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MOLECULAR ASPECTS OF THERAPEUTIC ACTION OF 3° TRIMESTER AMNIOTIC FLUID CELLS IN A MOUSE MODEL OF SPINAL CORD INJURY

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Tutor: Chiar.mo Professor Alfredo GORIO

Coordinatore del Dottorato: Chiar.mo Professor Alfredo GORIO

Tesi di Dottorato di:

Giuseppe SCESA

Matr. n° R09598

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INTRODUCTION

Spinal cord injury

Spinal cord injury (SCI) is an disabling disease caused in prevalence by a traumatic event against the spine, leading to a partial or total loss of conduction of nervous stimuli downstream of the injury site. From a pathophysiological point of view, it is possible to identify two main events that occur to define the damage: the first is defined as “primary damage”, deriving from the trauma and consisting in a contusion, compression or a laceration of the spinal cord, that results in a breaking of blood-brain barrier, ischaemia, necrosis of damaged cells; afterwards the “secondary damage” raises, including the inflammatory reaction of the lesioned tissue, as the releasing of toxic chemicals from necrotic cells and from activated immune cells, electrolytes unbalance, glutamate release, apoptosis of cells not directly involved in the primary damage, massive demyelination, hypoperfusion (McDonald JW and Sadowsky C, 2002).Later, the scar formation and epigenetic alterations determine the creation of a “non-permissive” environment towards the regenerations of nerve fibers (Cox A et al., 2014), thus increasing the initial damage, resulting in loss of fibers conductivity and finally in a partial or total loss of function.

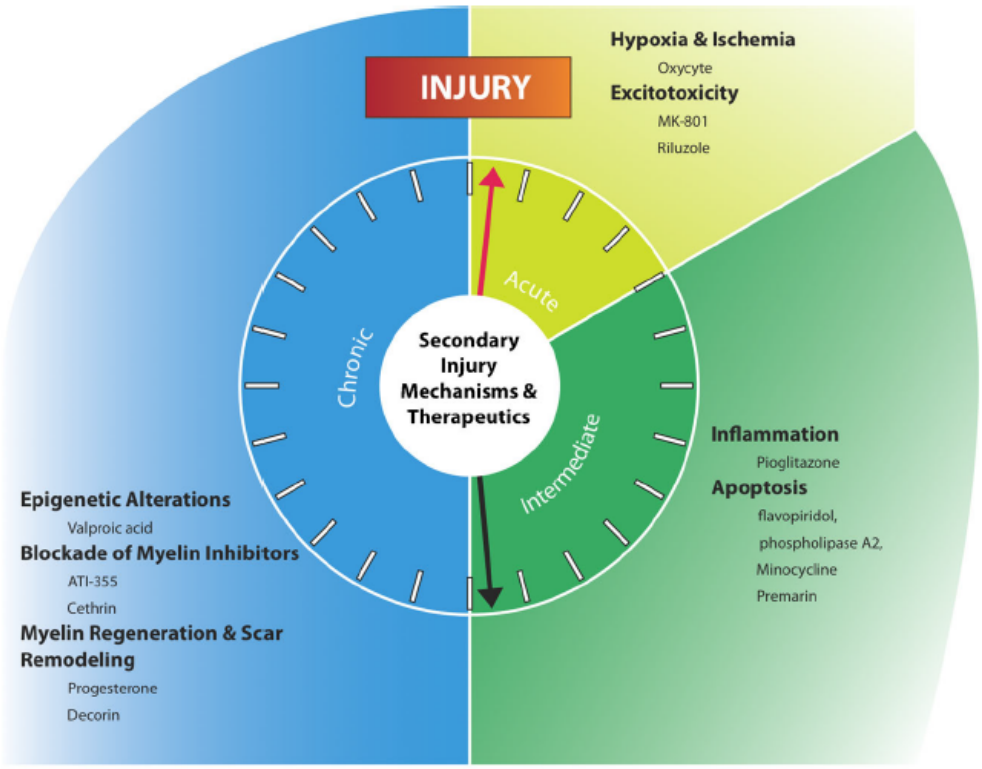

Figure 1: *Events contributing to damage formation in SCI (from Cox A. et al., 2014)*

So far the pharmacological treatment is based on steroids administration, especially methylprednisolone sodium succinate (MPSS), in order to achieve an overall reduction of the inflammatory processes that cause the secondary damage (Baptiste DC and Fehlings MG, 2006). There are evidences that MPSS is able to decrease the edema formation, reactive oxygen species (ROS) release and neural degeneration if administered by 8 hours from the traumatic event (Bracken MB et al., 1990, Anderberg L et al., 2007). However the use of MPSS raises some concerns because of its adverse effects, like hyperglycemia, massive immunodepression, and sepsis (Bracken MB et al., 1990; Bracken MB et al., 1997). Furthermore recent studies showed the beneficial effects of inflammation, especially for its pro-regenerative role: macrophages and T lymphocytes are known to elicit neuroregeneration by releasing neurotrophic factor (La Fleur M et al., 1996; Jameson J et al., 2002, Yin Y et al., 2006). Macrophages can also modulate the activity of T-lymphocytes, particularly after myelin debris removal (Bogie J et al., 2011), with an overall reduction of inflammation. Moreover it is known that the secondary phase of inflammation is fundamental for promoting a morpho/functional recovery (Beck KD et al., 2010; Baptiste DC and Fehlings MG, 2006) in SCI.

Due to all these concerns and to ensure patients the best therapies, others therapeutic approaches were taken into account, trying to treat all the different aspects of secondary damage. Over the past 20 years few clinical trials were undertaken, designed to assess the clinical feasibility and the safety of novel compounds. Nevertheless some of the studies showed a therapeutic efficacy for these molecules, albeit potential and still to be fully proven in order to be used in clinical.

Aminosteroids

Aminosteroids, also called *lazaroids*, are chemical compounds analogs to glucocorticoids able to decrease lipid peroxidation with no activity on steroid receptors. They could potentially be used in reducing tissue damage without eliciting the side effect of direct stimulation of the steroids pathway.

Lazaroids exert their beneficial effects *via* stabilization of the plasma membrane and neutralizing free radicals: in animal models of traumatic hypoxia/reperfusion, Tirilazad mesylate (TM) was able to limit the damage acting directly against free radicals and preserving vitamin E (a powerful antioxidant) (Hall ED et al., 1989; Sato PH et al., 1992).

Lazaroids are also effective in stabilizing plasma membrane: due to their chemical properties they are incorporated into the lipid bilayer reducing in loco lipid peroxidation (Hall ED et al., 1994).

Regarding these positive effects of Tirilazad, its use in clinical protocols is questionable, as some concerns raised following clinical studies. In NASCIS III the effects of Tirilazad mesylate were compared to diverse MPSS administrations, showing the same efficacy of a 48h MPSS administration in treating SCI, though with less adverse effects. However, after 6 months patients treated with methylprednisolone had a better outcome compared to TM (Bracken MB et al., 1997).

In another trial the use of Tirilizad did not exert any beneficial effect to patients with head injury (Marshall LF et al., 1998), or subarachnoid hemorrhage (Zhang S et al., 2010). In conclusion, there is no clinical evidence supporting use of TM in SCI, although further studies are necessary to elucidate the potentiality linked to the use of lazaroids as a clinical therapy.

Gangliosides

Gangliosides are sialic-acid containing glicosphingolipids present in great abundance in the nervous system. Here they locate in the outer part of the plasma membrane of cells, where they form clusters important for lipid rafts architecture and functions (Simons K and Toomre D, 2000; Furukawa K. et al., 2011). Biologically, gangliosides have a role in differentiation and cell growth, intracellular trafficking and in regulating the effects of neurotransmitters and neurotrophic factors (Prendergast J et al., 2014). Deficits in their production or metabolism could lead to neurological deficit and neurodegeneration (van den Berg B et al., 2014; Toshio Ariga 2014; Furukawa K et al., 2011).

The first trial using gangliosides was the Maryland GM1 Study, in which was compared the effects of using monosialotetrahexosylganglioside (GM1) vs. placebo in patients affected by cervical and thoracic SCI (Geisler FH et al., 1991): the study showed the effectiveness of GM1 administration in improving the recovery of lower extremities, probably due to a higher preservation of the axons in the white-matter tracts passing through the injury site.

In a more complete trial, the Sygen Multi-center Acute Spinal Cord Injury study, the use of GM1 was compared to MPSS, resulting in a higher recovery over the first 3 months from the injury (Geisler FH et al., 2001).

Thyrotropin-releasing hormone

Thyrotropin-releasing hormone (TRH) is a tripeptide produced by hypothalamus with endocrine activity towards the pituitary gland. Besides its well known biochemical effects over endocrine regulation of thyroid hormones, TRH has demonstrated a neuroprotective role, especially in animal models of SCI (Pitts LH et al., 1995; Faden A et al., 2005), *via* limitation of multiple secondary injury factors, including reduced blood flow, inflammation mediators such as peptidyl leukotrienes (Feuerstein G et al. 1983) and platelet-activating factor (Feuerstein G et al. 1984), metabolic changes, deregulation of ionic homeostasis (Na^+, K^+, Ca^{2+}, Mg^{2+}) (Faden AI et al., 1990) and blockade of opioids receptors (Faden AI et al., 1988).

Despite the strongly positive results deriving from animal studies, only a small clinical trial was undertaken to assess the safety and the potential efficacy on a limited cohort of 20 patients (Pitts LH et al., 1995). Compared to placebo, TRH was able to improve neurological and sensory functions in patients with an incomplete SCI after 4 months, while no differences were found in patients with complete SCI. However, due to the limited number of patients and the high fluctuations found in scores of the control group, the results should be considered promising rather than definitive.

Glutamate receptors antagonists

One of the events occurring after the trauma is the massive glutamate release, due to the necrosis of damaged neurons and to the deregulation of ions balance (especially Na^+ and Ca^{2+}) leading to a release of the synaptic vesicles from the excitatory pathways, resulting in cells death. Moreover, the Ca^{2+} influx causes the activation of cytoplasmic proteases, increasing the overall nerve damage.

To counteract these effects it might be useful the blockade of glutamatergic synaptic transmission, although some concerns might arise considering that glutamate is a key neurotransmitter possessing important physiological functions: any blockade of glutamatergic pathway may potentially result in unwanted psychomimetic side effects. Furthermore, the therapeutic potential of GluR antagonists has not yet been investigated for a clinical use in humans, due to the adverse effects associated with the large doses eventually required (Baptiste DC and Fehlings MG, 2006).

The feasibility and the effectiveness of ionotropic GluR (AMPA and NMDA) antagonists use were tested in preclinical studies (Wrathall JR et al., 1997; Gaviria M et al., 2000), in which was showed an attenuated neurotoxicity in rodents with almost no side effects. Regarding clinical trials, a

phase III study was completed in France in which the use of gacyclidine, a powerful non-competitive NMDA receptor antagonist, provided early benefits at 1 month from the injury, although not maintained after 1 year (Lepeintre JF et al., 2004). Metabotropic GluR antagonists administration showed an overall positive effects, albeit by different mechanisms: while 1-aminoindan-1,5-dicarboxylic acid (AIDA) and (1)-2-methyl-4-carboxyphenylglycine (LY 367385) were able to reduce mechanical allodynia and to improve locomotor function (Mills CD et al., 2002), the selective mGluR5 blockade by using 2-methy-6-(phenylethynyl)-pyridine (MPEP) resulted in no effects on allodynia or locomotor behaviour, but elicited a significant sparring of gray matter while reducing thermal hyperalgesia (Mills CD et al., 2002). Thus, the effects exerted by mGluRs suggest differences in the pathological roles of these receptors during progression of the injury, differences that might be selectively targeted for limiting the excitotoxic damage.

Ions channels blockers

To reduce the toxic effects of the ions unbalance following the spinal injury, the use of ions channels blocker might exert beneficial effects on the damaged spinal cord. The depolarization of plasma membranes lead to a direct Ca^{2+} influx, or as a consequence of Na^{+} massive entrance, that causes a reverse functioning of Na^{+}/Ca^{2+} pumps (that normally allow Na^{+} to enter while Ca^{2+} is pumped out of the cells).

As a consequence, there is the activation of calpains and others proteolytic enzymes leading to further cells death, amplifying the primary damage.

Nimodipine is a Ca^{2+} channel blocker originally used for treating hypertension and vasospasm, it showed a benefical effect in a rat and baboons model of SCI (Winkler T et al., 2003; Pointillart V et al., 1993), due to improvements of the axonal function in motor and somatosensory tracts of the cord.

In 1996 was held in France a small clinical trial in which the use of nimodipine was compared to methylprednisolone, both versus no medical treatment: after 1 year of follow-up, though there were some neurological ameliorations , no benefits were found in using nimodipine or MPSS vs controls (Pointillart V et al., 2000).

Regarding the use of sodium channel blockers, preclinical studies on rodents showed therapeutical effects exerted by tetrodotoxin (TTX) or amiloride (Rosenberg LJ et al., 1999;Jung GY et al., 2013; Durham-Lee JC et al., 2011). Tetrodotoxin, after focal administration by microinjections, was able to reduce functional deficits by sparing of large-diameter axons, to decrease the axonopathies by reducing the development of extensive periaxonal spaces (Rosenberg LJ et al., 1999). Furthermore,

TTX reduced early microglial activation, exerting a beneficial effect on the lesioned tissue (Jung GY et al., 2013).

Amiloride, a diuretic used as antihypertensive drug, was shown to have neuroprotective effects in neurological diseases (e.g. brain damage and brain ischemia). In 2011 its therapeutic potential was tested in a rat model of SCI: following a daily administration from 24 h to 35 days after the lesion, the animals exhibited a significant improvement in their motor behavior. The authors related the beneficial effects of amiloride to a higher sparing of oligodendrocytes in treated rats compared to controls (Durham-Lee JC et al., 2011).

Riluzole, a benzothiazole anticonvulsant Na^+ channel blocker, is known to exert a neuroprotective role, promoting functional recovery following SCI in rodents (Schwartz G and Fehlings MG, 2001). It was shown that riluzole is able to significantly reduce tissue loss in the area surrounding the lesion epicenter, macrophages infiltration and astrogliosis at lesion site. These effects reflected in a reduced apoptosis of nerve cells, concurring in sparing a higher amount of tissue (Wu Y et al., 2014). Furthermore, riluzole exerts beneficial effects also by reducing mechanical allodynia and thermal hyperalgesia, as resulted in a rat model of cervical radiculopathy (Nicholson KJ et al., 2014). In 2010 the North American Clinical Trials Network (NACTN) undertook a phase I clinical trial to investigate the safety and pharmacokinetics of riluzole, and to gain pilot data on its use on patients affected by cervical and thoracic spinal cord injury. The authors observed no adverse effects, as well a significantly higher motor score for riluzole-treated patients (Grossman RG et al., 2014).

Minocycline

Minocycline is a semi-synthetic derivative of tetracycline able to exert an anti-inflammatory activity as well as its classical antibiotic function. Its anti-inflammatory properties in SCI are due to a sum of actions, as a diminished lipid peroxidation (Sonmez E et al., 2013), a normalization of NMDA receptor activity with a reduced excitotoxicity (Nie H et al., 2010), a reduction in microglia activation and in Repulsive Guidance Molecule A (RGMa) expression (Kitayama M et al., 2011) and finally to an analgesic effect (Rojewska E et al 2014). In a rat model of SCI, minocycline showed a higher therapeutic effect compared to methylprednisolone (Wells JE et al., 2003), thanks to a diminished oligodendrocytes apoptosis, a lower microglial activation thus reducing the lesion and improving the neurologic and motor function.

In a clinical trial undertaken to assess dose optimization and treatment safety, patients treated with minocycline had an overall greater motor recovery compared to placebo (Casha S et al., 2012). While patients with a thoracic SCI did not show any benefit from drug administration, patients with cervical SCI showed a great improvements compared to controls, as well as a higher dose exerted the greater effects. The authors ascribe these results to a higher sensitivity of the ASIA motor exam for cervical injuries. The sensory function was also most preserved in minocycline treated group.

Erythropoietin

Besides its well-known effects on erythropoiesis, erythropoietin (EPO) was shown to possess a role also in neural development and neuroprotection (Alnaeeli M et al., 2012; Erbayraktar S et al., 2003): in animals models of neurodegenerative diseases, like Parkinson and Alzheimer, EPO was able to improve the neurological functions (Jia Y et al., 2014; Maurice T et al 2013) by reducing apoptosis and lipid peroxidation.

EPO was able to reduce ischemic/reperfusion and traumatic brain damage, improving recovery of sensorimotor function (Xiong Y et al., 2008; Cruz Navarro J et al., 2014; Wu SK et al., 2014). Similar effects were proven in animal models of spinal cord injury, in which EPO administration, or its vector-mediated overexpression, induced significant improvements in motor function, due to a marked attenuation of secondary inflammation, thus minimizing the injury size and enhancing tissue sparing, preserving large-caliber axons and with an improvement of both ascending and descending nerve pathways transmission (Gorio A et al., 2002; Wang S et al., 2012; Cerri G et al., 2012).

The safety for a use of EPO in humans was assessed in a clinical trial concluded in 2002, where erythropoietin was administered in patients affected by stroke: the study showed the beneficial effects of EPO in improving the outcome, acting over the later events (like apoptosis or inflammation) following the brain injury (Ehrenreich H et al., 2002).

However, several concerns are arisen by a chronic use of EPO, especially for its physiologic role on erythropoiesis, as the damage might be increased by high hematocrit levels (Wiessner C et al., 2001); for this reason researchers are focusing on developing EPO derivatives (e.g. asialo-EPO and carbamil-EPO) lacking activity on erythropoiesis (Erbayraktar S et al., 2003; Leist M et al., 2004).

Cell therapy

To date, the classical pharmacological treatments are designed to counteract the detrimental effects of inflammation and the other processes leading to secondary damage. However, enhancing the regeneration of the lesioned tissue could represent an improvement in the treatment of the pathology: in this scenario the cellular therapy could be regarded as a powerful tool, as it could grant the possibility to regenerate the lost fibers thanks to the integration of the transplanted cells into the circuitry of the spinal cord.

Embryonic stem cells

Embryonic stem cells (ES) are pluripotent cells derived from the inner cell mass of the blastocyst, characterized by the ability to proliferate and differentiate into all the cellular lineages deriving from the 3 germ layers. Potentially, ES are a powerful tool for cell therapy towards many diseases, due to their plasticity and differentiation abilities (Andres RH et al., 2008; Abdelli LS et al., 2012).

ES transplantation was shown to exert therapeutic effects towards many neurodegenerative diseases, where the positive effects were often mediated by production and release of trophic and anti-inflammatory factors (Kerr CL et al., 2010; Bottai D et al., 2010; Riess P et al., 2007). However, the use of embryonic stem cells rises both technical, due to the ability to form tumors (Riess P et al., 2007) once transplanted, and ethical concerns, that limits clinical use (Barker RA et al., 2013).

In 2006 the Nobel prize Shinya Yamanaka developed a technique that allows to reprogram fully-differentiated somatic cells into pluripotent cells, named iPS *(induced Pluripotent Stem cells)* (Takahashi K and Yamanaka S, 2006), allowing to overcome many issues bound to ES use in cell therapy and transplantation. One of the most important issue linked to the use of stem cells i*n vivo* concerns the histo-compatibility between donor and host, how it is dramatically seen for the graft vs. host disease (Blood. 2014Markey KA). Using pluripotent syngeneic cells should reduce the issue (Guha P et al., 2013; Araki R et al., 2013), although it is still not clear if iPS or iPS-derived cells are immunogenic (Zhao T et al 2011; Cao J et al., 2014).

Neural stem cells

Neural stem cells (NSC) are a specific subpopulations of cells derived from the subgranular zone (SGZ) of hippocampal dentate gyrus, the subventricular zone (SVZ) of the lateral ventricles, the spinal cord or, in embrionic stage, from the neuroepithelium. During fetal life, their primary role is the formation of the developing nervous system, while in the adult their role become more

complex: beside a classic neurogenic activity (in rodents neural stem cells from the SVZ migrates through the rostral migratory stream towards the olfactory bulb, where they fully differentiate into inter-neurons) (Lazarini F et al., 2014), in the last years NSC were found to be involved in immune modulation and immune surveillance (Mohammad MG et al., 2014; Butti E et al., 2014).

Due to their origin, NSC are the best candidate for transplantation in animal models of neurodegenerative diseases, where they showed therapeutic efficacy in improving the outcome (Zhang W et al., 2014; Butti E et al., 2012; Kang X et al., 2014), directly integrating in the tissue (Brilli E et al., 2013) or more often by releasing neurotrophic/immune-modulatory factors (De Feo D et al., 2012; Su P et al., 2014).

In spinal cord injury models, transplanted NSC were able to induce a higher motor recovery, by preserving the lesioned tissue and, in general, by reducing the secondary damage (Cusimano M et al., 2012; Bottai D et al., 2008) rather than integrating into the spinal cord. Despite the promising results, at least in animal models, the eventual use of NSC in human is affected by technical and ethical issues, due to the cells source (human foetuses) and its limited availability (need of multiple foetuses to get enough cells for transplantation in humans) (Barker RA et al., 2013). In order to obtain a high-proliferating cell population that could provide the great number of cells needed for human transplantation, some authors performed transplant of iPS-derived neural precursor in animal models of neurodegenerative diseases: these cells behaved similarly to classical NSC, improving the behavioral outcome (Romanyuk N et al., 2014;Tucker BA et al 2011; Wang S et al., 2013; Sareen D et al., 2014), and thus providing a useful tool for therapy.

Amniotic fluid cells

Amniotic fluid cells (AF) appears in the amniotic fluid starting from the 12° gestational week, and derive both from extra-embryonic structures (i.e., placenta and fetal membranes) and from embryonic and fetal tissues (Gosden CM, 1983). A morphological classification divides AF into three main groups: epithelioid, amniotic fluid, and fibroblastic type, though this classification reflect different characteristics regarding growth rate and markers expression (Cremer M et al., 1981). The use of amniotic fluid-derived cells was suggested for the first time in 2001 (Kaviani A et al.,2001): the cells used had a mesenchymal profile and were able to colonize a polimeric scaffold that might be eventually used in tissue engeneering. In 2006, AF cells were use in a animal model of spinal cord injury, where they succeeded in improving repair of the damaged tissue, ensuring a higher recovery (Wu ZY et al.,2006). Besides the aforementioned classification, in 2000s deeper

studies revealed the presence of two main population of AF, according to phenotipic characteristics: amniotic fluid mesenchymal stem cells and amniotic fluid stem cells (In't Anker et al., 2003; Prusa AR et al., 2003). While the first population expresses typical mesenchymal markers (e.g. CD 73,90,105) and is able to differentiate into cells belonging to the adipogenic, chondrogenic and adipogenic lineages, the second is characterized by the expression of Oct4, SSEA-4 and c-Kit, classical pluripotency markers, and by the capacity to differentiate into cells lineages representative of all three embryonic germ layers (Kim J et al., 2007; De Coppi P et al., 2007; Moschidou D. Mol Ther. 2012).

It was demonstrated that $CD117^{+}$ AF cells forms embryoid bodies (Oncogene. 2010 Valli A) and, after a minimal manipulation with Valproic acid they acquire embryonic characteristics, including the expression of the "Yamanaka factors" (OCT4, NANOG, SOX2 and c-MYC, KFL4), the formation of beating embryoid bodies *in vitro* and *in vivo* formation of teratomas (Moschidou D. Mol Ther. 2012).

Given this useful properties, recent studies using these cells were undertaken for diseases like myocardial infarction (Chiavegato A J Mol Cell Cardiol. 2007; Bollini S Stem Cell Rev. 2011), neuro-muscular diseases (Piccoli M Stem Cells. 2012), acute tubular necrosis (Perin L. PLoS One. 2010), lung damage (Buckley S Am J Respir Cell Mol Biol. 2011).

Besides these innate "pluripotency" properties, AF cells raise almost no ethical concerns, as they derive from a tissue otherwise discarded, especially for third trimester derived AF cells. For these reasons, AF could represent a useful and powerful tool for cell therapy.

METHODS

Cell culture

Cells belonging to cultures 1.1, 3.5, 3.6, 7.30 and 9.1 were grown in AFCS medium (containing DMEM, 10% fetal bovine serum [FBS], bFGF (fibroblast growth factor) 5 ng/ml, glutamin 2mM, penicillin/streptomicin 100 U/ml) in an incubator at 37° C, 5% CO_2 and O_2. Once confluent (every 4-5 days), the cells were splitted using a tripsin-like solution (TrypLE Express, Invitrogen), counted and seeded at a density of 10.000 cells/cm^2.

When necessary the cells were frozen by means of liquid nitrogen using a solution containing DMEM, 5% FBS and 10% dimethylsulfoxide (DMSO).

To make easier tracking the cells once transplanted, the ones used *in vivo* were marked immediately before their administration to the mice using Qtracker Cell Labelling Kit (Invitrogen), a fluorescent dye internalized by passive endocytosis. First it was prepared the labelling solution by adding 1 µl of reagent A with reagent B (both provided with the kit), incubating for 5' and then adding this solution to 200 µl of culture medium. The cells were incubated with the labelling solution for 45' at 37° C, washed twice with DMEM and finally suspended in PBS (Phosphate-Buffered Saline) 0,01M at a concentration of 10^4 cells/µl, ready for the transplant.

Spinal cord injury, transplant and animal care

Male, three months old CD1 mice were injured at T8 level by using an impactor (Infinite Horizon Device), in order to produce a contusive damage.

The animals were anesthetized using a 4% chloral hydrate in PBS solution, the skin cut and the dorsal muscles exposed. These were cut and moved to exhibit the spine. After the laminectomy of the vertebra, the spinal cord was exposed and mildly injured with a force of 50 kDyne on a vertical displacement range between 300 and 600 µm. Next the muscles and skin were sutured. After surgery and for the following week the mice received 2 ml of saline, painkillers (buprenorphine 0,03 mg/Kg) and antibiotics (Pen/Strep 100 U/ml 100 mg/ml) daily.

Immunosuppression started the day before cells transplantation: the mice received an intraperitoneal injection with Cyclosporin A (Sigma-Aldrich) 50 mg/Kg for one week, 25 mg/Kg for 3 days and 10 mg/Kg for 18 days. Cells belonging to cultures 1.1, 3.5, 3.6, 7.30 and 9.1 were transplanted 7 days after injury by means of 2 i.v. injections containing 500.000 cells each, while controls animals received the vehicle (PBS). The expression of mice's bladder was performed daily until bladder reflex recovered.

Behavioural analysis

In order to assess the motor recovery induced by the transplant, the motor behaviour was analyzed for 4 minutes in a open field by using the Basso Mouse Scale (Basso et al 2005), which allows to couple the motor performance to a numeric value. The analysis started the day following the lesion, the mice analyzed every 3 day until the 35 th day after the lesion (28 days from the transplantation).

SCORE	**ANIMAL MOVEMENT**
0	No ankle movement
1	Slight ankle movement (angle ≤ 90°)
2	Extensive ankle movement (angle ≥ 90°)
3	Plantar placing of the paw with or without weight support or occasional, frequent or consistent dorsal stepping but no plantar stepping
4	Occasional plantar stepping (number of plantar steps ≤ 50% of the overall movement time)
5	Frequent (number of plantar steps ≥ 50% of the overall movement time) or consistent (less than 5 missed steps in the overall movement time) plantar step with no coordination or frequent/consistent plantar stepping with some coordination) and paws rotated at inizial contact and lift off
6	Frequent or consistentplantar stepping with some coordination and paws parallel at initial contact or consistent plantar stepping mostly coordinated and paws rotated at initial contact and lift off
7	Frequent o consistent plantar stepping mostly coordinated, paws parallel at initial contact and rotated at lift off or frequent/consistent plantar stepping, mostly coordinated, parallel paws at initial contact and lift off with severe trunk instability
8	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off andmildt trunk instability or frequent/consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, normal trunk stability, tail up and down
9	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, normal trunk stability, tail always up

Table 1 *Basso Mouse Scale summary scheme*

Harvest of spinal cord samples and histochemical analysis

Some animals were sacrificed at 2, 7 and 28 days after transplantation in order to collect the spinal cord. Following a lethal anesthesia with 4% chloral hydrate, the mice were perfused with a solution of PBS with 4% formaldehyde, the spines collected, kept overnight in 4% formaldehyde and washed 3 times with PBS. Afterwards the tissues were kept in 15% sucrose for 3 h, 30% sucrose overnight, and finally included in OCT (Optimal Cutting Temperature compound Bio-Optica) and maintained at -80° C. The samples were cut, mounted on coverglasses and kept at -20° C until the analysis.

For the immunostaining the slides were rinsed with PBS, blocked with a solution of 2% normal goat serum (NGS), 1% bovine serum albumin (BSA) and Triton 0,1% in PBS and then incubated overnight with primary antibody at 4° C. Later, the sections were washed 3 times with PBS and incubated 45' with secondary antibody (Alexa 555/546 α-mouse/rabbit 1:1000; Alexa 488 α-rabbit, 1:1000) at room temperature. Finally, after labelling the nuclei with DAPI, a coverslip was mounted with FluorSave Reagent (Calbiochem).

Antigen	Antibody dilution	Brand
MOMA-2 *(Anti Monocyte-Macrophage Antibody-2)*	1:25	Chemicon
GFAP *(Glial Fibrillary Acidic Protein)*	1:500	Covance
B-tubulin III	1:200	Covance
NG-2 *(Neural/Glial Antigen-2)*	1:100	R&D
CD31 *(Platelet Endothelial Cell Adhesion Molecule-1)*	1:70	Santa Cruz
Nestin	1:100	R&D

Table 2 *List of antibody used for immunofluorescence*

The myelin preservation was assessed by use of FluoroMyelin (Life Technologies), a fluorescent dye, following the producer's instructions: briefly, the spine section spanning the lesion site for 5 mm were rinsed with PBS, incubated with the dye for 20' (1:300), washed again with PBS, counterstained with DAPI and finally mounted with FluorSave.

The evaluation was carried on comparing the ventral horns white matter of transplanted mice vs. controls; in this area it is present the reticular spinal pathway, involved in the regulation of hind limb movement in mice (Vitellaro Zuccarello 2007).

The angiogenesis assessment at 800 and 1600 µm from lesion epicenter was made as a double-staining with antibodies against CD31 and lectin (Texas Red Lectin, Vector Laboratories), a hemoagglutinin able to bind specifically endothelial glicoproteins.

Lectin staining was performed accordingly to producer's protocol: first, the samples were rinsed with PBS, then incubated with lectin (diluition 1:50) for 2h, wash 3 times with PBS, counterstained with DAPI and mounted as described before.

Plasma and tissues harvesting

The animals were sacrificed at 2 and 7 days from transplant using a lethal injection of 4% chloral hydrate. After a laminectomy of the vertebrae between T5 and T12, the spinal cord was collected, placed in Tri Reagent (Sigma) and kept at -80° C. The lungs were divided as follows: one was kept overnight in 4% paraformaldehyde, washed with PBS, treated with 15% sucrose for 3h and 30% o/n, then kept at -80° C in OCT with 30 % sucrose; the other part was put in TRI Reagent and stored at -80° C. The blood was harvested in tubes containing a solution of 3,8% w/v of citric acid in water and centrifuged at 1800 g for 10'. The plasma was then collected and saved at -80° C until analysis.

The protein quantification was performed using Lowry-Ciocalteau method.

First Lowry's solution was prepared, using solution A (NaOH 0,4% e Na_2CO_3 2% in H_2O) to dilute 1:50 solution B (sodium tartrate 1% e $CuSO_4$ 0,5%). Two µl of plasma were added to water, to a final volume of 200 µl , then 1 ml of Lowry's solution was added, incubating for 15'. After that 100 µl of Folin's reagent were added and the adsorbance red.

The standard curve was made using a solution of BSA in water, with a concentration from 0 to 35 µg.

RNA extraction, retrotrascription and analysis

The samples from spinal cord and lung collected 2, 7 and 28 days after transplantation were put into TriReagent and the RNA extracted following manufacturer's protocol: after adding 0,2 ml of Chloroform for each ml of TriReagent, the samples were shaken vigorously, incubated 10' at room temperature and centrifuged 15' at 4° C at 12000 g. The aqueous phase was transferred to a new tube containing 0,5 ml of isopropanol, mixed and incubated 10' at RT, then centrifuged at 12000 g for 10' at 4° C. The supernatant was discarded, the pellet containing the RNA was washed with 75% ethanol and then solubilized into 50 µl of RNase-free water. Afterwards the RNA was treated

with a Dnase, to remove any contamination with genomic DNA: 10X Dnase reaction mix was diluted tenfold with the RNA, than the reaction mix was incubated for 1h at 37° C. After the RNA was purified with PCI solution (Phenol,Chloroform, Isoamilic acid), precipitate with 75% Ethanol and resuspended in water; lately, it was quantified by NanoDrop (NanoDrop 2000c, Thermo Fisher Scientific Inc.). Afterwards 0,5 µg of RNA were retrotranscribed using the iScript cDNA Synthesis Kit (BioRad), following manufacturer's protocol; the reaction mix was then incubated 5' at 25° C, then 30 minutes at 42° C and lastly 5' at 85° C, in order to deactivate the retrotrascriptase. The cDNA was then brought to a final volume of 50 µl with H_2O.

The real-time PCR analysis was performed using iQ SYBR Green Supermix (BioRad), the primers concentration was 5 µM (see table 3 for primers sequences), while the choice of the housekeeping genes and the final analysis was made using qBase (Biogazelle) software.

Gene	Forward	Reverse	Product lenght
Mm GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	86
Mm HPRT-1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	94
Mm Ciclo	GCGTCTCCTTCGAGCTGTT	AAAGTCACCACCCTGGCA	146
Mm β-µTub	ATTCACCCCCACTGAGACTG	TGCTATTTCTTTCTGCGTGC	182
Mm BDNF	CATTACCTTCCTGCATCTGTTGG	CGTGGACGTTTACTTCTTTCATGG	158
Mm CNTF	ACATCAGTCTCCCGGTGGCATTAG	TTCTCCGTGGCTTTGGGGTTTC	151
Mm IL6	GACAACCACGGCCTTCCCTAC	CGTTGTTCATACAATCAGAATTGCC	169
Mm LIF	AACGTGGAAAAGCTATGTGCG	GCGACCATCCGATACAGCTC	101
Mm HIF	TCAGTGCACAGAGCCTCCT	GCGGAGAAAGAGACAAGTCC	209
Mm NGF	TGGGCCCAATAAAGGTTTTGCC	TGGGCTTCAGGGACAGAGTCTCC	165
Mm NT3	ACGTCCCTGGAAATAGTCACACG	TTGGATGCCACGGAGATAAGC	109
Mm TNFα	TCTATGGCCCAGACCCTCACAC	CAGCCACTCCAGCTGCTCCTC	100
Mm VEGF	ACACGGTGGTGGAAGAAGAG	CAAGTCTCCTGGGGACAGAA	145
Mm PGF	CACTTGCTTCTTACAGGTCC	CACCTCATCAGGGTATTCAT	174
Mm IL1β	CATGGAATCCGTGTCTTCCT	GAGCTGTCTGCTCATTCACG	100
HGF	TTGTCCCACATGGAACATGTAAG	CACTGACCCAAACATCCGAGTT	149
Hs IL1β	TTACAGTGGCAATGAGGATGA	TGTAGTGGTGGTCGGAGATT	131

Table 3 *Primer used for mRNA analysis*

Western blot of plasma proteins

Ten µg of proteins derived from plasma samples collected 2, 7 and 28 days after trasplantation were analyzed by Western Blot. After treating the proteins with 5% β-mercaptoethanol at 100 °C, in order to disaggregate protein complexes and to denaturate quaternary structures, they were run on a 10% polyacrilamide gel at 120 V. The transfer was performed on a nitrocellulose membrane for 1h at 100 V. The membrane was then coloured with Ponceau Red (Sigma) to assess the transfer, washed with T-TBS (Tris Buffer Saline Solution containing 0.05% Tween-20), treated 2.5h with a blocking solution (5% dry milk in T-TBS) and incubated at 4° C o/n with the primary antibody α-HGF (dilution 1:200, Santa Cruz Biotechnology). Afterwards the membrane was rinsed twice with T-TBS and incubated 1h with the secondary HRP-coniugated antibody (1:5000), washed again 3 times with T-TBS, once with TBS and finally it was added the substrate (SuperSignal West Femto, Pierce). The results were analyzed with Kodak MJ software.

Immunohistochemical analysis of the lungs

Lung samples were collected 2 and 7 days after transplantation, fixed overnight in 4% paraformaldehyde, washed with PBS, treated with 15% sucrose for 3h ad 30% o/n, included in OCT and cut with a cryostat. The slides were rinsed twice with PBS and then blocked with a blocking solution (2% NGS, 1% BSA and Triton 0,1% in PBS) and then incubated overnight with primary antibody α-HGF (1:200, Santa Cruz Biotechnology) at 4° C. Later, the sections were washed 3 times with PBS and incubated 45' with secondary antibody (Alexa 488 α-rabbit, 1:1000) at room temperature. Finally, after labelling the nuclei with DAPI, a coverslip was mounted with FluorSave Reagent (Calbiochem).

Proinflammatory stimulations of cells

Cells belonging to culture 7.30 and 9.1 were seeded at a density of 15000 cells/cm2 in a 48 wells plate. The next day the medium was changed with AFCS medium containing IL-1β (0,1 ng/ml) and LPS (200 ng/ml), the cells were kept in this medium for different times (2, 4, 8, 16 and 24 h). Negative controls were kept in the same medium not added with the chemicals. At the end the cells were fixed with 4% paraformaldehyde for 10', rinsed with PBS and kept at 4° C until analysis.

The cells were washed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS and then incubated o/n with primary antibody α-HGF (1: 300, Santa Cruz Biotechnology) diluted in a solution of NGS 10% in PBS. After this, the cells were washed twice with PBS, incubated with

secondary antibody (1:1000), counterstained with dapi, mounted with FluorSave and stored at -20°C.

RESULTS

Motor behavior analysis

According to our contusion model of spinal cord injury, mice transplanted with the vehicle (PBS) have a physiological recovery from the injury, allowing them to reach a score of about 3 in the BMS, corresponding to the capacity of moving the hind limbs, or using them to sustain the body weight. The mice transplanted with the cells reached different scores, depending on the cell line they received during the transplantation: some of the cell lines (3.5, 3.6, 7.30) caused a significant increase in the motor performance, while other (1.1, 9.1) had no differences respect to controls (Fig. 1A). It was decided to split the cells into a therapeutic group (T-AFs) and a non-therapeutic group (NT-AFs), according to the effects they have on the animals (Fig. 1B): the average score of mice treated with T-AFs is 4.83 ± 0.23 (n = 33) versus PBS 3.80 ± 0.22 (n = 33, p< 0.01), versus NT-AFs 3.36 ± 0.210 (n = 7, p<0.01). These results correspond to the possibility for the mice to use actively the hind limbs to walk, while controls or NT-AFs treated mice can barely sustain their body weight.

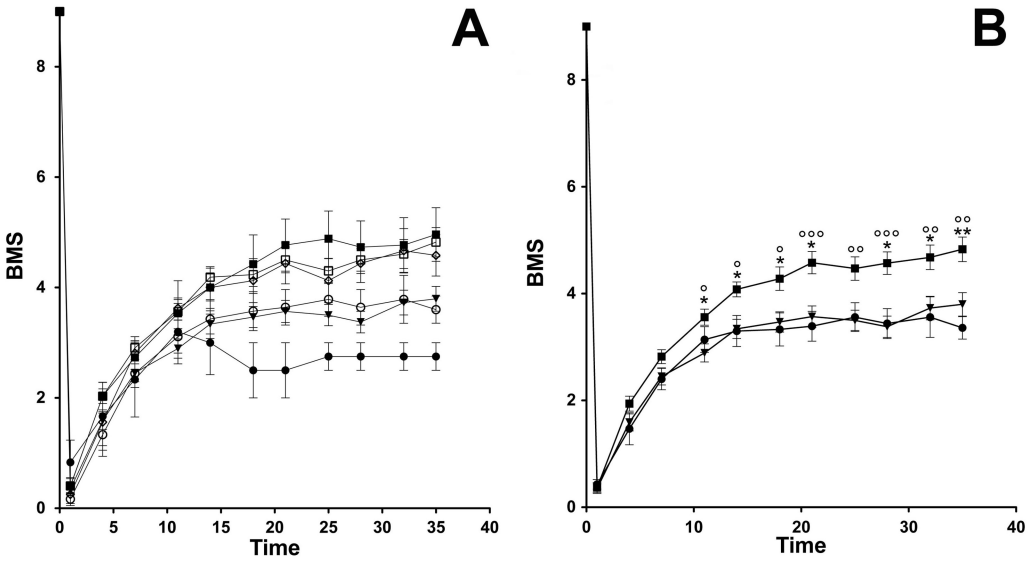

Figure 1*: Effect of PBS and AFCs treatments on motor activity after SCI. motor recovery was evaluated over one month period (time= days), and the assessment was based upon the 9-point BMS scale. Values represent means±SEM.* ***A****:*

Comparison of motor recovery between cell-treated and PBS-treated animals. ▼PBS treated mice (n = 33); ▪ #3.5 AFs treated animals (n = 16); ◊ #3.6 AFs-treated animals (n = 8); □ #7.30 AFs-treated animals (n = 15); ○ #9.1 AFs-treated animals (n = 9); ● #1.1 AFs-treated animals (n = 6). ***B:*** *Comparison of motor recovery after formation of the groups: ▪T-AFs-treated mice (n = 39); ● NT-AFs-treated mice (n = 15); ▼ PBS-treated mice (n = 33). Significance symbols: ***; °°°: p b 0.001; **, °°: p b 0.01; *, °: p b 0.05. ***, ** and * T-AFCs-treated mice versus PBS-treated mice; °°°, °° and ° T-AFCs-treated mice versus NT-AFCs-treated mice. BMS: Basso Mouse Scale.*

Surprisingly, the T-AFs cells (cultures #3.5, #3.6, #7.30) express the NG2 (Chondroitin Sulfate Proteoglycan) marker, while the NT-AFs (cultures #1.1, #9.1) do not (Fig. 2). This marker is normally associated to oligodendrocyte precursor cells, but it can be also found in endothelial cells, and it might have a role in the therapeutic effects observed (Stallcup WB, Huang FJ 2008).

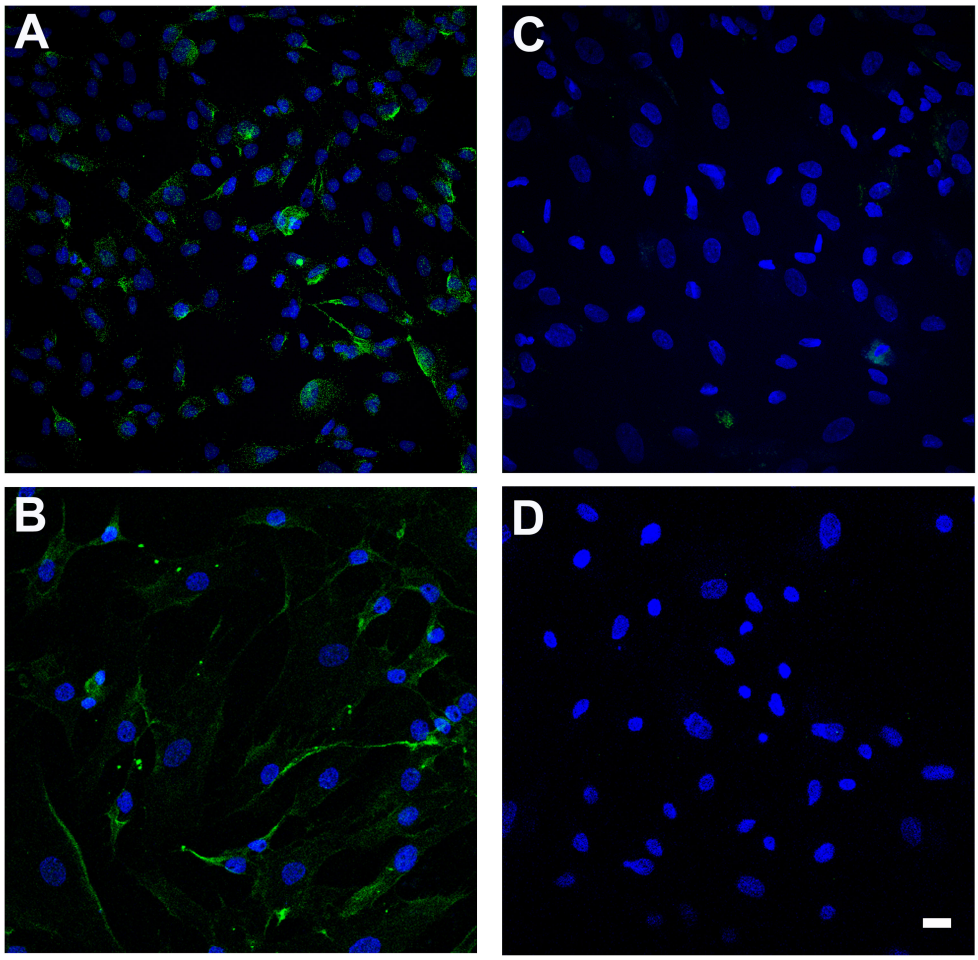

Figure 2: *NG2 expression in four of the transplanted cultures: A #7.30, B #3.5, C #9.1, D # 1.1.*

Histochemical analysis of spinal cord

In order to link the motor performance to eventual differences in tissue preservation, we performed a volumetric analysis of myelin content in a 5 mm region across the lesion site. The areas analyzed were located in the ventral horns of the spinal cord, as in that regions are located the descending portions of the reticulospinal tract, particularly important for coordination of the rhythmic stepping movements and in eliciting locomotion (Ballermann M. and Fouad K. 2006). It

was decided to focus on this particular region as it might be partially spared in our lesioning paradigm, affecting the dorsal and central part of the spinal cord . As result, it was observed a higher myelin content in mice transplanted with T-AFs respect to controls, probably due to a better preservation of myelin itself (Fig. 3): the myelin content is indeed 70% higher in T-AFs treated mice compared to PBS; no differences were found between PBS and NT-AFCs groups.

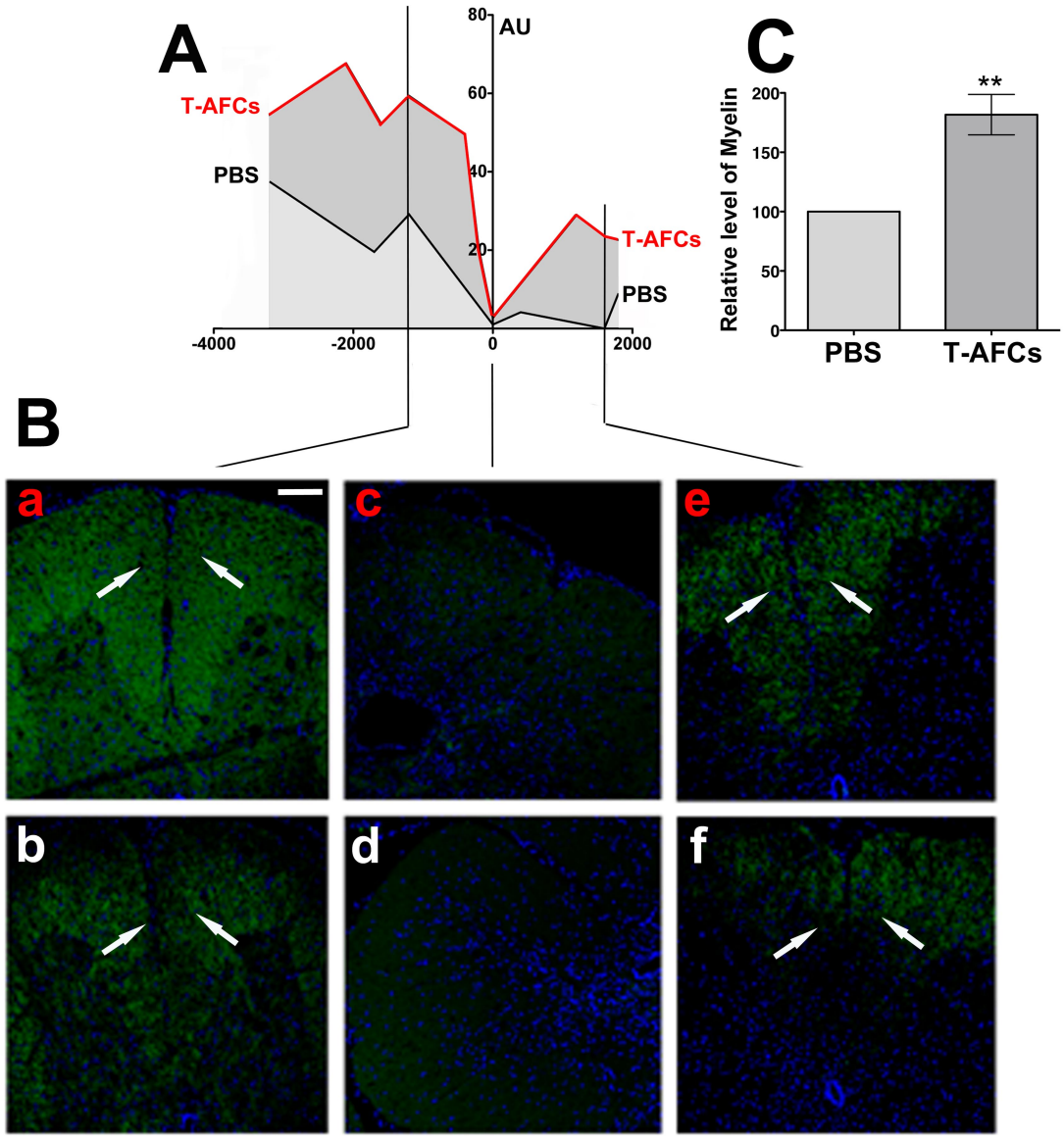

Figure 3: *Analysis of myelin. A: Myelin content in a 5mm region crossing the injury site, in a T-AFs (red) treated mouse and in a PBS-treated control (black), B: FluoroMyelin staining at 1.2 mm rostrally to the lesion (a and b), in the lesion epicenter (c and d) and 1.5 mm caudally to the lesion (e and f); a, c and e T-AFs treated animal, b, d and f PBS treated animal), arrows indicate the ventral funicoli were the ventral reticular spinal tract is located. C: Statistical analysis of the changes in myelin levels in transplanted animals compared to the control PBS-treated animals. These results represent the difference in myelin levels in a cord region spanning the lesion site; the data were obtained as average of*

*the outcome of different cellular treatments: #3.5, #3.6 and #7.30 compared to the vehicle treatment. Values represent means ± SEM. Significance symbols: **: p < 0.01. Scale bar 100 µm. AU: arbitrary unit.*

The number of cells found at lesion site is not high enough to sustain the integrative hypothesis, although there are some phenotypical changes in the cells, especially for the expression of typical neural marker such as GFAP and β-TUB III (Fig. 4A). We were able to find cell in which there was a total loss of the expression of the markers (Fig. 4-1), other in which was lost β-TUB III (Fig. 4-2) or GFAP (fig. 4-3) expression and finally cells which maintained the expression of both markers (Fig. 4-4).

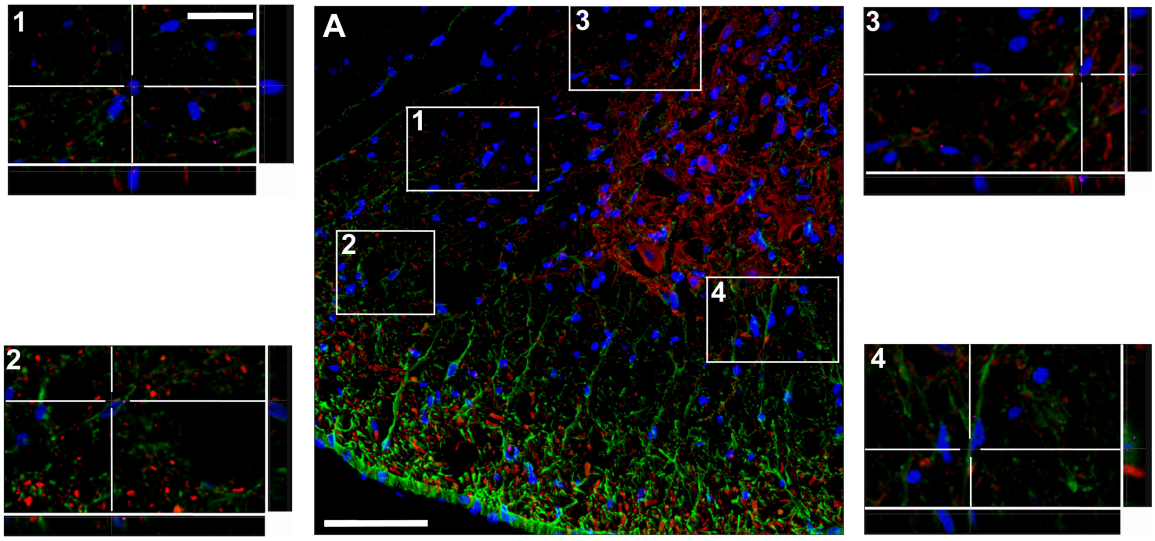

Figure 4: *Immunofluorescence staining of the spinal cord 1 mm rostrally to the lesion site. AFs labeled with Q-dot (magenta) were transplanted in lesioned mice one week after contusion. Immunostaining for GFAP (green), β-tubulin (red) nuclei (blue), and Q-dot staining (magenta). A: low magnification of the spinal cord ventral horn. Magnifications 1, 2, 3 and 4 indicated in Figure A by rectangles. Enlargements also reported their thickness (xz, yz) in order to highlight the cellular localization of the GFAP, β-tubulin, DAPI and Q-dot, Scale bar in A is 75 µm, in the enlargement 1 (representative for all the magnifications) scale bar is 25 µm.*

Following the double staining with CD31 and lectin it was found a statistically significant increase in volume of blood vessels in T-AFs treated mice compared to controls (Fig. 5) at 900 µm rostrally the injury site, while no differences were detected at 1600 µm between the two groups. Figure 5-A shows a lesioned not transplanted animal (PBS), while in fig. 5-B it is shown a lesioned and cell transplanted animal (T-AFs). It is clear the co-localization of CD31 (green) and lectin (red) (shown in the enlargements 1 and 2 respectively from panel A and B); some non-vessel staining is present for CD31 and most likely represents the staining of platelets and some sub-populations of T-lymphocytes. In 5-C is shown the quantification of total vessels volume in transplanted mice compared to controls: the increase detected was almost the 70% higher in T-cells treated animals vs. PBS.

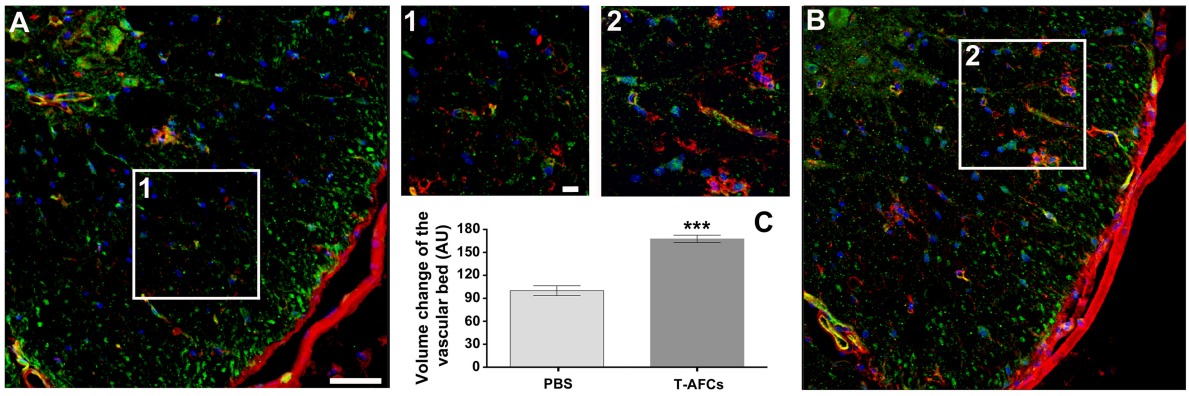

Figure 5: *Vascular compartment in the ventral horn 0.9 mm rostrally to the site of injury. A and B confocal analysis of lectin (red) and CD31 (green) co-staining of 10 µm thick transverse section. A: PBS-injected mouse; B: T-cells injected mouse; C: Statistical analysis of the relative changes in lectin levels in transplanted animals compared to the control animals. 1 and 2 enlargements respectively of panel A and B. Values represent means ± SEM. AU: arbitrary units. Scale bar 100 µm for A and B, scale bar 10 µm for enlargement 1 and 2. Significance symbols: **: $p < 0.01$.*

Regarding the inflammatory processes, we evaluated the presence of macrophages 400 µm from the lesion epicenter: the number of macrophages counted in the area is significantly lower in treated mice respect to controls (Fig. 6). In panel A is shown confocal analysis of a control mouse, while B is relative to a staining of a T-cells transplanted mouse. The estimated MOMA-2 positive cells number was 827 ± 244 (n = 3) in controls (Fig. 6C), and 300 ± 52 (n = 6) ($p<0.05$) in T-AFCs treated animals, corresponding to a 63.7% decrease.

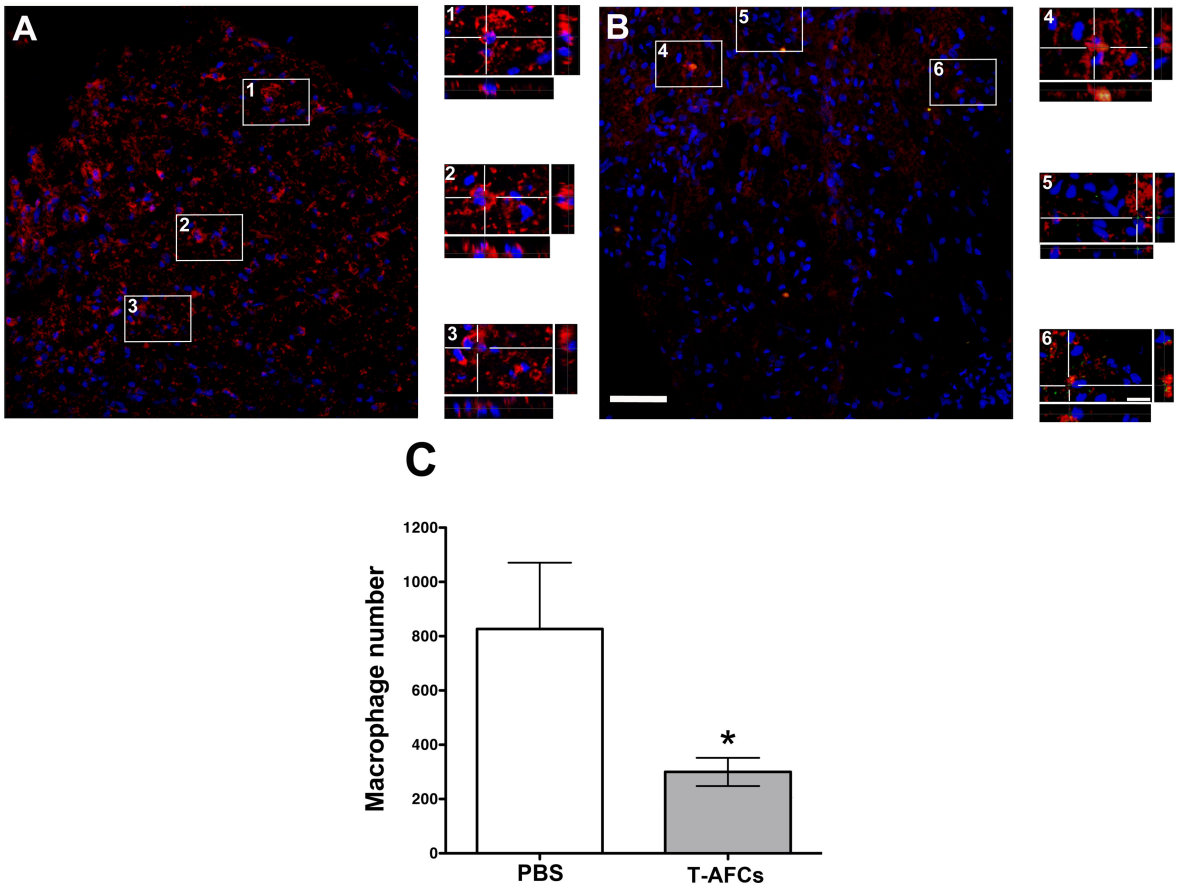

Figure 6: *Staining for MOMA-2 (macrophages staining) 100 µm rostrally to the lesion epicenter, four weeks after the transplantation. MOMA-2 in red, nuclei in blue, Q-dot in green. A) PBS-treated mouse; B) T-AFs-treated mouse; 1, 2, 3, 4, 5 and 6 are enlargements (located by rectangles on the panels A and B) indicating the distribution of the MOMA-2 staining and the presence (enlargements 4, 5 and 6) of Q-dot derived from phagocytosed transplanted cells. C) Relative number of macrophages. Values represent means ± SEM .Significance symbols: *: $p < 0.05$. Scale bar 50 µm in B is representative also for panel A. Enlargements 1, 2, 3, 4, 5 and 6 scale bar 10 µm.*

RNA extraction, retrotrascription and analysis

The mRNAs deriving from spinal cord and lung samples collected 2 and 7 days after transplantation were analyzed by means of Real-Time PCR. Regarding the spinal cord, no differences were found concerning the expression of genes related to neurotrophic factors (e.g. BDNF, NGF and others – data not shown), while we found an increase in the expression of VEGF (*Vascular endothelial growth factor*) at 2 and 7 days after the transplantation. The expression of HIF-1α (*Hypoxia Induced Factor 1α*), a regulator of VEGF transcription, was increased at 2 days while resulted decreased after 7 days from the transplantation.

Mesenchymal stem cells are known to produce, expecially after pro-inflammatory stimulation, many cytokines with anti-inflammatory, trophic and pro-angiogenic properties (Soleymaninejadian et al., 2012). Among these, we focused on HGF (*Hepatocyte growth* factor), because of its anti-inflammatory and angiogenic features. We then performed further Real-time PCR analysis, both in spinal cord and in lungs, site of cells homing: while no differences were found in the spinal cord, in the lungs HGF and IL-1β mRNA levels were significantly higher in mice that received T-AFs than controls (PBS and NT-AFs).

Gene of interest	sample	Tissue analised	Fold changes	Time (days) from transplant	Statistical significance
MmVEGF	Therapeutic/PBS	Spinal Cord	2.4	2	P=0.029
MmVEGF	Therapeutic/PBS	Spinal Cord	2.4	7	P=0.029
MmHIF	Therapeutic/PBS	Spinal Cord	1.7	2	P=0.014
MmHIF	Therapeutic/PBS	Spinal Cord	0.15	7	P=0.029
HGF	Therapeutic/PBS	Spinal Cord	none	2	
HGF	Therapeutic/PBS	Spinal Cord	none	7	
MmIL-1β	Therapeutic/Non Therapeutic	Lung	2.28	2	P=0.036
HGF	Therapeutic/Non Therapeutic	Lung	1.4	2	P=0.047

Table 1: *Summary of Real-Time PCR analysis and results in lungs and spinal cord*

Immunohistochemical analysis of the lungs

In order to confirm HGF expression in lungs, we perform a staining for HGF in samples deriving from mice transplanted with T-AFs, NT-AFs and PBS and collected 2 and 7 days after transplantation (Fig.7). As a result, HGF protein levels are higher for both groups (T/NT-AFs mice) after 2 days from transplant (20x for T-AFs and 12x for NT-AFs vs. PBS), while only the therapeutic

cells (represented here by culture 7.30) are able to sustain HGF production for a longer period (1 week) compared to culture 9.1 (representative of non-therapeutic cells) (8x T-AFs vs. PBS, 4x T-AFs vs. NT-AFs) (Fig 7).

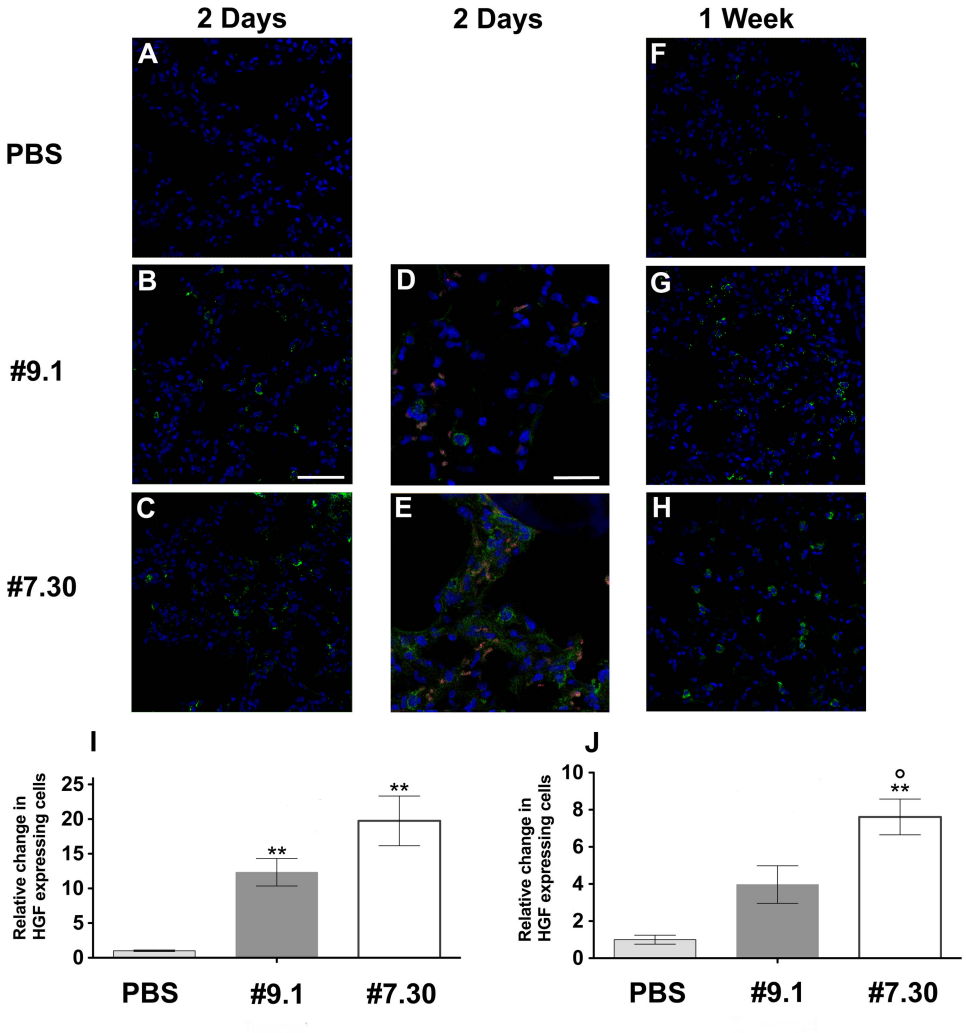

Figure 7: *HGF expressing cells in the lung two and seven days after transplantation.*

*In panels A, F are shown the stainings for a PBS-injected mouse; B, D and G: #9.1 (representative of NT-cells) and C, E, and H: #7.30 (representative of T-cells); D and E represent magnifications of panels B and C respectively. In blue are stained the nuclei, in green HGF positive cells, in red Q-dot positive cells. A, B, C, D and E: two days after transplantation. F, G and H: one week after transplantation. Lower graphs: quantification of the HGF expressing cells two days (I) and seven days (J) after transplantation. Scale bar 50 µm in B is representative for all the pictures with the exception of D where the bar scale is 30 µm (representative also for E). Significance symbols: *, °: $p < 0.05$, **: $p < 0.01$. * groups #9.1 and 7.30 compared to the PBS group; ° comparison between group #9.1 and 7.30.*

Western blot of plasma proteins

Given the previous results, we analyzed by western blot the HGF content in blood, as we were assuming that it might reach the injury site via bloodstream, in order to elicit the response of the damaged tissue. The results showed a significant increase of HGF plasma level in mice transplanted with T-AFs compared to NT-AFs and PBS 7 days after transplantation: mice receiving cells 7.30 (chosen as representative of T-AFs group) have almost 2.5 times more HGF in their blood than mice transplanted with cells 9.1 (chosen as representative of NT-AFs group) or controls (Fig. 8). No differences were found in plasma samples collected 2 days after cells transplant (data not shown).

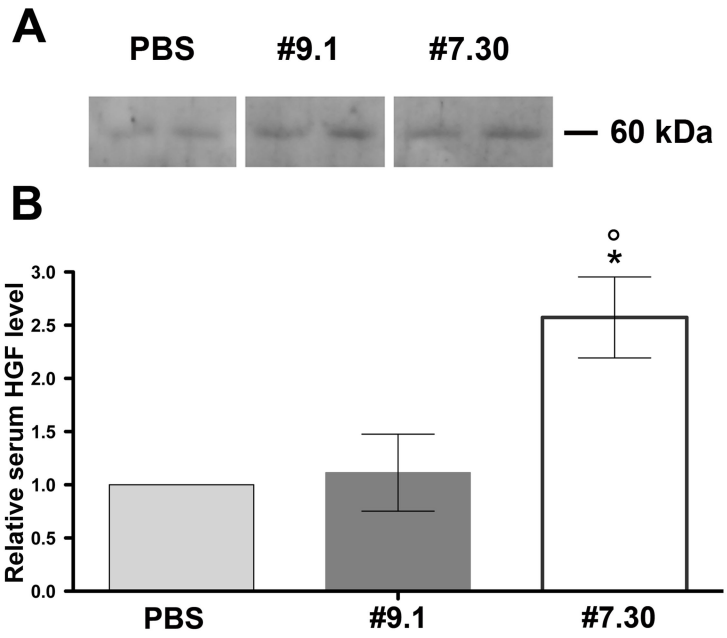

Figure 8: *HGF releasing in the blood circulation.*

*Western blot analysis of the HGF level in the circulating blood. A: western blot, in each lane was loaded the same amount of protein as obtained by means of protein quantification. B: quantification of the panel A (n = 4 for each sample), gray bar: PBS; dark gray bar: #9.1 and white bar: #7.30. Significance symbols: *°: $p < 0.05$.*

In vitro production of HGF

Cells belonging to culture 7.30 and 9.1, representative of T-AFCs and NT-AFCs groups, have a basal production of HGF. However, after a pro-inflammatory stimulation with IL-1 β and LPS the cells raised their HGF production, with a peak at 16 h after the stimulation, although the 7.30 cells have a higher increase, (4-fold vs. 1.7-fold) compared to 9.1 cells. Moreover, cells 9.1 are not able to significantly increase the HGF production after stimulation with LPS (Fig. 9).

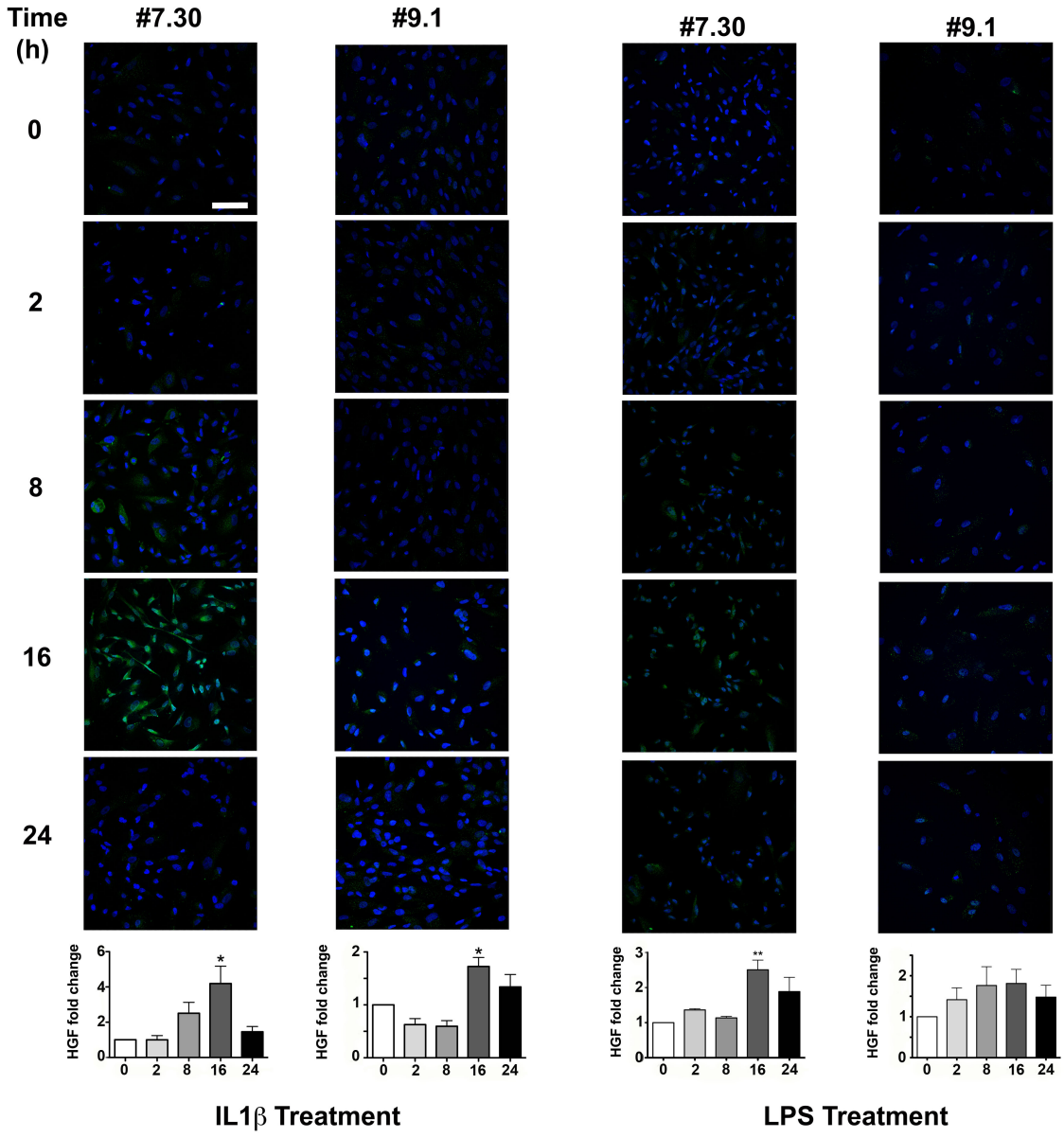

Figure 9: *In vitro induction of HGF by means of inflammatory stimuli.*

*Interleukin (IL)-1β and LPS treatments of the cultures #7.30 (T-AFCs) and #9.1 (NT-AFCs) for 0, 2, 8, 16 and 24 h. In the lower panels are represented the summary and the statistical analysis of the results obtained in four different experiments. Scale bar 75 µm is representative for all the pictures. Significance symbols: *: p < 0.05.*

DISCUSSION

Spinal cord injury (SCI) is a disabling degenerative disease that heavily impacts on patient's life from a physical, psychological, and social point of view. In a 2011 study it was estimated SCI global prevalence to be between 236 and 1,009 per million (Cripps RA et al., 2011), with a prevalence in young man (Singh A et al., 2014). To date, the only treatment available is thought to target the inflammatory processes sustaining the secondary damage: for this reason, finding new therapeutic approaches able to elicit regeneration of damaged fibers is of primary importance, as it might permit a complete recovery of the patient. In this regard, cell therapy might represent a useful tool, as the integration of transplanted cells might lead to a recover of tissue's lost functions. There are many different kind of cells that might be used with this purpose (for example embryonic or neural stem cells); however their use could raise some technical and ethical concerns, that might necessitate the use of novel cellular sources (Barker RA et al., 2013).

The aim of the present study was therefore to assess potential therapeutic activity of third trimester amniotic fluid cells once transplanted in a mouse model of spinal cord injury. Despite the expectations, only some of the transplanted culture were able to improve motor behaviour in the animals: mice transplanted with therapeutic culture #3.5, #3.6 and #7.30 (T-cells) had an average score of 4,83, corresponding to the ability to use the hind limbs to actively walk; while culture #1.1 and #9.1, resulted non-therapeutic (NT-cells), had an average score very similar to controls (3,36 vs. 3,80 respectively), allowing the mice to use the hind limbs only to sustain the body weight. Further analysis revealed that, among the analyzed markers, T-cells were able to express the surface marker NG2, while NT-cells were not. This fact is very important, as it might explain the reason for cellular effectiveness: NG2, also called chondroitin sulphate proteoglican 4 (CSPG4) or melanoma-associated chondroitin sulfate proteoglycan (MCSP) (Pluschke G et al., 1996), was first identified on human melanoma cells (Wilson BS et al., 1981), where it was able to increase the tumorigenic and metastatic properties of the cells. In nerve tissue, NG2 is normally expressed by a particular subgroup of cells, called Oligodendrocyte Precursor Cells (OPC) (Dawson MR et al., 2003), or Polydendrocytes (Nishiyama A et al., 2014), whose function is to produce remyelinating cells (Young KM et al., 2013), even though recent findings suggest a more complex role, especially in formation of non-myelinating oligodendrocytes and in pathogenesis of demyelinating neurodegenerative diseases (Nishiyama A et al., 2009). In spinal cord injury models, NG2 positive cells where thought to block endogenous regeneration, inhibiting assonal sprouting (Chen ZJ et al., 2002; Ughrin YM et al., 2006; Tan AM et al., 2006). Nevertheless, recent studies suggest a beneficial effect of NG2 in regenerating damaged fibers (de Castro R et al., 2006; Yang Z et al.,

2006), as well modulating neuronal networks (Sakry D et al., 2014) through synapses formation with neurons and partecipation into hippocampal long term potentiation (Nishiyama A et al., 2009; Ge WP et al., 2006). NG2 is also present on the surface of pericytes, a subgroup of perivascular cells able to make intimate connections with capillary endothelial cells (Birbrair A et al., 2014). These cells have a fundamental role in regeneration and formation of new vessels (Kabara M et al., 2014), as demonstrated in mouse models, in which NG2 absence leaded to aberrant tumor vascularization (Huang FJ et al., 2010). Furthermore, NG2 positive cells are important for immune suirvellance, as pericytes are able to attract leukocytes exiting through venules and "instruct" them through upregulation of adhesion molecules and chemoattractant factors (Stark K et al., 2013).

In the past years it was demostrated the ability for NG2 to bind growth factors, as FGF2 and PDGF-AA, serving as co-receptor and thus enhancing their signalling (Goretzki L J Biol Chem. 1999). In a corneal angiogenesis model, NG2 negative corneas showed a greatly reduced responsiveness to FGF2 respect to wild type controls (Ozerdem U et al., 2004).

The therapeutic effects could not be exerted by AF cells integration into spine ciruitry, as the number of cells in the lesion site at 1 month from the transplant was not high enough to sustain such hypothesis, even though some cells were induced to change their phenotype, altering GFAP and β-TUBIII expressions. At the same time, T-cells were able to induce a greater myelin sparing in a 5 mm perilesion region compared to NT-cells and PBS controls: the higher tissue preservation might underlie the motor differences between the animal groups. To explain these results, very similar to the ones previously obtained in our laboratory (Bottai D, et al 2008), it was performed a Real-Time PCR analysis on neurotrophic genes expression, such as NGF, BDNF, LIF, NT-3, CNTF, without finding any statistical difference between groups.

AF cells express typical mesenchymal markers (Bottai D, et al 2012), moreover mesenchymal cells are known to possess pro-angiogenic and immunomodulatory activity by producing and releasing trophic and immunoactive molecules (Uccelli A, 2008; Pacini S, 2014) directly, or by stimulating the production by endogenous tissues (Chen X et al., 2002). Thus, the positive action might be explaind with a pro-angiogenic and immunomodulatory action of the cells: as hypothesized, AF cells-induced angiogenesis is greater in T-cells treated mice compared to NT-cells or PBS treated animals, effect obtained through a higher expression of HIF-1α (*hypoxia induced factor 1 alpha*) and VEGF (*vascular endothelial growth factor*). This results were confirmed with a staining that

clearly showed a higher content of microvessels in a perilesion site: the increased number of vessels may probably result in a more efficient debris removal by the blood flow, contributing in sparing the tissue, as debris are known to be detrimental for nerve regeneration (Kang H and Lichtman JW, 2013). Furthermore, the immune reaction is also lower in these animals, as the number of macrophages is significantly lower if compared to NT or PBS transplanted mice: this may lead to a formation of a more pro-regenerative environment, resulting in the surviving of a higher number of nerve cells.

It is well known that MSC are almost entirely entrapped in the lungs when administered *i.v.* (Lee RH et al., 2009), though it is not clear if this is a passive process or MSC express adhesion molecules that determine a selective homing: it was shown that changes in materials or detachment procedures during *in vitro* culturing steps reduce lung entrapment, as the cells lose affinity for the endothelium (Deak E et al., 2010; Dreher L et al., 2013; Kerkelä E et al., 2013). Similarly, the homing site for both T and NT-cells were the lungs, thus in order to find out the molecular mechanism by which the cells exerted their therapeutic activity, it was performed a qPCR analysis of gene expression. Mesenchymal stem cells are known to produce HGF, a pro-angiogenic, trophic and immunomodulatory polypeptide (Soleymaninejadian E et al., 2012), able to supports the regeneration of organs like liver, kidneys, and lungs (Matsumoto K and Nakamura T, 1996), especially after their "activation" through several cytokines, like IL-1β, IL-6 and TNF-α (Ren G et al., 2008; Trusolino L et al., 2010). HGF and its receptor (c-Met) are up-regulated after injury mainly in reactive astrocytes around the injured region (Shimamura M et al., 2007), promoting functional recovery through immune suppression and induction of myelination. In an elegant study, Bai and colleagues clearly demontrated that, following MNS transplantation in an multiple sclerosis mouse model, the cells elicited functional recovery through production and release of HGF (Bai L et al., 2012). After qPCR analysis of the lungs, HGF and IL-1β levels resulted significantly higher in T-cells treated mice compared to NT-cells and PBS-treated controls, data confirmed with immunohistochemical analysis of HGF production in the lungs. Furthermore, also plasma HGF level was greater in these animals than in controls, significant of the ability for the cells to recognise stimuli from the environment and to react by changing their proteins production.

Further in vitro studies showed a correlation between inflammatory state (induced by IL-1β and LPS addition to culture media) and HGF production by T and NT-cells, with a significantly greater synthesis by T-cells, with a peak at 16 hours after the induction.

Thus, our hypotesis was that, after i.v. transplantation, the cells homed in the lungs, where they increased HGF production. Once released, HGF was able to reach the injury site through the blood stream, where it could exert its pro-angiogenic, trophic and immunomodulatory activity. The overall effects were then translated in a higher myelin preservation that was reflected in a better motor performance (Fig. 1).

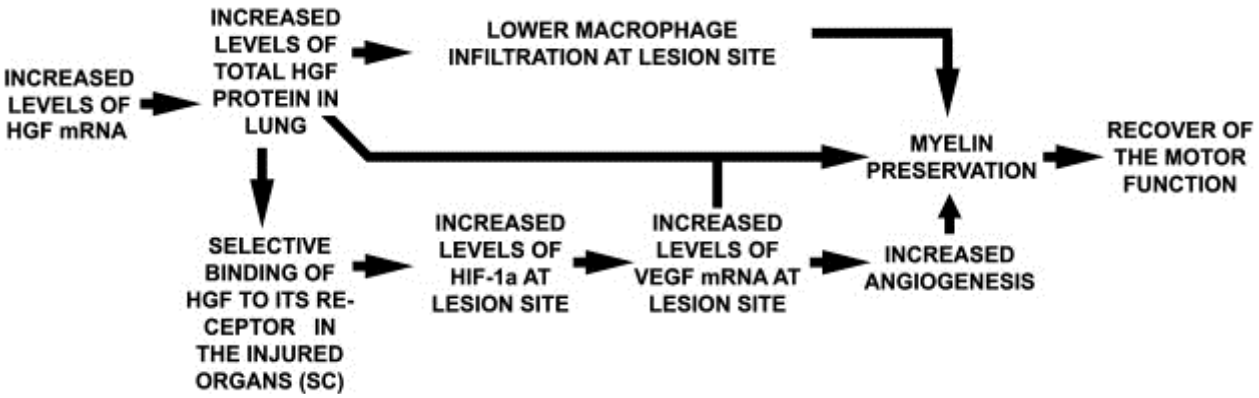

Figure 1: *Hypothesis motor recovery mechanisms. The increased levels of HGF mRNA and protein in lung sustained by T-AFCs transplantation is detected in the injured organs. This binding is able to induce an increase of HIF-1α and VEGF at the lesion site thus increasing angiogenesis. On the other site, HGF induces a lower macrophage infiltration at the lesion site. Finally, HGF possesses intrinsic antiapoptotic properties: all together, this actions reflect in higher tissue preservation with a consequent better motor performance. (Abbr. SC = spinal cord).*

Preliminary studies revealed a sort of correlation between NG2 presence and HGF production. We performed a gene silencing experiment in which we assessed HGF production after IL-1β and LPS stimulations of T-cells from culture #7.30 previously silenced for NG2 (as controls were used wild-type #7.30 cells and NT-cells from culture #9.1, that did not express the marker). Interestingly, we observed a diminished HGF production in the silenced cells, even though we were not able to find out if the cells were less responsive to the inflammatory agents or suffered an impairement in HGF production. Though the experiments were held as a pilot study, the results obtained suggest that it might be useful to perform further studies, in order to assess if and how NG2 is linked to HGF production.

The aforementioned results are very similar to the ones obtained by our group after neural stem cells (NSC) and embryonic stem cells (ES) transplantation (Bottai D. Et al., 2008, 2010) using the same mouse model of spinal cord injury. Even though AF cells resulted less effective than NSC, they are equivalent to ES in promoting motor recovery; however, it is important to consider that AF cells were transplanted one week after injury, while NSC soon after the spine contusion. This delay might explain the different scores obtained by AF-treated respect to NSC-treated mice. In all cases, the different cells did not integrate into spine circuitry, being able to elicit therapeutical effects by producing trophic/antiapoptotic/antiinflammatory factors. The observation that the beneficial and therapeutic effects are achieved through secretion of immune modulatory or neurotrophic paracrine factors has inspired an alternative outlook on the use of stem cells in regenerative medicine (Drago D et al., 2013). During years, the perspective has changed from integration into the damaged tissues, to a "bystander effect": in this case the transplanted cells are thought to act as "drugs factories", able to react to the environment releasing bioactive molecules (Martino G and Pluchino S, 2006; Fischbach MA et al., 2013; Pluchino S., et al 2013). Very recently, Cossetti and colleagues gave new insights on cellular cross-talks, identifying a mechanism of cellular signaling by which neural stem/precursor cells (NPCs) communicate with the microenvironment via vesicles release (Cossetti C et al., 2014).

In perspective, being able to induce a "pluripotent state" or a neuralization of AF (Moschidou D et al 2012), might represent a useful improvement of amniotic fluid cells properties, increasing their therapeutic potentiality, as it could affect cells homing and/or increase neurotrophic factors production: NSC in fact, seems to possess a higher "pathotropism" towards the injured tissue, as it is represented by nerve tissue (Butti E et al., 2014). Furthermore, a greater production of neural-derived neurotrophic factors by neuralized AF cells, might improve their efficacy over target cells.

To conclude, amniotic fluid cells possess therapeutic activity when transplanted in our model of spinal cord injury, representing an a tecnical and ethical alternative to the use of neural or embryonic stem cells.

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