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LABORATORY INVESTIGATION

# Schwann cells and mesenchymal stem cells in laminin- or fibronectin-aligned matrices and regeneration across a critical size defect of 15 mm in the rat sciatic nerve

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**OBJECTIVE** Artificial nerve guides are being developed to substitute for autograft repair after peripheral nerve injuries. However, the use of conduits is limited by the length of the gap that needs to be bridged, with the success of regeneration highly compromised in long gaps. Addition of aligned proregenerative cells and extracellular matrix (ECM) components inside the conduit can be a good strategy to achieve artificial grafts that recreate the natural environment offered by a nerve graft. The purpose of this study was to functionalize chitosan devices with different cell types to support regeneration in limiting gaps in the rat peripheral nerve.

**METHODS** The authors used chitosan devices combined with proteins of the ECM and cells in a rat model of sciatic nerve injury. Combinations of fibronectin and laminin with mesenchymal stem cells (MSCs) or Schwann cells (SCs) were aligned within tethered collagen-based gels, which were placed inside chitosan tubes that were then used to repair a critical-size gap of 15 mm in the rat sciatic nerve. Electrophysiology and algesimetry tests were performed to analyze functional recovery during the 4 months after injury and repair. Histological analysis was performed at the midlevel and distal level of the tubes to assess the number of regenerated myelinated fibers.

**RESULTS** Functional analysis demonstrated that SC-aligned scaffolds resulted in 100% regeneration success in a 15-mm nerve defect in this rat model. In contrast, animals that underwent repair with MSC-aligned constructs had only 90% regeneration success, and those implanted with acellular bridges had only 75% regeneration success.

**CONCLUSIONS** These results indicate that the combination of chitosan conduits with ECM-enriched cellular gels represents a good alternative to the use of autografts for repairing long nerve gaps.

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**KEY WORDS** peripheral nerve; chitosan; alignment; Schwann cells; extracellular matrix; fibronectin

The use of artificial nerve guides can be an alternative to autograft for the repair of peripheral nerve defects. However, the length of the gap to be bridged determines the success of axonal regeneration after tube repair of transected nerves. Axonal regeneration occurs within hollow tubes implanted in rat peripheral nerves if the gap is shorter than 10 mm, but not in longer gaps.[27] In a previous study of repair of long-gap peripheral nerve injury in the rat, we demonstrated that chitosan tubes may be a promising biomaterial for the treatment of critical peripheral nerve lesions.[17] However, in contrast with autograft, repair with chitosan tubes did not allow for effective nerve regeneration in all instances.

Tissue-engineered alternatives for the design of complex artificial nerve guides aim to mimic the structural and molecular microenvironment of a natural nerve graft, resembling the original tissue structure, topographical design, and composition.[6] For this purpose, prefilling hollow tubes with extracellular matrix (ECM) components[25,38] and addition of cells into these constructs has been demonstrated to enhance axonal regeneration. Schwann cells (SCs) have been the most common choice to promote regeneration through neural conduits. However, mesenchymal stem cells (MSCs), especially those isolated from bone marrow or adipose tissue, have emerged as an important alternative due to their higher potential for clini-

**ABBREVIATIONS** CMAP = compound muscle action potential; dpo = days postoperation; FBS = fetal bovine serum; MSC = mesenchymal stem cell; PBS = phosphate-buffered saline; SC = Schwann cell.

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cal application.[19,21,22] The therapeutic roles of SCs[12,35] and MSCs[6,14] have been studied previously with respect to the repair of peripheral nerve injuries. Their tissue repair activity has been attributed to their capacity for cell replacement, production of growth factors, ECM synthesis, and immune modulation.[39]

In addition, alignment of cellular and extracellular constituents of the composite graft has been shown to provide guidance and facilitate axonal regrowth when incorporated within nerve conduits. The incorporation of guiding elements, such as microchannels or nanofibers, and the prealignment of ECM components to direct axonal regrowth have been used previously.[7,38] More recently, engineered neural tissue, based on stabilized self-aligned cellular gels, has emerged as an alternative for guiding regrowing axons after peripheral nerve injuries.[15] When combining cell therapy with scaffolds to further improve the capabilities of neural guides, it is important to take into account the interactions between the grafted cells and the scaffolds within which these cells are embedded. The use of collagen-based scaffolds allows the further addition of other ECM molecules, such as laminin[38] and fibronectin,[8] that promote axonal regeneration. It remains, however, challenging to dissect the cooperative influence of multiple ECM parameters on cell behavior[34] when evaluating cellular scaffolds within conduits to repair nerve defects.

In the present work, we have compared the capability of nerve guides containing SCs or MSCs to support regeneration across a critical peripheral nerve defect of 15 mm in the adult rat sciatic nerve. The conduits were made of chitosan and prefilled with stabilized self-aligned cellular collagen Type I scaffolds enriched with either fibronectin or laminin and containing SCs or MSCs. Since the cells were allogenic, animals were treated with the immunosuppressant FK506 during the first 2 months to avoid immune rejection of transplanted cells. By means of functional and morphological methods we assessed the effects of these aligned cellular constructs to sustain regeneration over the critical gap during the 4 months after injury and repair.

## Methods

### Animals

Female Wistar Hannover rats (Janvier) were used in the experiment. The animals were housed in plastic cages and maintained under standard conditions with free access to water and food. The experimental procedures were approved by the ethics committee of the Universitat Autònoma de Barcelona and followed the rules of the European Communities Council Directive.

### Mesenchymal Stem Cell Culture

MSCs were obtained from 21-day-old Wistar Hannover rats (n = 4). Animals were euthanized with carbon dioxide. Under a dissecting microscope, tibias and femurs were collected, placed in cold phosphate-buffered saline (PBS), and epiphyses were removed. Diaphyses of tibias and femurs were flushed with PBS using a syringe, and the marrow was homogenized. The cell extract was filtered in a 70-μm nylon mesh and recovered by centrifugation for 10 minutes at 230*g*. Collected cells were grown in α-MEM medium supplemented with L-glutamine (Life Technologies), 20% fetal bovine serum (FBS; Lonza), and penicillin-streptomycin (Life Technologies, 100×), and plated in 100-mm culture dishes (Iwaki, Asahi Technoglass) at a density of $5 \times 10^6$ cells/cm$^2$. After 24 hours, the supernatant containing nonadherent cells was removed and fresh medium was added. When the cell culture was near confluence, the cells were detached and replated at 5000 cells/cm$^2$.

### Schwann Cell Culture

Dissociated SCs were also prepared from 21-day-old Wistar Hannover rats (n = 4). Animals were euthanized with carbon dioxide. Under a dissecting microscope, sciatic and median nerves were exposed, removed, and cleaned of connective tissue, then cut into small pieces that were dissociated in 10% collagenase (Invitrogen), 10% trypsin (Life Technologies), and 10% DNase (Invitrogen) in Hanks solution (Invitrogen) for 30 minutes at 37°C. Then, the nerves were mechanically dissociated by carefully resuspending the cell solution with a pipette, and the enzyme action was stopped by adding DMEM. After centrifugation, cells were plated in 35-mm culture dishes (Greiner Bio-One), pretreated with 10 μl/ml poly-D-lysine (Sigma), in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin, at a density of $1 \times 10^6$ cells/cm$^2$. After 24 hours, the supernatant was removed and fresh medium was added. When the cell culture was near confluence, the cells were detached and replated at a density of 1000 cells/cm$^2$.

### Cell Survival and Proliferation Assays

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) was used to evaluate mitochondrial activity as an indirect measure of cell survival. Approximately 8000 MSCs and SCs were cultured on 24-well plates previously treated with poly-D-lysine 10 μl/ml, laminin (5 μg/cm$^2$), or fibronectin (5 μg/cm$^2$) for 72 hours. Then, 0.15 mg/ml of MTT was added and the cells were incubated for 45 minutes at 37°C. The formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (DMSO) and 100 μl was passed to 96-well plates. The optimal density was determined with a microculture plate reader (BioTek Instruments) at 570 and 620 nm and analyzed with KCjunior software.

To analyze cell proliferation, MSCs and SCs were cultured on glass coverslips pretreated with poly-D-lysine 10 μl/ml, laminin (5 μg/cm$^2$), or fibronectin (5 μg/cm$^2$) for 48 hours at 8000 cells per coverslip; 1 μl of BrdU was added to the cultures at 20 μM. One day later, the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and then treated with HCl at 2 M for 20 minutes at 37°C. Afterward, the pH was re-equilibrated with Tris-buffered saline (TBS) to 8.5 and incubated overnight with primary antibodies anti-BrdU (1:500; Fitzgerald) and anti-S-100 (1:100; Immunostar). After washes, the cells were incubated for 2 hours with biotinylated IgG (1:200; Life Bioscience) and incubated overnight with Alexa 594 and Streptavidin 488 conjugated secondary antibodies (1:200;

Life Bioscience) and mounted with Mowiol (Sigma) containing DAPI. To label MSCs, we used CD90 conjugated with FITC (1:100; BD Pharmingen).

### Preparation of Cellular Constructs

For the preparation of the ECM, a volume of 800 μl of rat tail Type I collagen solution (BD Bioscience) at a concentration of 3 mg/ml was mixed with 100 μl of 10× Eagle's medium (Gibco) and 4 μl of 7.5% sodium bicarbonate solution. These matrices were enriched with 200 μl (20% final volume) of fibronectin (BD Biosciences) or laminin Type I (Sigma) at 1 mg/ml and kept in ice.

For the preparation of the cellular constructs, MSCs and SCs obtained from the cultures were resuspended in 1 ml of medium. Cells were counted using a Neubauer chamber. Then, appropriate volumes of medium containing cells were centrifuged to get 750,000 cells. The pellet was resuspended in 1 ml of each of the matrices prepared.

MSCs and SCs were aligned within tethered collagen-based gels enriched with either fibronectin or laminin in rectangular plastic ABS molds according to the method described previously.[18,32] The gels were integrated with a tethering mesh of nylon at opposite ends. Tethered gels were immersed in culture medium and incubated at 37°C in a humidified incubator (5% $CO_2$) overnight to allow alignment to develop. Control acellular matrices were subjected to the same procedure. The resulting constructs were separated from the nylon mesh and placed on absorbent pads to stabilize. Then they were rolled and placed in chitosan tubes (19 mm long) that had been longitudinally cut.

### Experimental Design and Surgical Procedure

For the in vivo experiments, 36 adult female Wistar Hannover rats (10 weeks old; 220–250 g) were used. Surgeries were performed under ketamine/xylazine anesthesia (90/10 mg/kg administered intraperitoneally).

Right sciatic nerves were exposed and cut 96 mm from the third toe, and a nerve segment of 6 mm was resected. Nerve stumps were fixed to 19-mm-long chitosan tubes (2-mm internal diameter) with 10-0 sutures, leaving a 15-mm gap. The wound was disinfected and the muscle plane and skin were sutured with absorbable silk sutures. The operated rats were given amitriptyline to prevent autotomy.[30]

Animals were distributed into 6 experimental groups, depending on the intraluminal contents of the tube: aligned collagen-fibronectin 20% and 750,000 MSCs (n = 6, FN-MSC group), aligned collagen-fibronectin 20% and 750,000 SCs (n = 7, FN-SC group), aligned collagen-laminin 20% and 750,000 MSCs (n = 7, LM-MSC group), and aligned collagen-laminin 20% and 750,000 SCs (n = 8, LM-SC group); stabilized and rolled FN enriched construct (n = 4, FN-St) and stabilized and rolled LM-enriched constructs (n = 4, LM-St group) served as controls.

Once the animals were operated on, they received an injection of the immunosuppressant FK506 (Fujisawa Pharmaceuticals) at 2 mg/kg, and then daily subcutaneous injection of 1 mg/kg for up to 2 months. To prevent possible infections, all of the rats were treated with 500 mg/L amoxicillin (Normon) in their drinking water during the entire postoperative period.

### Electrophysiological Tests

Electrophysiological tests were performed to evaluate reinnervation of the target muscles monthly up to 120 days postoperation (dpo). Animals were anesthetized and placed on a warm plate. The sciatic nerve was stimulated percutaneously by electrodes placed at the sciatic notch. The compound muscle action potential (CMAP) of the tibialis anterior, gastrocnemius, and plantar interosseus muscles was recorded.[3] The amplitude and the latency of the M-wave were measured.

### Functional Evaluation of Sensory Recovery

Mechanical (von Frey algesimeter from Bioseb) and thermal (plantar algesimeter from Ugo Basile) nociceptive responses were evaluated monthly up to 122 dpo. For both tests, the lateral area of the hind paw of the rats was stimulated and compared with the contralateral paw used as control. The saphenous nerve was resected at 122 dpo to avoid confounding responses due to collateral reinnervation of this nerve from medial to lateral areas of the hind paw.[9] Cutoff values were set at 40 g force for the von Frey and 20 seconds for the plantar test. Threshold responses were calculated as the mean of 3 measurements and expressed as percentage of response in the injured paw with respect to the contralateral intact paw of each animal.

### Histology and Morphometry

At the end of the observation period (122 dpo), the animals were killed and perfused with 4% paraformaldehyde in PBS (0.1 M, pH 7.4) and postfixed in 3% paraformaldehyde–3% glutaraldehyde in PBS. The nerves were postfixed in osmium tetroxide (2%, 2 hours, 4°C), dehydrated through ethanol series, and embedded in Epon resin. Semithin sections of the nerves (0.5 μm) were obtained using an ultramicrotome (Leica) and stained with toluidine blue. Representative images of the whole regenerated nerve were acquired at 10×, and at least 30% of the nerve cross-sectional area was acquired at 100× to allow for counting of the number of myelinated fibers at midsection and distal part of the tube by means of ImageJ software (National Institutes of Health).

### Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups for MTT assay were made using a 1-way ANOVA followed by a Bonferroni post hoc test. Statistical comparisons between groups and intervals for algesimetry and electrophysiological test results were made using a 2-way ANOVA followed by a Bonferroni test. Statistical comparisons between groups for histological results were made using nonparametric Kruskal-Wallis and Mann-Whitney U-tests. Differences were considered significant if the p value was less than 0.05.

## Results

### Response of SCs and MSCs Cultured on Different ECM Components

Cell survival on different ECM coatings was examined

after 72 hours in culture (Fig. 1A) by using the MTT assay, which measures mitochondrial activity. We observed a significant increase of mitochondrial activity in SCs grown on laminin (19.89% ± 0.45%, $p < 0.0001$) or fibronectin (8.57% ± 2.4%, $p < 0.0001$) compared with the ones grown on the control condition (poly-D-lysine) (Fig. 1B). The mitochondrial activity of SCs on laminin was also significantly higher than on fibronectin. When analyzing mitochondrial activity of MSCs, we found a significant increase on fibronectin (21.76% ± 0.54%, $p < 0.0001$) but not on laminin (10.76% ± 0.92%, $p > 0.05$) compared with poly-D-lysine (Fig. 1B).

We evaluated cell proliferation on the different coatings by BrdU staining. After 72 hours in culture, we observed similar proportions of BrdU-labeled (for both SCs or MSCs) on laminin and fibronectin compared with poly-D-lysine (Fig. 1C).

## Functional Reinnervation After In Vivo Implantation

### Motor Reinnervation

Nerve conduction tests performed 1 week and 1 month after sciatic nerve injury demonstrated complete denervation of the hindlimb muscles. Initial evidence of reinnervation of tibialis anterior and gastrocnemius muscles was found at 60 dpo in some animals of each group, with CMAPs of small amplitude. The CMAPs progressively increased in amplitude and were recorded in most animals over time, with a similar pattern for both muscles (Fig. 2A and B). During the last electrophysiological test (120 dpo), reinnervation of the tibialis anterior and gastrocnemius muscles was observed in 3 of 4 animals in the FN-St group, 3 of 4 in the LM-St group, 5 of 6 in the FN-MSC group, 6 of 7 in the LM-MSC group, 7 of 7 in the FN-SC group, and 8 of 8 in the LM-SC group. Significant differences for the tibialis anterior CMAP ($p < 0.0001$) were observed at the final time point between FN-SC and LM-SC groups versus the groups of animals that received acellular constructs (FN-St and LM-St). Significant differences ($p < 0.05$) were also observed for the FN-SC and LM-SC groups versus the MSC construct (FN-MSC and LM-MSC) groups (Fig. 2). At the final time point, significant differences in the gastrocnemius muscle ($p < 0.05$) were only observed between the FN-SC and the LM-St groups. Latencies of CMAPs were considerably longer than normal during the first stages of reinnervation and tended to shorten with time toward normal values. At the end of the postoperative observation period, the latencies of the CMAP recorded on tibialis anterior and gastrocnemius muscles were similar between groups (Fig. 2D and E).

Onset of reinnervation of the more distal plantar interosseous muscles was detected at 60 dpo. In these cases, CMAPs were of very small amplitude (< 0.5 mV). At 120 dpo, plantar CMAPs were recorded in 3 of 4 animals in the FN-St group, 3 of 4 in the LM-St group, 5 of 6 in the FN-MSC group, 6 of 7 in the LM-MSC group, 7 of 7 in the FN-SC group, and 8 of 8 in the LM-SC group. Significant differences ($p < 0.0001$) were observed between the FN-SC group and the rest of the groups (Fig. 2). The plantar CMAP latency followed a similar pattern to that described for the proximal muscles, returning toward normal values during the regeneration process, without significant differences between groups. Results are summarized in Table 1.

### Sensory Reinnervation

Withdrawal responses to mechanical and thermal stimuli were evaluated by means of the von Frey test and the plantar test, respectively. Values are presented as percentage of response of the injured versus the contralateral intact paw (to overcome possible variations between testing conditions) of each individual at each time point. Either mechanical or heat nociceptive thresholds were taken as the mean of 3 measurements per paw region per animal; values were grouped per individual animal and compared to the mean of the 3 measurements obtained for the contralateral intact paw of that animal. Absence of responses at the lateral part of the paw on the repaired side of the rats during the first 30 dpo demonstrated complete denervation. When animals did not respond to either mechanical or heat stimuli, these were penalized with cutoff values (40 g and 20 seconds, respectively).

Withdrawal responses to mechanical stimuli from 60 to 120 dpo showed that the paw on the repaired side responded to lower intensity stimulus compared with the contralateral paw, indicating some degree of hyperalgesia (Fig. 3A). After elimination of the saphenous nerve at 120 dpo, the mechanical withdrawal responses in the different groups compared with their contralateral side at 122 dpo were 74 ± 21 g in FN-St group, 63 ± 15 g in LM-St group, 70 ± 17 g in FN-MSC group, 67 ± 15 g in the LM-MSC group, 68 ± 14 g in the FN-SC group, and 53 ± 3 g in the LM-SC group, without significant differences between groups.

Similarly, withdrawal responses to heat stimuli in the plantar test showed no response on the denervated paw during the first 30 dpo. From 60 to 120 dpo, most rats showed withdrawal responses at lower stimulus intensity than in the contralateral paw (Fig. 3B). After cutting the saphenous nerve, the withdrawal time of the injured paw at 122 dpo increased to average values of 134 ± 60 seconds in the FN-St group, 124 ± 11 seconds in the LM-St group, 134 ± 58 seconds in the FN-MSC group, 117 ± 17 seconds in the LM-MSC group, 95 ± 6 seconds in the FN-SC group, and 134 ± 25 seconds in the LM-SC group, without significant differences between groups.

## Histological Results

Macroscopic examination of regenerated nerves after 4 months postinjury revealed that 3 of 4 animals in the FN-St and LM-St groups, 5 of 6 animals in the FN-MSC, 6 of 7 animals in the LM-MSC, 7 of 7 animals in the FN-SC, and 8 of 8 animals in the LM-SC presented a regenerated cable inside the chitosan tube (Fig. 4 and Fig. 5G). The repaired nerves were surrounded by a thick connective layer. Transverse sections of the regenerated nerves taken at the midpoint of the tube and 3 mm distal to the distal suture were analyzed under light microscopy (Fig. 5A and B). To compare the number of myelinated fibers, all rats were included in the statistical analysis, giving null values to nonregenerated animals. The mean number of myelinated fibers at the midpoint of the tube was higher in the FN-SC group (7656 ± 1379) followed by the LM-SC group (6677

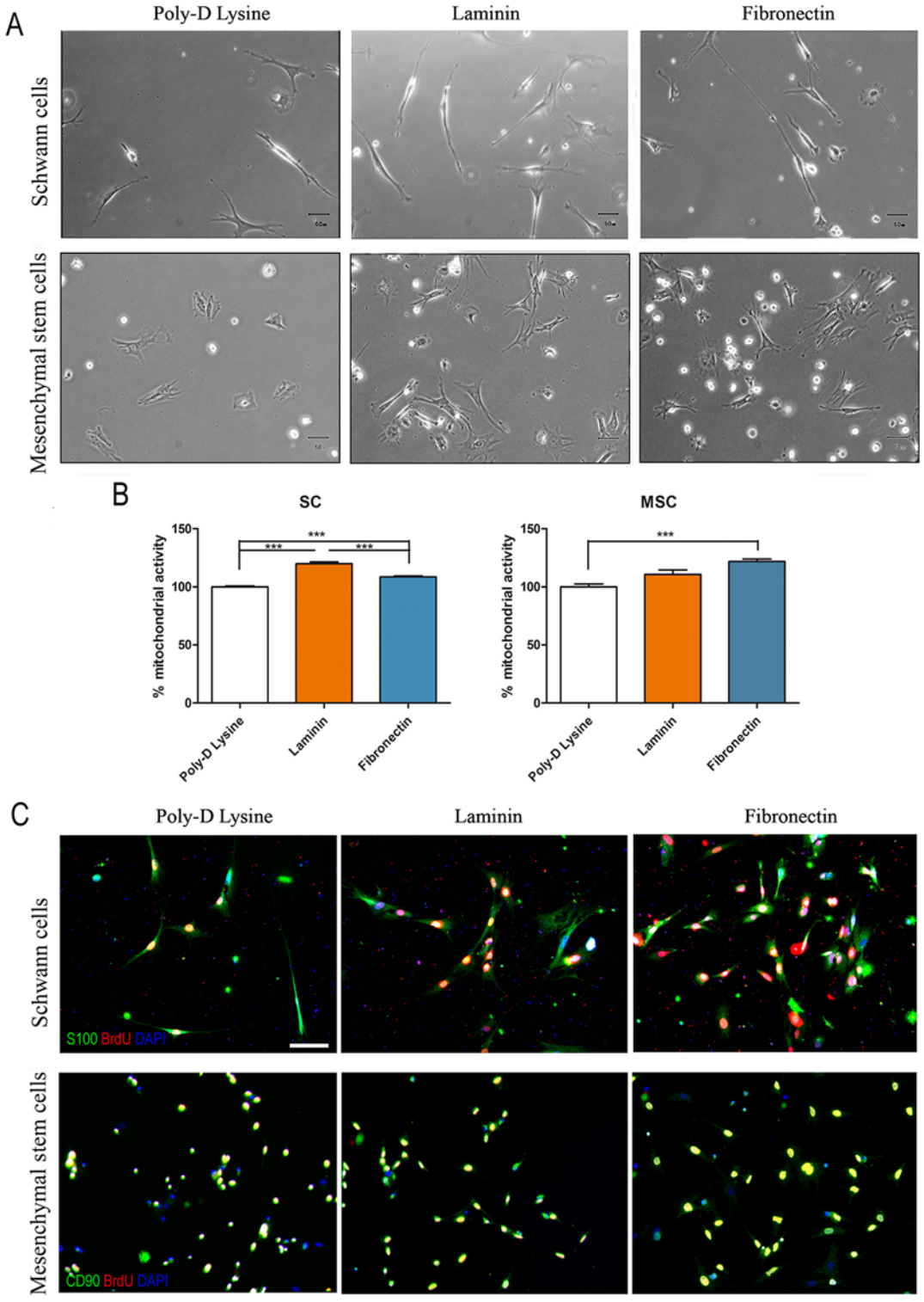


**FIG. 1. A:** Representative images of cultured SCs (*upper*) and MSCs (*lower*) on poly-D-lysine, laminin, and fibronectin coatings. Scale bar = 50 μm. **B:** Results of the MTT assay of dissociated SCs and MSCs cultured for 72 hours, presented as percentage of mitochondrial activity of the cells on laminin- or fibronectin-coated surfaces vs poly-D-lysine–treated wells. ***$p < 0.0001$. **C:** In vitro proliferation of SCs (*upper*) and MSCs (*lower*) grown on poly-D-lysine–, laminin-, and fibronectin-coated surfaces after 72 hours in culture. BrdU was incorporated in the media after 48 hours in culture and cells were fixed after 24 hours. Cultures were labeled for BrdU (*red*), DAPI (*blue*), and S-100 for SCs (*green*, *upper*) or CD90 for MSCs (*green*, *lower*). For group definitions, please refer to the *Experimental Design and Surgical Procedure* subsection of *Methods*. Scale bar = 100 μm. Figure is available in color online only.

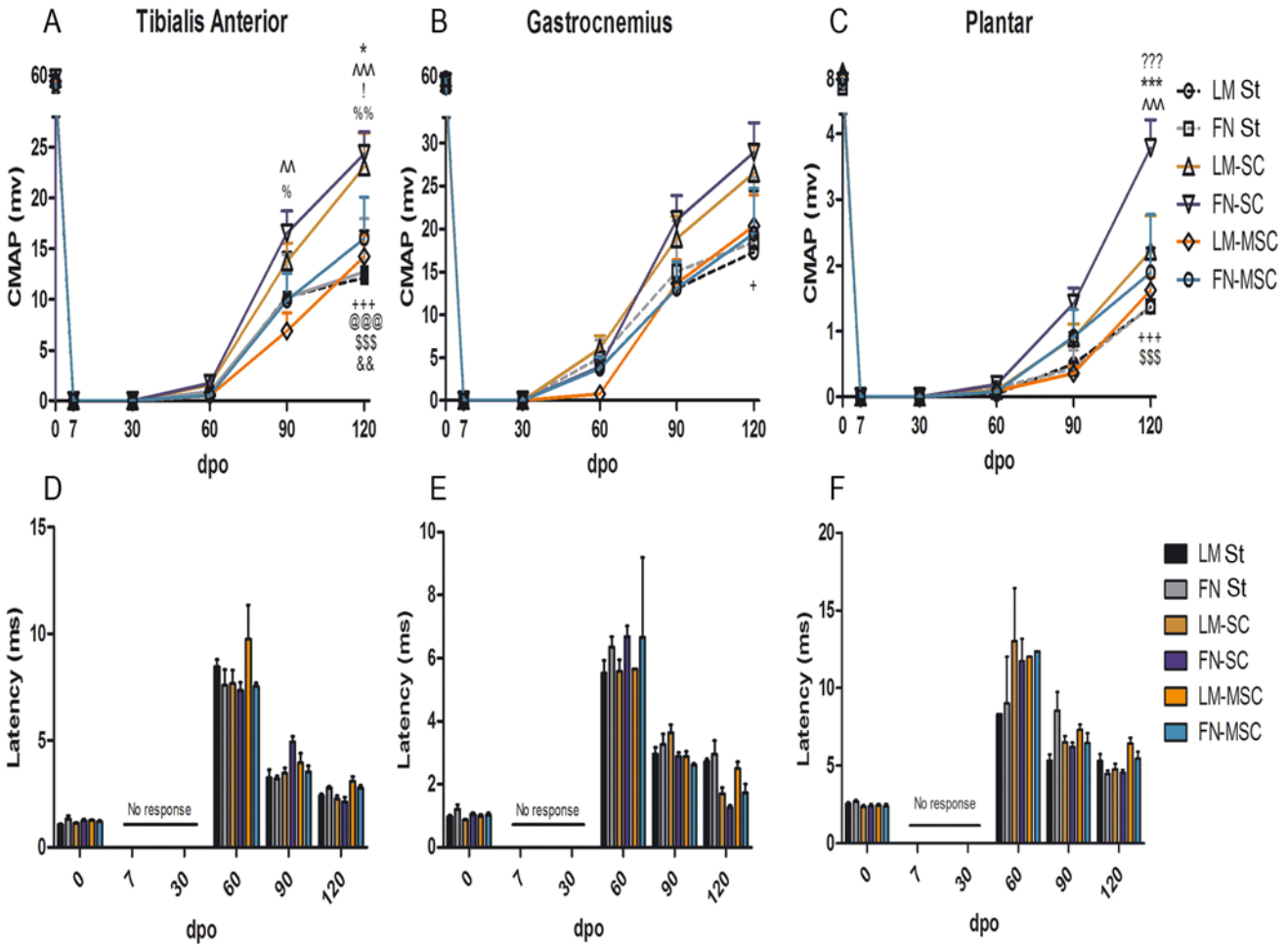


**FIG. 2. A–C:** Mean amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A), gastrocnemius (B), and plantar muscles (C) of the injured limb. $^{+}p < 0.05$ and $^{+++}p < 0.0001$ between FN-SC and LM-St groups; $^{@@@}p < 0.0001$ between LM-SC and LM-St groups; $^{\$\$\$}p < 0.0001$ between FN-SC and FN-St groups; $^{\&\&}p < 0.001$ between LM-SC and FN-St groups; $^{\%\%}p < 0.001$ between LM-SC and LM-MSC groups; $^{!}p < 0.05$ between LM-SC and FN-MSC groups; $^{\wedge\wedge\wedge}p < 0.0001$ between FN-SC and LM-MSC groups; $^{*}p < 0.05$ and $^{***}p < 0.001$ between FN-SC and FN-MSC groups; and $^{???}p < 0.001$ between FN-SC and LM-SC groups. **D–F:** Mean values of the CMAP latency for the same muscles. *Error bars* represent SEM. Figure is available in color online only.

± 1119), the FN-MSC group (6192 ± 1999), the LM-MSC group (5750 ± 2198), the LM-St group (3458 ± 1795), and the FN-St group (3105 ± 1348) (Fig. 5C). The same pattern was observed in the distal nerve, where the number of myelinated fibers was higher in the FN-SC group (4771 ± 683), followed by the LM-SC group (3927 ± 524), the FN-MSC group (3228 ± 907), the LM-MSC group (3076 ± 962), the FN-St group (2915 ± 837), and the LM-St group

**TABLE 1. Summary of data obtained in the present study and collected data from historical results in the 15-mm-autograft group from our laboratory in past studies**

| Group | % of Rats w/ Regeneration | % Recovery | | | | | No. of Myelinated Fibers | |
|---|---|---|---|---|---|---|---|---|
| | | TA CMAP | GM CMAP | PL CMAP | Von Frey Test | Plantar Test | At Midlevel | At Distal Level |
| AG | 100 | 60 | 60 | 33 | 61 | 128 | 14,409 | 6,865 |
| LM-St | 75 | 23 | 30 | 15 | 63 | 124 | 3,458 | 2,079 |
| FN-St | 75 | 23 | 35 | 23 | 74 | 137 | 3,105 | 2,915 |
| LM-SC | 100 | 41 | 51 | 24 | 60 | 134 | 6,677 | 3,927 |
| FN-SC | 100 | 43 | 54 | 49 | 68 | 94 | 7,656 | 4,771 |
| LM-MSC | 86 | 27 | 42 | 20 | 67 | 117 | 5,750 | 3,076 |
| FN-MSC | 83 | 32 | 34 | 23 | 69 | 134 | 6,192 | 3,228 |

AG = autograft; GM = gastrocnemius; PL = plantar interosseous; TA = tibialis anterior.

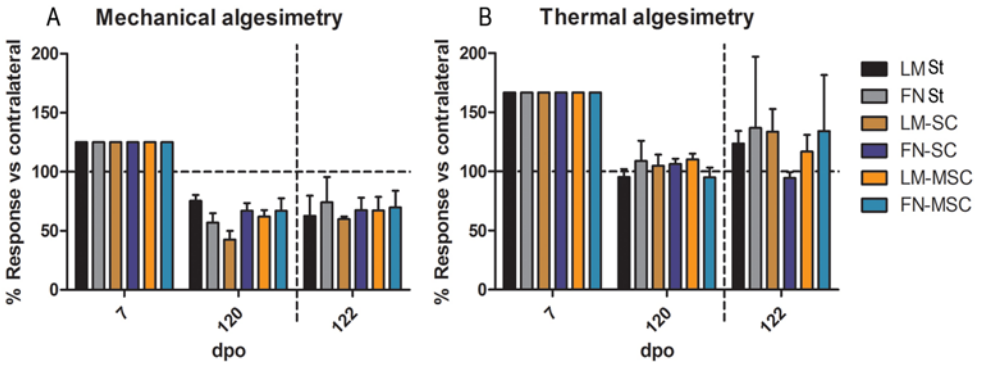


**FIG. 3.** Mechanical (**A**) and thermal (**B**) algesimetry test results. Values are the group mean of the percentage of withdrawal response to the noxious stimuli applied to the lateral side of the paw on the injured side versus the intact side. The *horizontal dotted lines* represent the normalized baseline values. The *vertical dotted lines* indicate when the saphenous nerve was cut, at 120 dpo; then the rats were tested again at 122 dpo. *Error bars* represent SEM. Figure is available in color online only.

(2079 ± 711) (Fig. 5D), but differences were only significant between the FN-SC group and LM-St and FN-St groups at the distal level.

The area of the regenerated nerves at the midlevel and the distal level did not show significant differences between groups (Fig. 5E and F). The highest values were found for both mid-tube and distal nerve levels in the FN-SC group (0.33 ± 0.08 μm² and 0.30 ± 0.05 μm²), followed by the LM-SC group (0.31 ± 0.10 μm² and 0.28 ± 0.03 μm²), the FN-MSC group (0.28 ± 0.10 μm² and 0.26 ± 0.10 μm²), the LM-MSC group (0.28 ± 0.16 μm² and 0.25 ± 0.18 μm²), the FN-St group (0.26 ± 0.10 μm² and 0.14 ± 0.10 μm²), and the LM-St group (0.24 ± 0.07 μm² and 0.12 ± 0.05 μm²). Again, differences were found significant only for the FN-SC group and LM-St and FN-St groups at the distal level.

## Discussion

In this study we have compared the effect of aligned constructs of SCs or MSCs in a collagen Type I–based matrix enriched with either fibronectin or laminin. These constructs were tested in chitosan conduits for their ability to promote axonal regeneration across a critical 15-mm gap of the sciatic nerve in rats. Our results show that inclusion of aligned SCs in either fibronectin- or laminin-enriched scaffolds was associated with regeneration of peripheral nerves in 100% of cases and provided the best levels of regeneration and functional recovery, compared with MSC-containing constructs and acellular scaffolds.

The use of artificial nerve guides has been proposed to replace the autograft in the repair of severe peripheral nerve lesions. Development of artificial nerve guides has progressed during the last decades to yield a variety of biodegradable and biocompatible devices.[10] In this study, we used a chitosan tube that has already been demonstrated to be more effective than the standard silicone tube for supporting axonal regeneration across limiting gaps in rats.[16,17,20] However, the percentage of success after repair with a hollow chitosan tube was still far from the 100% of regenerated cases reached when using an autograft to bridge such a long nerve gap. Regeneration in hollow conduits is highly dependent on the formation of an initial fibrin cable, which serves as a guidance structure for fibroblasts, SCs, and blood vessels to migrate and populate the cable and facilitate axonal regeneration. To further mimic the natural geometry and architecture of the endoneurium in nerve grafts, cellular and extracellular components should be longitudinally aligned. Among different techniques (gradients, electrical fields, magnetic fields, nanofibers, etc.), cells embedded in tethered ECM-based hydrogels can self-align in response to cell-generated tension to form tracts of engineered neural tissue.[15,33]

Immunosuppression with FK506 for the first 2 months after the operation was used to avoid rejection of allogeneic transplanted cells. Since administration of FK506 has been demonstrated to enhance regeneration and functional recovery after peripheral nerve injuries,[37] groups with acellular constructs were also treated with the drug. In these groups, the percentage of animals with a regenerating cable in the tube was about 75%, similar to the results obtained in a previous study in which animals were not treated with FK506 (data not published). In contrast, inclusion of aligned cells increased the success of axonal regeneration up to 100%.

Both SCs and MSCs have been used to promote nerve

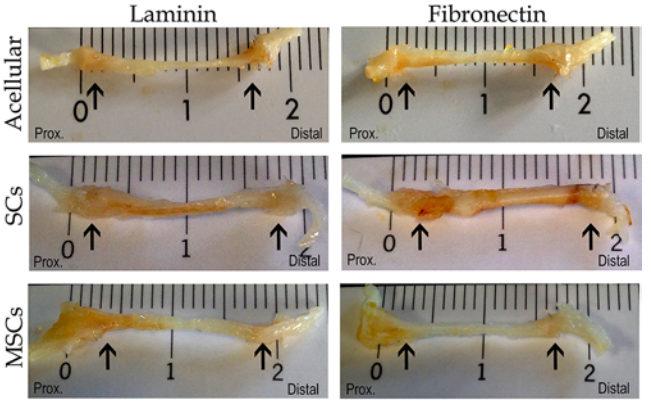


**FIG. 4.** Representative images of regenerated nerves 4 months after repair using constructs enriched with fibronectin or laminin and containing either aligned SCs, MSCs, or no cells (acellular). The chitosan tubes have been removed to aid visualization. *Black arrows* indicate the gap bridged with the formed regenerated cable. The values on the ruler below the nerves are in millimeters. Figure is available in color online only.

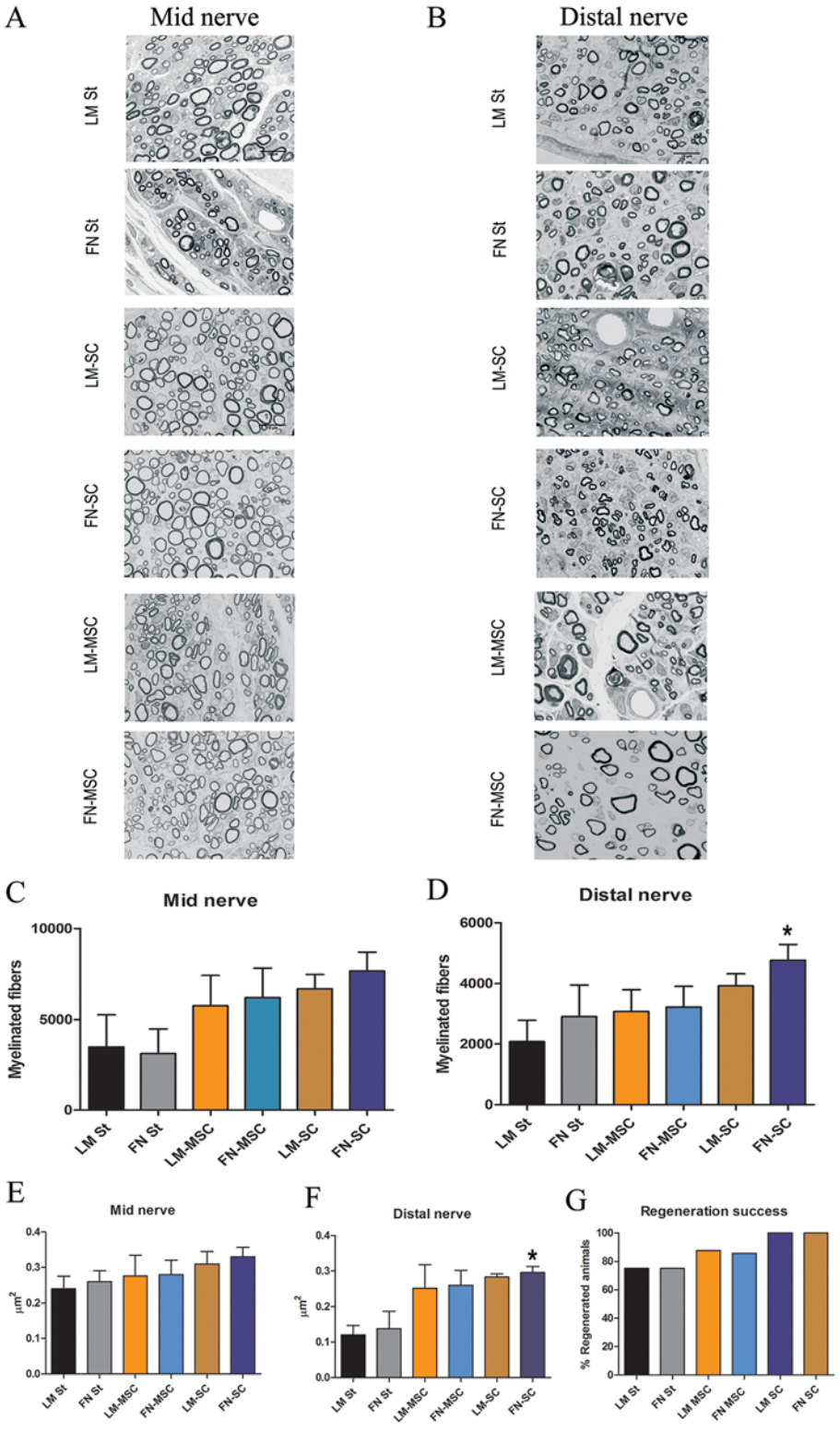


**FIG. 5. A and B:** Representative images of semithin sections of the regenerated nerves taken at the midpoint of the tube (A) and distal to the tube (B) 4 months after sciatic nerve resection and repair, from a representative animal of each group. Scale bar = 10 µm. **C and D:** Estimated number of regenerated myelinated fibers in the tibial nerve at the mid-tube (C) and 3 mm distal to the tube (D) 4 months after sciatic nerve resection and repair. *$p < 0.05$ vs Lm St and FN St groups. **E and F:** Cross-sectional area of the regenerated nerve at mid-tube (E) and 3-mm-distal (F) levels. Animals with no regenerated nerve were also included (with null values) in the calculations. *Error bars* represent SEM. **G:** The percentage of animals that presented a regenerated nerve at 120 dpo in the different experimental groups. Figure is available in color online only.

regeneration and provide support in long peripheral nerve gaps. The potential ability of those cells relies mainly on populating the neural guide and continuously producing neurotrophic factors.[26] Interestingly, SC-aligned hydrogels allowed regeneration in a slightly higher proportion of animals than MSC-aligned constructs. These differences may be determined by the different behavior of these cell types in the scaffolds. ECM molecules like laminin or fibronectin influence cell and tissue behavior by providing an adhesive substrate to anchorage-dependent cells and determine a variety of cell differentiation processes.[13] For instance, Abdeen and colleagues[1] demonstrated a positive correlation between fibronectin, stiffness, and proangiogenic signaling of MSCs in 2D hydrogels. Indeed, in another study, the angiogenic marker PECAM was decreased in MSCs when cultured on laminin matrices.[13] On the other hand, SC proliferation increased in laminin[2] and fibronectin[5] in vitro. In this study we also tested the performance of MSCs and SCs on laminin- and fibronectin-coated surfaces in vitro and found that MSCs survived better on fibronectin-coated surfaces and SCs on laminin-coated surfaces. However, in vivo SCs seeded in fibronectin-aligned matrices provided better functional results than SCs seeded in laminin-aligned matrices. The positive effect of fibronectin on SC proliferation and migration has been previously proved.[5,29] On the other hand, independent of the ECM matrix used for the aligned constructs, functional recovery was better in SC groups than in MSC groups.

Overall, our results indicate that SCs are better candidates than MSCs for populating neural conduits to promote nerve regeneration. This is not surprising, since SCs are the glial cells of the peripheral nervous system and thus responsible for guiding axons toward their target organs. However, their clinical use is limited, since acquisition of autologous primary SCs requires the extraction of a donor nerve and the consequent loss of its function. Moreover, to amplify the number of cells obtained, a period of preoperative cultivation is needed,[22] during which SCs lose their phenotypic characteristics in vitro[24] and increase the proregenerative potency.[35] Nevertheless, skin-derived stem cells have been reported as an accessible and autologous alternative source of SCs for therapeutic application to injured peripheral nerves. These skin precursor–derived SCs have regenerative attributes that resemble those of acutely denervated SCs and, when grafted in injured nerves, they have demonstrated positive effects on nerve regeneration.[23,36,40]

In contrast, MSCs are easily harvested from bone marrow or several other tissues and exhibit great plasticity for differentiating toward multiple cell types,[4] allowing for adjustment of the requirements needed to promote axonal regeneration. Probably the predifferentiation of these cells toward SC-like cells, acquiring the capacity of guiding and myelinating peripheral axons,[11,31] will improve their regenerative capacity when used to enrich artificial conduits to repair long nerve gaps. Although some studies have indicated that MSCs have positive effects when directly transplanted into injured nerves[28] or used to seed nerve grafts,[22,39] SCs are still the key element to consistently sustain nerve regeneration through limiting nerve gaps.

## Conclusions

The combination of cell therapy and tissue engineering resulted in marked improvement in the success of regeneration and the degree of functional recovery achieved after repairing a critical nerve gap with a chitosan guide, providing support for tube repair as a potential alternative to autograft in the repair of injured peripheral nerves. SC-seeded grafts are likely the best alternative to the autograft for repairing long-gap peripheral nerve injuries, since they allowed successful regeneration in all the animals included in the study and provided the highest values of regeneration reported with artificial nerve grafts.

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## Disclosures

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## Author Contributions

Conception and design: Navarro, Udina. Acquisition of data: Gonzalez-Perez, Hernández. Analysis and interpretation of data: Gonzalez-Perez. Drafting the article: Gonzalez-Perez, Udina. Critically revising the article: Navarro, Heimann, Phillips, Udina. Reviewed submitted version of manuscript: Navarro, Udina. Approved the final version of the manuscript on behalf of all authors: Navarro. Statistical analysis: Navarro. Administrative/technical/material support: Heimann, Phillips. Study supervision: Navarro, Udina.

## Supplemental Information

### Previous Presentations

Part of this work was presented in an oral communication to the 3rd International Symposium on Peripheral Nerve Regeneration, Hannover, Germany, September 24, 2015.

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