Stem Cells of the Apical Papilla Regulate Trigeminal Neurite Outgrowth and Targeting through a BDNF-Dependent Mechanism

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Regenerative endodontic procedures have become a valuable alternative for the treatment of immature teeth with pulp necrosis. In addition to resolution of periradicular pathosis and promotion of continued root development, positive vitality testing has been observed in some regenerative clinical cases. Importantly, the positive response to electric stimulation of the regenerated tissue requires targeting of periradicular axons into the previously empty root canal space. However, the mechanism by which this process occurs is largely unknown. Since stem cells of the apical papilla (SCAP) have been proposed to populate the root canal following regenerative endodontic procedures, we hypothesized that SCAP regulate neurite outgrowth and axonal targeting. To test this hypothesis, we established primary co-cultures of human SCAP and rat trigeminal neurons, and performed neurite outgrowth assays using ELISA and confocal microscopy to determine the effect of increasing concentration of SCAP on the total neurite outgrowth and axonal targeting. In addition, we evaluated whether SCAP evoked axonal targeting *in vivo* using a matrigel subcutaneous implant assay. Data were analyzed by ANOVA with Bonferroni's *post hoc* test, and significance was set at $p < 0.05$. The results demonstrated that SCAP release a soluble factor that regulates neurite outgrowth from cultured trigeminal neurons. Next, we demonstrated that this effect is completely abolished by pretreatment with a neutralizing antibody to brain-derived neurotrophic factor (BDNF), but not by antibodies to other neurotrophins. Further, SCAP release BDNF in a concentration-dependent manner as detected by ELISA, and trigger directed axonal targeting both *in vitro* and *in vivo* as demonstrated by microfluidic and matrigel implant experiments, respectively. Collectively, these results suggest that SCAP may be responsible for the chemical signal driving axons to target regenerated tissue via a BDNF-dependent mechanism.

Introduction

NOICEPTORS ARE A MAJOR class of specialized neurons of the peripheral somatosensory system that play the essential role of tissue surveillance and signaling of possible, or actual tissue damage.¹ The nociceptive innervation of oral peripheral tissues depends largely on the targeting of axons from sensory neurons located in the trigeminal ganglia into newly developed tissues during organogenesis.^{2,3} In addition, postnatal changes in innervation may occur, resulting in peripheral neuronal plasticity in response to tissue injury and inflammation.⁴⁻⁶ The human dentition has several unique innervation features: (1) innervation of the developing dental organ occurs at a late postnatal stage in organogenesis for both deciduous and permanent dentitions⁷; (2) the dental pulp is innervated primarily by noci-

ceptors^{8,9}; and (3) dental pulp is one of the most densely innervated tissues of the human body.^{10,11} This unique innervation has been shown to be directed through a concert of chemotactic signals released from the developing dental tissues.^{12,13} Neurotrophins, among other growth factors, are recognized as the main mediators of guided axonal growth and organization of the free nerve terminals in the dental pulp and surrounding tissues.^{14,15}

Several elegant studies have directly implicated nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) in the process of pulpal innervation.^{12,16-20} Other neurotrophins such as neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) are only expressed at early stages in tooth development before the ingress of trigeminal sensory axons into the apical papilla and dental mesenchyme, and, thus, appear

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to be more important in odontogenesis²¹ than in innervation of the developing dental organ.^{22,23} Among these neurotrophins, NGF has been identified as a key molecular signal for axonal targeting during dental development,²⁴ and later in response to injury such as caries and trauma.^{24–26} Importantly, both the putative NGF high-affinity receptor tyrosine kinase A (TrkA) and its low-affinity receptor p75 are expressed by a major subpopulation of dental nociceptors.^{25,26} Interestingly, although BDNF and its high-affinity receptor, TrkB, have been detected in the developing tooth,²⁷ mice carrying the genetic deletion of TrkB do not display deficiency in dental pulp innervation.²⁸ Conversely, BDNF mediates neuronal plasticity in the developed dental pulp with a significant percentage of trigeminal neurons expressing TrkB after injury.²⁹ Further, GDNF has been demonstrated to support dental pulp innervation by large-diameter sensory neurons that are not thermosensitive.³⁰ Thus, neurotrophins mediate a rich innervation of distinct classes of nociceptors within the developing dental pulp.

Despite being particularly equipped to detect potential tissue damage with an enriched nociceptive innervation, the dental pulp may succumb to infection or direct trauma, resulting in liquefaction necrosis. These untoward events are particularly detrimental for immature teeth with incomplete root development. Although traditional endodontic therapy in these immature teeth successfully controls infection and inflammation,³¹ it does not promote continued root development. The teeth remain with fragile underdeveloped roots, which are susceptible to fractures, and have a poor long-term survival prognosis.^{32,33} Regenerative endodontic procedures are a new treatment modality in the dental specialty of endodontics that use principles of bioengineering. Thus, it integrates knowledge on stem cell biology, scaffold, and growth factors.³⁴ These procedures have emerged as a viable alternative for treatment of immature teeth with pulp necrosis that would have otherwise a questionable prognosis. It has been recently demonstrated that these procedures deliver a substantial concentration of undifferentiated mesenchymal stem cells (MSCs) into previously disinfected root canal spaces.³⁵ The dental tissues are recognized as important sources of adult MSCs. These cells may be found in different compartments of dental structures such as the dental pulp stem cells (DPSCs),³⁶ periodontal ligament stem cells,³⁷ inflamed periapical progenitor cells,³⁸ and stem cells of the apical papilla (SCAP).³⁹ The latter (SCAP) likely represent the main MSC population involved in the regenerative endodontic procedures due to their anatomical location and relative abundance in immature teeth.⁴⁰

Most published regenerative endodontic clinical cases demonstrate continued root development, with approximately half of these cases showing normal physiological responses to cold or electric pulp testing, such as those observed in healthy intact teeth.³⁴ These responses require targeting of the periapical neuronal terminals into coronal-most areas of the repaired tissue nearly 1–1.5 cm away from the closest nerve trunks. Although significant advances have been made in understanding the crucial role of neurotrophins in dental development, the mechanism by which trigeminal apical neurons target the regenerated dental pulp-like tissue is largely unknown. In this study, we evaluated whether SCAP are capable of releasing soluble factors (neurotrophins) that direct axonal growth and targeting.

Methods

Stem cells of the apical papilla

The Institutional Review Board of the University of Texas Health Science Center at San Antonio approved the use of a previously characterized SCAP cell line called RP-89 in this study.⁴¹ All experiments were conducted with RP-89 from fifth to eighth passages. Cells were cultured in media composed of alpha-minimum essential medium (a-MEM; Gibco) supplemented with 10% fetal bovine (BenchMark™ Gemini), 1X L-glutamine (Gemini), penicillin (100 U/mL; Gemini), and streptomycin (100 mg/mL; Gemini) in 10 cm cell culture dishes at 37°C and with 5%CO₂. Cells were detached from culture dishes when they reached 80% confluency with 0.05% trypsin/EDTA (Gibco) for 3 min at 37°C, and passed at a lower density to new culture dishes or were used in experiments.

On the day of the experiments, cells from fifth to eighth passages were trypsinized, and used for neurite outgrowth quantification, axonal targeting microfluidic experiments, and *in vivo* matrigel implant experiments as described later.

Trigeminal neuronal primary culture

Adult male Sprague–Dawley rats (weight, 200–250 g each) (Charles River) were used in this study. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to the International Association for the Study of Pain (IASP) and Federal guidelines. Animals were housed for 1 week before the experiments with food and water available *ad lib*.

The trigeminal ganglia dissected from male rats were treated with collagenase-dispase (1 mg/mL; Roche), and neuronal cultures were prepared as previously described⁴² and used for the subsequent experiments as described next.

Microfluidic experiments (axonal targeting)

A microfluidic device was used to test the ability of trigeminal neuronal axons to follow a concentration gradient of chemotactic factors (AXIS™ Axon Isolation Device).⁴³ In this device, SCAP and TG neurons are separated by 120 microgrooves of 150 µm in length, 3 µm in width, and 5 µm in height. These microgrooves enable fluid exchange and neurite invasion into the adjacent channel (containing SCAP) while preventing neuronal cell bodies from entering. Each of the channels of individual microfluidic devices received no cells (control) or SCAP at a concentration of 10⁵ SCAP/mL (1×) or equivalent to 3×10⁵ cells/mL (3×). Primary trigeminal neurons were seeded at a concentration of 10⁴ cells/mL in the opposite channel of all microfluidic devices. The microfluidic devices containing cells were incubated at 37°C and 5% CO₂ for 3 days. Media was removed and cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer (PB) and incubated in 4% normal goat serum (Sigma Aldrich), 2% bovine gamma-globulin (Sigma Aldrich), and 0.3% Triton X-100 (Fisher Scientific) in PBS as a blocking solution for 60 min. After fixation, the cells were incubated with a primary antibody against neurofilament 200 kDa (N-52; cat # N0142 at a 1:2000 dilution in blocking solution—Sigma Aldrich). Cells were rinsed in PBS and then incubated in secondary antimouse immunoglobulin

G conjugated to Alexa-488 fluorophore (Molecular Probes). All cells were also incubated with the nuclear stain DAPI in Vectashield (Vector Labs). Immunoreactivity was visualized with a Nikon C1 si laser confocal imaging system equipped with 90i Nikon microscope (Nikon).

Neurite outgrowth experiments

A neurite outgrowth assay kit (Chemicon/Millipore - NS220) was used for these experiments. Briefly, this assay consists of a dual chamber culture system that enables co-culture of trigeminal neurons and SCAP while preventing direct contact of the two cell types. Approximately 5×10^3 neurons were placed in each culture insert and co-cultured with SCAP at concentrations of 10^3 , 10^4 , 5×10^4 , 10^5 , or 3×10^5 cells/mL. In addition, trigeminal neurons were cultured in the absence of SCAP (control), or in the presence of neurotrophin cocktail composed of NGF (50 ng/mL; Harlan), human recombinant GDNF (10 ng/mL; Promega), and recombinant human BDNF (1 ng/mL; Promega) as a positive control for neurite outgrowth.^{44,45} Further, nocodazole (500 ng/mL), a potent inhibitor of microtubules,⁴⁶ was added to the media of TG neurons and co-cultured in the presence of either 1×10^5 or 3×10^5 SCAP/mL, or neurotrophin cocktail as a negative control for neurite outgrowth. To test whether the effect on neurite outgrowth was specific for SCAP, independent experiments were carried out using this neurite outgrowth assay but replacing SCAP with either Chinese hamster ovary (CHO) cell or human embryonic kidney (HEK) cells at a density of 10^5 cells/mL. For all experiments, the trigeminal neuronal/SCAP co-culture was maintained for 3 days at 37°C and 5%CO₂ in the same media used for SCAP culture, but with the use of heat-inactivated fetal bovine serum to deplete potential growth factors present in the serum (Gemini).

For the antibody neutralization experiments, TG neurons were co-cultured with 10^5 SCAP/mL as described earlier but in the presence of either monoclonal antibody against human NGF (40 µg/mL; Abcam), BDNF (20 µg/mL; Promega), or GDNF (20 µg/mL; R&D Systems), or in the presence of a mixture of all three antibodies used at the concentrations stated earlier. The co-cultures were maintained under the same conditions as previous experiments for 3 days, followed by the quantification of neurite outgrowth.

Real-time RT-PCR

Following the neurite outgrowth experiments, total RNA was extracted from SCAP (density of 1×10^5) co-cultured with TG neurons or control (single culture in absence of TG neurons) using the RNeasy Mini Kit, and any potential genomic DNA contamination was removed by DNase treatment (DNA-free kit; Ambion). Total RNA (1 µg) was used in complementary DNA (cDNA) synthesis reactions using Superscript reverse transcriptase II kit (Invitrogen). The cDNA templates were then used in a real-time PCR reaction by using TaqMan Assays on Demand (Applied Biosystems) consisting of previously validated primers and probes specific for human NGF (NGF assay #Hs01113193_m1), human BDNF (BDNF assay #Hs03805848_m1), human GDNF (glial-derived neurotrophic factor assay #Hs01931883_s1), and the endogenous control (housekeeping gene) 18S (ribosomal 18S subunit gene; assay #Hs03003631_g1). The reactions

were run in triplicates of 25 µL, and cycle threshold (Ct) values obtained after the reactions were stopped at 45 amplification cycles. The Ct values for each target gene were normalized to the endogenous control (18S) values. The comparative delta-delta Ct method was used to normalize the data on the basis of the endogenous reference and to express it as the relative fold change in relation to the values obtained for the single SCAP cultures (control samples).

Measurement of immunoreactive NGF, BDNF, and GDNF

Media aliquots were collected from the co-culture experiments and concentrations of neurotrophins determined by an ELISA assay against human NGF (Abcam; cat #ab99986), human BDNF (Abcam; cat #ab99978), and human GDNF (Abcam; cat #ab100525) following the manufacturer's instructions. Data are presented as pg/mL of media normalized to levels measured in SCAP conditioned media in the absence of TG neurons (control).

Quantification of SCAP-mediated axonal targeting and innervation in vivo

Severe combined immunodeficient (SCID) mice (6–8 weeks old; strain: NOD.CB17-*Prkdc*^{scid}/J) (Jackson Laboratories) were used in this study. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to the International Association for the Study of Pain and federal guidelines. Animals were housed for 1 week before the experiments with food and water available *ad libitum*.

An *in vivo* matrigel (basement membrane hydrogel) implant model of innervation and axonal growth was used as previously described.⁴⁷ Briefly, after isoflurane (3%) inhalation anesthesia, SCID mice received 450 µL subcutaneous injections of ice-cold growth factor reduced (GFR) BD Matrigel™ (BD biosciences) containing a 1:10 volume of SCAP, CHO cells, or vehicle (cold MEM). For each injection, a total of 1×10^6 SCAP (5th passage), or CHO cells (10th passage) in 50 µL of ice-cold MEM was mixed with 400 µL of ice-cold GFR Matrigel. Injections were administered using a prechilled 1 cc syringe fitted with a 23G needle (Becton, Dickinson and Company). The matrigel containing suspended cells or vehicle (negative control) was slowly injected subcutaneously in the mid-dorsal area of each animal, allowing the matrigel to set at body temperature, forming a subcutaneous implant of irregular shape. Animals were euthanized with an overdose of isoflurane at 14 days post injections, and the matrigel implants were harvested after dissection from the surrounding tissue.

The matrigel implants were immediately fixed in 4% paraformaldehyde in 0.1 M PB for 30 min, rinsed in 0.1 M PB, and placed in 0.1 M PB with 30% sucrose at room temperature overnight. Next, the implants were embedded in Neg-50 (Richard-Allan Scientific) and serially sectioned with a cryostat (30 µm sections) in the transverse plane. Sections were placed onto Superfrost Plus slides (Fisher Scientific), air dried, and stored at -20°C until use.

Immunofluorescence staining was performed with primary antibody against neurofilament heavy (NFH; cat # ab4680 at a 1:1000 dilution in blocking solution-Abcam),

followed by visualization of immunoreactivity with an Alexa-633-conjugated secondary antibody and DAPI as a nuclear stain. Four evenly spaced sections (120 μm between each section) of each matrigel specimen were stained and examined using the confocal microscope. Images were collected using a Nikon Eclipse 90i microscope (Nikon) that was equipped with a Nikon C1si laser scanning confocal imaging system using standardized settings at 2 $\times$ and 20 $\times$ magnifications. Control preparations consisted of sections stained with secondary antibody but that lacked primary antibody. Acquired images at 2 $\times$ were opened in National Institutes of Health ImageJ on a GraphPad (GraphPad Software), enabling visualization of the whole matrigel implant. The NFH-positive nerve fibers were thresholded with ImageJ to enable visualization of specific staining, and the percent area of nerve fibers within the region of interest (matrigel implant) was determined. For each specimen, the total area occupied by nerve fibers was determined by the mean measurement of the four sections per specimen. Data were normalized to the percentage of negative control innervation (matrigel only group).

Data analysis

All experiments were conducted in triplicate, with $n=3$ /group for the microfluidic experiments, $n=9$ –12 wells *per* group for the neurite outgrowth experiments and ELISA measurements, $n=9$ samples/group for the RT-PCR experiments, and $n=4$ samples/group for the *in vivo* experiments. Data were analyzed with Prism software version 5 (GraphPad Software). For all experiments, results were analyzed by one-way ANOVA with Bonferroni's *post hoc* test, except the RT-PCR experiments analyzed by Student's *t*-test. The statistical significance was set at $p < 0.05$.

Results

Microfluidic experiments

First, to evaluate whether SCAP could mediate axonal targeting, a microfluidic culture system was employed. In

this proof-of-concept experiment, axonal projections from TG neurons seldom crossed the 150 μm in length channel to invade the adjacent chamber in the absence of SCAP. However, there was an increase in neurites targeting channels that contained SCAP. Importantly, this effect was greater when concentrations of SCAP were increased with several neurites crossing the channels of the microfluidic device, and often directly terminating adjacent to individual stem cells (Fig. 1).

Neurite outgrowth quantification

Next, we employed a quantitative neurite outgrowth assay to evaluate the trophic effect of different SCAP densities on TG neurons. There was an SCAP-mediated concentration-dependent increase in TG neuron neurite outgrowth with a peak increase (150.7%) evoked by 1×10^5 SCAP/mL ($p < 0.001$). The SCAP concentration of 3×10^5 /mL evoked a neurite outgrowth response statistically similar to the concentration of 1×10^5 /mL (Fig. 2A). Importantly, the increase in neurite outgrowth evoked by SCAP was greater than that of the neurotrophin cocktail (NC, Fig. 2B), a positive control for this experiment. The increase in neurite outgrowth evoked by both SCAP and the neurotrophin cocktail was completely blocked by treatment with the potent neurotubulin inhibitor nocodazole (500 ng/mL) (Fig. 2B). Further, the neurite outgrowth increase was specific for the co-culture with SCAP, as experiments performed with co-culture of TG neurons and either CHO cells or HEK cells at a cell concentration of 1×10^5 /mL failed to demonstrate any detectable neurite outgrowth increase (Fig. 2C).

Neurotrophin gene expression quantification

Cultures of SCAP alone had detectable levels of transcripts encoding NGF, BDNF, and GDNF (Fig. 3). Interestingly, the culturing of SCAP in the presence of TG neurons evoked a significant upregulation of BDNF (3.4-fold increase) and GDNF (2.6-fold increase) expression and a downregulation in NGF expression ($\sim 45\%$). Since the SCAP and neurons were not in physical contact, this finding

FIG. 1. Trigeminal ganglia (TG) neurons extend axonal projections toward stem cells of the apical papilla (SCAP) in culture. A microfluidic neurite isolation device was used to evaluate the targeting of trigeminal neurons toward a SCAP culture. Axonal projections into the opposing chamber of the isolation device were seldom seen in TG neurons cultured in the absence of SCAP (A). However, there were evident axonal projections toward the opposing chamber containing SCAP (10^5 and 3×10^5 cell/mL); (B and C, respectively). TG neurons are visualized by immunocytochemistry with staining against the neuronal marker N52 (green), whereas cellular nuclei were visualized by the nuclear stain, TOPRO (blue). Images were acquired by confocal microscopy with a 10 $\times$ objective as representative images of replicate experiments.

FIG. 2. SCAP mediate neurite outgrowth of trigeminal neurons in a concentration-dependent manner. A well-characterized assay was used to quantify the neurite outgrowth of TG neurons cultured in the absence (control) or presence of increasing concentrations of SCAP (**A**). There was a significant increase in the neurite outgrowth when TG neurons were co-cultured with SCAP at a concentration of $\geq 5 \times 10^4$ cells/mL, with a peak effect seen at 10^5 cells/mL (**A**). The neurite outgrowth evoked by SCAP (10^5 cells/mL) was greater than that evoked by an exogenous neurotrophin cocktail (NC) added to the culture (**B**). Further, the neurite outgrowth increase evoked by both SCAP and NC was completely blocked by treatment with the neurotubulin inhibitor nocodazole (Noco) (**B**). Importantly, the effect on neurite outgrowth was specific for SCAP, as Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells failed to promote neurite outgrowth (**C**). Data were analyzed with one-way ANOVA with Bonferroni *post hoc* test (* $p < 0.05$, *** $p < 0.001$, and n.s., no statistical difference compared with control).

demonstrates the existence of a soluble neuronal-derived factor capable of selectively altering neurotrophin expression in these MSCs.

Quantification of secreted neurotrophins

The measurement of BDNF, GDNF, and NGF levels in the media collected from the neurite outgrowth experiments revealed that there was a significant increase of BDNF levels with increasing SCAP densities (Fig. 4A). The peak BDNF level (217.9 ± 31.1 pg/mL) was detected in media collected from the 3×10^5 SCAP/mL group (Fig. 4A). Similarly, levels

of GDNF also increased with increasing densities of SCAP with the peak level (21.2 ± 5.2 pg/mL) detected in the 3×10^5 SCAP/mL group (Fig. 4B). Thus, peak BDNF levels were 10 times greater than GDNF levels in aliquots from the same SCAP-conditioned media (Fig. 4A, B). Conversely, there was no significant release of NGF from SCAP when co-cultured with TG neurons as seen by a constant basal measurement of approximately 8 pg/mL independent of the presence of SCAP (Fig. 4C). In addition, no significant difference from controls was detected in neurotrophin measurement in media collected from the CHO cells/TG neurons and HEK/TG neurons co-cultures (data not shown).

FIG. 3. Neurotrophin gene expression in SCAP is regulated by co-culture with TG neurons. Total RNA was isolated from SCAP cultured in the absence or presence of TG neurons and used in qRT-PCR experiments with primers specific for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF). There was a significant reduction ($\sim 45\%$) in NGF mRNA transcripts in SCAP when co-cultured with TG neurons. Conversely, there was a significant increase (upregulation) of transcripts for BDNF ($\sim 340\%$) and GDNF ($\sim 260\%$) in SCAP when cultured in the presence of TG neurons. Data were analyzed with Student's *t*-test for each evaluated target experiment compared with SCAP-only culture levels (** $p < 0.01$, *** $p < 0.001$).

Neurotrophin neutralization experiments

Experiments were conducted to determine which neurotrophin(s) mediates the SCAP-induced neurite outgrowth (Fig. 5). The addition of a neutralizing antibody against NGF or GDNF to the co-culture media did not significantly reduce SCAP-mediated neurite outgrowth. Conversely, treatment with a BDNF-neutralizing antibody completely blocked SCAP-mediated neurite outgrowth. Importantly, the combination of a mixture of antibodies against NGF, GDNF, and BDNF did not result in a greater reduction of neurite outgrowth than that observed with the anti-BDNF antibody alone (Fig. 5).

Quantification of *in vivo* axonal targeting

To evaluate whether SCAP would promote axonal growth and targeting *in vivo*, a subcutaneous matrigel implant model was used. The quantification of specific neuronal immunoreactivity was performed on confocal micrographs of the serially sectioned harvested implants (Fig. 6). Control matrigel-only implants had a macroscopic translucent appearance (Fig. 6A), and they revealed minimal innervation density with NFH-positive neuronal axons present in nerve bundles around the periphery of the implant (Fig. 6B, C). Similarly, matrigel implants containing CHO cells also had a translucent appearance, and they demonstrated minimal innervation as evidenced by NFH-positive neurons also present in bundles at the periphery of the matrigel implants. On the other hand, SCAP-containing matrigel implants had

FIG. 4. SCAP release BDNF and GDNF when cultured with TG neurons. The measurement of BDNF, GDNF, and NGF levels in the media collected from the neurite outgrowth experiments revealed that there was a significant increase of BDNF and GDNF levels with increasing SCAP densities (A and B, respectively). Conversely, there was no effect seen in NGF levels when SCAP was co-cultured with TG neurons (C). Data were analyzed with one-way ANOVA with Bonferroni *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s., no statistical difference compared with control).

FIG. 5. Neutralizing antibody against BDNF completely blocks SCAP-mediated increase in neurite outgrowth. Rat primary trigeminal neurons were cultured in the absence (control) or presence of 10^5 SCAP/mL. Further, the co-cultures were maintained in the presence of neutralizing antibodies against NGF (α -NGF), GDNF (α -GDNF), and BDNF (α -BDNF) or in the presence of a cocktail of these antibodies (Ab mix) for 3 days. A neurite outgrowth assay was used to quantify changes in axonal growth. Similar to previous experiments, co-cultures with SCAP resulted in neurite outgrowth increase. This effect was not significantly altered by treatment with α -NGF or α -GDNF neutralizing antibodies (a, no statistical difference from SCAP co-culture). However, treatment with α -BDNF completely blocked the increase in neurite outgrowth evoked by SCAP. Importantly, a mixture of the neutralizing antibodies (Ab mix) did not result in a further reduction in neurite outgrowth (b, no statistical difference between α -BDNF and Ab mix). Data were analyzed with one-way ANOVA with Bonferroni *post hoc* test (* $p < 0.05$, ** $p < 0.01$, and n.s., no statistical difference compared with control).

an opaque macroscopic appearance (Fig. 6G) and demonstrated substantial innervation density, including extensive axonal branching distributed throughout the matrigel implants (Fig. 6H). In addition, individual neuronal fibers were found in intimate proximity to SCAP organized three dimensionally in the matrigel scaffold (Fig. 6I).

Subcutaneous implantation of SCAP resulted in a statistically significant increase ($322.2\% \pm 16.5\%$) in innervation density compared with that observed in a matrigel-only implant ($p < 0.01$) (Fig. 7). Conversely, subcutaneous implantation of CHO cells did not result in significant innervation density difference when compared with matrigel-only implants ($133\% \pm 41\%$) (Fig. 7).

Discussion

MSCs have the ability of secreting numerous factors depending on the biological demand of the environment.⁴⁸ These factors include cytokines,⁴⁹ chemokines, growth factors,⁵⁰ and neurotrophins.^{51,52} This ability of MSCs to secrete needed factors on demand has been recently investigated as potential therapeutic targets that may mediate and modulate local tissue repair.^{53–56} In the present study, we demonstrated that SCAP promotes and directs axonal outgrowth of trigeminal sensory neurons in culture and *in vivo*. In addition, we demonstrated that there is a significant up-

regulation in the expression and release of BDNF and GDNF in SCAP in the presence of trigeminal neurons in a co-culture system. Collectively, these studies implicate a reciprocal secretory regulatory system between MSCs and sensory neurons with SCAP-derived BDNF serving as the chemical mediator of the neurite outgrowth.

Human dental development occurs late after embryogenesis extending into adult life. Thus, teeth undergo post-natal organogenesis. The dental pulp is uniquely innervated with a high density of trigeminal nociceptive primary afferents. Somatosensory innervation typically occurs during *in utero* embryonic development.^{57,58} However, the dental pulp becomes innervated much later in life, generally after initial tooth eruption.² Another unique feature of the dental pulp innervation is the high density of exclusively nociceptive primary afferents.^{59–61} Further, the innervation of the dental pulp is gained and lost as deciduous teeth exfoliate and permanent teeth erupt. Thus, there is a very dynamic pattern of axonal targeting through the shift from primary (deciduous) to permanent dentition, and during the maturation of each of these sets of teeth.⁶² The process of dental innervation during developmental maturation of teeth has been shown to be elegantly modulated by a cascade of molecular signals.¹⁸ Primary afferent branches accumulate around the apical papilla, but do not enter the dental pulp proper until late in the cap stage of tooth development to finalize the branching of the primary afferents on the periphery of the pulp-dentin complex. These molecular signals are primarily mediated by a concert of neurotrophins released with specific spatio-temporal resolution resulting in the unique highly specialized innervation of the dental pulp.^{13,63} Among these neurotrophins, NGF and BDNF have been implicated in the development of pulpal innervation.^{12,16–20}

Neurotrophins also participate in the robust neuronal plasticity seen in the dental pulp after dental injury due to infection (caries) or trauma.^{25,27} In particular, NGF has been shown to mediate nociceptive axonal targeting of the injured site, as well as upregulation and sensitization of pain receptors such as the ionotropic channel transient receptor potential vanilloid type-1 (TRPV1) and transient receptor potential ankyrin type-1 (TRPA1).^{64–66} Interestingly, similar detectable levels of NGF, GDNF, and BDNF were measured from media from SCAP cultured in the absence of TG neurons. These levels could not be attributed to exogenous neurotrophic factors, as they were undetected in the media used for the experiments (no cells control; data not shown). These results suggest a constitutive basal release of these neurotrophic factors by SCAP in culture. There was, however, a dramatic upregulation of BDNF and GDNF mRNA gene expression in SCAP when cultured in the presence of, but not in physical contact with, TG neurons. Interestingly, NGF gene expression was downregulated in SCAP when co-cultured. The selective regulation of gene expression was consistent with the significant increase in released neurotrophins in the media. It appears that SCAP alone are regulated by nearby TG sensory neurons, by a mechanism that does not require direct cellular contact; this is most likely due to neuronal secretion of soluble factors. Primary neuronal cultures are generated after axotomy of sensory neurons and enzymatic digestion. This process results in primary neuronal cultures expressing an injured phenotype.⁶⁷ Thus, SCAP act in a paracrine mechanism releasing increased levels of BDNF

FIG. 6. SCAP increases peripheral innervation of matrigel implants *in vivo*. Matrigel subcutaneous implants were harvested from SCID mice at 14 days post injection, and examined with confocal microscopy to visualize neurofilament heavy positive axons (seen in blue) and cellular nuclei (see in green). Matrigel implants are outlined in matrigel-only (control) implants, have a translucent appearance (**A**), and only minimal NFH-positive innervation is observed at the periphery of the implants (outlined in white in **B**, 2 $\times$ magnification), consisting mainly of larger nerve bundles (**C**), 20 $\times$ magnification of a yellow-square demarcated area in (**B**). Similarly, matrigel implants containing CHO cells appeared translucent (**D**), and had innervation mainly limited to the periphery of the implant (outlined in white in panel **E**), 2 $\times$ magnification), consisting of larger nerve bundles (**F**), 20 $\times$ magnification of a yellow-square demarcated area in (**E**). Conversely, matrigel implants containing SCAP had an opaque appearance (**G**) with innervation widespread throughout the implant (outlined in white in panel **H**), 2 $\times$ magnification) with both large nerve bundles and individual branched nerve fibers (**I**), 20 $\times$ magnification of a yellow-square demarcated area in (**H**). White arrows point to representative NFH+ neurons in the micrographs.

and GDNF, and basal levels of NGF to support neuronal survival, and axonal outgrowth and targeting. Importantly, SCAP maintained in co-culture with trigeminal neurons displayed a normal fibroblast-like morphology and proliferative potential. In addition, these cells could differentiate into mineralizing cells if exposed to osteogenic differentiation media (data not shown). Thus, the soluble signal that resulted in the release of supportive neurotrophins by the SCAP does not appear to evoke terminal differentiation of these cells in 3 days of co-culture. Nonetheless, the nature of the soluble signal released from cultured trigeminal neurons that mediates the modulatory effect on neurotrophin expression in SCAP is largely unknown.

The concentration of BDNF detected in SCAP/TG neuronal co-culture was approximately 10 times greater than the peak concentration of GDNF. Importantly, the concentrations of both neurotrophins in the conditioned media increased with greater SCAP densities. However, a plateau in the neurite outgrowth was detected with a SCAP density lower than the maximum concentration tested. Thus, the greatest concen-

tration of neurotrophic factors in the media did not correlate with the greatest level of neurite outgrowth. This is possibly due to reaching the limiting rate neurotubulin synthesis, neurite assembly and growth in the 3 days of experiment, or to saturation or desensitization of neurotrophin receptors at higher concentrations. Alternatively, the results seen could represent the detection limit of the neurite outgrowth assay utilized, or evidence of a tight negative feedback. An example of this feedback is the binding to low-affinity receptors with biologically opposing responses, as seen between the NGF high-affinity receptors (TrkA) and low-affinity receptor (p75).^{68,69} Nonetheless, the neurite outgrowth detected was significantly greater than that evoked by a cocktail of exogenous neurotrophins often used to provide maximum support to primary sensory neurons in culture. These results suggest that SCAP are able to release supportive neurotrophic factors of greater potency than exogenously applied factors.

We found that the robust increase in neurite outgrowth evoked by SCAP was completely blocked with treatment with a BDNF neutralizing antibody. Importantly, a combined

FIG. 7. SCAP promotes significant increase in peripheral innervation *in vivo*. SCID mice received injections (s.c) with matrigel by itself (control), or containing CHO cells or SCAP. The area occupied by neuronal axons (visualized by NFH) was quantified in subcutaneous control matrigel implants, and in implants containing CHO cells or SCAP. Subcutaneous implantation of CHO cells in a matrigel scaffold did not result in increased innervation. Conversely, SCAP evoked a significant increase in peripheral innervation of the subcutaneous matrigel implant. Data are presented as mean $\pm$ SEM innervation/control, and analyzed with one-way ANOVA with Bonferroni *post hoc* test (** $p < 0.01$, and n.s, no statistical difference compared with control).

mixture of antibodies against NGF, GDNF, and BDNF did not provide any additional blockade in neurite outgrowth. These results suggest that BDNF alone, without any clear synergism with either GDNF or NGF, is responsible for the neurite outgrowth mediated by SCAP. These results are consistent with the upregulation of BDNF seen in DPSCs used in an animal model, resulting in complete regeneration of the dental pulp innervation in a model of regenerative endodontics.⁷⁰ In addition, bone marrow stem cells have been shown to secrete BDNF, leading to increased neuronal survival of cortical neurons exposed to nitric oxide,⁵² and to promote functional recovery by promoting axonal sprouting and targeting in a spinal injury model.⁷¹ Thus, BDNF appears to be preferentially released by MSCs when in the presence of injured neurons, leading to increased survival and axonal targeting.

There has been a dramatic increase in both clinical and basic science reports in the field of regenerative endodontics since its first case report in 2001.^{34,72} The mechanisms that contribute to observed desirable outcomes such as continued root development and physiological nociception are largely unknown. Positive responses to clinical vitality tests are observed in approximately half of the performed cases.³⁴ These tests require a positive response to a noxious cold test using a refrigerant such as 1,1,1,2-tetrafluoroethane or neuronal activation of voltage-gated sodium channels when an electric pulp tester is used. Since these tests are applied to the crown of the tooth, a positive response requires that new primary afferents recruited from the periapical area extend projections to the coronal most area of the engineered pulp tissue located several millimeters away. Indeed, in the microfluidic experiment, several trigeminal neurons extended axons through the 150 μ m channel and much further away

into the opposing chamber where SCAP were located after only 3 days in culture, demonstrating the efficiency of directed axonal targeting. In addition, SCAP evoked a three-fold increase in innervation of a matrigel implant in SCID mice at only 14 days after implantation. Importantly, this robust response was specific to SCAP, as implantation of CHO cells resulted in innervation of a similar density seen in matrigel-only implants (negative control). Furthermore, in clinical cases of regenerative endodontics, the responses observed are similar to those normal physiological responses observed in healthy teeth. Thus, neurons of the correct subtype are recruited, and they extend their primary afferent axons into the regenerating dental pulp tissue.

We have demonstrated that regenerative endodontic procedures deliver a substantial concentration of undifferentiated MSCs into root canal spaces in young patients.³⁵ The prominent apical papilla in these patients likely represents the main source of MSCs in these procedures.⁴⁰ In the present study, we report that stem cells derived from the apical papilla (SCAP) mediate both *in vitro* and *in vivo* neurite outgrowth and axonal targeting. Although additional research is required to further elucidate the mechanisms of reinnervation of regenerated dental pulp-like tissue in patients, the results from this study suggest that SCAP may be the main source of trophic factors such as BDNF which mediate the re-innervation of the regenerated pulp. Finally, the findings of this study transcend the field of endodontics and may prove useful in the development of MSCs therapeutics for neuronal repair after ischemia, spinal cord injury, or even in neurodegenerative diseases such as Alzheimer's and Parkinson's disease.^{73–75}

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Disclosure Statement

No competing financial interests exist.

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