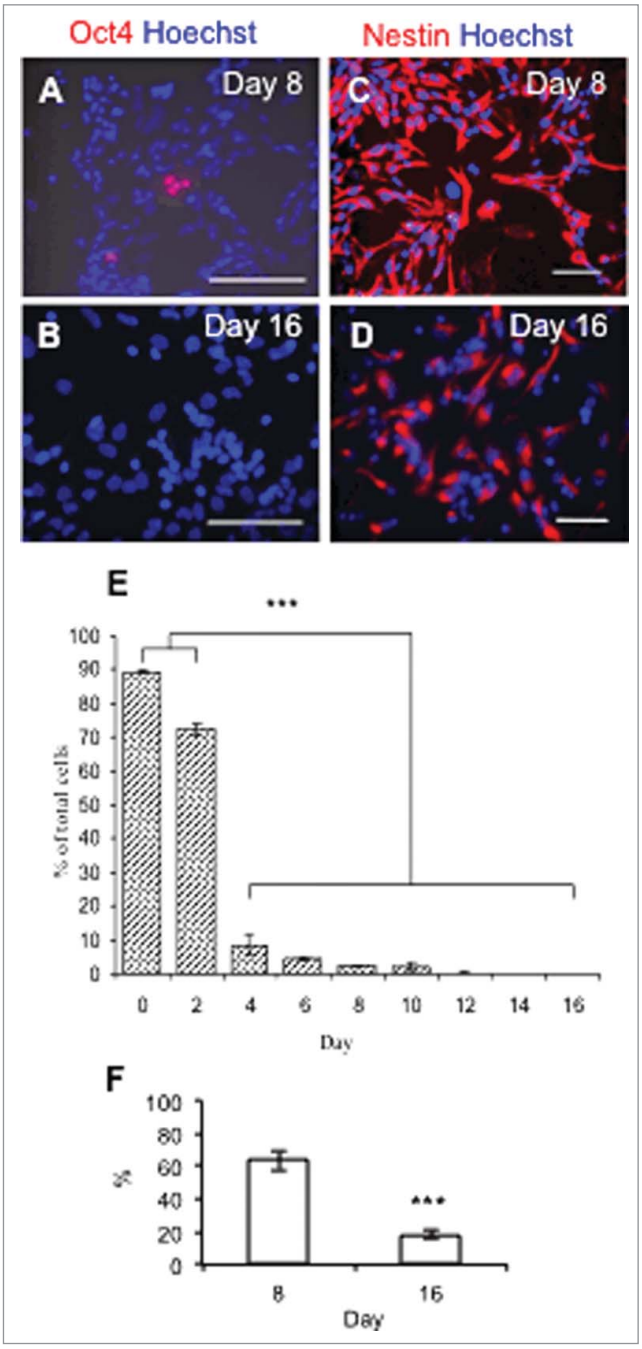

Figure 3. *In vitro* comparison of day 8 and day 16 FoxG1Z-derived precursors. FoxG1Z precursors were plated onto substrate at day 8 (A, C) and day 16 (B, D). Cultures were fixed after 4 hours and immuno-labeled with Oct4 (red) (A, B and E) or Nestin (red) (C, D and F) and Hoechst nuclear stain (blue). At day 8 few Oct4 positive cells were present (A) and at day 16 there were no Oct4 positive cells detected (B). The neural precursor marker Nestin was more highly expressed in precursors at day 8 (C) compared with day 16 (D). Oct4 and Nestin immuno-positive cells were counted and are represented as a percentage of total Hoechst cells (E and F, respectively). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. (***$p < 0.001$). Scale bars = 100μm (A and B) and 50 μm (C and D)

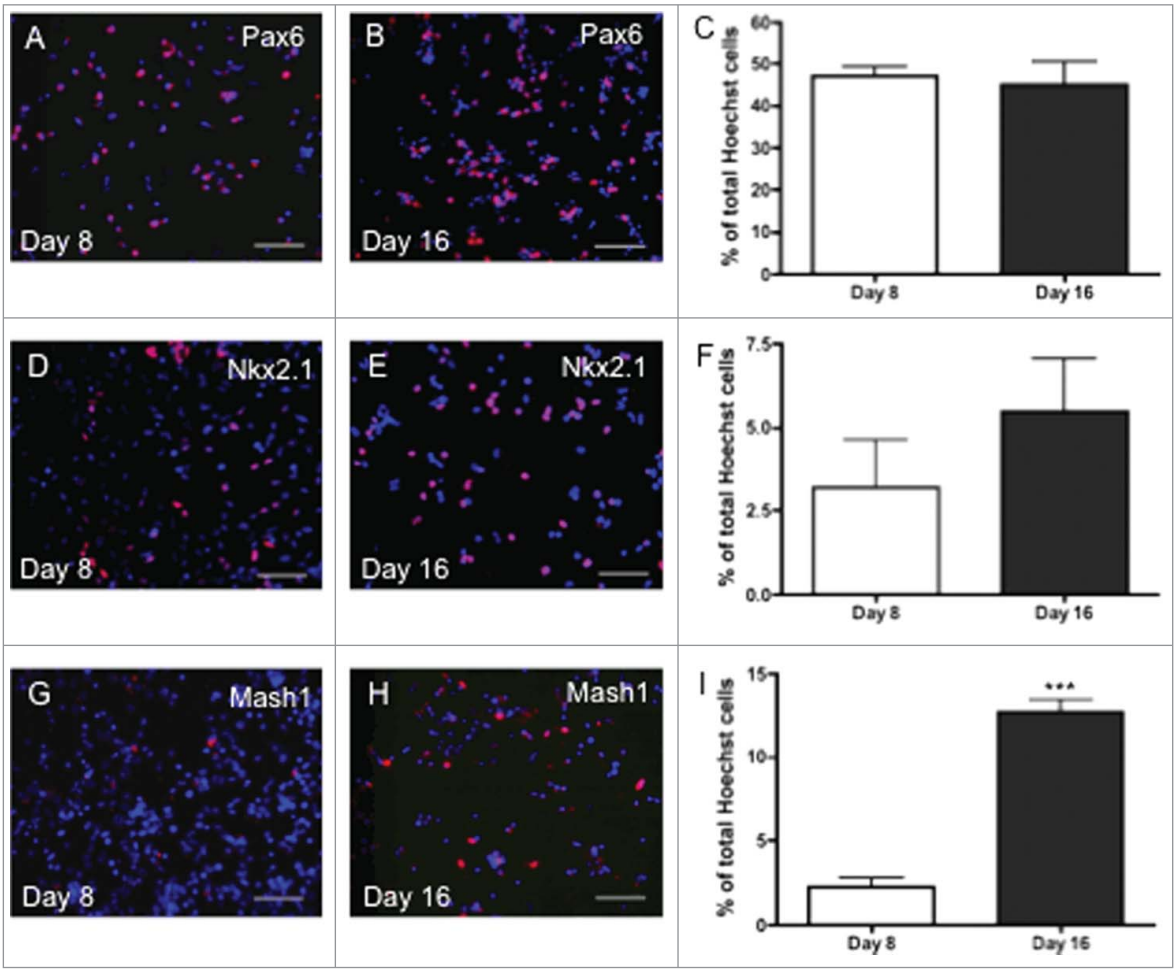

Figure 4. Dorso-ventral comparison of day 8 and day 16 FoxG1Z-derived precursors. FoxG1Z precursors were plated onto substrate at day 8 (A, D, G) and day 16 (B, E, H). Cultures were fixed after 4 hours and immuno-labeled with Pax6 (red) (A and B), Nkx2.1 (red) (D and E) or Mash1 (red) (G and H) and Hoechst nuclear stain (blue). Pax6, Nkx2.1 and Mash1 immuno-positive cells were counted and are represented as a percentage of total Hoechst cells (C, F and I, respectively). There was no significant difference in expression of Pax6 and Nkx2.1 between day 8 and day 16. There were significantly more Mash1 immuno-positive cells at day 16 than at day 8. Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. ($^{***}p < 0.001$). Scale bars = 50 μm.

decreased from 2% at day 8 to 0% at day 16. Nestin expression decreased significantly between day 8 and day 16 ($t_4 = 8.67$, $p<0.001$) (Fig. 3F).

Expression of dorso-ventral markers Pax6, Nkx2.1 and Mash1 was analyzed, comparing precursors at day 8 and day 16 (Figs. 4A-I). There was no significant difference in Pax6 expression ($t_5= 0.38$, p = n.s) (Figs. 4A-C) or Nkx2.1 expression ($t_4= 1.075$, p = n.s) (Figs. 4D-F) between these 2 time points. Mash1 expression was significantly different, with more immune-positive cells at day 16 (12.66 ± 0.82%) compared with day 8 (2.26 ± 0.56%) ($t_4 = 10.55$, $p < 0.001$) (Figs. 4G-I).

The next step was to evaluate neuronal differentiation; FoxG1Z precursors were plated onto substrate in neuronal differentiation medium and differentiated for 7 d Following differentiation FoxG1Z cells continued to express β-Gal and there was co-expression with the neuronal marker β-III-tubulin. Day 16 precursors yielded a comparable proportion of neurons (30.71 ± 4.89%) and a greater proportion of GFAP immune-positive cells (14.92 ± 1.48%) compared with day 8 precursors (data not shown).

Assessment of neuronal phenotype following 7 d in neuronal differentiation medium revealed the presence of GABAergic neurons, from both day 8 and day 16 cultures (Figs. 5A-D; 5E-H, respectively). DARPP32 was never detected in day 8 differentiated cultures but was found to be expressed at low levels at day 16 (4.16 ± 1.77%, as a percentage of β-III-tubulin positive cells) (Figs. 5I-L). The same was seen with FoxP1, where no expression

was evident at day 8, but by day 16 FoxP1 expression was detected (16.89 ± 0.81%, as a percentage of total Hoechst positive cells). Additionally, there were a small number of cells that co-expressed FoxP1 and DARPP32 (Figs. 5M-P).

Graft survival and differentiation

FoxG1Z precursors generated following neural induction in CDM with addition of FGF2 at day 4 were transplanted at either day 8 or day 16 into the QA-lesioned rat striatum. Day 8 grafts at 2 weeks post-transplantation had the appearance of uncontrolled proliferation. There were graft masses with well-defined borders, filling the striatum of the grafted side, and pushing into the cortex (Fig. 6A). All grafts in this group were similar in appearance, with 'swirling' patches of tissue throughout, based on Nissl staining. In comparison, all animals in the day 16 graft group survived for analysis at 6 weeks post-transplantation. Nissl-stained sections revealed the presence of cell-dense regions throughout the graft, with less well-defined borders than the day 8 grafts, suggesting a degree of integration of the day 16 grafts into the host brain, rather than the distinctly isolated cell mass of the day 8 grafts (Fig. 6B). Additionally, day 16 grafts exhibited less heterogeneous appearance than day 8 grafts.

Graft volumes were based on analysis of β-Gal immunoreactivity (Fig. 6C) of FoxG1-positive cells: mean graft volume for day 8 grafts was 336.32 ±

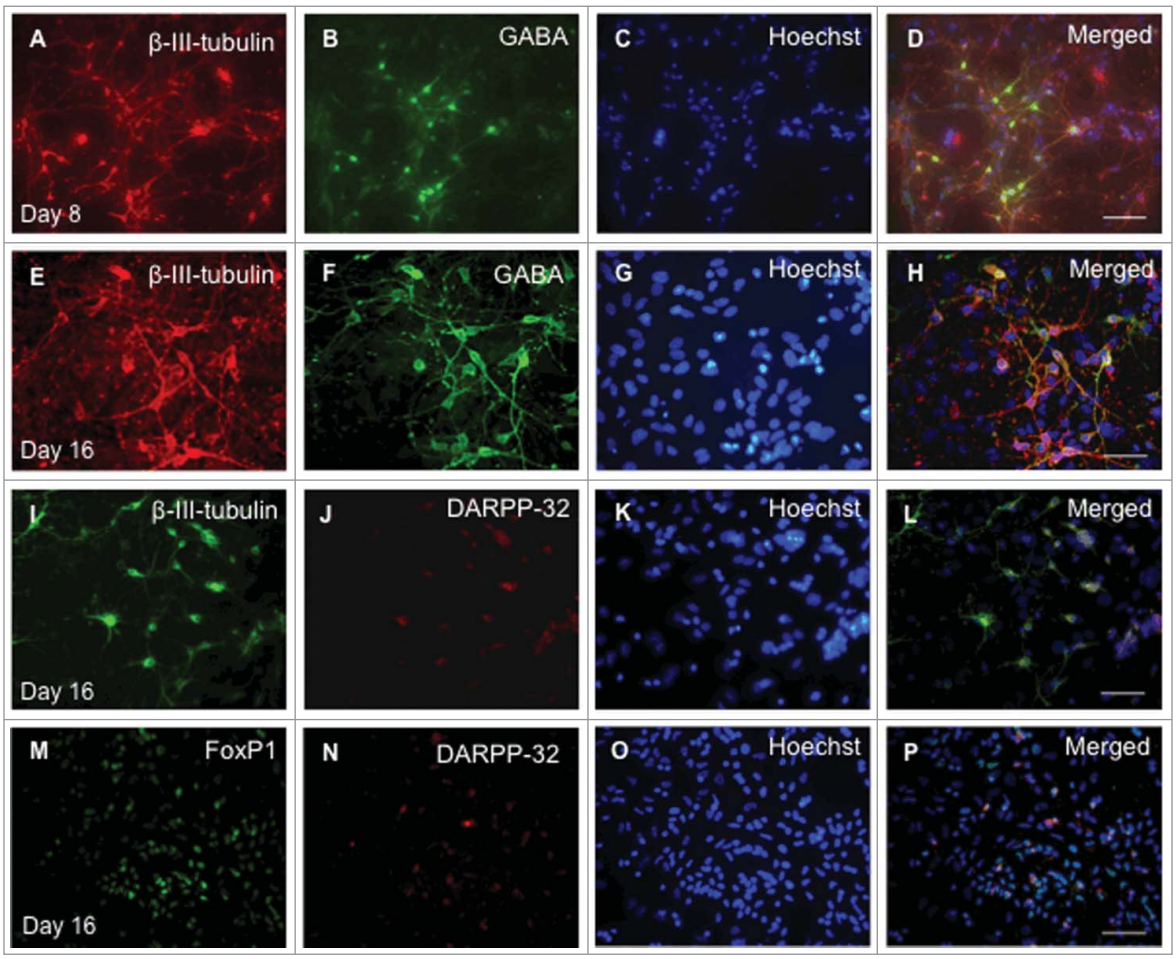

Figure 5. *In vitro* neuronal differentiation of FoxG1Z-derived precursors. FoxG1Z precursors were plated onto substrate at day 8 (A-D) and day 16 (E-P) and differentiated for 7 d in neuronal differentiation medium with no additional growth factors. Cultures were triple-labeled for β-III-tubulin (red) and GABA (green) at day 8 (A-D) and day 16 (E-H); β-III-tubulin (green) and DARPP32 (red) (I-L); and FoxP1 (green) and DARPP32 (red) (M-P); with Hoechst nuclear stain (blue). D, H, L and P represent merged images of the first 3 photomicrographs in each row. Scale bars = 100 μm (D) and 50 μm (H, L and P).

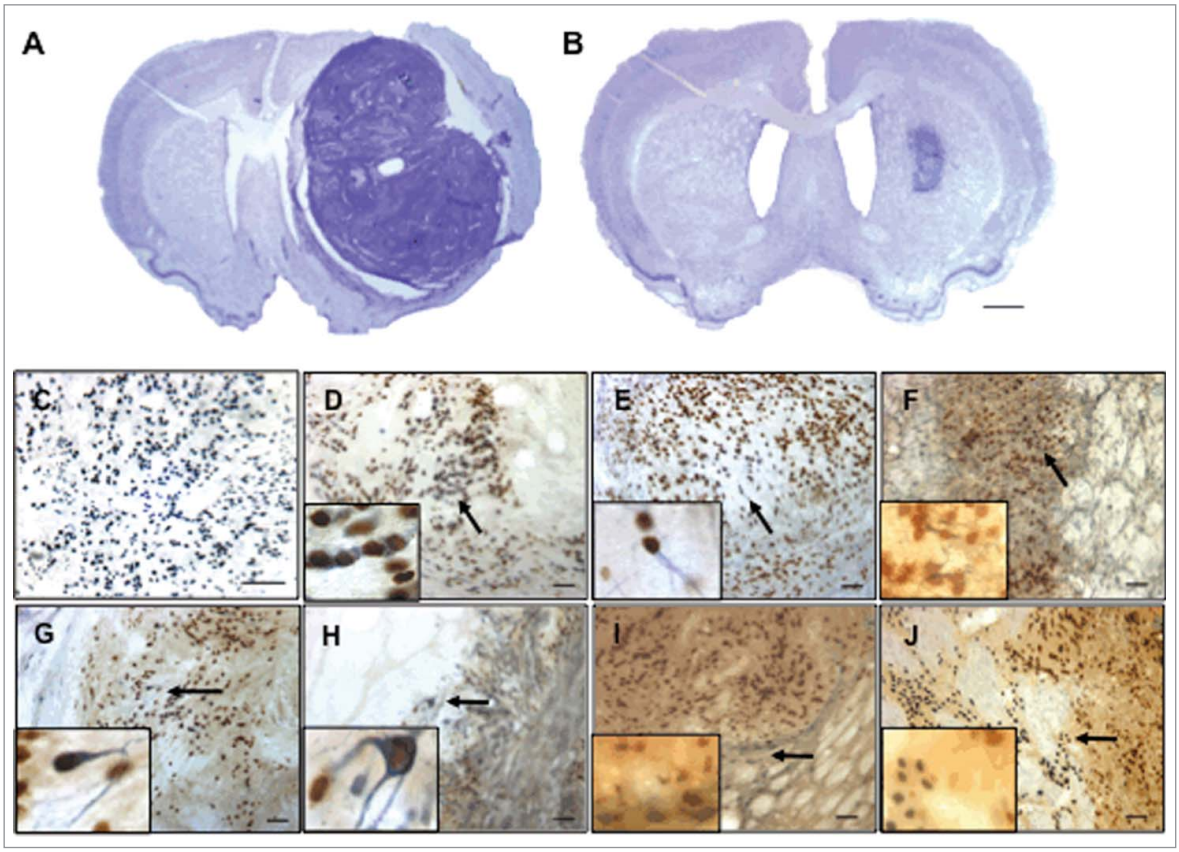

Figure 6. *In vivo* analysis of FoxG1Z-derived precursors. Photomicrographs of histological sections from brains transplanted with day 8 precursors (A) and day 16 precursors (B-J). Low magnification images of cresyl violet stained sections (A, B) show size and position of the grafts. Higher magnification images reveal β-Gal immunoreactive cells present in the day 16-derived grafts (C), which allows for subsequent analysis of FoxG1-positive cells. FoxG1-positive cells were shown to double-label with NeuN (D), DCX (E), GFAP (F), calbindin (G) and parvalbumin (H). There was no double-labeling of FoxG1-positive cells with DARPP32 (I) or FoxP1 (J). Scale bars = 1 mm (A and B); 100 μm (C); 50 μm (D-J).

129.16 mm^3, with a wide range between animals (19.24 to 962.32 mm^3); and mean graft volume for day 16 grafts was 21.87 $\pm$ 4.63mm^3, with a range from 13.14 to 56.5 mm^3.

Day 16 grafts were analyzed for co-expression of β-Gal with markers to demonstrate the neural lineage of graft-derived cells. β-Gal immune-positive cells were seen to double-label with NeuN (16.95 $\pm$ 1.44%) (Fig. 6D); DCX (8.24 $\pm$ 1.29%) (Fig. 6E); and glial marker GFAP (7.38 $\pm$ 1.12%) (Fig. 6F). Expression of other striatal neuronal markers was also assessed, and co-expression was seen with calbindin (1.62 $\pm$ 0.77%) (Fig. 6G) and parvalbumin (2.26 $\pm$ 0.76%) (Fig. 6H). There was no co-expression of β-Gal with DARPP32 (Fig. 6I) or FoxP1 (Fig. 6J), with no expression of either marker within the graft area and few immunopositive cells seen at the periphery of the graft only.

Discussion

Forebrain-like character of FoxG1Z-derived precursors

Serum-free neural induction suspension culture of mouse ES cells resulted in generation of neural precursor cells.[9,11,15,27] Over time in culture, neurogenicity is induced by default as demonstrated by upregulation of the neuroectodermal marker Sox1[10,11] and the neural precursor marker Nestin,[10] with a concurrent down regulation of the ES cell marker Oct4.[10,11,15] We have previously shown a downregulation of the non-neural markers *Oct4, Nodal, Brachyury* and *Gooscoid* over time in the CDM culture system between day 0 and 8,[15] with no expression of the muscle marker Desmin or the epidermal marker pan-Cytokeratin.[10,15]

The cell line used in this study enabled analysis of expression of the early forebrain marker FoxG1 via use of the *LacZ* reporter gene.[24] FoxG1Z ES cells

cultured in CDM spontaneously formed aggregates, grew as spheres and could be maintained under these conditions for multiple days without the addition of growth factors. When plated onto substrate, fixed as precursors without differentiation and stained with X-Gal, blue cells were visualized. Using this assay we showed an increase in FoxG1 positive cells over time in culture as expected, up to 25% at 8 days, which was higher than shown in a previous study.[11] Watanabe and colleagues showed that feeder-dependent cultures maintained for 5 or 10 d resulted in less than 2% FoxG1 positive cells; this was increased to 11% when cultures were feeder-free and in suspension for the initial 5 days, and further increased to 15% when all 10 d of culture were feeder-free and in suspension.[11] The CDM culture system used in this study appears to encourage induction of FoxG1 precursors more than other protocols,[10,11] yielding higher proportions of FoxG1 positive precursors, as determined using the FoxG1Z ES cell line in combination with the X-Gal assay. The extent to which these differences are due to the different ES cell culture medium components is currently uncertain.

Effect of addition of FGF2 and DKK1 on FoxG1 expression

FGF2 signaling is important for the induction and maintenance of normal mammalian telencephalic development.[28] *In vivo* studies have shown a relationship between expression of *Fgf8* and *FoxG1*, where loss of *FoxG1* results in reduced *FGF8* expression in the developing telencephalon.[29] Neural plate explants incubated with FGF8-soaked beads were shown to extensively express *FoxG1* in the area immediately surrounding the beads as compared with PBS-soaked beads, where no *FoxG1* was expressed.[26] Loss-of-function studies with *Fgf8* mutant mice revealed that *FoxG1* expression was reduced in the neural plate (9-10 somite stage) and also at the later gestational stage E9.[30]

Addition of increasing concentrations of FGF2 to FoxG1Z ES cells in neural induction cultures resulted in an increase of FoxG1-expressing cells. This was consistent with previous work that showed that treatment at day 4 with 20ng/ml FGF2 (as presented here) induced expression of *FoxG1* at day 8.[10] Previous work showed that addition of FGF2 to mouse ES cells undergoing neural induction within the CDM culture system resulted in increased BrdU incorporation, with a concomitant increase in expression of both Nestin and *FoxG1* at day 8.[10,15] This increase in proliferating cells with the increase in forebrain identity suggests that FGF2 is affecting proliferation rather than lineage selection. The lack of response to earlier FGF2 addition might be due to an absence of the appropriate FGF receptors. FGFR1 is not detected in mouse ES cells, but following initiation of neural induction it can be detected at low levels from day 2 with increasing expression through to day 8.[15]

During mouse development Wnt signaling is evident in the posterior region of the embryo from E6.5 and in EB development it is required for establishment of the anterior-posterior axis.[31] Inhibition of Wnt signaling within EBs promotes anterior expression and induces neuroectodermal differentiation.[31] We did not observe an effect of Wnt inhibition on FoxG1 expression in culture, neither when DKK1 was added at day 4 at a range of concentrations nor when the highest concentration (1000 ng/ml) was added on different starting days and maintained throughout. However, we did find that the Wnt inhibitor DKK1 exerted an effect on the Wnt signaling pathway within our cultures, but it was not acting on the FoxG1 population of cells. Our findings here are contradictory to a previous study, which reported a significant up-regulation of FoxG1 expression following addition of 1000ng/ml DKK1 between days 0–5.[11] In untreated neural induction cultures *Wnt3a* has been shown to be expressed from day 4 and its expression persists, albeit at a reduced level, through to day 8.[15] The same cells were shown to endogenously express *Dkk1* at day 4 through to day 8.[15] Therefore, although the presence of endogenous Wnt in neural induction cultures might suggest a requirement for supplementation of Wnt inhibitors to permit direction toward a neural lineage (to anteriorise cultures), the concomitant presence of endogenous *Dkk1* in these cultures might be enough to inhibit alternative cell fates, without addition of exogenous Wnt inhibitors.

In vitro *analysis of FoxG1Z-derived precursors and neurons*

Oct4 expression is exclusive to pluripotent cells.[32] It is expressed in ES cell lines derived from the inner cell mass when cells are in an undifferentiated state and when these cells begin to differentiate, expression of

Oct4 rapidly down regulates. ES cells subjected to our CDM protocol support this, and expression of Oct4 decreased over time in culture. Bouhon et al show continued expression of Oct4 following 8 d in CDM.[10,15] Following a longer period in CDM culture with continued FGF2 addition we demonstrated further down-regulation of Oct4 and by day 16 Oct4 is no longer expressed.

Expression of the neural precursor marker Nestin in suspension culture of mouse ES cells in CDM is significantly decreased between day 8 and day 16, which is consistent with a previous study reporting a decrease in Nestin expression between day 8 and day 12.[15] Addition of FGF2 to serum-free suspension cultures of ES cells has been shown to maintain the proportion of Nestin-positive cells over time in culture up to day 8, when compared with CDM alone, and cultures older than day 8 depend on addition of exogenous FGF2 for survival.[15] Analysis of Nestin-positive cells in neurally induced cultures of ES cells prior to transplantation revealed that the lower the proportion of Nestin-positive cells, the lower the risk of teratoma formation *in vivo*.[20]

Assessment of dorso-ventral identity of cells in CDM cultures revealed that neither Pax6 nor Nkx2.1 expression were significantly different between cultures at day 8 and day 16, but Mash1 expression was significantly higher in cultures at day 16 compared with those at day 8. Data from other studies reveals different expression patterns for these markers. Bouhon et al reported an increase in *Pax6* expression at day 8 compared with ES cells,[10] and showed downregulation of *Pax6* expression following addition of FGF2 and retinoic acid between day 4 and day 8 using RT-PCR.[15] Gaspard et al showed, an increase in expression of *Nkx2.1* from day 7 through to day 14, using CDM culture without addition of any morphogens.[33] In a previous study from our group, a cDNA expression array revealed an increase in expression of *Mash1* between ES cultures and day 8 CDM culture, which was confirmed by RT-PCR;[15] expression beyond day 8 was not analyzed. Taken together, these data present an unclear picture of the dorso-ventral expression pattern of ES cell-derived precursors over time in culture. It further highlights the importance of caution when interpreting results from studies using differing cell culture media and differentiation conditions, as well as different cell lines, and that reported findings might be restricted to each individual culture system.

FoxG1Z-derived precursors differentiated for 7 d in neuronal differentiation medium, in the absence of exogenous growth factors such as FGF, resulted in generation of β-III-tubulin positive neurons with few GFAP positive astrocytes. Here we demonstrate between 30-40% β-III-tubulin positive cells as a percentage of total cells, which is comparable with other studies, showing similar proportions of β-III-tubulin neurons depending on the neural induction method employed.[19] The increase in GFAP expression between cultures differentiated from day 8 and day 16 is consistent with previous work demonstrating a temporal neurogenic-gliogenic switch following increased time in neural induction culture.[10]

A proportion of the neurons generated were positive for GABA, the principle neurotransmitter of striatal MSNs. The expression of β-III-tubulin and GABA is consistent with previous *in vitro* differentiation analysis of neurally induced mouse ES cells at the same time points.[10] Although we did not detect expression of the striatal MSN marker DARPP32 in neurons generated from day 8 cultures, a small proportion of neurons generated from day 16 cultures did express DARPP32. However, the proportion of both GABA and DARPP32 positive neurons demonstrated here are less than previously reported; where between 78-95% of β-III-tubulin neurons expressed GABA and 32% of the GABAergic neurons expressed DARPP32.[19]

In vivo *analysis*

Precise post-mortem analysis requires the ability to distinguish between graft and host-derived cells following transplantation and *in vivo* differentiation. The use of the FoxG1Z ES cell line in this study allowed identification of cells expressing *LacZ* and therefore FoxG1. Only cells immunopositive for β-Gal were positively identified as donor cells, and thus, due to lack of another reliable marker, only these cells were further characterized. It is therefore not straightforward to draw conclusions regarding the origin of single-labeled cells, that is, whether graft or host derived.

Histological analysis revealed that all animals within the day 8 group exhibited grafts with massive overgrowth. The potential for ES cells to develop teratomas following transplantation is one major downfall

in their consideration as an alternative cell source for clinical applications. The appearance of non-neural-like areas within the day 8 grafts together with the overgrowth observed indicates the generation of teratomas, which can be recognized by the presence of a range of differentiated tissue types, representative of the 3 germ layers.[34] Encouraging observations revealing the potential of ES cell-derived precursors to generate desired phenotypes post-transplantation have not been without undesirable differentiation.[35-37] Indeed, within the same host brains, other findings include undifferentiated ES cells, non-neural tissue clusters,[35] including epithelium, muscle and cartilage,[36] and teratomas.[37]

In contrast, day 16 derived grafts displayed what appeared to be healthy, surviving grafts at 6 weeks post-transplantation, with no distortion of the brain and no signs of over-growth. There was an indication though that the grafts comprised heterogeneous cell types of both neural and non-neural lineage. However, this finding is not remarkable since non-neural differentiation has previously been reported following neural transplantation, as detailed earlier.[35-37]

Graft volumes were variable within the day 16 graft group and looked comparable to volumes from a previous study where mouse ES cell-derived precursors were grafted into the adult rat QA-lesioned striatum,[16] although this study reported no cell numbers or graft volumes. Primary rat striatal tissue grafts, with similar cell numbers transplanted, generated a range of volumes 20 times smaller than those reported here.[38,39]

Due to the problem of reliably identifying all donor cells, further graft analysis considered only those cells that were β-Gal positive. Grafted cells were shown to generate both immature and more mature neurons, shown by co-expression of β-Gal with the markers DCX and NeuN, respectively, and also glial cells, shown by co-expression with GFAP. Analysis of striatal-specific neurons revealed expression of donor-derived calbindin and parvalbumin, but neither DARPP32 nor FoxP1. The presence of calbindin immunopositive cells indicates striatonigral projection neurons characteristic of the matrix compartment of the striatum[40] and parvalbumin immunoreactivity is evidence of striatal medium aspiny neurons and interneurons within the graft.

There are only a handful of reports on the survival and phenotype of mouse ES cell-derived precursors transplanted into the QA-lesioned striatum (reviewed in ref. [6]). Most recently, mouse ES cells underwent neural induction using the monolayer method, were transplanted into the QA-lesioned striatum at day 12 and a proportion of grafted cells were shown to express DARPP32 at 4 weeks post-transplantation.[19] However, there was some continued expression of Oct4 at the time of transplantation and in some host animals tumor formation was evident.

DARPP32 expression has been reported following transplantation of human ES cell-derived neural precursors at both short and long-term survival times, but only when the precursors themselves were 'late' stage following addition of BDNF, SHH and DKK1, and not 'early' stage *in vitro*.[21] Indeed, DARPP32 positive cells within the graft comprised 21% of the NeuN population.[21] Human ES cells cultured in FGF2 and Noggin for 4 weeks prior to engraftment into unlesioned rat striata generated 30% DARPP32 positive neurons at 6 months post-transplantation, (expressed as a percentage of Human Nuclei immunopositive cells),[41] and human ES cell-derived precursors transplanted into the QA-lesioned striatum at 40 d following addition of SHH with subsequent exposure to valproic acid generated more than 50% DARPP32 positive cells, as a percentage of total grafted cells.[22] A dual SMAD inhibtion approach with subsequent modulation of SHH and Wnt pathways resulted in generation of a population DARRP32-expressing cells with concomitant reduction in apomorphine-induces rotations compared with non-grafted animals.[23] However, these studies also reported significant graft overgrowth;[21,22] with 35 fold expansion of grafted cells over the 4 month post-transplantation period.[22] Most recently, addition of Activin A has been shown to induce expression of markers of the lateral ganglionic eminence with 20-50% DARPP32 immunopositive neurons *in vitro*.[42] Following transplantation into the rat QA-lesioned striatum this protocol yielded high levels of DARPP32-expressing cells at 16 weeks post-transplant (50% of all Human Nuclei immunopositive cells), and, encouragingly, no sign of graft overgrowth.[42]

The precursors used in this study are likely too immature in terms of their gene expression profile and it is possible that precursors that have undergone additional patterning to further specify their fate would be more disposed to generation of DARPP32. Interestingly, Nasonkin et al reported no DARPP32

positive cells at 3 months post-transplantation but at 6 months DARPP32 was detected, suggesting that increased time *in vivo* may also play a part in inducing grafted cells to differentiate toward the DARPP32 phenotype.[41] This mirrors what has been reported following transplantation of primary human fetal striatal tissue into the rat QA-lesioned striatum, and further emphasizes the need for transplantation models that will allow for long-term assessment of grafts.[43] The absence of DARPP32 within the grafts presented here is most likely due to a combination of the aforementioned issues; cells were not exposed to all the necessary differential cues while in culture prior to transplantation, and also the *in vivo* maturation period was not long enough to encourage induction of more mature neuronal phenotypes such as DARPP32.

Conclusions

Using suspension cultures of ES cells we highlight the importance of concentration and timing of exposure to factors to endeavor to direct the fate of these cells. We show that increased time in induction culture down-regulates expression of stem cell markers, which results in reduced potential for teratoma formation. However, it is understandable that a combination of factors will be needed in order to generate striatal-like progenitor cells. Together, this demonstrates the need for further controlled refinement of protocols both *in vitro* and *in vivo*, to achieve the desired striatal phenotype population.

Materials and methods

Cell culture

The mouse ES cell line FoxG1-*LacZ* (FoxG1Z), derived from FoxG1^{lacZ} mice[24] was used (a gift from J. Quinn and D. Price, University of Edinburgh). ES cells were maintained in feeder-free culture conditions in Iscove's Modified Dulbecco's Medium supplemented with 15% knock-out serum replacement, 1% penicillin/streptomycin (PS), 1mM non-essential amino acids, 2mM L-glutamine (all Gibco, Paisley, Scotland, UK), 0.1mM β-mercaptoethanol (Sigma, Gillingham, Dorset, UK) and leukemia inhibitory factor (LIF) (produced 'in-house').

For neural induction, ES cells were enzymatically harvested with trypsin-EDTA (Gibco) and resuspended at a density of 5×10^5 cells/ml on bacteriological-grade culture dishes (Sterilin, Newport, UK) in CDM: Advanced DMEM/F12 supplemented with 1 × lipid concentrate, 1% PS, 2mM L-glutamine (all Gibco), transferrin (final concentration 150 μg/ml), insulin (final concentration 14 μg/ml) (both Sigma) and 0.1mM β-mercaptoethanol. The day of transfer to CDM was day 0 and medium was changed every 2 d FGF2 and DKK1 (both R&D Systems, Oxfordshire, UK) were used at 20ng/ml and 1000ng/ml, respectively, unless otherwise stated.

For neuronal differentiation of neurally induced cells, cells were harvested and incubated in accutase (PAA Laboratories, Somerset, UK) at 37°C for 15 min. A single-cell suspension was generated by trituration and cells were plated at a density of 25,000cells/cm^2 in neuronal differentiation medium (DMEM/F-12, 1% PS, 2% B-27 and 1% FCS) on poly-L-lysine (PLL)-laminin-coated coverslips.

X-Gal assay

The presence of β-Gal activity in cultures was detected by plating cells onto PLL-laminin-coated coverslips, partially fixing in 4% formaldehyde for 10 min followed by incubation in 1 mg/ml 5-bromo-4-chloro-3-indolyl galactopyranoside (X-Gal) (Fermentas, ThermoScientific). Cells were counter-stained with eosin.

Fluorescent immunocytochemistry

Cells were plated and fixed after 4 hours (as precursors) or 7 d (as differentiated cells), in 4% formaldehyde or 0.2% glutaraldehyde in 4% formaldehyde. Fluorescent immunocytochemistry was performed using standard protocols using the following primary antibodies: anti-β-Gal (1:1000, Promega), anti-Nestin (1:400, BD Pharm), anti-Oct4 (1:100, Santa Cruz), anti-Pax6 (1:50, DSHB), anti-Nkx2.1 (1:100, DAKO), anti-Mash1 (1:200, BDPharm), anti-β-III-tubulin (mouse 1:1000; rabbit 1:500, both Sigma), anti-GFAP (1:2000, DAKO), anti-GABA (1:500, Sigma), anti-DARPP32 (1:20,000, a gift from Paul Greengard) and anti-FoxP1 (1:500, Abcam). Secondary antibodies used were Alexa-fluor α 594 anti-mouse and α 488 anti-rabbit (both 1:200, DAKO).

Cells were visualised under UV fluorescence using a Leitz microscope and cell counts were performed using a grid randomly placed over 5 different fields per coverslip. The number of positive cells counted was expressed as mean ± SEM from replicates of 3–4

coverslips per condition. Images were processed using Optronics MagnaFire Software and Adobe Photoshop.

Transplantation experiments

All animal experiments were conducted in compliance with local ethical guidelines and approved animal care according to the UK Animals (Scientific Procedures) Act 1986 and its subsequent amendments.

Adult female Sprague-Dawley rats (Harlan, UK) received unilateral injections of 45nmol QA into the right striatum, as described previously.[44]

Cells were harvested on day 8 and day 16 of CDM culture and a single-cell suspension was generated using accutase (described above). Cells were grafted at: +0.6 mm AP, −2.8 mm ML and −5.0 and −4.0 mm below dura; 250,000 cells/μl at 1μl/min, 1 min at each of 2 heights. The needle was left at the graft site for a further 2 min before withdrawal. From the day prior to grafting and for the duration of the experiment, animals received daily immunosuppression; cyclosporin A (10 mg/kg; intraperitoneal) (Sandimmun, Novartis, Hampshire, UK). Post-operatively animals were administered 5ml 4% glucose/saline solution and analgesia in the form of Metacam (both subcutaneous).

Immunohistochemistry

Rats were transcardially perfused and tissue was prepared, as previously described.[44] Sections were processed for Nissl staining using cresyl violet and immunohistochemistry with the following antibodies: anti-β-Gal (1:1000, Promega), anti-GFAP (1:2000, DAKO), anti-NeuN (1:4000, Chemicon), anti-doublecortin (DCX, 1:500, Abcam), anti-DARPP32 (1:10,000, a gift from Paul Greengard), anti-FoxP1 (1:500, Abcam), anti-calbindin (1:20,000, Sigma) and anti-parvalbumin (1:4000, Sigma). The basic protocol was the same for each antibody.

Quantification and statistical analysis

Graft volumes were determined based on β-Gal immuno-reactivity. Cells expressing markers of interest were counted at 100x magnification using a Leitz light microscope and stereology software (Olympus CASTgrid stereology).

Statistical significance was assessed by t-test and ANOVA with *post-hoc* Tukey-Kramer (Minitab 15 statistical software).

Abbreviations

β-Gal	β-galactosidase
CDM	chemically defined medium
DARPP32	dopamine and cyclic adenosine 3′, 5′- monophosphate-regulated phosphoprotein, 32kDa
DKK1	Dickkopf-1
FGF2	fibroblast growth factor-2
GABA	gamma-amino butyric acid
HD	Huntington's disease
LIF	leukaemia inhibitory factor
MSN	medium-sized spiny neuron
QA	quinolinic acid
X-Gal	5-bromo-4-chloro-3-indolyl galactopyranosideneuron

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

SVP was involved in study design, carried out cell culture and surgical procedures, collected all data, performed data analysis and drafted the manuscript. CMK was involved in study design, carried out surgical procedures and critically revised the manuscript. NDA was involved in the concept and study design. AER was involved in study design and critically revised the manuscript. All authors approved the final manuscript for publication.

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