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Comparison of transplant efficiency between spontaneously derived and noggin-primed human embryonic stem cell neural precursors in the quinolinic acid rat model of Huntington's disease

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Abstract
Human neural precursors (hNP) derived from embryonic stem cells (hESC) may provide a viable cellular source for transplantation therapy for Huntington's disease (HD). However, developing effective transplantation therapy for the central nervous system (CNS) using hESC relies on optimizing the in vitro production of hNP to control appropriate in vivo posttransplantation neuronal differentiation. The current study provides the first direct in vivo comparison of the transplant efficiency and posttransplantation characteristics of spontaneously derived and noggin-primed hNP following transplantation into the quinolinic acid (QA) rat model of HD. We show that spontaneously derived and noggin-primed hNP both survived robustly up to 8 weeks after transplantation into the QA-lesioned striatum of the adult rat. Transplanted hNP underwent extensive migration and large-scale differentiation towards a predominantly neuronal fate by 8 weeks post-transplantation. Furthermore, in vitro noggin priming of hNP specifically increased the extent of neuronal differentiation at both 4 and 8 weeks posttransplantation when compared to spontaneously derived hNP grafts. The results of this study suggest that in vit ro noggin priming provides an effective mechanism by which to enhance hNP transplant efficiency for the treatment of HD.

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Brief Communication

Comparison of Transplant Efficiency Between Spontaneously Derived and Noggin-Primed Human Embryonic Stem Cell Neural Precursors in the Quinolinic Acid Rat Model of Huntington's Disease

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Human neural precursors (hNP) derived from embryonic stem cells (hESC) may provide a viable cellular source for transplantation therapy for Huntington's disease (HD). However, developing effective transplantation therapy for the central nervous system (CNS) using hESC relies on optimizing the in vitro production of hNP to control appropriate in vivo posttransplantation neuronal differentiation. The current study provides the first direct in vivo comparison of the transplant efficiency and posttransplantation characteristics of spontaneously derived and noggin-primed hNP following transplantation into the quinolinic acid (QA) rat model of HD. We show that spontaneously derived and noggin-primed hNP both survived robustly up to 8 weeks after transplantation into the QA-lesioned striatum of the adult rat. Transplanted hNP underwent extensive migration and large-scale differentiation towards a predominantly neuronal fate by 8 weeks post-transplantation. Furthermore, in vitro noggin priming of hNP specifically increased the extent of neuronal differentiation at both 4 and 8 weeks posttransplantation when compared to spontaneously derived hNP grafts. The results of this study suggest that in vitro noggin priming provides an effective mechanism by which to enhance hNP transplant efficiency for the treatment of HD.

Key words: Stem cell transplantation; Noggin; Neural progenitors; Huntington's disease

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant genetic neurodegenerative disorder (19) characterized by involuntary movements, cognitive deficits, and behavioral changes due to the progressive and selective degeneration of striatal GABAergic medium spiny projection neurons. HD is a good candidate for cell transplantation therapy because there is no effective treatment and there is the potential for early intervention. Primary fetal cells grafted into animal models of HD have demonstrated proof-of-principle evidence of neuroanatomical integration that alleviates clinically relevant motor and cognitive deficits (5,6,9–11,14,20,23,24,28,29,36). As a result, primary fetal tissue transplantation for HD is currently undergoing clinical evaluation (2,3,12,13,15–18,22,31–33).

However, ethical and technical issues associated with the use of primary fetal tissue for cell transplantation therapy have prompted studies to evaluate the potential of alternative renewable cell sources for transplantation therapy. Recent studies have identified the therapeutic value of various types of stem/progenitor cell transplants

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for HD (29,39,40). In particular, human embryonic stem cells (hESC) have the potential to provide a stable and unlimited cell source for transplantation therapy (35). The feasibility of transplanting hESC into the central nervous system (CNS) relies on the in vitro production of neural precursors (hNP). There are several methods for deriving hNP from hESC (1,21,27,30,37) but the transplant efficiency and posttransplantation characteristics of hNP derived with these methods have not been compared under the same in vivo conditions. One of the original methods for deriving NP from hESC relied on the spontaneous generation of rosette-like structures in differentiated colonies of hESC (30). Pera and colleagues (26) advanced this technique by the development of an efficient and controlled serum-free method for deriving hNP-rich cultures from hESC by priming cultures through the addition of the bone morphogenic protein antagonist noggin. Noggin-induced hNP can be maintained as free-floating neurospheres that differentiate in vitro into astrocytes, oligodendrocytes, and electrophysiologically mature neurons (21,26). In the current study we use both spontaneous derivation in neural medium (30) and noggin-primed derivation (21) to induce hNP from hESC. Transplant efficiency and posttransplantation characteristics of hNP derived from each method were directly compared in vivo using the quinolinic acid (QA) lesion model of HD.

MATERIALS AND METHODS

The GFP-expressing hESC line ENVY (7) was used for posttransplantation identification of hNP. This line expresses GFP at sustained, high levels throughout the entire cell in all differentiated progeny. ENVY hNPs were derived either by noggin treatment (500 ng/ml) of hESC (8) or by spontaneous hESC differentiation (30). In both conditions, rosette structures were mechanically harvested and cultured in suspension in neural medium supplemented with growth factors to allow neurospheres formation as previously described (30). hESC-derived neurospheres were cultured for 3 weeks prior to the day of transplantation, upon which they were collected, gently dissociated using Cell Dissociation Buffer (Invitrogen), and suspended in neurobasal medium without growth factors at 37,500 cells/μl for transplantation. All experiments were performed in strict compliance with the Florey Neuroscience Institute Animal Ethics Guidelines and conformed to international guidelines on the ethical use of animals.

Adult male Wistar rats (250–300 g at QA lesion) received surgery under IP anesthesia using 0.1 ml/kg of a 7:3 mix of ketamine (100 mg/ml) and xylazine (20 mg/ml). Transplantation of 2 μl/site of hNPs across two sites in the striatum (relative to bregma: AP +0.7 mm and ML +2.5 mm, DV −5.0 mm and −4.0 mm) was undertaken 1 week after 50 nM QA lesioning (at coordinates AP +0.5 mm, ML +2.7 mm, DV −5.0 mm). Cyclosporine (10 mg/kg, SC) was administered three times weekly for the duration of the experiment beginning 48 h prior to transplantation. Animals were transcardially perfused with 4% paraformaldehyde after terminal anesthesia with sodium pentobarbitone (120 mg/kg, IP) either 4 ($n = 8$) or 8 ($n = 9$) weeks after transplantation, brains were then postfixed in 4% paraformaldehyde overnight before further processing.

Brains were cryoprotected in 30% sucrose and sectioned on a Microm HM450 sliding microtome set at 40 μm. Eight series of sections (320 μm between consecutive sections within a series) were collected and stored in cryoprotective solution at −20°C. For immunofluorescence, sections were incubated with the appropriate primary antibodies. Antibodies were used at the following concentrations: mouse anti-MAP2 1:1000 (Millipore, USA), mouse anti-Pax6-s 1:20 (Developmental Studies Hybridoma Bank, IA, USA) mouse anti-NeuN 1:500 (Millipore), rabbit anti-GFAP 1:500 (DAKO, Sweden), rabbit anti-DARPP-32 1:500 (Millipore), rabbit anti-GAD67 1:1000 (Millipore), mouse anti-Ki67 1:200 (MIB-1 clone, DAKO), and mouse anti-Oct3/4 1:40 (Santa Cruz). Secondary goat anti-rabbit and goat anti-mouse Alexa Fluor™ 594 antibodies (1:200, Invitrogen) were used. Fluorescently labeled sections were imaged using a confocal laser-scanning microscope (Leica TCS SP2) equipped with UV, argon, argon/krypton and helium/neon lasers (Biomedical Imaging Resource Unit, University of Auckland).

GFP and the fluorescent labels were imaged serially to eliminate detection of bleed through and other artifactual fluorescence. To confirm colocalization confocal images were captured in a *Z*-series with an interslice gap of 0.3 μm. Graft volume was quantified from a set of serial coronal sections (distance 320 μm) from each animal. Graft core GFP expression was analyzed using Stereo Investigator™ (Microbrightfield, Williston, VT, USA) linked to a Nikon e800 upright microscope with epiflourescent capabilities. All statistical analysis was undertaken using Graphpad Prism v4.02 (Graphpad Software Inc., CA, USA). Analysis between groups at the same time point was undertaken using two-tailed unpaired Student's *t*-tests, whereas comparisons between multiple groups at different time points were undertaken using two-way ANOVA analysis with Bonferroni post hoc analysis. All results are presented as mean ± SEM and deemed statistically significant if $p < 0.05$.

RESULTS

Both spontaneously derived and noggin-primed ENVY hNPs transplants produced robust dense graft cores in the QA-lesioned striatum that survived up to 8 weeks

posttransplantation (Fig. 1A, B). Occasional GFP-positive hNPs were observed to extend up the needle tract and into the ipsilateral cortex (boxed region in Fig. 1A). Human nuclei staining confirmed that grafts expressing GFP were of human origin (Fig. 1C). Grafts were present in 7/8 recipient rats at 4 weeks posttransplantation and 7/9 recipient rats by 8 weeks posttransplantation; overall grafts survived in 14/17 of transplanted rats. By 8 weeks posttransplantion, 1/4 of the spontaneously derived hNPs grafts had developed into a large hyperplasic growth. The severely hyperplasic spontaneously derived hNP graft in the 8 week cohort compressed the ventricle walls and contained intragraft rosettes (Fig. 1D). This excessive overgrowth was not seen in any of the noggin-primed grafts, suggesting that highly proliferative cells remained in the neurospheres of spontaneously derived hNP even after 3 weeks induction in neural medium. Within the hyperplasic growth Oct3/4-positive pluripotent hESC (Fig. 1E) and a large numbers of human Ki67-positive proliferating cells (Fig. 1F) were found. The animal with spontaneously derived hNP that formed severe hyperplasia was excluded from subsequent analyses.

Epifluorescence microscopy and the Microbrightfield Stereo Investigator™ system were used to quantify the volume of the graft core. Quantification identified that at both 4 and 8 weeks posttransplantation, spontaneously derived hNP grafts were larger than noggin-primed hNP grafts ($p = 0.03$ and $p = 0.04$, respectively, two-tailed t-test) (Fig. 1G). We also observed a nonsignificant trend for the volume of both types of graft to decrease with time (Fig. 1G). After transplantation, GFP-positive cell bodies from both spontaneously derived and noggin-primed grafts were found not only in the graft core at the site of injection, but also distributed rostrocaudally throughout the lesioned striatum with GFP-positive cells migrating up to 3 mm from the injection site (Fig. 1H). However, the distribution of hNP cells varied greatly between animals and both spontaneously derived and noggin-primed hNP were observed overall to have a similar migratory capacity ($p = 0.74$, two-way ANOVA) (Fig. 1H). In addition, GFP-positive processes were found to extend up to 3.5 mm from the injection site in both 4 and 8 weeks posttransplant with no difference observed over time between transplants ($p = 0.30$, two-way ANOVA) (Fig. 1I).

Double fluorescent labeling and confocal microscopy was used to identify the phenotypic fate of grafted hNP. Cells within the striatum were randomly sampled and analyzed for coexpression of Pax-6, NeuN, MAP2, and GFAP. At both 4 and 8 weeks posttransplantation the neural progenitor cell marker Pax-6 was absent from all but a few transplanted cells in some animals (data not shown), indicating that most grafted hNP had developed a mature phenotype. Analysis of expression of neuronal markers MAP2 (Fig. 2A–C) and NeuN (Fig. 2D–F) confirmed that grafted hNP were highly directed towards a neuronal fate after transplantation into the lesioned striatum. Many GFP-positive cells coexpressed the neuronal marker MAP2 (isoforms a, b, and c) at both 4 and 8 weeks posttransplantation (Fig. 2A, B), with variable staining intensity. The number and proportion of MAP2 coexpressing cells (in both noggin-primed and spontaneously derived transplants) increased between 4 and 8 weeks posttransplantation ($p = 0.02$ spontaneously derived, $p = 0.008$ noggin primed) (Fig. 2C). Furthermore, in noggin-primed hNP grafts there were more GFP-positive cells coexpressing MAP2 than in spontaneously derived hNP grafts at both 4 weeks (21.50 ± 1.71 noggin primed vs. 4.00 ± 1.78 cells per 375 μm^2 of the graft core spontaneously derived; $p = 0.0004$) and 8 weeks (48.00 ± 6.58 noggin primed vs. 22.75 ± 5.65 cells per 375 μm^2 of the graft core spontaneously derived; $p = 0.03$) posttransplantation (Fig. 2C).

We also observed a population of grafted hNP differentiated into mature neurons. These cells were located mostly at the graft/host interface and were identified by NeuN coexpression (Fig. 2D, E). More noggin-primed grafted hNP expressed NeuN than spontaneously derived hNP at both 4 weeks (9.50 ± 1.56 noggin primed vs 4.00 ± 1.68 spontaneously derived cells per 375 μm^2 of the graft core; $p = 0.05$) and 8 weeks (22.00 ± 5.22 noggin-primed vs. 6.67 ± 0.88 spontaneously derived cells per 375 μm^2 of the graft core; $p = 0.04$) posttransplantation (Fig. 2F). Astrocytes were also observed to surround the edges of the ENVY hNP grafts and occasionally entered the grafts with most appearing to be host derived. Occasionally astrocytic differentiation of grafted hNP was seen within the main body of the ENVY hNP grafts as demonstrated by low levels of GFAP coexpression at 8 weeks (3.50 ± 0.65 spontaneously derived and 2.50 ± 0.65 noggin primed cells per 375 μm^2 of the graft core) (Fig. 2G–I). These results demonstrate that spontaneously derived and noggin-primed hNP undergo large-scale differentiation predominantly towards a neuronal fate by 8 weeks posttransplantation, with in vitro priming of hNP with noggin specifically increasing the extent of neuronal differentiation. The extensive neuronal differentiation of grafted ENVY hNP prompted a search for the expression of striatal markers that are selectively lost in animal models of HD. However, neither DARPP-32 nor GAD67 was coexpressed by grafted ENVY hNP in either group at 4 or 8 weeks after transplantation (data not shown).

DISCUSSION

This study has directly compared the transplant efficiency and posttransplantation characteristics of hNP de-

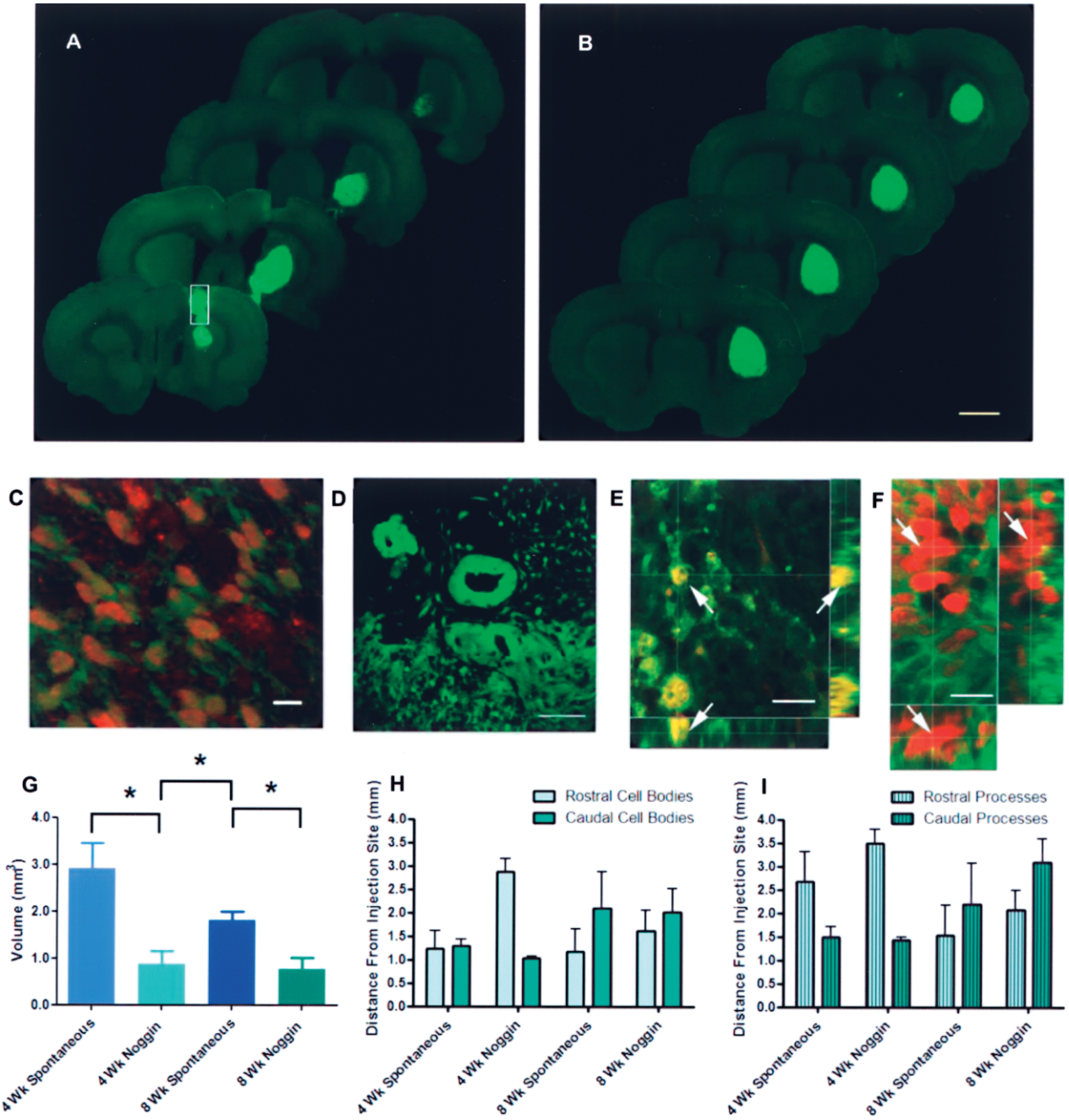

Figure 1. Survival, migration, and tumor formation of transplanted ENVY hNPs in the QA-lesioned striatum. Endogenous GFP expression in spontaneously derived (A) and noggin-primed (B) hNP grafts 8 weeks after transplantation into the QA-lesioned striatum; boxed region in (A) denotes grafted ENVY cells in needle tract. Scale bar: 2 mm for both (A) and (B). (C) Merged confocal image of GFP (green) and HuNu (red) coexpression confirming that GFP expression is maintained in xenotransplanted hNPs after 8 weeks in vivo. Scale bar: 8 μm. (D) Confocal slice through hyperplasic overgrowth with characteristic rosette structures from spontaneously derived hNP graft. Scale bar: 40 μm. (E) Oct3/4 (red) and (F) Ki67 (red) expression within hyperplasic overgrowth from a spontaneously derived hNP graft (green). Scale bar: 16 μm for both (E) and (F). (G) Graph of relative graft core volume for noggin-primed and spontaneously derived grafts at 4 and 8 weeks posttransplantation. (H) The migration of GFP-positive cells and (I) extent of GFP-positive processes from injection site.

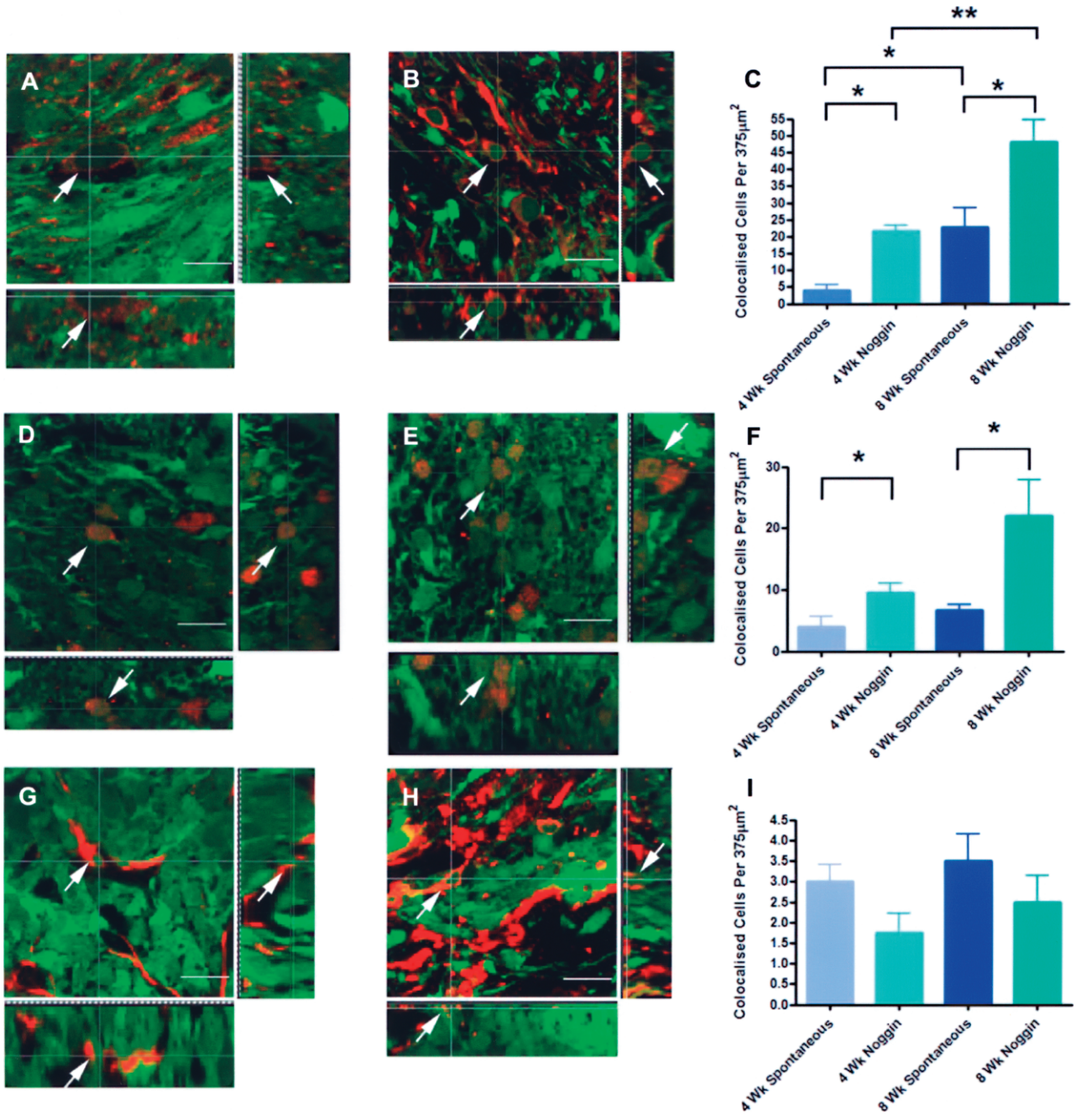

Figure 2. Neural differentiation of transplanted ENVY hNP in the QA-lesioned striatum. (A, B) Representative MAP2 expression (red) in spontaneously derived hNP grafts (A; green) and noggin-primed hNP grafts (B; green) 8 weeks after transplantation. (C) Graph demonstrating the number of MAP2-positive hNP cells per 375 μm^2 in the graft core. (D, E) Representative NeuN expression (red) in spontaneously derived hNP grafts (D; green) and noggin-primed hNP grafts (E; green) 8 weeks after transplantation. Graph demonstrating the number of NeuN (F) positive hNP cells per 375 μm^2 in the graft core. (G, H) Representative GFAP expression (red) in spontaneously derived hNP grafts (G; green) and noggin-primed hNP grafts (H; green) 8 weeks after transplantation. Graph demonstrating the number of (I) GFAP-positive hNP cells per 375 μm^2 in the graft core. Bonferroni post hoc pairwise comparisons. $*p < 0.05$, $**p < 0.01$. Scale bars: 16 µm.

rived by two different methods. We have established in the QA-lesioned rat striatum that noggin-primed hNP have a greater capacity to survive long term and differentiate into neurons than spontaneously derived hNP. In addition, hyperplasic growths and aberrant differentiation was not observed to accompany grafting of noggin-primed hNP even up to 8 weeks posttransplantation, whereas grafts of spontaneously derived hNP had the capacity to form tumors. This suggests that noggin priming of hESC may provide an important safety advantages for hNP transplantation. We suspect this is because noggin priming induces hNP further toward a neuronal fate while in vitro and still within neurosphere aggregates. At the time of transplantation, spontaneously derived hNP likely comprise a more heterogeneous cell population, including residual pluripotent cells, increasing the potential for tumor formation. Hyperplasic overgrowth of hESC derived neural grafts and teratoma formation are commonly encountered; indeed, other studies have reported either high rates of tumor formation or reduced rates of survival with the transplantation of extensively differentiated hESC-derived dopaminergic neurons in animal models of Parkinson's disease (4,25, 34,38).

A small number of studies have recently been published on hESC-derived stromal cell-induced hNP transplants in animal models of HD (1,37). Song and colleagues (37) examined transplanted hNP generated by PA6 stromal coculture up to 3 weeks posttransplantation. They reported mainly immature (nestin and TUJ1 positive) neuronal differentiation of PA6-generated hNP following transplantation into the QA-lesioned striatum. This is in contrast to the current study where the majority of transplanted hNP were highly neuronally directed, and even at 4 weeks expressed mature neuronal markers, such as MAP2 or NeuN. While Song and colleagues (37) did not report the presence of hyperplasia, the study did not investigate hNP cell fate beyond 3 weeks posttransplant, which may have misrepresented the potential safety of their PA6-generated hNP. In the current study the overgrowth and appearance of rosette-like formations was not apparent until 8 weeks posttransplantation and was only observed in grafts of spontaneously derived hNP. Disparities between these studies may reflect differences in cell fate determined by noggin priming of hNP versus PA6 stromal coculture methods, and the extent of differentiation that will be induced by longer periods that the graft is in vivo (3 vs. 4 and 8 weeks posttransplant). In support of this later point, a recent investigation (1) using MS5 stromal-induced hNP grafted into the QA-lesioned striatum found that it was not until 8 weeks posttransplantation that significant numbers of transplant-derived overgrowths could be identified.

Although ENVY hNP grafted into the QA-lesioned striatum differentiated into mature neurons relatively early, expression of striatal-specific markers such as DARPP-32 expression was not observed by 8 weeks posttransplantation. In contrast, we have previously demonstrated that 8 weeks is sufficient for DARPP-32-positive neurons to develop following the transplantation of adult rodent NP into the QA-lesioned striatum (39). This suggests either that region-specific neuronal phenotypes are slower to develop from xenotransplanted hNP than in allogenic transplanted progenitors, or that NP from embryonic stem cells are slower than those from adult sources to develop a mature phenotypic identity. Future transplantation studies using embryonic and/or human stem cell sources should be sure to contain an extended period of investigation in order to confirm the generation of region-specific striatal phenotypes.

In this study we have made the first direct comparison of the transplant efficiency and differentiation potential of spontaneously derived and noggin-primed hESC-derived NP in the QA lesion rat model of HD. By establishing the efficiency and safety of noggin-primed hNPs against traditional neuronal direction paradigms in vivo in a lesioned environment, we are able to identify the value of larger future investigations using noggin primed hNPs. The safety and efficiency of noggin-primed neuronal direction we have shown in the current study decreases the likelihood of arresting future investigations due to hyperplasic hNPs, as has recently been required in some studies (1). The assessment of spontaneously derived or noggin-primed hNPs on sensorimotor function was not included in this current study due to the small group sizes. However, additional long-term studies elucidating the functional effects of noggin-primed hNP on a variety of sensorimotor deficits produced by QA striatal lesioning, and the ability for transplanted hNP to form appropriately integrated mature medium spiny striatal neurons, will be necessary to confirm and further establish the therapeutic potential of hESC-derived hNP transplantation for HD. The QA lesion model was used in this initial study to provide a rapid, reliable, and well-substantiated model of the selective and extensive striatal cell loss observed in HD. Transgenic mouse models of HD are more predictive to the clinical situation, and future studies using transgenic models of HD are also necessary in order to assess whether the diseased host brain affects the long-term efficiency of hESC-derived hNP transplants.

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