Universidade do Minho
Escola de Ciências da Saúde

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Functionalized Hydrogels and Cell based Therapies: A Tissue Engineering Approach for Spinal Cord Injury Regeneration

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Work performed under the supervision of:
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Declaration

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RESUMO

As Lesões da Medula Espinal (LME) continuam a ser das doenças mais devastadoras do Sistema Nervoso Central (SNC), devido aos graves danos funcionais causados, bem como o fardo que representam para os pacientes. A falta de uma terapia eficaz para tratar esta condição ou pelo menos atenuar a sua extensão levou a comunidade científica a procurar novas estratégias possíveis para abordar esta doença. Desta forma, hoje em dia terapias moleculares e celulares têm sido aplicadas em modelos animais de LME com resultados promissores. Contudo, terapias singulares, normalmente falham quando aplicadas na clínica, portanto a combinação de mais do que uma técnica poderá ser a solução. Assim sendo, neste trabalho conjugamos um hidrogel (Gellan Gum-GRGDS) com células mesenquimais do tecido adiposo (ASCs) e células gliais do bolbo olfativo (OECs), com o intuito de promover a recuperação de ratos com LME. Em primeiro lugar, as interações *in vitro* entre os dois tipos de células foram avaliadas através de co-culturas de contacto direto e através do secretoma de cada tipo celular. Depois, foi determinado o crescimento de ASCs e OECs quando encapsuladas no hidrogel GG-GRGDS. Também foi verificado o potencial regenerativo do secretoma de ambas as células em estudo, com recurso a um modelo *in vitro* de regeneração axonal, baseado em Gânglios da Raiz Dorsal (DRGs). Finalmente, o valor terapêutico da nossa abordagem foi determinado usando um modelo *in vivo* de LME. Os ratos foram sujeitos a uma hemissecção do lado esquerdo da medula espinal (T10-T11) e divididos em diferentes grupos de acordo com o respetivo tratamento: animais não tratados (grupo HS); animais tratados com células (Cells); com GG-GRGDS (GG); e com células encapsuladas em GG-GRGDS (GG+cells). Foi também incluído um grupo de animais sem lesão, apenas sujeito a uma laminectomia (Sham). Os resultados demonstraram que as ASCs e OECs foram capazes de crescer em co-culturas de contacto direto sem alterações significativas da sua morfologia e do seu número total; ambas as células apresentaram uma taxa de crescimento normal quando encapsuladas em GG-GRGDS, o que demonstra a capacidade deste último como veículo de transporte celular. Relativamente aos explantes de DRGs, os fatores secretados pelas ASCs e OECs parecem potenciar a regeneração axonal. Finalmente, os animais tratados com a conjugação de hidrogel com ASCs+OECs apresentaram melhorias significativas das suas capacidades motoras e ao mesmo tempo menor astrogliose. Em suma, estes resultados indicam que ao conjugar GG-GRGDS com ASCs e OECs, poderá ser possível desenvolver terapias alternativas direcionadas para a recuperação de LME.

ABSTRACT

Spinal Cord Injury (SCI) remains one of the most devastating diseases of the Central Nervous System (CNS) due to the drastic functional impairments caused, as well as the burden it represents for patients. The lack of an effective therapy to treat this condition or at least minimize the injury extent, led the scientific community to search for new possible strategies to address this question. Consequently, nowadays molecular and cell based therapies are often applied on SCI animal models with promising results. However single therapies usually fail when translated to the clinics, so the combination of more than one technique appears to be the best solution. Having this in mind, in the present work, we aimed at conjugating a hydrogel based scaffold (gellan gum-GRGDS) with Adipose tissue derived Mesenchymal Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) in order to promote the recovery of SCI rats. Firstly, the *in vitro* interactions between both cell types were assessed through direct co-cultures and using the secretome of each cell. Then, the growth of ASCs and OECs while encapsulated within the GG-GRGDS hydrogel was evaluated. We also verified the regenerative potential of both cells' secretome, using an *in vitro* model of axonal regeneration based on Dorsal Root Ganglia explants (DRGs). Finally, the therapeutic value of our proposed strategy was determined in an *in vivo* model of SCI. Rats were subjected to an hemisection injury on the left side of the spinal cord (T10-T11) and divided in different groups according to the respective treatment: no treated animals (HS group); cells treated (Cells); GG-GRGDS-treated (GG); and GG-GRGDS with cells-treated animals (GG+cells). An additional group of animals without injury, only subjected to a laminectomy was also included (Sham). The results showed that ASCs and OECs were able grow together on direct co-cultures without significant alterations in cell morphology and cell numbers; both cells also exhibited a standard growth profile while inside the GG-GRGDS hydrogel, which proves the suitability of this hydrogel as a vehicle for cell transplantation. Regarding DRGs explants, ASCs and OECs secreted paracrine factors seemed to potentiate axonal regeneration. Finally, the animals treated with the conjugation of GG-GRGDS hydrogel with ASCs+OECs presented significant improved motor skills, while simultaneously disclosing an evident decrease in astrogliosis. All together, these results indicate that by using GG-GRGDS conjugated with ASCs and OECs, it may be possible to develop alternative therapeutic routes for SCI repair/regeneration

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List of Abbreviations

AIS – ASIA Impairment Scale

ANS – Autonomic Nervous System

ASCs – Adipose Stem Cells

ASIA – American Spinal Injury Association

BBB_1 – Blood Brain Barrier

BBB_2 – Basso, Beattie and Bresnahan

BDNF – Brain-Derived Neurotrophic Factor

bFGF – basic Fibroblast Growth Factor

BM-MSCs – Bone Marrow Mesenchymal Stem Cells

CM – Conditioned Media

CNS – Central Nervous System

CSPGs – Chondroitin Sulphate Proteoglycans

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DRG – Dorsal Root Ganglia

ECM – Extracellular matrix

EPO – Erythropoietin

ESCs – Embryonic Stem Cells

FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

FDA – Food and Drug Administration

FGF – Fibroblast Growth Factor

GAG - Glycosaminoglycan

GDNF – Glial Derived Neurotrophic Factor

GFAP – Glial Fibrillary Acidic Protein

GG – Gellan Gum

GTPase – Guanosine Triphosphatase

HBSS – Hank's Balanced Salt Solution

HGF – Hepatocyte Growth Factor

HNA – Human Nucleotide Antibody

HUCPVCs – Human Umbilical Cord Perivascular Cells

ICC – Immunocytochemistry

IFNγ – Interferon gamma (γ)

IGF-1 – Insulin like Growth Factor

IL-1 – Interleukin 1

IL-10 – Interleukin 10

iPSCs – induced Pluripotent Stem Cells

MAG – Myelin-Associated Glycoprotein

MEM – Minimum Essential Medium

MES – 2-(*N*-morpholino)ethanesulfonic acid

MSCs – Mesenchymal Stem Cells

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NGF – Nerve Growth Factor

NK – Natural Killer

NMDA – N-methyl-D-aspartate

NSAIDs – Non-Steroidal Anti-Inflammatory Drugs

NSCs – Neural Stem Cells

NSPCs – Neural Stem/Progenitor Cells

NT3 – Neurotrophin 3

NT4/5 – Neurotrophin 4/5

OECs – Olfactory Ensheathing Cells

OF – Open Field

OPCs – Oligodendrocyte Progenitor Cells

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PNS – Peripheral Nervous System

RT – Room Temperature

SEMA3 – Semaphorin 3

SCF – Stem Cell Factor

SCI – Spinal Cord Injury

SCs – Schwann Cells

SGZ – Subgranular Zone

SVZ – Subventricular Zone

TE – Tissue Engineering

TGFβ – Transforming Growth Factor beta (β)

UV – Ultraviolet

VEGF – Vascular Endothelial Growth Factor

WJ-MSCs – Wharton's Jelly Mesenchymal Stem Cells

% - percent

°C – Celsius degrees

µg – micrograms

µl – microliters

cm – centimeters

cm^2 – square centimeters

h – hours

mg – milligrams

min – minutes

ml – milliliters

mm – millimeters

ng – nanograms

nm – nanometers

rpm – rotations per minute

v/v – volume per volume

w/v – weight per volume

Figures and tables

Chapter 1
INTRODUCTION

1.1 The Central Nervous System

An important component of the nervous system, the CNS is responsible for integrating all the information received and coordinating the activity of most cells, tissues and organs. Having this in mind, it is not hard to imagine that an injury affecting this system will have a tremendous impact on physiologic functions, as well as at the psychological level.

1.1.1 CNS composition: brain and spinal cord

The CNS consists of the brain and the spinal cord. The brain is divided into two hemispheres in addition to the brainstem, cranial nerves and the cerebellum, being responsible for processing the information received (the exogenous stimuli), and controlling all the organs and actions of the human body. [1]

However, the spinal cord (fig. 1) is also very important, since it makes the direct connection from the brain to the rest of the body. This long, thin, tubular bundle of nervous tissue extends from the medulla oblongata (part of the brainstem) to the space between the first and second lumbar vertebrae. The spinal cord gives rise to a total of 31 pairs of spinal nerves, which exit the spinal cord between the vertebral bones of the spine. The spinal nerves possess two roots, a ventral root that innervates skeletal muscles and a dorsal root carrying sensation to the spinal cord. Dorsal root fibers have the particularity of having their cell bodies located in structures called Dorsal Root Ganglia (DRG), which lie just outside the spinal canal. [1] Although spinal cord serves as a conduit of descending motor information and ascending sensorial information, it also participates actively in the transmission of information from the Autonomic Nervous System (ANS), responsible for controlling involuntary actions like heart rate, digestion, among others. [1] Moreover spinal cord helps to form the reflex arc, where the information from the periphery is carried by a sensory neuron to the spinal cord and is directly transmitted to a motor neuron for a motor response, without involvement of the brain. [2]

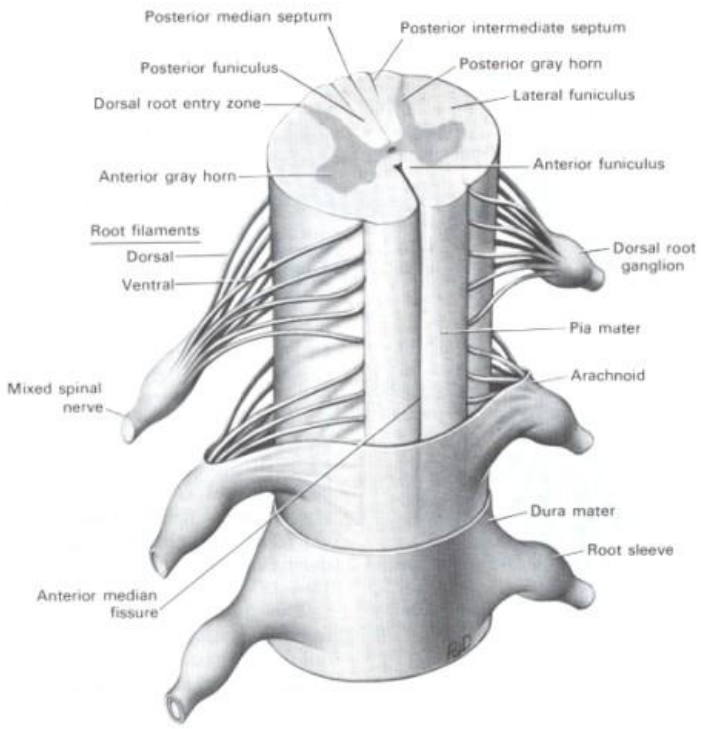

Figure 1 – Illustration of the human spinal cord. Image obtained from: http://www.laesieworks.com/spinal/images-spinal/SpinalCord.jpg

1.1.2 CNS cells: neurons and glial cells

Neurons and glial cells are the building blocks of the nervous system. [3] Neurons are highly differentiated cells, assuming a variety of shapes. In general they have multiple extensions from the nerve cell body – called dendrites – and possess one axon, characterized by the transmission of chemical and electrical signals to other cells (most commonly other neurons). This process is called neurotransmission. Moreover, in some neurons the axon is surrounded by an insulating myelin sheath, which facilitates rapid impulse conduction. The sites of interneuronal communication in the CNS are termed synapses and it is through the connection of neurons that neural networks are formed. [3] On the other hand, glial cells are known for their supportive functions, maintenance of homeostasis and more recently, to participate actively in the process of neurotransmission. [4] Among the different non-neuronal cells of the CNS, there are astrocytes, oligodendrocytes (both generally called macroglia) and microglia. Astrocytes mostly provide metabolic supporting roles; oligodendrocytes are myelin producing cells, and microglia are the mediators of immune responses in nervous tissue, becoming activated under pathological states. [3]

1.1.3 CNS protection and Neurogenesis

Both brain and spinal cord are protected by the skull and the vertebral column, respectively. In addition, both structures are enclosed by a system of membranes (the meninges) and protected by the Blood Brain Barrier (BBB_1). The BBB_1 provides a very restrictive selective permeability between the CNS and the blood stream, conferring protection against inappropriate signals. Thus, there is a diminished rate of access of most lipophobic chemicals, diffusional barriers retard the movement of substances and only specific energy-dependent transport systems permit selected access. [3]

However, and despite this kind of protection, the CNS is not immune to injury, either trauma-induced or not. To this, it aggravates the fact that the CNS possesses a limited regenerative capacity. [5] Since neurons do not undergo cell division, the generation of new neurons is dependent on the differentiation of a particular subset of stem cells – neural stem progenitor cells (NSPCs). [6] This process is called neurogenesis. In adults, it happens in particular regions of the brain, namely the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. [5] Furthermore, neurogenesis can also be found in the spinal cord. [7]

1.2 Spinal Cord Injury

Knowing about the limited regenerative capacity of the CNS and taking into account the utmost importance of the spinal cord in this system, an injury in this vital organ usually leads to drastic consequences for the patient. Spinal Cord Injury (SCI) is a condition with a growing interest for scientists and medical researchers. This fact is clearly evident due to the increasing number of publications on this topic (fig. 2).

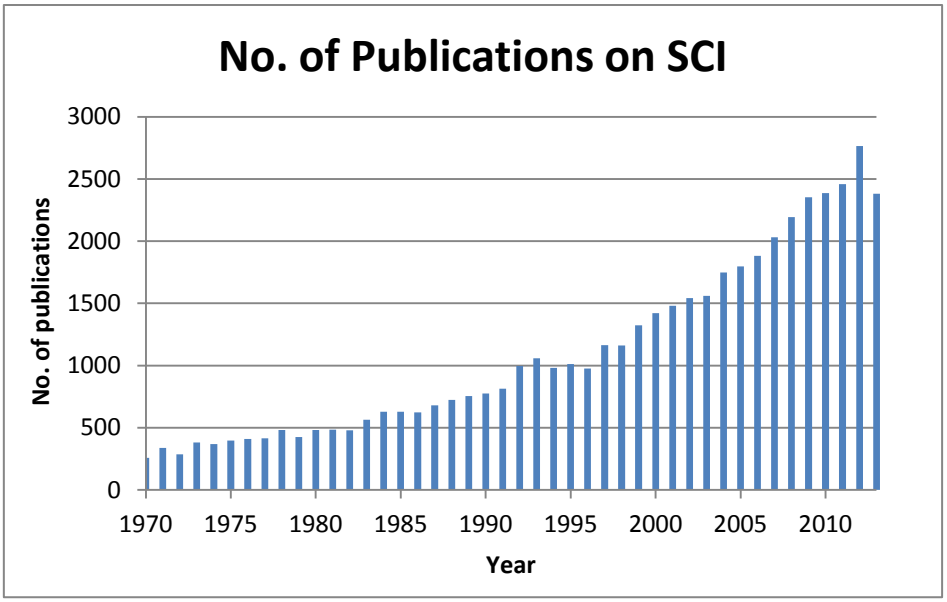

Figure 2 – Number of publications concerning Spinal Cord Injuries, since 1970 to date. Source: Pubmed, October 2013.

1.2.1. SCI incidence, prevalence and causes

Annual incidence rates in traumatic SCI vary from 12.1 per million in The Netherlands to 57.8 per million in Portugal. [8] There can also be distinguished two age-peak groups, concerning SCI incidence: i) young adults (between 15 and 29 years old) most likely due to motor vehicle accidents and sport-related injuries, and ii) older adults (more than 65 years old) due to falls and non-traumatic complications. This study also showed a trend to increase SCI incidence among the elderly. [8] A more recent systematic review [9] showed that annual incidence varies from 2.3 per million in a Canadian study to 83 per million in Alaska. This study also showed that prevalence ranges from 236 per million in India to 1800 per million in the USA. Patients with traumatic SCI have a higher lethality compared with the normal population, and the main causes of death are respiratory problems, heart diseases and suicide. The last reason highlights the impact of SCI at the psychological level.

As we can see, different studies on SCI epidemiology show that SCI incidence varies substantially all over the world. This may be due to a lack of standardization regarding the inclusion strategy used in these studies, namely heterogeneity in population-age intervals and the inclusion or not of different injury stages, pre-hospital deaths and non-traumatic derived lesions. [8]

Nevertheless, SCI still affects millions of people worldwide and the reason behind the lack of treatment is its particular aggressive pathophysiology.

1.2.2 SCI consequences

SCI can occur by reasons other than traumatic events. For instance, it can derive from an internal force like the growth of a tumor, internal bleeding, illnesses causing inflammation such polio or transverse myelitis, osteoporosis and cervical spondylosis, which may cause damage to the vertebrae. [10]

In general, people lose sensation and movement of all parts of the body below the injury. For instance, if the injury occurs in the lumbar region of the spinal cord, the patient will probably lose sensation and/or movement from the waist down. This means that the higher the location of the damaged area, the greater the impairment is (fig. 3). [10]

Furthermore, more specific types of lesion can occur. For example, the damaged area can only affect some type of nerves. So, damage to motor nerves results in paralysis, or loss of control of movement. On the other hand, damage to somatosensory nerves leads to loss of sensation and perception (loss of feelings of touch, pain, temperature, among others). [10]

In addition, spinal cord damage can be classified as complete or incomplete, being the complete lesions the ones with more pronounced effects. If the autonomic nervous system (ANS) is affected, one can lose control over important body function aspects, like heart rate, digestive and respiratory systems, reducing the probability of survival. [10]

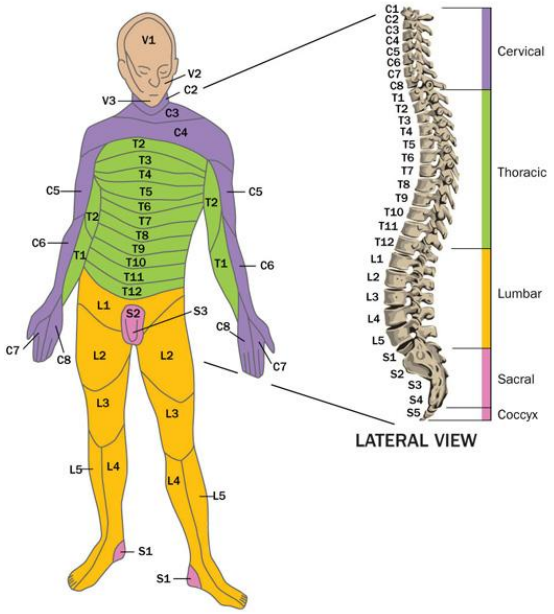

Figure 3 – Illustration of the body parts affected after SCI, depending on the site of injury. Image obtained from: http://sci-bc.ca/wp-content/uploads/2011/10/spine-art-affected-areas.jpg

1.2.3 Pathophysiology of SCI

Spinal Cord injuries can be roughly divided in four types of lesion: 1) cord maceration; 2) cord lacerations (from gunshot or knife wounds); 3) contusion injury and 4) solid cord injury (no central focus of necrosis). In the latter two types of injury, the spinal cord surface is not breached and the invasion by connective tissue is minimal. [11]

SCI can be divided in three distinct phases: the **primary** or acute phase, the **secondary** and the **chronic** phases.

The primary injury comprises the immediate effects resulting after an injury to the spinal cord. Sharp penetrating objects and compression by a blunt force are the main "external" reasons behind SCI – the so called traumatic SCI.

Hence, the initial injury depends on the type of impact made to the spinal cord. The primary mechanical trauma usually causes damage to neural and other soft tissues, like endothelial cells of the vasculature, resulting in necrosis, edema, hemorrhage and vasospasm. [11, 12] This is then followed by a barrage of action potentials accompanied by ionic imbalances. This contributes to a failure in normal neural function and spinal shock, which can last for 24 hours. In addition to this, there is often a continuous compression force exerted by a displaced vertebra, which increases edema and fibrotic responses. [11]

After the initial injury, a cascade of molecular and cellular alterations is triggered, leading to the **secondary phase**, which starts minutes after injury and can last for weeks. The ischemic cellular death, ionic imbalances and edema continue from the acute phase to this one. Moreover, cell lysis (as well as synaptic and non-synaptic transport) leads to an extracellular increase of glutamate and other excitatory amino acids, reaching cytotoxic levels. As a result of glutamate excitotoxicity, lipid peroxidation and free-radical production also occur. Additionally, programmed cell death – apoptosis – is triggered, involving reactive gliosis and astrocytic proliferation. Within the first 24 hours there is also an infiltration of immune cells, namely neutrophils and lymphocytes. All these changes lead to further neural damage. The expression of inhibitory factors and barriers to regeneration also occurs. During the secondary phase it is notorious an increase in lesion size, represented by a larger region of cell death. [11]

In the **chronic phase** (lasts from days to years), apoptosis continues in both anterograde (towards synapses) and retrograde directions (towards cell bodies), including brain regions. There are several alterations in expression levels and activation states of receptors and ion channels. In addition, several other phenomena might occur like: scarring and tethering of the cord (mostly in penetrating injuries); conduction deficits as a result of demyelination; formation of a cyst that continues to enlarge (a condition called syringomyelia); alterations in neural circuits due to changes in inhibitory and excitatory inputs and development of hyperexcitability in some cells, resulting in chronic pain syndromes. Although some axons exhibit regenerative and sprouting responses, they do not go farther than one millimeter. [11]

The large fluid-filled cavity (or cyst) that is formed in the center of the cord is surrounded by a subpial rim containing some preserved axons (Fig. 4). [12] In that region there is also an activation of astrocytes and macrophages, which along with other cells secrete extracellular matrix (ECM) and inhibitory molecules that constitute the **glial scar**, resulting in a physical and chemical barrier to regeneration. [13]

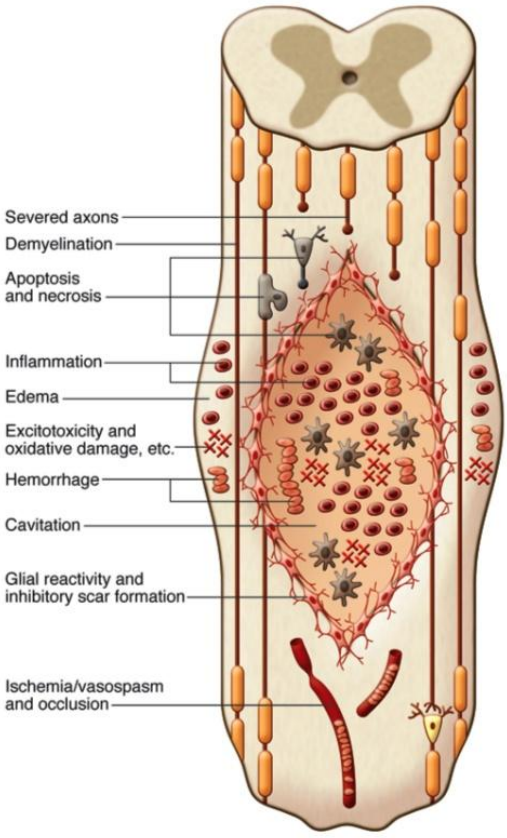

Figure 4 – Representation of an injured spinal cord, with the respective pathophysiological events occurring after injury. Image adapted from Mothe *et al.* [12]

The Glial Scar consists predominantly of reactive astrocytes and proteoglycans, but in severe lesions that open the meninges, there is also an invasion of connective tissue elements. [14]

The astrocytic response after CNS injury is called reactive gliosis, characterized by a concentration of hypertrophic astrocytes around the lesion core. [15] This can be observed by an increased production of intermediate filaments, like the glial fibrillary acidic protein (GFAP). [16] Eventually, this astrocytic response forms a physical wall, which acts as a barrier to axonal growth (axons take a dystrophic appearance). [14]

However, and despite the glial scar inhibits regeneration, some evidences indicate that it might also have a protection role in some CNS tissue. [15]

For instance, Faulkner and colleagues [15] used a transgenic mouse model that allowed the ablation of the reactive astrocytes response. After a mild SCI, transgenic animals presented a failure of BBB_1 repair, leukocyte infiltration, local tissue disruption, severe demyelination, among others. On the other hand, control animals presented little tissue disruption and little demyelination. This emphasizes the importance of reactive astrocytes in protecting CNS tissue and preserving function after SCI. Therefore the glial scar seems to isolate the injury site from healthy tissue, preventing the spreading of a wave of uncontrolled tissue damage.

The rupture of the BBB_1 is a key factor in glial scar formation, since areas of most extensive BBB_1 breakdown correlate with areas of greatest glial scarring, as well as the largest numbers of activated macrophages. [14] Nevertheless, neurons still cannot grow across the glial scar and recover their function. This indicates that the glial scar formation may be important in the acute phase, but after the BBB_1 integrity is restored, the degradation of the glial scar may be fundamental to promote axonal regrowth.

At the molecular level, transforming growth factor β 1 and 2 (TGFβ1 and TGFβ2) expression levels are augmented after CNS injury [17] and when both molecules were experimentally attenuated, glial scar formation was reduced. [18] Another molecular mediator implicated in glial scarring is interleukin 1 (IL-1), a protein that is known for initiating inflammatory responses in various cells, including astrocytes. [19] Additionally, the interaction between interferon γ (IFNγ) and fibroblast growth factor 2 (FGF2) also has a role in the induction of the glial scar. [14]

As mentioned before, axons near to the glial scar take a dystrophic appearance and although there is an evident stalled growth at the injury site, neurons still have the capacity to regenerate or at least return to active growth states. [14] This was proved in different experiments, where peripheral nerve grafts were able to promote axonal regrowth of CNS injured neurons. [20, 21]

Moreover, even after long time periods post-injury, dystrophic endings were capable of sprouting, being some of the sprouts myelinated through the action of infiltrated Schwann cells. [22]

Growth cone collapse is another form of axonal stalled growth near to the glial scar. When injured neurons find myelin products, their growth cones arrest, collapse and often retract. [14]

Inhibition of axonal growth

Astrocytes are known to produce a class of molecules, known as proteoglycans. [23, 24] Components of the ECM, these molecules have a protein core, linked by four sugar moieties to a sulphated glycosaminoglycan (GAG) chain that contains repeating disaccharide units. [14] Among the proteoglycans produced, chondroitin sulphate proteoglycans' (CSPGs) expression is increased after an injury in the brain and spinal cord. [25, 26]

CSPGs have been implicated as barriers to axonal growth. [14] They repel both embryonic as well as adult axons *in vitro* and are potent inhibitors of several growth promoting molecules. [27, 28] In addition, cold-blooded species, capable of long tract regeneration, have a minimal upregulation of CSPGs on reactive glia. [14] Furthermore, when the enzyme chondroitinase ABC was applied to *in vitro* cultures, it allowed a greater extension of the neurites of retinal ganglion cells, since this enzyme is able to partially degrade CSPGs in culture. [29] However, it is also known that different neuronal populations respond differently to CSPGs and it seems that in general, neurons are able to grow on CSPGs, until a critical threshold level of CSPGs is reached and consequently axonal growth ceases. [30] This is also true for different neuronal populations in the spinal cord. [31]

In addition to CSPGs, other molecules have been implicated as inhibitory for axonal regeneration, namely semaphorin 3 (SEMA3), [32] ephrin-B2 [33] and Slit proteins, [34] which further increase the complexity of the inhibitory environment that is created after CNS injury.

Another well studied group of growth-inhibitory molecules are the myelin-associated molecules.[35] In this group we can find Nogo-A, a membrane protein expressed by oligodendrocytes and some neurons, which causes growth inhibition and growth cone collapse. [36] Myelin-associated glycoprotein (MAG) also belongs to this group and it is one of the inhibitors of white matter regeneration. [35] Several studies show MAG inhibitory capacity. [37, 38] Finally, Tenascin R is an inhibitory molecule present in the ECM of the CNS. It is produced by oligodendrocytes and is upregulated after injury. [35]

So, apparently neurons still have some capacity to regenerate, however physical barriers and inhibitory signals avoid the actual regrowth across the lesion. Therefore, it seems that it must be created the appropriate environment in order to allow neuronal regeneration.

1.3 SCI treatment

1.3.1 Current therapies in the clinics

Nowadays, a SCI patient is classified according to the severity of its lesion, using a grading scale developed by the American Spinal Injury Association (ASIA). [39] The standard treatment procedures in the acute phase are based on a decompression of the cord, spinal stabilization or realignment of displaced vertebrae [40] to prevent further injury. In addition, intensive care monitoring to treat cardiovascular instability and respiratory insufficiency is often needed. [12] Regarding pharmacology, the drug methylprednisolone is commonly used due to some possible neuroprotective effects. [41] Nevertheless, its efficacy is quite limited, revealing simultaneously some harmful side effects, such as an increase in wound infections, gastrointestinal hemorrhages, sepsis, severe pneumonia, among others. [12, 35] In this sense, the search for a therapeutic approach that can improve the quality of life of SCI patients is still open, being many promising therapies under evaluation.

1.3.2 Molecular therapies

New molecular therapies, tested mostly in animal models, are based on the principles of: 1) neutralizing the growth inhibitory environment of the damaged CNS; 2) stimulating axon regeneration; or 3) promoting intracellular molecular signals that allow growth cones to ignore growth inhibitory proteins. [35]

Inhibition of inflammatory reactions

One strategy used to diminish inflammatory reactions is the systemic administration of Interleukin-10 (IL-10), an anti-inflammatory cytokine. IL-10 significantly reduced the lesion volume of SCI-rats, [42] promoted neuronal survival and provided trophic support to neurons. [43, 44] However functional improvements are not consensual. [45]

The broad-spectrum antibiotic minocycline has also been used as a therapeutic agent and it is known for inhibiting microglial activation and the release of pro-inflammatory mediators. It also has an anti-apoptotic action. [46] However there are also several contradictory results concerning the action of this agent. [47, 48]

To avoid the side effects of steroids, non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin and ibuprofen have been used. Decreased edema, less tissue damage and less microglia and astrocytes are among the effects obtained. [49, 50]

Furthermore, we can find in the literature the use of atorvastatin, a drug used for lowering high cholesterol levels, which is also capable of reducing the expression of inflammatory cytokines, macrophage invasion, reactive astrocytes and apoptotic cells. [51] Erythropoietin (EPO), known for its hematopoietic effects, has also been used since it appears to have a neuroprotective effect, by reducing apoptosis, inflammation and lipid peroxidation. [52]

Overall, these molecular approaches seem to significantly decrease inflammatory reactions in animal experimental models. However inconsistent results between different groups exist. Moreover, usually the doses applied in animals are much higher (in proportion) than what is clinically safe to humans. Therefore, particular care should be taken when advancing to clinical trials.

Inhibition of excitotoxicity

Due to the high glutamate levels present in the ECM after injury, constant excitation of NMDA receptors occurs. Therefore, an interesting molecular approach is based on the blockage of sodium channels using riluzole, or NMDA antagonists such as magnesium. [35] Both compounds present several benefits in SCI animal models, such as promoting tissue sparing, decreasing lipid peroxidation and reducing neuronal loss. [53, 54]

Blocking growth inhibition signals

One obvious step towards reverting inhibitory signals to axonal growth is to inactivate growth inhibitory molecules. With this objective, anti-Nogo antibody was administered in SCI models, leading to improved behavioral outcomes, as well as substantial axonal sprouting and long-distance corticospinal regeneration. [55]

A common intracellular pathway of myelin ligands that leads to growth inhibition is based on the activity of the Rho molecule. [56] This guanosine triphosphatase (GTPase) molecule, when activated, leads to the depolymerization of actin filaments and subsequently growth cone collapse. [35] On the other hand, if Rho is inhibited, polymerization of actin occurs, enabling axonal growth. [56] Therefore, the inhibition of Rho pathways has shown to lead to axonal outgrowth and functional recovery. [57]

Stimulating axonal growth

Several neurotrophic factors have been applied in experimental models, with the objective of promoting neuronal growth. Among the different factors used, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are the most popular. In general they promote sprouting and regeneration of different motor and sensory tracts. [58-60] Others factors like basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and glial-derived neurotrophic factor (GDNF) have also been used with some beneficial effects in SCI models. [35]

1.3.2 Cell therapies in SCI

Cell therapies in the CNS are generally characterized by the transplantation of a certain cell type with the objective of providing neuroprotection and also restoring lost tissue through regeneration. [12]

Some of the first promising results in transplantation techniques came from peripheral nerve grafts that promoted regeneration of CNS axons [61] and also fetal spinal cord grafts, which supported the regrowth of host axons. [62]

Stem Cells, including Embryonic Stem Cells (ESCs), Mesenchymal Stem Cells (MSCs), Neural Stem Cells (NSCs) and induced Pluripotent Stem Cells (iPSCs) are among the possible sources for SCI transplantation. Moreover, glial cells have also been used, namely Schwann Cells (SCs), oligodendrocytes (or their precursors) and Olfactory Ensheathing Cells (OECs). Other strategies, such as the use of fetal tissue and fibroblasts modified to express trophic factors, have also been explored. [35]

Stem Cells

Stem cells are by definition undifferentiated cells that can proliferate continuously, generating daughter cells committed to differentiation. [12]

Generally speaking, stem cells have been used in order to target different injury-related phenomena like oligodendrocytes or neurons replacement, remyelinate spared axons, restore the neuronal circuitry, enhance the preservation of host neuronal and glial cells, increase the expression of neurotrophins/cytokines by transplanted or host cells, promote angiogenesis, bridge cysts or cavities, reduce inflammation or gliosis, stimulate endogenous precursor cells and create a favorable environment for plasticity and axonal regeneration. [12]

Embryonic Stem Cells (ESCs)

ESCs are derived from the inner cells mass of developing blastocyst embryos and have the ability to differentiate into practically all cell types. [63] *In vivo* studies revealed that predifferentiated ESCs into neurons and glia promoted partial functional recovery of SCI-rats, upon transplantation. [64] Furthermore, ESCs predifferentiated into oligodendrocyte progenitor cells (OPCs) remyelinated spared axons and improved recovery of SCI-rats. [65]

However, despite their obvious potential, ESCs do have several disadvantages such as their possible tumorigenic character, due to incomplete or aberrant differentiation. Moreover the ethical concerns raised with the use of human-derived embryos always represent an obstacle for the use of these cells in clinical regenerative medicine. [12]

Even so, a human clinical trial using oligodendrocytes derived of ESCs was approved in 2009, but in 2011 Geron Corp. discontinued this trial due to funding complications.

Nevertheless, no safety issues were reported in the five patients submitted to ESCs transplants and no further results are available. [12]

Induced Pluripotent Stem Cells (iPSCs)

iPSCs are generated by reprogramming mature, differentiated cells into a pluripotent state. iPSCs were developed by Takahashi and Yamanaka in 2006 [66], by taking somatic cells like fibroblasts, from a mouse and reprogramming them to be pluripotent. Later, the establishment of iPSCs from human source became also possible. [67, 68]

iPSCs can be easily compared to ESCs [69], since besides their pluripotency, they also share identical morphology, self-renewal capacity and similar gene expression. [12] Moreover, iPSCs cell lines, similarly to ESCs, often acquire abnormal karyotypes and genetic amplification associated with oncogenic transformation. [69] In addition, although iPSCs can differentiate into neural lineages, this occurs at lower frequency than with ESCs. [70]

Since transplanted undifferentiated iPSCs increase the likelihood of tumor formation, there is the need to screen and select safe iPSCs-derived clones before transplantation. [71, 72] This step is crucial to avoid robust teratoma formation. [72] Recently, Okano and colleagues grafted human-derived iPSCs into SCI-mice, promoting motor recovery with synapse formation between host and grafted cells, expression of neurotrophic factors, angiogenesis, axonal regrowth and increased myelination. [73]

Neural Stem Cells (NSCs)

Neural Stem Cells (NSCs) or Neural Stem/Progenitor Cells (NSPCs) are multipotent cells committed to the neural lineage. They can self-renew and are readily expanded *in vitro*. NSCs are usually grown as free-floating neurospheres, which are 3D aggregates composed by a mixture of progenitor cells, stem cells and small numbers of more differentiated cell types. NSCs are able to differentiate into neurons, oligodendrocytes and astrocytes. [12]

NSCs can be found in fetal and adult CNS [6], namely within specific niches including the subventricular zone lining the lateral ventricles of the forebrain [74], the dentate gyrus of the hippocampus [75], and the region of the central canal of the spinal cord. [7] In animal models of SCI, transplanted NSCs were capable of promoting functional recovery and had neuroprotective

and neuroregenerative effects. [76] However in other works, the recovery induced by transplanted NSCs was only modest. [77] *In vivo* studies also showed that transplanted NSCs have the ability to differentiate into oligodendrocytes [78] and neurons [79] promoting also functional recovery in SCI-rats. Immunomodulatory properties [80] and secretion of neurotrophic factors and cytokines [81] are other features of these cells.

The great disadvantage of NSCs is that human-NSCs are not readily available and are very difficult to grow. [12] Nevertheless, NSCs with human origin have been successfully transplanted into SCI rats, differentiating into neurons and glia [82] and in SCI-mice, human NSCs induced some recovery. [83]

Regarding clinical trials, NSCs use is more limited since human NSCs cannot be autologously transplanted; therefore fetal-derived cells are an alternative (though it raises ethical concerns), as well as adult-derived cells from organ donors, which are more difficult to grow. Nevertheless, a phase I/II (safety/efficacy) trial is ongoing in Switzerland, using fetal brain stem cells. [12]

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) are a multipotent stem cell population that has emerged in the last years as a promising tool in regenerative medicine of different tissues. [84] MSCs are identified according to three important criteria: 1) adherence to plastic in standard culture conditions; 2) positive expression of markers CD73, CD90, CD105 and negative expression of hematopoietic markers like CD34, CD45, HLA-DR, CD14, CD11B, CD79α or CD19; and 3) *in vitro* multipotent differentiation profile. [85, 86]

MSCs can be found in a variety of tissues [87] namely bone marrow, umbilical cord, adipose tissue and even in adult muscle and dental pulp of deciduous baby teeth. [12] It is believed that, in response to injury, MSCs have the capacity to migrate to the damaged site and help in the repair process, mostly due to the secretion of growth factors, cytokines and antioxidants. [86, 88] The presence of these bioactive molecules in MSCs' **secretome**, make it a very promising tool in regenerative medicine. The secretome can be defined as the proteins released by a cell, tissue or organism, being afterwards crucial in the regulation of different cell processes. [89] The application of the secretome in different experiments is obtained through

collection of conditioned media (CM) of some type of cells under culture, for a certain period of time. [90, 91]

Another property of MSCs is their interaction with different types of immune cells, presenting a general immunosuppressive role. MSCs (isolated from the bone marrow in most studies) were shown to suppress T lymphocyte activation and proliferation *in vitro*. [92, 93] It is accepted that this inhibition of proliferation is related to the secretion of soluble factors [92, 94], mainly when MSCs are stimulated by lymphocytes presence. [93] Besides T cells, MSCs were also shown to interfere with dendritic cells differentiation, maturation and function. [95] Furthermore, inhibition of B cells proliferation [96] and inhibition of NK cells cytotoxicity and proliferation [97] are other features presented by MSCs. Figure 5 summarizes the major interactions between MSCs and different immune cells.

These cells also present immunomodulatory properties *in vivo*, namely prevention of Graft-versus-host disease formation [98], decrease in graft rejection [99] and protection against renal ischemia/reperfusion injuries. [100]

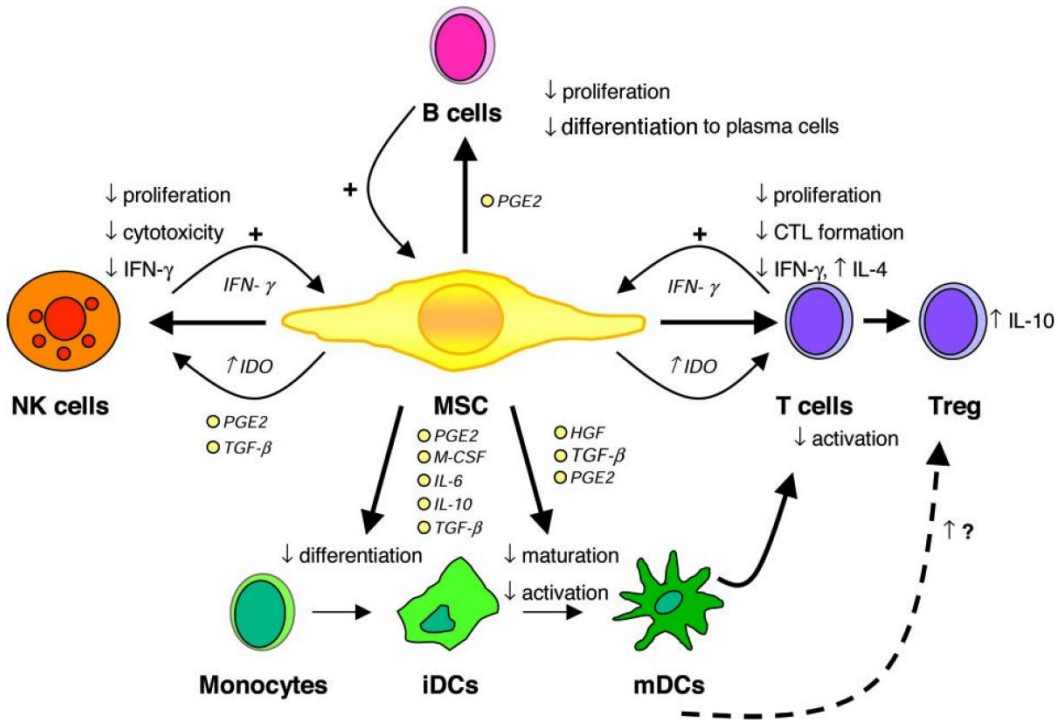

Figure 5 – Immunomodulatory effects of MSCs. Illustration by Paulette Dennis, adapted from *Nauta et al.* [95]

Bone Marrow derived-MSCs (BM-MSCs) are the most common MSCs in cell transplantation techniques in SCI animal models. [101] Isolated from the mononuclear cellular fraction of the bone marrow, BM-MSCs are expanded in culture based on their adherence to

tissue culture plastic and can be distinguished from hematopoietic cells due to the expression of distinct cell-surface antigens. [12]

As other MSCs, BM-MSCs can be autologously transplanted and are able to secrete several neurotrophic factors that are beneficial for repair. [86] In fact, secretion of BDNF and beta-NGF by BM-MSCs was shown to promote neuronal survival and neuritogenesis. [102] In another study, it was demonstrated that BM-MSCs conditioned media (CM) was composed of Insulin like Growth Factor (IGF-1), Hepatocyte Growth Factor (HGF), VEGF and TGFβ, which were related with higher levels of neuronal survival. [103] In addition to the neurotrophic properties, BM-MSCs capacity of *in vitro* differentiation into neural lineages was reported, although these findings are still quite controversial. [104]

In animal SCI-models, some studies showed that transplantation of rat BM-MSCs is capable of improving motor function [105], as opposed to other similar works, which failed to demonstrate significant motor benefits. [106, 107] In addition, approximately 50% of the studies with transplanted human BM-MSCs presented some behavioral benefits. [108] It is generally accepted that the effects observed may result from neuroprotection and recruitment of endogenous cells rather than MSCs differentiation into neural cells. [104, 108] Expression of growth factors or cytokines, vascular effects and remyelination of spared axons are other mechanisms of recovery potentially induced by BM-MSCs transplantation. [104]

Regarding clinical trials, there are some works with a small number of patients, and at least no adverse effects were found after BM-MSCs transplantation. Part of the patients showed some improvements in their neurological status. [109, 110]

The **umbilical cord** started to gain interest as a source of MSCs when it was reported the isolation of MSC-like cells from the **umbilical cord blood**. [111] More recently, MSC niches were found in other tissues of the umbilical cord like the **umbilical cord Wharton's jelly** (WJ-MSCs) [112] and the **umbilical cord perivascular layer** (HUCPVCs). [101]

MSCs derived from the umbilical cord are very attractive because it is a tissue readily accessible and frequently discarded. [12] Like other MSCs, the secretory profile of umbilical cord-derived cells seems to be important. For instance, it is known that WJ-MSCs secrete several cytokines and chemoattractants [113] that can be associated with their immunomodulatory properties. In addition, the secretome of HUCPVCs also presents trophic properties, namely the

capacity of modulating the action of CNS cells [91], possibly related to the abundant presence of NGF in their secretory profile. [90]

In SCI experimental models, transplantation of MSCs derived from human umbilical cord Wharton's Jelly promoted significant improvements in locomotion, accompanied by increased numbers of regenerated axons and fewer microglia and reactive astrocytes. [114] In other study, neurospheres derived from WJ-MSCs in conjugation with BDNF lead to functional improvements, increased axonal regeneration and reduced cavitation. [115] Interestingly, very few grafted cells survived, which suggests that the effects observed were due to a neuroprotective role, rather than cell replacement. [115]

In humans, a clinical trial was performed in China, where MSCs isolated from the umbilical cord Wharton's Jelly were injected intrathecally by lumbar punctures. In 13 of the 22 treated patients the treatment was effective, translated into improved motor and sensory functions, as well as better bowel and bladder control abilities. [116]

Adipose Stem Cells (ASCs) are a population of multipotent stem cells that can be found in the adipose tissue. Human ASCs can be easily obtained after digestion of adipose tissue derived from liposuction surgeries or biopsies.

As well as other MSCs found in the body, ASCs are able to adhere to tissue culture plastic, acquiring a fibroblast like morphology similar to the one observed for BM-MSCs [117]; in addition they also express some cell-specific surface markers such as CD29, CD44, CD71, CD90, CD105/SH2 and SH3 and lack the expression of CD31, CD34 and CD45. [118] Furthermore, ASCs are capable to differentiate into different lineages, under the appropriate conditions in vitro, namely osteogenic, adipogenic, myogenic and chondrogenic lineages. [119]

ASCs have some particular advantages, comparing to other MSCs. Unlike BM-MSCs for instance (considered the gold standard in the field [120]), ASCs can be obtained from a simple surgical procedure; therefore the access to adipose tissue is easy and repeatable and the isolation procedure is not complicated. In addition, adipose tissue is also routinely discarded in liposuction surgeries, making it a very accessible source. [121]

All of these reasons make ASCs quite appealing for research and/or biomedical applications. [122] Moreover, ASCs can be transplanted in an autologous manner, avoiding the risk of immune rejection.

ASCs delivered into an injured tissue may secrete cytokines and growth factors that stimulate recovery in a paracrine manner. [121] It is believed that ASCs stimulate the recruitment of endogenous stem cells to the site of injury and promote their differentiation along the required lineage pathway. [121] ASCs might also provide antioxidant chemicals, free radical scavengers and chaperone/heat shock proteins at an ischemic site. [121] Additionally, ASCs are known for their immunomodulatory capacity and suppressing immunoreactions. [123, 124]

In terms of experimental studies, predifferentiated ASCs (pASCs) transplanted into SCI-rats promoted the protection of denuded axons and also induced significant motor improvements. [125]

A phase I clinical trial carried out by Ra *et al.* revealed that autologous transplantation of ASCs in SCI-patients did not induce serious adverse effects. [126]

ASCs secretome has been reported to be composed by different growth factors, such as VEGF, HGF, bFGF, IGF1, TGF-β1, among others. [120, 127] In vitro experiments revealed that ASCs conditioned media (ASCs-CM) protects PC12 cell line against the induction of glutamate excitotoxicity, mainly due to the presence of VEGF, HGF and BDNF. [128] Moreover, ASCs secretome proved to be valuable in the induction of neuritogenesis of the same PC12 cell line, through the action of secreted NGF. [129] ASCs-CM also provided increased protection of cerebellar granule neurons against apoptosis. [130] Recent work from our group revealed that ASCs secretome acts positively on neuronal and glial cell populations, by increasing their viability. [90] The presence of NGF, Stem Cell Factor (SCF), HGF and VEGF in the secretory profile of ASCs seems to be important in this effect. [90]

Glial cells: Olfactory Ensheathing Cells (OECs)

The olfactory system is particularly special in the CNS, since it is known that neurons in that region are constantly renewed throughout life. [131] This remarkable capacity is believed to be closely related to the presence of a unique subtype of glial cells: the Olfactory Ensheathing Cells (OECs). [132]

OECs are derived from neural crest cells (like Schwann cells) [133] and are mainly characterized by participating in the growth and guidance of primary olfactory neurons from the olfactory epithelium to the olfactory bulbs. In fact, their common origin with Schwann cells may

explain some similarities observed between these two cell types, namely OECs capacity to surround olfactory axons, form fascicular processes and synthesize peripheral-like myelin. [134]

The potential of the transplant of OECs as a therapy for CNS damage has already been tested in animal models. For instance OECs were able to remyelinate axons in spinal cord injured rats [135, 136], while leading to functional improvement of conduction properties in previously demyelinated axons. [136] Therefore, it is considered that OECs can create a permissive environment for axonal regeneration, in the usually hostile milieu of the damaged CNS. [137] The reasons behind this OECs ability might be the secretion of neurotrophic factors that promote neuronal survival, providing also extracellular matrix and substrates required for axonal elongation and myelination. [138] Moreover, OECs seem to create a less adverse reaction in astrocytes after transplantation than other common cell transplants in SCI. [137] For instance, Schwann cells transplants might induce hypertrophy of astrocytes surrounding the wound region, as well as an upregulation of inhibitory molecules such as CSPGs, compared to OECs transplants. [139]

Different studies have shown that transplanted OECs are able to promote functional and morphological preservation of the spinal cord in SCI animal models. [140, 141] They also were able to induce axonal regeneration of different spinal tracts. [142]

OECs grafts have also demonstrated to induce beneficial effects in a rat model of chronic SCI. Among others, they lead to a partial restitution of supraspinal pathways and modest recovery in hindlimb movements. [143] Moreover, the pioneer work of Ramón-Cueto and Nieto-Sampedro [144] showed that the regeneration of injured dorsal root axons into the adult spinal cord was possible after OECs transplantation. In fact, axonal growth was possible even though the inhibitory action of the glial scar.

However there are also studies, where OECs transplants were not as efficient in promoting sparing/regeneration in injured spinal cords as Schwann cell grafts, for instance. [145]

Although the autologous transplantation of OECs in humans might be limited, some clinical trials have already been performed. Therefore, autologous use of OECs into SCI-patients proved to be safe after one [146] and three years [147] post-transplantation, with no significant functional improvements. [147] In contrast, work from Lima *et al.* [148] showed that after transplantation of olfactory mucosa autografts, 11 (out of 20) SCI chronic patients had some improvements, as assessed by the ASIA impairment scale (AIS).

It is believed that part of the role exerted by OECs is also due to the release of neurotrophic substances. [149] For instance, it has been shown that a clonal cell line of OECs is able to express and secrete NGF, BDNF and neuregulins. [150] They also express NT-4/5 but retain this trophic factor intracellularly. [150] In addition, other study confirmed that OECs also express GDNF. [151]

Concerning the action of the neurotrophic factors, it was demonstrated that conditioned media secreted by OECs induced significant increases in neurites outgrowth in both cortical (268%) and DRG (270%) neurons. [152]

1.4 Tissue Engineering

Tissue Engineering (TE) is a fast growing and one of the most promising fields in science. It is an interdisciplinary field that combines the knowledge from physics, materials science, engineering, chemistry, biological sciences and medicine in an integrated manner with the ultimate goal of restore, maintain or improve tissue function. [153] For that, TE tries to develop a functional substitute for the damaged tissue. This substitute is based on a 3D scaffold, which usually is a biomaterial that plays a central role in every TE approach. The concept of TE is depicted in figure 6.

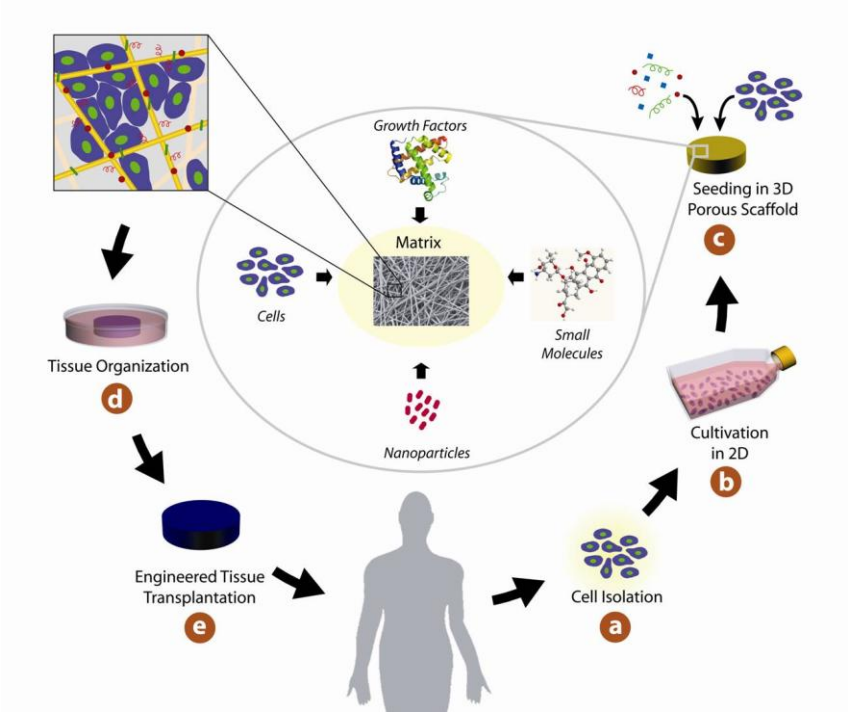

Figure 6 – Tissue Engineering concept. Image obtained from:
http://www.tau.ac.il/lifesci/departments/biotech/members/dvir/dvir.html

Therefore, ideally TE applications intend to isolate cells from the own patient, cultivate and expand them on 2D surfaces, then seed the cells on a 3D scaffold (which can be further enriched with drugs or growth factors) and finally implant the scaffold in the damaged tissue. This hopefully allows cellular and tissue reorganization and eventually function recovery.

Having this in mind, the chosen scaffold should have some important characteristics, such as biocompatibility, appropriate mechanical properties (similar to the target tissue), biodegradability and also adequate cell adhesive and proliferative properties. [35] The 3D devices most widely used include hydrogels, sponges, guidance tubes and nanofibrous scaffolds. [154]

1.4.1 Hydrogels

As mentioned before, one of the biggest concerns in tissue engineering is the type of scaffold to be applied. Therefore the scaffold should mimic as best as possible the size and the mechanical properties of the target tissue. Moreover, it should be delivered in a minimal invasive manner.

Hydrogels, for instance, have physical properties that allow them to be injected into the body in a non-invasive manner. Moreover, they can be administered in a localized manner and are also able to fill the defects provoked by injury. Therefore, they act as depots, for a sustained release at the injury site. As cell delivery agents, hydrogels also improve cell survival and integration. [155] Structurally, they are very similar to macromolecular-based components in the body and are considered biocompatible, especially when derived from natural polymers. [156]

Hydrogels derived from natural polymers are widely used in tissue engineering applications due to their biocompatibility. Some derive from major ECM molecules like collagen (and its derivative gelatin) and also hyaluronate. Fibrin-derived hydrogels are also a common option, as well as algae-derived polymers such as alginate, agarose and even chitosan, a polysaccharide derived from chitin. In general, these hydrogels are biodegradable, but also present some disadvantages, mainly related with their limited range of mechanical properties. Some also might be potentially immunogenic. [157]

Synthetic polymers offer a variety of possibilities concerning hydrogel formulation. Among the myriad of synthetic hydrogels available for biomedical applications, poly(acrylic acid) and its derivatives, poly(ethylene oxide) and its copolymers, poly(vinyl alcohols) and polyphosphazenes are probably the most used. This type of hydrogels has the possibility of being tailored and

designed to match the needs of a certain biomedical application, either structurally or functionally. However, most of them do not degrade in physiological conditions and might have in their composition toxic elements that require extra purification steps. [157]

Gellan Gum (GG) is a natural polysaccharide that is produced by the bacterium *Pseudomonas elodea*. [158] Its structure consists in repeating units of a tetrasaccharide, composed by two residues of D-glucose and one of each residue of L-rhamnose and D-glucuronic acid [D-Glc(β1→4)D-GlcA(β1→4)D-Glc(β1→4)L-Rha(α1→3)]n. [159]

This linear anionic polysaccharide exists both in the acetylated and deacetylated forms, forming thermoreversible gels with different mechanical properties according to the degree of deacetylation. [158]

Gellan Gum is noncytotoxic and particularly resistant to heat and acid stress [160], being useful in the culture of extremophile organisms.

The gelation process is ionotropic, which means that the presence of cations is necessary for the formation of a stable hydrogel structure. [161] In this process divalent cations promote a more efficient gelation than monovalent cations. [162] Several structural changes take place in this process. When at high temperatures, GG is in the coil form. Then, as temperature decreases, there is a thermoreversible transition from coil to double-helix structures. These structures form oriented bundles by self assembly, which are called junction zones. Untwined regions of polysaccharide chains can also link with the junction zones, leading to the formation of a three dimensional network, that creates the gel. [162]

It is used as media in microbiological cultures, as well as gelling agent in plant cell cultures, because it provides a very clear gel, thus facilitating light microscopical analysis of the cells and tissues.

Among its applications, gellan gum is frequently used as a food additive, namely as thickening agent or stabilizer, being FDA approved since 1992. [163]

In what concerns biomedical applications, gellan gum hydrogels have already been used as drug delivery systems. [164]

Gellan Gum also proved to be adequate for the formation of a functional cartilage tissue-engineered construct, since human chondrocytes were kept viable for 14 days while encapsulated in the gel and being also capable of producing extracellular matrix (ECM) molecules. [161]

A common modification employed in these types of hydrogels is the addition of different peptide sequences that mimic the extracellular matrix (ECM). [165, 166] This is done with the objective of improving phenomena like cell adhesion, growth and development. [167] Therefore, the use of cell therapies targeted for CNS repair will be more efficacious if the transplanted cells are encapsulated in a ECM-like biomaterial, leading to greater cell survival.

Therefore, Silva *et al.* modified the Gellan Gum (GG) hydrogel with a fibronectin-derived synthetic peptide (GRGDS). This GRGDS-modified gellan gum (GG-GRGDS) promoted greater adhesion and proliferation of NSPCs than GG unmodified controls. [168] This finding is essential since it supports the idea of the application of GG-GRGDS as a scaffold for SCI treatment, as well as other CNS related injuries.

Despite all the work that has been done on SCI treatment, from molecular therapies to cellular transplantation, there is still no efficient therapeutic strategy to this condition. This is mainly related to the complexity of the events that follow the primary injury, which need a multidisciplinary approach in order to be efficiently modulated. Therefore, a combinatorial strategy, in which more than one aspect of SCI can be addressed, could represent a valuable alternative to the existing methodologies.

Chapter 2
OBJECTIVES

2.1 Gellan Gum with cells – a combinatorial approach for SCI treatment

The main goal of this project is to develop a novel therapeutic strategy designed for SCI recovery. The modified gellan gum hydrogel (GG-GRGDS) will be used as a scaffold for neuronal outgrowth while ASCs and OECs will be applied with the purpose of promoting neural regeneration and providing trophic support in injured spinal tracts. We chose OECs mainly because of their guidance role in their native environment and their ability to grow through the glial scar. Moreover, considering the work from Silva *et al.* [169], we also chose to combine the OECs with ASCs. In this study, OECs were co-cultured with different types of MSCs and seemed to interact positively and most evidently with ASCs, inducing increases in metabolic activity and proliferation. [169]

Having this in mind, the experimental approach was divided into three main objectives:

Objective 1 – Determine how ASCs and OECs interact when in contact with each other or when under the influence of factors secreted by the other cell type. Moreover the ability of these cells to grow in the GG-GRGDS hydrogel will also be assessed.

Objective 2 – Assessment of the therapeutic potential of ASCs and OECs secretome on dorsal root ganglion explants, which will act as an *in vitro* model for axonal migration.

Objective 3 – *In vivo* proof of concept in a SCI model. The objective will be to ascertain the effects of the application of the combinatorial therapy at functional and anatomical levels.

Chapter 3
MATERIALS AND METHODS

3.1 Cell isolation and expansion

3.1.1 Olfactory Ensheathing Cells

Olfactory Ensheathing Cells (OECs) were harvested from olfactory bulbs of 5 to 6-days-old Wistar rats, according to the protocol described by Ramón-Cueto and Nieto-Sampedro. [170] Briefly, upon dissection, the meninges and blood vessels were removed (using an adherent paper) and the tissue was digested with Collagenase type I (2.5 mg/ml, Sigma) for 30min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5ml pipette and centrifuged at 1000rpm for 5min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation at 1000rpm for 5min, cells were resuspended and seeded on fibronectin coated surfaces at the density of 60.000 cells/cm^2. Cells were cultured in DMEM/F12 (Gibco) with 10% Fetal Bovine Serum (FBS, Biochrom AG) and 1% antibiotic-antimycotic solution – penicillin-streptomycin (Gibco) at 37 °C and 5% CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5,36 µg/ml, Gibco) and Forskolin (1,4 µg/ml, Sigma).

OECs purification steps

A. 24h upon OECs seeding, the culture media was replaced for a serum-free media for a period between 24h-48h. This serum-free media contained FGF (20 ng/ml, Gibco) instead of FBS. This step is aimed at drastically reducing the percentage of fibroblasts and astrocytes in culture.
B. As an alternative, cells can be plated after isolation in uncoated plates for 18h. A posterior change to uncoated plates for 36h is made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second periods, respectively. Then after this purification protocol, OECs can be plated in fibronectin coated surfaces.

3.1.2 Human Adipose-derived Stem Cells

Human Adipose-derived Stem Cells (ASCs) were kindly provided by Professor Jeffrey Gimble and isolated according to the protocol described by Dubois *et al.* [171] These cells were

cultured and maintained in α-MEM (Gibco), with 10% FBS (Biochrom AG) and 1% antibiotic-antimycotic solution (Gibco) at 37 °C and 5% CO_2 (v/v).

3.2 2D direct co-cultures

3.2.1 OECs and ASCs co-cultures

In order to assess the potential positive or negative interactions between OECs and ASCs, a direct co-culture system with these two cell types was used. After isolation (described in section **3.1.1**), OECs were seeded (60.000 cells/cm^2) on fibronectin coated coverslips. Following a OECs purification step (described in section **3.1.1 A**), ASCs were seeded (10.000 cells/cm^2) over the OECs culture. Cells were allowed to grow in OECs culture medium (DMEM/F12 with supplements) since previous experiments proved its suitability for the culture of ASCs (data not shown). After 1 and 7 days of incubation, cell growth was assessed by immunocytochemistry (ICC). OECs and ASCs monocultures were used as controls.

3.3 Conditioned Media experiments – OECs vs ASCs

As mentioned before, OECs and ASCs interact positively through the secretion of paracrine factors. [169] Therefore, in order to confirm the effects of OECs and ASCs secretome on the viability and proliferation of each cell type, 24h CM of both cells were collected.

In the case of ASCs CM effects on OECs, the latter were isolated as described in **3.1.1.** Then cells were seeded at the density of 60.000 cells/cm^2 on fibronectin coated surfaces. OECs were allowed to grow in their normal growth medium (**section 3.1.1**) for 10-14 days and followed that period, ASCs CM was added. Normal growth medium was used as a positive control. After 4 and 7 days in culture, ICC for p75 and MTS test were performed in order to evaluate OECs proliferation and metabolic activity.

Concerning OECs CM effects, ASCs were seeded at 5.000 cells/cm^2 on poly-D-lysine coated surfaces and allowed to grow for 2 days in their normal growth medium (**section 3.1.2**). After that time, OECs CM was added and cells were maintained in culture for 5 days. Normal growth media of ASCs was used as a positive control. After 5 days in culture, phalloidin and DAPI staining and MTS test were performed to assess ASCs proliferation and metabolic activity.

3.4 Immunocytochemistry (ICC)

Immunocytochemistry (ICC) is a technique used to determine the presence, localization and relative abundance of an antigen of interest, most commonly a protein, in cultured cells. [172]

ICC procedures were used to identify OECs and ASCs in culture. For OECs, rabbit anti-NGF receptor (p75) antibody (Chemicon) was used, being also useful for discriminating OECs from fibroblasts (common contaminators in OECs cultures). Mouse anti-Human Nuclear Antigen antibody (HNA, Chemicon) was employed to identify ASCs, since this antibody specifically stains the nuclei of human cells. Controls were made by the omission of the appropriate primary antibody. Briefly, cells were fixed with a solution of 4% PFA in PBS for 20min at room temperature (RT). Then, after wash with PBS, cell membranes were permeabilized (just in the case of HNA antibody) with 0,3% Triton X-100 (Sigma) for 5min. Non-specific binding sites were blocked using a solution of 10% Fetal Calf Serum (FCS, Biochrom AG) in PBS for 1h. The respective primary antibodies were then added for 1h, after which cells were exposed to the specific secondary antibody for 1h (Alexa Fluor 488 goat anti-rabbit for OECs; Alexa Fluor 488 goat anti-mouse for ASCs, Invitrogen). Following another washing step with 0,5% of FCS, cell nuclei were counterstained with 1 μg/ml DAPI (Invitrogen) for 15min. Imaging was performed using an Olympus BX61 fluorescence microscope.

3.5 Phalloidin/DAPI staining

Phalloidin is a toxin extracted from the fungus *Amanita phalloides*. It has the particularity of binding to F-actin filaments. Thus when conjugated with a fluorophore it has been widely used as an imaging tool for the cytoskeleton.

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain synthesized in 1971 by Otto Dann and binds to A-T regions of DNA. The use of these two compounds allows the performance of cell counts. This staining also enables to look for possible alterations at the cytoskeletal level.

Cells that were stained with phalloidin and DAPI were subjected to a 20min period of fixation with 4% PFA at RT. Then, cells' membranes were permeabilized with 0.3% Triton X-100 and the cells washed 3 times with PBS (1x). After the washes, a PBS (1x) solution with 0,1 μg/ml phalloidin (Sigma) and 1 μg/ml DAPI (Invitrogen) was added to the cells for 30min at RT.

For phalloidin and DAPI staining of cells within hydrogels, all time periods of incubation were multiplied by 1,5x. Therefore, fixation with PFA was performed for 30min and staining with phalloidin and DAPI for 45min, all at RT.

3.6 MTS test

The MTS test is based on the reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) to formazan by viable cells. The reaction only takes place when reductase enzymes are active, and therefore the conversion can be directly related to the viability of cells in culture.

Formazan is soluble in culture medium and its concentration can be indirectly measured through spectrophotometry.

After each specific time point, complete culture medium was replaced by serum-free medium containing MTS in a 5:1 ratio and incubated in a humidified atmosphere at 37 °C and 5% CO_2. After 3h of incubation, the optic density for triplicates of each sample (n=3) was measured at 490 nm in a microplate reader.

3.7 Hydrogel formulation

3.7.1 Gellan Gum

Gellan Gum (GG, fig. 7) is a natural polysaccharide that is particularly resistant to heat and acid stress [160]. Gellan Gum for all experiments was purchased from Sigma-Aldrich.

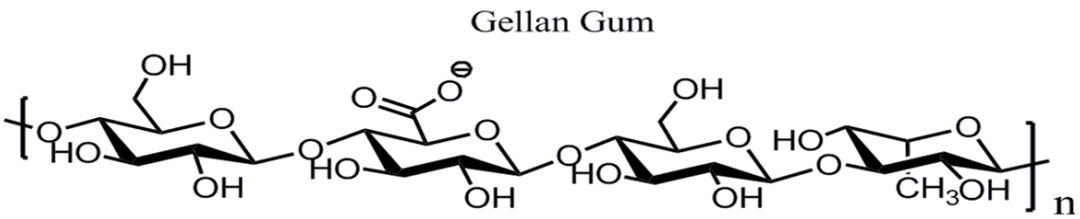

Figure 7 – Chemical structure of Gellan Gum

3.7.2 Functionalization of Gellan Gum with a GRGDS peptide

Fibronectin is a high molecular glycoprotein of the extracellular matrix that facilitates processes like cell adhesion and migration. Particularly, the fibronectin-derived GRGDS sequence is known to bind to integrins V3 and IIb3 and enhance cell attachment and migration.[173] The synthesis of the GRGDS peptide was ordered to Anaspec.

GG-GRGDS binding – Diels-Alder Click Chemistry

The binding of GRGDS to Gellan Gum was performed using a click chemistry reaction[174], namely the Diels-Alder reaction.[175] This last one is an organic chemical reaction between a conjugated diene and an alkene to form a substituted ciclohexene. [35] In this work we used a GG previously modified with a furan group (diene) and we ordered to Anaspec, the synthesis of the peptide GRGDS modified with a maleimide group (alkene). The high affinity of these two molecules was used to indirectly bind the GRGDS peptide to the GG hydrogel (fig. 8).

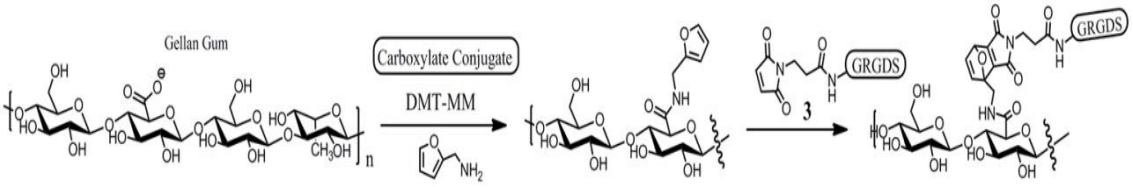

Figure 8 – Representation of the Diels Alder reaction used to bind the GRGDS peptide to Gellan Gum

Both GG-Furan and GRGDS-Maleimide were obtained as lyophilized powders. After weighing the two compounds in a 5:1 molar ratio (Maleimide:Furan), they were dissolved and mixed in MES buffer (pH 5.5) for 48h, at 40 °C. Then, a dialysis procedure was done to remove the excess of GRGDS-Maleimide that did not bind to GG-Furan. For that, alternate washes of distilled water and PBS (1x) were performed during 5 days. Finally, the excess of water was removed by lyophilization, obtaining the GG-GRGDS hydrogel as a white powder.

3.7.3 Hydrogel preparation

The Gellan Gum-GRGDS hydrogel used for the *in vitro* 3D cultures and for in vivo studies was prepared as follows:

1. Gellan Gum-GRGDS lyophilized powder was weighed in order to prepare a solution at 1 % w/v in ultra pure water.
2. Before adding the appropriate volume of water, the gel was sterilized by exposure to UV lights for 15min.
3. After the addition of water, the gel preparation was heated at 40 °C until we obtained a homogenous solution.
4. Before encapsulating the cells, PBS 10X was added to get a 0.5X concentration in solution that allows a strength increase of the connections inside the hydrogel.

3.8 3D cultures – OECs and ASCs

Direct co-cultures of OECs and ASCs encapsulated within the GG-GRGDS hydrogel were performed with the objective of assessing the interactions of these cells while in a 3D environment. Moreover this experiment was also crucial to determine if the hydrogel is suitable for the growth of both cells, being a good vehicle for cellular transplantation.

OECs and ASCs were obtained as described in **3.1.** At the same time, GG-GRGDS hydrogel was prepared according to **3.7.3.** Then, pellets from both cells were resuspended in complete DMEM/F12 and pipetted into the hydrogel. A total of 10.000 ASCs/100µl and 35.000 OECs/100µl were placed inside the hydrogel and monocultures of each cell type were used as controls, using the same cellular densities as co-cultures. Cells were allowed to grow for 7 days, with medium changes every 2 days. After 1 and 7 days of incubation, phalloidin and DAPI staining protocol was performed in order to assess cells' growth and morphology. Samples were analyzed by confocal microscopy using a confocal point-scanning microscope, Olympus FV1200.

3.9 DRG explants

As mentioned before, Dorsal Root Ganglia (DRG) are structures located just near to the spinal cord that are composed mostly of sensory neurons' cell bodies. DRG explant cultures can be used as an excellent model of axonal regeneration, since these structures when isolated and plated under the appropriate culture conditions are able to produce extensive neurite outgrowth. Therefore, DRG explant cultures were performed to evaluate the effect of conditioned media collected from both ASCs and OECs on neurite outgrowth.

3.9.1 DRG explants on 2D surfaces

DRGs isolated from neonatal rat pups (P5-P12) were dissected from the thoracic region and placed in cold HBSS (1x) without Ca^{2+} and Mg^{2+}, after which they were placed on laminin coated surfaces and further cultured in neurobasal medium (Gibco) supplemented with 6mg/ml D-glucose (Sigma), 1% antibiotic-antimycotic solution (Gibco), 1x B27 (Gibco), 2mM L-Glutamin (Gibco) and 3% FCS (Biochrom AG). After 24h, FCS was replaced by 50ng/ml NGF (Promega), to promote neurite extension. DRGs were kept in a humidified atmosphere at 37 °C, 5% CO_2.

Addition of conditioned media

DRGs were maintained in complete culture media, till the number and extension of neurites formed was considerably high. DRGs were then used to compare the effects of conditioned media (CM) collected from OECs, ASCs and a mixture of both CM (50:50) collected individually. For collecting cells CM, neurobasal (Gibco) supplemented only with 1% antibiotic-antimycotic solution (Gibco) and glucose (Sigma) was used.

Complete culture media was replaced by the respective CM or controls. As a control group, complete culture media with NGF was used. After 5 days with CM, DRGs were fixed and subjected to an immunohistochemistry (IHC) for neurofilament, a major component of axons' cytoskeleton. Imaging was done in a fluorescence microscope, Olympus IX81. Images were analyzed in ImageJ (NIH) software.

3.9.2 DRG explants on 3D surfaces – collagen gels

After some difficulties in the culture of DRGs on 2D surfaces, mostly related with DRG adherence to the surface, we decided to perform explant cultures using the method described in Allodi *et al.* [176]. Basically these authors utilized a 3D matrix based on collagen, to seed the DRG explants.

In summary, collagen gels were prepared in a proportion of 450 µl of collagen I (BD Biosciences) mixed with 50 µl of 10x DMEM (Sigma) and 2 µl of 7.5% $NaHCO_3$ solution. Then, single drops of 30 µl of the mixture were placed in each well and kept in the incubator at 37 °C

and 5% CO_2 for 2h. After that, the DRGs were placed on top of the gels and a second drop of 30 µl was positioned over the DRGs, being incubated again for further 45min. Finally, complete culture medium with NGF was added to explants. Following 6 days in culture (medium changed every 2 days), complete culture medium was replaced by CM from OECs and the respective control. This time, the neurobasal medium (Gibco) used to collect OECs CM was enriched with 1x B27 (Gibco) and 2mM L-Glutamin (Gibco), besides 6mg/ml glucose (Sigma) and 1% antibiotic-antimycotic solution (Gibco). Complete culture media with NGF was used as a control.

After 3 days in culture with CM, DRGs were fixed and stained with phalloidin and DAPI. Samples were analyzed by confocal microscopy using a confocal point-scanning microscope, Olympus FV1200. Images were analyzed using ImageJ (NIH) software.

3.10 Collection of Conditioned Media

Conditioned Media used in experiments described in sections **4.1** and **4.2** was collected using the following protocols. For ASCs CM, cells were seeded at a density of 5.000 cells/cm^2 and allowed to grow in α-MEM (Gibco), with 10% FBS (Biochrom AG) and 1% antibiotic-antimycotic solution (Gibco) for 72h. Then, cells were washed thoroughly with PBS (1x) and neurobasal media was added, only supplemented with 6mg/ml glucose (Sigma) and 1% antibiotic-antimycotic solution. 24h later, the CM was frozen in liquid nitrogen, only to be thawed and filtrated in the day of the experiments.

For collection of OECs CM, cells isolated were seeded at a density of approximately 60.000 cells/cm^2 and allowed to grow in DMEM/F12 (Gibco) with different supplements (as described in **3.1.1**). Since the adhesion time of OECs to the cell culture flasks varied between the different isolation procedures, the period of time before starting conditioning varied from 8-13 days. After that period, cells were washed thoroughly with PBS (1x) and it was added neurobasal supplemented with 6mg/ml glucose (Sigma) and 1% antibiotic-antimycotic solution (for experiments described in **3.9.1** and **3.3**) or neurobasal supplemented with 6mg/ml glucose, 1% antibiotic-antimycotic solution (Gibco), 2mM L-Glutamin (Gibco) and 1x B27 (Gibco) (in experiments described in **3.9.2**). Following 24h of conditioning, the CM was frozen in liquid nitrogen and only thawed and filtrated in the day of the respective experiments.

3.11 DRGs area analysis with Image J software

After obtaining the micrographs of DRG explant cultures (experiments described in **section 3.9**) through confocal or fluorescence microscopy, the pictures were opened with the Image J (NIH) software. Before starting the analysis, the scale had to be determined and the area occupied by the DRG itself was eliminated. Then, the images were converted to 8 bits and were processed in the menu "make binary". Finally, using the menu "analyze particles" the software automatically calculated the areas occupied by the neurites, using the dark background as a contrast.

3.12 *In vivo* testing

The *in vivo* proof of concept of our proposed therapy was performed using a rat hemisection model of SCI. This model is particularly useful because it allows a better perception of regenerating fibers, unlike contusion models for instance. Moreover, since the lesion only affects half of the spinal cord, it allows a greater rate of animal survival after injury.

3.12.1 Animals and groups

Eight weeks old female Wistar rats (Charles River), housed in light and temperature controlled rooms and fed with standard diet, were used in the studies described in **section 4.3**. The Animal Care Committee of the Research Institute approved the animal protocols in accordance with standardized Animal Care Guidelines. [177] Handling was performed for 3 days before the surgeries.

The animals were divided in 5 different groups according to the respective treatment/procedure: 1) Animals subjected to SCI with no treatment (SCI, n=10); 2) SCI animals treated with a transplantation of ASCs and OECs (Cells, n=8); 3) SCI animals treated with GG-GRGDS implantation at the injury site (GG, n=8); 4) SCI animals treated with local administration of ASCs and OECs encapsulated in GG-GRGDS (GG+cells, n=8); and 5) Animals with laminectomy only, no SCI (Sham, n=5).

A total of 21.400 OECs and 60.600 ASCs per animal were injected rostrally to the lesion using a hamilton syringe. In animals of the "GG" group, a total of 4 µl of GG-GRGDS per animal were implanted at the injury site, using a micropipette. Hydrogel formulation was done as

described in section **3.7.** Finally, animals of the "GG+cells" group were subjected to an implantation of the hydrogel GG-GRGDS with OECs and ASCs. Both cells were encapsulated within the hydrogel, as described in section **3.8.** Each rat received a total of 21.400 OECs and 60.600 ASCs encapsulated in 4 µl of GG-GRGDS.

3.12.2 Spinal Cord Injury surgery

All animals were anesthetized by intraperitoneal injection of a mixture (1,5:1) of ketamine (100mg/ml, Imalgene/Merial) and medetomidine hydrochloride (1mg/ml, Dormitor/Pfizer). Once anesthetized, fur was shaved from the surgical site and the skin disinfected with chlorohexidine (AGB). Then a dorsal midline incision was made from T7-T13 and the paravertebral muscles retracted. A laminectomy was performed at the junction T10-T11 in which the spinous processes were removed and the spinal cord exposed. A unilateral defect on the left side of the spinal cord was done, removing 2-3 mm tissue. After the respective treatment, paravertebral muscles and skin were separately closed with Vicryl sutures (Johnson and Johnson). The incision of SCI control animals was closed after injury without treatment.

After SCI/laminectomy, SPCL scaffolds* were implanted at the vertebral bone level, juxtaposed to the spinal cord. Bone cement (Biomet) was used to fix the scaffolds margins to bone.

* 3D scaffolds based on a blend of starch with polycaprolactone (SPCL) were developed by Silva *et al.* [178] with the objective of providing spine stabilization in animal experimental models. Spine stabilization with SPCL scaffolds demonstrated to be important for improving motor deficits and even preventing infiltration of the injury site by connective tissue.

3.12.3 Post-Operative care

Following SCI surgery, rats were kept under heat lamps and received subcutaneous injections of vitamins (Duphalyte/Pfizer), 0.9% NaCl, the anti-inflammatory drug carprofen (5mg/ml, Rimadyl/Pfizer), the analgesic butorphanol (10mg/ml, Butomidor/Richter Pharma AG) and the antibiotic enrofloxacin (5mg/ml, Baytril/Bayer), besides atipamezole (5mg/ml, Antisedan/Pfizer) a drug used in order to revert anesthesia. Bladder evacuation was done manually. Then, during the first week post-injury, rats received daily subcutaneous injections of

all the above mentioned components with the exception of atipamezole. Carprofen administration was stopped 3 days post-injury.

Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment. The diet was changed to a diet with higher caloric-content.

3.12.4 Motor Behavior Analysis by BBB Score

The motor behavior of all rats was assessed with the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB_2) [179] on day 3 post-injury and every week, starting exactly 1 week post-injury until a total of 8 weeks. The BBB_2 is a 21-point scale designed to assess hindlimb locomotor recovery following thoracic spinal cord injury. A BBB_2 score of 0 indicates no hindlimb movement. A BBB_2 score of 1 through 8 indicates joint movement, but no weight support. A BBB_2 score of 9 through 20 indicates an ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB_2 score of 21 corresponds to the locomotion of a normal rat. Detailed information can be found in Table 1. In summary, animals were placed in an open arena with no obstacles and allowed to move freely. During the 4min of the test, 2 observers (blinded to the animal groups) evaluated the animal locomotion profile, recording and attributing a score to the animal performance.

Table 1 – Basso, Beattie, Bresnahan Locomotor Rating Scale [179]

0	No observable hindlimb movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL/HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL/HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL/HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL/HL coordination
14	Consistent weight-supported plantar steps; consistent FL/HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL/HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL/HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
17	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift-off
18	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
19	Consistent plantar stepping and consistent FL/HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift-off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift-off, and trunk instability; tail consistently up
21	Consistent plantar stepping and consistent gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

3.12.5 Open Field Test

The open field (OF) test is commonly used to assess exploratory and anxiety-like behavior in laboratory animals. Moreover, the open field is also a versatile test that permits the assessment of locomotor behavior by measuring the amount of rearing activity and the total distance travelled by the animals. [180] The test was performed in an arena (43.2 cm × 43.2 cm) with transparent acrylic walls (Med Associates Inc.) placed in a brightly illuminated room. Animals started the test at the arena's centre and were given 5min to explore it. The total distance travelled in the arena and the number of rearings were automatically registered by equipment sensors.

3.13. Histological analysis

After the last BBB_2 and OF tests and approximately 10 weeks post-injury, the animals were sacrificed in order to perform a histological analysis to the spinal cord tissues.

3.13.1 Tissue preparation

Rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (200mg/ml, Eutasil/Ceva Saúde Animal) and perfused through the ascending aorta with PBS (1x) followed by 4% PFA. A rough dissection of the spine and spinal cord was performed, centered on the site of hemisection and scaffolds placement, and the tissues were fixed in a solution of 4% PFA. On the next day, a more detailed dissection of the spinal cord was done and the tissues were carefully placed on a solution of saccharose at 30%. After 24h 2,5-3 cm length of spinal cord tissues, centered on the lesion, were involved in frozen section medium (Neg-50, Thermo Scientific), frozen with liquid nitrogen and stored at -20 °C. Later on, cross sections of 20 µm thickness of each sample were performed using a Leica CM1900 cryostat.

3.13.2 Hematoxylin-Eosin (HE) staining

Hematoxylin-Eosin (HE) stain is a popular staining method in histology and the most widely used in medical diagnosis. Hematoxylin and eosin are two separate dyes; the first one

stains cells nuclei in a deep purplish-blue color; the second one stains the cytoplasm and connective tissue in shades of red, pink and orange. [181]

HE staining of spinal cord cross sections was performed using an automatic processor (Leica TP1020-1). Basically, the slides were immersed in hematoxylin and eosin solutions and then washed in distilled water, dehydrated in increasing concentrations of ethanol and finally cleared in xylene substitute. In the end, slides were mounted using Microscopy Entellan® (Merk & Co., Inc.). Imaging was done using an Olympus SZX16 microscope.

3.13.3 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a technique that follows the same principles of ICC, but it is used in tissue sections. IHC was performed in order to assess the levels of regeneration possibly promoted by our proposed therapy, as well as glial scar formation.

Therefore, spinal cord cross sections were permeabilized with 0.2% TBS-T for 10min. Then, the slides were blocked with a solution of 5% FBS in PBS for 30min. After that, the samples were incubated overnight with the following primary antibodies: i) mouse anti-CD11B (Pharmigen); ii) rabbit anti-rat GFAP (Dako); and iii) rabbit anti-TH. On the next day, samples were incubated for 2h with the respective secondary antibodies, alexa fluor 488 goat anti-rabbit (Invitrogen) and alexa fluor 588 goat anti-mouse (Invitrogen). In the end, all samples were counterstained with DAPI (Sigma) for 10min. Between steps, 3-5 washes with PBS (1x) were performed. Finally, the slides were mounted in Immu-Mount® (Thermo Scientific) and observed at an Olympus BX61 fluorescence microscope and an Olympus FV1000 confocal microscope. All images were treated using ImageJ (NIH) software.

3.14 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by one way ANOVA test or by the two-way ANOVA test followed by the Tukey post-hoc test or by the Bonferroni post-hoc test. A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance.

Chapter 4
RESULTS AND DISCUSSION

4.1 In vitro assessment of ASCs and OECs interactions

2D direct co-cultures

SCI treatments using cellular transplantation techniques often focus on the use of only one type of cell. [106, 114, 125] When using more than one cell type, it is essential to look for possible negative interactions between the cells under question. So, after direct co-cultures of ASCs/OECs and regarding cell morphology, assessed by ICC, ASCs maintained their fibroblast-like shape, typical of MSCs, while OECs kept their bipolar fusiform shape (similar to Schwann cells). [182] (Fig. 9)

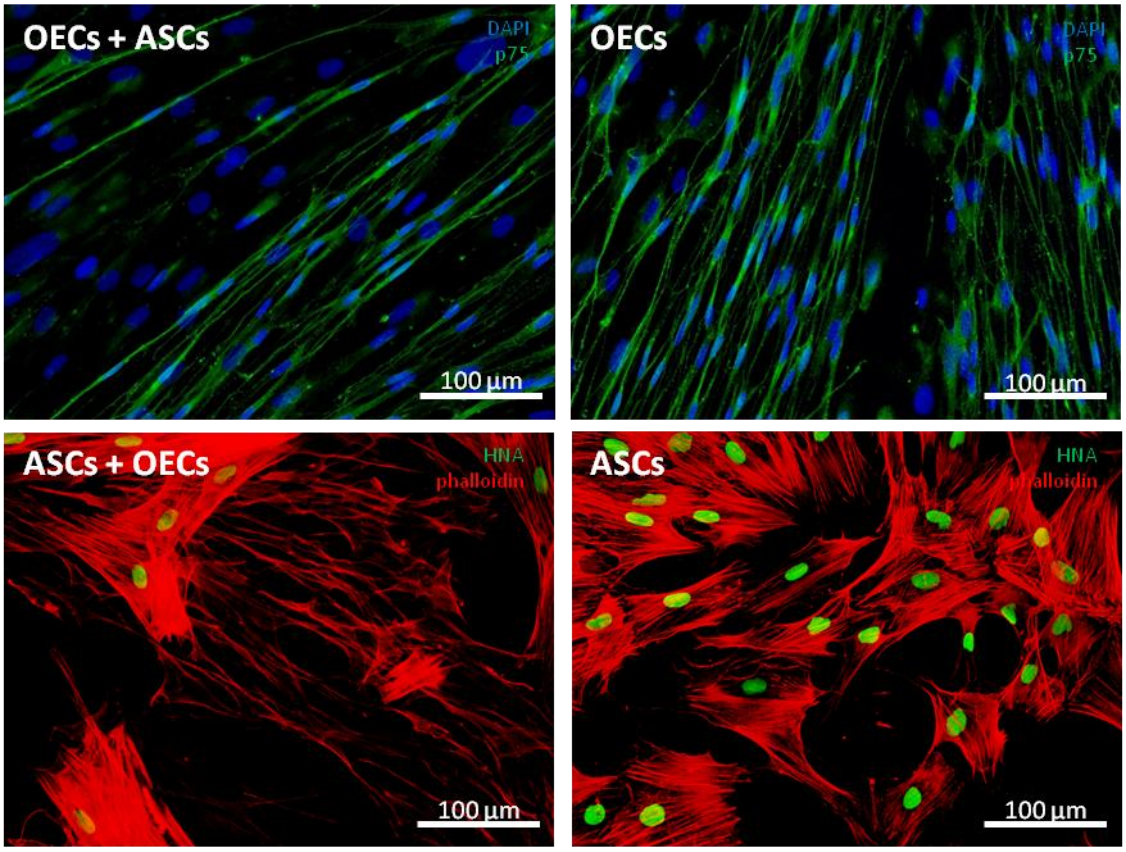

Figure 9 – OECs and ASCs in co-culture (left micrographs) or in monoculture conditions (right micrographs). OECs were stained with p75 (in green) and nuclei counterstained with DAPI (upper micrographs). ASCs were identified through HNA staining (in green) and phalloidin (in red) was used to verify cytoskeleton morphology (bottom micrographs).

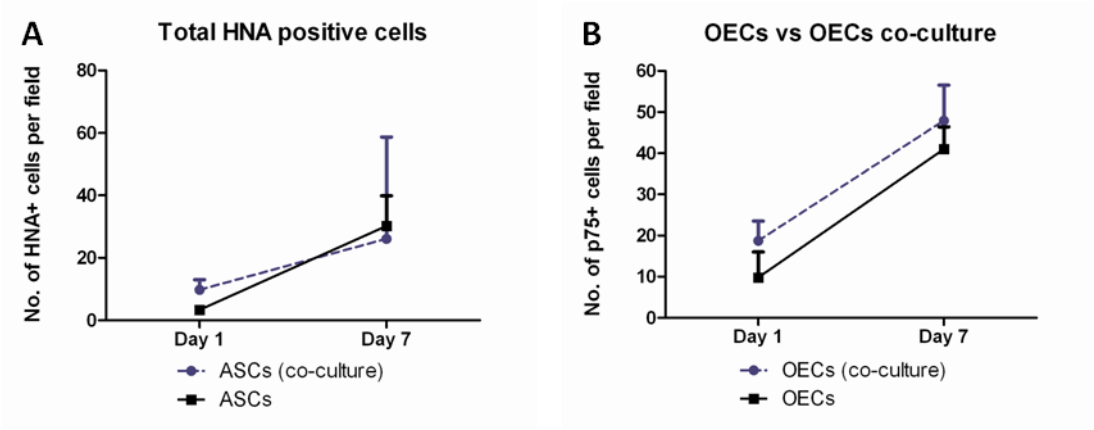

Figure 10 – Cell counts of ASCs and OECs in 2D direct co-cultures after 1 and 7 days. Monocultures are represented in black solid lines and co-cultures in blue dashed lines. (A) Number of HNA positive cells. (B) Number of p75 positive cells. A total of 10 fields/coverslip were counted to obtain the mean number of cells per field (n=3, mean ± standard deviation).

Regarding cell numbers, ASCs co-cultured with OECs were capable to proliferate as much as ASCs grown in monocultures (Fig. 10A), while OECs tend to proliferate more when in co-culture, both at 1 and 7 days. This indicates that both cells can grow together and possibly benefit from cell-cell interaction.

3D direct co-cultures on GG-GRGDS

The 2D co-culture of ASCs and OECs did not provoke particular harmful effects on both cell populations; hence we culture these cells in a 3D environment. A qualitative analysis, after confocal microscopy, of cells encapsulated within the GG-GRGDS hydrogel revealed that ASCs and OECs were able to grow and extend their processes, either alone, or in co-culture (fig. 11). It is important to note that only few cells presented a round shape, characteristic of non-viable cells. This is also an indicator that the modification of GG with the GRGDS peptide was successful and useful to support the *in vitro* (and possibly *in vivo*) survival of the cell populations under study.

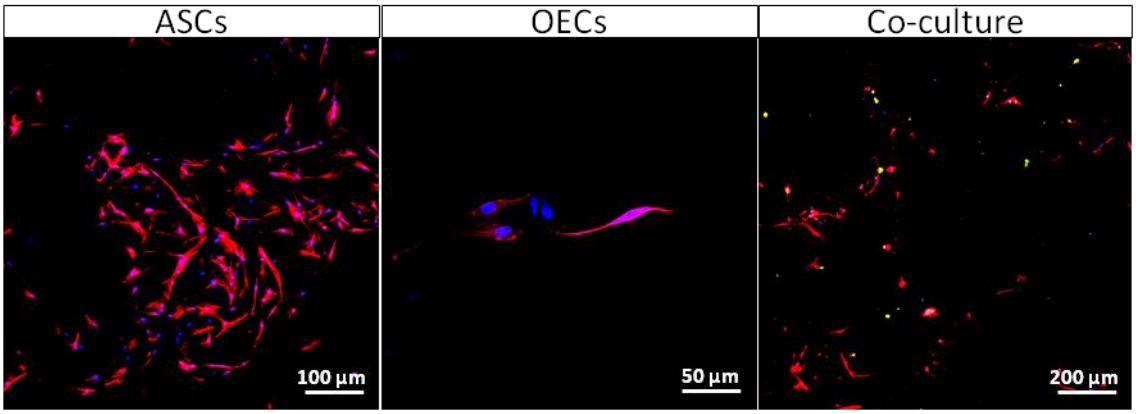

Figure 11 – Confocal images of OECs and ASCs while encapsulated on GG-GRGDS hydrogel, after 7 days in culture. Phalloidin (red) and DAPI (blue) were used to stain both cell types. In co-culture ASCs were additionally stained with a green cell tracer (Cell Trace™ CFSE, Invitrogen).

Secretome interactions

24h conditioned media (CM) from both ASCs and OECs were collected in order to evaluate the secretome role in the proliferation and metabolic activity of the other cell type.

Regarding ASCs CM effect on OECs, the number of p75 positive cells after 4 days in culture (13.5 ± 6.3 cells/field) was identical to the control group (15.3 ± 2.9 cells/field), as it can be seen in figure 12A. In terms of metabolic activity (fig. 12B), OECs grown in ASCs CM presented similar levels to the control group, as assessed by the MTS test. This is very interesting, because unlike the control group, ASCs CM does not contain serum, which is a major source of proteins for cultured cells. Nevertheless, it still provides enough support to OECs reach identical levels of proliferation and metabolic activity.

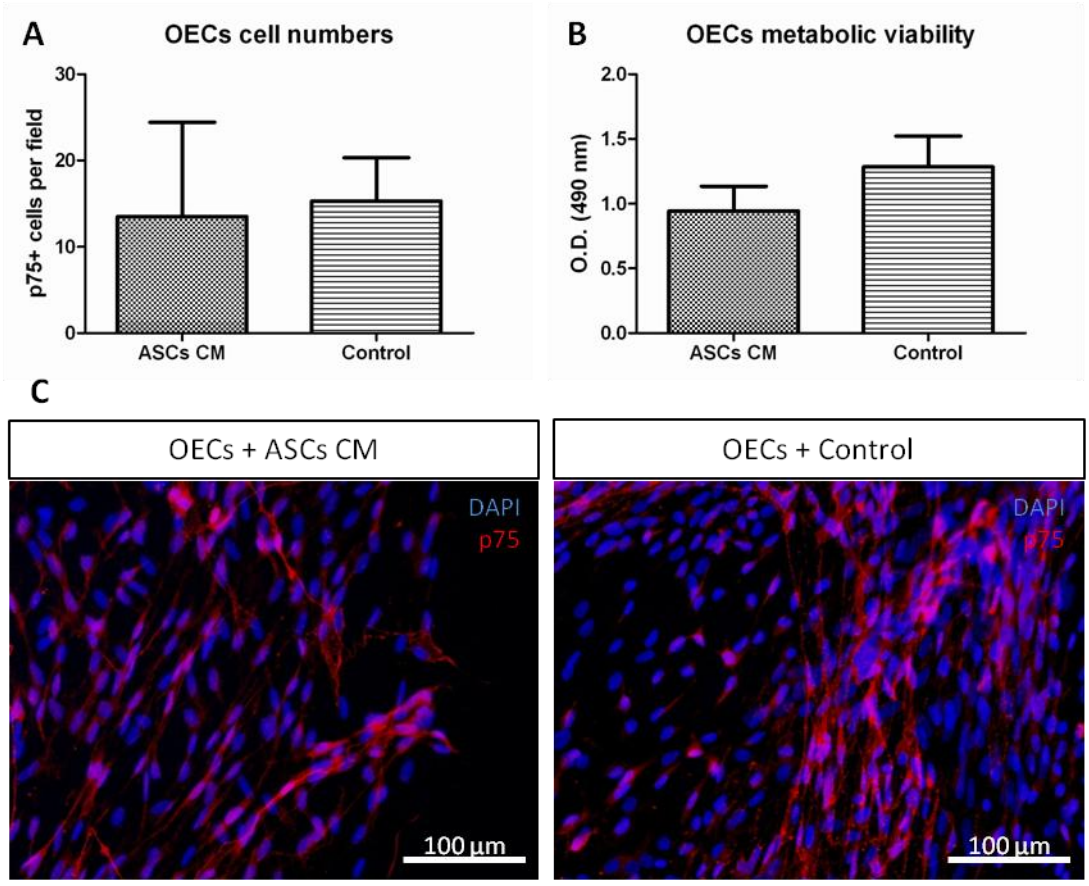

Figure 12 – Effect of ASCs 24h CM on OECs proliferation (A,C) and metabolic viability (B), after 4 days in culture. ICC for p75 was used to determine the number of OECs. Cells metabolic viability was assessed through MTS test (n=3, mean ± standard deviation). (C) Representative images of OECs grown in ASCs CM and control medium.

Concerning the OECs CM role on ASCs, it is notorious that the number of cells in this condition after 5 days in culture is less than in the control group (fig. 13A; 10.5 ± 2.0 against 75.0 ± 3.6 cells/field). In addition, the metabolic activity of ASCs grown in OECs CM was also significantly lower than controls (fig. 13B; 0.08 ± 0.02 against 1.31 ± 0.02 O.D. levels).

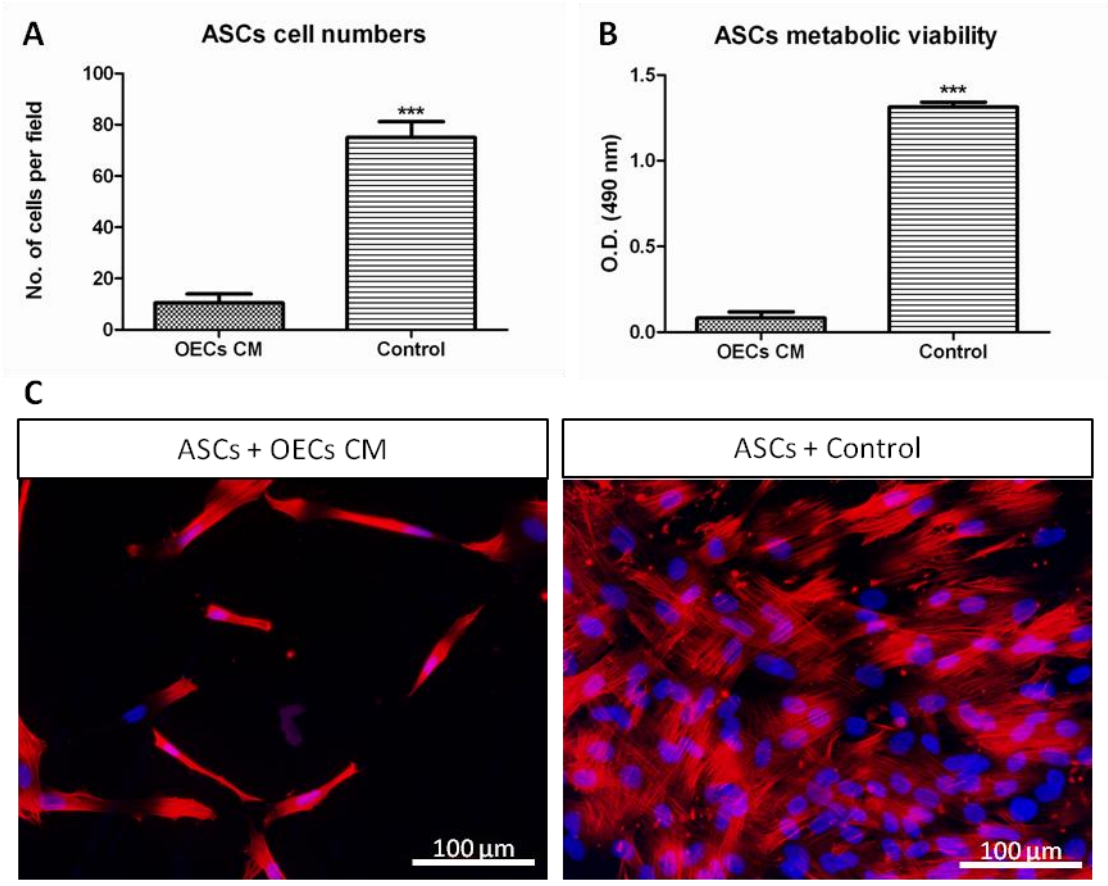

Figure 13 – Effect of OECs 24h CM on ASCs proliferation (A,C) and metabolic viability (B), after 5 days in culture. Phalloidin and DAPI staining was used to determine the number of ASCs and the MTS test was done to assess the metabolic viability of ASCs (n=3, mean ± standard deviation, ***P < 0.001). (C) Representative images of ASCs grown in OECs CM and control medium.

Previous work from our group has already provided some hints on ASCs and OECs secretome interactions. [169] Silva *et al.* demonstrated that both cells benefited from an indirect co-culture system, since their proliferation and metabolic activity was significantly increased.

However, in our studies, cells did not present the same kind of improvements. This can be explained in part by the different experimental set ups of these two studies. In the case of Silva *et al.* work, ASCs and OECs although separated physically, had constant access to the factors secreted by the other cell type, during all the time of the experiment. This creates a sort of a loop mechanism, in which cells are secreting factors that affect the other cell type, but at the same time are being influenced by the factors secreted by the other cell. On the other hand in our experiments, the CM of each cell type was collected at a specific time point, with a secretome composition with low variability. Moreover, the medium used to collect the secretome was serum-deprived, which clearly influenced cells response.

Nevertheless, OECs response to ASCs CM was particularly positive. A curious aspect of ASCs CM effects on OECs is that OECs grown in this medium presented less total number of

cells (fig. 14A). This means that even though there were fewer cells as compared to the control group, they had similar levels of metabolic activity and identical numbers of p75 positive cells (fig. 12). In others words, OECs cultured in ASCs CM were more pure (higher percentage of p75 positive cells, fig. 14B) and their metabolic viability/cell had a 2.6-fold increase, as seen in the ratio MTS/total number of cells (fig. 14C).

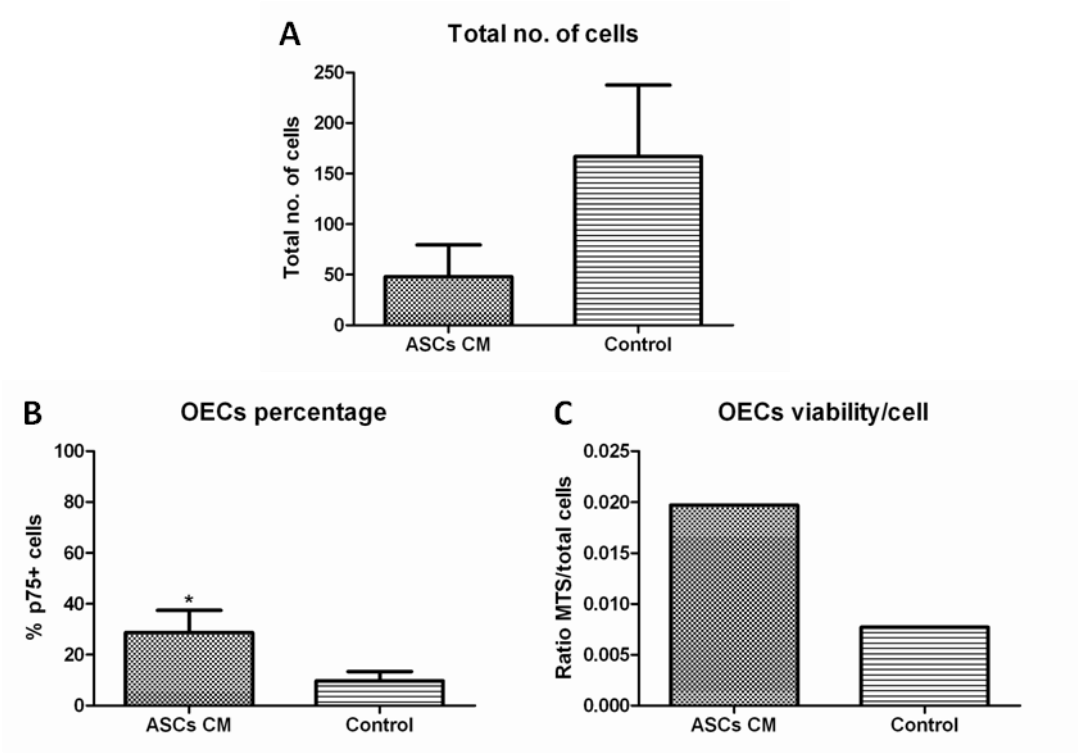

Figure 14 – OECs cultured in ASCs CM or control medium. (A) Total number of cells in culture; (B) Percentage of p75 positive cells in culture (n=3, mean ± standard deviation; *P < 0.05); (C) Ratio between the average O.D. obtained through the MTS test and the average number of cells in culture.

In the opposite direction, OECs CM seemed to exert a negative influence on ASCs proliferation and metabolic activity (fig. 13). Once again, the fact that the medium used to collect OECs CM was serum-deprived might partially explain the results observed. On one side, serum was not available for ASCs as it was in controls; on the other hand, the medium used to collect OECs secretome was also deprived of other supplements essential for OECs growth. Therefore, during the conditioning period, OECs might have entered in an initial stage of cell death, hence do not producing neurotrophic factors. Future studies should focus on a proteomic analysis on the CM collected, as this will allow obtaining valuable information on the composition of the secretome of each cell type.

4.2 ASCs and OECs secretome effects on a model of in vitro axonal regeneration

DRG cultures on laminin coated surfaces

Dorsal Root Ganglia (DRG) explants have been used as an *in vitro* approach to test the neuroregenerative potential of neurotrophic factors. [176] Based on the principle that cell bodies of sensory neurons are able to produce new neurites *ex vivo*, under the appropriate conditions, this technique holds great promise as a preliminary trial, before moving to *in vivo* studies.

Therefore, in order to test the regenerative potential of ASCs and OECs, we used DRG explant cultures. The DRGs were exposed to both cells conditioned media and the respective neurite outgrowth was measured. As it can be seen in figure 15, ASCs CM was the group that promoted the greatest neurite regeneration (0.38 ± 0.21 mm^2), reaching levels similar to the control (0.33 ± 0.05 mm^2). On the contrary, OECs CM almost did not induce regeneration of neurites (0.004 ± 0.004 mm^2), being that 3 of the 4 samples analyzed did not have any neurites. The medium 50:50, which was a mixture of ASCs and OECs CM, induced some level of neurites regeneration, in what we can consider an intermediate result between the other CM used (0.14 ± 0.01 mm^2).

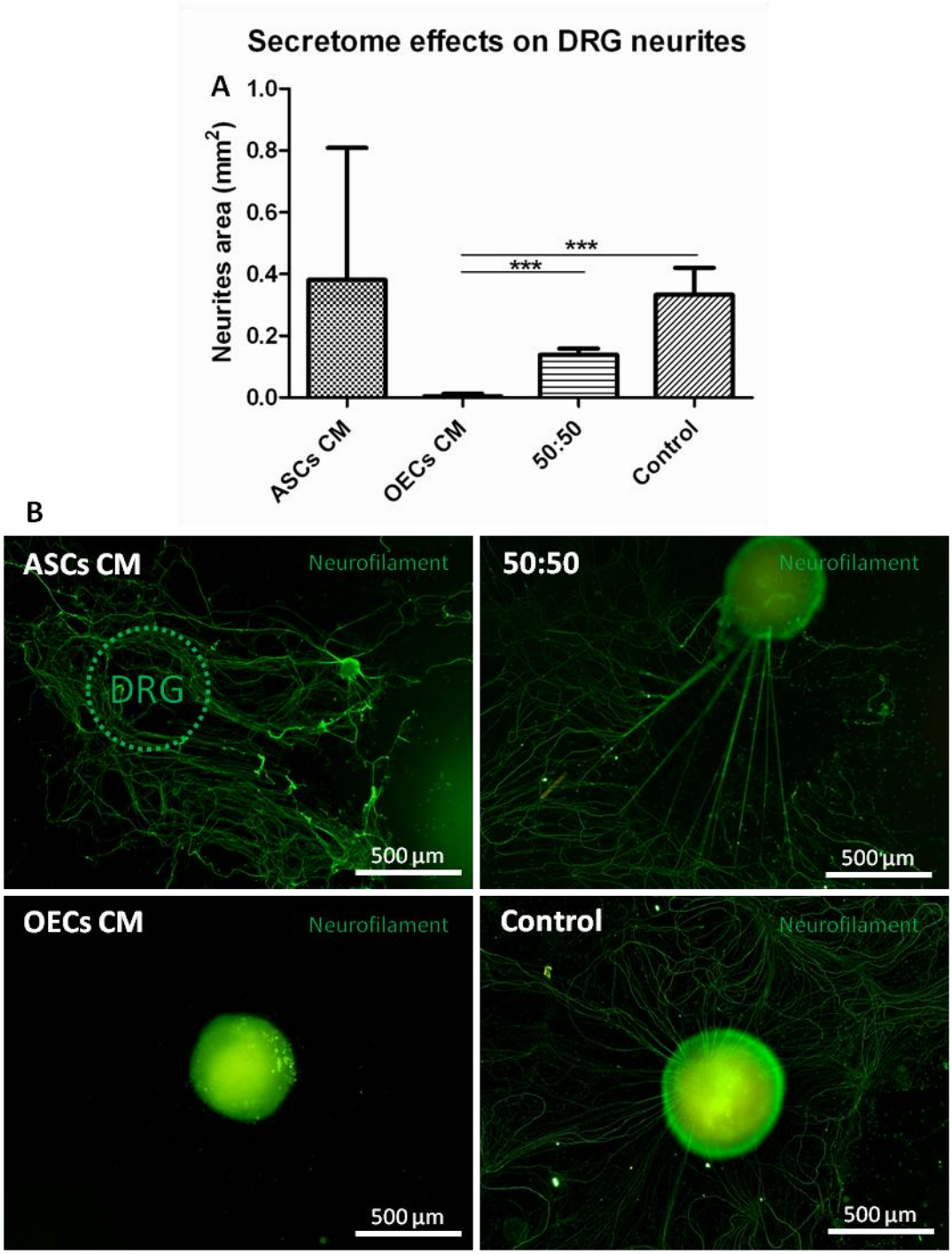

Figure 15 – Effects of different secretomes on DRG neurites outgrowth. Groups: ASCs CM 24h; OECs CM 24h; 50:50 – mixture of OECs CM 24h and ASCs CM 24h; Control – Complete culture media. The mean area occupied by the neurites in each condition was calculated using ImageJ (NIH) software (n=3, mean ± standard deviation; ***P < 0.001).

We tried to implement the DRG explants technique in our lab, by using a simple experimental set up. Basically, after DRGs isolation, the samples were placed on laminin coated 2D surfaces and cultured with the respective media of interest. As it can be seen in figure 15, ASCs CM was capable to induce as much neurite outgrowth as the control, which has NGF and other supplements. This supports the neurotrophic effect of ASCs' secreted paracrine factors, as

evidenced in neuronal cultures. [90] 50:50 group also had some positive results and most importantly they were consistent, as it can be seen by the short error associated. However, there were two major aspects that we concluded from this experiment. The first was related with the poor results observed once again with OECs CM. The secretome that we collected from OECs was not only negatively affecting ASCs proliferation and metabolic activity (fig. 13), but also almost did not promote any neurite regeneration (fig. 15). Therefore, we hypothesized that the medium used to collect OECs secretome was not providing the nutrients needed for cells to grow and secrete bioactive factors. In this way, we changed the method of OECs CM collection by adding B27 (1x) and L-glutamin (2 mM) supplements to the medium formerly used (**section 3.9.2**). The second aspect had to do with the difficulties observed in the DRGs adhesion process. The time of adhesion varied greatly between samples and consequently the first DRGs to adhere were the ones with longer neurites and more in number. We always selected DRG explants within the same state of development before adding the CM, but there was a clear need to standardize the experimental procedures. In this sense, we opted to culture the DRG explants according to Allodi *et al.* [176] By using two layers of a collagen-based gel to involve the DRGs, we abrogated the problem of explants adhesion. After having the DRGs "trapped" in this sort of collagen "sandwich", the growth medium was added.

DRG cultures on collagen gels

Taking into account the results obtained, collagen-based gel was prepared and the DRGs were involved in this 3D matrix. Preliminary results (fig. 16) show that the new OECs CM was capable of inducing neurite regeneration to a level close to the control group (0.18 ± 0.07 against 0.23 ± 0.13 mm^2).

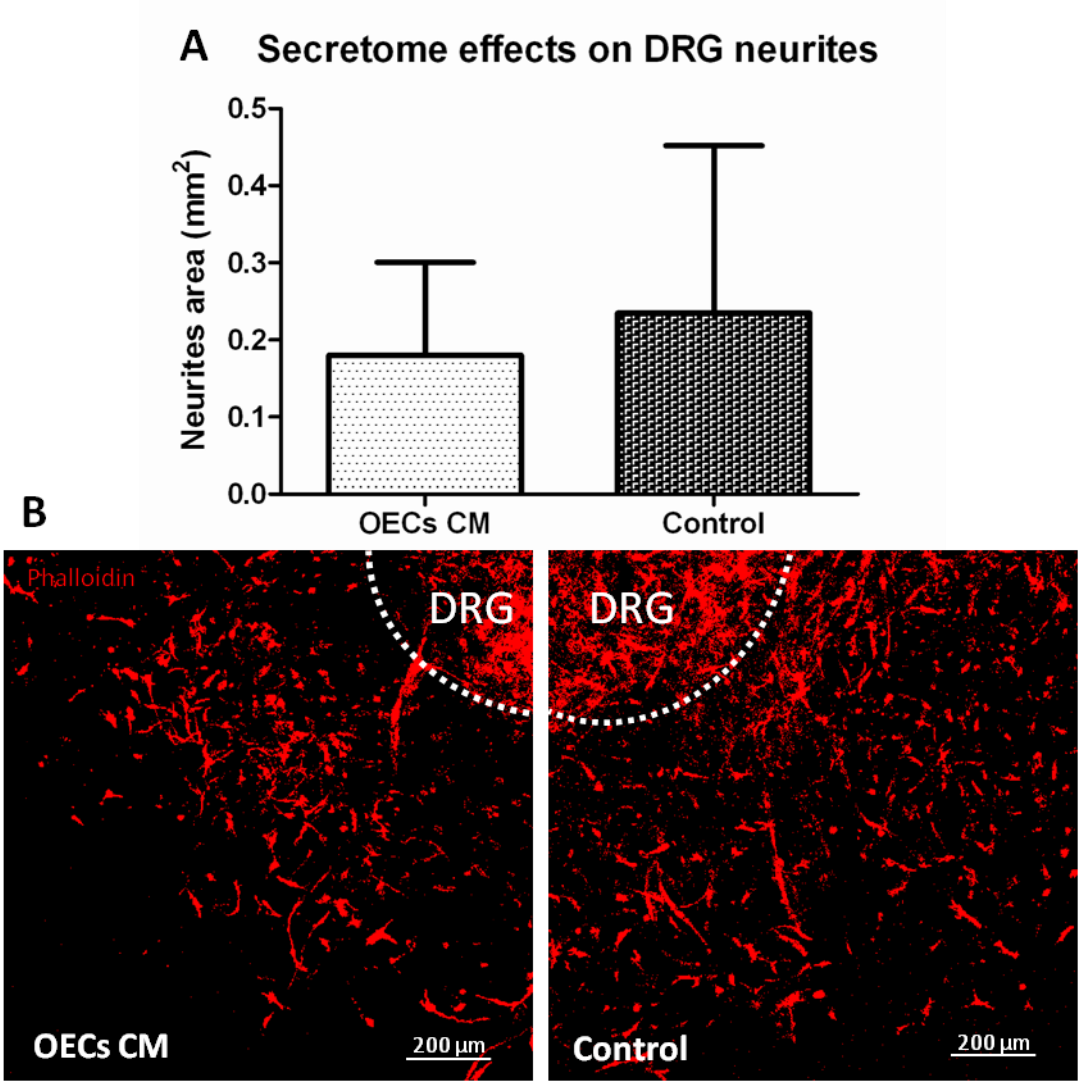

Figure 16 – Effect of OECs 24h CM on DRG neurites outgrowth. The mean area occupied by the neurites in each condition was calculated using ImageJ (NIH) software. The control group was constituted by complete culture media (n=3, mean ± standard deviation).

Having optimized the technique of DRGs culture, in further experiments other CM can be tested. ASCs CM, 50:50 or even a medium collected from a direct co-culture of ASCs and OECs are other options in order to determine which the best combination is. Furthermore, our combinatorial proposed therapy might also be tested using this technique. For instance, DRGs can be seeded on the GG-GRGDS hydrogel and the growth of neurites evaluated. Other experimental designs could also be applied, like encapsulating ASCs or OECs in the GG-GRGDS and then test this combination using the DRG explants. Many different treatments are possible, from CM of other cells, to other biomaterials or even drug testing, which make this in vitro model very interesting.

4.3 In vivo assessment of SCI rats after treatment with GG-GRGDS with cells

BBB test

In order to assess the locomotor recovery of SCI rats, we used the BBB_2 test. As it can be seen on figure 17, the only group that presented statistical significant improvements was the one whose animals were treated with ASCs and OECs encapsulated in the GG-GRGDS hydrogel. The mean score attributed to this group was 0.67 in the first days post-injury, evolving to a maximum of 7.00 after 7 weeks, with some animals regaining movement of the three joints of the affected left hind leg and even some weight support. These animals presented a BBB_2 score statistically different from the score of animals with no treatment (HS group) at 2, 3, 4, 7 and 8 weeks. In addition the improvement was also significant comparing to the group of animals treated only with GG-GRGDS at 4 weeks. Although the cells-treated group was not statistically different from the GG-GRGDS with cells-treated one, it also did not show significant improvements to any of the other experimental groups. Finally, sham animals presented minor locomotor difficulties in the first BBB_2 evaluation (20.60 score) but after that they showed a normal motor behavior as evidenced by the 21 point score attributed to all animals.

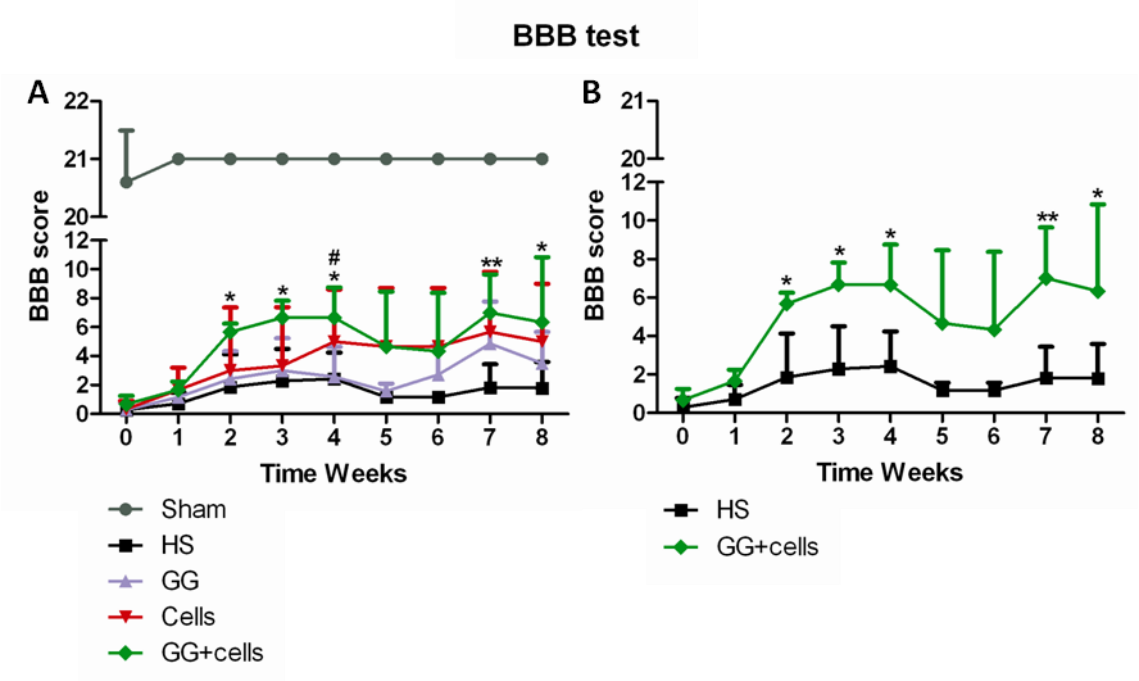

Figure 17 – (A) Locomotor behavior evaluation through the BBB test of SCI rats without treatment (HS, n=7), treated with GG-GRGDS (GG, n=7), treated with cells (Cells, n=3), treated with GG-GRGDS and cells (GG+cells, n=3) and animals only subjected to a laminectomy (Sham, n=5). (B) BBB score for HS and GG+cells groups. Values are shown as mean ± standard deviation. * - differences between GG+cells and HS; # - differences between GG+cells and GG (*P < 0.05; **P < 0.01; #P < 0.05).

Open Field test

The open field test was also performed in order to evaluate the total distance travelled by SCI rats and also to determine the total number of rearings, both indicators of motor behavior. Animals treated with ASCs and OECs encapsulated in GG-GRGDS had once again the best locomotor performance (fig. 18A), travelling a total distance (2902.0 ± 343.8 cm) significantly higher than that of animals without treatment (HS group, 1818.0 ± 264.8 cm) or animals treated with GG-GRGDS hydrogel (2012.0 ± 190.6 cm). GG-GRGDS with cells-treated animals even travelled a similar distance to sham animals (2902.0 ± 343.8 against 2675.0 ± 218.0 cm).

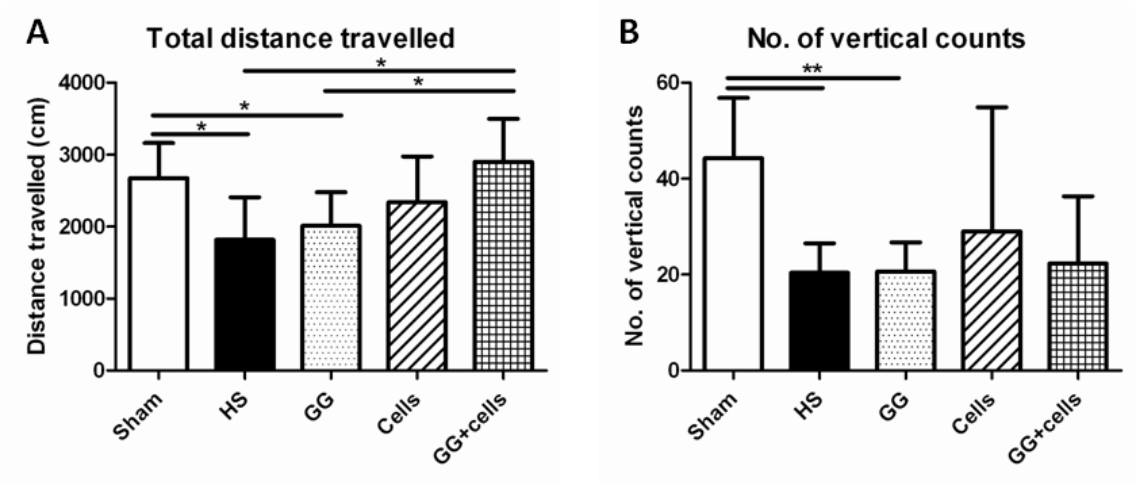

Figure 18 – Total distance travelled (A) and total number of rearings performed (B) by SCI rats in the OF arena, after 8 weeks. Values are shown as mean ± standard deviation (Sham n=5, HS n=5, GG n=6, Cells n=3, GG+cells n=3; *P < 0.05; **P < 0.01).

Regarding the number of rearings, only sham animals had a performance significantly different from other experimental groups. Therefore, none of the treatments assessed provided significant improvements on this particular motor behavior, 8 weeks post-injury.

The in vivo testing of our proposed combinatorial therapy was a crucial step to reinforce and validate the clinical potential of this tissue engineered based strategy. The results of the GG-GRGDS with cells group were very promising. These animals subjected to the combinatorial treatment showed evident signs of improved locomotor function, which was translated into better performances in the motor behavioral tests (fig. 17 and 18A). On the other hand, the cells-treated group presented an increase in the mean BBB_2 score from 0.33 in the first days post-injury to a maximum of 5.67 at 7 weeks after the lesion. However, in none of the time points evaluated, this group presented statistically significant differences compared to the other groups. This is mainly

due to the high variability existent in this group. Concerning the other 2 groups, GG-GRGDS-treated and non-treated animals (HS), they presented a similar pattern of motor behavior, with the exception of the last 3 weeks (fig. 17). This suggests that the implantation of the GG-GRGDS hydrogel by itself does not create an immediate benefit for SCI-rats. Another important fact is that non-treated animals kept a very low BBB_2 score throughout all the experiment, which means that the lesion inducted was correctly performed and the animal model consistent. In addition, sham animals did not present any motor deficits, showing that the laminectomies that were performed did not negatively influence the locomotor skills of the animals.

Regarding the open field test, the data on total distance travelled is a reflex of the benefits provided by the treatment with GG-GRGDS and cells; once again, animals of this group performed better than the other experimental groups (fig. 18A). Like it happened on the BBB_2 test, animals treated only with cells or GG-GRGDS demonstrated minor improvements (not statistically significant) as compared to non-treated animals, being that, only when these 2 strategies were combined, we could obtain a significant difference. However, the data obtained on the total number of rearings showed no significant differences between all groups, except for sham animals. This can be explained because the rearing activity is highly dependent on the ability of supporting weight in both hind legs, and most animals (except for sham group) were not capable of supporting their weight on the left hind leg. Therefore, this behavior might be useful for discriminating improvements in animals with better motor skills, but not in animals in this particular stage.

Histological evaluation

Hematoxylin and eosin staining was performed on spinal cord sections at the injury site, in order to determine any major alterations at the morphological level. The most evident observation is that there was a greater integrity of the spinal cord tissue in animals treated with ASCs and OECs encapsulated in GG-GRGDS (fig. 19). Unlike the non-treated or cells-treated animals, there was either preservation or a reorganization of the tissue, which may have contributed to the better locomotor profiles presented in behavioral tests. In the spinal cord tissue of animals treated with GG-GRGDS only, there is also some tissue preservation, which indicates that hydrogel implantation might have been essential in this process. However the reorganization

of the tissue by itself, in this case, did not correlated with significant motor improvements (fig. 17).

Immunohistochemistry (IHC) procedures were also performed against GFAP and CD11B (markers of astrocytes and inflammatory cells respectively). After confocal microscopy we observed higher areas of astrocytosis in non-treated (HS) and cells-treated animals (Cells). Additionally, rats treated with GG-GRGDS with cells presented less density of astrocytes and less expression of CD11B (fig. 20).

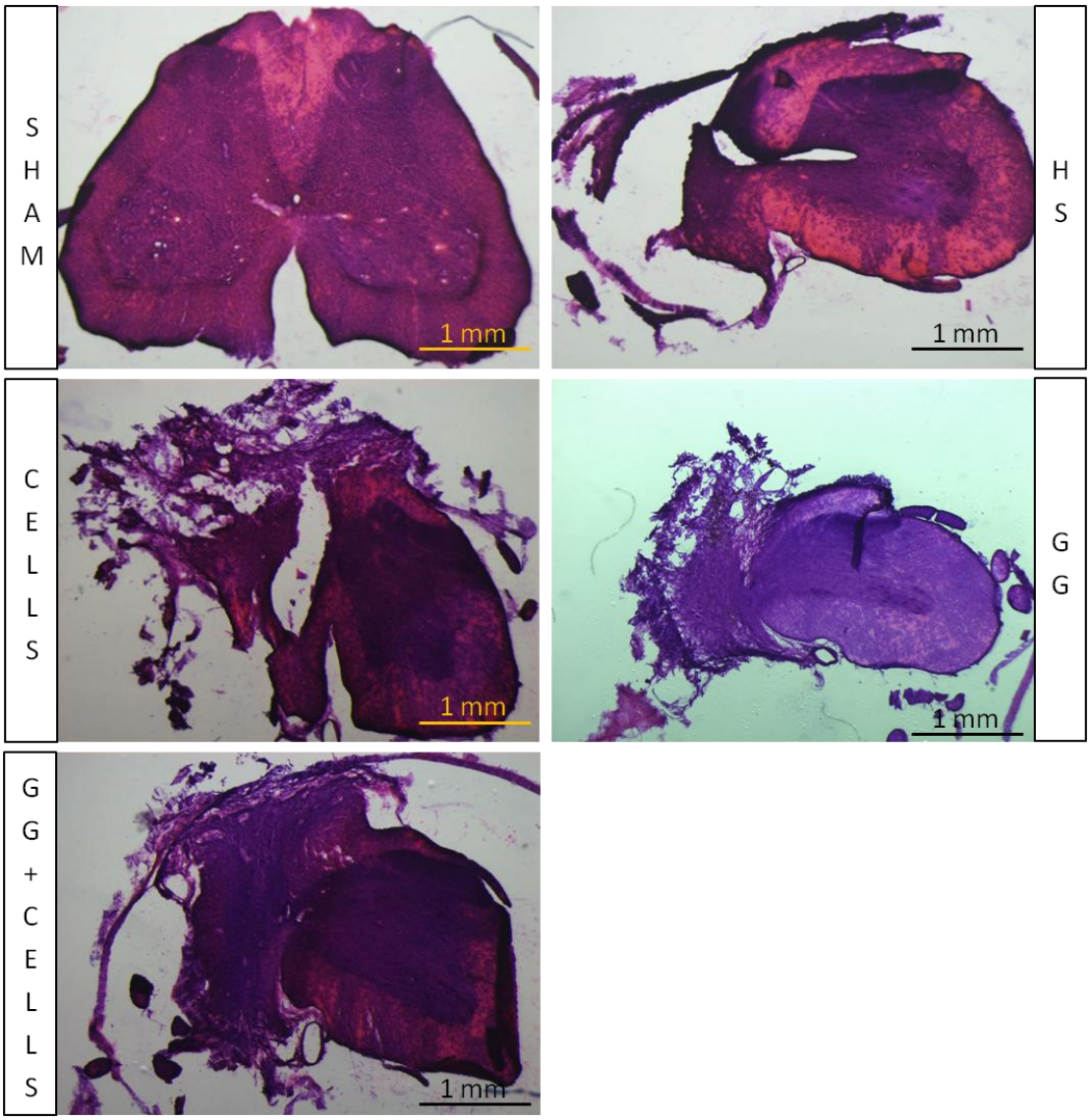

Figure 19 – Hematoxylin and Eosin staining of spinal cord sections at the lesion site.

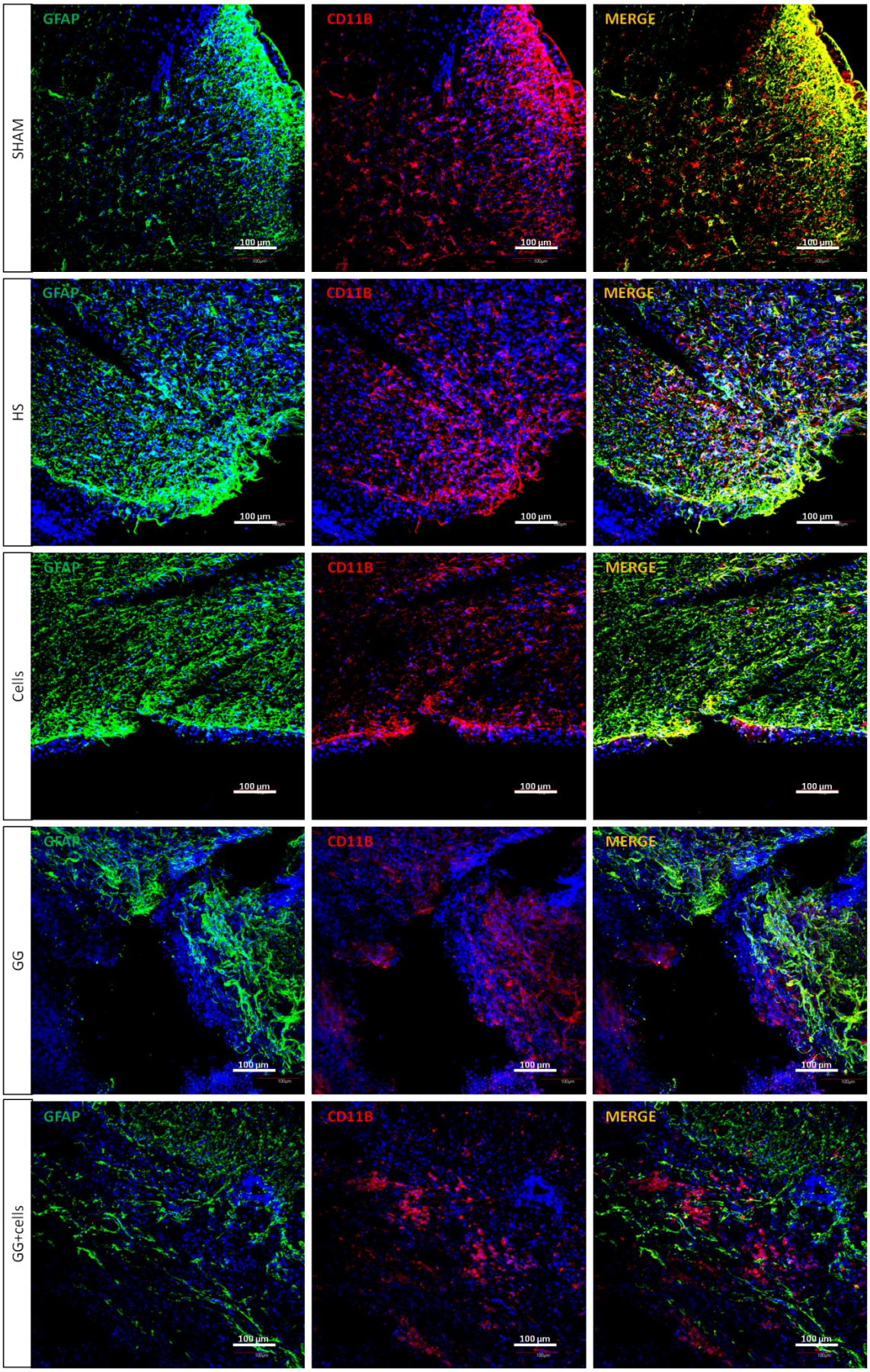

Figure 20 – Confocal images obtained following an IHC against GFAP (left panel) and CD11B (center panel). On the right panel we can find the merge of the two markers. Nuclei were counterstained with DAPI.

The histological analysis performed so far is still limited. These results are preliminary and a thorough analysis of all samples will be done in order to obtain a good characterization of the histological consequences of our proposed therapy.

The first major observation was that treatment with our combinatorial strategy of GG-GRGDS hydrogel with cells seemed to promote tissue preservation after SCI. Although animals treated only with GG-GRGDS also presented some tissue preservation, this was more notorious in the combinatorial treatment approach. The reorganization of the tissue is very important, otherwise the regeneration of the neuronal circuitry will not happen as well as the recovery of its function.

In addition to assessment of the general spinal cord morphology, we also did a preliminary analysis for astrogliosis (GFAP staining) and other inflammatory cells such us microglia and dendritic cells (CD11B staining). Reactive astrocytes are usually associated to impairment of axonal regrowth due to the formation of a physical barrier [14]; taking this into account, the lower levels of GFAP expression in GG-GRGDS with cells-treated animals (fig. 20) might partially justify their better performance in the motor behavioral tests. At the same time the concentration of GFAP positive cells is higher in non-treated, GG-GRGDS-treated and only cells-treated groups, which can be related to the poor locomotor performances of these animals. Regarding the expression of CD11B marker, it is observed a homogenous expression in non-treated and cells-treated animals. Moreover, GG-GRGDS-treated and GG-GRGDS with cells-treated groups present a smaller CD11B expression, although concentrated in some regions of the spinal cord. So far, it is not fully understood the role of inflammation on tissue regeneration after SCI. [183] Considering the good motor performance of animals treated with our proposed therapy (GG-GRGDS + cells), we assume that the inflammatory response observed in these rats was not detrimental. It might have just been a response to the hydrogel. Moreover, the number of inflammatory mediators present at this stage (approximately 10 weeks post-injury) is considerably lower, when comparing to the first hours or days post-injury. [183] Therefore the importance of inflammation at this stage could be smaller. In order to conclude the histological analysis, neuronal markers, as well as markers for the transplanted cells will be used. This will provide a better perception about the neuronal regeneration levels and at the same time, enlighten how the transplanted cells integrated in the spinal cord tissue. Moreover, after

analyzing all the samples it will be possible to correlate the histological profile with individual performances in the locomotor tests, which will further elucidate the role of our propose therapy.

Chapter 5
CONCLUSIONS

Patients suffering from spinal cord injuries still have a dismal prognosis. Despite all the efforts developed in this area, single therapies usually fail when translated to the clinics. Therefore, a combinatorial strategy like the one herein proposed represent a viable alternative for the current treatments employed. The use of a natural gellan gum based hydrogel in combination with cells with therapeutic value demonstrated to improve motor deficits of SCI-rats. Moreover, with this strategy, spinal cord tissues were better preserved and the presence of reactive astrocytes was less evident. Additional improvements to this approach can be made, namely the addition of other peptides with bioactive roles. For instance, laminin peptides associated with neuronal migration and differentiation might help in the process of axonal regeneration, by increasing the affinity between hydrogel and nervous tissue. Other bioactive molecules such as neurotrophic factors are other options that can be included in a combinatorial strategy. By using an integrative approach, based on tissue engineering principles, we might be one step closer to tackle the enormous complexity of SCI, providing a treatment with a significant and clinical value.

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