

Transplantation of pig stem cells into rat brain: proliferation during the first 8 weeks

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Abstract

Previous work indicated that pig umbilical cord matrix (pUCM) cells are a type of primitive stem cell and that these cells could be recovered after central or peripheral injection into rats that did not receive immune suppression therapy. To determine the safety and proliferation potential of pUCM cells after brain transplantation, approximately 150 pUCM cells were transplanted into the brains of rats that previously received a striatal injection of the neurotoxin 6-hydroxydopamine (6-OHDA). The pUCM cells were previously engineered to express enhanced green fluorescent protein (eGFP); in this way, the graft cells were identified. The rats did not receive immune suppression therapy. There were no postsurgical complications and the animals thrived following transplantation. At 2, 4, 6, and 8 weeks after transplantation, two rats were sacrificed and the morphology, size and number of graft cells, and the percentage of tyrosine hydroxylase (TH)-positive graft cells were determined. The size distribution of the grafted pUCM cells was unimodal and normal, and the average size increased significantly over the 2- to 8-week survival period. The number of pUCM cells increased from approximately 5400 cells at the 2-week survival period post-transplantation to approximately 20,000 cells at the 8-week survival period. There was an increase in the percentage of TH-positive pUCM cells from approximately 1% at the 2-week survival period to approximately 6% at the 8-week survival period. There was no evidence of a significant host immune response at any time; for example, no accumulation of CD-4, CD-8, CD-11b, CD-161 cells in the transplantation site. These results suggest that pUCM cells engraft and proliferate without requiring immune suppression. These findings also suggest that a subset of pUCM cells can differentiate into TH-positive cells within 8 weeks after transplantation into the 6-OHDA lesioned rat brain.

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Introduction

In Parkinson's disease (PD), human fetal mesencephalic transplants were once used as a source of replacement cells. Moral and ethical concerns associated with the use of fetal tissue, the scarcity of that tissue, and other problems associated with obtaining enough tissue were barriers to the widespread use of human fetal mesencephalic transplantation. Mesencephalic cells obtained from fetal pigs is one treatment modality used for therapeutic transplantation in PD

patients, which is yet to be approved for clinical purposes (Bjorklund and Lindvall, 2000; Deacon et al., 1997). Xenotransplantation has problems such as graft versus host disease (GVHD) and immune rejection (Larsson et al., 2000). For therapeutically useful numbers of the xenografted cells to survive, the host's immune system must be suppressed (Deacon et al., 1997). Despite immune suppression, it was estimated that about 5–10% (Bjorklund, 1991) or about 25% (Brundin et al., 2000) of the harvested cells survive transplantation. Experimental work with fetal pig mesencephalic tissue grafted into the rat brain indicates that the grafted tissue is rejected in about 4–6 weeks (Larsson et al., 2001). The rejection response affects the function of the engrafted tissue because the grafts with a lower immune rejection response have better survival and more extensive

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fibers compared to the grafts with stronger immune rejection response (Larsson et al., 2001). Furthermore, immunosuppression treatment slows but does not prevent the rejection of pig tissue grafts in rat brain (Duan et al., 1996). At the same time, serious complications can arise from immunosuppression and from the secondary effects of the immunosuppressive drugs, such as cyclosporine-A. Thus, two ideal characteristics of therapeutic grafts would be (1) the capacity to treat the neurodegenerative disease, that is, differentiate into the appropriate replacement cells or produce factors that slow or reverse the disease process and (2) the ability to not stimulate a robust immune rejection response.

The umbilical cord matrix (UCM), or Wharton's jelly, is a gelatinous connective tissue of umbilical cord, which consists of myofibroblast-like stromal cells, collagen fibers, and proteoglycans (Kobayashi et al., 1998; McElreavey et al., 1991). The umbilical cord matrix is a source for primitive stem cells and differentiation signals cause pig UCM (pUCM) cells to exhibit a neuronal morphology and express neural and glial markers in vitro (Mitchell et al., 2003). Two to 6 weeks following transplantation into rat brain, a subset of pUCM cells were found to morphologically resemble neurons and to express pig-specific neuronal markers (Weiss et al., 2003). Here, the safety of pUCM cells for transplantation is evaluated by testing their potential to proliferate and the ability of pUCM cells to express tyrosine hydroxylase (TH) following transplantation into the brain of rats with a previous catecholaminergic (6-OHDA) lesion, a rodent model of PD.

Materials and methods

Cell culture and counting

Pig UCM cells were cultured and maintained as described previously (Mitchell et al., 2003; Weiss et al., 2003). Manipulation of pUCM cells to express enhanced green fluorescent protein (eGFP) has been described previously (Weiss et al., 2003). Briefly, pUCM cells that were cultured in vitro 60 passages were transfected to express eGFP. After several selection passages, the eGFP expressing pUCM cells were lifted by a trypsin solution. The cells were counted by a hemocytometer and were adjusted to a final concentration of approximately 150 cells per microliter. The number of cells in 1 μ l was verified by spreading a 1 μ l drop on a plastic Petri dish and manually counting the cells at 10 \times in bright field of a light microscope. The cell concentration was confirmed before and after the injection to insure that approximately 150 cells were delivered.

Transplantation procedure

A guide cannula was implanted in the brain of anesthetized male Lewis rats (Harlan) via stereotaxic

surgery into the right striatum (Bregma + 0.5, Lateral 3.4, Ventral 5.0 mm from the surface of the brain) (Paxinos and Watson, 1986). The cannula was attached to the skull with screws and dental acrylic. At least 3 days later, anesthetized animals received a single injection of 10 μ l of 7 mg/ml 6-hydroxydopamine (6-OHDA, Sigma) over 5 min. At least 1 week after 6-OHDA injection, approximately 150 eGFP-pUCM cells in 1 μ l of the sterile media were injected over 5 min. Rat which received guide cannula, but did not receive a transplant or 6-OHDA, served as control. At 2, 4, 6, and 8 weeks post-transplantation, two rats were randomly selected, anesthetized, and sacrificed by transcardial perfusion with heparinized isotonic saline rinse followed by 10% buffered neutral formalin. The brains were removed, postfixed, and cryoprotected in 20% sucrose overnight. Frozen sections of the brains were cut coronally at 40 μ m, and the sections were collected into three sets of adjacent sections, each set consisting of every third serial section.

Tissue processing and immunocytochemical processing

Immunocytochemical (IC) detection of a single antigen was performed on one set of sections as previously described (Weiss et al., 2003) and the adjacent sets of sections were held in reserve in a cryoprotectant solution (Watson et al., 1986). The free-floating tissue sections were stained with primary antibodies for GFP (rabbit host, 1:1000; Santa Cruz Biotechnology, Inc), TH (rabbit host, 1:2000; East Acres Biologicals), CD-4 (mouse host, 1:500, Serotec), CD-8 (mouse host, 1:500; Serotec), CD-11b (mouse host, 1:250; Serotec), CD-161 (mouse host, 1:250; Serotec). The antigens were visualized either with diaminobenzidine (DAB) and hydrogen peroxide using a commercially available ABC kit (Vectastain) or with immunofluorescence. For immunofluorescence localization, 7-amino-4-methylcoumarine-3-acetic acid (AMCA)-Avidin D (Vector Laboratories) was used with the biotinylated secondary antibody. The IC-stained sections were mounted on subbed microscope slides, air-dried, and rinsed with distilled water. To detect the immunofluorescence and eGFP staining, the sections were observed using epifluorescence illumination with the appropriate filter combinations on a Leica DMRD microscope after clearing and coverslipping with glycerol containing *N*-propyl gallate (3 parts 2% *N*-propyl gallate in 0.1 M Tris buffer, pH 9.0 and 7 parts glycerol). IC-stained cells were considered positive if (1) the signal in the cytoplasm is above background and (2) if the signal was absent in tissues in which the primary antibody had been omitted. To be considered double-labeled, the morphology and location of the cells must appear identical in both bright-field (DAB) and fluorescence (eGFP) for immunoperoxidase detected cells, or in both filter combinations for immunofluorescence (UV filter set for AMCA versus FITC filter set for eGFP).

Cell size and number

To measure cell size and number, one set of sections were stained with anti-GFP antibody and localized with DAB. A design-based unbiased stereological method was used for counting the cells. Individual cells were identified in bright-field illumination and measured using a morphometry and/or image analysis system (Bioquant Nova Prime, R&M Biometrics). Graft cells that were identified in both bright-field (IC for GFP) and epifluorescence (GFP fluorescence) were measured. With the experimenter blind to survival group, the area of at least 75 cells (cells which did not overlap with any other graft cell and had an obvious nucleus) was measured (this area represents cell size and the term “cell size” is used for cell area throughout the manuscript), and the cell size distribution was analyzed for outliers and normality (StatView 5.0), and a frequency histogram was generated for each individual animal. To estimate the number of transplanted cells, the morphometry and/or image analysis software was used to measure the area occupied by graft cells in each section (the area occupied by the graft cells in a particular section is assumed to be directly proportional the volume of the graft in that section). After measuring the graft cell area in all sections containing GFP IC-positive cells, the total area of the graft was represented as the sum of the area in each section. No correction factors were applied. The total area of the graft was divided by the average cell size to yield an estimate of the number of graft cells detected in each set of sections. Because there are three sets of brain sections for each animal, the estimate of the total number of graft cells in the respective animal was three times the total number of graft cells in one set of sections.

Assessment of TH-positive cells

A second set of brain sections was IC-stained for tyrosine hydroxylase (TH) and localized using AMCA. The TH-positive graft cells appeared in both the FITC filter set (eGFP) and the UV filter set (AMCA). Again, with the experimenter blind, an estimate of the percentage of graft cells that stained for TH was made by evaluating a minimum of 10 fields per animal (fields were selected for counting based upon the distribution of cells in the field; e.g., fields were selected that did not have clumps of graft cells). In each field, the number of eGFP cells (FITC) and the eGFP-TH positive cells (AMCA and FITC) were counted. The percentage of TH-positive graft cells was calculated and averaged to yield an estimate of the percentage of TH-positive graft cells in each animal. After the analysis was completed, the individual animals were assigned to the appropriate survival groups. To estimate the total number of TH cells, the percentage of TH graft cells was multiplied by the graft cell number for each animal.

Assessment of host immune response

The frozen brain sections were IC-stained using primary anti-rat CD4, CD8, CD11b, or CD161 antibodies. These primary antibodies were localized using biotinylated secondary antibodies and Avidin-AMCA.

Statistical analysis

All tissue manipulations were conducted in large batches to avoid batch to batch differences in tissue IC staining. Tissue processing and data collection were conducted in an experimenter-blind fashion. After measurements and counting, the survival group status was decoded before statistical analysis. The histogram of the cell size was inspected for outliers (visual inspection of the distribution compared to a normal one). If no outliers were observed, then the Kolmogorov–Smirnov (K–S) test was used to compare the distribution of measured cell size to an idealized normal distribution (Statview 5.0). When outliers were observed, the Mann–Whitney *U* test was used to compare the distributions. In all cases, no outliers were observed; thus, the K–S test was used here. The K–S test revealed that all distributions were normal. Thus, ANOVA was used to test interactions between the independent variable (survival period after grafting) and the dependent variables (cell size, graft cell number, percentage of TH graft cells and number of TH graft cells). Significance for ANOVA was set at $P < 0.05$ (two-tailed). Following significant ANOVA, post hoc analysis using Scheffe’s *F* test was used to examine planned comparisons. Significance for post hoc testing was set at $P < 0.05$ (two-tailed). The means ± 1 standard error are presented on graphs.

Results

Cannula placement

In one of the eight animals, the guide cannula was misplaced. This animal was excluded from further analysis (6-week survival). Because the 6-week survival period had only one animal with a good cannula placement, no data from that survival period is included. Thus, the results presented are from the 2-, 4-, and 8-week survival periods averaged from two animals at each survival period.

Behavioral and histological findings

None of the animals showed any behavioral abnormalities following the implantation of cannula. Following 6-OHDA lesion, the animals demonstrated rotation (spontaneous) towards the damaged hemisphere during periods of excitement. Rotational behavior was not quantified. Following 6-OHDA lesion, the animals did not show other

behavioral signs or changes in their health status. After the transplantation with eGFP-pUCM cells, the animals acted normally and appeared in robust health throughout the 2- to 8-week survival period (there was no indication of sickness behavior, weight loss, etc). Midbrain sections, when stained for TH, revealed destruction of most of the TH-positive dopaminergic cells in substantia nigra of the ipsilateral side of the 6-OHDA lesion, but not on the contralateral side (Fig. 1).

Histological findings within the graft

There was no evidence of tumor, teratoma, or scar formation in the transplant recipients. Withdrawal of host tissue was noted around the cannula implantation site in both control and grafted animals. The pUCM-eGFP cells were identified by their endogenous candy apple green fluorescence (FITC filter cube, see Fig. 2A). To control for the possibility of autofluorescence by host cells, the sections were IC-stained using an antibody to GFP and visualized with AMCA. These results are shown in Fig 2B. Virtually, all the graft cells exhibiting GFP fluorescence were localized with AMCA. When the primary antibody is omitted, the graft cells were not observed using the UV filter set (Figs. 2C and D).

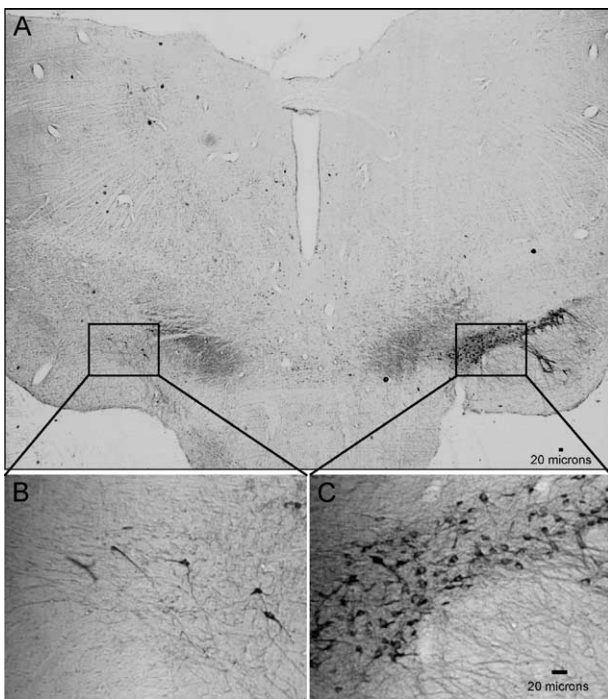


Fig. 1. Histological findings in substantia nigra (SN) of the lesioned rats. A unilateral 6-OHDA lesion is created by injecting 6-OHDA in to the right striatum of all the rats under study. The midbrain sections were stained for TH to evaluate the effect of the lesion. Most of the TH-positive dopaminergic neurons in the ipsilateral (right) SN are destroyed (A) (magnified view in B), but not in the contralateral (left) SN (A) (magnified view in C).

The graft cells were localized by IC staining for GFP followed by DAB visualization. Two weeks after transplantation, the graft cells were found along the sides and at the tip of the guide cannula tract. Most of the graft cells were clustered. Occasionally, individual cells were observed; most individual graft cells appeared small and spherical with a granular cytoplasm (Fig. 3A). Four weeks after transplantation, the graft cells were found farther from the guide cannula tract in the surrounding host brain tissue. At this time, more of the graft cells were dispersed and a greater percentage of the graft cells were elongated or bipolar in appearance. Some of the graft cells had short, primary processes attached to the cell body (Fig. 3B). Eight weeks after transplantation, the GFP staining in the graft cells was less intense and the graft cells appear larger and morphologically more complex when compared to the graft cells recovered 2 weeks post-transplantation (Fig. 3C). No further migration was noticed from 4 to 8 weeks post-transplantation. Short processes were observed on a subset of the population of pUCM cells 4, 6, and 8 weeks after transplantation (see Fig. 4).

Cell size

Graft cells were identified by IC staining for GFP (visualized with DAB) and epifluorescence of GFP (FITC filter set). From 75 to 110 cells per animal were measured and a frequency histogram of the cell size was created. No outliers were observed. All distributions were normal (K–S normality test) and unimodal. Next, the size distributions between animals at a given survival period were compared; there was no significant difference between cell size distributions at each survival period; those data were blocked for subsequent analysis by survival period (data not shown). At 2 weeks post-transplantation, the average size of the graft cells was $140.0 \pm 3.7 \mu\text{m}^2$. At 4 weeks post-transplantation, the average size of the graft cells was $160.2 \pm 12.1 \mu\text{m}^2$. At 8 weeks post-transplantation, the average size of the graft cells was $171.9 \pm 2.3 \mu\text{m}^2$. Thus, the graft cells at 8 weeks after transplantation were 22% larger than those at 2 weeks; this represents a significant increase in cell size (see Fig. 5).

Number of graft cells

To estimate the proliferation of the graft cells in each individual, the total GFP-IC positive area in one set of sections (set A) was divided by the average cell size for that individual. The estimated number of graft cells at each survival period is shown in Fig. 5. At 2 weeks after transplantation, the estimated number of graft cells was 1825 ± 163 (yields a total of about 5475 graft cells per animal). At 4 weeks after transplantation, the estimated number of graft cells was 5758 ± 400 (yields a total of 17274 graft cells per animal). At 8 weeks after transplantation, the number of graft cells was estimated to be

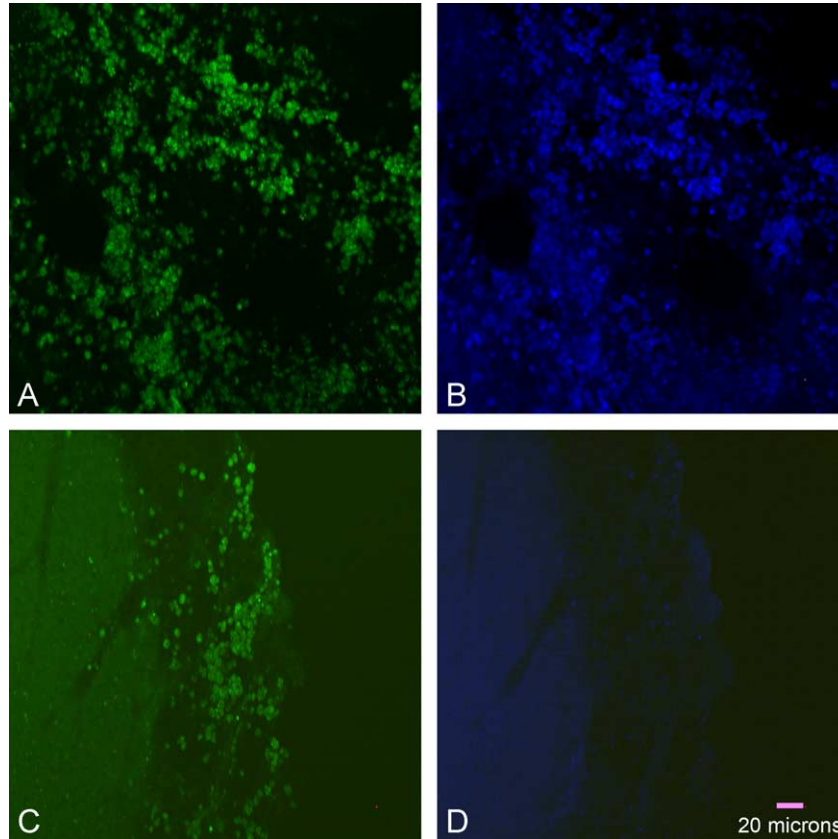


Fig. 2. Appearance of pig UCM cells after transplantation into rat brain. Pig UCM cells that were engineered to express eGFP were transplanted into the brains of rats that had a previous unilateral 6-OHDA lesion. The pUCM graft cells were recovered after 2, 4, and 8 weeks post-transplantation. (A) The eGFP-pUCM graft cells were identified by the epifluorescence (FITC filter set). (B) The brain sections were immunostained with anti-GFP serum and visualized using AMCA. The colocalization of AMCA with eGFP confirms the recovery of the graft cells. Virtually all of the graft cells show positive reaction with AMCA. (C and D) Control staining performed without the primary antibody for GFP shows no reaction for AMCA.

6904 ± 1000 (yields an estimated total of 20712 graft cells per animal). The number of graft cells increases significantly from 2 to 4 weeks and 2 to 8 weeks (see Fig. 5). This indicates that there is roughly a five- to sixfold expansion 2 weeks after transplantation and a seven- to eightfold expansion of the original 150 graft cells by 8 weeks after transplantation.

TH-positive graft cells

The sections in set B were IC-stained using anti-TH antibody and visualized with AMCA. The graft cells that colocalized with green fluorescence for GFP (FITC filter set) and blue fluorescence for AMCA (UV filter set) are considered to be TH-positive graft cells (Figs. 6A and B). When the TH primary antibody was omitted, no positive staining for TH was observed (Figs. 6C and D). The percentage of TH-positive graft cells is shown in Fig. 7. Two weeks post-transplantation, $1.0 \pm 0.6\%$ of the graft cells were positive for TH. Four weeks post-transplantation, $3.4 \pm 0.6\%$ of the total graft cells were positive for TH. Eight weeks post-transplantation, $6.0 \pm 0.3\%$ of the total graft cells were positive for TH. Thus, there was a significant increase in the percentage of TH-positive graft

cells at the 8-week survival period compared with the 2-week survival period. The estimate of total number of TH-positive graft cells at each survival period is shown in Fig. 7. The total number of graft cells previously calculated was multiplied by the percentage of TH-positive graft cells. It was estimated that a total of 54 TH-positive graft cells were found in the 2-week survival animals, 587 TH-positive graft cells in the 4-week survival animals, and approximately 1242 TH-positive graft cells in the 8-week survival animals (see Fig. 7). Thus, there was a significant increase in the number of TH-positive graft cells at the 8-week survival period compared with the 2-week survival period.

Host immune response

There was no significant gross or histological evidence of immune rejection in the brain of the animals 2–8 weeks following transplantation; for example, there was no vacuolization, perivascular cuffing, or cellular infiltrate. In addition, the frozen brain sections were IC-stained using anti-rat CD4, CD8, CD11b, and CD161 antibodies, and visualized with AMCA. IC staining for anti-rat CD4 showed negligible staining; in contrast, the control animal, which received a cannula only, had variable and conspicuous

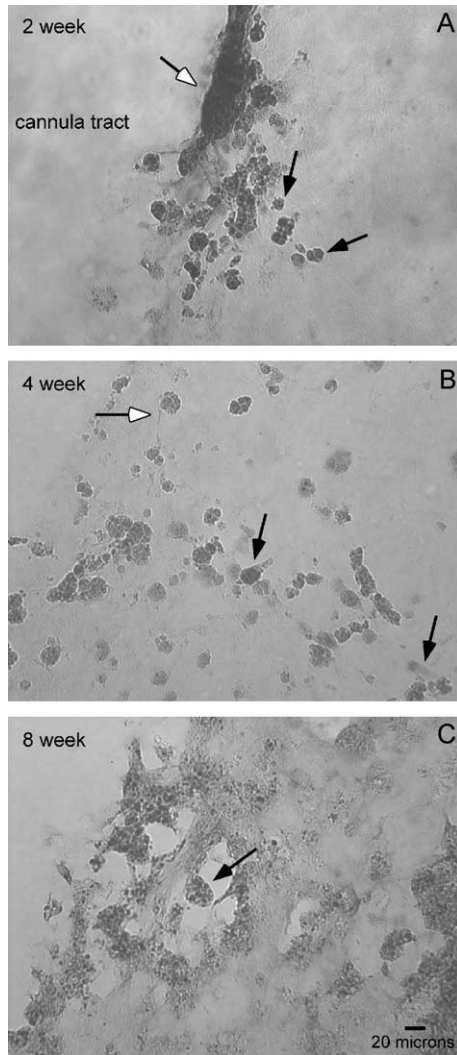


Fig. 3. Pig UCM graft cells after 2, 4, and 8 weeks post-transplantation. The frozen brain sections of all the rats were immunostained with GFP antibody and localized with DAB. (A) After 2 weeks post-transplantation, the pUCM cells can be recovered and they appear round (black arrow), granular, tend to form clumps (white arrow) and are located just next to the cannula tract. (B) After 4 weeks post-transplantation, the pUCM cells can be recovered. Some of the cells appear elongated (black arrow) and some appear to be extending small processes (white arrow). The graft cells are more scattered into the brain tissue around the cannula tract. (C) After 8 weeks post-transplantation, the pUCM cells can be recovered and appear to be bigger in size (black arrow) than after 2 and 4 weeks. A more diffused staining is noticed in the brain tissue around the cannula tract.

staining for anti-CD4 antibody (see Fig. 8). Similar results were observed when the sections were IC-stained using primary antibodies directed at CD8, CD11b, and CD161 (data not shown).

Discussion

Here, approximately 150 pig UCM cells were transplanted into the brains of previously 6-OHDA lesioned rats and characteristics of the graft cells were determined over a



Fig. 4. Pig UCM cells extend processes. A subset of pUCM graft cells extends processes (black arrow) into the host's brain. This may indicate that the graft cells respond to the local cues and differentiate into a neural phenotype.

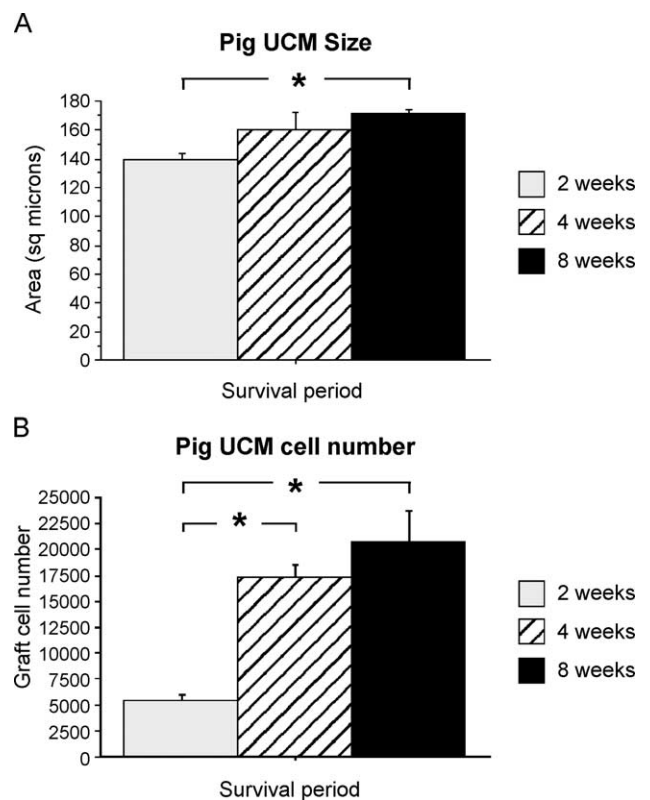


Fig. 5. Pig UCM cell size and cell number. One hundred fifty pUCM cells were transplanted into rats with previous 6-OHDA lesions. The cell size and number of grafted cells was evaluated at 2, 4, and 8 weeks post-transplantation. (A) Cell size measured in 75–110 graft cells. The distribution of size was tested and found to be normal with a single mean (data not shown). The cell size is calculated for each animal and cell size at each survival period was calculated. (B) The number of pig UCM cells was estimated in each animal. The graft cells undergo about a fivefold expansion in the first 2 weeks and increase to a maximum of about a sevenfold expansion by the eighth week. $N = 2$ at 2, 4, and 8 weeks. $*P < 0.05$.

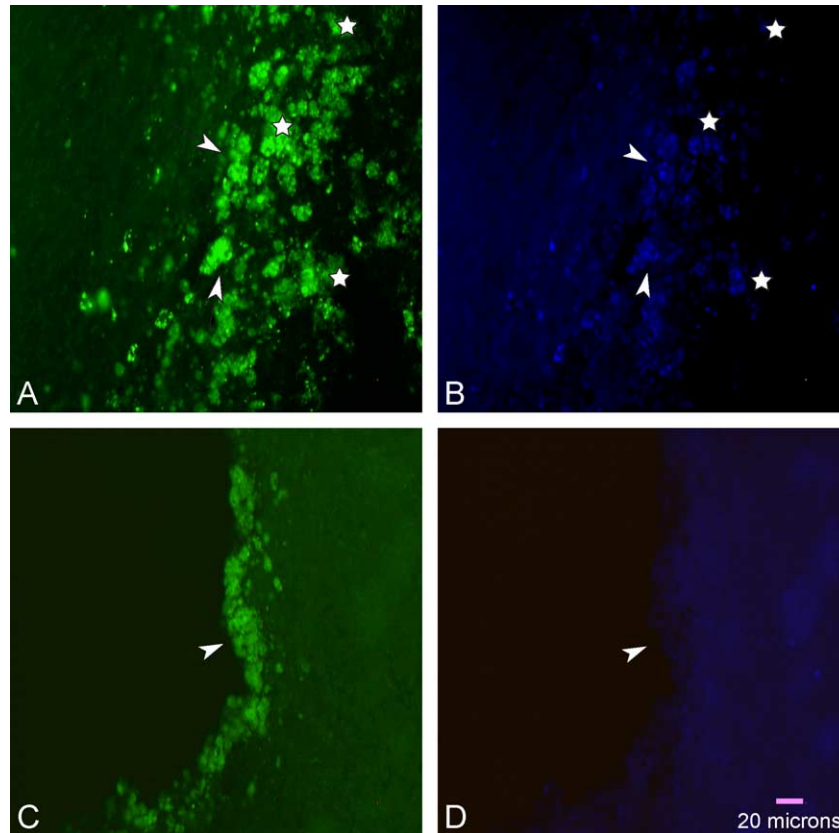


Fig. 6. Pig UCM cells stain for TH. Approximately 150 pig UCM cells expressing eGFP are transplanted into rats with a previous unilateral 6-OHDA striatal lesion. The pUCM graft cells were recovered after 2, 4, and 8 weeks post-transplantation. (A.) The pUCM graft cells can be identified by the green fluorescence of the GFP. (B) The frozen brain sections were IC-stained using anti-TH serum and visualized by AMCA. The colocalization of AMCA with eGFP confirms the TH-positive graft cells (white arrows). TH-negative graft cells are indicated by white asterisks. (C and D) Control staining performed without the primary antibody for TH shows no reaction for AMCA (white arrowheads).

2- to 8-week survival period. Specifically, the size and morphology of the graft cells, the total number of graft cells, and the percentage and number of pig UCM cells that synthesize tyrosine hydroxylase (TH) was determined at 2, 4, and 8 weeks after transplantation. Four observations were made. First, pig UCM cells increased in size over the 2- to 8-week period and a subset of the grafted cells extended short processes into the parenchyma of the host's brain. Second, pig UCM cells proliferated rapidly in the first 4 weeks after transplantation, but the proliferation rate appears to slow over the next 4 weeks after transplantation (weeks 4–8 after transplantation). Third, the percentage and total number of pig UCM cells that stain for TH significantly increased over the 2- to 8-week survival period. Fourth, there was no apparent evidence of a significant host immune response to the transplantation of pig UCM cells. These results indicate that pig UCM cells proliferated after transplantation and that a subset of the grafted cells that synthesize TH increased over the 2- to 8-week survival period. Additionally, the pig UCM cells appear to engraft without stimulating a significant immune rejection response over the 2- to 8-week period.

Pig UCM cells were detected by GFP fluorescence and IC staining for GFP. No positive GFP-IC staining, nor

GFP fluorescence was found in the control animal which had a cannula but no graft cells (data not shown). The expression of a marker protein such as GFP over the 2- to 8-week survival period following transplantation supports the notion that pig UCM cells have not been scavenged by the immune system. In previous work, lysed pig UCM cells were injected into the rat brain and no stained cells (neither host nor graft cells) were detected at the injection site (Weiss et al., 2003). This indicates that either cellular debris or the leakage of GFP from dead or dying cells does not produce nonspecific staining. It was observed that GFP staining was less intense in animals with the longest survival times compared to those animals that survived 2 weeks. The reason for the decrease in intensity is unknown. Additionally, the grafted cells increased in average size over time; the reason for this is not understood.

Here, 150 cells were transplanted because previous work has shown that the number of cells in a graft may influence graft survival or function. For example, Ostenfeld et al. (2000) compared human neural precursor cells that were transplanted at 200,000 cells (low density), or high-density grafts (1–2 million cells). They reported that low-density grafts had significantly fewer host immune cells compared

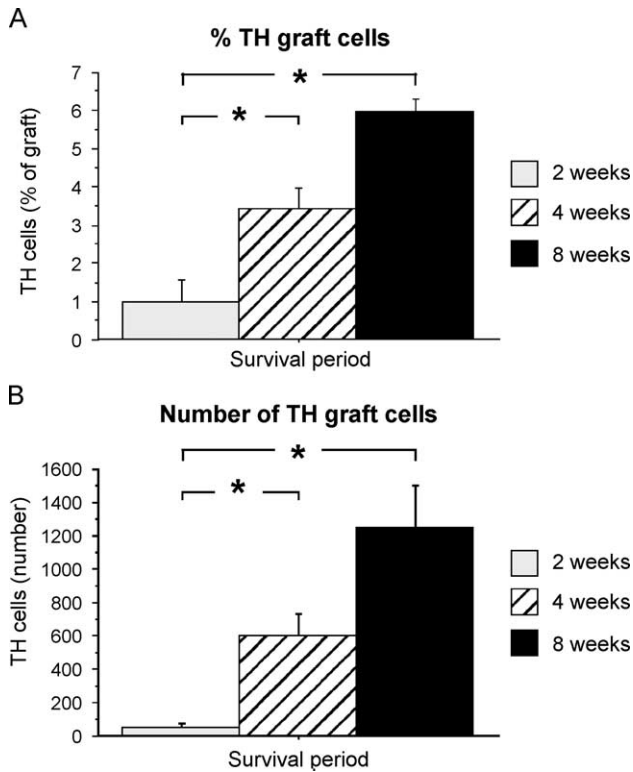


Fig. 7. Percentage and number of TH-positive graft cells. About 150 pig UCM cells were transplanted into the striatum of rats with 6-OHDA lesion. The percentage and number of TH-positive graft cells are evaluated at 2, 4, and 8 weeks post-transplantation. (A) The percentage of TH stained graft cells was determined in at least 10 fields per animal. The percentage of TH-positive graft cells increases over the time (1% at 2 weeks to about 6% at 8 weeks post-transplantation). (B) Based on the total number of graft cells and the percentage of TH stained graft cells, the number of TH-positive graft cells was calculated. The number of TH-positive graft cells increases from about 50 at 2 weeks to about 1200 cells at 8 weeks post-transplantation. * $P < 0.05$.

to animals with high-density grafts. They found that the low-density grafts have a higher proliferation rate 2–6 weeks after transplantation, and compared to the high-density grafts, the low-density grafts had more fiber outgrowth (Ostenfeld et al., 2000). Bjorklund et al. (2002) transplanted 1000–2000 undifferentiated ES cells and found

proliferation in the rat brain. These authors noted that when a high concentration of ES cells were transplanted, there was a greater likelihood of teratoma formation (Bjorklund et al., 2002). They speculated that when cells are transplanted at low concentration, the cell-to-cell contact will be less and the cells will be influenced by the local host signals leading to increased differentiation to neural cells. In their work, 2000–2500 TH-positive cells were recovered 14–16 weeks after transplantation (Bjorklund et al., 2002). Thus, similar to the present findings, previous work demonstrates that stem cells can proliferate and differentiate into TH-positive cells following xenografting. In contrast to the present findings, the work reviewed above was conducted in animals that were receiving immune suppression therapy.

We have shown that UCM cells are a type of primitive stem cell that can differentiate into neural cells in vitro (Mitchell et al., 2003) and pig UCM cells engraft following central or peripheral injection of 10,000 cells into rats without requiring immune suppression (Weiss et al., 2003). Pig UCM cells, after transplantation into rat brain, were shown to stain for pig-specific neurofilament and other neural markers (Weiss et al., 2003). In those experiments, the number of cells in the graft could not be determined because of the high-density grafts and wide distribution of the cells in the host brain tissue. The present work follows the previous work and demonstrates that UCM cells apparently proliferate during the first 4 weeks following transplantation, but this growth rate tapers apparently during the next 4 weeks. The decrease in the rate of proliferation after 4 weeks allows us to speculate that the cells might stop proliferating after a while, and we intend to address this speculation in our future work with longer survival period. Furthermore, over the 2- to 8-week survival period, the percentage and number of TH-positive graft cells increases. In future work, the number of graft cells undergoing apoptosis and division will be assessed using TUNEL staining and Ki67 staining over time.

Tissue transplantation has associated complications such as graft versus host disease (GVHD) and immune rejection of the graft by the host. The rat is a model system to study rejection of grafted tissue. For example, transplantation of

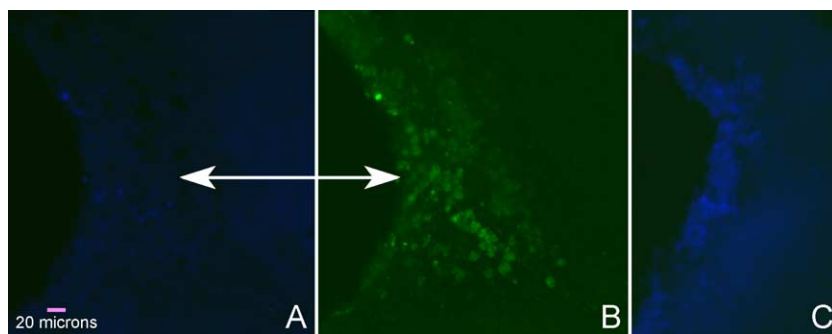


Fig. 8. Host immune cell infiltration into the injection site. The frozen brain sections were IC-stained using anti-CD4 antibody and visualized by AMCA. (A) No significant evidence of immune cell infiltration; few CD4-positive cells are found in the site of pig UCM cell transplants. (B) Pig UCM graft cells expressing GFP. (C) A variable and conspicuous staining for anti-CD4 antibody was found around the cannula tract of control animal, which had no graft cells. Similar staining was obtained for CD8, CD11b, and CD161 (data not shown).

fetal pig brain tissue into rat brain results in a significant host immune response and the grafted material is cleared from the rat brain in the 2- to 6-week period after transplantation (Borlongan et al., 1996; Duan et al., 1995, 1997; Larsson and Widner, 2000; Larsson et al., 2000, 2001; Wennberg et al., 2001). The clearance of the grafted tissue is correlated with an infiltration of host immune cells. In contrast, transplantation of tissues that are generated from the same species, an allograft, has less tissue rejection problems than a xenograft (Duan et al., 1995). In the rat, treatment with immune suppressive drugs is not successful at blocking the immune response to a xenograft (Duan et al., 1996), but immune suppression does increase the survival rate of the graft significantly (Borlongan et al., 1996; Pakzaban and Isacson, 1994). Rejection of the grafts remains a hurdle to therapeutic neural xenografting (Borlongan et al., 1996).

In contrast to the rejection problems observed during xenotransplantation of tissue, here, approximately 20,000 GFP-positive graft cells were recovered 8 weeks after transplantation with no significant host immune response. There are several possible explanations for this observation. First, pig UCM cells may have immunosuppressive effects like another type of stem cell (Le Blanc et al., 2003). Second, the low number of graft cells used here may not provide a sufficient stimulus for rejection. Indeed, previous work has indicated that the number of cells in the transplant can affect differentiation to neurons and survival (Bjorklund et al., 2002; Nishimura et al., 2003). Third, in humans, cells located at the feto-maternal interface contain HLA-G which has been suggested to play a role in dampening the mother's inflammatory and immune response against the fetus (Carosella et al., 2001; Marchal-Bras-Goncalves et al., 2001; Paul et al., 1998; Rouas-Freiss et al., 1997, 1999, 2003, 2000). In preliminary work, human UCM cells stained for HLA-G (Traas et al., 2003). Hypothetically, pig UCM cells may synthesize an analogue of HLA-G which may dampen the rat's immune response. Further work is needed to understand how pig UCM cells avoid immune surveillance.

Therapeutic potential of stem cells for neurodegenerative diseases has been demonstrated in rodent models (Kim et al., 2002). One hypothesis for their therapeutic role is that stem cells are attracted to the neurodegenerative environment, where they replace dead cells (Bjorklund and Lindvall, 2000) or rescue sick or dysfunctional cells (Ourednik et al., 2002). Here, rats with a previous unilateral 6-OHDA lesion received 150 pig UCM cells. From these 150 transplanted cells, the number of TH-positive pig UCM cells increased from about 50 at 2 weeks post-transplantation to more than 1200 cells after 8-week survival. Exceeding 1000 TH-positive cells may be significant because it was previously estimated that 1000 dopaminergic cells are needed for behavioral recovery in a rat model of Parkinson's disease (Studer et al., 1998). This finding, together with the lack of significant host immune response

to the xenograft, would suggest that transplantation of pUCM cells may provide a therapeutic approach for the treatment of neurodegenerative diseases such as Parkinson's disease.

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