STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

Implanted Adult Human Dental Pulp Stem Cells Induce Endogenous Axon Guidance

AGNIESZKA ARTHUR,[a] SONGTAO SHI,[b] ANDREW C. W. ZANNETTINO,[c] NOBUTAKA FUJII,[d] STAN GRONTHOS,[a] SIMON A. KOBLAR[e,f]

[a]Mesenchymal Stem Cell Group and [c]Myeloma Research Program, Bone and Cancer Laboratories, Division of Haematology, Institute of Medical and Veterinary Science/Hanson Institute, CSCR University of Adelaide, Adelaide, South Australia, Australia; [b]Division of Craniofacial Sciences and Therapeutics, School of Dentistry, University of Southern California, Los Angeles, California, USA; [d]Department of Bioorganic Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan; [e]Stroke Research Programme, School of Medicine and [f]School of Molecular and Biomedical Science (Genetics), University of Adelaide, Adelaide, South Australia, Australia

Key Words. Mesenchymal/Neural crest stem cells • Neuroplasticity • SDF-1 • Stem Cell transplantation

ABSTRACT

The human central nervous system has limited capacity for regeneration. Stem cell-based therapies may overcome this through cellular mechanisms of neural replacement and/or through molecular mechanisms, whereby secreted factors induce change in the host tissue. To investigate these mechanisms, we used a readily accessible human cell population, dental pulp progenitor/stem cells (DPSCs) that can differentiate into functionally active neurons given the appropriate environmental cues. We hypothesized that implanted DPSCs secrete factors that coordinate axon guidance within a receptive host nervous system. An avian embryonic model system was adapted to investigate axon guidance in vivo after transplantation of adult human DPSCs. Chemoattraction of avian trigeminal ganglion axons toward implanted DPSCs was mediated via the chemokine, CXCL12, also known as stromal cell-derived factor-1, and its receptor, CXCR4. These findings provide the first direct evidence that DPSCs may induce neuroplasticity within a receptive host nervous system. STEM CELLS *2009;27:2229–2237*

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

INTRODUCTION

There is significant evidence that stem cell-based therapies enhance functional recovery in animal models of neurological injury or disease [1–5]. Two paradigms for stem cell-based therapies currently are available: transplantation of exogenous progenitor/stem cells or the recruitment of endogenous neural stem cells. In transplantation of exogenous progenitor/stem cells to enhance functional recovery the underlying mechanisms of action remain unknown. A parsimonious idea is that progenitor/stem cells replace the neural cells lost in the damaged nervous system. However, it is becoming evident that a more complex neuroregenerative process is likely to underlie functional recovery. This may involve implanted progenitor/stem cells secreting a combination of signaling molecules that act upon the damaged nervous system through varied mechanisms: inflammation, programmed cell death, angiogenesis, and/or neuroplasticity. The emerging concept of "bystander" or paracrine mechanisms of activity induced by implanted progenitor/stem cells requires further investigation [6]. The findings that transplanted neural stem cells act via immunomodulation of an inflammatory plaque in a murine model of multiple sclerosis in addition to replacement of the lost oligodendrocyte to improve neurological function favors the concept of a more complex underlying neuroregenerative process [7, 8]. In this article we investigate the role of adult human progenitor/stem cell-mediated neuroplasticity in a receptive host nervous system.

The trigeminal ganglion (TG) has been an important vertebrate neural structure within which to investigate axon guidance because of its highly patterned trifasciculate branching [9, 10]. In the avian embryo the TG is bilobed and during maturation extends three axonal processes into the developing face; the ophthalmic, maxillary, and mandibular processes, which innervate the forehead, cheek, and lower jaw, respectively. The trifasciculate branching of the TG is accurately coordinated owing to a combination of attractant and repellent factors mediated over short- or long-range distances [10].

We have demonstrated that a population of human progenitor/stem cells persists in the pulp tissue obtained from impacted third molar teeth in young adults [11]. Adult human

Author contributions: A.A.: concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.S. and N.F: provision of study material, manuscript review; A.C.W.Z.: provision of study material; S.G. and S.A.K.: concept and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript. S.G. and S.A.K. are senior co-authors on this work.

Correspondence: Simon A. Koblar, M.D., Ph.D., Stroke Research Programme, School of Medicine, University of Adelaide, Adelaide, 5000, SA, Australia. e-mail: simon.koblar@adelaide.edu.au Received December 15, 2008; accepted for publication May 20, 2009; first published online in STEM CELLS *EXPRESS* June 4, 2009. 1066-5099/2009/$30.00/0 doi: 10.1002/stem.138

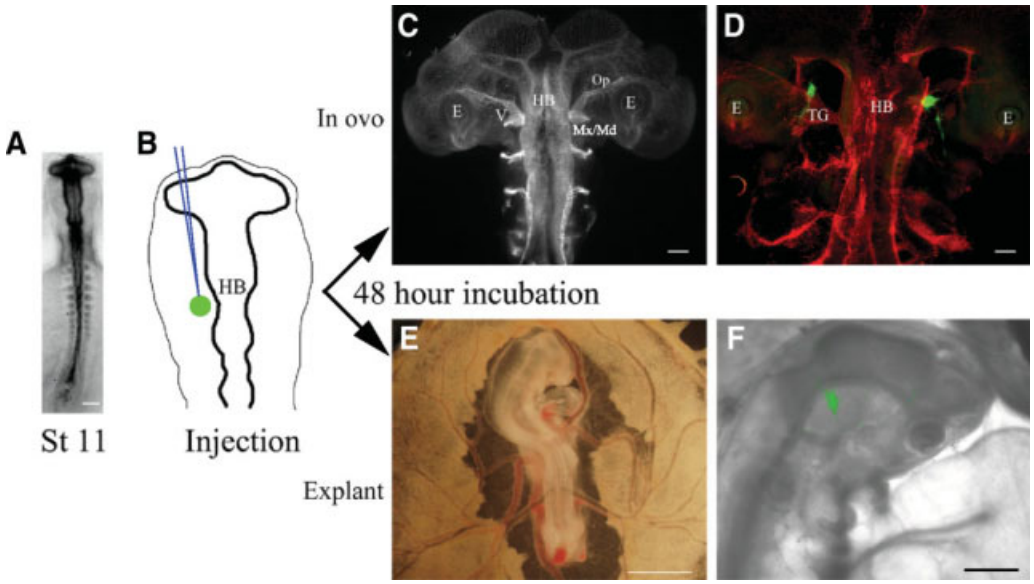

Figure 1. Human-chick xenotransplantation model system. **(A):** St 10-12 avian embryos were prepared for human cell xenotransplantation in ovo. **(B):** Green fluorescent protein (GFP)-transduced human cells were injected into the mesodermal tissue lateral to rhombomere 2 of the developing avian hindbrain. Embryos were allowed to develop normally in ovo for **(C)** 48 hpi which correlated to St 20, after which time embryos were removed from the egg and stained for pan-neuronal markers, class III β-tubulin or neurofilament-medium chain (NF-M); here NF-M staining is shown. Embryos were mounted in an open-book fashion with the HB in the midline of the embryo, the E bilateral to the HB, and the TG bilateral to the HB. The TG has two lobes and three distinct axonal outgrowths: the Op process above the eye and the Mx/Md processes that innervate the lower face. **(D):** Representative image of St 20 embryo stained with NF-M (red) and GFP (green). **(E):** Alternatively, at 2 hpi the embryos were explanted and grown ex ovo for a further 48 hpi on a Millipore membrane. **(F):** Human-chick chimeric embryos were imaged by transmitted and fluorescent light to demonstrate the site of the human GFP^+ cells relative to the host TG. Scale bar **(A, C, D, F)** = 200 μm Scale bar **(E)** = 1 mm. Abbreviations: E, eye; HB, hindbrain; Mx/Md, maxillary/mandibular; Op, ophthalmic; St, stage; TG, trigeminal ganglion; V, trigeminal ganglion bilateral to the hindbrain.

dental pulp progenitor/stem cells (DPSCs) are putatively neural crest (NC) cell derived [12] and thus may have neurogenic potential that relates to the generation of neurons and their connections, that is, axon guidance. Consistent with this proposition, it has been demonstrated that DPSCs, given the appropriate environmental cues, differentiate into functionally active neurons [13], influence endogenous recruitment of neural stem cells [14], and may themselves generate neurospheres [15]. Tissue from the dental pulp is a source of neurotrophic factors capable of promoting neuronal survival and neurite outgrowth in vitro and in vivo [16, 17]. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and CXCL12, also known as stromal cell-derived factor-1 (SDF-1), are expressed by dental pulp cells [16–18]. These factors have also been implicated in axon guidance of the TG, which subserves sensation to the face and motor control to the masseter muscles of the jaw [9, 10]. CXCL12 belongs to the chemokine family of molecules that was originally characterized as having primary roles within the immune system [19]. However CXCL12, interacting via its cognate receptor CXCR4, has been shown to possess axon guidance activities in the nervous system [20] and to instigate recruitment of dental pulp cells after injury [21]. Furthermore, CXCL12/CXCR4 interactions pattern TG motor and sensory development [22, 23].

In the present study, we developed a cross-species xenotransplantation model system to investigate progenitor/stem cell-mediated axon guidance. This was based on Le Douarin's quail-chick chimeric experiments that used microsurgical approaches, which have provided fundamental insights into NC cell ontology over the past quarter century [24]. In our human-chick xenotransplantation experiments we found that implanted adult human DPSCs induced chemoattraction of TG axons via CXCL12/CXCR4 signaling interactions. This finding provides the first direct evidence that implanted dental pulp stem cells cause neuroplastic change within a receptive host nervous system.

Materials and Methods

In Ovo Transplantation of Human Cells into Avian Embryos

Ethical approval was obtained from the University of Adelaide (approval number S-59-2003). Chicken eggs (white leghorn; HiChick Breeding Company, Bethal, SA, Australia) were incubated in a humidified 37°C incubator containing 5% CO_2 for approximately 40 hours to reach stage 10-12 [25] (Fig. 1A) before injection. DPSCs isolated as described previously [11] were retrovirally transduced with a green fluorescence protein (GFP)-encoding gene as reported previously [26]. Stable GFP-positive transduced adult human DPSCs or human foreskin fibroblasts (HFFs) were selected by fluorescence-activated cell sorting using a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA, http://www.bd.com). Transduced DPSCs and HFFs (5×10^3 cells/μl) were injected into the developing avian embryo as described previously [13]. In brief, a window was cut into the top of the egg, and the embryo was visualized by injecting Indian ink (prepared in Ringer's solution; Winsor & Newton, Harrow, Middlesex, UK, http://www.winsornewton.com) below the embryo to improve contrast. Fast green dye was added to the cells to visualize during the injection procedure. The vitelline membrane was removed from around the head of the embryo. The cells were placed in a glass capillary needle (GC100TF-10; SDR Clinical Technology, Sydney, Australia, http://www.sdr.com.au/), attached to a micromanipulator and pressure injector

(Narishige, Tokyo, Japan, http://www.narishige.co.jp), set at 25 psi. The micromanipulator was used to guide the needle into the region directly adjacent to rhombomere 2 in the developing hindbrain (Fig. 1B). Cells were injected into the embryo using a foot pump attached to the pressure injector. After this manipulation the egg was sealed and incubated further to mature the human-chick chimeric embryo.

Explanting of Human-Avian Chimeric Embryos

GFP-transduced adult human DPSCs (5×10^3 cells/μl) were maintained in growth media alone or in the presence of CXCL12/SDF-1, inhibitor T140 [27–29], or control peptide (RTVAHHGGLYHTNAEVK) (5 μM) (Mimotope, Victoria, Australia) before transplantation into the avian embryo. DPSCs were injected as described above, embryos were then dissected from the egg 2 hours postinjection, washed in Ringer's solution, and explanted onto a Millipore membrane (Millipore, Billerica, MA, http://www.millipore.com), as described previously [30]. The embryos were orientated so that the vitelline membrane (ventral side down) was in contact with the Millipore membrane bathed in Neurobasal Medium (GIBCO BRL, Grand Island, NY, http://www.invitrogen.com), B27 supplement (GIBCO BRL), and 0.5 mM L-glutamine (GIBCO BRL) with T140 or control peptide. Explanted human-chick chimeric embryos were incubated for a further 48 hours postinjection (hpi).

Dissection and Staining Procedure

Human-chick chimeric embryos grown in ovo or explanted were generally dissected 48 hours postinjection. The head was cut through the midline as in an open-book manner, that is, cut from the nose toward the hindbrain down the length of the head on the ventral side. Dissected embryos were fixed in 4% paraformaldehyde and then were washed with phosphate-buffered saline (PBS) before immunofluorescent staining. Embryos were washed five times with PBS and 0.3% Triton X-100 (PBS-T) and then were incubated in blocking solution (10% heat-inactivated horse serum in PBS containing 1% Triton X-100) for 5 hours at room temperature. Embryos were next incubated overnight at room temperature with primary antibodies, goat anti-GFP (4 μg/ml, 600-101-215; Rockland Immunochemicals, Inc., Gilbertsville, PA, http://www.rockland-inc.com), neural crest markers rabbit anti-Sox9 (1:6000 dilution; gift from D. Newgreen, Murdoch Children's Research Institute, Parkville, VIC, Australia) and mouse anti-Pax7 (supernatant, 1:10; gift from M. Ziman, Edith Cowan University, Perth, WA, Australia) and neuronal markers, class III β-tubulin (TUJ1)(4 μg/ml, MMS-435P; Covance, Princeton, NJ, http://www.covance.com) and neurofilament-medium chain (NF-M) (5 μg/ml, 13-0700; Zymed Laboratories, Inc., South San Francisco, CA, http://www.invitrogen.com), in PBS containing 10% horse serum and 0.1% Triton X-100. Samples were washed five times with PBS-T and then incubated in the dark overnight at 4°C with secondary antibodies, donkey anti-goat Alexa 488 (10 μg/ml, A11055; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) and donkey anti-mouse Cy3 (7.5 μg/ml, 715-165-150, Jackson ImmunoResearch, West Grove, PA, http://www.jacksonimmuno.com), diluted in PBS containing 10% horse serum and 0.1% Triton X-100. Embryos were washed as described above, placed in 80% glycerol, and then mounted onto glass slides and cover-slipped with ProLong Gold anti-fade reagent with 4,6-diamidino-2-phenylindole (P36931; Invitrogen). Embryos were imaged with a Radiance 2100 Confocal (Bio-Rad, Hercules, CA, http://www.bio-rad.com) or TE 2000-E confocal (Nikon, Melville, NY, http://www.nikon.com) microscope. Images were processed with Adobe Photoshop; only the brightness and contrast were altered. Axonal perturbation was counted using the following criterion: aberrant axon guidance defined as host trigeminal ganglion fasciculated axons deviated from their normal position (i.e., above the eye for ophthalmic branches and below the eye and toward branchial arches for maxillary and mandibular branches) and directed toward implanted DPSCs. Statistical significance was determined using a two-tailed Fisher's exact test.

Real-Time Polymerase Chain Reaction

The level of CXCL12 gene expression was determined using real-time polymerase chain reaction (PCR) as described previously [26, 31]. In brief, RNA was isolated using the TRIzol (Invitrogen) method and reverse transcribed with Superscript III reverse transcriptase (Invitrogen). Real-time PCR reactions were performed using TaqMan Master Mix on an ABI SDS 7000 light cycler driven by ABI prism SDS v1.1 (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The following primers were synthesized locally (GeneWorks, Hindmarsh, SA, Australia, http://www.geneworks.com.au): CXCL12 (accession number NM_199168.2), forward (ATGCCCATGCCGATTCTTCG) and reverse (GTCTGTTGTTGTTCTTCAGCC); control gene TBP (TATA box-binding protein), forward (CTGGAAAAGTTGTATTAACAGGTGCT) and reverse (CCATCACGCCACAGTTTCC); GDNF (accession number NM_000514), forward (GGGCACCTGGAGTTAATGTC) and reverse (GCCACGACATCCCATAACTT); BDNF (accession number NM_170731), forward (TGGAGTTGGCATTGCATTTA) and reverse (TCCACCTAGACCTTGGGATG); NGF (accession number NM_002506.2), forward (AGGGAGCAGCTTTCTATCCTG) and reverse (GGCAGTGTCAAGGGAATGC), neurotrophin-4/5 (NT-4/5) (accession number NM_006179), forward (GCCACCTGTGTCCTCCAC) and reverse (AGAAAAGGGGGCAATGTTGA); and neurotrophin 3 (NT-3) (accession number NM_002527), forward (CAGAGACGCTACAACTCACCG), reverse (CCGTGATGTTCTGTTCGCC).

Enzyme-Linked Immunosorbent Assay Protein Analysis

The protein levels of CXCL12 expressed by DPSCs and HFFs were determined using a commercial CXCL12 specific immunoassay (Human CXCL12/SDF-1α Quantikine Colorimetric Sandwich ELISA; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), as described previously [26]. In brief, DPSCs and HFFs were plated at 1×10^5 cells per well in a six-well plate and incubated in growth media for 48 hours. The supernatant was filtered through a 0.2-μm filter and analyzed by the CXCL12 specific immunoassay. The cells within the plate were detached and counted. The level of CXCL12 production per cell was determined and is represented as pg/cell.

Results

Implanted Adult Human DPSCs Induce Chemoattraction of Host Avian TG Axons

In the present study, we injected ex vivo expanded human DPSCs, isolated from adult third molar teeth [11], adjacent to the second rhombomere of avian embryos in ovo at stage 10-12 (Fig. 1A, 1B) [25]. The xenotransplanted human-chick chimeric embryos were allowed to develop in ovo for 48 hpi and then were removed for detailed characterization. The chimeric embryos were analyzed in an open-book preparation after pan-neuronal staining for either NF-M or TUJ1 (Fig. 1C). The transplanted human DPSCs had been previously retrovirally transduced to express GFP to formally identify implanted human cells within the background of the host chick tissue (Fig. 1D).

Studies focused on the examination of the host chick TG, which is a highly patterned trifasciculated cranial nerve. In normal chick embryos extrafasciculated axonal branching away from the main ophthalmic and maxillomandibular lobes is never seen, however, with the rare exception of a stray single axon projecting from the trifasciculate processes (Fig. 2A). Our studies showed a marked perturbation at 48 hpi to

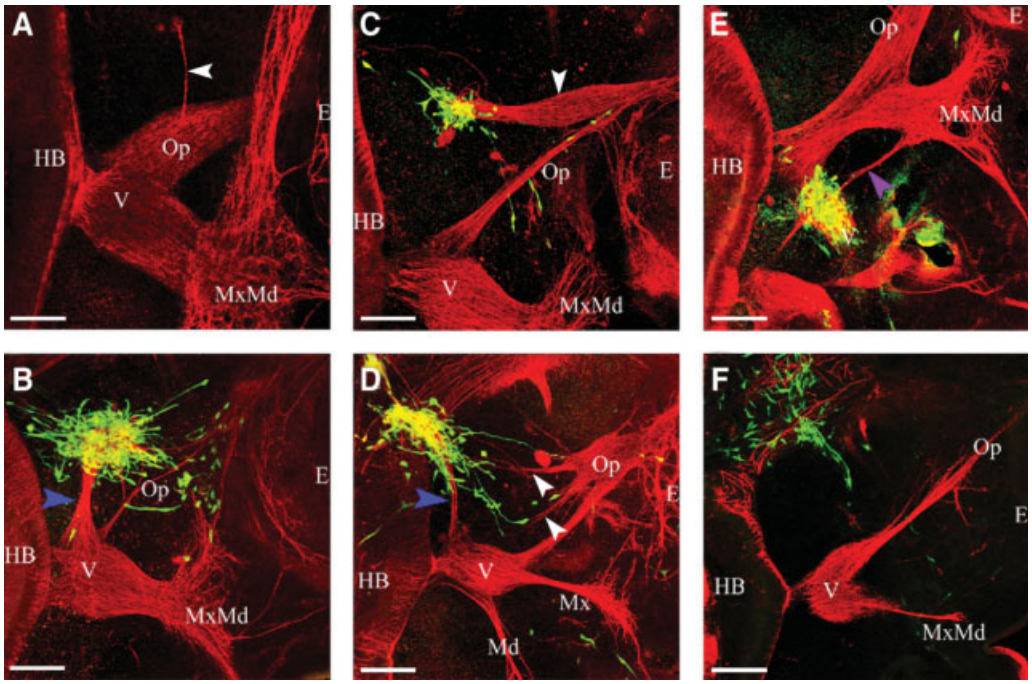

Figure 2. Implanted adult human dental pulp progenitor/stem cells (DPSCs) induce chemoattraction of host avian trigeminal ganglion (TG) axons. Images of the host avian embryo 48 hpi stained by class III β-tubulin (red) showed patterning of the host TG in relation to the site and type of implanted human cells identified by transduced green fluorescent protein expression (green). **(A):** Normal patterning of avian TG 48 hpi in an uninjected control avian embryo. **(B–F):** Human DPSC-chick chimeric embryos at 48 hpi. **(B–E):** Implanted adult human DPSCs induce chemoattraction of host chick TG axons. **(F):** Implanted human foreskin fibroblasts did not alter axonal projections of the host TG. Arrowheads indicated normal stray single axons **(A)** or perturbed host TG axonal projections **(B–E)**. All images are z-serial confocal images compressed to a single plane. Scale bars = 200 μm. Abbreviations: E, eye; HB, hindbrain; Mx/Md, maxillary/mandibular; Op, ophthalmic; V, trigeminal ganglion bilateral to the hindbrain.

the normal patterning of the host TG in 30 of 43 (70%; $n = 3$ human donors) avian embryos xenotransplanted with human DPSCs (Figs. 2B–2E, 4C; Table 1). In the human-chick chimeric embryos 48 hpi the DPSCs appeared to aggregate in close proximity to the host TG. Depending on the site of the DPSC aggregate, three patterns of aberrant host TG branching were observed (Fig. 2B–2E). When DPSCs were positioned superior to the host TG, the outgrowth of the ophthalmic process was altered in either one of two patterns: first, the DPSCs induced branching of TG axons proximally from the ophthalmic lobe (Fig. 2B, D); and second, the DPSCs induced redirection of TG axons from distal ophthalmic and/or maxillomandibular axonal projections (Fig. 2C, 2D). Alternatively, when DPSCs were positioned inferior to the host TG, the maxillomandibular lobe and processes were altered with proximal and distal axonal projections directed toward the DPSCs (Fig. 2E). Therefore, the altered guidance of host TG axonal outgrowth toward the implanted adult human DPSCs in the chimeric embryos suggested the presence of DPSC-secreted putative soluble factor(s), which exerted chemoattractive effects on axonal guidance.

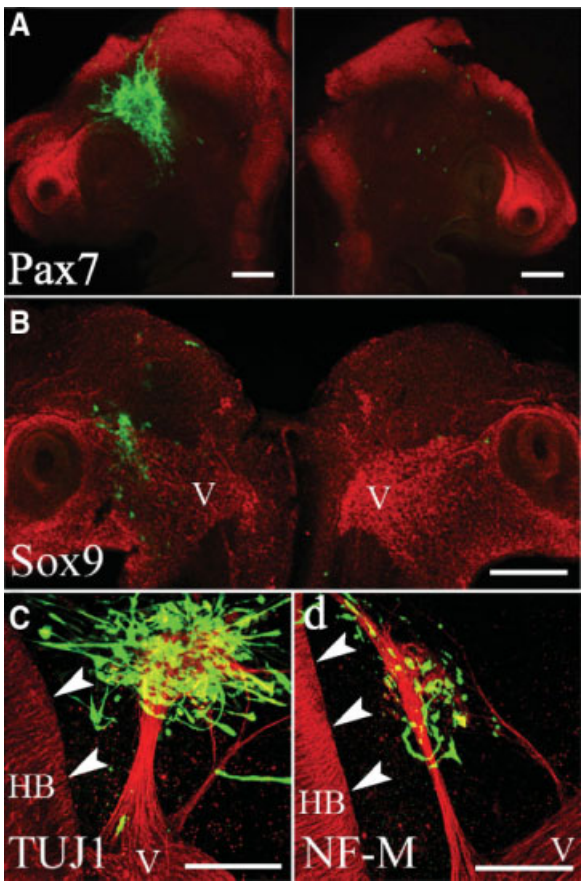

Figure 3. Neural crest cells and motor axons are not responsive to implanted dental pulp progenitor/stem cells (DPSCs). Embryos injected with DPSCs (green) stained with early neural crest markers Pax7 (red) **(A)** and Sox9 (red) **(B)** show normal development and patterning of cranial neural crest cells. Furthermore, TUJ1 **(C)** and NF-M **(D)** staining of motor axons (arrowheads) demonstrated normal patterning of axons within the HB of DPSCs (green)-injected embryos. Scale bars = 200 μm. Abbreviations: HB, hindbrain; NF-M, neurofilament-medium chain; TUJ1, class III β-tubulin; V, trigeminal ganglion bilateral to the hindbrain.

Table 1. Avlan embryo endogenous axonal perturbation in response to DPSC and CXCL12

Sample	No. of embryos	Perturbed axons	Nonperturbed axons	% perturbation	p^a
HFFs	17	0	17	0	
DPSCs	43	30	13	70	<.0001
HFF-control	10	1	9	10	
HFF-CXCL12$^+$	19	17	2	89	<.0001
Explants					
DPSC + control	28	20	8	71	
DPSC + T140	26	9	17	35	<.01

[a]Statistical significance was determined using a two-tailed Fisher's exact test by comparing DPSC to HFF, HFF-CXCL12$^+$ to HFF-control, and DSPC + T140 to DSPC + control peptide explants.
Abbreviations: DPSC, dental pulp progenitor/stem cell, HFF, human foreskin fibroblast, HFF-control, HFFs expressing a empty vector, HFF-CXCL12$^+$, HFFs expressing CXCL12, DPSC + control, DPSC-explanted embryo bathed with a control peptide, DPSC + T140, DPSC-explanted embryo bathed with a CXCL12 inhibitor (T140).

Next we investigated whether the observed changes to host chick TG axonal outgrowth was a specific response to adult human DPSCs or was due to a nonspecific response mediated by other ectodermal-derived human cells after xenotransplantation in ovo. To investigate this question, we injected HFFs, previously transduced to express GFP, into the head mesoderm lateral to the second rhombomere and analyzed the avian embryos 48 hpi. HFFs were chosen as a control human cell population due to their ectodermal origins, consistent with DPSCs, which have served as controls in other studies [16, 32]. The analyses showed that HFFs behaved in a different manner than DPSCs, in that none of the 17 human HFF-chick chimeric embryos displayed aberrant TG axonal branching (Fig. 2F). Human DPSCs exhibited a significant ($p < .0001$, two-tailed Fisher's exact test) (Fig. 4C; Table 1) chemoattractive axonal guidance, which did not appear to be evident in HFFs, in which chemoattraction was defined as host trigeminal ganglion fasciculated axons deviating from their normal position and directed toward implanted DPSCs.

To substantiate that the perturbed TG axonal migration was in response to the injected DPSCs and not a consequence of aberrant cranial neural crest migration, DPSC-injected embryos 24 hpi were stained with early neural crest markers, Pax7 and Sox9 (Fig. 3A, 3B). These embryos did not display perturbed neural crest patterning or TG formation (Fig. 3A, 3B). Furthermore, when embryos were stained at 48 hpi with different neuronal markers, perturbed axonal migration was only observed from the TG lobes or its axonal branches (Figs. 2B–2E, 3C, 3D). Moreover, it was noted that motor axons from the hindbrain did not migrate toward the injected DPSCs even when the hindbrain axons were in close proximity to the injected DPSCs. Together these observations suggested that the perturbed host TG axonal migration was in response to the injected DPSCs and was not influenced by cranial neural crest settling or migration. In addition, the axons that were responsive to the chemoattraction in the presence of DPSCs appeared to originate distal and not proximal to the TG.

DPSC-Induced ChemoAttraction of TG Axons Mediated via CXCL12/CXCR4 Interactions

We next investigated the molecular basis of the DPSC-induced TG axon chemoattraction by examining the chemokine CXCL12/CXCR4 signaling system, which is known to be involved in axon guidance activities in dental pulp tissue, the nervous system, and patterning of TG motor and sensory axons [20–23]. Real-time PCR analysis demonstrated that DPSCs expressed significantly greater levels of CXCL12 transcripts (14-fold or 93% greater expression ($p < .0017$, Student's t test) (Fig. 4A) compared with HFFs. Enzyme-linked immunosorbent assay (ELISA) analysis also confirmed that DPSCs expressed significantly higher levels of CXCL12 (27-fold or 96% greater expression, $p < .003$, Student's t test) compared with HFFs (Fig. 4A, 4B). These observed levels of CXCL12 expression were similar to those described previously for bone marrow-derived mesenchymal stem cells [26]. Because HFFs failed to demonstrate a measurable level of chemoattraction of host TG axons, HFFs were transduced to stably overexpress CXCL12 at a protein level comparable to that of human DPSCs (Fig. 4B). HFFs transduced to overexpress CXCL12 (HFF-CXCL12$^+$) or vector control HFFs were injected as described previously, adjacent to the second rhombomere in the avian embryo and were analyzed at 48 hpi (Fig. 1A–1D). HFF-CXCL12$^+$ cells induced a pattern of aberrant branching from the host TG similar to that exhibited by implanted DPSCs (Fig. 4C, 4F; Table 1). This resulted in a proportionally similar frequency of significantly ($p < .0001$, two-tailed Fisher's exact test, compared with HHF-vector control cells) perturbed TG branching with 89% (19 of 21 human-chick chimeric embryos) induced by HFF-CXCL12$^+$ cells compared with 70% (30 of 43 embryos) exhibited by DPSCs. The control HFF population transduced with empty vector alone failed to induce chemoattraction of the host TG axons (Fig. 4C, 4E; 1 of 10 embryos; Table 1).

Studies were performed to confirm whether the human DPSC-derived CXCL12 was interacting with endogenous CXCR4 on the avian TG axons using a small peptide, T140, a potent inhibitor of CXCL12/CXCX4 interactions [27–29]. Dose-dependent analyses showed that optimal T140 inhibition of DPSC-mediated TG axon guidance occurred at 5 μM (data not shown). The human-chick chimeric embryos 2 hpi were explanted onto Millipore membranes and grown ex ovo in serum-free defined media [30] supplemented with T140 or control peptide (Figs. 1E, 1F, Fig. 4D, 4G, 4H; Table 1). After 48 hours of incubation on the Millipore membranes bathed in media containing control peptide, 71% of embryos (20 of 28) demonstrated a chemoattraction of host chick TG axonal projections (Fig. 4D, 4G). However, when xenotransplanted embryos were grown ex ovo in T140 supplemented media, there was a significant ($p < .01$, two-tailed Fisher's exact test) reduction in DPSC-induced chemoattraction with 35% axonal perturbation ($n = 9$ of 26) (Fig. 4D, 4H; Table 1). These data strongly suggest that CXCL12 is a potential factor involved in DPSC-mediated chemoattraction of host TG axons.

To investigate other putative molecules responsible for the DPSC-mediated perturbation of TG axons, we next examined the expression of other candidate molecules that have been identified previously as axon guidance factors during development. Real-time PCR analysis of axon guidance molecules GDNF, BDNF, NGF, NT-3, and NT-4/5 demonstrate that GDNF is also significantly up-regulated in DPSCs compared with HFFs ($p < .005$, Student's t test) (supporting information

Fig. 1). These observations suggest that other guidance molecules in combination with CXCL12 may influence axonal migration in response to DPSCs.

Discussion

The molecular mechanisms underlying the process of neuroplasticity are thought to involve a complex interaction of molecules that coordinate axonal guidance and/or synaptogenesis [33]. In the present study, we developed a human-chick xenotransplantation model system and showed that transplanted human dental pulp-derived progenitor/stem cells alter a host nervous system during development through direct action on axons rather than a secondary phenomenon due to changes in host NC migration. Development of this human-chick xenotransplantation model was based on Le Douarin's quail-chick chimeric experiments using microsurgical approaches that have provided invaluable insight into NC ontology over the

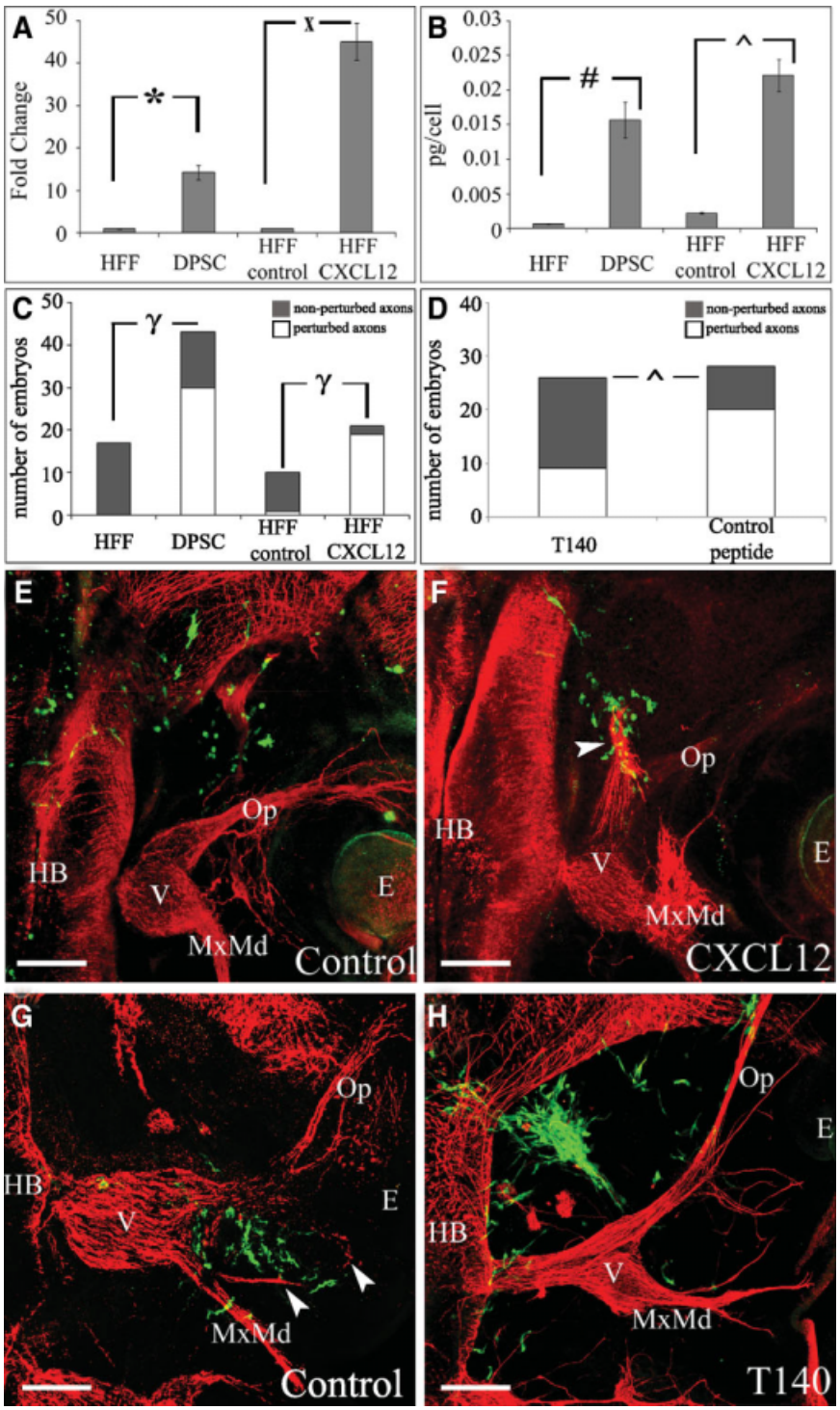

Figure 4.

last 25 years [24]. Recently, human melanoma cells, which are NC-derived, tracked along host NC migratory pathways after injection into chick embryos [34]. Furthermore, a subpopulation of transplanted human metastatic melanoma cells appeared to reprogram to a nonmetastatic phenotype owing to the embryonic microenvironment [35]. It has also been demonstrated that DPSCs themselves have the potential to differentiate into melanocytes because of their putative NC origin [36]. Thus, xenotransplantation studies may provide significant insights into basic cellular and molecular mechanisms underlying development, disease, and therapeutics [24, 37].

The typical axon guidance molecules that have been studied previously in relation to TG axon guidance include chemoattractant and contact attractant and repellent molecules, for example, semaphorin family members, Eph/ephrin receptor tyrosine kinases, BDNF, NT-3, and fibroblast growth factor-8 [9, 10, 33], and some of these factors are known to be expressed by dental pulp-derived populations [16–18, 38]. Another family of molecules that constitute the chemokines are well known for their importance in the inflammatory response [39] and are now thought to also mediate a number of other processes including cell survival and correct migration of neural progenitors during brain development, in addition to stimulating stem cell migration after neuronal injury, such as stroke [26, 40–45]. CXCL12 belongs to the chemokine family of molecules and interacts predominantly via its cognate receptor, CXCR4. Both CXCL12- and CXCR4-deficient mice die at late gestation, at which time these mice display abnormal migration of neuronal progenitor cells in the cerebellum, dentate gyrus, cortex, and dorsal root ganglion and aberrant projections of axonal processes [40–42, 46], highlighting the potential role of CXCL12/CXCR4 in neuronal migration and axon guidance. Although these studies do not directly relate to the TG, two elegant studies have since demonstrated that a particular subset of neural progenitors that form part of the TG in zebrafish express CXCR4 and are responsive to CXCL12 expressed by cells located posterior to the forming TG [22, 23]. CXCL12/CXCR4, interactions are now thought to help mediate axon guidance in the developing nervous system [42] and more specifically facilitate chemoattraction of TG axons [22, 23]. In the present study, we demonstrated that altered patterns of axonal migration were due, in part, to the interaction between exogenous CXCL12 derived from human DPSCs via avian CXCR4 receptors on the TG, suggesting that CXCL12 and CXCR4 are highly conserved across different species.

The finding in the present study that almost one-third of chimeric embryos, when grown in the presence of the CXCL12-CXCR4 small peptide inhibitor (T140), still exhibited an altered host TG phenotype suggested that other factors are also likely to be involved, and preliminary studies have eluded to the possible involvement of GDNF. Dental pulp tissues express BDNF and GDNF [16–18], which also possess axon guidance activity and have previously been shown to influence the migration of TG axons during development [47]. Alternatively, it has been suggested that CXCR4 is not expressed by all axons of the TG in zebrafish [22], and, therefore, only those axons expressing CXCR4 would be responsive to the T140 inhibitor. Lieberam et al. [23] concluded that all motor neurons within the dorsal root ganglion (DRG) default to the dorsal motor neuron (dMN) migratory pathway. However, ventral motor neurons (vMNs) and not dMNs within the dorsal root ganglion express CXCR4 at the time of their initial migration, whereas CXCL12 is expressed by the mesenchymal cells that line the ventral spinal cord [23]. The subsequent chemoattractant response after the interaction of CXCR4 expressing vMNs with CXCL12 overrides any repellent guidance signals, mediated predominantly by semaphorins and netrins, and, therefore, vMNs migrate ventrally through the mesenchymal tissue. Whereas the TG is a sensory ganglion composed of dMN, the neurons of the TG were found to respond in a similar manner to the vMNs of the DRG [23]. Furthermore, our observations suggest that it was the sensory neurons located distal of the TG that were responsive to the DPSCs as no abnormal migration of motor axons from the hindbrain was noted. We suggest that the TG axons are more responsive to the transplanted DPSCs than to the endogenous guidance cues via a mechanism similar to that for vMNs, for which the attractant signaling of CXCL12/CXCR4 is stronger than the signaling of the endogenous guidance molecules.

The observation that endogenous axonal processes are more responsive to exogenous sources of CXCL12 may be important for future stem cell-based therapies, for which DPSCs may assist in repair in a two-pronged approach. Not only do DPSCs express neurotrophic factors, as rat dental pulp cells have been shown to assist in the survival of damaged axons after hemisection of the spinal cord [16, 17], but

Figure 4. CXCL12 secreted by adult human DPSCs mediates chemoattraction of host avian trigeminal ganglion (TG) axons. **(A):** DPSCs express significantly higher levels of CXCL12 mRNA than HFFs (14-fold increase, *, $p < .0017$, Student's t test, $n = 3$ independent donors). Real-time polymerase chain reaction analysis indicated a significantly higher level of CXCL12 mRNA expression by HFFs transduced to express CXCL12 than HFFs transduced with a control vector (46-fold increase, x, $p < .0005$, Student's t test, $n = 3$ independent donors). **(B),** CXCL12 enzyme-linked immunosorbent protein assay demonstrated that DPSCs express significantly higher levels of CXCL12 protein than HFFs (27-fold increase, #, $p < .003$, Student's t test, $n = 3$ independent donors). HFFs transduced to express CXCL12 demonstrated a significant upregulation of CXCL12 protein secretion compared with HFFs transduced with a control vector (10-fold increase, [caret], $p < .01$, Student's t test, $n = 3$ independent donors). There was no significant difference in CXCL12 protein levels between DPSCs and HFFs transduced with CXCL12. **(C, D):** Quantitation of axon perturbation. **(C):** DPSCs ($n = 30$ of 43 embryos) and HFF-CXCL12$^+$ cells ($n = 19$ of 21 embryos) significantly (γ, $p < .0001$, two-tailed Fisher's exact test) perturb normal axonal migration compared with HFFs ($n = 0$ of 17 embryos) or HFF-control vector cells ($n = 1$ of 10 embryos), respectively, which do not influence endogenous axonal migration. **(D):** Axonal perturbation in response to DPSCs was significantly ([caret], $p < .01$, two-tailed Fisher's exact test) inhibited when embryos were cultured with the CXCL12/CXCR4 inhibitor T140 ($n = 9$ of 26 embryos) compared with embryos cultured with control peptide ($n = 20$ of 28 embryos). **(E-H):** Images of human-chick chimeric embryos 48 hpi demonstrated the host avian TG and its axonal projections stained for NF-M (red) and the human cells transduced to express GFP (green). **(E):** Implanted HFFs transduced with a control vector did not alter the host avian TG axonal projections. **(F):** Implanted HFFs transduced to express CXCL12 resulted in chemoattraction of the host avian TG ophthalmic process (arrowhead) towards HFF-CXCL12$^+$ cells. **(G, H):** Human DPSC-chick chimeric embryos were explanted and grown for 48 hpi ex ovo in media supplemented with control peptide **(G)** or with T140 **(H)**. **(G):** In media supplemented with control peptide a representative chimeric embryo exhibited the usual chemoattraction of TG axons toward implanted DPSCs (arrowheads). **(H):** Media supplemented with T140, a representative chimeric embryo, demonstrated a loss of chemoattraction by host TG axons toward implanted DPSCs. All images are z-serial confocal images compressed to a single plane. Scale bars = 200 μm. Abbreviations: DPSC, dental pulp progenitor/stem cell; E, eye; HB, hindbrain; HFF, human foreskin fibroblasts; Mx/Md, maxillary/mandibular; Op, ophthalmic; V, trigeminal ganglion bilateral to the hindbrain.

also human DPSCs express CXCL12 at a level that can alter the migration path of axonal processes toward the transplanted DPSCs. Importantly, CXCL12 has been shown to be up-regulated at sites of neuronal injury after stroke and induce the migration of endogenous neuronal stem cells to the injury site [43]. Furthermore, bone marrow stem cells transplanted after stroke have also relied on CXCL12 for their correct migration and homing into the injury site [48]. Therefore, DPSCs may not only provide a paracrine/bystander affect on the surrounding tissue but also may assist in the homing of endogenous neural stem cells to the site of transplantation/injury.

Acknowledgments

We thank Dr. Peter Diamond for his assistance with retroviral transduction of cells, Kate Pilkington for her assistance with fluorescent cell sorting (DETMOLD Imaging Core Facility, Hanson Institute, Adelaide, SA, Australia), and Dr. Gafar Sarvestani for his assistance with confocal microscope imaging (DETMOLD Imaging Core Facility, Hanson Institute). This work was supported in part by the Australian Research Council (ARC) Special Research Centre for the Molecular Genetics of Development (Grant S00001531 to S.A.K.), The University of Adelaide, Faculty of Science Postgraduate Scholarship, and the ARC Special Research Centre for the Molecular Genetics of Development Postgraduate Scholarship (to A.A), and the National Health and Medical Research Council (Project Grant 242804 to S.G.).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. Nature 2006;441:1094–1096.
2 Curtis MA, Eriksson PS, Faull RLM. Progenitor cells and adult neurogenesis in neurodegenerative diseases and injuries of the basal ganglia. Clin Exp Pharmacol Physiol 2007;34:528–532.
3 Khanna A, Shin S, Rao MS. Stem cells for the treatment of neurological disorders. CNS Neurol Disord Drug Targets 2008;7:98–109.
4 Zhao CM, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008;132:645–660.
5 Kondziolka D, Wechsler L. Stroke repair with cell transplantation: neuronal cells, neuroprogenitor cells, and stem cells. Neurosurg Focus 2008;24:E13.
6 Martino G, Pluchino S. The therapeutic potential of neural stem cells. Nat Rev Neurosci 2006;7:395–406.
7 Pluchino S, Quattrini A, Brambilla E et al. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 2003;422:688–694.
8 Pluchino S, Zanotti L, Rossi B et al. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 2005;436:266–271.
9 O'Connor R, Tessier-Lavigne M. Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. Neuron 1999;24:165–178.
10 Jayasena CS, Flood WD, Koblar SA. High EphA3 expressing ophthalmic trigeminal sensory axons are sensitive to ephrin-A5-Fc: implications for lobe specific axon guidance. Neuroscience 2005;135:97–109.
11 Gronthos S, Mankani M, Brahim J et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci UlSlA 2000;97:13625–13630.
12 Chai Y, Jiang X, Ito Y et al. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 2000; 127:1671–1679.
13 Arthur A, Rychkov G, Shi S et al. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 2008;26:1787–1795.
14 Huang AH-C, Snyder BR, Cheng P-H et al. Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. Stem Cells 2008;26:2654–2663.
15 Sasaki R, Aoki S, Yamato M et al. Neurosphere generation from dental pulp of adult rat incisor. Eur J Neurosci 2008;27:538–548.
16 Nosrat IV, Widenfalk J, Olson L et al. Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. Dev Biol 2001;238:120–132.
17 Nosrat IV, Smith CA, Mullally P et al. Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system. Eur J Neurosci 2004;19:2388–2398.
18 Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. Bone 2001;29:532–539.
19 Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. Immunol Rev 2003;195:58–71.
20 Klein RS, Rubin JB. Immune and nervous system CXCL12 and CXCR4: parallel roles in patterning and plasticity. Trends Immunol 2004;25:306–314.
21 Jiang L, Zhu YQ, Du R et al. The expression and role of stromal cell-derived factor-1α-CXCR4 axis in human dental pulp. J Endod 2008; 34:939–944.
22 Knaut H, Blader P, Strahle U et al. Assembly of trigeminal sensory ganglia by chemokine signaling. Neuron 2005;47:653–666.
23 Lieberam I, Agalliu D, Nagasawa T et al. A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. Neuron 2005;47:667–679.
24 Le Douarin NM. The avian embryo as a model to study the development of the neural crest: a long and still ongoing story. Mech Dev 2004;121:1089–1102.
25 Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Embryol Exp Morphol 1951;88:49–92.
26 Kortesidis A, Zannettino A, Isenmann S et al. Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. Blood 2005;105:3793–3801.
27 Zannettino AC, Farrugia AN, Kortesidis A et al. Elevated serum levels of stromal-derived factor-1α are associated with increased osteoclast activity and osteolytic bone disease in multiple myeloma patients. Cancer Res 2005;65:1700–1709.
28 Tamamura H, Fujisawa M, Hiramatsu K et al. Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent. FEBS Lett 2004;569:99–104.
29 Tamamura H, Xu Y, Hattori T et al. A low-molecular-weight inhibitor against the chemokine receptor CXCR4: a strong anti-HIV peptide T140. Biochem Biophys Res Commun 1998;253:877–882.
30 Koblar SA, Krull CE, Pasquale EB et al. Spinal motor axons and neural crest cells use different molecular guides for segmental migration through the rostral half-somite. J Neurobiol 2000;42:437–447.
31 Stokowski A, Shi S, Sun T et al. EphB/ephrin-B interaction mediates adult stem cell attachment, spreading, and migration: implications for dental tissue repair. Stem Cells 2007;25:156–164.
32 Shiba H, Nakamura S, Shirakawa M et al. Effects of basic fibroblast growth factor on proliferation, the expression of osteonectin (SPARC) and alkaline phosphatase, and calcification in cultures of human pulp cells. Dev Biol 1995;170:457–466.
33 Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 1996;274:1123–1133.
34 Schriek G, Oppitz M, Busch C et al. Human SK-Mel 28 melanoma cells resume neural crest cell migration after transplantation into the chick embryo. Melanoma Res 2005;15:225–234.
35 Kulesa PM, Kasemeier-Kulesa JC, Teddy JM et al. Reprogramming metastatic melanoma cells to assume a neural crest cell-like phenotype in an embryonic microenvironment. Proc Natl Acad Sci UlSlA 2006; 103:3752–3757.
36 Stevens A, Zuliani T, Olejnik C et al. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 2008;17: 1175–1184.
37 Hendrix MJ, Seftor EA, Seftor RE et al. Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer 2007;7:246–255.
38 Nosrat CA, Fried K, Ebendal T et al. NGF, BDNF, NT3, NT4 and GDNF in tooth development. Eur J Oral Sci 1998;106(suppl 1):94–99.

39 Tran PB, Miller RJ. Chemokine receptors: signposts to brain development and disease. Nat Rev Neurosci 2003;4:444–455.
40 Ma Q, Jones D, Borghesani PR et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA 1998;95:9448–9453.
41 Zou YR, Kottmann AH, Kuroda M et al. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 1998;393:595–599.
42 Chalasani SH, Sabelko KA, Sunshine MJ et al. A chemokine, SDF-1, reduces the effectiveness of multiple axonal repellents and is required for normal axon pathfinding. J Neurosci 2003;23:1360–1371.
43 Imitola J, Raddassi K, Park KI et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1α/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci UlSlA 2004; 101:18117–18122.
44 Claps CM, Corcoran KE, Cho KJ et al. Stromal derived growth factor-1α as a beacon for stem cell homing in development and injury. Curr Neurovasc Res 2005;2:319–329.
45 Robin AM, Zhang ZG, Wang L et al. Stromal cell-derived factor 1α mediates neural progenitor cell motility after focal cerebral ischemia. J Cereb Blood Flow Metab 2006;26:125–134.
46 Miller RJ, Tran PB. Chemokinetics. Neuron 2005;47:621–623.
47 Lillesaar C, Eriksson C, Johansson CS et al. Tooth pulp tissue promotes neurite outgrowth from rat trigeminal ganglia in vitro. J Neurocytol 1999;28:663–670.
48 Shyu WC, Lin SZ, Yen PS et al. Stromal cell-derived factor-1α promotes neuroprotection, angiogenesis, and mobilization/homing of bone marrow-derived cells in stroke rats. J Pharmacol Exp Ther 2008;324: 834–849.


See www.StemCells.com for supporting information available online.