Final report on the NKFI project 117031 entitled “Fostering regeneration and functional improvement in the injured spinal cord by a novel, stem cell secretome-based combined treatment

The aim of the project was to investigate whether a treatment of a spinal cord contusion injury induced in rats with a lesion-induced secretome of the clonal neuroectodermal stem cell line NE-GFP-4C can produce the same morphological and functional improvement as grafting of the stem cells directly into the lesion cavity. The significance of this study is marked by the possibility to replace an ethically and/or clinically potentially risky stem cells treatment with a controllable pharmacological application.

We have provided evidence that the NE-GFP-4C stem cell line is very strongly supports the morphological and functional improvement after a contusion injury in the rat spinal cord. The mechanism of action is based upon the composition of the secretome that the grafted cells produce in the injured cord and therefore this set of secreted molecules is called lesion-induced secretome. Cellular replacement by the derivatives of the grafted stem cells play little if any effect in the recovery after such lesion. Moreover, it is clear that the secretome is produced only in the first 7 to 10 days after grafting, ie. as long as the majority of the grafted cells remain undifferentiated. Accordingly, we have set up our experimental model in a way that we could mimic the beneficial effects of the stem cells by simultaneous expression/delivery of the four secretome factors (GDNF, IL-6, IL-10 and MIP1-alpha) for 10-14 days. In order to investigate the effects of the lesion-induced secretome the following experimental groups have been set up:

a) NE-GFP-4C grafted animals (positive control group)

b) contusion injury only (negative control for all groups)

c) all the 4 factors delivered into the contusion cavity by means of miniature osmotic pumps

d) IL-10 alone delivered via osmotic pumps

e) saline delivered through the use of osmotic pumps (control of the groups c and d)

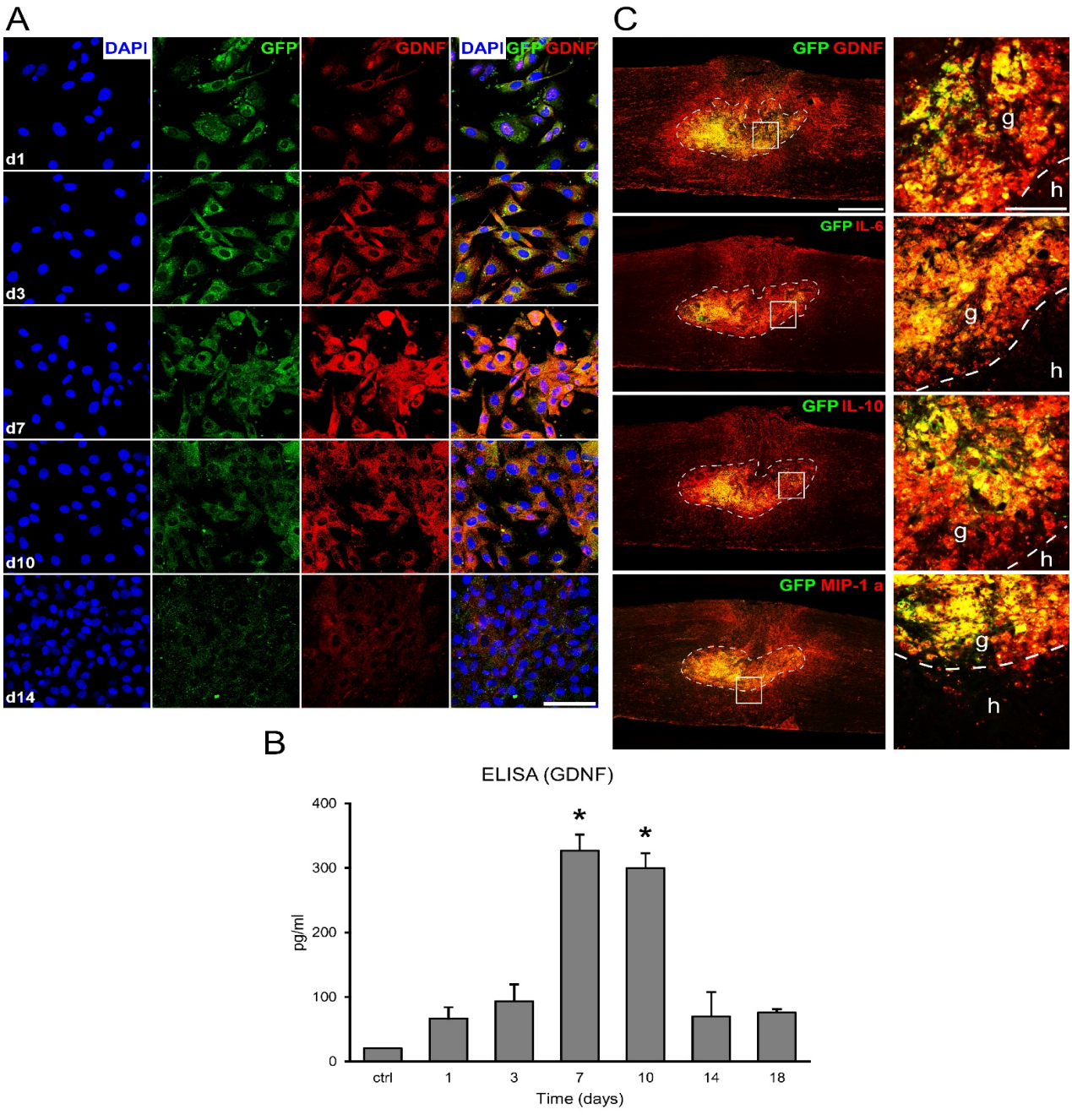
f) transfected fibroblast (Sleeping Beauty-transfection) grafted into the cavity

g) untransfected fibroblasts grafted into the cavity (control of the Sleeping Beauty-transfected fibroblasts)

To find out the dose of the factors to be delivered to the site of lesion (ie. into the contusion cavity) we have set up a dose-response curve of IL-10 administered to the injured cord via osmotic pumps (Alzet, type 1020). Based upon the literature it was expected that all the four factors are effective in the dose of IL10. Administration of various doses of IL-10 (7.5 to 45 ng/day) produced a dose-dependent increasing functional recovery of the treated animals with little difference between doses of 22.5 and 45 ng/day of IL-10 (Suppl. Fig. 1). Therefore we decided to use in these experiments 22.5 ng/day of each factor.

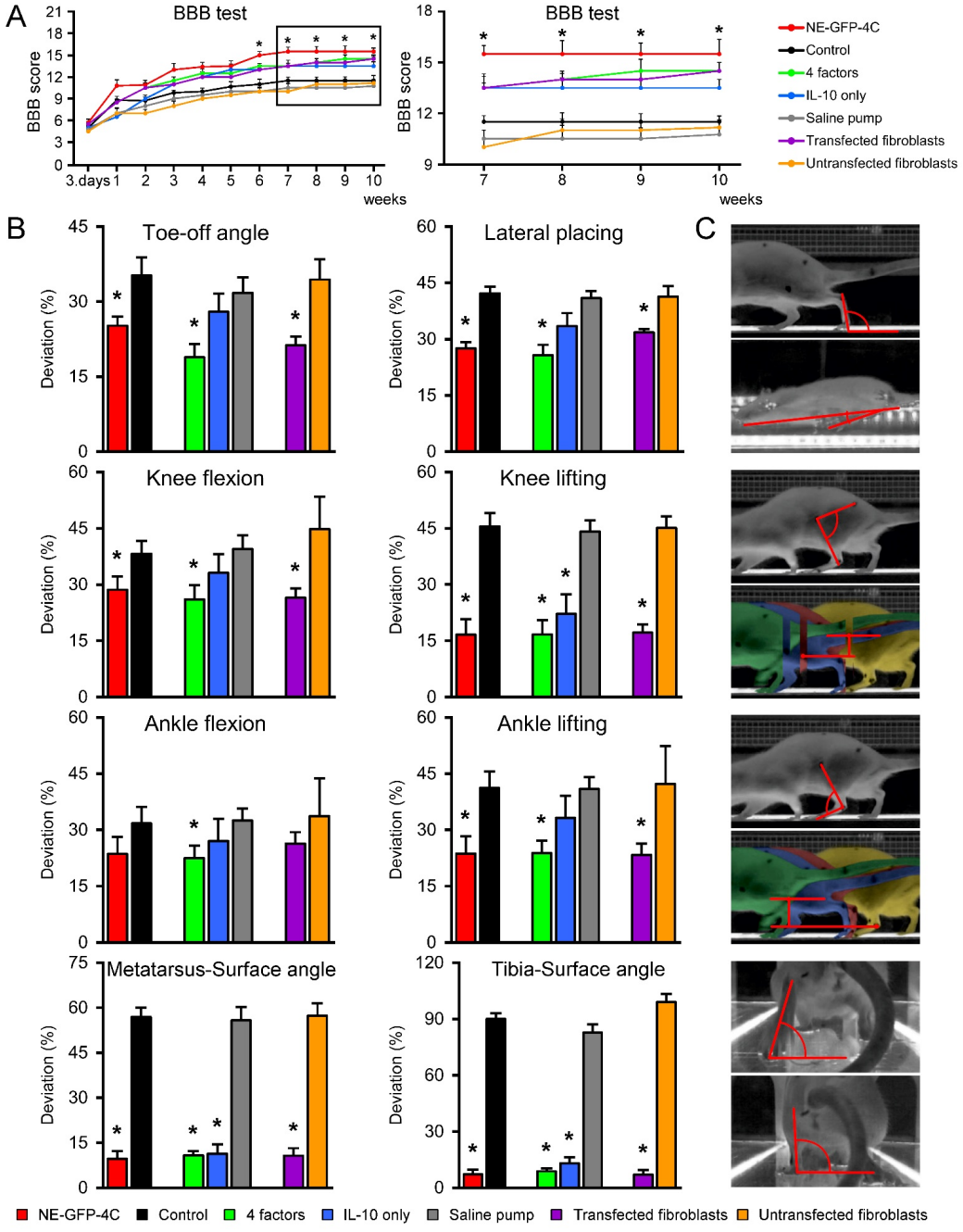
To verify the biological action of the Sleeping Beauty vector, first in vitro experiments have been performed to determine the amount and the time period of secretion of the factors. Embryonic rat fibroblasts transfected with the Sleeping Beauty vector carrying the genes for all the 4 factors expressed all factors simultaneously for approximately 12 days. The increasing and then decreasing gene expression pattern was verified immunhistochemically in vitro and also after grafting the transfected cells into the cord (Fig. 1A,C). ELISA measurement of GDNF expression from transfected fibroblasts showed a peak expression on days 7 and 10 after transfection (Fig. 1B). As all the 4 genes were coupled to a common promoter in the Sleeping Beauty vector, all genes were equally and simultaneously expressed (Fig. 1A).

Fig1.



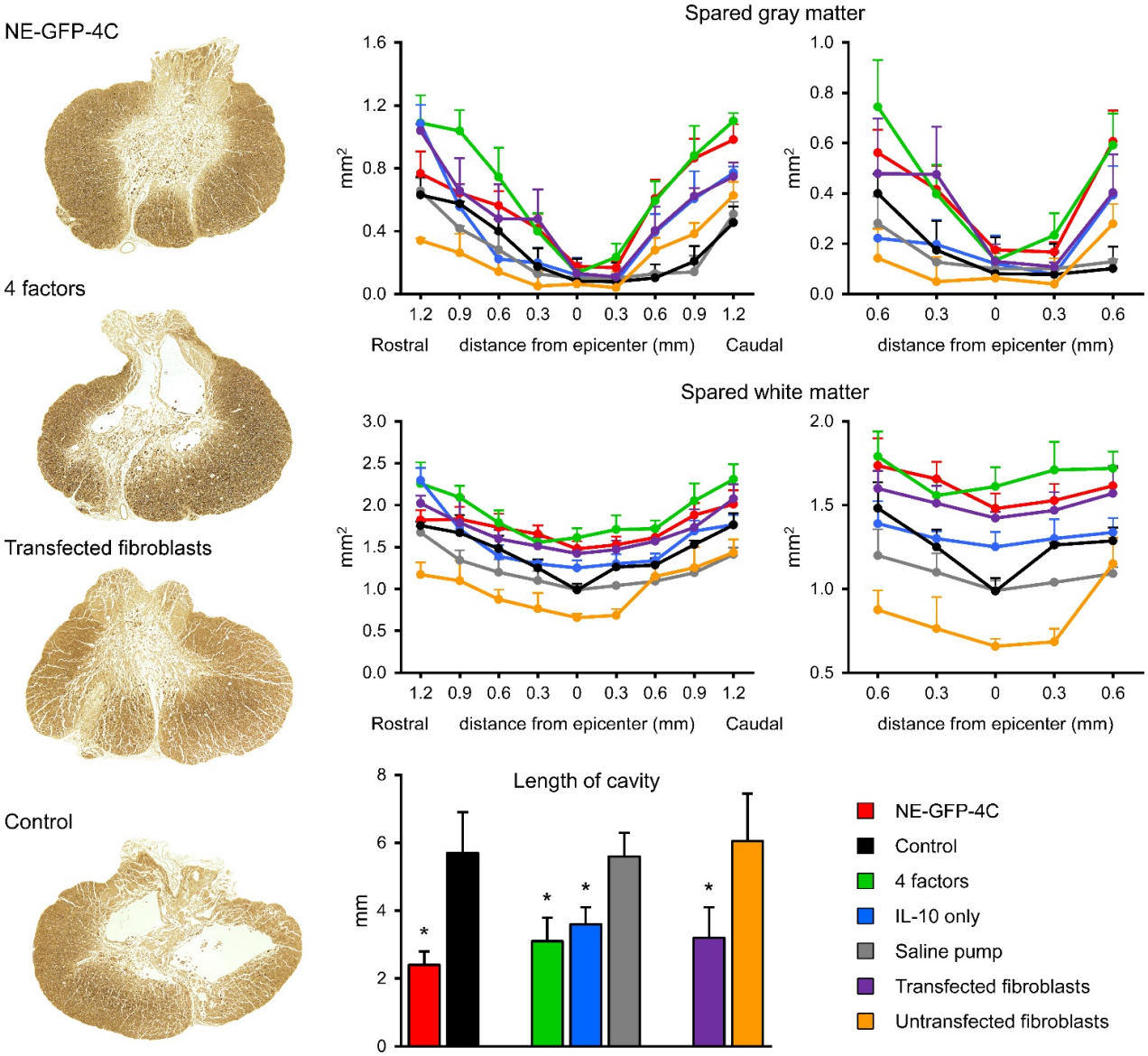
Next the functional and morphological improvement induced by the various therapeutical approaches were studied. For functional analysis we used a general open-field test (BBB) and a detailed kinematic lower limb locomotor analysis (our custom-developed method). All the 3 pharmacological treatment groups (IL-10 alone, 4 factor-treatment and transfected fibroblast grafting) performed significantly better compared to their controls and the grafted NE-GFP-4C cells seemingly improved the general locomotor pattern to a slightly better extent in the BBB test than the secretome-based administrations (Fig. 2A). However, this differential improvement between the cellular and secretome-based therapies have not been confirmed by the detailed locomotor test, where both therapeutic approaches showed equally prominent improvement compared with their controls (Fig. 2B).

Fig2.



The functional improvement was justified by the morphological features of the contusion cavity. Three major parameters (amount of spared white and grey matter and length of cavity) proved to be significantly improved in the treatment groups as compared with their controls. Moreover, treatment of the injured cords with the 4 factors, the NE-GFP-4C cells and the transfected fibroblasts were superior to the IL-10 treatment alone (Fig. 3).

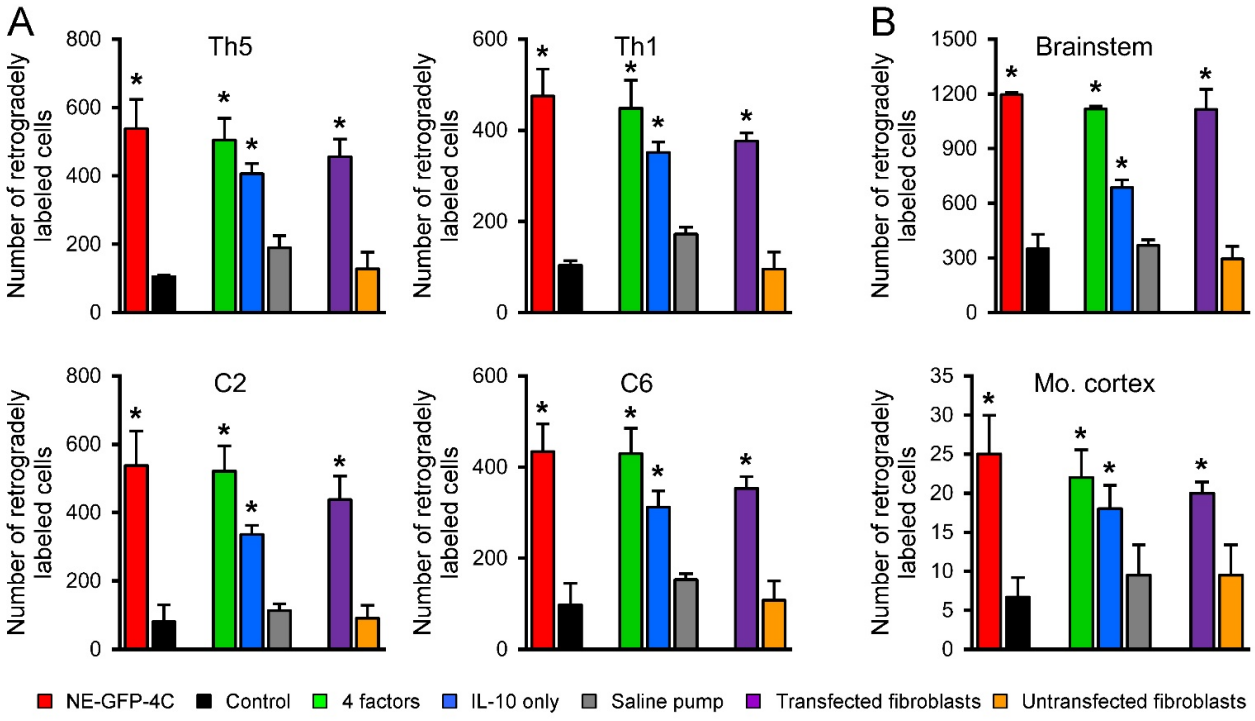
Fig3.



The extent of regenerating and spared spinal axons of various tracts was examined by retrograde labelling from the right L3 hemisegment of the injured cord. Neurons retrogradely labelled with the fluorescent Fast Blue tracer have been mapped and counted in the C2,C6,Th1 and Th5 spinal segments (Fig. 4A), brainstem and motor cortex (Fig. 4B). Both the stem cell-grafted and the secretome-treated animals (4-factor treatment and transfected fibroblast grafting) had significantly more retrogradely labelled cells in all investigated areas, indicating a far better proprio/supraspinal control of the spinal cord segments caudal to the injury in the treated animals compared with their

controls. IL-10 treatment alone significantly augmented the number of retrogradely labelled cells in the above areas, but to a lesser extent as compared with the other treatment study groups (Fig. 4A-B).

Fig4.

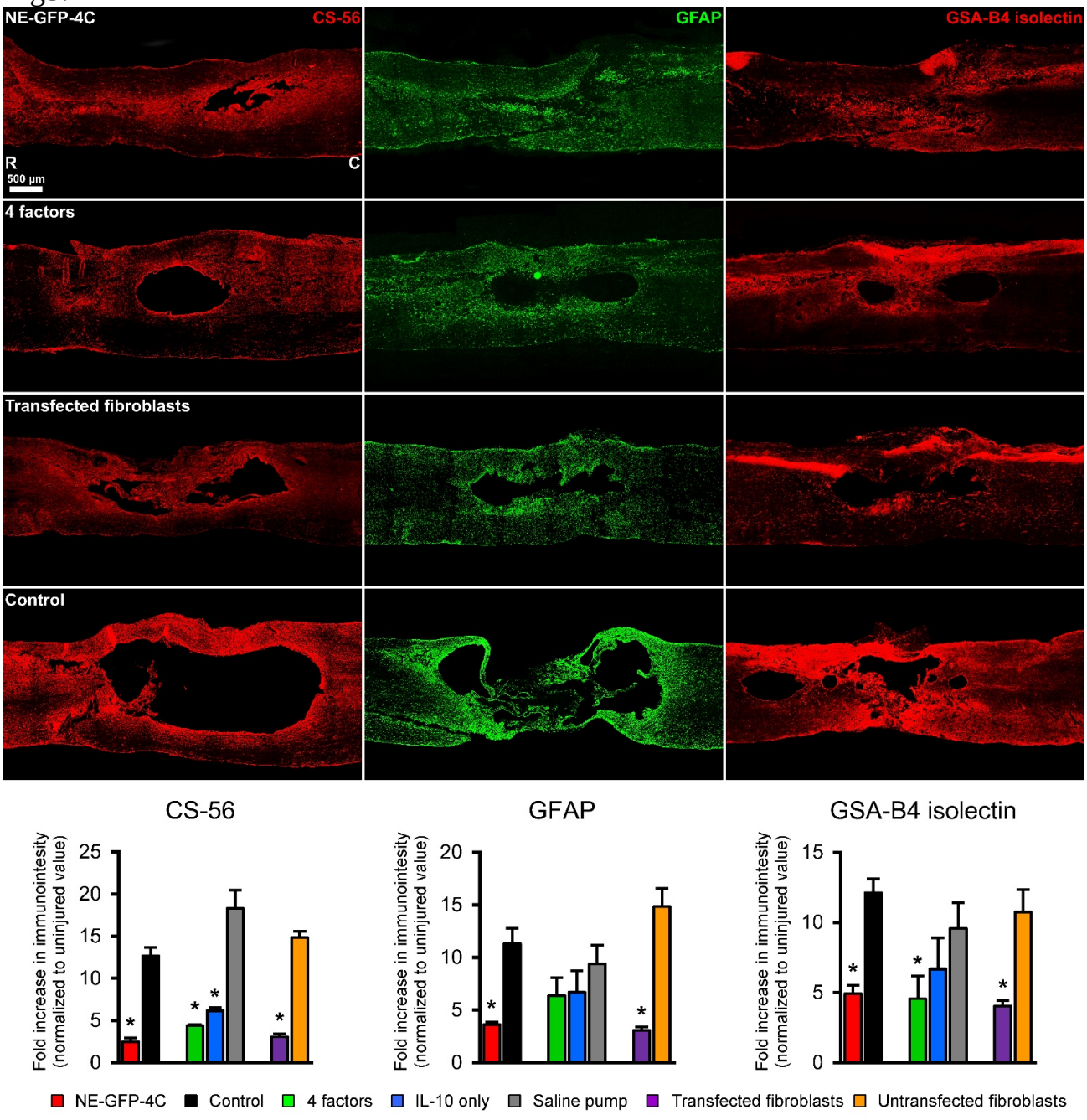


To detect the effects of the various treatments on the glial changes and the deposition of chondroitin sulphate, a major regeneration-inhibiting molecule in the lesioned area we determined the levels of chondroitin sulphate deposits, astro- and microgliosis by using the markers CS56, GFAP and GSA-4B isolectin, respectively (Fig. 5). All the 3 relevant markers showed significant downregulation due to the treatment effects. Both the cellular therapy and the secretome delivered by osmotic pumps or produced by transfected fibroblasts prevented the development of serious chondroitin sulphate deposits. On the other hand, both NE-GFP-4C stem cell grafting and administration of transfected fibroblasts successfully downregulated astro- and microgliosis in the injured cord, while the 4-factor treatment was effective only in reducing microgliosis. Interestingly, IL-10 treatment alone had some, but non-significant effect on the glial response.

To further analyze the number of remaining axons at the level of the epicenter, the blocks of epicenter regions have been paraffin-embedded and osmicated. The number of myelinated axons has been determined and normalized to the total area of the spare white matter. All treatment strategies have resulted in significantly higher numbers of myelinated axons as compared with their controls, except for the IL-10 only treatment which did not produce significant improvement in the sparing of axons. Accordingly, successful treatments yielded minimum twice as many myelinated axons as found in the control spinal cords. This finding has clearly shown that all the 4 factors are

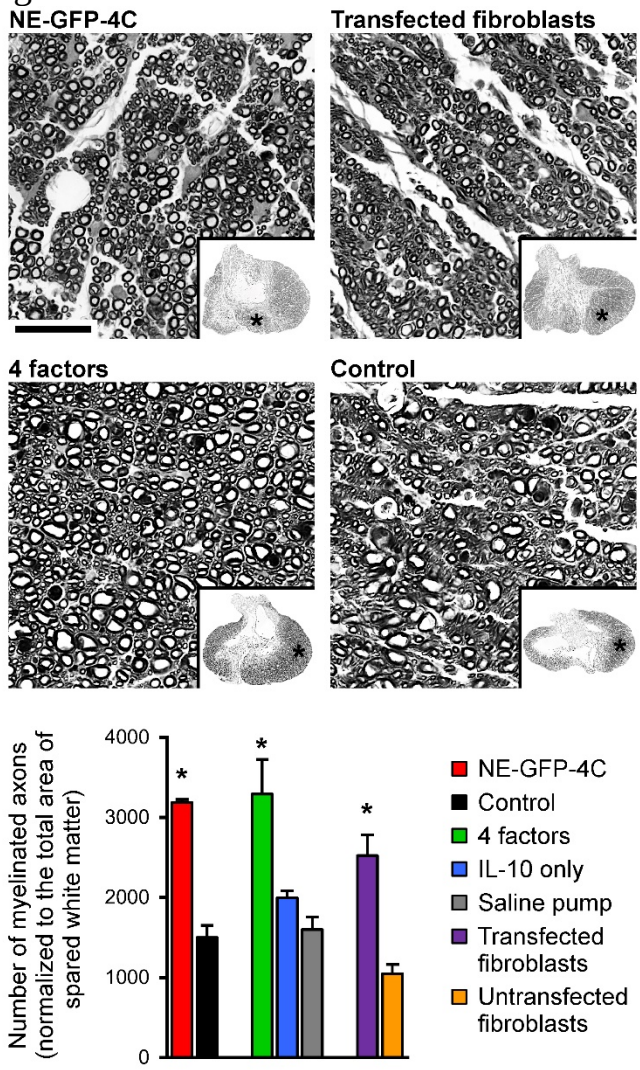
required for the promotion of the complete regenerative and restorative processes, and IL-10 therapy alone is although successful, but not satisfactory to foster the long axonal regeneration and sparing without the other 3 factors. Moreover, it has been proven that these factors are able to provide a powerful remedy for the injured cord and initiate both neuroprotection and axonal regeneration.

Fig5.



The first set of results of the above project have already been published (J. Neurotrauma 2019, 36:2977-2990. doi: 10.1089/neu.2018.6332. Epub 2019 Jul 10. A second manuscript detailing the effects of successful humoral treatment strategies is currently being written up and will be published soon.

Fig6.



SuppFig1.