Adult Human Dental Pulp Stem Cells Differentiate Toward Functionally Active Neurons Under Appropriate Environmental Cues

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Key Words. Dental pulp stem cells • Neuronal differentiation

ABSTRACT

Human adult dental pulp stem cells (DPSCs) reside within the perivascular niche of dental pulp and are thought to originate from migrating cranial neural crest (CNC) cells. During embryonic development, CNC cells differentiate into a wide variety of cell types, including neurons of the peripheral nervous system. Previously, we have demonstrated that DPSCs derived from adult human third molar teeth differentiate into cell types reminiscent of CNC embryonic ontology. We hypothesized that DPSCs exposed to the appropriate environmental cues would differentiate into functionally active neurons. The data demonstrated that ex vivo-expanded human adult DPSCs responded to neuronal inductive conditions both in vitro and in vivo. Human adult DPSCs, but not human foreskin fibroblasts (HFFs), acquired a neuronal morphology, and expressed neuronal-specific markers at both the gene and protein levels. Culture-expanded DPSCs also exhibited the capacity to produce a sodium current consistent with functional neuronal cells when exposed to neuronal inductive media. Furthermore, the response of human DPSCs and HFFs to endogenous neuronal environmental cues was determined in vivo using an avian xenotransplantation assay. DPSCs expressed neuronal markers and acquired a neuronal morphology following transplantation into the mesencephalon of embryonic day-2 chicken embryo, whereas HFFs maintained a thin spindle fibroblastic morphology. We propose that adult human DPSCs provide a readily accessible source of exogenous stem/precursor cells that have the potential for use in cell-therapeutic paradigms to treat neurological disease. STEM CELLS 2008;26:1787–1795

INTRODUCTION

Regeneration of the disrupted human nervous system from disease or trauma is a challenge for stem cell-based therapeutic paradigms (reviewed by Lindvall and Kokaia [1]). Thus, the goal of neuroregeneration may entail a complex process of neuroprotection, immunomodulation of inflammation or gliogenesis, neuroplasticity, and neurogenesis. Understanding the underlying cellular and molecular mechanisms that may facilitate this complex process is paramount to achieve an improvement in neurological function.

In model organisms, both endogenous and exogenous neural stem cells (NSCs) have been investigated for their capacity to regenerate a damaged nervous system. Due to the low incidence of human adult NSCs and problems with accessibility, the use of exogenous sources of stem cells with neural potential has been suggested as a plausible approach to stem cell therapy. Although embryonic stem cells [2] (reviewed by Lindvall et al [3] and Lindvall and Kokaia [4]) and bone marrow stem cells have been suggested as a plausible approach to stem cell therapy. Although embryonic stem cells [2] (reviewed by Lindvall et al [3] and Lindvall and Kokaia [4]) and bone marrow stem cells have been assessed as potential candidates for neuronal therapy, adult stem cell populations derived from cranial neural crest (CNC) cells may possess a greater propensity for neuronal differentiation and repair.

Dental pulp tissue is thought to be derived from migrating neural crest cells during development [5, 6], and has been shown to harbor various populations of multipotential stem/progenitor cells [7–10]. In vitro neuronal differentiation studies of rat and human adult dental pulp cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs) demonstrated that these stem/precursor cell populations were able to differentiate into neurons based on cellular morphology and the expression of specific markers at both the gene and protein levels. Culture-expanded DPSCs also exhibited the capacity to produce a sodium current consistent with functional neuronal cells when exposed to neuronal inductive media. Furthermore, the response of human DPSCs and HFFs to endogenous neuronal environmental cues was determined in vivo using an avian xenotransplantation assay. DPSCs expressed neuronal markers and acquired a neuronal morphology following transplantation into the mesencephalon of embryonic day-2 chicken embryo, whereas HFFs maintained a thin spindle fibroblastic morphology. We propose that adult human DPSCs provide a readily accessible source of exogenous stem/precursor cells that have the potential for use in cell-therapeutic paradigms to treat neurological disease. STEM CELLS 2008;26:1787–1795

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

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early neuronal markers [8, 9]. Furthermore, in vivo studies demonstrated that rat DPCs and SHEDs when transplanted into the adult rodent brain survived and expressed neuronal markers [8, 9].

We have previously reported that multipotential dental pulp stem cells (DPSCs) [7] retain their neural crest properties following ex vivo expansion [11–15]. Preliminary investigations into the neural potential of human adult DPCs and DPSCs have shown that under non-neuronal inductive conditions, these cells expressed the neural progenitor marker, Nestin, and glial marker, glial fibrillary acidic protein (GFAP), at both the gene and protein levels [12, 16]. Subsequent examinations documented that DPSCs expressed the postmitotic neuron-specific marker, neuronal nuclei (NeuN), when cultured under neural inductive conditions [11]. We hypothesized that DPSCs exposed to the appropriate environmental cues would differentiate into functionally active neurons and that neural crest-derived adult DPSCs may provide an alternative stem cell source for therapy-based treatments of neuronal disorders and injury.

The present study examined the neuronal differentiation potential of adult human DPSCs both in vitro and in vivo, using factors known to initiate neuronal differentiation [17–20]. The findings of the present study demonstrated that adult human DPSCs have a predisposition to a neuronal fate that is further enhanced when exposed to neuronal differentiation and environmental factors.

**MATERIALS AND METHODS**

**Isolating DPSCs and Human Foreskin Fibroblasts**

DPSCs and SHEDs were isolated as described by [7, 8, 13]. Briefly, discarded normal human impacted third molars were collected from adults (19–35 years of age) or exfoliated teeth (7–8 years of age) with informed consent of patients undergoing routine extractions at the Dental Clinic of the University of Adelaide, under approved guidelines set by the University of Adelaide and Institute of Medical and Veterinary Science Human Subjects Research Committees (H-73–2003). Tooth surfaces were cleaned and cracked open using a M strainer. Cultures were established by seeding single-cell suspensions into T-25 flasks in growth media (o-modification of Eagle’s medium supplemented with 20% fetal calf serum, 100 μM l-ascorbic acid 2-phosphate, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), then incubated at 37°C in 5% CO2.

Human foreskin fibroblasts (HFFs) were isolated by collagenase/dispase digestion from explants of foreskin biopsies from neonatal male donors, with informed parental consent.

**Neural Induction Assay**

Tissue culture flasks or wells were coated with polyornithine (10 μg/ml final concentration) overnight at room temperature. Flasks and wells were washed twice with water and then coated with laminin (5 μg/ml final concentration) overnight at 37°C in a humid incubator. Flasks and wells were washed with phosphate-buffered saline (PBS), and then with growth media before cells were added. Cells were thawed and grown in T25-coated flasks until 80% confluent. Flasks and wells were washed twice with water and then coated with Neurobasal A Media (10888-022; Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 100 U/ml penicillin, 1X B27 supplement, 100 μg/ml streptomycin, 20 ng/ml epidermal growth factor (EGF, 100-15; PeproTech, Rocky Hill, NJ, http://www.peprotech.com), and 40 ng/ml basic fibroblast growth factor (FGF, no. 104FGFB01; Prospec Tany Techno Gene, Rehovot, Israel). Media B consists of three separate media conditions for the duration of the 3-week period: the first incubation was media A for 7 days, followed by a change in media consisting of 50:50 ratio of Dulbecco’s modified Eagle’s medium (DMEM, 12100-046; Invitrogen) and F12 media (21700-035; Invitrogen), and insulin-transferrin-sodium-selenite supplement (ITTS, 11074547001; Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com), 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 ng/ml FGF for 7 days. The final 7 days of incubation consisted of media containing 50:50 ratio of DMEM and F12 media, ITTS, 100 U/ml penicillin and 100 μg/ml streptomycin, 40 ng/ml FGF, and 0.5 μM retinoic acid (RA, R2625; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). Control conditions were maintained in the absence of any neuronal inductive assay. The media for all conditions were replaced twice weekly. Following the final incubation, the 8-well coated chamber slides were fixed with 4% paraformaldehyde (PFA), and cells in 6-well plates were lysed with TRIZol (Invitrogen) and stored for RNA isolation and real time-polymerase chain reaction (RT-PCR) analysis or replated for electrophysiological analysis.

**2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-Tetrazolium, Monosodium Salt Assay**

Cell proliferation of human adult DPSCs undergoing neuronal differentiation was performed as follows. Human adult DPSCs from each donor were seeded at 8 × 103 cells/well in 96-well plates in quadruplicate for each of the media conditions and for each media change time points and were incubated at 37°C in a 5% CO2-containing humidified atmosphere. Cells were cultured in control media for 2 days and then changed to the corresponding media conditions as per the neuronal differentiation method. Cell number and viability were quantitated at each media change using the colorimetric reagent 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) (Roche Molecular Biochemicals, Indianapolis). The absorbance was measured directly with a plate reader (BIO-RAD Model 3550 microplate reader; Bio-Rad, Hercules, CA, http://www.bio-rad.com) using the test wavelength of 450 nm. Results of representative experiments are presented as the mean ± SEM.

**Real-Time Polymerase Chain Reaction**

RNA was extracted from DPSC neuronal differentiation assays. Briefly, total RNA was extracted using the TRizol method. RNA samples were quantified by spectrophotometer (Eppendorf, Hamburg, Germany, http://www.eppendorf.com), and RNA integrity was checked on 1% agarose gels using a deionized formamide-based loading buffer. Reverse-transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen). cDNA samples were diluted to a uniform concentration of 50 ng/μl. Real-time PCR reactions were performed using TaqMan master mix on an ABI SDS 7000 light cycle driven by ABI prism SDS v1.1 (Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com). TaqMan primers were designed using Primer Express v2.0 (Applied Biosystems) and synthesized locally (GeneWorks, South Australia, Australia, http://www.geneworks.com.au). Primers TBP (TATA box-binding protein) forward (CTG GAA AAG TTG TAT TAA CAG GTG CT), reverse (CCA TCA GGC AAC AGT TTC C); Nestin (NM (006617) forward (5’ GTG- CATCTTCATGTGTCGCA 3’); reverse (5’ CCGATTGACTCCACATC 3’)); β-III tubulin (accession NM (006806) forward (5’ GGGCGAAGCTGTTGGGAAGATC 3’); reverse (5’ ATCCGCTC-CAGCTGCAAGT 3’)); neurofilament-medium chain (NF-M; accession NM 005382) forward (5’ GACGGCGCTAGAAGGAAATC 3’); reverse (5’ CTCTTGCGCTTCGCTCAT3’); and neurofilament-heavy chain (NF-H; accession NM 021076) forward (5’ CAGCCAGAGTGAACAGAC 3’); reverse (5’ GCTGCT-GAATTGCTTCT3’)) were used at a final concentration of 300 nM, and reactions for each sample were performed in triplicate.
Electrophysiology
At the completion of the 3-week neuronal differentiation assay, cells were liberated with trypsin and replated onto glass coverslips treated with hydrochloric acid at a concentration of 2 × 10⁻⁷ cells/500 μL in neuronal differentiation media or growth media and incubated for 24 hours. Whole-cell patch clamping was performed at room temperature using a computer-based patch clamp amplifier (EPC-9; HEKA Electronics, Lambrecht/Pfalz, Germany, http://www.heka.com) and PULSE software (HEKA Electronics). The bath solution contained (in mM): NaCl, 140; KCl, 4; CaCl₂, 2; MgCl₂, 2; glucose, 10; and HEPES, 10; adjusted to pH 7.4 with NaOH. To block K⁺ channels and separate Na⁺ currents, K glutamate and KCl in the internal solution were replaced with equimolar amounts of Cs glutamate and CsCl. Patch pipettes were pulled from borosilicate glass and fire polished; pipette resistance ranged between 2–4 MΩ. All voltages shown were corrected for liquid junction potential (~14 mV and ~18 mV for K⁺ and Cs⁺-based internal solutions, correspondingly), estimated by JCable (provided by Dr. P.H. Barry, University of New South Wales, Sydney, Australia, 1994). The holding potential was ~90 mV throughout.

Avian Embryo Injections
Ethical approval was obtained from the University of Adelaide (approval number S-59–2003). Chicken eggs (white leghorn; HICHEICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use. Chicken eggs (white leghorn; HICHICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated in a humidified incubator at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use. Ethical approval was obtained from the University of Adelaide (approval number S-59–2003). Chicken eggs (white leghorn; HICHEICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated in a humidified incubator at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use. Ethical approval was obtained from the University of Adelaide (approval number S-59–2003). Chicken eggs (white leghorn; HICHEICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated in a humidified incubator at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use. Ethical approval was obtained from the University of Adelaide (approval number S-59–2003). Chicken eggs (white leghorn; HICHEICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated in a humidified incubator at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use. Ethical approval was obtained from the University of Adelaide (approval number S-59–2003). Chicken eggs (white leghorn; HICHEICK Breeding Company, Bethal, Australia) were placed longways in egg trays and incubated in a humidified incubator at 37°C for approximately 40 hours to reach stages 10–12 [21] prior to use.

Imaging and Image Processing
The bright-field images of the neuronal differentiation cultures were acquired using Olympus CKX41 fitted with an Olympus DP11 camera (Olympus, Tokyo, http://www.olympus-global.com). The in vitro neuronal differentiation culture-stained images were acquired using the Olympus AX70 microscope fitted with a cooled CCD camera (Olympus), and V + + v.4 software (Nikon Australia, Lidcombe NSW, Australia, http://www.nikon.com.au). Whole-mount chicken embryo images were taken using confocal microscopy (Bio-Rad Radiance 2100). Confocal images and z-images were processed and analyzed using Confocal Assistant v.4.02 (Bio-Rad). All digital images were processed using Adobe Photoshop 6 (Adobe Systems, San Jose, CA, http://www.adobe.com) and only brightness and/or contrast were altered.

RESULTS
Neural Induction of DPSCs In Vitro
In the present study, we examined the effect of different factors and media formulations to facilitate neural differentiation of human adult DPSCs. We assessed two different neuronal media conditions previously described to promote the neural differentiation of postnatal SHEDs and embryonic stem cells [8, 22, 23]. Following 3 weeks of induction, DPSCs exposed to either neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons. Following 3 weeks of induction, DPSCs exposed to either neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons. Following 3 weeks of induction, DPSCs exposed to either neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons. Following 3 weeks of induction, DPSCs exposed to either neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, neuronal inductive media A or B acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons.
following each media change (supplemental online Fig. 1); whereas dead or dying cells uptake trypan blue, living or healthy cells remain clear.

The protein expression patterns of early (Nestin, PSA-NCAM), intermediate (β-III tubulin), and late (NF-M or NF-H) neuronal-associated markers expressed by DPSCs cultured in the different neuronal inductive conditions were determined by immunocytochemical analysis. The data demonstrated a progressive increase in the early/intermediate (Nestin, PSA-NCAM, and β-III tubulin) and mature (NF-M and NF-H) neuronal markers (Fig. 1A, 1C). In contrast to the protein expression of the Nestin, the proportions of PSA-NCAM-, β-III tubulin-, NF-M-, and NF-H-positive cells were significantly lower (media A: p < .0005, p < .000002, p < .0004, and p < .003, respectively; media B: p < .0005, p < .000005, p < .005, and p < .003, respectively; Student's t test; n = 3 DPSC donors) in control media compared with DPSCs cultured under both neuronal inductive conditions (Fig. 1C).

Supportive real-time PCR analysis indicated that the gene expression profile of DPSCs was more consistent with that of mature neuronal cells, following 3 weeks of neuronal induction. When DPSCs derived from three different donors were cultured in neuronal inductive media, the transcript levels of the neuronal precursor marker, Nestin, appeared to be unchanged or slightly downregulated (Fig. 1D). Similarly, there was a minor decrease observed in the expression of the early/intermediate neuronal gene, β-III tubulin, over the same time period. Conversely, it was found that when DPSCs were cultured with either media A or media B, there was significant increase (p < .015 and p < .0004, respectively, Student’s t test), in the transcript levels for NF-M compared with cells maintained in control media (Fig. 1D). Furthermore, a significant increase in the gene expression of the mature neuronal marker, NF-H, was also observed when DPSCs were cultured under both media A and B (p < .02 and p < .00001, respectively, Student’s t test), in comparison with control cultures (Fig. 1D). Collectively, these data suggest that in response to the neuronal inductive stimuli, a greater proportion of DPSCs had stopped proliferating and had acquired a phenotype resembling mature neurons.
Functional Analysis of the Neuronal Properties of DPSCs In Vitro

To confirm that adult human DPSCs were differentiating into functionally active neurons, electrophysiology was performed on cells grown under normal and neuronal inductive conditions (Fig. 2A). The presence of the ionic currents was first investigated by applying voltage ramps between \(-120\) and \(120\) mV to the cells patch clamped using K-based pipette solution. DPSCs cultured in standard growth media (Fig. 2C, nondifferentiation [ND]) displayed a small inward current, whereas DPSCs cultured in neuronal differentiation media produced a current that increased approximately 8.5-fold (Fig. 2C, 2E, differentiation [D]). To separate inward currents, we used Cs-based pipette solution. Moreover, differentiated DPSC (D) voltage steps revealed large, fast, inactivating inward current with a threshold at approximately \(55\) mV and a maximum at approximately \(5\) mV (Fig. 2D, 2E). This current was approximately 65% blocked by 30 nM tetrodotoxin (TTX), although 300 nM TTX blocked it completely (Fig. 2E). This confirmed that differentiated DPSCs express voltage-gated Na channels. To investigate further the ionic composition of the inward current, Na in the external solution was replaced with BaCl\(_2\), the inward current completely disappeared (not shown), which confirmed that this current was carried by Na and not Ca\(^{2+}\) channels, thus validating the presence of voltage-gated Na\(^{+}\) channels in DPSCs. In contrast, HFFs (Fig. 2A) showed no significant inward currents and high-threshold outward currents in either ND or D media (Fig. 2B). Furthermore, no difference in current amplitude was observed by HFFs cultured in control or neuronal induction media (Fig. 2B). In addition, although K was the main cation in the pipette solution, neither HFFs nor DPSCs showed appreciable outward K\(^{+}\) currents in the physiologically relevant range of membrane potentials (Fig. 2B, 2C). The size of the outward current was not affected by differentiation. Our in vitro data suggested that DPSCs have a predisposition to differentiate into functionally active neurons, which is augmented when cultured in neuronal inductive media.

Neural Induction of DPSCs In Vivo

A developmental avian embryo model was adapted to investigate the neuronal differentiation capacity of human DPSCs during a time of active neurogenesis in vivo [24]. Stably transduced GFP-expressing DPSCs (\(n = 7\) donors) and control HFFs (\(n = 1\) cell line) were injected into regions adjacent to the developing neural tube at stages 10–12 [21]. At this stage of development, CNC cells coalesce, migrate to their target tissue,
and differentiate into neural derivatives [25]. In the present study, the neuronal differentiation of transplanted DPSCs and HFFs was investigated at 24, 48, and 72 hours after injection. The detection of injected human DPSCs was predominantly assessed using an antibody to GFP; however, to further confirm that the injected cells were of human origin, injected embryos were also stained with human-specific integrin-β antibody at 48 hours after injection (supplemental online Fig. 2). At 24 hours after injection, DPSCs exhibited extended processes and localized to regions normally followed by migrating endogenous CNC cells, but did not colocalize with neuronal-specific markers (data not shown). However, the response of DPSCs (n = 53 embryos) to the endogenous neuronal environment was more pronounced at 48 hours after injection. DPSCs displayed the morphology of bipolar cells (Fig. 3A, asterisk) and neurons with multiple neurites (Fig. 3B, 3D, arrows; supplemental online Fig. 3A, 3B), which appeared to have migrated into the central nervous system (CNS) based on merged z-series confocal image analysis. Importantly, the DPSCs that displayed the multiple neurite morphology also colocalized with both early and mature neural markers, β-III tubulin (Fig. 3A, 3B; supplemental online Fig. 3A, 3B) and NF-M (Fig. 3C, 3D, arrows; supplemental online Fig. 3C, 3D), respectively. NF-M was lowly expressed (Fig. 3D, arrows) by the differentiated DPSCs in comparison with GFP staining. Growth cone-like structures were also observed with NF-M staining, suggesting that these DPSCs had differentiated into neuron-like cells (Fig. 3C, 3D, asterisks), which appeared similar to endogenous avian axonal processes. SHEDs also responded in a similar manner to DPSCs (n = 15 embryos), which migrated closely to the axons of the trigeminal ganglion (TG) (data not shown).

To validate that the response of adult human DPSCs and SHEDs to the in vivo neural environment was specific to these cell types or whether all ectodermal-derived cells were able to respond to the same embryonic environmental cues, transduced GFP-expressing HFFs (n = 17 embryos) were injected into the avian embryo. In these studies, the HFFs were observed to migrate toward the epidermal layer of the embryo rather than within the CNS or peripheral nervous system (PNS) or along axonal processes of the TG. Although the morphology of HFFs was thin and spindle-like, similar to bipolar neural cells, GFP-labeled HFFs lacked expression of β-III tubulin and NF-M (Fig. 3E–3H; supplemental online Fig. 3E, 3F) at 24, 48, and 72 hours PI.

Seventy-two hours after injection, adult human DPSCs survived and displayed bipolar processes, which, in close proximity to endogenous sensory axons (Fig. 4A, 4B, asterisks; supplemental online Fig. 4A), whereas the DPSCs exhibited multineurite processes when localized to the CNS (Fig. 4C, 4D, arrowheads; supplemental online Fig. 4B). Furthermore, 7 days after injection, GFP could no longer be detected, however, the injected human DPSCs were still viable, as demonstrated with integrin-β1 staining of frozen sections (supplemental online Fig. 5). These observations suggested that human DPSCs were capable of responding to endogenous migratory and neuronal differentiation cues and followed CNC pathways.

**SUMMARY**

In the present study, we investigated the neuronal potential of human adult DPSCs that have previously been shown to retain properties characteristic of neural crest cells following ex vivo expansion [15, 26, 27]. Ex vivo-expanded DPSCs were cultured in different media formulations previously reported to induce neuronal differentiation of embryonic stem cells [22] and postnatal stem cell populations [8, 28] when cultured in the presence of growth factors such as EGF [17], FGF [18], and/or RA [19]. Similar media conditions have also been reported to be capable of maintaining neuronal cell types in culture [29–31]. Our data showed that DPSCs were receptive to neuronal differentiation using both media conditions over a 3-week duration. These conditions are in accord with other investigations that examined the neural differentiation potential of SHEDs and embryonic stem cells [8, 22, 23]. However, although these conditions do vary, both media were able to induce both a morphological and a gene expression response, suggesting that factors present in both media conditions (EGF and FGF) were predominately responsible for the neuronal induction.

Following neuronal induction in vitro, DPSCs were shown to constitutively express the immature markers Nestin and PSANCAM, which are associated with neuronal precursors and immature neuronal-committed progenitors through to neuroblasts, respectively. This confirms our original findings that ex vivo-expanded postnatal dental pulp-derived stem cells constitutively express Nestin and the GFAP [8, 12], where these two markers are also expressed within fibrous dental pulp tissue in situ [32, 33]. Whereas Nestin protein expression continued to be detected by the majority of DPSCs following neuronal induc-
of action potentials. These include voltage gated K⁺, Na⁺, and Ca²⁺ [40]. In our study, only functional voltage gated Na⁺ channels and not Ca²⁺ were present in adult DPSCs when exposed to neuronal inductive conditions. Importantly, the presence of functional Na⁺ channels, and the size and the properties of the voltage-gated Na⁺ currents in differentiated DPSCs were consistent with those reported for neuronal cell types [41]. The expression of neuronal voltage-gated Na⁺ channels is a critical stage for the generation of action potentials [41]. More recently, voltage-dependent Na⁺ currents have been used as an electrophysiological marker for assessing neuronal maturation of differentiating embryonic stem cell-derived NSCs toward the generation of fully functional neurons [42]. Biella et al were able to demonstrate that there was a correlation between cell excitability and the Na⁺ channel system during neuronal differentiation. Cells cultured in neuronal inductive media up to 21 days resulted in a shift in the steady-state inactivation, whereby a greater percentage of Na⁺ current activation was available with greater duration in neuronal inductive media. Furthermore, these changes in Na⁺ channel system were associated with the ability of differentiating NSCs to generate action potentials [42]. The present study observed a correlation between the gene and protein expression of mature neuronal markers, NF-M and NF-H, with a 8.5-fold increase in inward current following 3 weeks of neuronal induction conditions, consistent with the findings of Biella et al. [42]. However, undifferentiated DPSCs were also able to produce a minor inward current, implying that DPSCs or a proportion of the total cell population may have a predisposition to differentiate into neuronal cell types.

Previous studies have demonstrated that cultured rat and human DPCs survived and expressed some neural-associated markers when transplanted into the rodent brain [8, 9]. In the present study, the in vivo neural differentiation potential of human adult DPSCs was assessed using an avian embryo model as a host environment due to the absence of a functional immune system and because of the presence of active sites of early neuronal development. The chicken embryo has previously been used to investigate cellular migration and differentiation using quail-chicken [43] and mouse-chicken chimera [44] experiments and following transplantation of rat mesenchymal stem cells [45] and mouse embryonic endothelial progenitor cells into the chicken embryo [46]. In the present study, GFP-labeled DPSCs were found to coexpress neuronal-associated markers within 48 hours after injection into 4-day-old chicken embryos, indicating a rapid response by DPSCs to endogenous environmental cues. It must be noted that the possibility of the neuronal multiple neurite morphology being attributed to multiple GFP-expressing DPSCs was highly unlikely, as the figures represent a composite of merged z-series confocal images, and individual images from the z-series, with XZ and YZ axis (supplemental online Figs. 3 and 4). Furthermore, the robustness and reliability of the avian xenogenic assay was demonstrated with more than 200 embryos injected with either adult human DPSCs, SHEDs, or HFFs, which survived for the duration of the experiments at 24, 48, and 72 hours.

Importantly, the location of DPSCs within the embryo appeared to be essential for their specific neural morphology and differentiation capacity. DPSCs and SHEDs located near sensory TG neurons displayed a bipolar morphology characteristic of sensory neurons, whereas in the CNS, DPSCs exhibited multidendritic processes associated with motor neurons. The significance of this observation suggests that transplanted human adult DPSCs have a propensity to differentiate into CNS-type neurons when exposed to the correct environmental conditions. Furthermore, these DPSCs were not manipulated prior to transplantation, and therefore demonstrate their ability to respond directly to their surrounding environment. Although it

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is not common for CNC cells to exhibit CNS neural morphology. Ruffins et al have observed that neural crest cells exhibit the capacity to differentiate and express markers specific for neurons of the CNS, when transplanted into the ventral neural tube [47]. It has been proposed that neural crest and neural tube derivatives originate from the same precursor within the dorsal neural tube, which contributes to both CNS and PNS derivatives [48]. Since DPSCs retain neural crest properties in vitro [15], it is not surprising then, that DPSCs displayed characteristics of both PNS and CNS neurons in response to the environmental cues when transplanted in vivo. Our study showed that DPSCs were able to survive and differentiate into neuronal derivatives and potentially integrate into neuronal networks within the chicken embryo up to 7 days after injection. In contrast, the control cell type, HFFs, derived from the same germ layer as the pulp tissue did not respond to the in vitro neuronal inductive conditions or the in vivo microenvironment. Overall, these studies suggest that DPSCs are responsive to the surrounding microenvironment, surviving, migrating, and differentiating according to the appropriate cell types within the avian host neural tissue. These observations confirm the specificity of the neuronal differentiation response by adult human DPSCs as previously reported for SHEDs. The data suggest that DPSCs and SHEDs are appropriate candidates for further evaluation as stem cell therapy-based treatments using animal models representative of neurological diseases and injury.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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