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# Schwann cells promote neurite outgrowth of dorsal root ganglion neurons through secretion of nerve growth factor

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The transplantation of Schwann cells (SCs) could successfully promote axonal regeneration. This is likely to attribute to the adhesion molecules expression and growth factors secretion of SCs. But which factor(s) play a key role has not been precisely studied. In this study, an outgrowth assay using dorsal root ganglia (DRG) neuron-SC co-culture system *in vitro* was performed. Co-culture of SCs or application of SC-conditioned medium (CM) substantially and significantly increased DRG neurite outgrowth. Further, nerve growth factor and NGF receptor (TrkA) mRNA were highly expressed in Schwann cells and DRG neuron, respectively. The high concentration of NGF protein was detected in SC-CM. When K-252a, a specific inhibitor of NGF receptor was added, DRG neurite outgrowth was significantly decreased in a concentration-dependent manner. These data strongly suggest that SCs play important roles in neurite outgrowth of DRG neurons by secreted NGF.

**Keywords:** Dorsal root ganglia, Nerve growth factor, Neurite outgrowth, Schwann cells

Mounting evidence suggests that Schwann cells (SCs) play a central role in the repair of both central nervous system (CNS) and peripheral nervous system (PNS) injuries[1]. Transplanted SCs successfully bridged injured spinal cord and promoted axonal regeneration across the lesion gap following SCI[2,3]. Both, cell adhesion molecules expression and growth factors secretion by SCs are likely to contribute to their capacity to enhance axonal outgrowth[4].

SCs could express and secret abundant growth factors such as glial cell line-derived neurotrophic factor (GDNF)[5,6], brain-derived neurotrophic factor (BDNF)[7,8], neurotrophin-3 (NT-3)[9], nerve growth factor (NGF)[8,10] and basic fibroblast growth factor (bFGF)[11]. SCs also synthesize and secrete many extracellular matrix (ECM) molecules[12] and express a variety of cell adhesion molecules[1,13]. All these components are implicated in supporting neuronal survival and axonal growth[1,4]. Although the secretion of these growth-promoting factors from SCs may facilitate the outgrowth of axons and also could aid in the regeneration of axon regeneration after spinal cord injury, however which factor(s) play a key role has not been precisely studied.

In the present study, the effects of Schwann cells and its conditioned media on the neurite outgrowth of dorsal root ganglia (DRG) neurons have been investigated and provide evidence that the secreted factor of SCs, NGF, contributes to SC-promoted DRG neurite outgrowth.

## Materials and Methods

*Purification and culture of schwann cells*—SCs were purified and amplified as described previously[14]. Briefly, sciatic nerves were obtained from adult rats anaesthetized with pentobarbital sodium (40 mg/kg, ip.) under aseptic conditions. After epineurium and connective tissue were removed, the nerves were cut into 1 $mm^2$ explants. The explants were placed in

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35 mm Corning tissue culture dishes (Baxter, Stone Mountain, GA, USA) with low levels of Dulbecco's Modified Eagle's Medium (DMEM; Invirogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). When the outgrowth of migratory cells (predominantly fibroblasts) reached a near-confluent monolayer around the explants (about 7 days), the explants were transferred to new culture dishes with fresh medium. After four to six such passages (about 4–6 weeks), the cells that emerged from the explants were primarily SCs. The explants were then transferred to a 35-mm dish containing 1.25 U/mL dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), 0.05% collagenase (Worthington Biochemicals Corp., Freehold, NJ, USA) and 15% FBS in DMEM for incubation overnight at 37° in 5% $CO_2$. On the following day, the explants were dissociated and the cells were plated onto poly-L-lysine-coated 100 mm dishes in DMEM/10% FBS. Later, the cultures were re-fed with the same medium supplemented with 20 μg/mL pituitary extract (BTI, Stoughton, MA, USA) and 2 μM forskolin (Sigma, St Louis, MO, USA) for dividing. When the SCs reached confluence (about $4 \sim 5 \times 10^6$ cells/dish), they were rinsed in $Ca^{2+}$- and $Mg^{2+}$ free Hanks balanced salt solution (CMF-HBSS; Gibco, USA) and briefly treated with 0.05% trypsin (Gibco, USA) and 0.02% EDTA (Gibco, USA) in CMF-HBSS. Cells were washed twice in DMEM/10% FBS and passed into new dishes at a density of $2 \times 10^6$ cells/100 mm dish. The purity of the SCs was quantified according to the methods described previously[2]. All SCs used in this study were P2 cells.

*Preparation of schwann cell-conditioned culture medium (SC-CM)*—To prepare the SC-conditioned medium (CM), purified P2 Schwann cells were grown on poly-L-lysine-coated 75 $mm^2$ flask in DMEM/10% FBS for 1~2d firstly. When SCs obtained good growth state and reached 80% confluence, the cultures were washed with D/F12 medium, and then 12 ml neurobasal medium was added. After 2 days, the CM was removed from the cultures and immediately passed through a membrane filter (0.2 μm pore diameter) to remove cells and debris. The SC-CM was stored at -80°C for use.

*DRG explant culture*—Explant cultures of rat dorsal root ganglion (DRG) cultures were established from DRGs obtained from the embryonic (E) day 15 SD rats. Briefly, DRGs were dissected and digested with 0.25% Trypsin/EDTA (Invitrogen, Grand Island, NY) for 5 min. The ganglia were placed into 24 well plates coated with rat tail collagen (4 mg/ml) as described previously[15] or onto monolayer of Schwann cells in 24 well plates. The DRG or DRG/SC co-cultures then were maintained in Neurobasal medium with B27 Supplement (Invitrogen) and and 1.5 ng/ml recombinant human nerve growth factor (NGF; Invitrogen), hereafter designated NB Medium (control), NB Medium plus NGF (50 ng/ml, Roche, Indianapolis, IN) or NB Medium plus SC-CM (half to half). For blocking experiment, DRG was pre-incubated with K252a (10 and 50 nM; Biosource, Camarillo, CA), a high-affinity nerve growth factor receptor blocker[16], for 45 min before treatment of SC-CM. DRG cultures were allowed to grow for 2 days *in vitro* before being fixed and immunostained, as described below.

*Immunocytochemistry*—The DRG cultured in 24 well plates were rinsed in PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature (RT). After three rinses in PBS (10 min each), the cells were incubated with 10% normal goat serum (NGS) in PBS in the presence of 0.3% Triton X-100 for 1 hour at RT and then with monoclonal primary antibodies against mouse anti-SMI31 (1:1000; Chemicon), a marker for phosphorylated neurofilaments, S100 (1:100; Sigma), a marker for Schwann cells overnight at 4°C. On the second day, the cultures were incubated with rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA; 1:200; for SMI31), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab; 1:200; for S100) for 1 h at 37°C. The labeled DRG explants were examined and Images were captured using an Olympus BX60 microscope.

*Quantification of DRG explant outgrowth*—Average neurite carpet radius was used to quantify explant outgrowth as described previously[17]. Briefly, the center of the ganglion and the outer edge of the explant were defined. A series of equidistant points are laid along the outline, and the distances from the center of the ganglion to each of the points were measured. The distances were averaged to produce an average outer radius (R1). A similar analysis was performed outlining the ganglion itself to give the inner radius (R2). R2 is subtracted from R1 to get the average axon radius.

*Reverse transcription polymerase chain reaction (RT-PCR)*—For RT-PCR assay, 2 μg of total RNA extracted from cultures of SCs was reverse transcribed to cDNA and PCR was performed by a routine method[18]. The sequences of specific primers for RT-PCR are as follows: NGF, forward primer: GCCCACTGGACTAAACTTCAGC, reverse primer: CCGTGGCTGTGGTCTTATCTC; TrkA, forward primer: AATGCTCGTCAGGACTTCCATC, reverse primer: TCTTGACCACTAGTCCCTGACC; β-actin, forward primer: ATTGTAACCAACTGGGACG, reverse primer: TTGCCGATAGTGATGACCT. The sizes of amplification products used by the above PCR primers are 349 bp for NGF, 343 bp for TrkA and 533 bp for β-actin, respectively. β-actin was used here as an internal control. PCR products were analyzed on 2% agarose gel.

*Measurement of NGF by ELISA*—The protein concentration of NGF in SC-CM was assessed using a two-site ELISA (NGF EmaxTM ImmunoAssay System; Promega Corp., Madison, WI). ELISA assay was performed according to the manufacturer's instructions. Briefly, the 96-well flat-bottomed immunoplates were coated with 100 μl/well of 0.5 mg/ml polyclonal anti-NGF antibody (NGF pAb) at 4°C for 18 h and washed once with 1× PBS. Blocking of non-specific binding was done with 200 μl of 1× blocking and sample buffer for 1 h at room temperature. SC-CM (100 μl) was added to the coated wells, incubated with immobilized anti-NGF pAb for 6 h at room temperature, and washed five times. Afterward the plates were incubated with monoclonal anti-NGF antibody at 4°C for 12 h. After five washings, the plates were incubated with anti-rat IgG conjugated with horseradish peroxidase for 2.5 h at room temperature. After five washings, TMB peroxidase substrate was added for 10 min at room temperature. The colour reaction was stopped with 1 *M* phosphoric acid, and the light absorbance was measured at 450 nm using a microplate reader. A standard curve using recombinant rat NGF was included in each ELISA experiment. The concentration of NGF in SC-CM was interpolated from the standard curve.

*Statistical analysis*—Data are presented as mean±SD. One-way analysis of variance (ANOVA) with post hoc Tukey t-test was used to determine statistical significance. A $P$ value of $< 0.05$ was considered statistically significant.

## Results

*SCs/SC-CM promote DRG neurite outgrowth*—In this study, a baseline NGF concentration (1.5 ng/ml) was determined at which the DRG neurons within explants consistently survived with similar frequency to the survival index recorded at higher concentrations (up to 5 ng/ml) but with minimal neurite outgrowth (unpublished data). To determine whether SCs affects on neurite outgrowth of DRG neuron, SMI31 staining was performed and average neurite carpet radius calculated following SCs/DRG co-culture. The result showed that DRGs, plated on SC monolayers for 2 days, substantially and significantly increased neurite outgrowth (534.76 ± 45.68 μm; Fig. 1C, E) when compared to the control (86.45 ± 21.32 μm; Fig. 1A, E; $P < 0.05$). To test whether this outgrowth could be attributed to SC-secreted factors, SC-CM was prepared. Application of SC-CM to DRGs (Fig. 1D, E) stimulated a similar degree-increase in neurite outgrowth (565.89 ± 48.57 μm) when compared to SC monolayers, suggesting that SC-derived diffusible factors promoted DRG neurite outgrowth.

*The mRNA expression of NGF in SC and NGFR in DRG neuron, and the concentration of NGF protein in SC-CM*—It was reported that SCs can secrete many kinds of factors, including nerve growth factor

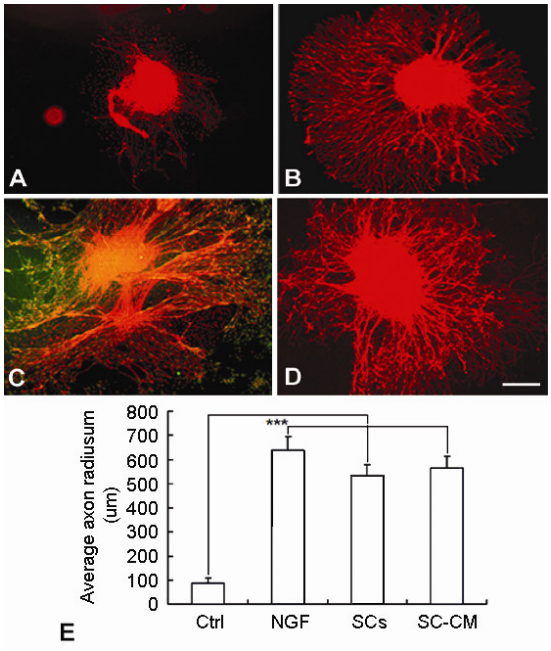


Fig.1—Schwann cells and SC-CM promote neurite outgrowth. A–D, Representative photomicrographs of embryonic DRG explants grown in NB Medium (Ctrl, A), NB Medium plus 50 ng/ml NGF (NGF, B), co-cultured on SC monolayer (SCs, C) or NB Medium plus SC-CM (SC-CM, D). E, Quantitative analysis shows the effect of SCs/SC-CM on neurite outgrowth of DRG. (***: $P < 0.001$). Note: The anti-SMI31 (red) was used to label neurofilaments, and the anti-S100 (green) was used for Schwann cells.

(NGF)[8,10], and NGF was able to promote axon growth[19]. To explore the possibility that NGF mediates the neurite outgrowth effect of SC-CM, first NGF expression by SC was examined. By RT-PCR, it was found that NGF mRNA was expressed substantially in Schwann cells (Fig. 2A). More importantly, high level of NGF protein was detected in SC-CM by ELISA. The concentration of NGF in SC-CM (without being concentrated) was 13.4 ng/ml and 20.2 ng/ml in SC-CM prepared from SCs cultured for 1 day and 2 day, respectively (Fig. 2B). Next mRNA expression of TrkA, a receptor of NGF was examined. As shown in Fig. 2C, the obvious expression of TrkA mRNA was observed in DRG neurons.

*SCs promote DRG neurite outgrowth by secreted NGF*—K-252a, a specific inhibitor of NGF receptor was used, to examine whether NGF protein in SC-CM is responsible for SC-mediated DRG neurite outgrowth. When K-252a was added, the average axon radius of DRG neurite outgrowth was significantly decreased in a concentration-dependent manner (Fig. 3; $P < 0.001$). This finding strongly supports the hypothesis that SCs promote DRG neurite outgrowth by secreting NGF.

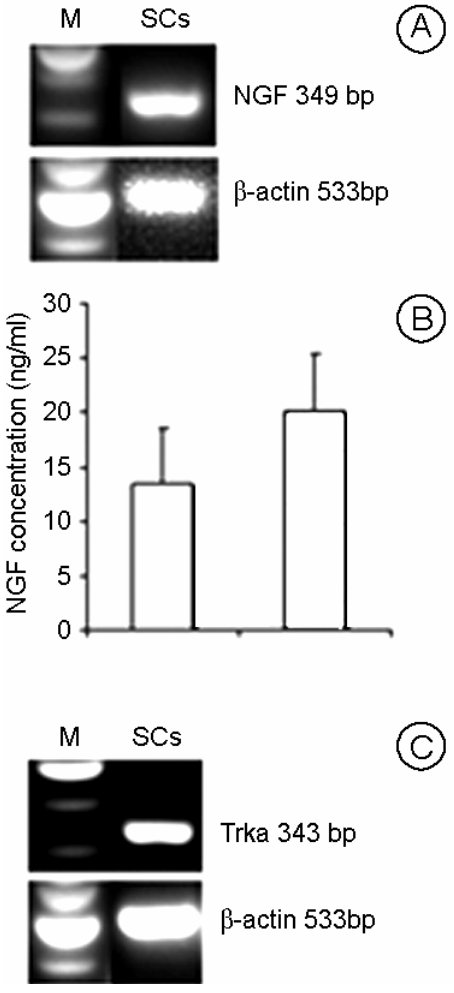


Fig.2—The mRNA expression of NGF in SCs and TrkA in DRG neurons, and the concentration of NGF protein in SC-CM. A, RT-PCR analysis of NGF mRNA expression in SCs. B, NGF concentration in SC-CM was determined by ELISA. C, RT-PCR analysis of TrkA mRNA expression in DRG neurons.

## Discussion

In animal models of spinal cord injury (SCI), grafting Schwann cells or peripheral nerve into the lesion site has been shown to promote axonal regeneration[1,4]. SCs have the capacity to promote the survival and growth of 5-HT neurons[20+] and PC12 Cells[16]. But the precise mechanism was not identified. To explore the possible mechanism which SCs enhance axonal regeneration, in the present study the influence of adult rat sciatic nerve-derived SCs was investigated on DRG neurite outgrowth by using DRG explant model.

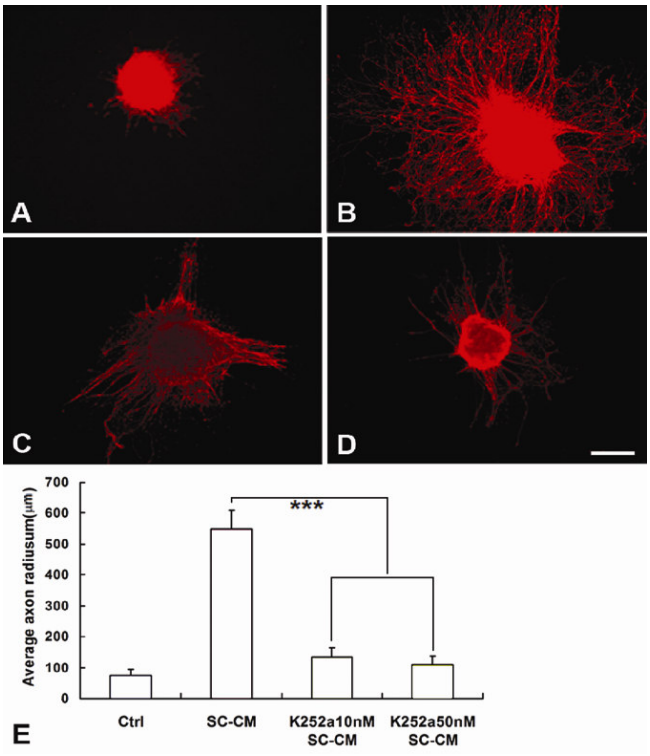


Fig.3—K252a inhibits the neurite outgrowth of DRG neuron enhanced by SC-CM. A–D, Representative photomicrographs of embryonic DRG explants grown in NB Medium (Ctrl, A), NB Medium plus SC-CM (SC-CM, B), K252a 10 nM + SC-CM (C) or K252a 50 nM + SC-CM (D). E, Quantitative analysis shows the effect of K252a on SC-CM promoted-neurite outgrowth of DRG neurons (***:$P < 0.001$). Note: The anti-SMI31 (red) was used to labelled neurofilaments.

To exclude the effect survival-promoting factor on DRG neurite outgrowth, a baseline NGF concentration (1.5 ng/ml) was used to support the survival of DRG but with minimal neurite outgrowth. As expected, SCs strongly stimulated neurite outgrowth of DRG neuron when DRG was plated on SC monolayers. Because SC-CM is sufficient to promote DRG neurite outgrowth without the presence of Schwann cells themselves, the neurite outgrowth-promoting factor(s) produced by Schwann cells appear to be diffusible molecules. However, which factor(s) play a role in here remains unknown and requires to be identified.

SCs can express a variety of trophic factors and adhesion molecules[1,4]. Among these neurotrophic factors, NGF has been reported to promote survival and neurite outgrowth of DRG neuron[19,21]. Thus, whether NGF is a key factor responsible for SC-induced DRG neurite outgrowth required be confirmed. As expected, the expression of NGF mRNA in SCs was observed. More importantly, our result confirmed that there was considerable level of NGF in SC-CM by ELISA. Moreover, the expression of NGF receptor TrkA in DRG neuron was also confirmed. Together, these results indicate that NGF could be an important candidate responsible for SC-induced DRG neurite outgrowth. To further confirm the possible effect of NGF in SC mediated- DRG neurite outgrowth, the action of NGF in SC-CM was blocked using K-252a, the specific inhibitor of NGF receptor TrkA. The present finding showed that the effect of SC-CM on DRG neurite outgrowth was significantly blocked by K-252a in a concentration-dependent manner, supporting that SCs promote DRG neurite outgrowth by secreting NGF.

## Conclusion

The present present study has provided convincing evidence to suggest that SCs promote DRG neurite outgrowth by secreting NGF. This result not only reveal the mechanism which SCs enhance DRG neurite outgrowth, but also enrich our understanding of the reparative role of SCs in CNS injury.

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