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### Putative Dental Pulp-Derived Stem/Stromal Cells Promote Proliferation and Differentiation of Endogenous Neural Cells in the Hippocampus of Mice

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### ABSTRACT

Until now, interest in dental pulp stem/stromal cell (DPSC) research has centered on mineralization and tooth repair. Beginning a new paradigm in DPSC research, we grafted undifferentiated, untreated DPSCs into the hippocampus of immune-suppressed mice. The rhesus DPSC (rDPSC) line used was established from the dental pulp of rhesus macaques and found to be similar to human bone marrow/mesenchymal stem cells, which express Nanog, Rex-1, Oct-4, and various cell surface antigens, and have multipotent differentiation capability. Implantation of rDPSCs into the hippocampus of mice stimulated proliferation of endogenous neural cells and resulted in the recruitment of pre-existing Nestin<sup>+</sup> neural progenitor cells (NPCs) and  $\beta$ -tubulin-III<sup>+</sup> mature neurons to the site of the graft. Additionally, many cells born during the first 7 days after implantation proliferated, forming NPCs and neurons, and, to a lesser extent, underwent astrogliosis, forming astrocytes and microglia, by 30 days after implantation. Although the DPSC graft itself was short term, it had long-term effects by promoting growth factor signaling. Implantation of DPSCs enhanced the expression of ciliary neurotrophic factor, vascular endothelial growth factor, and fibroblast growth factor for up to 30 days after implantation. In conclusion, grafting rDPSCs promotes proliferation, cell recruitment, and maturation of endogenous stem/progenitor cells by modulating the local microenvironment. Our results suggest that DPSCs have a valuable, unique therapeutic potential, specifically as a stimulator and modulator of the local repair response in the central nervous system. DPSCs would be a preferable cell source for therapy due to the possibility of a "personalized" stem cell, avoiding the problems associated with host immune rejection. STEM CELLS 2008;26: 2654-2663

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Adult stem cells (ASCs) and embryonic stem cells are the two major divisions in stem cell research. ASCs have been isolated from various tissues including dentin, bone marrow, umbilical cord blood, and amniotic fluid [1–4]. Due to their ability to self-renew and differentiate into multiple cell lineages, ASCs are thought to have great therapeutic potential. For example, bone marrow stem/progenitor cells (BMSCs) may be beneficial for the treatment of tumors [5–6], neuro-degenerative diseases [7–8], and diabetes mellitus [9]. Although few to no BMSCs were detected 7 days after implantation in a neurogenic niche of the adult mouse brain, changes including the increase in endogenous neurogenesis and the

secretion of growth factors may promote recovery [10]. These studies suggest the therapeutic potential of BMSCs in the central nervous system (CNS).

Human DPSCs (hDPSCs) were first reported in 2000 from adult human dental pulp [11–12]. DPSCs share similar functions to BMSCs, which can differentiate into osteoblasts and adipocytes [11–15]. Recent studies suggest that DPSCs can also differentiate into neurons [4, 11, 16]. When implanted subcutaneously in mice, DPSCs secrete dentin, differentiate into odontoblasts, and form teeth-like structures [11–12]. When DPSCs were injected intramyocardically after myocardial infarct, there was a significant increase in the size of the anterior wall and a significant decrease in the size of the infarct [17]. Recent study has further demonstrated the potential of DPSCs in preventing the progression of liver fibrosis of rat treated with carbon

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tetrachloride and restoring liver function [18]. Similar to BMSCs in the CNS [10], the DPSC graft did not differentiate into the tissue, but rather induced angiogenesis at the graft site, thereby reducing infarct size.

Here we report the establishment of a rhesus DPSC (rDPSC) line and determine the similarity between BMSCs and DPSCs, specifically in therapeutic potential for CNS disorders. Due to the complexity of matching BMSC donors to hosts and the severity of graft-versus-host disease, DPSCs would be a preferable cell source with the potential of a "personalized" stem cell via cell banking. To determine the response of the microenvironment of the brain to rDPSCs, rDPSCs were grafted into the dentate gyrus of the hippocampus of immune-suppressed mice. The results of this study suggest that implantation of rDPSCs stimulates proliferation, differentiation, maturation, and recruitment of endogenous neural cells by modulating the microenvironment in the brain. Thus DPSCs may be an alternative source in cell therapy for neurological diseases.

### **MATERIALS AND METHODS**

All reagents were acquired from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) unless specified.

### Isolation and Culture of rDPSCs

A molar of a 4-month-old rhesus macaque, euthanized for clinical reasons at Yerkes National Primate Research Center, was recovered during necropsy. The tooth was stored in Dulbecco's phosphate buffered saline (DPBS) on ice and delivered to the laboratory. The isolation and culture of DPSCs was described by Gronthos et al [12].

### **Culture of Human BMSCs**

Human BMSCs (hBMSCs) were acquired from the Tulane University Health Sciences Center. In brief, hBMSCs were cultured in  $\alpha$  minimum essential medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, http://www.atlantabio.com), 100 units/ml penicillin (Invitrogen), 100  $\mu$ g/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). Cells were then cultured at 37°C with 5% humidified CO<sub>2</sub>. The hBMSCs were passaged and maintained at low density. hBMSCs were replated at 50 cells per centimeter squared and were harvested at 70% confluence.

### **Proliferation Rate of rDPSCs**

The proliferation rate was determined by plating 25,000 rDPSCs per well of a 6-well plate and three replicas were performed for each passage and time point. Cells were passaged and counted at 72 hours. Cells were counted before the next passage and 25,000 cells were replated for a total of four passages. The proliferation rate was calculated by dividing the total cell number at 72 hours of culture by the initial plating number: 25,000.

### **Telomere Length Assay**

To determine telomere length, the TeloTAGGG Telomere Length Assay kit (Roche Diagnostics, Basel, Switzerland, http://www. roche-applied-science.com) was used. In brief, genomic DNA was extracted at passages 5 and 17, and digested with a mixture of HinfI and RsaI restriction enzymes. Digested DNA was then separated in a 0.8% agarose gel followed by depurination, denaturation, and neutralization, and DNA was transferred onto a positively charged nylon membrane. The membrane was hybridized with a DIGlabeled telomere probe at 42°C for 3 hours. The membrane was washed in a high-stringency buffer and incubated with anti-DIG-AP solution. After the final wash, AP substrate was applied and exposed on x-ray film.

### **Colony-Forming Unit Assay**

The number of colony-forming units (CFUs) was determined by plating 30 cells in a 35-mm culture dish followed by 2-week culture in DPSC medium. After 14 days in culture, the cells were fixed in 100% methanol for 20 minutes followed by 3% crystal violet staining. All colonies larger than 2 mm in diameter were counted. The CFU equals the total number of colonies divided by the initial number of cells multiplied by 100%.

### **Differentiation of rDPSCs**

Adipogenic Differentiation. For adipogenic differentiation, the cells were seeded at 400 cells per 35-mm culture dish and cultured for 11 days in DPSC medium. On day 11, the DPSC medium was supplemented with 5.0  $\mu$ g/ml insulin, 50  $\mu$ M indomethacin, 1  $\mu$ M dexamethasone, and 0.5  $\mu$ M isobutylmethylxanthine, which was subsequently replaced every 3 to 4 days for 3 weeks. The culture was then fixed using 4% paraformaldehyde (PFA) and stained with 0.0125% oil red O in isopropanol for 20 minutes at room temperature followed by thorough wash and microscopic examination.

**Osteogenic Differentiation.** For osteogenic differentiation, the cells were prepared as described for adipogenic differentiation until day 11. On day 11, the DPSC medium was supplemented with 1 nM dexamethasone, 50  $\mu$ M L-ascorbic acid 2-phosphate sequimagnesium salt, 20 mM  $\beta$ -glycerol phosphate, and 50 ng/mL L-thyroxine sodium pentahydrate, which was subsequently replaced every 3 to 4 days for 3 weeks. The culture was then fixed in 4% PFA and stained with 1% alizarin red S, pH 4.1, for 20 minutes at room temperature followed by thorough wash and microscopic examination.

For chondrogenic differentiation, Chondrogenic Differentiation.  $2.5 \times 10^5$  rDPSCs were centrifuged in a 15-ml conical tube at 1,000 rpm for 5 minutes. The pellet was maintained in Dulbecco's modified Eagle's medium (high glucose) medium supplemented with ITS-plus premix (BD Biosciences, San Diego, http://www. bdbiosciences.com) to final concentration 6.25  $\mu$ g/ml insulin, 6.25  $\mu$ g/ml transferrin, 6.25 ng/ml selenious acid, 5.35  $\mu$ g/ml linoleic acid, 1.25 mg/ml bovine serum albumin, 50 µg/ml ascorbate 2-phosphate, 40 µg/ml L-proline, 100 µg/ml sodium pyruvate, 100 nM dexamethasone, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin, and 10 ng/ml transforming growth factor  $\beta$ 3 (R&D) Systems Inc., Minneapolis, http://www.rndsystems.com). Medium was replaced every 3 to 4 days for 4 weeks. The pellets were then fixed in 4% PFA overnight, and paraffin-embedded sections (4–5  $\mu$ m) were stained with 1% Alcian Blue in 10% sulfuric acid solution for 15 minutes followed by thorough wash and microscopic examination.

### **Reverse-Transcription–Polymerase Chain Reaction**

cDNA from cell samples of rDPSCs were prepared with RNeasy Mini kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) and SuperScript III Reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using a specific primer set targeting stem cell markers: Oct 4 (Oct4-foward [F]: 5'-GCA ACC TGG AGA ATT TGT TCC T-3' and Oct4-reverse [R]: 5'-AGA CCC AGC AGC CTC AAA ATC-3' with an amplicon of 182 bp), Rex-1 (Rex1-F: 5'-GTG AAC AGA ACA GAA GAG GCC-3' and Rex1-R: 5'-GAA ATC GTC CTC TCC AAC AGC-3' with an amplicon of 350 bp), and Nanog (Nanog-F: 5'-TCT CTC CTC TTC CTT CCT CCA-3' and Nanog-R: 5'-GGA AGA GTA GAG GCT GGG GT-3' with an amplicon of 389 bp); and differentiation markers: osteonectin (osteonectin-F: 5'-ATC TTC TTT CTC CTT TGC CTG G-3' and osteonectin-R: 5'-GCA CAC CTC TCA AAC TCG CC-3' with an amplicon of 323 bp), osteocalcin (osteocalcin-F: 5'-AGG TGC GAA GCC CAG CGG T-3' and osteocalcin-R: 5'-GCC AGC AGA GCG ACA CCC T-3' with an amplicon of 258 bp) and osteopontin (osteopontin-F: 5'-CAG TGA TTT GCT TTT GCC TCC T-3' and osteopontin-R: 5'-CAT TCA ACT CCT CGC TTT CCA T-3' with an amplicon of 507 bp). Glyceraldehyde-3phosphate dehydrogenase (GAPDH) was used as an internal control (GAPDH-F: 5'-ATG GGG AAG GTG AAG GTC GG-3' and GAPDH-R: 5'-CCA TCA CGC CAC AGT TTC CC-3' with an amplicon of 596 bp).

### **Flow Cytometry**

rDPSCs were divided into 15 fluorescence-activated cell sorting (FACS) tubes (BD Biosciences) at  $2 \times 10^5$  cells per tube and stained with fluorescein isothiocyanate- or phycoerythrin-conjugated anti-CD14, -CD45, -CD59, -CD73, -CD90, -CD150, -CD166, -IgG1k, and -IgG2ak (BD Pharmingen, San Diego, http://www. bdbiosciences.com), anti-CD18, -CD24, -CD29, -CD34, and -CD44 (BD Biosciences), or anti-CD105 (eBioscience, San Diego, http:// www.ebioscience.com). After a 20-minute incubation at ambient temperature in the dark, cells were washed with 2 ml FACS wash solution (DPBS + 1% bovine serum albumin + 0.1% NaN<sub>3</sub>) and centrifuged for 5 minutes at 230g. The supernatant was removed and cells were fixed with 1% formaldehyde (in phosphate-buffered saline [PBS]). All data was acquired using a FACS Calibur (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and analyzed using CellQuest (Becton, Dickinson and Company) and Flowjo (TreeStar, Inc., Ashland, OR, http://www.treestar.com) software.

### Stereotaxic Surgery in Mice

All surgeries were done using 8-week-old ICR mice and at least three mice were used for each group. Cyclosporine (CsA) injections were given daily, beginning 3 days prior to surgery. All protocols involving animal care and handling were approved by Emory University's Institutional Animal Care and Use Committee. Mice were anesthetized, and their heads were shaved and secured in the stereotaxic frame. The surgery site was cleaned using disinfectant. Burr holes were drilled at the following coordinates with respect to bregma: -2.3 anterior/posterior,  $\pm 1.3$ medial/lateral, -2.0 dorsal/ventral [19]. rDPSCs were implanted at a density of 25,000 cells per microliter for a total of 100,000 cells per hemisphere. Cells were infused at a rate of 500 nL/minute. Five minutes were allowed after implantation to limit any backflow of the cells up the needle tract. The wound was closed using Vetbond (Fisher Scientific International, Hampton, NH, http://www. fisherscientific.com). The damage caused by the needle tract could be sufficient to promote an endogenous cellular response; therefore, 4 µL of PBS was injected into the contralateral hemisphere. PBS was the vehicle for the DPSC delivery, therefore, the only difference between the PBS and hMSC hemispheres should be the presence of the hMSCs.

### **Bromodeoxyuridine Administration**

Mice receiving bromodeoxyuridine (BrdU) treatment were administered 50 mg/kg BrdU in PBS with 0.007 N NaOH intraperitoneally at 12-hour intervals for up to 7 days. There is currently some speculation that BrdU is incorporated into the DNA during apoptosis and repair. However, several relatively recent papers have concluded that BrdU does not effectively label these populations when used at the standard dose [20–22]. To prevent cytotoxicity due to BrdU labeling, we used the standard rodent dose of 50 mg/kg.

### Perfusion and Processing of Brain Tissue

Mice were anesthetized and ice-cold PBS was pumped through the left ventricle followed by ice-cold 4% PFA. Brains were removed and postfixed in 4% PFA overnight at 4°C. Brains were equilibrated using 30% sucrose and then frozen.

### Immunohistochemistry

Each antibody combination was tested in every 1:12 sections, based on the distribution of the cell graft in the green fluorescent protein (GFP)/BrdU study. Sections were cut at 40  $\mu$ m and placed in serial order in a 12-well plate. Therefore, each well contained every 12th section. Staining was performed on every 12th section. Due to the relatively subjective nature of immunostaining, each antibody was analyzed in at least four different mouse brains and a representative image was used. Sections were permeabilized. All sections stained using anti-BrdU first underwent BrdU treatment: 30 minutes in 2 N HCl at 37°C and 15 minutes in a borate buffer. Sections were blocked, incubated in the specific primary antibody overnight at 4°C, and incubated in the secondary antibody for 1 hour at room temperature. Confocal imaging was performed using Zeiss LSM 510 Meta (Carl Zeiss, Jena, Germany, http:// www.zeiss.com). All images of the brain sections were captured using the same conditions (e.g., exposure time for contralateral hemispheres).

### **RESULTS**

### Isolation and Characterization of rDPSCs

The isolation of DPSCs from humans and other species has previously been reported [11-12, 16]. However, establishing rDPSCs is the first step in developing a comparative model for studying the therapeutic value of DPSCs in nonhuman primates (NHPs). A molar from a 4-month-old rhesus macaque was attained and the dental pulp was retrieved to establish an rDPSC line. The resulting cell line has a high initial proliferation rate. Their morphology is similar to BMSCs and hDPSCs, which are predominantly long and spindle shaped (Fig. 1Ea). After the first or second passage, a homogenous population of DPSCs was established and maintained. One of the primary criteria for defining a stem cell is the ability to self-renew and propagate, demonstrated by the formation of a colony from a single cell [23-24]. After 14 days in culture, the CFU efficiency was 90% (data not shown). This result demonstrates that rDPSCs are capable of self-renewal and readily form colonies in vitro. Additionally, rDPSCs had a high proliferation rate during early passages, but decreased gradually in culture. At passage 3, rDPSCs proliferated at 18.8-  $\pm$  5-fold per 72 hours and by passage 6 the rate had decreased to 3.8-  $\pm$  0.69-fold per 72 hours (Fig. 1B).

# Expression of Stem Cell and Differentiation Markers by rDPSCs

rDPSCs were also tested to determine their expression of stem cell-specific transcription factors. The expression of Nanog, Rex-1, and Oct-4 was confirmed by reverse-transcription (RT)-PCR (Fig. 1A). Three markers of osteogenesis: osteonectin, osteocalcin, and osteopontin, were also detected by RT-PCR (Fig. 1A). A similar expression pattern has previously been reported in hDPSCs [11–12] and rhesus BMSCs (rBMSCs) [23]. These results indicate similarities between rDPSCs and other ASCs.

### Differentiation of rDPSCs

Multipotent differentiation is instrumental in defining a stem/ progenitor cell. Differentiation into osteoblasts, adipocytes, and chondrocytes is considered a trademark of DPSCs [11– 12, 25] and BMSCs [23–24]. rDPSC osteogenesis (Fig. 1Eb) was comparable to that of hDPSCs [11–12, 25], hBMSCs [24], and rBMSCs [23]. Adipogenesis was clearly confirmed by the formation of fat droplets (Fig. 1Ec), which were morphologically comparable to those of hBMSCs [24] and hDPSCs [11]. Furthermore, rDPSCs differentiated into chondrocytes (Fig. 1Ed). These results confirm that rDPSCs are capable of differentiating into cells of multiple lineages similar to hBMSCs and hDPSCs.



**Figure 1.** Characterization of rhesus monkey dental pulp stem/stromal cells (rDPSCs). (A): Expression of stem cell (Nanog, Rex-1, Oct-4) and osteogenic (osteonectin, osteocalcin, and osteopontin) markers was determined by reverse-transcription polymerase chain reaction. (B): The proliferation rate of rDPSCs was compared between passages 3, 4, 5, and 6. (C): rDPSC samples were collected at passages 5 (P5) and 17 (P17) for telomere analysis. No obvious change in telomere length was observed. (D): Cell surface antigen profile was determined by using a total of thirteen cell surface antigens. The expression profile of rDPSCs was comparable with human bone marrow stem/progenitor cells (hBMSCs) and the published profiles of rhesus BMSCs (rBMSCs) [23], hBMSCs [24], and human DPSCs (hDPSCs) [11, 15]. Both DPSCs and BMSCs derived from rhesus monkey and human share almost identical expression profiles on common cell surface antigens. rDPSCs also share similar morphology to that of rBMSCs, hBMSCs and hDPSCs. (**Ea**): rDPSCs are spindle and fibroblast like cells, and are capable of differentiating into (**Eb**) osteogenic, (**Ec**) adipogenic, and (**Ed**) chondrogenic lineages. Scale bar = 5  $\mu$ m. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

#### **Cell Surface Antigen Profile of rDPSCs**

The expression profiles of a total of 13 cell surface antigens were compared in rDPSCs and hBMSCs (Fig. 1D), which were also compared with published profiles of hDPSCs [15], hBMSCs [24], and rBMSCs [23]. rDPSCs were CD29<sup>+</sup>, CD73<sup>+</sup>, CD90<sup>+</sup>, CD166<sup>+</sup>, CD59<sup>+</sup>, CD44<sup>+</sup>, and CD105<sup>+</sup>, all of which are common hBMSC markers. However, markers specific for hematopoietic cells, including CD14, CD34, and CD45, were not expressed. Both rDPSCs and hBMSCs were CD18<sup>-</sup>, CD24<sup>-</sup>, and CD150<sup>-</sup>. These results are comparable to the expression profiles of hDPSCs [15], hBMSCs [24], and rBMSCs [23]. Although the percentage of some surface antigens was lower than that of the hBMSCs, such ratio in hDPSCs and rBMSCs has not been reported or determined. In fact, a homogenous population of rDPSCs with specific surface markers could be enhanced by flow cytometry using specific antibodies. Based on the overall expression pattern, rDPSCs are similar to hDPSCs, hBMSCs, and rBMSCs.

### Assessment of Telomere Length in rDPSCs

Telomere length is commonly used to determine the impact of aging on a cell. Telomere length decreases as cells divide; therefore, older cells have shorter telomeres [23]. In general, long telomeres (10–20 kb) are observed in stem cells or cells that vigorously proliferate [26], whereas shorter lengths, such as 5–15 kb, are typical of somatic cells [27]. Telomeres were not significantly shortened in early (P5) or late (P17) passages (Fig. 1C). rDPSC telomeres remained at approximately 21 kb in length until p17. Telomere lengths were comparable to those observed in stem cells and cells that vigorously proliferate [26], even the growth rate at passage 5 was slower than that of earlier passages (Fig. 1B).

### Gene Transfer in rDPSCs

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rDPSCs that expressed the *GFP* gene were first created using lentivirus to facilitate detection. Gene transfer and expression

were more than 95% efficient. Stable gene transfer was confirmed by invariant expression of GFP after several passages after infection (data not shown) and observation of GFP cells at the graft site (Fig. 2A–2E). We have demonstrated that rDPSCs can be genetically modified efficiently and maintained in culture with no detectable adverse effect.

### Immunosuppressants Prevent Immune Rejection of the rDPSC Graft

To determine the role of the immune response despite CsA administration, astrocyte, microglia, and macrophage number were monitored over 30 days, rDPSCs were grafted unilaterally into the hippocampus and PBS was injected into the contralateral hemisphere to control for the effect of the injection itself on the immune response. BrdU was injected every 12 hours, beginning postoperatively. Immunostaining for astrocytes (glial fibrillary acidic protein [GFAP]) and microglia/macrophages (Iba1) revealed an increase in astrocytes, but no overt difference in the quantity of microglia/macrophages in rDPSC-implanted (Fig. 3C, 3K [GFAP] and 3D, 3L [Iba1]) or PBS-injected (Fig. 3G, 3O [GFAP] and 3H, 3P [Iba1]) hemispheres, either at 7 or 30 days after implantation. The lack of a heightened immune response suggests that CsA treatment was sufficient to prevent a robust immune response to the rDPSC graft and the surgery itself for up to 30 days.

### rDPSC Graft Promotes Proliferation of Endogenous Cells

To determine whether rDPSCs could promote neural cell proliferation, 100,000 rDPSCs expressing GFP (GFP-rDPSCs) were implanted unilaterally into the dentate gyrus of the hippocampus of immune-suppressed mice. PBS was injected into the contralateral hemisphere to control for the effect of the surgery itself. Mice were perfused at 1, 3, 5, 7, and 30 days after implantation to study the time course of the effect. BrdU was used here to label proliferating endogenous neural cells during



**Figure 2.** Rhesus dental pulp stem/stromal cells (rDPSCs) increase neural cell proliferation. Green fluorescent protein-rDPSCs were implanted into the right dentate gyrus of the hippocampus. The size of the graft was assessed at 1, 3, 5, 7, and 30 days after implantation. The graft was successful (1 day, **A**), but subsequently diminished in size (3–30 days, **B–E**). (**A–E**, inset images): High-magnification confocal images reveal the disruption of the rDPSC cell body into smaller fragments between 5–30 days, suggesting apoptosis or phagocytosis. (**G–J**): rDPSC implantation stimulated neural cell proliferation at days 3–30 after implantation, (**L–O**) compared with the contralateral, phosphate-buffered saline-injected hemisphere. There were no changes in the number of proliferating cells one day after rDPSC (**F**) or PBS implantation (**K**). Bromodeoxyuridine was injected every 12 hours for the first 7 days after implantation to label all dividing cells. New cells survived for at least 30 days. Images at contralateral sides were captured using the same exposure time. Scale bar = 100  $\mu$ m.

the first 7 days after implantation; therefore, all experiments during the first 7 days after implantation describe actively dividing cells and those at 30 days describe surviving cells that were born during the first week after implantation. GFP-rDPSCs appeared to decrease by day 3 (Fig. 2B) after implantation. Whereas intact, spindle-shaped DPSCs can be easily found at 1-3 days after implantation (Fig. 2A, 2B, inset), there is a change in cell morphology by 5 days to smaller GFP fragments (Fig. 2C, inset). A recent article has elucidated this phenomenon using MSCs, describing the transfer of GFP and other cell labels to surrounding neural cells after the death of the grafted cells [28]. By day 30, only remnants of the GFP<sup>+</sup> cells could be found (Fig. 2E, inset). On the contrary, the number of BrdU<sup>+</sup> cells gradually increased, peaking at day 5 after implantation (Fig. 2H) and surviving for the 30-day period (Fig. 2J). Some BrdU<sup>+</sup> cells were also found in the contralateral, PBS-injected hemisphere, proliferating in response to the injection itself (Fig. 2K-2O). However, the number of BrdU<sup>+</sup> cells was significantly less than in the hemisphere receiving the rDPSC graft at all time points (Fig. 2F-2J). To verify the rDPSC graft itself was stimulating proliferation, GFP-rDP-SCs were killed using ethanol treatment prior to implantation. After implantation of dead rDPSCs, there was no increase in BrdU staining (supplemental online Fig. 1A-1D) compared with the contralateral, PBS-injected hemisphere (supplemental online Fig. 1E-1H). This suggests that rDPSCs stimulate endogenous cell proliferation.

### rDPSCs Promote Differentiation and Maturation of Endogenous Cells into Neural Progenitor and Mature Neural Cells

An rDPSC graft increases proliferation of endogenous neural cells, which survive for up to 30 days (Fig. 2F-2J). Cells that proliferated during the first week after implantation can easily be identified for up to 30 days due to BrdU labeling. A variety of cell-specific markers were used for immunostaining to determine the fate of BrdU-labeled cells. There are two therapeutically relevant pathways that these cells could follow; neurogenesis into a neuron or astrogliosis into a glial cell. There was an overwhelming increase in the overall number of Nestin<sup>+</sup> cells at the site of the rDPSC graft at 7 days after implantation (Fig. 3A), which did not occur in the contralateral hemisphere (Fig. 3E). At 30 days after implantation there was still a large increase in Nestin<sup>+</sup> cells at the site of the graft (Fig. 3I) compared with both surrounding brain regions and the contralateral, PBS-injected hemisphere (Fig. 3M). At 7 days after implantation, many of the BrdU<sup>+</sup> cells colabeled with Nestin (Fig. 3A, inset), which is expressed by neural progenitor cells (NPCs), suggesting these newly divided cells were expressing a neural phenotype. However, the majority of NPCs did not colabel with BrdU, suggesting additional recruitment of endogenous NPCs and neural cells to the site of the graft after rDPSC implantation. Similar results were found at 30 days (Fig. 3I, inset).

Immunostaining for mature neurons ( $\beta$ III-tubulin) revealed a large increase in the number of neurons in the region



**Figure 3.** Rhesus dental pulp stem/stromal cells (rDPSCs) recruit endogenous neural cells and stimulate differentiation of new cells to mature neural cells. The rDPSC graft recruited pre-existing endogenous neural cells at 7 (**A–D**) and 30 (**I–L**) days compared with the contralateral, phosphate-buffered saline-injected hemisphere (**E–H** [7 days], **M–P** [30 days]). rDPSC implantation stimulated recruitment of Nestin<sup>+</sup> (**A, I**) and  $\beta$ III-tubulin<sup>+</sup> (**B, J**) neural cells. (**C, K**): There was an increase only in glial fibrillary acidic protein-positive (GFAP<sup>+</sup>) astrocytes (**D, L**) and no increase in Iba1<sup>+</sup> microglia/macrophages, (**G, O** and **H, P**, respectively) compared with the contralateral hemisphere. Insets show high-power confocal images at the heart of the cell graft as indicated by a white box. New cells had differentiated into (**A, I**) Nestin<sup>+</sup>, (**B, J**)  $\beta$ III-tubulin<sup>+</sup>, and (**C, K**) GFAP<sup>+</sup> at 7 and 30 days and into (**D**) Iba1<sup>+</sup> cells at 7 days. Images at contralateral sides were captured by using the same exposure time. Scale bar = 100  $\mu$ m. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole.

immediately surrounding the cell graft at 7 days after implantation (Fig. 3B). This increase in neurons had slightly decreased by 30 days (Fig. 3J). However, at both time points the number of neurons at the site of the graft was higher than in the contralateral, PBS-injected hemisphere (Fig. 3F, 3N). Many BrdU<sup>+</sup> cells colabeled with neurons at 7 days; however, neural processes were short, if present at all (Fig. 3B, inset). By 30 days these BrdU<sup>+</sup> neurons had grown elaborate neurites (Fig. 3J, inset). These results suggest that although many BrdU<sup>+</sup> cells differentiated by 30 days, the majority of the "new" neurons already existed at the time of implantation and were recruited to the site of the graft. Due to the high number of NPCs and immature neural cells at the site of the graft, it is conceivable that these cells were NPCs at the time of implantation, but were stimulated to differentiate into mature neurons by the rDPSC graft.

Although only a slight increase in astrocytes (GFAP; Fig. 3C, 3K) and negligible differences in macroglia/microglia (Iba1; Fig. 3D, 3L) were observed at 7 or 30 days after implantation,

some BrdU<sup>+</sup> cells did differentiate into these phenotypes. Highpower confocal imaging revealed BrdU<sup>+</sup>/GFAP<sup>+</sup> cells at both 7 (Fig. 3C, inset) and 30 (Fig. 3K, inset) days as well as BrdU<sup>+</sup>/ Iba1<sup>+</sup> cells at 7 days (Fig. 3D, inset).

### Expression of Neurotrophins After rDPSC Implantation

To stimulate the proliferation and differentiation of endogenous neural cells, rDPSCs must be able to modulate the local microenvironment via direct interaction or cell signaling. One possible mechanism, explained here, is the regulation of the synthesis and release of neurotrophins in adjacent endogenous cells. To determine whether rDPSCs upregulated the expression of neurotrophins in the newly divided cells or cells neighboring the rDPSC graft, double immunostaining of BrdU and specific neurotrophins including ciliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and fibroblast growth factor (FGF) was performed at 7



**Figure 4.** Rhesus dental pulp stem/stromal cells (rDPSCs) increase growth factor signaling. The rDPSC graft increases the amount of growth factor signaling at both 7 (**A–D**) and 30 (**I–L**) days compared with the contralateral, phosphate-buffered saline-injected hemisphere (**E–H** and **M–P**, respectively). (**A**): There was a robust increase in ciliary neurotrophic factor (CNTF) at 7 days and (**J**) vascular endothelial growth factor (VEGF) at 30 days, (**K**) substantial increases in nerve growth factor (NGF) at 30 days and (**D**) fibroblast growth factor (FGF) at 7 days, and small increases in (**I**) CNTF at 30 days and (**B**) VEGF at 7 days. There were no overt differences in (**C**) NGF at 7 days and (**L**) FGF at 30 days. Images at contralateral sides were captured by using the same exposure time. High-power confocal images show the expression of each growth factor by bromodeoxyuridine-positive (BrdU<sup>+</sup>) and surrounding cells at 7 and 30 days (inset images). Scale bar = 100  $\mu$ m.

and 30 days after implantation. CNTF increases adult endogenous neurogenesis [29]. CNTF was robustly upregulated at 7 days (Fig. 4A) and only slightly by 30 days (Fig. 4I) at the site of the rDPSC graft, whereas no significant upregulation was observed in the contralateral, PBS-injected hemisphere, even at the point of highest intensity, which was just dorsal to the injection site (Fig. 4E, 4M).

VEGF has neuroprotective [30-33] and neurogenic [30, 34] effects on pre-existing cells. VEGF was slightly upregulated at 7 days after implantation (Fig. 4B). This effect was extremely robust at 30 days (Fig. 4J). A slight increase in VEGF expression was found dorsal to the injection site at 7 days (Fig. 4F), which was negligible by 30 days (Fig. 4N), in the contralateral, PBS-injected hemisphere.

NGF promotes survival in neural cells [35]. NGF was upregulated at 30 (Fig. 4K), but not 7 (Fig. 4C) days after implantation. There was no increase in NGF expression in the contralateral, PBS-injected hemisphere at either time point (Fig. 4G, 4O).

FGF-2 increases adult neurogenesis, as well as the percentage of these cells that mature into neurons [36]. FGF expression in-

creased at 7 (Fig. 4D), but not 30 (Fig. 4L) days after implantation compared with the contralateral, PBS-injected hemisphere (Fig. 4H, 4P).

These results suggest that elevated CNTF, VEGF, and FGF-2 secretion resulting from DPSC implantation could be responsible for the increase in endogenous neurogenesis. The increase in local VEGF, and to a lesser extent NGF, may promote the survival of these cells for at least 30 days, with FGF-2 levels prompting differentiation into mature neurons.

### DISCUSSION

NHPs play an important role in the development of human stem cell research and therapy [37–39]. NHPs hold great promise for understanding the mechanisms of differentiation, establishment of neural circuitry, and local cellular responses for tissue repair [23, 37, 40]. Here we describe the establishment and characterization, both in vitro and in vivo, of an rDPSC line. This is the first report of a DPSC line from an NHP. rDPSCs are multipo-

tent, capable of differentiating into at least osteoblasts, adipocytes, and chondrocytes (Fig. 1Eb–1Ed). rDPSCs express similar cell surface antigens to rBMSCs, hBMSCs, and hDPSCs (Fig. 1D). They also display a high proliferation rate at early passages, retain long telomere lengths, and express stem and progenitor cell markers similar to rBMSCs [23], hBMSCs [24], and hDPSCs [11, 15] (Fig. 1A–1C). However, the expression level of the stem cell markers was gradually decreased in extended culture (A.W.S. Chan, unpublished data). Although DPSCs have been primarily considered for applications in paradontology, implantology, and calcified tissue reconstruction [11, 13, 15], these results suggest that clinical applications of DPSCs should not be limited to hard tissue engineering.

DPSCs are considered a subgroup of BMSCs at a different differentiation hierarchy, thus committed to a specific function under the influence of the local microenvironment [15]. Although the therapeutic potential of DPSCs has not been fully investigated, recent studies on BMSCs [41–42] suggest that BMSCs can stimulate neurogenesis [10]; produce and secrete growth factors [10, 41, 43]; and treat tumors [5–6], neuro-degenerative diseases [7–8], and diabetes mellitus [9]. Based on a high similarity in gene expression profiles and differentiation potentials between DPSCs and BMSCs, it is logical to consider similar uses for BMSCs and DPSCs [11–12, 15, 25, 35, 44].

This study strives to answer whether DPSCs have the same effect on neural cell proliferation, differentiation, and neurotrophin release as BMSCs. BMSCs may be used for therapy in the CNS via three possible mechanisms: (a) engraftment and differentiation into local cell types [9, 18, 42-43], (b) recruitment of adjacent or distal endogenous cells [9-10, 17-18, 43], and (c) formation of trophic support for cell graft or adjacent endogenous cells [9-10, 17-18, 42-43, 45]. BMSCs may serve as trophic bundles, bridging the epicenter of a spinal cord injury [46]. Caplan and Dennis suggest the ability of BMSCs to provide trophic support is multifaceted, involving suppression of the local immune response, fibrosis, and apoptosis meanwhile enhancing angiogenesis, mitosis, and differentiation [47]. It has been hypothesized that BMSCs stimulate neurogenesis and recruit cells by secreting neurotrophins [48]. Due to the high similarity shown here between BMSCs and DPSCs, DPSCs should be considered in each of these therapeutic methods.

rDPSCs grafted into the hippocampus of immune-suppressed mice stimulated neural cell proliferation, shown by a significant increase in proliferating cells at the site of the graft (Fig. 2F–2J). The rDPSC graft also caused the recruitment of pre-existing neural cells to the site of the graft (Fig. 3). Finally, the rDPSC graft elicited an increase in the secretion of CNTF, VEGF, NGF, and FGF-2 (Fig. 4). These findings further support the theory of similar biofunctions between rDPSCs and BMSCs; however, this study does not exclude the capability of the other cell types to promote similar changes in vivo.

Our hypothesis is that implantation of rDPSCs in the hippocampus alters the local microenvironment of the brain. The rDPSC graft causes an increase in the secretion of CNTF, VEGF, NGF, and FGF-2 that stimulates neurogenesis. The graft also causes an increase in the production of VEGF and NGF that promotes survival of these cells. These new cells differentiate into NPCs, mature neurons, and glial cells due to elevation of FGF-2 levels (Fig. 5). The rDPSC graft also recruits pre-existing NPCs and mature neurons to the site of the graft. This overall boost in the number of NPCs and neurons at the site of the graft makes rDPSC implantation as potential treatment of stroke and neurodegenerative diseases.

In addition to our results, a recent study has further demonstrated the therapeutic potential of DPSCs in a disease model of acute myocardial infarct. The DPSC graft improves ventricular



**Figure 5.** Rhesus dental pulp stem/stromal cells (rDPSCs) have therapeutic potential in the central nervous system. The rDPSC graft increases ciliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and fibroblast growth factor 2 (FGF-2), and thus increases endogenous neurogenesis. The increase in VEGF and NGF signaling promotes survival of these cells. FGF-2 signaling increases the differentiation potential to neural progenitor cells (NPCs), neurons, and mature glial cells. The rDPSC graft also recruits pre-existing, endogenous NPCs and neurons. By increasing local growth factor signaling, the rDPSC graft increases the overall number of NPCs and neurons at the site of the graft.

function in acute myocardial infarcted rats by the induction of angiogenesis at the graft site [17]. Although DPSCs may have comparable therapeutic potential to BMSCs, DPSCs have an advantage over BMSCs as an alternative cell source for therapy. A patient is rarely able to use their own BMSCs for treatment but must rely on compatible marrow from donors or close relatives. Unfortunately, the marrow is not always immune compatible and the risk of immune rejection remains. Furthermore, bone marrow retrieval is an invasive procedure and most people are adverse to the procedure itself. A major advantage to the use of DPSCs is relatively easy access [15, 44]. Teeth can be recovered during routine dental procedures at any time throughout life [15, 44]. A homogenous population of DPSCs can be established from one's own dental pulp in a relatively short time [11-12, 15, 44]. In fact, supernumerary teeth, deciduous teeth, and wisdom teeth are often extracted for physiological, medical, or cosmetic reasons; however, they are the most convenient and valuable source of DPSCs [44]. DPSCs can be cryopreserved and revived as needed [49], thus "personal" DPSCs can be established and preserved for future medical needs.

In conclusion, we report the first DPSC line established from NHPs. This cell line is similar to rBMSCs, hBMSCs, and hDPSCs. Implantation of rDPSCs into the hippocampus of mice stimulates cell proliferation, endogenous cell recruitment, and maturation of neuronal cell types by modulating the local microenvironment. Our results suggest the therapeutic potential of DPSCs, which can be used as an alternative source for therapy of neurological disorders. Most importantly, rDPSCs can be easily established from anyone at any age and preserved for future need, without the concern of immune compatibility. Thus the potential of personal DPSCs merits further investigation for the advancement of clinical treatment.

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### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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