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SCHWANN CELLS TRANSDUCED WITH A LENTIVIRAL VECTOR ENCODING FGF-2 PROMOTE MOTOR NEURON REGENERATION FOLLOWING SCIATIC NERVE INJURY

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Main points:
LV-FGF2 infected Schwann cells promote motor but not sensory neurite outgrowth in vitro
Overexpressing FGF-2 in silicone conduits to repair a sciatic nerve transection enhances axonal regeneration and muscle reinnervation

Key words: trophic factor, axonal growth, muscle reinnervation, gene therapy

Abstract

Fibroblast growth factor 2 (FGF-2) is a trophic factor expressed by glial cells and different neuronal populations. Addition of FGF-2 to spinal cord and dorsal root ganglia (DRG) explants demonstrated that FGF2 specifically increases motor neuron axonal growth. To further explore the potential capability of FGF-2 to promote axon regeneration, we produced a lentiviral vector (LV) to overexpress FGF-2 (LV-FGF2) in the injured rat peripheral nerve. Cultured Schwann cells transduced with FGF-2 and added to collagen matrix embedding spinal cord or DRG explants significantly increased motor but not sensory neurite outgrowth. LV-FGF2 was as effective as direct addition of the trophic factor to promote motor axon growth *in vitro*. Direct injection of LV-FGF2 into the rat sciatic nerve resulted in increased expression of FGF-2, which was localized in the basal lamina of Schwann cells. To investigate the *in vivo* effect of FGF-2 overexpression on axonal regeneration after nerve injury, Schwann cells transduced with LV-FGF2 were grafted in a silicone tube used to repair the resected rat sciatic nerve. Electrophysiological tests conducted for up to 2 months after injury revealed accelerated and more marked reinnervation of hindlimb muscles in the animals treated with LV-FGF2, with an increase in the number of motor and sensory neurons that reached the distal tibial nerve at the end of follow up.

Introduction

After peripheral nerve injury, axotomized neurons are able to regenerate. Denervated Schwann cells in the distal stump proliferate and support axon regeneration, by secreting several trophic factors and expressing cell adhesion molecules (Allodi et al., 2012). Axons that enter the distal stump are able to grow within this permissive environment, find the target organs and reinnervate them. After complete nerve transection, reconnection of the two nerve stumps is therefore essential, because this will allow axons to regenerate through scar tissue into the distal stump. The use of an autograft to connect both stumps, when direct suture is not possible, is the gold standard repair technique. However, even when axons are able to reach the distal nerve stump, they are often misdirected and innervate the incorrect target organs. In these cases, although a significant degree of regeneration does occur, functional recovery is quite limited.

Artificial nerve guides, although widely studied in experimental models, are inferior to autografts (reviewed in Lundborg, 2000). Nerve repair with guides containing different matrices and/or glial cells has been extensively studied experimentally (reviewed in Deumens et al., 2010). Gene therapy is a relatively recent approach to study the regenerative effects of over-expression of specific therapeutic proteins on the injured nerve. The introduction of genetically engineered glia cells overexpressing trophic and tropic factors in nerve guides may improve their pro-regenerative properties and guides filled with genetically modified cells more closely resemble the autograft (Eggers et al., 2013; Godinho et al., 2013; Gravvanis et al., 2005; Hu et al., 2010; Hu et al., 2005; Kokai et al., 2011; Li et al., 2006; May et al., 2013; Santosa et al., 2013; Shakhbazau et al., 2012a; Shakhbazau et al., 2012b; Yu et al., 2009). Adenoviruses were the first viral vectors used to successfully transduce Schwann cells in a peripheral nerve (Dijkhuizen et al., 1998; Shy et al., 1995). Later, the development of lentiviral (LV) vectors (Naldini et al., 1996) has resolved the problems related to vector-induced immune responses and neurotoxicity. Moreover, LV vectors are able to infect Schwann cells in the peripheral nerve (Eggers et al., 2013; Eggers et al., 2008; Tannemaat et al., 2008); reviewed in (Mason et al., 2011). Overexpression of factors that selectively enhance regeneration of motor or sensory neurons may help to overcome the limited functional recovery after nerve injury and surgical repair, by enhancing appropriate regeneration towards muscle and sensory targets respectively. *In vitro* studies performed in our laboratory revealed a potent effect of the low molecular weight isoform of fibroblast growth factor 2 (FGF-2) on motor but not sensory neuritogenesis (Allodi et al., 2013). FGF-2 is secreted by

fibroblasts and Schwann cells and interacts with components of the extracellular matrix, in contrast to most other neurotrophic factors that diffuse over relatively long distances (Ornitz and Itoh, 2001). FGF-2 has been suggested as a promising trophic factor to enhance motor axon regeneration *in vivo* (Haastert et al., 2006). The aim of the present study was to produce a LV vector to direct the expression of FGF-2 to Schwann cell transplants, and to assess the effect of FGF-2 overexpression in these transplants on axon regeneration in a model of sciatic nerve injury and repair.

Material and Methods

LV vector production

A lentiviral vector encoding the FGF-2 low molecular weight (18 kDa) isoform (LV-FGF2) was produced using the pRRL-MCS vector containing the Woodchuck post-transcriptional regulatory element (Brun et al., 2003). The NheI/XmaI FGF-2 cDNA excised from a pCI-neo-bFGF vector (a generous gift of Prof. Claudia Grothe) was cloned into an XbaI/MscI opened LV transfer vector. FGF-2 expression was under the control of the human cytomegalovirus (CMV) promoter. The LV plasmid was sequenced to verify the sequence and orientation of the insert.

Lentiviral vectors were generated as described previously (Naldini et al., 1996). pRRL-MCS, encoding FGF-2 (20 μg), the VSV-G envelope protein vector pMD.G.2 (7 μg) and the viral core-packaging construct pCMVdeltaR8.74 (13 μg) were co-transfected in 293T cells with Isocove's Modified Dulbecco's Medium (IMDM) (Sigma) containing 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin and 2 mM glutamine (all from Gibco). The next day, medium was replaced. Viral particle-containing medium was harvested 30h later and cellular debris were removed from the medium through low-speed centrifugation at 1000 rpm in an Eppendorf centrifuge (Eppendorf 5810R, Hamburg, Germany) for 5 min and filtering through a 0.22 μm cellulose acetate filter. The filtered supernatant was then concentrated by ultracentrifugation in an SW-28 rotor (Beckman Coulter BV, Mijdrecht, The Netherlands) at 20.000 rpm for 2.5 h. The viral particle-containing pellet was resuspended in 0.1 M phosphate buffered saline pH 7.4 (PBS) and aliquoted and stored at -80°C until further use. The number of transducing particles of the viral stocks (titer units) was defined by infecting 293T cells upon serial dilution and determining the number of transducing units per ml (TU/ml) by immunocytochemistry. 293T cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and after several washes and 1h blocking in

TBS/5% calf serum/0.4%Triton X-100 incubated with primary antibody against FGF-2 (Upstate – Millipore, 1:200) for 2h at room temperature. After several washes, cells were incubated with rabbit anti-mouse HRP secondary antibody (Dako, 1:100) for one hour and reaction was developed with the VIP kit (Vector Laboratories). The cell counting gave a titer in the order of 10^9 TU/ml. For additional titering, viral vector stocks were analyzed for p24 content with an ELISA assay (ZeptoMetrix Corporation, 0801111). The ratio between the TU/ml and p24 content of the LV-FGF2 stock was used and the final titer was estimated to be at the range of $2x10^9$ viral particles/ml.

Schwann cell culture

Dissociated Schwann cells were prepared from inbred adult female Fischer F344 rats (Harlan, the Netherlands), as described previously by Morrissey et al. (1991). Sciatic nerves were dissociated and kept in cold Leibovitz-15 (L15) medium (Gibco) and cleaned from connective tissue. Nerves were cut into small pieces and stripped off from the epineurial sheaths and transferred in 35mm cell culture dishes (Greiner Bio-one) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin (1%P/S) (all from Gibco). Once a week (for approximately 5 weeks) the nerve pieces were transferred in new culture dishes and fresh medium in order to eliminate the presence of migrating fibroblasts. Then the nerve pieces were incubated overnight in DMEM-10%FCS-1%P/S supplemented with 1.25 U/ml dispase (Roche) and 0.05% collagenase (Invitrogen). The day after nerves were washed twice in DMEM-10%FCS-1%P/S and dissociated by pipetting thoroughly the explants through a 10 ml stripette (Greiner bio-one) and seeded into 10 cm^2 Poly-L-Lysine (Sigma) coated culture dishes in DMEM-10%FCS-1%P/S supplemented with mitogens (2 uM/ml Forskolin and 20 μg/ml pituitary extract, all from Sigma). The purity of the cultures (>90%) was confirmed with rabbit anti-S100 direct immunofluorescent staining (1:500 from Dako) followed by 1h incubation with donkey anti-rabbit Cy3 conjugated secondary antibody (1:700 from Jackson ImmunoResearch). LV-FGF2 was added at a multiplicity of infection (MOI) of 0, 5, 10, 20, 50 and 100. Cells were kept at 37°C and 5% CO2. Subsequently, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and stained for FGF-2 as described above. Images were taken with a confocal laser scanning microscope (SP5, Leica).

LV-FGF2 expression in vitro

293T and Schwann cells were passaged to 60-70% confluence and then infected with LV-FGF2. The vector was added at a MOI of 0, 5, 10, 20, 50 and 100. Medium was changed after 24 h. After 7 days, cells were lysed with RIPA buffer (25 mM Tris-HCl pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1mM EDTA) containing 10 mg/L protease inhibitors (Roche Diagnostics GmbH), and the protein suspension obtained was analyzed by Westerblot (WB). The protein extract of medium and lysated cells was separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Whatman). The membranes were blocked 0.1M PBS supplemented with 5% non-fat dry milk for 1h at RT and incubated with primary antibodies anti FGF-2 and anti-β-actin (1:5000, Sigma) at 4°C overnight. Following several washes with 0.1M PBS-0.05%Tween20 blots were probed with Cy-5-conjugated anti goat IgG secondary fluorescent antibody (Jackson ImmunoResearch) and anti mouse IgG secondary IR Dye 800 (LI-COR biosciences). Bands were visualized using the Odyssey Infrared Imaging System (LI-COR biosciences).

Quantitative analysis of FGF2 was performed in both 293T and Schwann cells transduced at the same MOIs described above (0 to 100). Schwann cells were cultured for 7 or 15 days, and 293T cells for 7 days. Conditioned medium and cell lysated were collected and the concentration of FGF-2 was measured using an FGF-2 ELISA kit (R&D, DuoSet DY233) according to the manufacturer's instructions. The absorbance was read at 450nm using a microplate reader (Varioskan Flash, Thermo, Finland).

Spinal cord organotypic slices and DRG explants co-culture

Co-cultures of spinal cord slices and DRG explants were prepared as described previously (Allodi et al., 2011). Schwann cells were cultured for 4 days and then transduced with FGF-2 or GFP encoding LVs using a MOI of 50 during one night. Medium was refreshed the next day and the cells kept at 37°C and 5% CO2 for 3 more days. Then cells were trypsinized, centrifuged and re-suspended in 1 ml of medium. Cells were counted in a Neubauer chamber and a number of $10x10^4$ or $50x10^4$ was kindly mixed into the collagen solution. Spinal cord slices and DRG explants were placed within the collagen matrix and kept in culture for 4 days. Then, they were fixed and immunolabeled with RT97 antibody (Developmental Studies Hybridoma Bank, Iowa, USA). Pictures were taken with a confocal microscope (SP5, Leica) and neurite elongation and arborization measured as previously described (Allodi et al., 2011).

Quantification of LV-FGF2 expression in vivo

All rats were housed under standard conditions, maintained in a 12 hr light/dark cycle, and had *ad libitum* access to water and food. All experimental procedures were conducted in accordance with the guidelines of the local animal welfare committee for use and care of laboratory animals.

For *in vivo* quantification of LV-FGF2 expression, the LV-vector was injected into the sciatic nerve as previously described (Tannemaat et al., 2008), and transgene expression was analyzed 1 week later. Two months old Wistar rats (Harlan, Horst, The Netherlands) were deeply anesthetized with Isofluorane (Isoflo, Abbott, Hoofddorp). Sciatic nerves were exposed under a dissection microscope and 1 µl of $2x10^9$ TU of LV-FGF2 vector solution was injected inside the nerve using a glass capillary with 80 µm diameter tip attached to a 10 µl Hamilton syringe. The same volume of saline was injected in the contralateral side as sham control. Fast green (Sigma) was added to the viral vector solutions at a final concentration of 0.5% to visualize the spread of the solution during injection. The distal point of injection was marked with an epineural 10-0 nylon suture. The wound was sutured and disinfected.

One week after the injection the sciatic nerves of 5 animals were harvested in a segment 1 cm proximal to the microsuture. From the nerve that received the saline solution, a piece of 1 cm obtained 5 cm away from the injection site was used as an additional control condition. All the segments were snapfrozen on dry-ice and stored at -80° C. For FGF-2 quantification by ELISA, the frozen nerves were grounded with an ice cold mortar containing liquid nitrogen. The tissue was resuspended in 250 µl lysis buffer (TBS containing 1% NP-40 substitute, 10% glycerol, 0.1% Tween-20, 0.5 mM Sodium Orthovanadate and Roche total protease inhibitor). Samples were vortexed 3 times at 4°C, centrifuged and the supernatant stored in 50 µl aliquots at -20° C. The concentration of FGF-2 was measured by ELISA as indicated above. The concentration of FGF-2 in the nerve was calculated and expressed as pg FGF2/cm nerve. The detection limit of the ELISA was 1.5 ng/ml.

Immunohistochemistry of transduced nerves

Two other rats were anesthetized, the sciatic nerves exposed and injection of LV-FGF2 in one nerve and saline in the contralateral nerve was performed as above. One week later, animals were deeply anesthetized with pentobarbital and perfused

transcardially with 4% paraformaldehyde in PBS. Both sciatic nerves were carefully harvested and cryoprotected in 30% sucrose at 4°C. Nerves were cut in 15 μm thick longitudinal sections with a cryostat (Leica) and collected onto gelatin-coated glass slides. Slices were incubated overnight with primary mouse anti-FGF-2 (1:200) and rabbit anti-laminin antibodies (1:1000, AbSerotec). After several washes, the secondary antibody (FITC antirabbit, 1:200, Vector, and Cy3 antimouse, 1:200, Jackson) was applied to the sections and incubated for 2 h. Sections were washed and coverslipped with Mowiol (Sigma).

In vivo effects of transduced Schwann cells

To evaluate the effects of LV-FGF2 *in vivo*, primary Schwann cells transduced with LV-FGF2 or LV-GFP were added to a collagen solution supplemented with DMEM 10X at a density of 18,000 cells/μl. A volume of 23 μl was gently placed into a silicone tube of 8 mm length and 2 mm internal diameter, and kept at 37ºC for two hours to allow the matrix to gel. Two months old Sprague Dawley rats (from the Animal Facilities of the Universitat Autonoma de Barcelona) were anesthetized and the right sciatic nerve exposed. A 6 mm long segment was resected and the gap repaired with a silicone tube prefilled with a collagen matrix containing 400,000 transduced Schwann cells. The cut stumps were fixed into the ends of the silicone conduits with 9-0 suture leaving a 6 mm long gap (measured with a microruler).

To corroborate that Schwann cells were effectively transduced, two animals per group were sacrificed after one week and the content of the tube (regenerating cable and matrix) was snap-frozen and the protein extract was collected as previously described. FGF-2 expression was quantified by ELISA.

The remaining animals (n=10 in group LV-GFP and n=9 in group LV-FGF2) were reanesthetized after one month, the sciatic nerve was exposed again and sectioned 4 mm distal to the tube. The proximal stump was maintained in a basin containing True Blue retrotracer (Invitrogen) for 1h. One week later, the animals were transcardically perfused with a solution of 4% paraformaldehyde in PBS. The lumbar segment of the spinal cord, the right L4 and L5 DRG and the tube containing the regenerated nerve were removed. After 1 h post-fixation all the samples were moved to a cryo-protective solution of 30% sucrose in PBS. Longitudinal sections of the spinal cord (40 μm thickness) and of the DRG (15 μm) were obtained with a cryostat and collected on glass slides. Retrogradely labeled motor and sensory neuron cell bodies were counted under an epifluorescence

microscope. The obtained number was corrected with Abercrombie factor (Abercrombie, 1946) to estimate the total number of motor and sensory neurons traced with True Blue.

In addition, the silicone tube was carefully removed and the regenerated nerve was fixed in glutaraldehyde-paraformaldehyde (3%/3%) in PBS, postfixed in OsO4 (2%) and dehydrated through ethanol series. The samples were then processed for embedding in Epon. Transverse semithin sections of the entire nerve at mid-tube were made with an ultramicrotome (LKB 6802), stained with toluidine blue and examined under light microscope. Images were acquired with an Olympus DP50 camera connected to a computer. For estimating the total number of regenerated myelinated fibers, fibers were counted in systematically selected fields covering 40% of the total nerve area (Gomez et al., 1996). Counting and measurement of the transverse area of the nerve were made using ImageJ software (NIH). The total number of myelinated fibers in the nerve was estimated from the area occupied by the fibers in the counted fields.

Another subset of Sprague Dawley rats (n=5 for each group) was followed for 67 days after the surgical procedure, and weekly electrophysiological tests were performed to evaluate reinnervation of tibialis anterior and plantar interosseus muscles. Briefly, animals were anesthetized, placed on a warm plate, and the sciatic nerve was stimulated percutaneously through a pair of needle electrodes at the sciatic notch, and the compound muscle action potential (CMAP) was recorded from both muscles. The evoked CMAPS were amplified and displayed on a digital oscilloscope (Tektronix 450S). At the end of follow up, True Blue tracing was applied as described above to the distal tibial nerve (at the ankle level) to estimate the amount of neurons regenerating axons to the paw. After one week, animals were perfused and the lumbar segment of the spinal cord and L4-L5 DRG were harvested to analyze the number of traced motor and sensory neurons (as described above).

Statistical analyses

Statistical analyses were performed with Prism software (GraphPad). One and two ways ANOVA with Bonferroni posthoc test (where needed) were used. A p value lower than 0.05 was considered significant.

Results

Characterization of LV-FGF2 in vitro

The titer of the LV-FGF2 stock used in this study was $2x10^9$ tu/ml. Schwann cells infected with increasing MOI of LV-FGF2 expressed increasing amounts of FGF-2, measured by immunohistochemistry (Fig. 1A-E) and Western blot (Fig. 1F). Schwann cells infected with LV-FGF2 showed increased proliferation, morphological elongation and boundary formation. We also found an increase in the levels of actin with increasing MOIs, thus corroborating that cell proliferation was dependent on the amount of viral particles per number of cells (Fig. 1G). This increased proliferation by FGF2 was specific for Schwann cells, since when 293T cells were transduced, we did not observe increased levels of actin as a function of increasing MOIs (Fig. 1H). Medium and lysates collected from cells 7 days post-infection, medium (Fig. 2A) and lysates (Fig. 2B) of 293T cells contained increased levels of FGF-2 already at low MOIs of 5 as measured with an ELISA. In Schwann cells, the amount of FGF-2 present in the cell lysates increased from MOI of 20, and reached the same level as observed in 293T cells at a MOI of 100 (Fig. 2B). In contrast, media of transfected Schwann cells contained relatively low levels of FGF-2, even following transduction at high MOIs (50 or 100; Fig 2A). The low levels of FGF-2 in Schwann cell media compared to lysated is probably due to the fact that basal lamina of these cells can trap FGF-2 (see discussion for details). To study the time course of FGF-2 levels in Schwann cells we therefore measured levels in cell lysates. We found that FGF-2 levels were still significantly higher at 15 days when using MOIs from 20 to 100 (Fig. 2C).

Biological activity of LV-FGF2 in vitro

To investigate the effect of LV-mediated overexpression of FGF2 on motor and sensory neuron outgrowth, Schwann cells infected with LV-FGF2 or with LV-GFP (MOI of 50) as control, were mixed in the collagen matrix embedding spinal cord slices and DRG explants. In co-cultures of spinal slices and Schwann cells overexpressing FGF-2 neurite elongation and arborization of motoneurons was enhanced, whereas co-culturing with DRG explants did not promote sensory neuron outgrowth (Fig. 3). Co-cultures of spinal cord explants with Schwann cells that expressed lentiviral vector-derived FGF-2 exhibited significantly enhanced neurite elongation (40% increase; $p<0.05$), and arborization (55% increase; $p<0.05$) (Fig. 3E-H) as compared to co-cultures with Schwann cells expressing GFP.

LV-mediated FGF2 expression in vivo

To determine the capability of LV-FGF2 to direct FGF2 expression *in vivo* and to study the distribution of FGF2 in the nerve, LV-FGF2 was injected in the intact rat sciatic nerve. FGF-2 expression was studied by immunohistochemistry (Fig. 4A-E) and ELISA (Fig. 4G) and compared with expression levels in intact nerves injected with LV-GFP or saline. Seven days after the injection of the vector, FGF-2 expression was increased as observed in longitudinal sections of the nerve. FGF-2 co-localized with laminin (Person's correlation of 0.52 ipsilateral side, 0.20 contralateral side), indicating that a relatively high amount of the protein was localized in the basal lamina of the Schwann cells, in contact with the axons (Fig. 4E, F vs B, C). At the same time point, the amount of FGF-2 in the nerve was significantly increased as measured in nerve protein extracts by ELISA, reaching a concentration of 15.4 ± 2.5 ng per cm of nerve (Fig. 4G; $p<0.001$).

In vivo effects of transduced Schwann cells

In order to investigate the potential role of FGF2 on motor neuron regeneration after peripheral nerve injury, we used an *in vivo* model of axotomy and repair. Proximal and distal stumps of a rat transected sciatic nerve were repaired with a silicone conduit filled with collagen solution that contained a suspension of Schwann cells transduced with LV-FGF2 or LV-GFP at a density of 18,000 Schwann cells/µl. LV-GFP transduced Schwann cells were used as control condition and to visualize the distribution of the Schwann cells into the tube under a fluorescence inverted microscope prior to implantation of the conduit. One week after the surgery, two animals were sacrificed and their neural conduits collected in order to evaluate FGF-2 expression in the graft. FGF-2 levels were 6.3 ± 2.8 ng in the LV-GFP conduits and 15.7 ± 5 ng in the LV-FGF2 conduits, thus corroborating FGF-2 over-expression by transduced Schwann cells present in the silicone tube.

Four weeks after surgery, the regenerative cable in the middle of the silicone tube consisted of a rounded nerve, with a thick perineurium and multiple mini-fascicles corresponding to an early stage of regeneration. In the group treated with Schwann cells transduced with LV-GFP all but one of the animals presented regenerated myelinated fibers in the cable, and in all animals there were numerous unmyelinated fibers at the mid level of the tube (Fig. 5A). The density of myelinated fibers in the LV-GFP group was quite variable, with 5 of the 10 rats having less than 1000 fibers with a mean estimated number of axons in the group of 3250±1014 (mean±SEM). In the LV-FGF2 group the caliber of regenerated nerves inside the tube was larger and contained higher number of

axons (Fig. 5B); all the animals had more than 1000 regenerated myelinated fibers, with a mean number of axons of 4187±809. However, this increase was not statistically significant.

To evaluate the number of motor and sensory neurons that regenerated their axons, we applied True Blue tracer 4 mm distal to the conduit. The quantitative analysis of labeled neurons, one week after applying the tracer, revealed marked variability of regeneration between animals. Over-expression of FGF2 resulted in a slight increase in the number of motoneurons but not of sensory neurons, but these differences were not statistically significant. Therefore, we also evaluated regeneration at a longer post-lesion time (two months), and at a more distal level. The electrophysiological results showed that reinnervation of tibialis anterior muscle (Fig. 7A) was higher in the LV-FGF2 than in the LV-GFP group, being significantly higher from 50 days until the end of the experiment ($p=0.01$). For the more distal plantar muscle, values of CMAP amplitude were significantly higher in the LV-FGF2 group, (final values of 0.5 ± 0.2 mV), than in the LV-GFP group (0.3 ± 0.1 mV, $p=0.001$; Fig. 7B). Application of the retrograde tracer at the tibial nerve at the ankle at the end of the electrophysiological analysis confirmed that animals of the LV-FGF2 group had a significantly higher number of labeled motoneurons (719±110) than animals of the LV-GFP group (386±37, $p=0.047$). The number of labeled sensory neurons was also higher in the LV-FGF2 (3637±193) compared to the LV-GFP group (2539±75, $p=0.006$). Comparatively, the percentage of increase of regenerating neurons promoted by FGF2 transduced Schwann cells had a trend to be higher for motor (46%) than for sensory (30%) neurons.

Discussion

In this study we show that a tubular nerve guide filled with Schwann cells that overexpressed FGF-2 was able to accelerate regeneration and functional recovery when used to repair the transected sciatic nerve in rats. To overexpress this factor, we generated and characterized a LV vector encoding FGF2. Lentiviral vectors are known to transduce Schwann cells and fibroblasts in the rat sciatic nerve with high efficiency (Eggers et al., 2008; Tannemaat et al., 2008). Previous *in vitro* and *in vivo* studies demonstrated that FGF2 stimulates motor axon regeneration (Allodi et al., 2013; Haastert et al., 2006). In our in vivo experiments, we found that overexpression of FGF-2 enhanced muscular reinnervation and promoted both motor and sensory regeneration. Therefore, gene

therapy to induce overexpression of FGF-2 in the injured nerve appears to be a powerful strategy to enhance axonal regeneration after peripheral nerve injury.

To study the ability of the vector to overexpress FGF-2, we used two types of cells, 293T and Schwann cells. The fact that in a confluent culture there are about 4 to 6 times more 293T cells than Schwann cells that expressed the transgene can explain why 293T cells secreted significant amounts of FGF2 in the medium when transduced at low MOIs, whereas Schwann cells needed higher MOIs and levels of FGF-2 released in the medium were lower. Interestingly, levels of FGF-2 were high in both lysates of 293T and Schwann cells at the same MOI of 100. The low levels of FGF-2 in Schwann cell media is due to the fact that this factor is not secreted as observed for most trophic factors, but it is associated with the basal lamina of Schwann cells within the extracellular matrix where it interacts with other molecules (Ornitz and Itoh, 2001). Indeed, in the *in vivo* study co-labeling of secreted FGF-2 and laminin, the main component of the Schwann cells basal lamina, indicates that FGF2 is captured in the extracellular matrix following secretion by Schwann cells. However, the fact that FGF-2 is trapped in the basal lamina of Schwann cells does not affect its ability to influence neurite outgrowth. When spinal cord organotypic slices were co-cultured with Schwann cells infected with the LV-FGF2, we corroborated the effects found in the same model when directly adding the recombinant protein, i.e. promotion of neuritogenesis in motor neurons (Allodi et al., 2013).

To study the functional role of increased FGF-2 in axonal regeneration *in vivo*, we repaired a 6 mm sciatic nerve gap in rats with silicone tubes filled with a collagen gel containing primary Schwann cells modified *ex vivo* with LV-FGF2 or LV-GFP. We found that high numbers of motor and sensory axons were able to cross the tube with overexpression of FGF2. Other studies overexpressing trophic factors, in particular GDNF, have described that regenerating axons were trapped into the region where the factor was increased, the so-called candy-store effect, and axons were unable to cross the area of high neurotrophic factor expression. Thus, although GDNF promoted local axon growth the trapping of axons in the nerve was detrimental for regeneration (Eggers et al., 2013; Santosa et al., 2013; Tannemaat et al., 2007). We did not observe this phenomenon when using FGF-2 and two main reasons can explain this difference. First of all, the trapping of axons has been mainly described when using GDNF (Eggers et al., 2013; Santosa et al., 2013; Tannemaat et al., 2007), but not other factors like NGF (Hu et al., 2010; Shakhbazau et al., 2012a), BDNF (Godinho et al., 2013) or CNTF (Hu et al., 2005).

GDNF-trapping of axons could be due to too high amounts of this factor at the site of injection. In fact, carefully timed overexpression of this factor appears to prevent this phenomenon (Shakhbazau et al., 2013). Secondly, some experimental models could be more prone to trapping of axons. When a vector was used to directly transduce the distal stump (Eggers et al., 2013; Tannemaat et al., 2007), high levels of GDNF occur in that region, exceeding the levels of GDNF and other factors from more distal segments. In contrast, when overexpressing GDNF using microspheres (Kokai et al., 2011; Yu et al., 2009) or in tubular models containing genetically modified Schwann cells (Li et al., 2006; May et al., 2013), the trapping phenomenon was not observed. In these cases, the amount of overexpressed GDNF could be lower than the levels of trophic factors found in the distal nerve stump. In fact, when a higher amount of transduced Schwann cells overexpressing GDNF was injected in an acellular nerve graft (Santosa et al., 2013), trapping of axons and reduced distal regeneration was observed.

Although we did not observe trapping of axons into the tube, we could neither detect an increased number of regenerating axons 4 mm distally to the tubes containing LV-FGF2 transduced Schwann cells. It is likely that, after one month, the regenerative front has already passed the site of tracer application in both groups of animals. In fact, we used a short gap that allows strong regeneration *per se* (Udina et al., 2004), and denervated Schwann cells are an important source of trophic factors, among them FGF-2 (Hoke et al., 2006). Furthermore, in the tubes with SC transduced with LV-GFP we detected increased levels of FGF2 compared to an intact nerve. Even though the increase in expression of FGF2 achieved by transduction with LV-FGF2 was only about 2 folds, the advantage of this extra supply of FGF-2 in this model did result in faster regeneration throught the tube and, therfore, also faster and higher reinnervation of the distal targets. When studying the effects of FGF-2 overexpression in distal segments of the nerve, we observed that rats of the LV-FGF2 group had earlier and more marked reinnervation of the denervated muscles. Reinnervation of the distal plantar muscle was more markedly increased by FGF-2 over expression than for the more proximal tibialis anterior muscle. This is important, since this suggests that the effects of overexpression of FGF-2 at the injury site improves reinnervation of distal muscles, which are usually the ones with poorer recovery (Navarro and Udina, 2009).

Our electrophysiology findings were confirmed by applying retrograde tracers at the level of the ankle at 67 days, since we observed an increased number of regenerating motoneurons. The number of DRG sensory neurons regenerating distally was also

increased in the LV-FGF2 group. Therefore, FGF-2 promoted both motor and sensory regeneration. However, when comparing the magnitude of the increase for both types of neurons, FGF-2 tends to favor motor regeneration (46% increase) over sensory regeneration (30% increase). These results suggest that FGF-2 has some preference to promote motor regeneration as observed *in vitro*. Taken together, our findings demonstrate that LV-FGF-2 is a powerful tool to enhance axon regeneration after peripeheral nerve injury when over expressed in the injured site.

It is important to note that the use of LV-FGF2 was an effective way to engineer Schwann cells used to populate an artificial nerve conduit. Moreover, direct injection of LV-FGF2 in the nerve also resulted in enhanced FGF2 expression. Interestingly, LV-vectors have been shown to transduce cells in chronically denervated nerves (Eggers et al., 2013). Chronically denervated nerves have a poor regenerative capabilities, since Schwann cells decrease trophic factor secretion after long periods of denervation. (Sulaiman and Gordon, 2000). Therefore, by transfecting these chronically denervated Schwann cells to overexpress trophic fators, we could facilitate regeneration in these situations. In fact, chronic denervation often occurs after severe injuries, and the deterioration of the distal nerve stump is one of the main limitations of successful motor recovery (Gordon et al., 2011). Chronic denervation of the most distal nerve branches is almost unavoidable in proximal injuries, as the front of regenerating axons may need months to reach these branches. Therefore, application of the LV-vector into the distal motor branches could be an approach to both reduce the effects of chronic denervation and also favor attraction of motor axons towards the correct pathway. On the other hand, LV-FGF2 can be used to engineer Schwann cells added to artificial nerve guides to improve the capability of these guides to sustain regeneration over long gaps.

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Figure legends

Figure 1. In vitro proliferation of transfected Schwann cells. Confocal micrographs of Schwann cell cultures (taken at 40x), immunostained for FGF-2 in red and DAPI (nuclear marker) in blue, revealing the different expression of the trophic factor depending on the multiplicity of infection (MOI) used to infect the cells *in vitro*. (**A)** MOI of 0, (**B**) MOI of 10, (**C**) MOI of 20, (**D**) MOI of 50, (**E**) and MOI of 100. (**F**) Western blots of lysated Schwann cells after 7 days *in vitro*. Bands at 18 (FGF-2) and 42 (actin) kDa were detected. FGF-2 expression only reached detectable levels at MOI of 50 and 100 in Schwann cells. (**G**) Quantification of actin fold increase in lysated Schwann cells. The same amount of cell lysate was added in the Western blot. Actin increased with the MOIs (represented in x axis), indicating that cell proliferation was dependent on the amount of viral particles per number of cells.(**H**) Western blots of lysated 293T cells after 7 days *in vitro*. In contrast to FGF-2 levels (band 18), actin (band 42) was not increaseing with the MOIs, suggesting that FGF2 selectively increased proliferation of Schwann cells and not other type of cells.

Figure 2. Overexpression of FGF-2 in transfected 293T and Schwann cells (**A**) Quantification of FGF-2 present into the culture media by ELISA. A higher amount of FGF-2 was secreted by 293T cells than by Schwann cells after 7 days post infection. The secretion depended on the MOIs. (**B**) Expression of FGF-2 quantified by ELISA in lysated Schwann and 293T cells at 7 days post infection. In 293T cells a plateau was reached already with a MOI of 5. In Schwann cells, the amount of FGF-2 present in the cells increased from MOI of 20, but only with a MOI of 100 reached the same level observed in 293T cells. (**C**) Levels of FGF-2 in lysated Schwann cells at 7 and 15days post infection with MOIS of 0, 20, 50 and 100. (* $p<0.05$)

Figure 3. In vitro effects of LV-FGF-2 transfected Schwann cells. DRG co-cultured with Schwann cells infected by LV-GFP (**A**) and by LV-FGF2 (**B**). Both lentiviral vectors were used at a MOI of 50. RT97 (marker for phosphorylated heavy chain neurofilament) in red, labels neurite growth from sensory neurons. Spinal cord slice co-cultured with Schwann cells infected with LV-GFP (**C**) and LV-FGF2 (**D**). Plots showing the quantification of neurite elongation and arborization of sensory (**E** and **F**) and motor neurons (**G** and **H**). Motoneuron outgrowth was significantly enhanced in co-culture with

LV-FGF2 infected Schwann cells, compared to LV-GFP (* $p<0.05$; ** $p<0.01$). In contrast, sensory outgrowth was not affected by FGF-2 secretion.

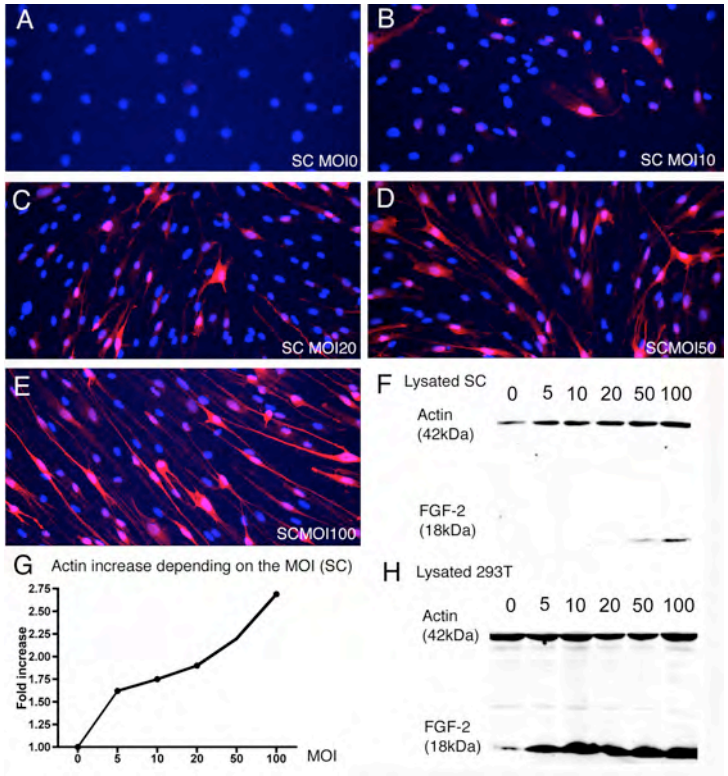
Figure 4. FGF-2 expression in naive sciatic nerves after LV-FGF-2 infection. Laminin expression in the nerve after injection of saline (**A**) and LV-FGF2 (**D**) solutions. Co-labeling for FGF-2 was performed to look at the expression of the trophic factor 1 week after infection (**B** and **E**). FGF-2 immunoreactivity was higher in the infected nerve**. (C)** and **(F)** are merged images showing co-localization of FGF-2 and laminin. Histogram in (**G)** shows the quantification of FGF-2 in control nerves and 7 days after saline injection (sham) or LV-FGF2 infection. * $p<0.05$.

Figure 5. Regenerative myelinated fibers at midtube after one month. Representative semithin transverse sections at mid tube of a rat of LV-GFP (**A**) and LV-FGF2 (**B**) groups. **(C)** Estimated number of regenerated myelinated fibers in the mid tube 37 days after repair in LV-GFP and LV-FGF2 groups. Bar=10 μm.

Figure 6. Regenerated axons distal to the tube (sciatic level) one month after surgery. (**A**) Corrected number of backlabeled motoneurons in the spinal cord and (**B**) primary sensory neurons in the DRG in animals that regenerated their axons 4 mm distal to the tube one month after the repair in the LF-GFP and LV-FGF2 group.

Figure 7. Functional recovery after LV-FGF2 application. Percentage of the amplitude of Tibialis anterior (**A**) and plantar muscles (**B**) over time in rats with sciatic nerve resection and repair with a Silicone tube filled with SC transfected with LV-FGF2 or LV-GFP. Backlabeled motor (**C**) and sensory (**D**) neurons at 67 days, after applying true blue tracer at the distal tibial nerve (ankle level). Animals with LV-FGF2 reinnervated faster their muscles and had a higher number of regenerating neurons at distal level. * $p<0.05$ group LV-FGF2 vs. group LV-GFP.

Fig 1


A
SC MOI0
B
SC MOI10
C
SC MOI20
D
SCMOI50
E
SCMOI100
F
Lysated SC
0 5 10 20 50 100
Actin
(42kDa)
FGF-2
(18kDa)
G
Actin increase depending on the MOI (SC)
Fold increase
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0 5 10 20 50 100
MOI
H
Lysated 293T
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Actin
(42kDa)
FGF-2
(18kDa)

Fig 2

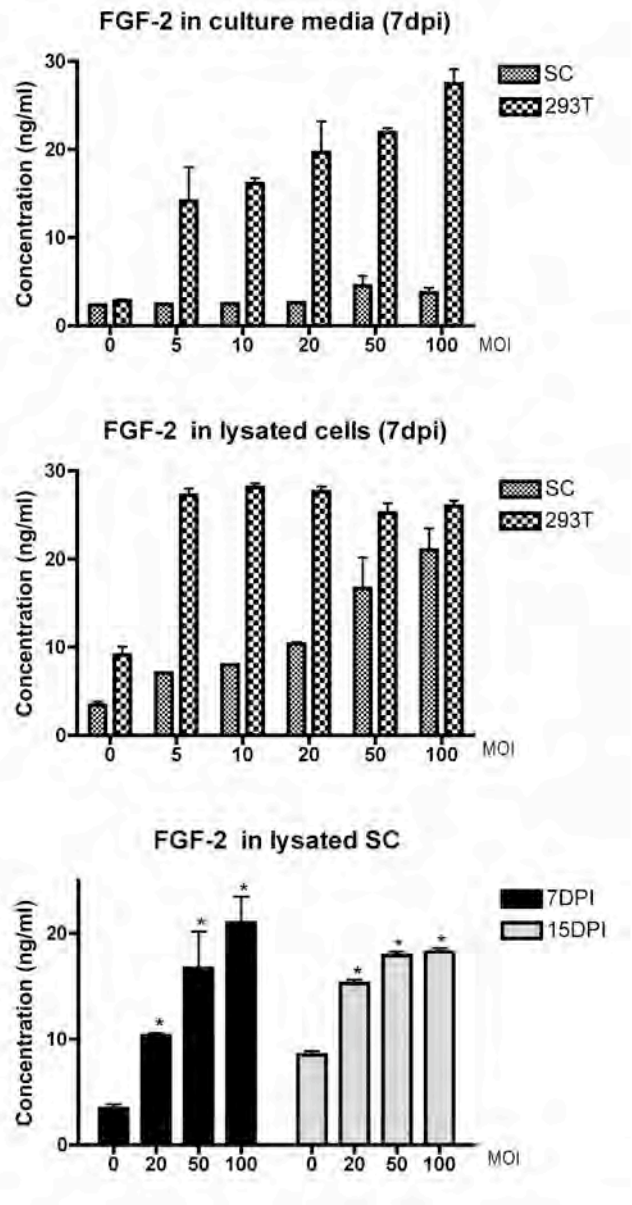

FGF-2 in culture media (7dpi)
Concentration (ng/ml)
30
20
10
0
SC
293T
0
5
10
20
50
100
MOI
FGF-2 in lysated cells (7dpi)
Concentration (ng/ml)
30
20
10
0
SC
293T
0
5
10
20
50
100
MOI
FGF-2 in lysated SC
Concentration (ng/ml)
20
10
0
7DPI
15DPI
*
0
20
50
100
0
20
50
100
MOI

Fig 3

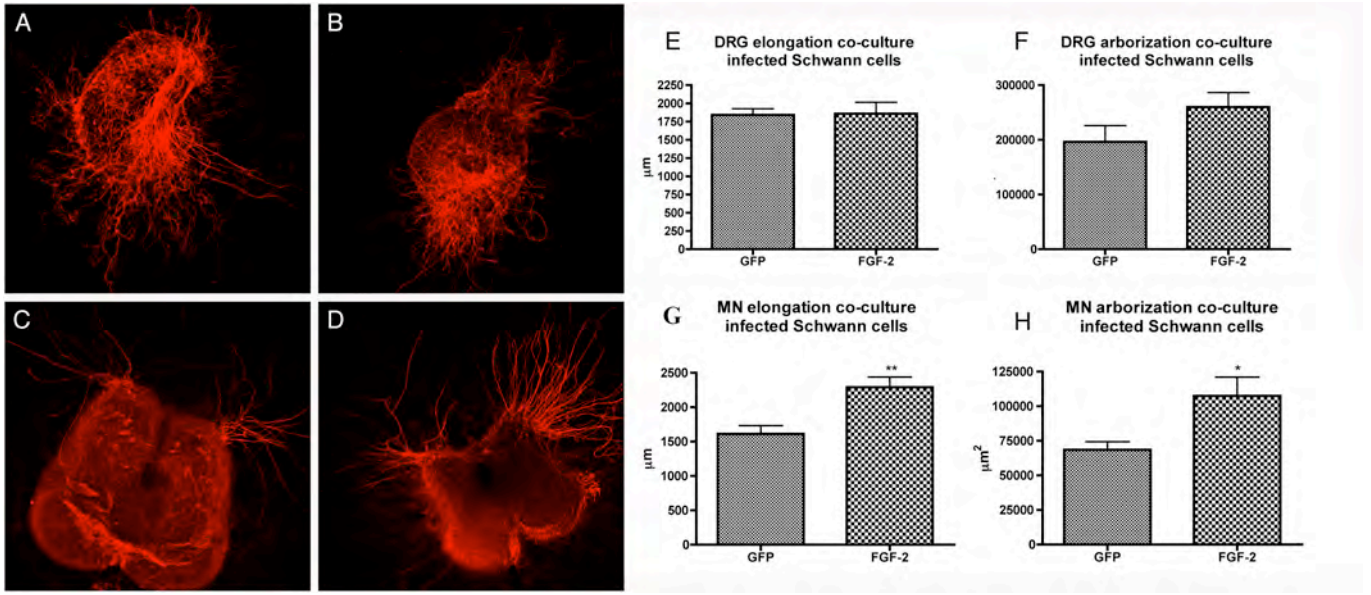
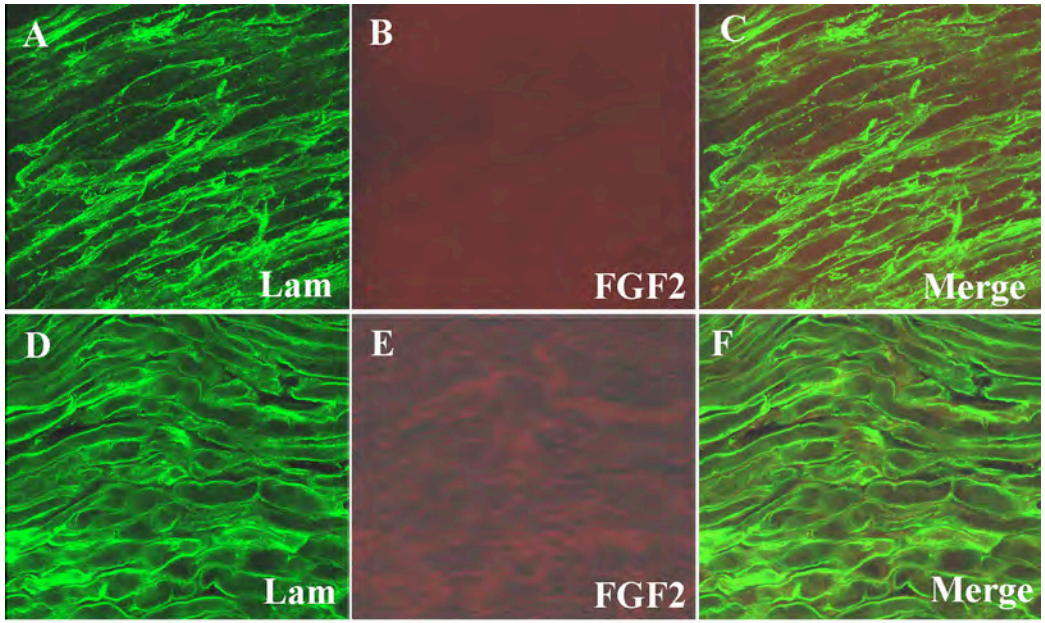

A
B
C
D
E
DRG elongation co-culture infected Schwann cells
µm
2250
2000
1750
1500
1250
1000
750
500
250
0
GFP
FGF-2
F
DRG arborization co-culture infected Schwann cells
300000
200000
100000
0
GFP
FGF-2
G
MN elongation co-culture infected Schwann cells
**
µm
2500
2000
1500
1000
500
0
GFP
FGF-2
H
MN arborization co-culture infected Schwann cells
*
µm²
125000
100000
75000
50000
25000
0
GFP
FGF-2

Fig4


A
Lam
B
FGF2
C
Merge
D
Lam
E
FGF2
F
Merge

G

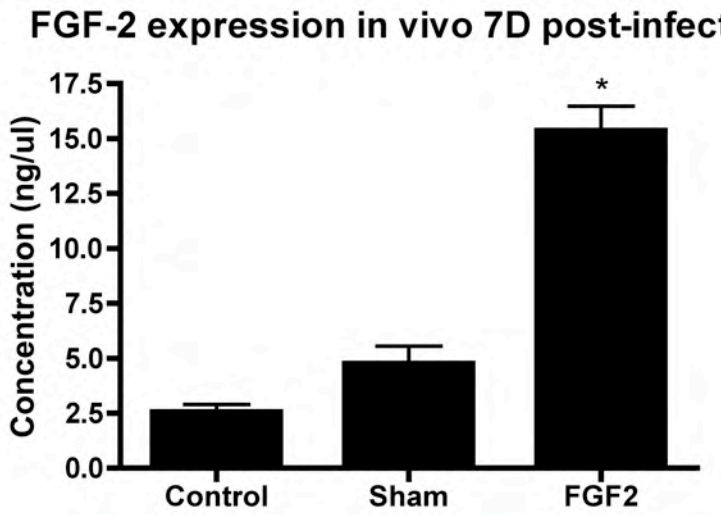

FGF-2 expression in vivo 7D post-infection
Concentration (ng/ul)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
Control
Sham
FGF2

Fig 5

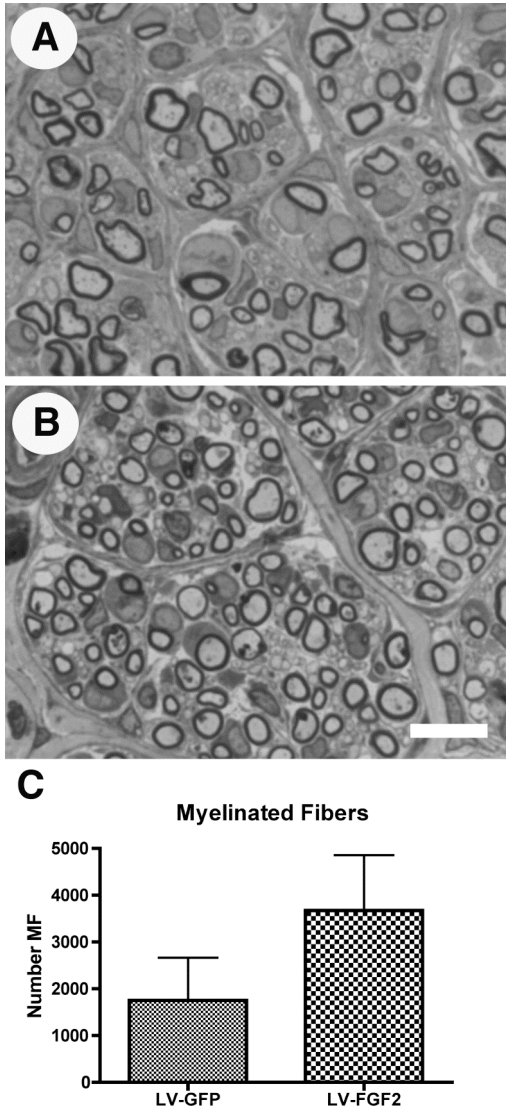
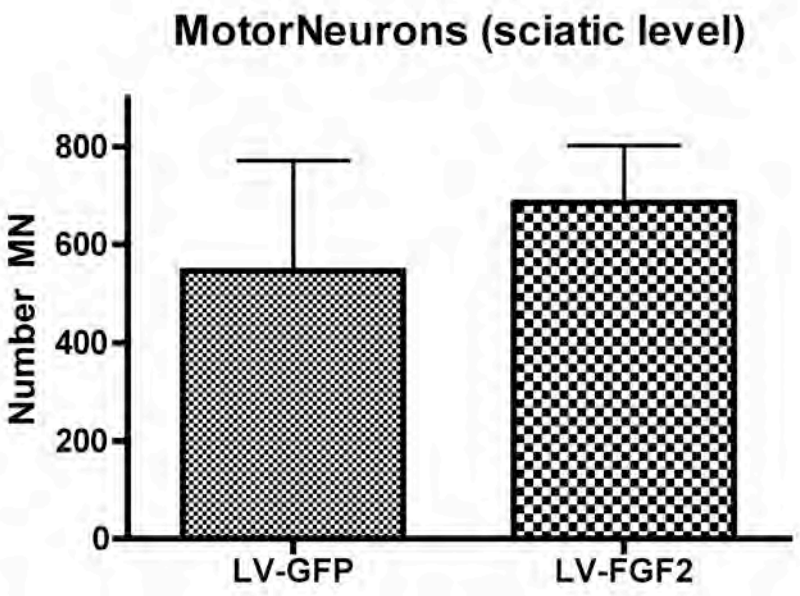

A
B
C
Myelinated Fibers
Number MF
5000
4000
3000
2000
1000
0
LV-GFP
LV-FGF2

Fig 6


MotorNeurons (sciatic level)
Number MN
800
600
400
200
0
LV-GFP
LV-FGF2

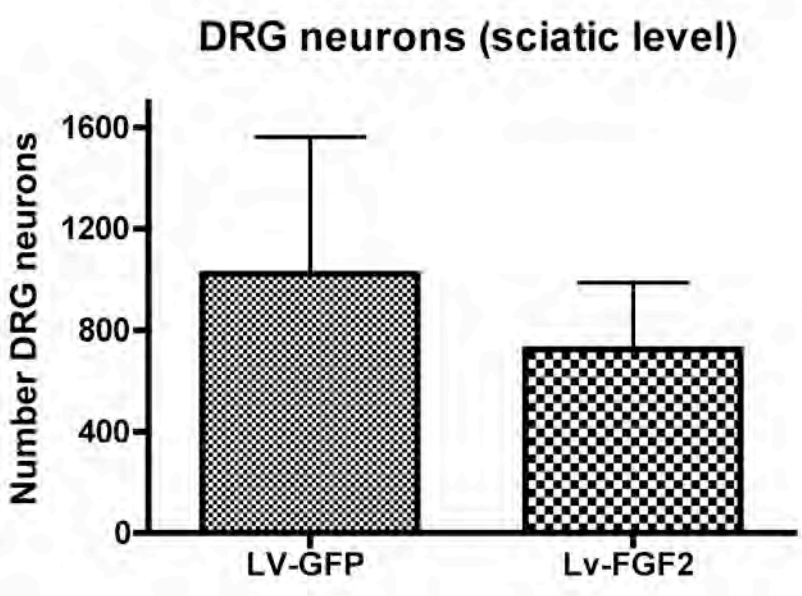
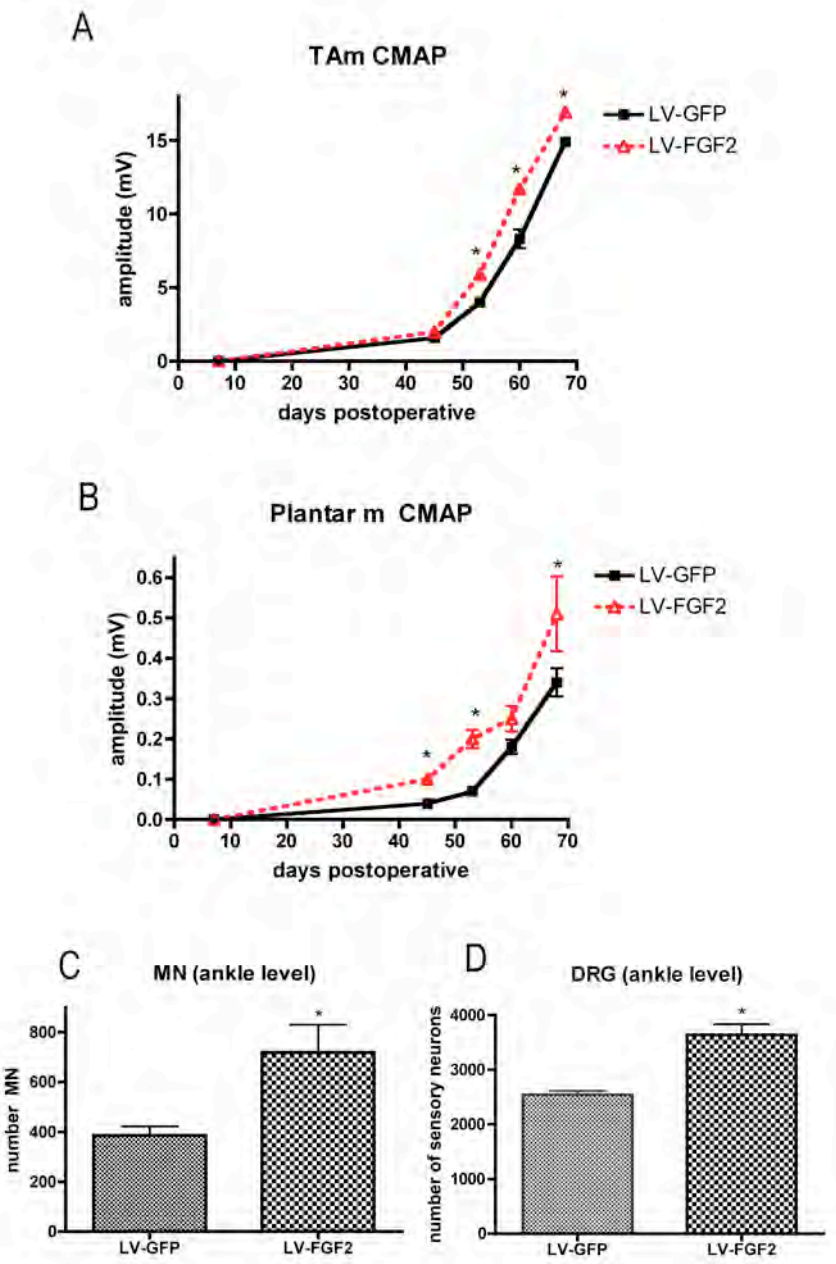

DRG neurons (sciatic level)
Number DRG neurons
1600
1200
800
400
0
LV-GFP
Lv-FGF2

Fig 7


A
TAm CMAP
amplitude (mV)
days postoperative
LV-GFP
LV-FGF2
B
Plantar m CMAP
amplitude (mV)
days postoperative
LV-GFP
LV-FGF2
C
MN (ankle level)
number MN
LV-GFP
LV-FGF2
D
DRG (ankle level)
number of sensory neurons
LV-GFP
LV-FGF2