Universidade do Minho
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Combination of Cellular Therapy with Peptide-modified Gellan Gum Hydrogels for Spinal Cord Injury Repair

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Tese de Doutoramento
em Ciências da Saúde

Trabalho Efetuado sob a orientação de
Doutor António J. Salgado
Doutor Nuno Silva

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Declaração

Nome: Eduardo Domingos Correia Gomes

Endereço eletrónico: id5788@alunos.uminho.pt; **Telefone:** +351 961 659 231

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RESUMO

As lesões vertebro-medulares (LVM) são uma condição debilitante, com consequências devastadoras quer para pacientes como para as suas famílias. A perda parcial ou total das capacidades motoras e sensoriais abaixo do local da lesão é a consequência mais notória. Contudo, problemas cardíacos e respiratórios, assim como disfunção dos sistemas urinário e gastrointestinal, entre outros, acontecem frequentemente, reduzindo significativamente a qualidade de vida. Atualmente não existem tratamentos para as LVM, com a maioria das abordagens a ser utilizada apenas de modo a minimizar a progressão da lesão.

A falta de tratamentos pode ser atribuída à agressiva fisiopatologia da doença. O impacto inicial leva a um dano tecidular e morte neuronal imediatas, com formação de edema e hemorragia. Durante os minutos seguintes, até várias semanas, uma série de alterações celulares e moleculares é ativada, conduzindo a um aumento do dano neuronal. Por fim, na fase crónica, são formadas a cicatriz glial e a cavidade cística, criando uma barreira física e química à regeneração. Tendo em conta a complexidade e a panóplia de alterações secundárias que podem ser usadas como alvo terapêutico, é provável que tratamentos baseados em abordagens combinatórias terão um maior impacto do que estratégias singulares. Dentro das várias possibilidades, os conceitos usados em engenharia de tecidos e medicina regenerativa poderão ser de uma grande relevância para as LVM.

O transplante celular é um dos pilares da engenharia de tecidos e tem vindo a ser aplicado na investigação em LVM desde há décadas. Não obstante, e apesar do elevado número de trabalhos neste campo, a grande maioria falha quando tenta ser aplicado na clínica. Combinar estratégias baseadas em biomateriais com terapias celulares poderá resolver alguns dos problemas associados a esta abordagem, nomeadamente a baixa sobrevivência das células após transplante. Deste modo, o trabalho desenvolvido durante esta tese teve como objetivo desenhar uma abordagem combinatória, conjugando o transplante de duas populações celulares distintas com um hidrogel modificado, que serve tanto como uma matriz física para o crescimento neuronal, assim como um veículo para o transplante celular.

Neste sentido, o hidrogel goma gelana, previamente modificado com péptidos derivados de fibronectina (GG-GRGDS), foi combinado com células estaminais do tecido adiposo (ASCs) e células envolventes do bolbo olfativo (OECs). Ambos os tipos celulares têm sido associados a efeitos neuro-protetores e neuro-regenerativos, em diferentes modelos de LVM.

Neste trabalho, primeiramente demonstramos que ASCs e OECs crescem *in vitro*, em sistemas de co-cultura direta, sem que sejam afetadas as suas morfologias, proliferação e viabilidade. Para além disso, a sua combinação foi benéfica, levando a um crescimento de neurites, num modelo *in vitro* de regeneração axonal. De seguida, ASCs/OECs foram transplantadas para um modelo de rato de hemisecção torácica (nível T10). O tratamento induziu uma recuperação significativa da função das patas traseiras, o que esteve associado a níveis reduzidos de inflamação no local da lesão.

Mais tarde, as células foram também encapsuladas em hidrogéis de GG-GRGDS e apresentaram uma morfologia e crescimento normais *in vitro*. Adicionalmente, a sua aplicação conjunta num modelo de hemisecção lombar (mais agressivo) levou a uma melhoria significativa da locomoção. A análise histológica permitiu perceber uma redução da inflamação, da astrogliose e aumento da marcação para neurofilamento, possível indicador de regeneração axonal ou de maior proteção neuronal.

Por fim, a mesma estratégia foi implementada num modelo de lesão cervical, que induz défices respiratórios, afetando a função do diafragma. Após hemisecção em C2, hidrogel e células foram imediatamente transplantados, no local de lesão. O tratamento combinado levou a uma maior recuperação da função do diafragma, avaliada por eletromiograma do músculo ipsilateral do diafragma. As regiões mais dorsal e medial do diafragma demonstraram uma recuperação parcial da função. Apesar de não terem sido observadas diferenças na função motora das patas dianteiras, a terapia combinatória ajudou a recuperar a função sensorial, da pata dianteira contralateral, após avaliação com filamentos de Von Frey.

Resumindo, estes resultados revelam um grande potencial terapêutico para o uso de hidrogéis de GG-GRGDS com ASCs e OECs, dada a sua aplicação com sucesso a diferentes modelos de lesão, com melhorias de função significativas. Não só comportamentos como a locomoção foram melhorados, mas também o controlo do diafragma foi recuperado parcialmente, o que é essencial para a função respiratória, e ainda a perceção sensorial. Mais ainda, todas as componentes desta abordagem podem ser facilmente aplicadas na clínica, visto ser possível obter ASCs e OECs de uma maneira autóloga (do próprio paciente) e ao facto da goma gelana ser biocompatível, com baixa probabilidade de provocar uma reação imune. Este trabalho apresenta uma abordagem relevante para as LVM, que após mais estudos pré-clínicos em animais de maior porte, poderá um dia vir a ser aplicado em pacientes.

ABSTRACT

Spinal Cord Injury (SCI) is a highly debilitating condition, with devastating consequences for the patients and their families. Partial or complete loss of motor and sensorial functions below the level of injury are the main visible outcomes. However, cardiac and respiratory compromise, together with bladder and bowel dysfunctions, among others, are frequently observed, impairing significantly the quality of life. Currently there are no treatments for SCI, with most approaches being used in order to minimize injury progression.

The lack of treatments can be attributed to the aggressive pathophysiology of SCI. The initial impact immediately leads to massive tissue damage and neuronal death, with edema formation and hemorrhages. For the next minutes up to several weeks, a cascade of cellular and molecular secondary alterations is triggered, leading to further neuronal damage. Finally, in the chronic phase, a glial scar and a cystic cavity are formed, creating a physical and chemical barrier to regeneration. Considering the injury complexity and the multitude of secondary alterations that can be targeted, it is more likely that a combinatorial therapeutic approach will have more impact than single-based therapies. Among the different approaches, tissue engineering (TE) and regenerative medicine concepts could be of extreme relevance for SCI.

Cellular transplantation, one of the cornerstones of TE, has been applied in SCI research for decades. Nonetheless, and despite the numerous studies in the field, most of them fail when translated into the clinics. Combining biomaterial-based strategies with cell therapies could solve some of the associated problems, such as the low cell survival after transplants. In this way, the work developed during this thesis aimed to develop a combinatorial approach, conjugating the transplantation of two different cellular populations with a functional hydrogel, serving both as a physical matrix for neuronal regrowth and as a vehicle for cell transplantation.

Thus, gellan gum hydrogel, previously modified with fibronectin-derived peptides (GG-GRGDS), was combined with adipose tissue-derived stem/stromal cells (ASCs) and olfactory ensheathing cells (OECs). Both cell types have been associated with promising protective and regenerative effects, in different models of SCI.

In this work, we firstly showed that ASCs and OECs could grow *in vitro*, in direct co-culture systems, without affecting their morphology, growth and viability. In addition, their combination was beneficial *in vitro*, resulting in increased axonal growth using a model of axonal regeneration (Dorsal Root Ganglia – DRG – explants). Then, ASCs/OECs were applied as a single

transplantation into a rat thoracic hemisection model of injury (T10 level). The treatment led to significant recovery of hindlimb function, which was associated with reduced levels of inflammation at the lesion site.

Later, cells were encapsulated within GG-GRGDS hydrogels and demonstrated a normal morphology and growth *in vitro*. Moreover, their combined application into a lumbar hemisection model, led to a significant recovery of locomotor function. The histological analysis showed reduction in inflammation, astrogliosis and an increased number of neurofilament positive staining, possible indicative of axonal regeneration or neuronal protection.

Finally, the same strategy was implemented in a cervical model of injury, which induces respiratory deficits, affecting diaphragmatic function. Following a C2 hemisection, hydrogel and cells were transplanted at the injury site, immediately after lesion. The combinatorial treatment induced the most significant recovery of diaphragmatic function, as assessed by electromyogram activity of the ipsilateral hemi-diaphragm. Both ventral and medial portions of the diaphragm were partially recovered. Moreover, and despite no differences were seen in forelimb motor function, GG-GRGDS and ASCs/OECs induced a significant recovery of sensorial perception, in the contralateral forelimb, as measured by the Von Frey test.

All together, these results disclose a great therapeutic potential for the application of GG-GRGDS hydrogels with ASCs and OECs, since its application to different models of injury resulted in significant and relevant improvements of function. Not only motor behaviors associated with locomotion were recovered, but also motor control of the diaphragm, essential for respiration, and also sensorial perception recovery. In addition, all components of this approach could be translated to the clinics, as ASCs and OECs can be obtained from autologous sources and gellan gum proved to be biocompatible, with low immunogenic potential. This work presents an important strategy for SCI repair, that with further pre-clinical testing in larger animal models, could be one day applied to the clinics.

CONTENTS

List of Abbreviations

2D – Two Dimensional

3D – Three Dimensional

5-HT – 5-Hydroxytryptamine

Akt – protein kinase B

AIS – ASIA Impairment Scale

ANOVA – Analysis of Variance

ASCs – Adipose Stem Cells

ASIA – American Spinal Injury Association

BBB – Basso, Beattie and Bresnahan

BDNF – Brain-Derived Neurotrophic Factor

bFGF – basic Fibroblast Growth Factor

BM-MSCs – Bone Marrow Mesenchymal Stem Cells

BMS – Basso Mouse Scale

BSCB – Blood-Spinal Cord Barrier

cAMP – cyclic Adenosine Monophosphate

CD – Cluster of Differentiation

CGRP – Calcitonin Gene Related Peptide

ChABC – Chondroitinase ABC

CINC-1 – Cytokine-induced Neutrophil Chemoattractant 1

CM – Conditioned Media

CMAPs – Compound Muscle Action Potentials

CNS – Central Nervous System

CNTF – Ciliary Neurotrophic Factor

COX2 – Cyclooxygenase

CPG – Central Pattern Generator

CSPGs – Chondroitin Sulphate Proteoglycans

CST – Corticospinal Tract

DAPI – 4',6-diamidino-2-phenylindole

DIV – Days *In Vitro*

DMEM – Dulbecco's Modified Eagle's Medium

DMT-MM – 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride

DRG – Dorsal Root Ganglia

ECM – Extracellular matrix

ED1 – anti-CD68 antibody

EGF – Epidermal Growth Factor

ELISA – Enzyme-linked Immunosorbent Assay

EMGs – Electromyograms

ERK – Extracellular signal-regulated Kinases

ESCs – Embryonic Stem Cells

FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

FDA – Food and Drug Administration

FGF – Fibroblast Growth Factor

GALC – Galactosylceramidase

GAP43 – Growth Associated Protein 43

GCSF – Granulocyte Colony-stimulating Factor

GDN – Glia-derived Nexin

GDNF – Glial cell-line Derived Neurotrophic Factor

GFAP – Glial Fibrillary Acidic Protein

GG – Gellan Gum

GRGDS – Glycine-arginine-glycine-aspartic acid-serine

HBSS – Hank's Balanced Salt Solution

HGF – Hepatocyte Growth Factor

HLA-DR – Human Leukocyte Antigen DR isotype

HNA – Human Nucleotide Antibody

HO-1 – Heme Oxygenase 1

HR – Horizontal Rung

ICC – Immunocytochemistry

IGF-1 – Insulin like Growth Factor

IHC – Immunohistochemistry

IL- – Interleukin

IP – Inclined Plane

iPSCs – induced Pluripotent Stem Cells

IT – Intrathecal

IV – Intravenous

LP – Lamina Propria

MAG – Myelin-Associated Glycoprotein

MAP2 – Microtubule-associated Protein 2

MEM – Minimum Essential Medium

MEPs – Motor Evoked Potentials

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

MP – methylprednisolone

MSCs – Mesenchymal Stem Cells

N/A – information Non-Available

NeuN – Neuronal Nuclei

NF – Neurofilament

NG2 – Neural/Glia antigen 2

Ngn2 – Neurogenin 2

NGF – Nerve Growth Factor

NMJs – Neuromuscular Junctions

NSCs – Neural Stem Cells

NSPCs – Neural Stem/Progenitor Cells

NT-3 – Neurotrophin 3

OECs – Olfactory Ensheathing Cells

OEG – Olfactory Ensheathing Glia

ONL – Olfactory Nerve Layer

OPCs – Oligodendrocyte Progenitor Cells

p75 – Low-affinity Nerve Growth Factor Receptor

PBS – Phosphate Buffered Saline

PDGFRα – Platelet-derived Growth Factor Receptor α

PEDF – Pigment Epithelium-derived Factor

PEG – Poly(ethylene glycol)

PFA – Paraformaldehyde

PHB – Poly-β-hydroxybutyrate

PHB-b-DEG – Polyhydroxybutyrate - polyethylene glycol

PhMN – Phrenic Motor Neurons

PHPMA – Poly[N-2-(hydroxypropyl) Methacrylamide

PLA – Poly(lactic acid)

PLGA – Poly(lactic-co-glycolic acid)

PNS – Peripheral Nervous System

PSD95 – Postsynaptic Density protein 95

pSTAT3 – Phopho Signal Transducer and Activator of Transcription 3

qPCR – quantitative Polymerase Chain Reaction

ROS – Reactive Oxygen Species

rVRG – Rostral Ventral Respiratory Group

RT – Room Temperature

SCI – Spinal Cord Injury

SCs – Schwann Cells

SEM – Standard Error of the Mean

SKPs – Skin-derived Precursors

SSEPs – Somatosensory Evoked Potentials

TE – Tissue Engineering

TGFβ – Transforming Growth Factor beta (β)

TNFα – Tumor Necrosis Factor Alpha

TrkC – Tropomyosin receptor kinase C

Tuj1 – class III β-tubulin

USA – United States of America

UV – Ultraviolet

VEGF – Vascular Endothelial Growth Factor

WB – Western Blot

% - percent

°C – Celsius degrees

µg – micrograms

µl – microliters

Ca^{2+} – calcium

cm – centimeters

cm^2 – square centimeters

dpi – days post-injury

h – hours

hpi – hours post-injury

K^+ – potassium

mg – milligrams

min – minutes

ml – milliliters

mm – millimeters

mopi – months post-injury

mpi – minutes post-injury

ms – milliseconds

mV – millivolts

Mw – Molecular weight

Na^+ – sodium

ng – nanograms

nm – nanometers

rpm – rotations per minute

v/v – volume per volume

wpi – weeks post-injury

w/v – weight per volume

Figures and tables

Thesis aims and Layout

The main aim of this thesis was to develop a TE strategy aimed for SCI repair, by combining human adipose tissue-derived stem/stromal cells (ASCs) and rat olfactory ensheathing cells (OECs) with GRGDS-modified gellan gum hydrogels (GG-GRGDS).

This strategy was evaluated in different models of SCI, addressing both functional and histological outcomes, following acute treatment protocols. Each model tested corresponds to a different chapter.

This thesis is therefore divided into five chapters:

Chapter 1 includes a general introduction to the main topics addressed in this study. An overview is provided for SCI epidemiological data, as well as the main pathophysiology components of this condition. In addition, the main cellular- and biomaterial-based therapeutic strategies are presented, together with the most recent studies combining both approaches. Portions of sections 1.4 and 1.5 were adapted from the author's previous publication: Assunção-Silva RC, **Gomes ED** et al. *Stem Cells International* 2015/948040.

Chapter 2 describes the transplantation of ASCs and OECs into a rat thoracic hemisection model (T10). Locomotor function and histological analyses were performed to assess the recovery up to eight weeks post-injury. This work unveils an important role for ASCs/OECs in SCI repair.

Chapter 3 presents one of the main objectives of the thesis, which is the combination of ASCs/OECs with GG-GRGDS hydrogels. Following a L1 lumbar hemisection injury, rats were followed for two months, for locomotion and histological evaluations. Only the combination of hydrogel and cells could lead to significant improvements of hindlimbs motor function, in a particularly aggressive model of injury.

Chapter 4 reports the application of the same combinatorial approach, GG-GRGDS hydrogel with ASCs/OECs, into a cervical model of injury. After a C2 hemisection and treatment, respiratory function, forelimb movements and sensorial perception were assessed. The combined treatment demonstrated the most significant improvements in diaphragmatic function, as well as sensorial tests.

Chapter 5 contains a general discussion, integrating all the findings from the three previous chapters. It further explores possible explanations for the results observed, discusses the limitations of this work and infers about the eventual implications of this thesis in the context of the SCI research field.

Chapter 1
Introduction

1.1 Spinal Cord Injury

The Central Nervous System (CNS) is probably the most intricate and astonishing achievement of the evolution of life on Earth. The development of the CNS and its increase in complexity allowed animals to evolve at a quicker pace [1]. In humans (and most vertebrates) the CNS is comprised of the brain and the spinal cord. In simple terms, the brain receives and integrates sensorial information, producing a motor response. The spinal cord mediates most of this transmission of information between the brain and the periphery. Neurons are considered the building blocks of the CNS, but glial cells, including astrocytes, oligodendrocytes and microglia, play important roles in the overall homeostasis of this system [2]. Therefore, it is easy to understand that when a degenerative process affects the CNS, either induced by disease or trauma, it could lead to a tremendous impact on the life of an individual. Injuries affecting directly the spinal cord may cause heavy motor deficits, sensorial impairments, and a shutdown of many functions controlled by the autonomic nervous system, including bladder and bowel function, heart rate and breathing [3]. A more serious injury could even result in death. However, how often Spinal Cord Injury (SCI) happens? What is the impact of SCI in the modern society?

1.1.1 Incidence/Prevalence/Social impact

The first reports of a SCI come back to the ancient Egypt. At the time, a traumatic event to the spinal canal, was considered, “a disease to not be treated” [3]. But despite the early recognition of the complexity of this type of injuries, there is still today, a huge lack of epidemiologic data on SCI. The USA and Canada are the countries where there is more data, and registry of SCI events.

Incidence and causes

The incidence in those countries varies quite significantly among states and provinces, from 25.2 per million in Northwest Kentucky/South Indiana (1993-98 period) [4] to 83 per million in Alaska (1991-93) [5]. More recent studies also place SCI incidence levels between those values, for example, 25.6 per million in the province of Manitoba, Canada (2003-07) [6].

SCI data from Asia/Pacific is scarcer. From the studies available, SCI affects 10 per million people in Fiji (1985-94) [7] up to 56.1 per million in Hualien county in Taiwan (1986-90) [8]. South America and Africa have no information available, while middle-East has very little information on SCI epidemiology, being the most relevant studies from Jordan, with an incidence of 18 per million (1988-93) [9] and 44 per million in Tehran, Iran (2007-08) [10]. Finally, in Europe, incidence varies from 9.2 per million in Denmark (1975-84) [11] up to 58 per million in Central Portugal (1989-92) [12].

These numbers are based on the most recent studies, however many of them were based in data collected from the 1980s and 1990s, which highlights the need for new surveys and the creation of registries, in order to get a better understanding of the real status of SCI globally. Nevertheless, how these differences in incidence levels around the globe can be explained? And what are the main causes of traumatic SCI?

To start, one should consider the different methodologies used in each of these studies. SCI definition, inclusion and/or exclusion criteria or even the time period under evaluation, differ among studies which could prevent direct comparisons between regions. For example, the inclusion of people that die from SCI at the accident scene or during transport to the hospital, is not present in all studies, altering significantly incidence numbers [13].

Then, the causes of SCI might also help to partially explain incidence levels. In general, motor vehicle accidents account for most of the SCI cases worldwide (~30-60%) [13]. This is true for both developed but also developing countries, where the use of motor vehicles is increasing, despite safety measures and road infrastructures are still poor. The second most common cause of SCI are falls (~10-50%) [13]. The constantly aging population, especially in developed countries, is leading to a fast increase in the number of SCI cases due to falls among the elderly. With improvement of safety regulations and infrastructures for motor vehicles circulation, falls might become the main cause of SCI worldwide. Finally, suicide attempts, violence-related injuries and sports/recreational accidents represent most of the remaining cases of SCI (~1-30%) [13]. Some particular aspects are worth to highlight. For example, violence-related SCI is more common in countries such as the USA (~20% in some states) [14, 15] or Jordan (27.8%) [9]. Suicide attempts resulting in SCI are frequent in Nordic countries, such as Denmark (8%) [11] or Greenland (26%) [16], even though the number of studies reporting SCI due to suicide attempts are very low. In Novosibirsk, Russia [17] or in Fiji [7], sports/recreational activities lead to more than 30% of SCI cases, mostly related with diving accidents.

A common aspect to all studies is the higher SCI incidence in men, going from a male:female ratio of 1.5:1 in Ontario, Canada [18] to a 7:1 in Thessaloniki, Greece [19]. Additionally, there are two age groups that typically present higher incidence of SCI: the 15-30 and >60 years old groups. If motor vehicle accidents and sports-related injuries are more frequent in the first group, falls are the main SCI cause in the second one [13]. Anatomically, cervical lesions represent the majority of SCI cases, followed by thoracic and lumbar injuries [13].

Prevalence

Data of SCI prevalence worldwide is even less available than incidence levels. It can vary from 250 per million in Rhone-Alpes in France (1970-1975) [20], up to 906 per million in the USA (1980) [21].

There are several reasons for the differences in prevalence numbers observed. First, differences in methodological approaches and the calculation of prevalence itself, since it can be based on estimated values. Then, obvious differences arise when comparing developed to developing countries, for instance. In developing countries, the lack of access to medical care leads to a very high one-year mortality rate, particularly in the sub-Saharan Africa [22], resulting in very low prevalence numbers. On the other hand, the increased survival after SCI in developed countries, associated with a significant improvement in the life expectancy of these patients also poses another problem: the sustainability of the medical care service. For example, in Olmsted county, Minnesota USA, the prevalence of patients with SCI increased from 220 per million in 1950, to 583 per million in 1981 [23]. This could mean an overload of the health system, and an economic burden for the patients and their families.

The increase in survival and life expectancy after a SCI also raises an important aspect: a growth observed in the number of psychological disorders in this vulnerable population, namely depression [24].

From a very recent report on the global incidence and prevalence of SCI worldwide (Fig. 1.1) we can conclude that, as of 2016, countries under war present the highest incidence rates of SCI (Syria, Yemen, Iraq or Afghanistan) [25]. Western countries maintain high-incidence rates in comparison to previous reports. In addition, another aspect that can be highlighted is the

increased SCI incidence in the elderly and the dilution of male predominance in SCI cases, in the same population (Fig. 1.2).

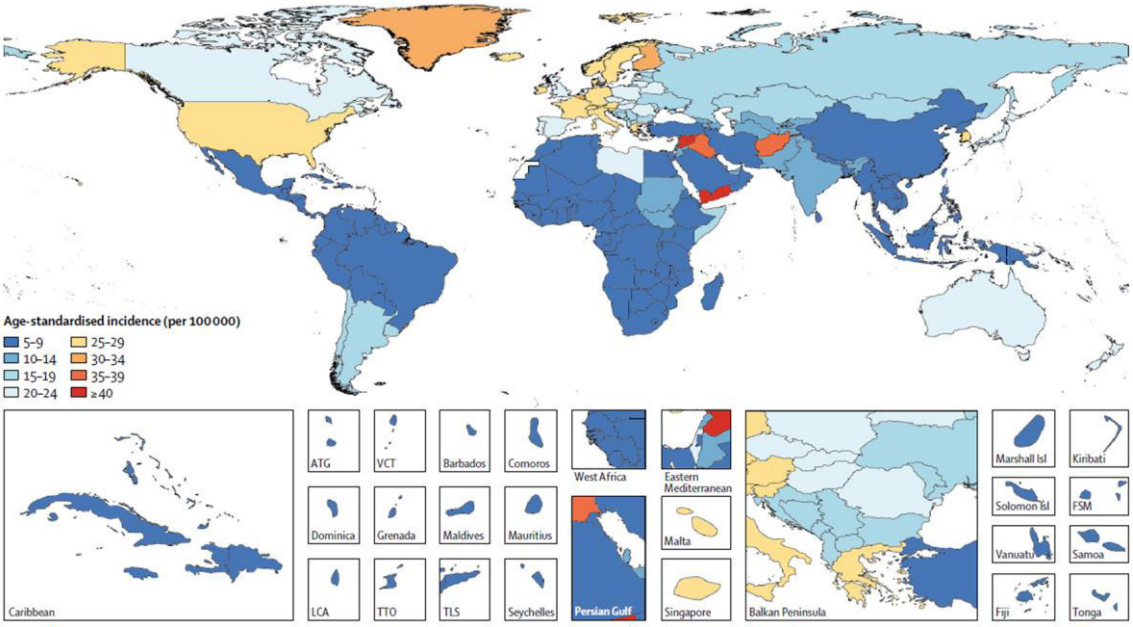

Figure 1.1 – Age standardized incidence of SCI per 100 000 people, by location and for both sexes in 2016. Figure adapted from [25].

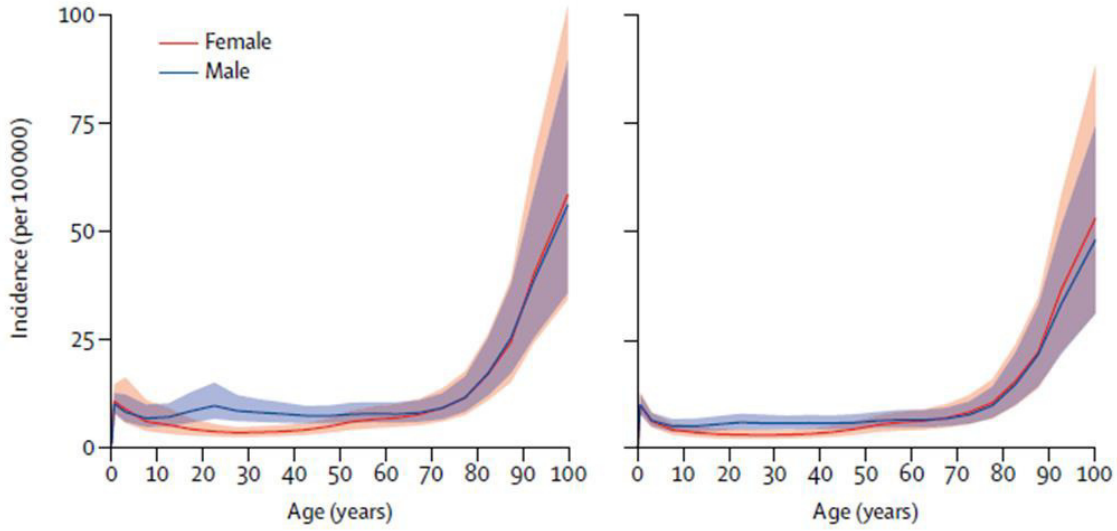

Figure 1.2 – Global incidence of SCI at neck level (left graph) and below neck level (right graph), by age and sex, 2016. Figure adapted from [25].

The case of Portugal

In Portugal there is an evident lack of studies on SCI epidemiology. The most relevant work on this subject was already mentioned above [12] and focuses its analysis on the central region of Portugal. An incidence of 57.8 per million was obtained, divided by two main age peaks, 15-24 and 55-74 years-old groups. There was a predominance of males (3.4:1 ratio) and the majority of the injuries were due to traffic accidents (57.3 %) followed by falls (37.4 %) [12]. Moreover, this study shows a very high mortality rate (56 %) in the initial hours after injury. However, this evaluation was performed during the 1989-1992 period (more than 25 years ago) and the epidemiology of SCI in the central region may not be representative of the country overall SCI population. A more recent and smaller study, focused on neurogenic sexual dysfunction, followed 178 paraplegic patients at the Alcoitão rehabilitation center [26]. 87.1 % of the patients were men, and most of them suffered a trauma-induced SCI (79.2 %).

More recently, a report from Campos et al. (2017) [27] evaluated the Portuguese SCI situation and based on their medical experience, they perceive a reduction in traffic accidents as the main cause, together with a trend to increase in falls and nontraumatic cases, even though they do not possess precise epidemiologic data. On the same line, they noticed that survival after SCI seems to be increasing, with patients living longer, but again no recent data is available. In addition, the same authors discuss the difficulties faced by SCI patients every day, usually observing an unemployment rate higher than the general population, and probably experiencing higher divorce rates, although no data is available. The presence of physical barriers to SCI patients, and people with disability in general, is another major problem. Architectural obstacles in public and private buildings are still common, therefore the implementation of the current legislation regarding this issue needs a closer supervision. Finally, according to the authors, an effort is being made to perform an epidemiological study at the national level, which will reduce the existing knowledge gap about this condition in Portugal.

1.1.2 Pathophysiology of SCI

External consequences

Injuries to the spinal cord resulting from a trauma have very characteristic responses that can vary according to the type of impact. The most common type of lesions to the spinal cord are contusions, compressions or laceration/maceration of the tissue. Depending on the spinal cord level affected, different symptoms and neurological systems can be damaged (Fig. 1.3). Cervical injuries (the most common ones) are the deadliest. According to the severity of the lesion (complete or incomplete injury) they could lead to paralysis of all body below the injury site (tetraplegia), compromising patients' autonomy. Associated with motor problems, different vital systems for body homeostasis can be damaged. Respiratory insufficiency and cardiac problems are the most obvious ones, threatening the survival of SCI patients, but other problems frequently arise from cervical injuries, such as, autonomic dysreflexia, bladder and bowel control. All these alterations strongly decrease patients' quality of life, demanding a constant need for a caretaker. Thoracic and lumbar injuries also lead to relevant motor deficits (paraplegia), associated with little to no control of bowel and bladder functions.

All these symptoms derive from damage to the spinal cord tissue. The adult CNS has a very limited regenerative capacity. Commonly accepted neurogenic niches in the CNS are restricted to the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles [28], although others have been described [29], including the spinal cord [30]. However, neurogenesis in the spinal cord occurs at a low rate [30] and the pathophysiological events after a SCI are a constant process of increased neural degeneration, with spontaneous formation of barriers to regeneration [3].

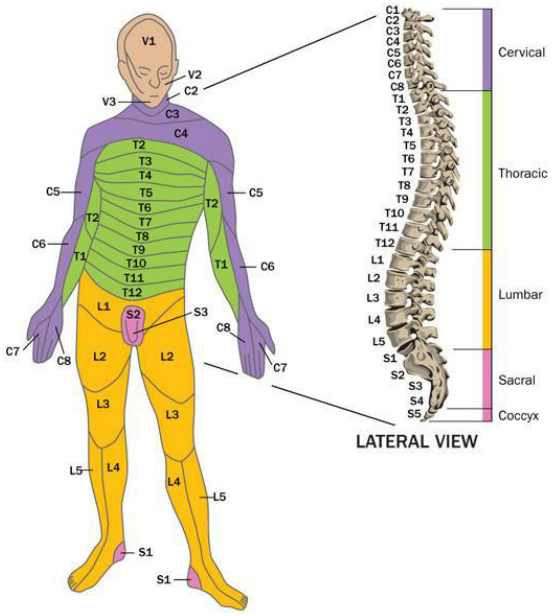

Figure 1.3 – Illustration of the body parts affected after SCI, depending on the site of injury. Image obtained from: http://sci-bc.ca/wp-content/uploads/2011/10/spine-art-affected-areas.jpg

Internal damaging events

Primary injury

The initial impact to the spinal cord causes by itself a great volume of neural death [31]. The nervous tissue is very soft, therefore the physical attrition between solid objects or bone fragments with the spinal cord easily destroys neural cells. Nearby blood vessels are also damaged resulting in blood leakage (hemorrhage) and edema formation [32]. Due to breakage of the blood-spinal cord barrier (BSCB), peripheral inflammatory cells infiltrate the nervous tissue [33]. These initial events occur in the first seconds to minutes after injury, being overall considered the primary injury [3].

Secondary injury

Minutes after injury, in a continuous process, numerous secondary alterations occur leading to an extended neuronal loss. Other vascular changes are initiated such as vasospasm and thrombosis, which leads to ischemia and necrosis [32]. Free radicals are formed, causing oxidative stress to the remaining neurons and reacting with polyunsaturated fatty acids of cellular membranes leading to lipid peroxidation [34]. This event, besides disrupting the normal phospholipid architecture also results in the formation of aldehyde products, which in turn impair

metabolic enzymes such as the Na^+ and K^+-ATPase, responsible for maintaining neuronal excitability. This accumulation of events also produces an ionic imbalance of K^+, Na^+ and Ca^{2+}, depolarizing cell membranes [35]. In addition, one of the most harmful secondary events following SCI is the excessive release of glutamate (along with other amino acids and neurotransmitters), causing an overactivation of glutamate receptors and consequently increasing neuronal death (due to excitotoxicity) [36]. Programmed cell death mechanisms are triggered (apoptosis) and different populations are affected, not only neurons, but also oligodendrocytes, microglia and astrocytes [3]. The death of oligodendrocytes contributes to demyelination, hampering signal conduction [37]. The inflammatory response is another key secondary event following SCI. Resident microglia become activated, peripheral macrophages invade the CNS tissue through the disrupted BSCB and an overall inflammatory state is triggered [33]. Even though, inflammation is a normal response to tissue injury, its chronic activation after trauma is dysfunctional, leading to extended neuronal damage [38]. All these events persist for several weeks, even months, increasing neuronal death over time.

Chronic phase

Several weeks after the injury, there is an increased demyelination of the white matter and dissolution of the grey matter [39]. In addition, the deposition of connective tissue, together with astrogliosis leads to the formation of a glial scar [40]. This is composed by reactive astrocytes, as well as microglia/macrophages and extracellular matrix (ECM) molecules such as chondroitin sulfate proteoglycans (CSPGs) [3]. It acts both as a physical and as a chemical inhibitory barrier to axonal regrowth [41]. Frequently, a cystic cavity is also formed at the lesion epicenter (syringomyelia), further preventing neuronal regeneration [40] (Fig. 1.4).

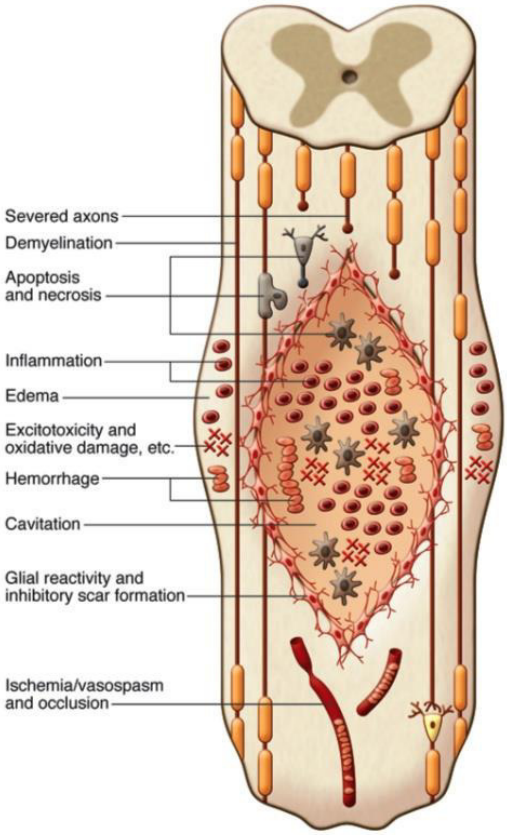

Figure 1.4 – Representation of an injured spinal cord, with the respective pathophysiological events occurring after injury. Image adapted from Mothe *et al.* [31].

1.1.3 Current treatments in the clinics

Despite all the technological improvements in medical equipment for diagnosis and patients' assistance, and despite better post-operative care procedures, there is not one effective therapy for SCI nowadays. The current approach after admission at the hospital, is to stabilize the patient, decompress the spinal cord and probably administer some anti-inflammatory drugs [42]. One of the most widely used for several years was methylprednisolone (MP), a corticoid drug that is known for inhibiting the lipid peroxidation (acting as a free radical scavenger), maintaining the BSCB, enhancing blood flow and limiting the inflammatory response [3]. However, the secondary effects of its use, have many times overcome its potential benefits. Increase in wound infections, gastrointestinal hemorrhages, sepsis, pulmonary embolism and pneumonia are among the main side effects, therefore MP application is still questionable [43].

1.2 Different models of SCI

The lack of novel therapies to SCI led researchers to develop different animal models of injury to test their therapeutic hypotheses. Rodents are by far, the most widely used model to study SCI [44]. The use of mice and rats allows a rapid and relatively cost-effective way of studying different mechanisms of injury, therapeutic-targeted or not [45]. Larger animal models such as cats, dogs or pigs have also been used to test therapies and understand mechanisms, in models more similar to humans [46]. Although less common, rhesus monkeys have also been used, most of the times as a final stage pre-clinical study before moving to human clinical trials, as non-human primates present the most similarities with humans [47, 48]. On a different perspective, smaller vertebrates such as zebra fish [49] or xenopus [50] and salamander [51], have been tested as they present remarkable regenerative properties of the spinal cord, being used mostly as a model to understanding molecular differences to humans and other mammals that lead to their great regenerative potential.

Due to the different characteristics of a traumatic SCI, models of injury have been designed to reflect this variability existing in humans. Cervical, thoracic and lumbar injury models have been tested with different mechanical insults to the spinal cord [52]. Hemisection injuries or total transection are used, even though they are not of great clinical relevance [46]. These models allow a precise study of axonal regeneration, since the lesion produced is very precise. On the other hand, compression and contusion injury models are crucial, since they do mimic very closely what happens in the clinics, for instance the formation of a glial scar or a cystic cavity [53]. Therefore, the right animal and injury model for each study must be chosen in accordance to the scientific question of interest.

1.3 Cell therapies

The transplantation of cells to treat a pathology, either isolated from the own individual (autologous) or from another person (allogeneic) is a reality for some diseases, mostly leukemias and lymphomas [54]. The rationale behind such approach has been for many years, to replace the damaged cell population or injured tissues. In CNS applications, one of the first experiments was done by Jorge Francisco Tello from Ramon y Cajal's lab, attempting to transplant pre-degenerated peripheral nerve segments onto proximal stumps of severed optic nerves [55].

Amazingly, retinal axons could grow into these transplanted nerves. Similarly, in the early 1980s, this observation was again put to the test, but this time in a SCI context. Richardson et al. (1980) [56] demonstrated that severed spinal cord axons could regrow onto autologous peripheral nerve grafts (from sciatic nerve), and in some rats even further to the caudal stumps of 10 mm gap injuries. In addition, after performing a peripheral graft bridge between the medulla oblongata and the spinal cord, some CNS axons could grow more than 30 mm into the grafts [57].

These experiments were made with the rationale of understanding the reason why peripheral nervous system (PNS) neurons have a regenerative capacity in opposition to CNS neurons. One of the main hypotheses was that the environment influenced neuronal regrowth, explaining why CNS neurons could easily grow into peripheral nerve grafts. Another, and a quite relevant one, was the presence of Schwann cells (SCs) in the PNS. Thus, even before Richardson, David and Aguayo's experiments, Richard Bunge and others have explored the properties of SCs *in vitro*, with the final objective of using these cells as a potential therapeutic strategy to CNS repair [58, 59]. In this case, the transplanted SCs would not serve as a replacement for neuronal populations, but as a supportive substrate for inducing endogenous neuronal repair.

Based on this dichotomy of purposes (cell replacement vs cell supportive), different cell populations have been proposed as candidates for SCI repair. Neural stem cells (NSCs), embryonic stem cells (ESCs) and more recently induced pluripotent stem cells (iPSCs) have been applied with the main objective of replacing lost neural cells, while SCs, olfactory ensheathing cells (OEC) or mesenchymal stem cells (MSCs) have been mostly used as a strategy to support endogenous neuronal regrowth.

1.3.1 NSCs, ESCs and iPSCs

The concept of stem cells can be traced back to the end of the 19th century [60]. At the time, it started as a theoretical postulate, trying to explain why some tissues had the capacity to self-renew, such as blood or skin for example.

ESCs

In 1981, Martin Evans and Kaufman were the firsts to successfully culture embryonic pluripotent cells from mouse blastocysts [61]. This breakthrough experiment opened a huge door to gene targeted therapies but also for the potential application of these cells to numerous degenerative diseases. Later in 1998, the first human ESCs were successfully cultured, maintaining proliferation and differentiation potential *in vitro* for several months [62].

The ability of ESCs to differentiate into neural and glial cells in *in vitro* culture systems has been extensively explored using different strategies. Retinoic acid- and embryoid body-based protocols have been used to induce neural differentiation of ESCs in culture, resulting in the activation of a complex system of neuronal gene expression provided by neuronal-like cells [63] and in the production of oligodendrocytes, capable of producing myelin for the myelination of neurons in culture [64]. Another approach, consisting in the use of specific factors in mouse ESCs culture, was found to efficiently direct cell differentiation into dopaminergic and serotonergic neurons [65, 66]. The use of cell culture media specifically defined for ESCs commitment to the neural fate is also an alternative method [67]. Of particular interest is the possibility to genetically modify the ESCs, in order to obtain neuronal precursors-enriched cultures [68].

The suitability of ESCs-based approaches for SCI treatment has also been investigated in a number of SCI models. Keirstead et al. [69] transplanted NSCs obtained from mouse ESCs into a rat spinal cord, after an induced thoracic SCI. Most transplanted cells survived, migrated away from the injury site, and were shown to preferentially differentiate into oligodendrocytes and astrocytes. Still, induced ESC-derived oligodendrocyte progenitor cells transplanted into demyelinated spinal cords were found to contribute to the remyelination of host axons. Moreover, cells transplantation also led to functional improvements [64]. Finally, ESC clinical applications in SCI patients started through a Phase I clinical trial provided by Geron's company in 2011. A cohort of patients with complete subacute thoracic SCI was transplanted with pre-differentiated oligodendrocyte precursor cells derived from human ESCs for safety studies. Unfortunately, Geron's program was aborted later in that year [70]. Nevertheless, to date no safety issues were reported in five patients submitted to ESCs transplants.

iPSCs

Recently, another type of pluripotent stem cells, known as induced pluripotent stem cells (iPS cells or iPSCs), emerged as a possible alternative to obtain stem cells directly from adult tissues for autologous transplantation. The iPSCs technology resulted from a pioneer work developed by Yamanaka's lab in Japan in 2006, which showed that the introduction of four transcription factors reverted the phenotype of differentiated adult cells into pluripotent stem cells [71]. iPSCs are often compared to ESCs, as they share similar characteristics, such as pluripotency, self-renewal capacity, and gene expression [72]. Moreover, the potential to acquire abnormal karyotypes and genetic amplification associated with teratoma formation is also a common feature between the two cell types [73]. However, iPSCs differentiation into neural lineages occurs at a lower frequency than for ESCs [74].

The fact that iPSCs can be derived directly from adult tissues offers an unlimited supply of autologous cells, which could be used to generate transplants without the risk of immune rejection. However, safety issues such as those related to tumor formation should be determined prior to their clinical application. Therefore, it is crucial to carefully test iPSCs for tumorigenicity [75].

A preclinical study investigated the therapeutic potential of transplanting pre-evaluated neural stem/progenitor cell (NSPC) clones derived from murine and human iPSCs into a nonhuman primate model of contusive SCI [76]. Similarly to previous studies, the grafted cells were found to survive and differentiate into neurons, astrocytes, and oligodendrocytes, without evidence of tumor formation. In addition, there was an enhancement in axonal sparing/regrowth and angiogenesis at the lesion site and the prevention of the lesion epicenter demyelination. At the end of the treatment, a functional recovery of the animal after SCI was observed. Nevertheless, more preclinical studies have yet to be performed, in order to investigate the true potential and safety of iPSCs, before moving to a clinical setting.

NSCs

Another cell population with a possible interest for SCI research are adult multipotent NSCs, which are particularly appealing due to their CNS origin. These cells have been shown to generate the three main neural cell lineages of the mammalian CNS in culture [77]. Thus, they

can hypothetically allow the replacement of spinal neurons lost after injury and differentiate towards astrocytes, to restore the nonneuronal milieu of the pre-injured spinal cord, or towards oligodendroglia, to allow remyelination [78]. In fact, previous studies have confirmed this theory.

In a cervical contusion-induced SCI in primates, *in vitro*-expanded human neural stem/progenitor cells (NSPCs) were grafted nine days after injury and were shown to survive and differentiate into the neural lineages. In addition, there was a decrease in the injury cavities extent, as well as a significant increase of the spontaneous motor activity of the transplanted animals [79]. Furthermore, demyelinated axons in immunodeficient mice with traumatic SCI were remyelinated after transplantation of human CNS stem cells (derived from fetal brain) grown in neurospheres. These cells also differentiated into neurons that exhibited the ability of synapse formation with host neurons [80].

The experimental ground work regarding NSCs as cellular-based therapy has shown promise in repairing damaged cells and tissues after SCI and ultimately led to the attempt of applying this therapy to humans. In line with this, Stem Cells Inc. Company (Switzerland) established the world's first clinical trial in spinal cord injured humans using these cells [81]. In 2011, the company initiated a Phase I/II clinical trial designed to assess both safety and preliminary efficacy of a single transplantation of purified fetal human neural stem cells, as a treatment for chronic thoracic SCI, for both complete and incomplete injuries. The study enrolled seven patients with complete injuries [American Spinal Injury Association (ASIA) Impairment Scale A – AIS A) and five patients with incomplete injuries (AIS B). The cells were directly injected into their spinal cords, and they were temporarily immunosuppressed. Clinical updates were reported on a total of eight of the twelve patients enrolled in the clinical trial. With regards to AIS A patients, there was significant posttransplant gain in sensory function in four patients up to date. Concerning AIS B subjects, two of three patients had significant gain in sensory perception, the third remaining unaltered [81]. Interestingly, most of these improvements were lost once the immunosuppression regiments were interrupted (non-published data). This highlights the relevance of autologous transplantation in SCI context.

1.3.2 Glial Cells

Schwann Cells (SCs)

As mentioned above, SCs have been studied in SCI research for decades [82-84]. SCs are glial cells with a myelinating function, only present in the peripheral nervous system, where spontaneous regeneration occurs. There, SCs are known to surround peripheral axons with a spirally wrapped myelin sheath, leading to increased signal conduction [85].

Applying SCs to treat the damaged spinal cord appeared to be logic, therefore the use of dissociated SCs in SCI pre-clinical models became frequent and often with promising results [82, 84]. One of the first studies using SC cultures to transplant to the injured CNS was performed by Kromer and Cornbrooks (1985) [86], moving away from experiments using complete peripheral nerve grafts [57].

Mary Bartlet Bunge's group from The Miami Project to Cure Paralysis has been working with SCs for SCI repair for several years now. The first preliminary data came in 1991, with Paino and Bunge (1991) [87] showing that SCs enclosed in collagen rolls led to axonal ingrowth in SCI-rats, similar to what was seen before with peripheral nerve grafts. In another study, two million SCs transplanted at the injury site, one week after a moderate midthoracic contusion (T9) led to increased spared tissue, higher numbers of myelinated axons and also more brainstem and propriospinal axons reaching longer distances beyond the graft [88]. This translated into function, resulted in modest, but significant improvements in motor function [88]. Later, Barakat et al. (2005) [89] transplanted SC cellular grafts into chronically-injured rats (T8 contusion; 8 weeks after injury). The survival of SC grafts was 17.1 % and in addition, SC grafts supported the ingrowth of numerous neurofilament (NF)-positive axons within the injury site, besides the growth of several ascending and descending fibers [89]. Once again, SCs led to significant improvements in motor function.

There have been works, differentiating other cells into SCs, broadening the availability of cell sources. In a very interesting work, Biernaskie and Sparling et al. (2007) [90], using a thoracic rat SCI model, demonstrated that mouse skin-derived precursors (SKPs) that were differentiated towards SCs were more effective in bridging the lesion site, myelinating spared axons or enhancing locomotor recovery than the same non-differentiated SKPs or neurospheres from the forebrain subventricular zone. More recently, the same authors demonstrated that both

rat SKP-derived or nerve-derived SCs promoted neural repair and functional recovery after incomplete cervical spinal cord crush injury [91].

SCs have also been genetically modified to express and secrete neurotrophic factors. Menei et al. (1998) [92] showed that brain-derived neurotrophic factor (BDNF)-secreting SCs enhanced axonal regrowth following SCI transection in the rat.

SC transplantation has also been coupled with other therapeutic strategies. Pearse et al. (2004) [93], again from Mary Bartlet Bunges's group, combined SC transplantation with cyclic adenosine monophosphate (cAMP) modulation treatment, in SCI rats. Using rolipram to inhibit cAMP hydrolysis, in combination with SC grafts, they successfully prevented the decrease of cAMP levels after SCI, promoting at the same time axonal sparing and myelination. By further adding an injection of dibutyryl-cAMP near the graft, they could significantly enhance axonal sparing and myelination and also improving locomotion. A recent study, tried to combine SC transplantation with macrophage depletion following contusive SCI in rats, observing a reduced cystic cavity size and lesion volume, in comparison to SC transplants alone [94].

Overall, these studies demonstrated interesting positive effects of SC transplants. The proposed mechanisms of action include neuroprotection, axon regeneration and myelination. All these positive pre-clinical results led to a recent clinical trial [95]. The Miami Project to Cure Paralysis team tested autologous transplants of SCs from the sural nerve in subacute SCI patients (four to seven weeks after injury). After transplantation into the injury epicenter in a total of six subjects, there were no adverse events and no evidence of additional spinal cord damage, mass lesion or syrinx formation, up to one year post-transplantation. These results show that SCs transplantation can be safe and further studies must be pursued.

Olfactory Ensheathing Cells (OECs)

Olfactory ensheathing cells (OECs) are a special type of cells present in the olfactory system, known for ensheathing and surrounding axons from olfactory neurons. They are specifically located in the lamina propria (LP) of the olfactory mucosa (at the PNS) and in the external layer of the olfactory bulb, the olfactory nerve layer (ONL, at the CNS, Fig. 1.5) [96]. They are glial cells that support the growth of olfactory receptor neurons in the olfactory bulb [97] and participate in the continuous renewal of neurons in that system [96]. More specifically, OECs communicate with olfactory axons by evoking calcium signals via glutamatergic and purinergic

pathways [98]. OECs' cytoplasmic projections envelop packages of axons, acting as insulators, avoiding contact with CNS inhibitory molecules [96].

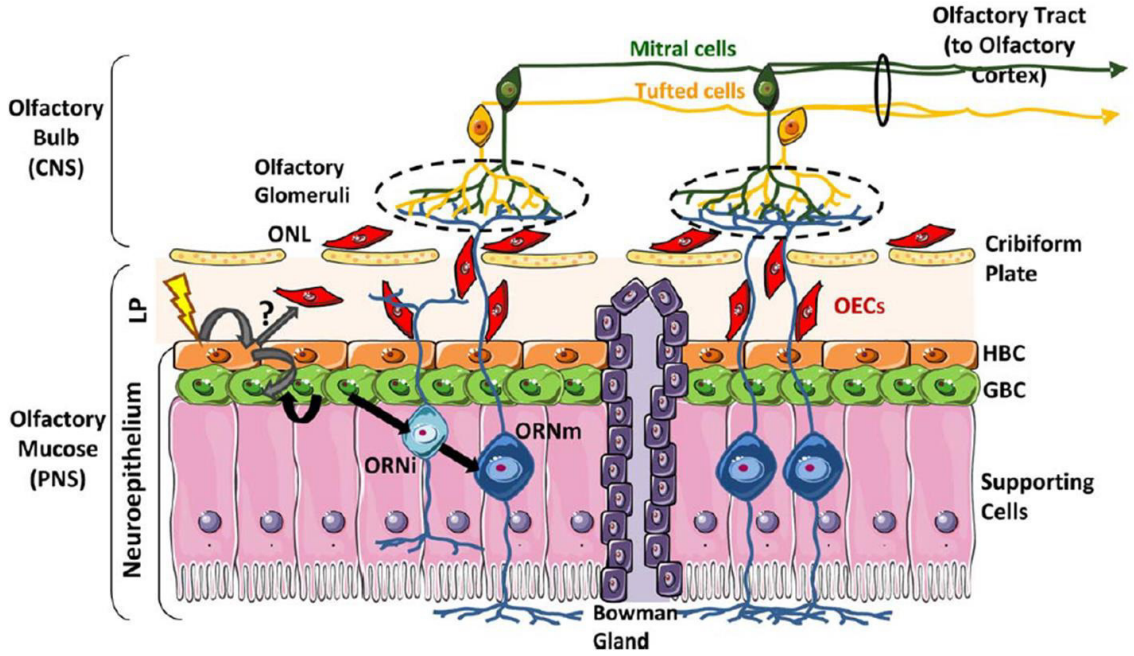

Figure 1.5 – Diagram illustrating the transition between the peripheral and central nervous system, in the olfactory system where OECs reside. OECs (in red) are located in the LP of the olfactory mucosa (PNS) and in the outer layer of the olfactory bulb (CNS). Figure adapted from Gómez et al. [96].

Based on cellular morphology and expression of different markers, it is believed that different OEC subpopulations exist. For example, all OECs in the ONL express S100β and glial fibrillary acidic protein (GFAP) markers, but the ones sitting in the inner layer express neuropeptide Y, while the ones in the outer layer express the low-affinity nerve growth factor receptor (p75) and neural cell adhesion molecule (E-NCAM) [96].

However, it is difficult to draw a clear separation of OEC populations, because the same cell can change its markers' expression [96]. Moreover, OECs are migratory cells and their morphology and gene expression might differ, in response to the environment [96]. *In vitro* cultures can be used as a model to understand this phenomenon, although the conclusions are limited due to the different stimulus that OECs are subjected to, in comparison to the *in vivo* scenario. Isolated OECs *in vitro* may assume predominantly two phenotypes: i) astrocyte-like in shape, with low or no expression of p75; ii) SCs-like morphology, with high expression of p75.

Work from Doucette [99, 100] was fundamental to identify OECs as a distinct type of glial cell present in the olfactory system, and also to characterize their importance in that

environment. The discovery of their role on neuronal growth led researchers to hypothesize their potential beneficial application to other CNS lesions.

Ramón-Cueto and colleagues were pioneers in the use of these cells for CNS applications. They started to characterize these cells in 1992, using cultures of rat olfactory bulbs and olfactory nerves [101]. At the time, the term olfactory ensheathing glia was often applied. Two years later, the same authors transplanted OECs into rhizotomized spinal cords at lower thoracic level (T10 dorsal root) [102]. Three weeks after transplantation, it was observed the growth of dorsal root axons into the spinal cord, which did not happen in non-transplanted rats. Regarding SCI applications, Li et al. (1997) [103] published one of the first works describing the transplantation of OECs, which led to an improved regenerating capacity of corticospinal tract (CST) fibers following a unilateral cervical injury in rats. The transplant also led to improved forepaw reaching skills in treated rats. Later, Ramón-Cueto et al. (1998) [104] tested OECs transplantation coupled with channels (made of synthetic polymers) filled with SCs, after a T9 transection injury in rats. Six weeks after injury, numerous NF-, growth associated protein 43 (GAP43)-, calcitonin gene related peptide (CGRP)- and serotonin-positive fibers traversed the glial scars previously formed. Interestingly, the growth of serotonin fibers avoided the interior of the channels, growing instead supported by connective tissue bridges formed on the exterior of the channels. Importantly, they verified that OECs migrated after transplantation, overcoming traditionally inhibitory factors such as the glial scar, and were present in the same locations of regenerated axons. The following years witnessed a great volume of studies using OECs as a tool for SCI repair (table 1). Either transplanted immediately following injury [105] or at a chronic stage [106], applied into transection or contusion injuries at different spinal levels [107, 108], transplants of OECs have shown a regenerative potential and the capacity to induce functional recovery in several cases. Moreover, OECs isolated from both adult and young animals have been used [109, 110], and obtained from two distinct regions: the lamina propria (from the olfactory mucosa) and the outer layers of the olfactory bulb. Overall both types of OECs are capable of inducing beneficial effects in SCI models [111, 112]. From a translational point of view, important studies have used OECs from other species other than rodents, including dogs [113], pigs [114] or human cells [115]. In this last work the authors demonstrated that OECs transplanted one-week post-injury reduced lesion volume and GFAP intensity, while improving hindlimb function.

There are several mechanisms associated with OECs' effects. For instance, OECs interact with astrocytes and meningeal cells, being able to grow with astrocytes in culture and in the glial scar, which is an advantage in comparison in SCs [116]. Some studies even report that OECs reduce astrocytic reactivity and CSPGs expression [117]. OECs can also produce ECM proteases, which might help in their migratory capacity within the nervous tissue [118].

Table 1.1 – Most relevant studies using OECs as a cellular therapy in animal models of traumatic SCI.

Model	Injury	Olfactory Ensheathing Cells						Main outcomes	Reference
		Source	Zone	DIV purification	No. Cells	Administration	Timing		
Rat	C1-C2 cervical hemisection (focal electrolytic lesion)	Adult rat	Olfactory bulb (outer nerve and glomerular layer)	14-17 days No purification	N/A	Intraspinal (lesion site)	Immediately	CST axons grew through the transplant into the denervated caudal host tract; directed forepaw reaching on the lesioned side	[103]
	C1-C2 cervical hemisection (focal electrolytic lesion)		Olfactory bulb (outer nerve and glomerular layer)	14-17 days No purification	1 × 10^5	Intraspinal (lesion site)	Immediately	3 wpi, regenerating axons are ensheathed by peripheral myelin, and reenter the caudal part of the host CST; OECs form a "bridge" over the lesion site	[119]
	C1-C2 cervical hemisection (focal electrolytic lesion)		Olfactory bulb (outer nerve and glomerular layer)	14-17 days No purification	1.25 × 10^5	Intraspinal (into the lesion site)	8 wpi	Regenerating CST axons crossed the OECs bridge and entered the host distal spinal cord, forming terminal arborizations in the gray matter; OEC transplants bridging the lesion site were associated with forepaw reaching abilities	[106]
	C1-C2 CST unilateral lesion (focal electrolytic lesion)		Olfactory mucosa (lamina propria)	14 days No purification	1 × 10^5	Intraspinal (3 injections, at lesion site)	8 wpi	No evidence of CST axonal growth across the lesion; minimal migratory ability of OECs; no formation of bridges across the lesion; restored direct forepaw retrieval	[120]

Model	Injury	Olfactory Ensheathing Cells						Main outcomes	Reference
		Source	Zone	DIV purification	No. Cells	Administration	Timing		
Rat	C2 hemi-contusion	Adult rat	Olfactory mucosa (lamina propria)	N/A NT-3 supplemented cultures	3 × 10^5	Intraspinal (rostral, center and caudal to the injury – x2)	2 wpi	Increased axonal sprouting in the injury site; improved breathing movements, diaphragm and phrenic nerve activity;	[107]
	T7-T8 transection		Olfactory bulb (first two layers)	12-14 days Purified by immunoaffinity (p75 antibody)	4 × 10^5	Intraspinal (4x rostral and caudal to the injury site)	Immediately	More NF+ axons at injury site and more 5-HT axons crossing the rostral scar border; axonal connectivity across the injury; no changes in motor behavior	[105]
	T8 transection		Olfactory bulb (olfactory nerve layer) Modified to secrete GDNF	P6-P7 OECs Purified by serial adhesion (2x 12h-periods and immunoaffinity (p75 antibody)	4 × 10^5	Intraspinal rostral and caudal (4 injection sites each)	Immediately	OECs increased GDNF expression *in vivo* (qPCR); survived 2 months post-transplantation; increased NF-positive fibers; functional recovery (BBB scores and IP test)	[108]
	T8-T9 transection		Olfactory bulb (outer nerve and glomerular layer)	15 days Purified by immunoaffinity (p75 antibody)	4 × 10^5	Intraspinal rostral and caudal (4 injection sites each)	Immediately	Regeneration of motor axons for long distances within caudal cord stumps; Recovered motor functions and sensorimotor reflexes	[109]
	T8-T9 transection		Olfactory bulb	Purified by immunoaffinity (p75 antibody)	4 × 10^5	Intraspinal (4x rostral and caudal)	Immediately	Improved stepping ability and use of hindlimbs during climbing; recovery of MEP	[121]

Model	Injury	Olfactory Ensheathing Cells						Main outcomes	Reference
		Source	Zone	DIV purification	No. Cells	Administration	Timing		
Rat	T9-T10 transection	Adult rat	Olfactory bulb (first two layers)	12-14 days Purified by immunoaffinity (p75 antibody)	4 × 10^5	Intraspinal (4x rostral and caudal to the injury site)	Immediately	OECs survive longer than fibroblast transplants; preserve axons and neurons; reduce inhibitory molecules; limit immune cells activation and infiltration	[122]
	T9-T10 contusion		Olfactory bulb (nerve layer)	> 9 days Purified by immunoaffinity (p75 antibody)	2 × 10^6	Intraspinal (lesion epicenter)	30 mpi or 1 wpi	Grafts filled the lesion site, reducing cavitation; promoted tissue sparing; increased serotonergic innervation; spared supraspinal axons; improved hindlimb performance (BBB scores)	[123]
	T10 transection		Olfactory mucosa (lamina propria)	0 days No purification	Small pieces of lamina propria tissue	At lesion gap	Immediately	Regeneration of serotonin-positive fibers; retrograde labeling of brainstem raphe gigantocellularis neurons; recovered movement of hindlimbs and joints; recovered spinal reflex circuitry	[124]
	T10 transection		Olfactory mucosa (lamina propria)	0 days No purification	Small pieces of lamina propria tissue	At lesion gap	4 wpi	Axonal regrowth across the transplant site; improved BBB scores	[111]

Model	Injury	Olfactory Ensheathing Cells						Main outcomes	Reference
		Source	Zone	DIV purification	No. Cells	Administration	Timing		
Rat	T8 transection	Postanal rats (P22-P33)	Olfactory bulb	7-9 days Purified by immunoaffinity (p75 antibody) Thawed 2h before transplant	1.5 × 10^6	Intraspinal (2 injections rostral and 2 caudal to the injury)	Immediately or 1 wpi	Long axonal outgrowth; reduced GFAP and NG2 expression (IHC); Returned MEPs; reduced hindlimb hyperreflexia; recovered movements of hindlimb joints (BBB scores); Earlier recovery, better functional and histological results with acute OECs transplants	[110]
	T8 transection		Olfactory bulb	7-9 days Purified by immunoaffinity (p75 antibody) Thawed 2h before transplant	1.5 × 10^6	Intraspinal (2 injections rostral and 2 caudal to the injury)	6 wpi	Regrowth of CST and raphespinal axons; no reduced astrogliosis; Partial recovery of MEPs and hindlimb movements (BBB scores); reduced lumbar reflex hyperexcitability;	[125]
	C3-C4 Dorsolateral funiculus crush	Neonatal mice (P5)	Olfactory bulb (nerve fiber layer) or mucosa (lamina propria)	14-16 days Purified by immunoaffinity (p75 antibody) or eliminating fibroblasts by specific lysis (2 times)	7.5-9.0 × 10^4	Intraspinal into lesion site (3x at different depths) or Rostral and caudal (2 injection sites each)	Immediately	Both types of OECs attenuate lesion and cavity formation, promote angiogenesis, SCs infiltration and axonal sprouting; LP-OECs migrate more, reduce cavity formation and lesion size and differentially stimulate axonal subpopulations outgrowth; LP-OECs increase autotomy behaviors	[112]

Model	Injury	Olfactory Ensheathing Cells						Main outcomes	Reference
		Source	Zone	DIV purification	No. Cells	Administration	Timing		
Rat	C4 dorsal column crush (CST lesion)	Dog	Olfactory mucosa	N/A Genetically modified to secrete ChABC	8 × 10^4	Intraspinal (rostral and caudal to the injury site)	Immediately	OECs survived and digested CSPGs; more CST axons rostral and caudal to the injury	[113]
	T11 transected dorsal columns	Pig	Olfactory bulb	0 days (freshly isolated cells) No purification	6 × 10^4	Intraspinal Rostral and caudal to the injury	Immediately	Transplanted cells migrated into the denervated host tract; axons thickly ensheathed by peripheral-like myelin; restored impulse conduction across and beyond the lesion; faster conduction in regenerated axons than normal axons	[114]
	T10 contusion	Human	Olfactory mucosa biopsies (lamina propria)	N/A Cultured in NT-3 Frozen and thawed	1 × 10^6	Intraspinal (at injury site, rostral and caudal)	1 wpi	Reduced lesion and cavities volume; reduced GFAP intensity adjacent to the lesion; improved hindlimb movements (BBB scale and HR walking test)	[115]
Dog (clinical setting)	T10-L4 chronic injuries	Autologous transplants	Olfactory mucosa	3-5 weeks Purified by differential trypsinization and mechanical detachment	~5 × 10^6	Intraspinal (rostral, center, and caudal to the injury)	~3 mopi	Better fore-hind coordination; no differences in measures of long tract functionality	[126]

Abbreviations: 5-HT – 5-hydroxytryptamine; BBB – Basso, Beattie and Bresnahan test; ChABC – chondroitinase ABC; DIV – days *in vitro*; GDNF – glial cell line-derived neurotrophic factor; HR – horizontal rung; IHC – immunohistochemistry; IP – inclined plane; MEPs – motor evoked potentials; mpi – minutes post-injury; mopi – months post-injury; N/A – information non-available; NG2 – neural/glia antigen 2; NT-3 – neurotrophin 3; qPCR – quantitative polymerase chain reaction; wpi – weeks post-injury.

The relevance of pre-clinical data led several groups to attempt OECs transplantation into patients. Lima et al. (2006) [127] transplanted olfactory mucosa autografts into seven chronic SCI patients, demonstrating that the procedure was feasible and relatively safe. Later, Mackay-Sim et al. (2008) [128] also demonstrated that autologous transplantation of mucosal OECs was safe, free of serious adverse effects after a three-year follow-up, even though no significant improvements were seen in transplanted patients. More recently, Tabakow et al. (2013) [129] obtained similar results and very interestingly, published a clinical case of OECs transplantation from the olfactory bulbs, which resulted in significant recovery of function [130].

1.3.3 Mesenchymal Stem Cells

The term mesenchymal stem cell (MSC) was used for the first time by Caplan et al. (1991) [131] but these cells were already well described, particularly by the work of Friedenstein and colleagues in the 1960s and 1970s [132, 133]. However, before the recognition of the MSCs' existence, some experiments dating back to the 19th century showed that transplants of bone marrow could induce ectopic bone formation in other regions of the body, suggesting an important role for bone marrow in osteogenesis. That role was later confirmed by Tavassoli and Crosby (1968) [134]. Friedenstein's work helped to clarify that a subset of cells from the bone marrow stroma was responsible for the osteogenic potential [132]. Moreover, he and his colleagues showed that these cells could be distinguished from hematopoietic cells due to their adherence to surfaces, fibroblast-like morphology and capacity to form colonies from a single cell [133]. Later, Friedenstein named these cells as osteogenic stem cells [135], or bone marrow stromal stem cells [136]. As abovementioned, Caplan suggested the name mesenchymal stem cell, a term which is still today under debate [60]. The essential characteristics of these cells were later defined by Dominici et al. (2006) [137]. In a statement of the International Society for Cellular Therapy, where the word "stem" was replaced for "stromal" to highlight the origin of these cells, it was defined that MSCs should: 1) be plastic-adherent; 2) express the clusters of differentiation (CD) CD105, CD73 and CD90, lacking the expression of CD45, CD34, CD14 or CD11b, CD79-α or CD19 and human leukocyte antigen DR isotype (HLA-DR) surface markers; and 3) differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts [137]. Based on these criteria, MSC-like cells have been identified in numerous other tissue sources besides bone marrow, including the adipose tissue [138], the umbilical cord [139], skeletal muscles [140],

dental tissues [141], synovial fluid [142], palatine tonsil [143], parathyroid gland [144], fallopian tube [145], brain [146], placenta [147], endometrial tissues [148] and uterine cervix [149].

Despite all these sources, bone marrow-derived MSCs (BM-MSCs) are the most studied and their potential application to SCI has been addressed for many years. One of the first studies was performed by Chopp et al. (2000) [150] demonstrating that rat-derived BM-MSCs transplanted into the lesion epicenter, one week after a contusion SCI led to functional improvements of injured rats, as assessed by the Basso, Beattie, Bresnahan (BBB) locomotor test [150]. Others had similar results [151] and the main mechanisms seem to be related with neuroprotection, immunomodulation and axonal regeneration and sprouting. Nevertheless, alternatives sources to the bone marrow are needed, since BM-MSCs isolation is still a painful, invasive procedure, accompanied by a risk of infection [152]. Adipose tissue-derived stem/stromal cells (ASCs) emerge as a potential candidate for SCI therapy, as they can be obtained in larger quantities in comparison to BM-MSCs and are equally suited for biomedical applications [153].

ASCs

The existence of cells with multipotent differentiation potential in the adipose tissue was suspected following some pathological observations such as osseous heteroplasia, where ectopic bone formation occurs within adipose tissue layers [154]. The histological analysis of these tissues revealed the presence of chondrocytes and osteoblasts, besides adipocytes [155]. In this sense, many authors started to explore the potentialities of this cell population, which later confirmed to fulfill the criteria defined for MSCs [137]. Adipose-derived Stem or Stromal Cells (ASCs), as they are mostly recognized, are responsible for replacing approximately 10 % of mature adipocytes that turn over each year [156]. However, they have many other properties, that can be useful when applied to neurodegenerative diseases.

ASCs are very attractive for regenerative medicine applications, mostly because while maintaining most of the characteristics of MSCs from the bone marrow, they can be obtained from an accessible source, in a relatively simple procedure that can be repeated over time. Besides their multipotent potential, ASCs are known for their paracrine activity, secreting several growth factors, cytokines, ECM molecules, among others [157]. ASCs have immunomodulatory

properties and tend to migrate into injured tissues (homing). All these properties make ASCs a possible solution for different medical conditions (Fig. 1.6).

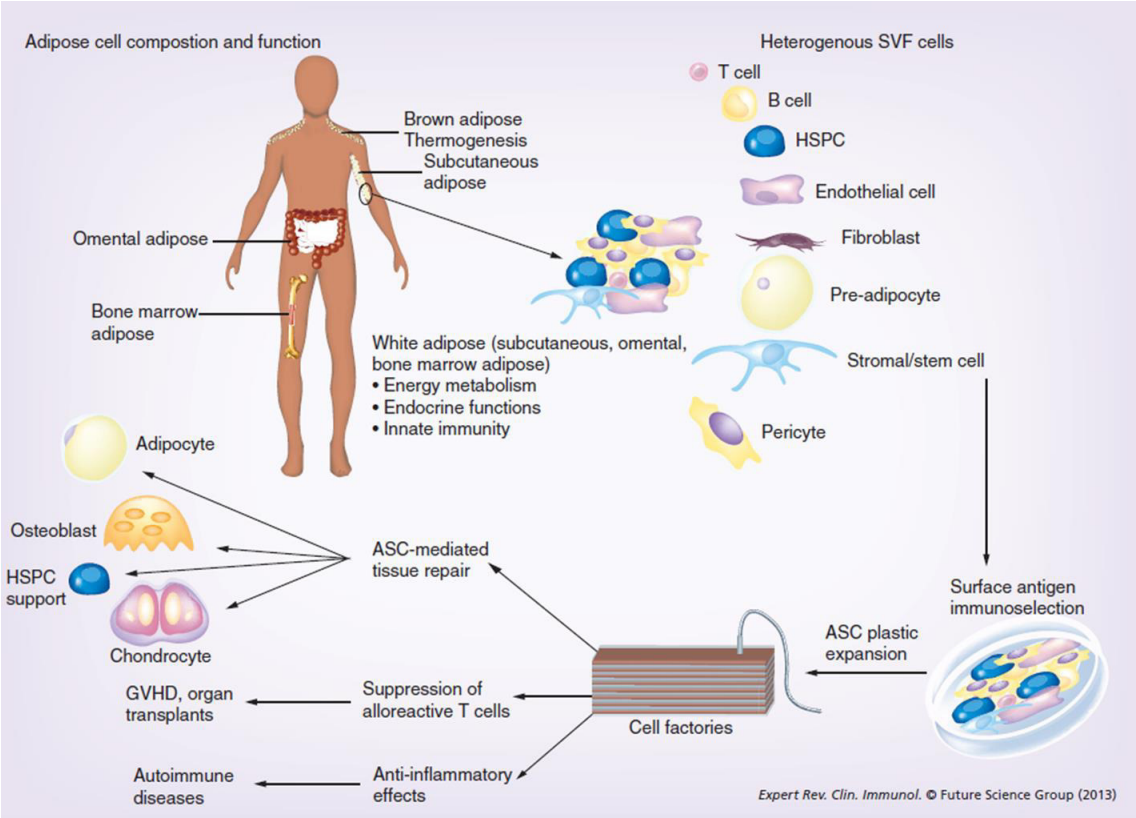

Figure 1.6 – Schematic representation of ASC sources, cell composition of adipose tissue, together with properties and possible applications of ASCs. Image adapted from McIntosh et al. [158].

Wound healing [159], cardiac disease [160] or ischemic stroke [161] are some of the conditions where ASCs have been most widely used. The capacity of ASCs to modulate the environment, rather than their differentiation properties have been associated to the positive results observed. The application to neural disorders has also been addressed, such as Alzheimer's disease [162] among others.

One of the first works describing the transplantation of ASCs into a SCI model was performed by Kang and colleagues, in 2006 [163]. In this study, the authors tried to differentiate rat ASCs towards oligodendrocyte progenitor cells (OPCs), before transplantation. In fact, ASCs increased the expression of platelet-derived growth factor receptor α (PDGFRα), an OPCs-associated marker. Then, three days after a complete CST transection at T9-T10 level, two million ASCs-OPCs were autologously injected through the tail vein. At four weeks post-injury, injected cells survived and migrated preferentially to the injured spinal cord, although they could

also be found in other organs such as the brain, liver, kidneys or lungs. More importantly, transplanted rats demonstrated improved locomotor functions, as assessed by the BBB test. Using a different approach, the same authors isolated cytoplasmic extracts of rat ASCs and transplanted them directly into the spinal cord, immediately after injury [164]. This resulted in significant suppression of apoptotic death and less astrogliosis, together with improved hindlimb function. The content of the extracts was not determined, however it seemed to influence apoptotic mediators, by reducing caspase-3 and Bcl2-associated X protein (Bax), while increasing B-cell lymphoma 2 (Bcl2) expression.

The main logic behind ASCs transplantation into SCI was, at the beginning, to find an alternative stem cell population that could replace damaged neurons and glial cells. However, as seen in other disease models, the main mechanism of action of ASCs does not seem to be related with neural differentiation, but rather with a general support and protection of endogenous cells. An interesting study by Zhang and colleagues (2009), pointed in that direction [165]. Even though different neural differentiation protocols were implemented to ASCs before transplantation, the fact was that undifferentiated ASCs induced similar recovery of locomotor functions and identical number of NF positive cells, in comparison to ASCs submitted to differentiation protocols, following a thoracic contusion injury in rats. Similar findings were reported by Arboleda et al. (2011) [166]. In this study, undifferentiated or ASCs submitted to sphere-differentiation protocols were transplanted into a rat model of SCI compression, one-week post-injury. Functional improvements were observed in both groups, indicating a possible paracrine mechanism for the effects observed, rather than differentiation.

Even though BM-MSCs are still the gold standard and the most widely used MSCs for different applications, there is a considerable number of studies using ASCs as a therapy for SCI (table 2). Both rat and human-derived ASCs have been addressed in different rodent models, from transection to contusion injuries, mostly at the thoracic region but some also at cervical level [167]. ASCs have been mostly administered intraspinally, but also intrathecally and intravenously with positive results [168, 169], which in this last case highlights the homing capacity of these cells to injured areas. Cells are usually transplanted one week after injury in order to avoid the aggressive environment and cell death often associated with immediate transplants. However, some works opted for treatments right after lesion [167, 170], demonstrating not only significant cell survival, but also positive functional and histological effects of this approach. This unveils an important neuroprotective character of ASCs. In an interesting

study, from the mechanistic point-of-view, Menezes et al. (2014) [171] transplanted human ASCs 30 minutes after a T8-T9 balloon compression injury in rats and observed functional recovery, tissue preservation and axonal regeneration. These results seemed to be associated with laminin deposition.

ASCs have already been applied to larger animal models such as dogs. The group of Dr. Kweon in South Korea has published several studies where canine-derived ASCs are transplanted into dogs with lumbar compression injuries [172-174]. Either alone [172] or combined with other strategies [173, 175], ASCs have demonstrated positive results in this animal model. At a veterinary setting, transplantation of ASCs proved to be a safe procedure, with no major complications and some preliminary effects on locomotion and electromyogram (EMG) activity [176, 177].

Considering such data, at least two SCI clinical trials have been put forward using ASCs [178, 179]. No serious adverse events were observed, and some preliminary improvements were seen in some patients.

As abovementioned, the factors and cytokines secreted by ASCs (the secretome) are the main responsible for the effects observed until now. The cell secretome can be used in fact as a novel tool for the treatment of different conditions. Our group and others, have shown that ASCs' secretome has a neuroprotective effect on cortical neurons subjected to inflammation *in vitro* [180]; it also favors the metabolic viability and cell density of hippocampal neuronal cultures, which seems to be related with the presence of nerve growth factor (NGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and stem cell factor in the secretome [181]; presents an anti-inflammatory effect over human monocytes, reducing the production of tumor necrosis factor alpha (TNF-α), nitric oxide (NO) or prostaglandin E_2 (PGE_2) and inducing the production of transforming growth factor β1 (TGF-β1) and interleukin 10 (IL-10) by M2 macrophages [182]; and more recently ASCs secretome demonstrated to promote increased neurite outgrowth from Dorsal Root Ganglia (DRG) explants, when compared to that mediated by MSCs isolated from the bone marrow [183]. In this sense, the future application of factors secreted by ASCs to a SCI context is a promising strategy, avoiding phenomena such as cell rejection or low cell survival.

Table 1.2 – Most relevant studies published regarding the application of ASCs in SCI models.

Model	Injury	Adipose Stem Cells					Co-treatments	Main outcomes	Reference
		Source	Passage (derivation)	No. Cells	Administration	Timing			
Mice	T9-T10 contusion	mice	N/A	1 × 10^5	Intraspinal (epicenter)	3 dpi	No	ASCs survived more than BM-MSCs; preserved axons and enhanced vascularization (IHC); improved BMS scores	[184]
Rat	T9-T10 CST transection	rat	N/A (derived into OPCs)	2 × 10^6	IV – tail vein	3 dpi	No	Cell Survival and integration at lesion site; recovery of locomotor functions (BBB)	[163]
	T7 contusion		N/A	1 × 10^7	IT (rostral to the epicenter)	3 dpi, 7 dpi and 14 dpi	GCSF	Increased expression of MAP2 (IHC), GAP43 (IHC, WB, qPCR) and NGF (qPCR); improved motor performance (BBB and ladder rung)	[168]
	T8-T9 contusion		N/A (modified to express Ngn2)	1 × 10^6	Intraspinal (epicenter)	9 dpi	No	Less cavitation; improved expression of BDNF and VEGF, reduced GFAP (IHC); improved BBB scores	[185]
	T10 contusion		P2	2.5 × 10^6	IV – tail vein	1 wpi	No	Reduced cavitation; ASCs accumulated at lesion site (no long-term survival); CINC-1 transient increase (cytokine array); increased GDNF (WB); ERK 1/2 and Akt phosphorylation (WB); improved BBB scores	[169]
	T10 contusion		N/A ASCs obtained from cryopreserved tissue	2.5 × 10^6	IV – tail vein	1 wpi	No	Improved BBB scores	[186]

Model	Injury	Adipose Stem Cells					Co-treatments	Main outcomes	Reference
		Source	Passage (derivation)	No. Cells	Administration	Timing			
Rat	T10 contusion	rat	N/A (derived into neural-like cells)	N/A	N/A	1 wpi	No	Low number of proliferating cells at lesion site; increased NF200 expression (IHC); improved BBB scores	[165]
	T8 balloon compression		P2 (undifferentiated >P3 (differentiated into spheres)	5 × 10^5	Intraspinal (rostral, central and caudal to the injury)	1 wpi	No	Differentiated ASCs survived more, wrapped more host axons; improved BBB scores	[166]
	T10 balloon compression		P4-P5	1 × 10^6	IV – tail vein	3 hpi	No	Decreased expression of CD68 and GFAP, while increased NeuN (IHC); reduced white matter degeneration; increased expression of IL-10, GDNF, VEGF, while decreased TNF-α and TGF-β; improved BBB scores	[187]
	T9-T11 clip compression		N/A (derived into SCs)	5 × 10^5	Intraspinal (epicenter)	9 dpi	No	Axonal myelination; increased neurotrophic expression by ASCs-SCs (qPCR); modest improvement of BBB scores	[188]
	T10-T11 clip compression		N/A	3 × 10^5	Intraspinal (epicenter)	Immediately or 9 dpi	No	Increased expression of βIII-tubulin, BDNF, CNTF (IHC); improved BBB scores	[189]

Model	Injury	Adipose Stem Cells					Co-treatments	Main outcomes	Reference
		Source	Passage (derivation)	No. Cells	Administration	Timing			
Rat	C3-C4 hemisection	human	P2-P6	7.5 × 10^4	Intraspinal (rostral and caudal to the injury – lateral funiculus)	Immediately	No	ASCs expressed BDNF, VEGF and FGF2 (qPCR); induced 5-HT-positive fiber growth into lesion area and some caudal to the injury, together with contralateral sprouting (IHC); decreased astrocytic network (IHC); less microglia activity (IHC); no alterations in motor scores (cylinder test)	[167]
	T9 dorsal transection		P3-P5	2 × 10^5	Intraspinal (rostral and caudal to the injury)	Immediately	No	Increased BDNF levels (WB and ELISA), angiogenesis and preserved axons (IHC); decreased ED1 levels and smaller cavities (IHC); improved BBB scores	[170]
	T10 right hemisection		P2-P14	1 × 10^6	Intraspinal (rostral and caudal to the injury)	1 wpi	17β-Estradiol (E2)	E2 increased ASCs survival after transplantation; enhanced the expression of neurotrophic factors by ASCs (qPCR); improved BBB scores	[190]
	T8-T9 contusion		N/A	1 × 10^6	Intraspinal (rostral and caudal to the injury)	1 wpi	No	Reduced cavity size; improved BBB scores	[191]
	T8-T9 contusion		N/A	1 × 10^6	Intraspinal (rostral and caudal to the injury)	1 wpi	ChABC	Cavities filled with myelinated areas and less chondroitin sulfate content (IHC); increased expression of βIII-tubulin, GFAP and MAP2 (IHC); improved BBB scores (better in combinatorial group)	[192]

Model	Injury	Adipose Stem Cells					Co-treatments	Main outcomes	Reference
		Source	Passage (derivation)	No. Cells	Administration	Timing			
Rat	T8 balloon compression	human	P3-P5	1.2 × 10^6	IV – tail vein	1 and 2 wpi	No	ASCs concentrated mostly in the tail and lungs; improved bladder function in 83.3% of the rats; no differences in motor function	[193]
	T8-T9 balloon compression		N/A	N/A	Intraspinal (rostral the injury)	30 mpi	No	Reduced cavitation (GFAP, IHC); increased axonal regeneration (5-HT, Tuj1, GAP43, IHC); deposition of laminin (IHC)	[171]
	T9 clip compression		P3-P5 (concentrated in a 3D cell mass)	3 × 10^5	Intraspinal (epicenter)	Immediately	No	ASCs differentiated into CD31-positive cells; increased vascular formations and axonal outgrowth at lesion site (IHC); improved BBB scores	[194]
Dog	L1 balloon compression	dog	N/A	1 × 10^6	Intraspinal (rostral, central and caudal to the injury)	1 wpi	No	Increased myelin staining; ASCs-derived cells expressed GFAP, Tuj-1, NF160 (IHC); increased SSEP and motor scores (Olby scores)	[172]
	L1 balloon compression		P3	1 × 10^7	IV	6 hpi, then once a day, for 3 consecutive days	MP	Less infiltrating microglia; reduced levels of IL-6, TNF-α and pSTAT3 (WB); improved hindlimb function (Tarlov scores)	[173]
	L1 balloon compression		N/A	1 × 10^7	Intraspinal (rostral, central and caudal to the injury)	3 wpi	ChABC	Increased levels of digested CSPGs, βIII-tubulin and NF (WB); increased COX2 (WB); improved motor scores (BBB and Tarlov)	[175]

Model	Injury	Adipose Stem Cells					Co-treatments	Main outcomes	Reference
		Source	Passage (derivation)	No. Cells	Administration	Timing			
Dog	L1 balloon compression	dog	P3 (modified to express HO-1)	1 × 10^7	Intraspinal (rostral, central and caudal to the injury)	1 wpi	No	Reduced fibrotic changes and microglia infiltration; reduced levels of TNF-α, IL-6, COX2, pSTAT3 and GALC (WB); improved motor scores (BBB and Tarlov)	[174]
	L1 balloon compression		P3 (modified to express BDNF)	1 × 10^7 + 1 × 10^7	Intraspinal (rostral, central and caudal to the injury) + IV	3 wpi + 4 and 5 wpi	ChABC	Reduced fibrotic changes; reduced levels of TNF-α, IL-6, COX2, GFAP and GALC (WB); increased expression of BDNF, NF and nestin (WB); improved motor scores (BBB and Tarlov)	[195]
	L1 balloon compression		P3 (modified to express HO-1 and BDNF)	1 × 10^7	Intraspinal (2 epicenter and 1 in the center of the spinal cord)	1 wpi	No	Increased levels of Tuj1, NF, GAP43 (WB and IHC); decreased IL-6, TNF-α and increased IL-10 (WB); improved motor scores (BBB)	[196]
	Chronic lesions (thoraco-lumbar regions)		P3	1 × 10^7	Intraspinal (at lesion site)	>6 mpi	No	ASCs remained at lesion site for at least 24h; no adverse effects or complications following transplantation; 3 out of 6 dogs improved locomotion (Olby scores)	[176]
	Chronic lesions (paraplegic dogs only)		N/A	1 × 10^7	IT (L7-S1) 2 lumbar punctures, 1 week interval	Between 6 mpi and 2 ypi	ES	Some dogs recovered EMG activity, but no differences observed between groups	[177]

Abbreviations: 3D – three dimensional; Akt – protein kinase B; BMS – Basso mouse scale; CINC-1 – cytokine-induced neutrophil chemoattractant 1; CNTF – ciliary neurotrophic factor; COX2 – cyclooxygenase; dpi – days post-injury; ED1 = anti-CD68 antibody; ELISA – enzyme-linked immunosorbent assay; ERK – extracellular signal-regulated kinases; FGF2 – fibroblast growth factor 2; GALC – galactosylceramidase; GCSF – granulocyte colony-stimulating factor; HO-1 – heme oxygenase 1; hpi – hours post-injury; IT – intrathecal; IV – intravenous; MAP2 – microtubule-associated protein 2; NeuN – neuronal nuclei; Ngn2 – neurogenin 2; pSTAT3 – phopho signal transducer and activator of transcription 3; SSEP – somatosensory evoked potential; Tuj1 – class III β-tubulin; WB – western blot.

1.4 Hydrogel-based therapies

The limited regenerative capacity of the CNS is well known. Besides the inhibitory environment that is created after damage, as it occurs in SCI, there is a lack of a physical matrix where neurons and endogenous repairing cells can adhere. These are two of the main reasons supporting the use of biomaterials in SCI-related research. In this sense, biomaterials and TE approaches have been in the forefront of new strategies to approach SCI treatment (Fig. 1.7). Among the biomaterials available, hydrogels appear as an excellent option, mainly due to their physical properties, which can closely mimic the soft tissues environment and the architecture of the CNS. Also, their chemical composition can be adapted to integrate ECM molecules as well as other adhesion proteins, aiming at efficiently support and guide axonal regeneration. Interestingly, the development of hybrid matrices is also an approach used for SCI repair, since one can benefit from the properties of different materials to promote SCI recovery [197, 198].

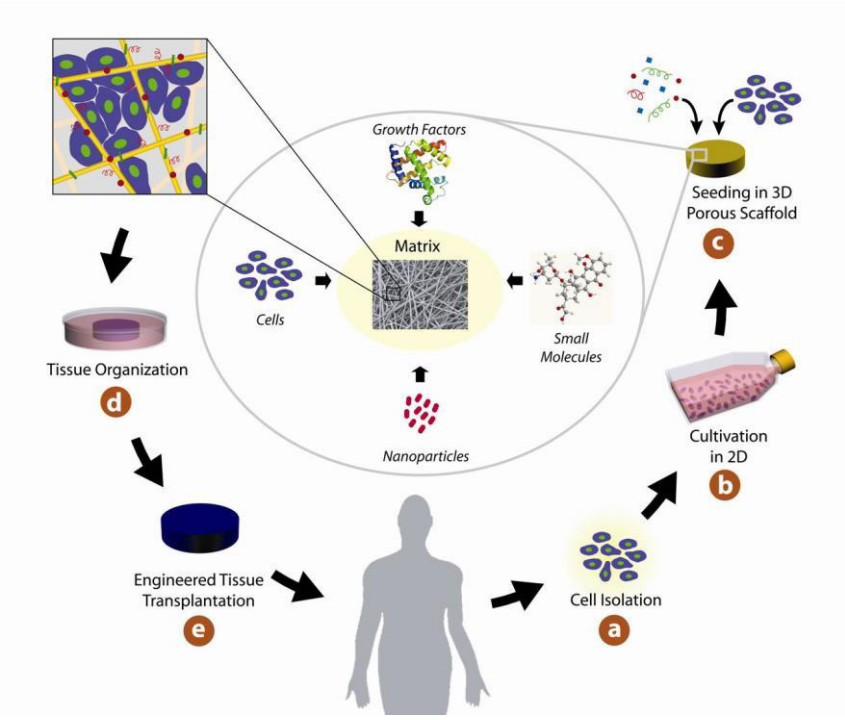

Figure 1.7 – Tissue Engineering concept. Image obtained from: http://www.tau.ac.il/lifesci/departments/biotech/members/dvir/dvir.html

For clinical applications, the design of a biomaterial must satisfy some essential criteria, such as biocompatibility, so it does not trigger any immune response from the host; specific tailored mechanical and physicochemical properties that allow both spinal cord stabilization, cell

attachment and growth; porosity and permeability for the diffusion of ions, nutrients, and waste products; and biodegradability, so the biomaterial degrades as new tissue grows, thus mimicking the natural mechanisms of breakdown and synthesis of ECM in the natural tissues [3, 199]. Among the variety of available materials for TE, hydrogels are particularly appealing for neural tissue repair, because their properties match all these requirements. Actually, hydrogels have physical properties that allow them to be injected into the body in a minimally-invasive manner. Moreover, they can be used for onsite administration and are also able to fill the defects caused by injury [200]. Therefore, they act as depots for a sustained release of cells and molecules at the injury site. As cell delivery agents, hydrogels may also improve cell survival and integration [201]. Structurally, they are very similar to macromolecular-based components in the body [202] and the high-water content has an advantage over other matrices, by better mimicking the aqueous environment of the ECM [203].

1.4.1 Natural-Based Hydrogels

An important aspect to be considered when developing a hydrogel is its integration and interaction with the host tissue. Therefore, many of the hydrogel formulations used in biomedical applications include natural polymers or molecules present in living tissues. For neural tissue repair, natural-based hydrogels are composed by molecules that normally exist in the natural ECM or have certain properties that are recognized by cells, facilitating their integration within the host [204], thus being preferred for SCI repair. Moreover, they exhibit similar properties of the soft tissues they are replacing. However, since these materials derive from natural sources, they may elicit immune reactions from the host where they will be implanted and heterogeneity between batches may also be observed [205].

Agarose

Agarose is a polysaccharide of D-galactose and 3,6-anhydro-L-galactopyranose that has interesting mechanical properties and has been widely used for drug delivery strategies due to its porous nature [206]. Being derived from cell walls of red algae, agarose is a biocompatible component, which enables it to be used in TE approaches. One aspect of agarose gels that makes them particularly interesting for CNS-related diseases is their ability to polymerize *in situ*,

so they can fill different types of neurological defects, adapting to the shape of the lesion [206]. Moreover, this type of hydrogel has already shown the capacity of supporting neurite extension *in vivo* [206]. In two different rat models of SCI (contusion and dorsal over hemisection), agarose gels were used as reservoirs for MP-loaded nanoparticles [207, 208]. This kind of construct allowed for a local and gradual release of the drug, with improved effects on reduction of the lesion volume and expression of proinflammatory proteins, when compared to systemic MP delivery. Agarose-based hydrogel has also been used for harboring lipid microtubes loaded with different drugs, namely ChABC [209]. This system facilitates a local sustained release of ChABC, consequently reducing the deposition of CSPGs and obviating the use of more invasive, continuous drug delivery systems (such as pumps or catheters). In another study, agarose gels were modified to become photolabile and then, after the exposure to a focused laser, physical and chemical channels were created, by simultaneously immobilizing a fibronectin peptide of glycine-arginine-glycine-aspartic acid-serine (GRGDS) into their structure. These channels were found to provide guidance in cell migration and neurite outgrowth [210].

Alginate

Another polysaccharide derived from cell walls of algae (brown algae) is alginate, which is able to absorb 200–300 times its own weight in water [211]. Composed of repeating units of (1–4)-linked β-D-mannuronate and α-L-guluronate [212], it has been used as a substrate for cell encapsulation, cell transplantation, and TE applications [213, 214]. The gelation of this hydrogel occurs upon interactions between the carboxylic acid moieties and different counterions, like calcium [215]. However, the gelation procedure can be also based on the existence of a physical network, stabilized by intermolecular hydrophobic interactions between alkyl chains linked to the alginate backbone [213]. In acute cervical spinal cord lesions of adult rats, alginate-based highly anisotropic capillary hydrogels induced directed axon regeneration across the implanted scaffold [198]. Since mammals do not possess enzymes capable of degrading high molecular polymers of alginate, the addition of poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with alginate lyases to the gel can provide a tunable and controlled enzymatic degradation of this natural hydrogel [216]. In another study, alginate hydrogels were used as deposits of GDNF (either free or inside microspheres) and injected into an injury of a hemisection model of SCI in rats. After either six weeks or three months, more NF-positive cells were observed in the lesion of the

animals treated with free GDNF loaded hydrogels, as compared to microspheres-GDNF-treated or untreated controls. In addition, the same group of animals presented less GFAP staining and more endothelial and nerve fiber infiltration at the lesion site. Superior functional recovery was also observed in free GDNF-treated rats, as assessed by gait analysis [217].

Collagen

Collagen is one of the major proteins found in the ECM of different tissues in mammals [218]. It is mainly synthesized by fibroblasts and there are up to 29 different collagen types, the type I being the most common [218]. In addition, gel formation can be induced just by changing the pH of a collagen solution [204]. Collagen-derived materials are therefore highly biocompatible, biodegradable and noncytotoxic, having the ability to support cellular growth [218]. In this sense, collagen has been widely used in clinics, in different applications such as recovery of tissue defects, burns, wound dressings, and nerve regeneration [219]. As major drawbacks, collagen mechanical behavior *in vivo* may be variable and sometimes it may elicit an antigenic response, namely, if cross-species transplantation is used [220]. In what concerns collagen application to SCI, Jimenez Hamann et al. (2005) [221] developed a concentrated collagen solution for the localized delivery of different growth factors. Collagen with epidermal growth factor (EGF) and FGF-2 was injected into the subarachnoid space of injured Sprague-Dawley rats. This resulted in less cavitation at the lesion epicenter (and also in other caudal areas), associated with more white matter sparing, as compared to non-treated animals. In another study, collagen filaments were grafted parallel to the spinal cord axis of SCI rats, working as a bridge to foster neuronal regeneration. After four weeks, regenerated axons crossed the proximal and distal spinal cord-implant interfaces. Following twelve weeks, rats presented improved locomotor behavior and SSEPs were observed [222]. More recently, multichannel collagen conduits were used as reservoirs for neurotrophin-3 (NT-3) gene delivery in SCI rats. One month after injury, an aligned axonal regeneration was observed, and a higher number of regenerating axons were found in the conduits delivering NT-3 [223].

Fibrin

Hydrogels based on fibrin have also been extensively explored for SCI treatment. Fibrin is a fibrous protein that is involved in blood clotting. It is produced during the coagulation cascade, when fibrinogen is cleaved by thrombin, giving origin to fibrin monomers. Thereafter, these monomers spontaneously polymerize and create a 3D matrix [224]. One important aspect of fibrin is the possibility to control their gelation process by varying the concentration of thrombin used. This feature offers the possibility of maintaining fibrin at a liquid state during injection, while forming a solid scaffold *in vivo* [225]. However, there are also some disadvantages. Fibrin gels from mammalian origin tend to degrade rapidly [226] and may be easily contaminated by blood-derived pathogens or prion proteins [227]. In addition, some reports show that autologous mammalian fibrinogen inhibits neurite outgrowth [228] and activates resident astrocyte scar formation [229]. Regarding the use of fibrin in SCI applications, Iwaya et al. (1999) [230] showed that it was an effective intermediate for intraspinal delivery of neurotrophic factors. In the same line of thought, Taylor et al. (2006) [231] managed to deliver NT-3 within fibrin scaffolds to SCI rats. Nine days after injury, this treatment elicited a more robust neuronal fiber growth into the lesion, in comparison to control groups. A dramatic reduction of glial scar formation was also observed. However, no differences in motor recovery were found between groups.

Chitosan

The linear polysaccharide chitosan is also a good alternative as a regenerative biomaterial-based strategy for SCI. This polysaccharide is composed of randomly distributed β-(1–4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It can be derived from chitin found in crustacean shells, which is the second most abundant biopolymer available, after cellulose [232]. Chitosan is able to form a gel by itself, without the need of additives [233]. That may happen via hydrogen bonds, hydrophobic interactions, and chitosan crystallites [234]. These hydrogels can also be formed by blending chitosan with other water-soluble nonionic polymers [235] or polyol salts [236]. Since it is of polycationic nature in acidic conditions, chitosan can also form hydrogels through interaction with negatively charged molecules [237]. Another type of chitosan hydrogels can be formed via covalent bonds with metal ions [238], though these gels are less suitable for biomedical use [233]. Finally, the

gelation of chitosan could also be obtained through covalent bonding between polymer chains. These bonds make the hydrogel more stable because the gelation is irreversible. Nevertheless, this approach may alter the primary structure of chitosan, which will lead to changes in its properties [233]. Chitosan hydrogels are pH-sensitive, being soluble in dilute aqueous conditions and precipitating into a gel at neutral pH [239]. The fact that this polymer is biodegradable and biocompatible is also very important for being used as a scaffold in TE applications. In vertebrates it is mainly degraded by lysozyme and some bacterial enzymes in the colon [240]. In what concerns neuronal repair, chitosan is commonly applied in the production of tubular structures most frequently used in peripheral nervous system [241]. However, chitosan hydrogels have also been applied in neural TE. For instance, the use of chitosan/glycerophosphate salt hydrogels showed that this type of gels provides a suitable 3D scaffolding environment for neurons, namely, fetal cortical mouse cells [239]. Addition of peptides, like poly-D-lysine, also showed the capacity to improve scaffold biocompatibility and nerve cell affinity for chitosan materials [242].

Gellan-Gum (GG)

Finally, the recent use of gellan-gum (GG)-based hydrogels for CNS applications has already been shown to be promising. GG is a natural polysaccharide that is produced by the bacterium *Pseudomonas elodea* [243]. Its structure consists of repeating units of a tetrasaccharide, composed by two residues of D-glucose, one residue of L-rhamnose and another of D-glucuronic acid [D-Glc(β1→4)D-GlcA(β1→4)D-Glc(β1→4)L-Rha(α1→3)]n [244]. This linear anionic polysaccharide exists in both the acetylated and deacetylated forms, originating thermo-reversible gels with different mechanical properties according to the degree of deacetylation [243]. GG is noncytotoxic and particularly resistant to heat and acid stress, being useful in culture of extremophile organisms [245]. The gelation process of this biomaterial is ionotropic, meaning that the presence of cations is necessary for the formation of a stable hydrogel structure [246]. In this process, divalent cations promote a more efficient gelation than monovalent cations [247]. At higher temperatures, GG is in a coil form, and as the temperature decreases, there is a thermo-reversible 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 assembles the gel [247]. Regarding SCI applications, our group has developed different strategies based on GG hydrogels [248, 249]. In 2010, Silva et al. [249] conjugated GG with three-dimensional tubular structures made of a biodegradable blend of starch. This construct was revealed to be noncytotoxic and capable of supporting the *in vitro* culture of oligodendrocyte-like cells. Moreover, when applied *in vivo* in a hemisection rat SCI model, it was shown that the scaffold was well integrated in the lesion site without eliciting any chronic inflammatory processes. A common modification employed in this type of hydrogels is the addition of different peptide sequences that mimic the ECM [210, 250], with the purpose of improving phenomena like cell adhesion, growth, and development [251]. By grafting GRGDS peptides onto the GG backbone, our group has shown that GG-GRGDS hydrogels significantly improve NSCs morphology and growth [252], as well as BM-MSCs' proliferation, metabolic viability and secretome properties [253], in comparison to unmodified GG. More recently, GG-GRGDS hydrogels promoted the growth and viability of ASCs, together with neurite outgrowth from DRG explants [254].

1.4.2 Synthetic Hydrogels

Regarding synthetic hydrogels, their biggest advantage is the fact that they can be tailored to fit the needs for a certain application. From physical and chemical properties to degradation rates, many aspects of their structure can be modulated in order to improve their biocompatibility and degradation rate [255].

Poly(lactic acid) (PLA) and Poly(lactic-co-glycolic acid) (PLGA)

PLGA/PLA polymers are members of the α-hydroxy acid class of compounds and are composed of synthetic biodegradable aliphatic polyesters [256]. For controlling the degradation rate and mechanical properties of these polymers, it is possible to vary the ratio of monomer units and their stereochemistry (either D- or L-form), as well as the molecular weight distribution of their chains [257]. Since PLGA and other similar polymers have been approved by the Food and Drug Administration (FDA) for use in the repair of human peripheral nerves, their translation into CNS-related injuries seems promising [258]. In SCI applications, Patist et al. (2004) [259] tested the effects of poly(D,L-lactic acid) macroporous guidance scaffolds (in the form of foams),

with or without BDNF, on a model of transected rat spinal cord. Foams were embedded in fibrin glue containing acidic-FGF, resulting in some gliotic and inflammatory response in the cord-implant interfaces. In addition, in BDNF-containing foams, 20% more NeuN-positive cells (marker for neurons) were present in the spinal nervous tissue in the rostral stump, as compared to controls, four and eight weeks after implantation, respectively. These same foams showed a significant higher level of vascularization. Curiously, treatment with fibrin only yielded more axons than the other groups. Through behavioral analysis, similar functional improvements in all groups were found. Furthermore, PLA microfibers, in an aligned or random form, were implanted in rats subjected to a complete transection of the spinal cord. Four weeks after injury, both types of microfibers facilitated the infiltration of host tissue and allowed the closure of the initial three millimeters gap. However, aligned PLA fibers promoted longer distance of rostro-caudal axonal regeneration as compared to random PLA fibers or film controls [260]. Regarding PLGA, nano- and microparticles of this hydrogel have been widely used as delivery agents for TE applications [261]. In a SCI animal model, Fan et al. (2011) [262] used PLGA nerve conduits in combination with recombinant human NT-3 (rhNT-3). Rats were subjected to a complete thoracic transection of the spinal cord and then PLGA was implanted together with an rhNT-3 single dose administration. Animals treated with the combinatorial approach presented significantly improved performances in the BBB rating locomotor scale and grid walk tests.

Methacrylate-Based Hydrogels

Poly[N-2-(hydroxypropyl) methacrylamide (PHPMA) hydrogels were first described by Woerly and colleagues [263, 264]. They synthesized a biocompatible and heterogeneous hydrogel, with an open porous structure that allowed the transport of both small and large molecules, as well as the migration of cells and blood vessels [264]. This hydrogel also presented viscoelastic properties similar to the neural tissue [263]. When implanted into a transected rat spinal cord, the hydrogel successfully bridged the tissue defect favoring cell growth, angiogenesis, and axonal growth within the microstructure of the network [263]. This hydrogel was showed to be permissive to the growth of a reparative tissue, composed of glial cells, blood vessels, axons, and dendrites and even ECM molecules, such as laminin and/or collagen [264]. Other features of PHPMA hydrogels include a reduction of necrosis and cavitation in the adjacent white and gray matter of transected rat spinal cords [265]. Furthermore, using

this type of hydrogels in cats subjected to a transection lesion provided some motor benefits, as compared to non-treated cats [266]. More recently, PHPMA hydrogels were used as a matrix in order to create an appropriate microenvironment for axonal regeneration in SCI rats. Hydrogel implanted animals exhibited an improved locomotor BBB score and an overall better coordination in neuromuscular evaluations, such as breathing adjustment to electrically evoked isometric contractions and H-reflex recovery. After immunohistochemistry analysis, ED1 positive cells accumulation (macrophages/monocytes) was evident at the border of the lesion. At the same time, a larger number of neurofilament-H positive axons penetrated the matrix. In addition, there was also myelin preservation rostrally and caudally to the lesion [267].

Poly(ethylene glycol) (PEG)

Poly(ethylene glycol) (PEG) is a non-toxic polyether compound that is water soluble and known to resist protein adsorption and cell adhesion [268]. These properties make PEG polymer highly resistant to recognition by the immune system after implantation. Besides this, PEG helps to seal cell membranes after injury, limiting cell death [205]. Depending on the cross-links created, PEG hydrogels can be designed with varying degradation rates and can be used as drug releasing vehicles [269, 270]. Moreover, they can be additionally modified in order to increase cell adhesion [271, 272]. It is also known that PEG exhibits rapid clearance rates and has already been approved for a wide range of biomedical applications [269], including SCI. In an *in vivo* model of SCI, treatment with a PEG solution by itself was capable of accelerating and enhancing the membrane resealing process, restoring neuronal membrane integrity. This led to suppressed levels of reactive oxygen species (ROS) elevation and lipid peroxidation [273]. Furthermore, a study performed on adult guinea pigs showed that, six hours after a spinal cord contusion, a single subcutaneous injection of PEG (in saline) produced a rapid recovery of SSEP propagation through the lesion. This was followed by a significant recovery of the cutaneous truncimuscle reflex, which is a good index of white matter integrity [274]. In another study, using dogs as an animal model of SCI, PEG injection in the acute phase was shown as clinically safe and induced a rapid recovery in different outcome measures, as compared to conventionally treated dogs [275]. In conclusion, it seems that PEG action has two main pathways: one is based on the protection against membrane damage, which leads to reduced necrosis and apoptosis,

while the other is preventing the effects of mitochondria-derived oxidative stress, showing a reduction in ROS formation and lipid peroxidation [276].

1.5 Combining Biomaterials and Cells

In spite of the experimental groundwork regarding cell transplantation and biomaterial-based therapies for SCI, their use as single approaches presents some limitations. Considering biomaterials alone, it is not easy to find a balance between biodegradability, biocompatibility and physical/chemical properties, so they respond exactly as expected. Moreover, they are not able to replace lost cells, after SCI. On the other hand, cell transplantation by itself is not always capable of recreating spinal cord complex architecture and stability, or even direct axonal regrowth [277]. Hence, taking advantage of what both therapies offer to overcome the multiple hurdles of SCI, synergistic effects on regeneration and functional recovery of the injured spinal cord can be provided if combined strategies are employed [3]. In this sense, researchers have been focused on the use of biomaterials, such as hydrogels [214, 252], sponges [278], films [279] or conduits [280], as systems for cell encapsulation and delivery into the injured spinal cord. NSCs for instance, have been combined with PLGA-based scaffolds with the objective of increasing both cell survival and differentiation towards neurons [281]. Several studies, have also demonstrated a functional and histological benefit of conjugating BM-MSCs with different materials, potentiating the cellular effect [282, 283]. SCs are also commonly used for SCI repair together with biomaterials, particularly tubular scaffolds, to attempt a directed and oriented axonal regrowth [280]. These and other studies are summarized on table 3.

Table 1.3 – Most relevant studies combining Cellular Therapies and Biomaterials in SCI repair applications, both *in vitro* and *in vivo*.

Cell	Biomaterial	Model	Main outcomes	Reference
NSCs	PLGA (polymer scaffold)	*In vitro*	NSCs modified with NT-3 or TrkC, were widely distributed and viable in the scaffold; 70 % of NSCs differentiated to MAP2+ neurons; formation of functional synaptic structures (positive for syapsin-I and PSD95, with active release of vesicles)	[281]
NSCs/ OECs	Gellan Gum (GG-GRGDS hydrogel)	*In vitro*	NSCs adhered and proliferated within modified GG hydrogels, when compared to unmodified ones; in co-cultures with OECs, NSCs presented significantly greater survival and proliferation compared to monocultures of NSCs	[252]
NSCs	PLGA (polymer scaffold)	Hemisection SCI rats	Improved functional recovery (BBB test); reduced tissue loss and glial scarring; fibers crossed injury epicenter; local GAP43 expression	[284]
	Alginate (sponge)	Transection SCI rats	Survival and differentiation (GFAP+, βIII-tubulin+) of rat hippocampus-derived neurosphere cells; implanted cells migrated and integrated within the host tissue	[278]
BM-MSCs/ OECs	Alginate + Matrigel (hydrogels)	*In vitro*	Alginate inhibited cell proliferation and viability of OECs, SCs and BM-MSCs; the addition of fibronectin allowed for OECs proliferation; alginate also inhibited neurite outgrowth of DRG explants, although the addition of fibronectin, or SCs/BM-MSCs attenuated this effect; matrigel stimulated both cell proliferation and DRG neurite outgrowth, in either the absence or presence of cells	[214]
BM-MSCs	Gellan Gum (GG-GRGDS hydrogel)	*In vitro*	GG hydrogel modified with GRGDS fibronectin-derived peptide increased cell proliferation and metabolic activity of BM-MSCs; BM-MSCs' secretome cultured in modified hydrogels enhanced the survival and differentiation of primary cultures of hippocampal neurons	[253]
	PLGA/SIS (polymer scaffold)	Transection SCI rats	Significant functional recovery (BBB test and MEPs); axonal regeneration; BM-MSCs survival eight wpi; induced axonal regeneration	[282]
	Agarose (scaffolds)	Dorsal column transection SCI rats	BM-MSCs modified with NT-3; Long-tract sensory axonal regeneration with increased linear organization; 83 % of axons grew the full length of the lesion cavity; a host reactive cell layer in the interface of the scaffold prevented axonal penetration into spinal cord parenchyma	[283]
		Transection SCI rats	BM-MSCs modified to secrete BNDF; supported motor axon regeneration, with axons organized into fascicles; the presence of BDNF secreting-BM-MSCs significantly enhanced axonal regeneration	[285]

Cell	Biomaterial	Model	Main outcomes	Reference
BM-MSCs	Fibrin (matrix scaffold)	Hemisection SCI rats	BM-MSCs survive in fibrin matrices *in vitro*; pronounced recovery of rats' neurological function; improved survival and migration of transplanted cells	[286]
SCs	PHB (scaffold conduit)	Hemisection SCI rats	Tubular conduits were well integrated, inducing modest astroglial reaction; regenerating axons found mainly outside the scaffold; NF+ axons filled the conduits, as well as numerous raphaespinal and CGRP-positive axons	[280]
	laminin/collagen (hydrogels)	Contusion SCI rats	The 3D matrix enhanced long-term cell survival, graft vascularization was improved, and the degree of axonal ingrowth; was also increased; some level of functional recovery was also achieved	[287]
OECs	PHB-b-DEG (films)	*In vitro*	OECs proliferation was enhanced when cultured in PHB-b-DEG films; no cytotoxic responses were observed, and cell viability and membrane integrity were maintained; downregulation of neurite growth inhibitory Nogo protein	[279]
OECs + ASCs	Serum-derived albumin scaffold	SCI rats	Both cell types adhered to the scaffold and remained viable; SCI rats improved locomotor skills at different time points; reduced glial scar formation and presence of cells expressing markers of neurons and axons at the injury site	[288]

Abbreviations: PHB – Poly-β-hydroxybutyrate; PHB-b-DEG – Polyhydroxybutyrate - polyethylene glycol; PSD95 – postsynaptic density protein 95; TrkC – tropomyosin receptor kinase C.

1.6 Concluding remarks

Overall, many works have been focused on single-based therapies, with some promising results. However, the complexity of SCI requires a multimodal approach that could target several aspects of the disease. Biomaterials such as hydrogels represent a viable alternative, being degradable and in general non-immunogenic. Together with the application of cells with neuroprotective and neuroregenerative properties, a combinatorial approach could be of interest for future clinical applications. From the possible candidates of cell populations to use in a SCI context, adult cells such as ASCs or OECs are of great relevance; these cells have shown potential in SCI repair in several works, and can be obtained from adult individuals, opening a door for autologous transplantation (reducing the risk of immune rejection). In addition, natural-based biomaterials, such as hydrogels also represent a suitable option as they can be designed to meet CNS physical and chemical properties, working both as a matrix for nervous regeneration and as a vehicle for cellular transplantation. From the vast list of candidates, GG is very interesting, considering that it is already FDA approved in the food industry and its application to nervous TE, even though never explored, presents great potential. In this sense, a combinatorial therapy conjugating GG hydrogel with cells such as ASCs and OECs would represent a novel approach for SCI repair.

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Chapter 2
Co-Transplantation of ASCs and OECs for Spinal Cord Injury Repair

The results presented in this chapter were published as an original article in an international peer reviewed journal:

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Co-Transplantation of Adipose tissue-derived Stromal Cells and Olfactory Ensheathing Cells for Spinal Cord Injury Repair

Running Head Title: *Co-Transplantation of ASCs and OECs for SCI Repair*

Eduardo D. Gomes,[1,2]* Sofia S. Mendes,[1,2]* Rita C. Assunção-Silva,[1,2]
Fábio G. Teixeira,[1,2] Ana O. Pires,[1,2] Sandra I. Anjo,[3,4] Bruno Manadas,[4]
Hugo Leite-Almeida,[1,2] Jeffrey M. Gimble,[5] Nuno Sousa,[1,2]
Angelo C. Lepore,[6] Nuno A. Silva,[1,2] António J. Salgado[1,2#]

[1] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[2] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3] Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, 3004-514 Coimbra, Portugal

[4] CNC – Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal

[5] Center for Stem Cell Research and Regenerative Medicine, Tulane University, 1430 Tulane Avenue, SL-99, New Orleans, LA 70112, USA

[6] Vickie and Jack Farber Institute for Neuroscience, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, 19107, USA

* Equally contributing authors

[#] Corresponding author: A. J. Salgado, PhD, Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: asalgado@med.uminho.pt; Tel: +351 253 60 49 47; Fax: +351 253 60 48 20

Author contributions

E. D. Gomes: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

S. S. Mendes: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

R. C. Assunção-Silva: Collection and/or assembly of data

F. G. Teixeira: Collection and/or assembly of data

A. O. Pires: Collection and/or assembly of data

S. I. Anjo: Collection and/or assembly of data

B. Manadas: Collection and/or assembly of data, Manuscript writing

H. Leite-Almeida: Collection and/or assembly of data

J. M. Gimble: Provision of study material or patients

N. Sousa: Manuscript writing

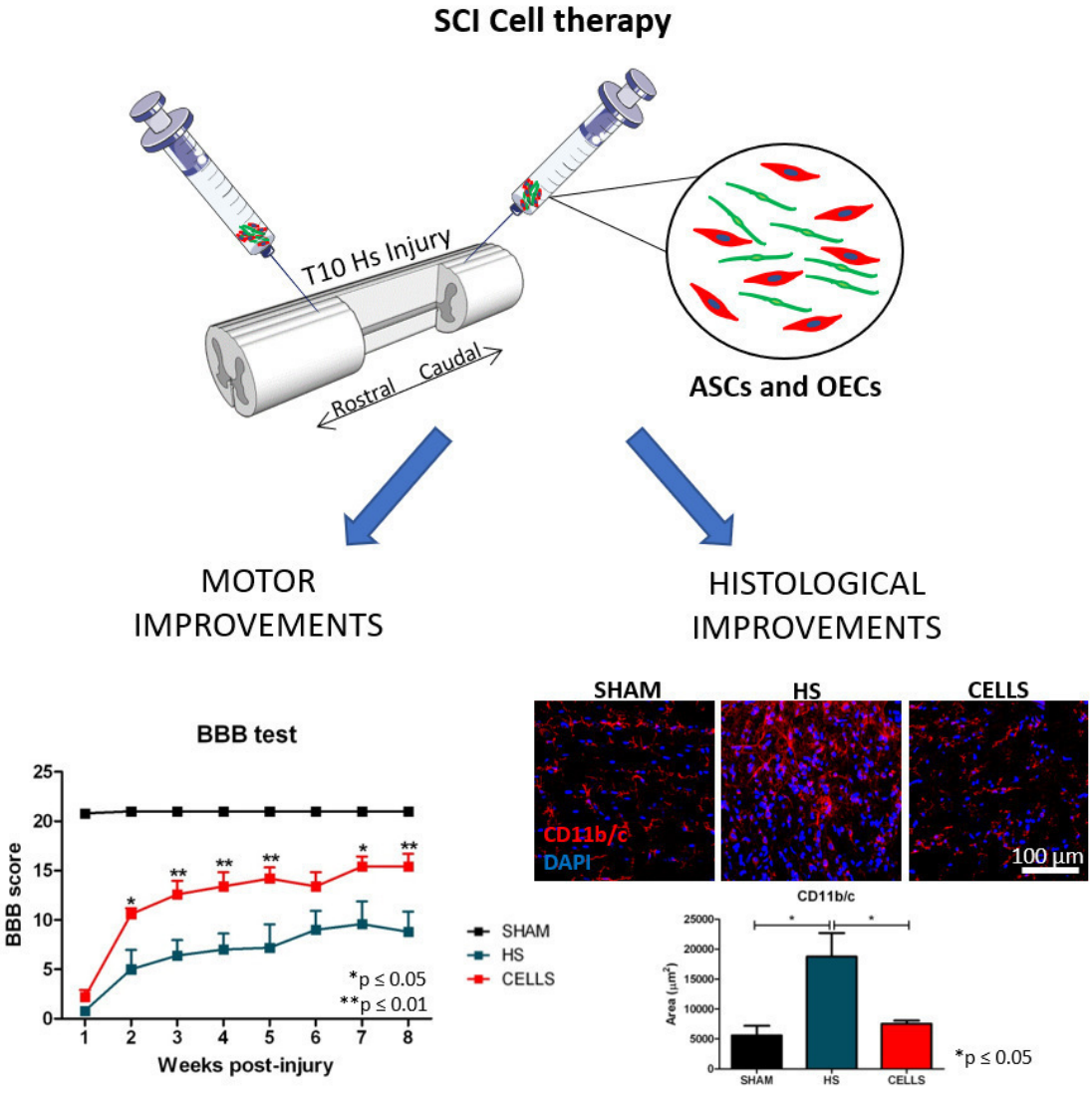
A. C. Lepore: Provision of study material or patients

N. A. Silva: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

A. J. Salgado: Conception and design, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Key Words: Spinal Cord Injury; Adipose tissue-derived Stem Cells; Olfactory Ensheathing Cells; Axonal Regeneration; Regenerative Medicine

Graphical Abstract



Locomotor recovery assessed by the BBB test, during eight weeks of follow-up. (A) – Schematic representation of the T10 left hemisection injury induced. (B) – Mean BBB score of the different experimental groups. (C) – Evaluation of three different parameters used in the BBB subscore: stepping, coordination and toe clearance. Cells-treated animals presented significant improvements of BBB score, in different time points, in comparison to non-treated (HS) animals. Values are shown as mean ± SEM (n=5); *p≤0.05, **p≤0.01.

Abstract

Patients suffering from Spinal Cord Injury (SCI) still have a dismal prognosis. Despite all the efforts developed in this area, currently there are no effective treatments. Therefore, cell therapies have been proposed as a viable alternative to the current treatments employed. Adipose tissue-derived Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) have been used with promising results in different models of SCI, namely due to the regenerative properties of the secretome of the first, and the guidance capability of the second. Using an *in vitro* model of axonal growth, the Dorsal Root Ganglia explants (DRGs), we demonstrated that OECs induce neurite outgrowth mainly through cell-cell interactions, while ASCs' effects are strongly mediated by the release of paracrine factors. A proteomic analysis of ASCs' secretome revealed the presence of proteins involved in VEGF, PI3K and Cadherin signaling pathways, which may be responsible for the effects observed. Then, the co-transplantation of ASCs and OECs showed to improve motor deficits of SCI-rats. Particular parameters of movement such as stepping, coordination and toe clearance were improved in rats that received the transplant of cells, in comparison to non-treated rats. A histological analysis of the spinal cord tissues revealed that transplantation of ASCs and OECs had a major effect on the reduction of inflammatory cells close the lesion site. A slight reduction of astrogliosis was also evident. Overall the results obtained with the present work indicate that the co-transplantation of ASCs and OECs brings important functional benefits to the injured spinal cord.

Significance Statement

Spinal Cord Injury (SCI) still has no cure and cellular transplantation has been considered a valid alternative for SCI treatment. Herein, we demonstrate that Adipose tissue-derived Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) induce neurite outgrowth of dorsal root ganglia explants through distinct mechanisms. The co-transplantation of ASCs and OECs into a rat model of SCI led to functional improvements. Additionally, there was a reduction of inflammatory cells close to the injury site and ASCs were integrated in the spinal cord tissue, eight weeks post-injury. Overall, the transplantation of ASCs and OECs is a promising strategy for SCI repair.

Introduction

Spinal Cord Injury (SCI) is one of the most devastating conditions of the CNS for which there is still no cure. Sensorial and motor function deficits, cardiac and respiratory complications, sexual and urinary dysfunctions and sometimes depression are among the consequences of SCI. The treatment to this condition is mainly based on palliative care. However, there are new therapies currently under investigation aiming to promote functional recovery after SCI through different neurorestorative mechanisms [1, 2]. From those, cellular transplantation emerges as a promising tool, either by replacing the damaged nervous tissue or alternatively potentiating endogenous neuronal regeneration by exerting a neurotrophic and/or neuroprotective role.

In this context, Mesenchymal Stromal Cells (MSCs) have gained increased notoriety, much due to their supportive character [3]. These cells, firstly characterized by Friedenstein et al. (1974) [4], are identified by their multipotency, adhesion to plastic and positive/negative expression of some specific markers [5]. Adipose tissue-derived Stromal Cells (ASCs) are a type of MSCs derived from an abundant and accessible source (adipose tissue). Moreover, ASCs have shown to possess an immunomodulatory profile [6, 7], in addition to a neurotrophic role, providing protection, survival and differentiation of different cells and tissues [8, 9]. All these effects have been associated to ASCs' secretome, that is, the set of molecules that these cells secrete to the extracellular milieu [6, 10, 11]. ASCs have been applied to animal models of SCI with promising results [12, 13]. In a cervical SCI model, transplanted ASCs modulated the structure of the glial scar and stimulated axonal sprouting [12]. In a thoracic compression injury model, ASCs were capable of promoting functional recovery in addition to tissue preservation and axonal regeneration [13]. Regarding human clinical trials, the autologous transplantation of ASCs, obtained from lipoaspirates, into SCI patients was free of serious adverse events and some patients showed mild improvements in motor and sensory scores [14].

Olfactory Ensheathing Cells (OECs) have also gathered particular attention due to their potential for SCI treatment [15]. OECs are a specific type of cells present in the olfactory system that support the constant neuronal regeneration in the transition between the PNS and the CNS [16]. Considering OECs' role in their native olfactory system, Ramon-Cueto and colleagues [17, 18] transplanted these cells into different models of CNS injury in rats, demonstrating improved axonal regeneration after a lesion. Following their pioneering work, different pre-clinical studies managed to reproduce the beneficial effects of OECs grafts, including partial recovery of motor

function [19-21], even under a chronic SCI lesion [22]. Among several interesting properties, OECs are capable of: 1) bridging the lesion site, by interacting with endogenous astrocytic processes [23]; 2) myelinating previously demyelinated regions of the spinal cord [24]; 3) having a phagocytic activity, removing degenerating axons [25, 26]; and 4) modulating the glial scar as well as promoting angiogenesis [27]. Transplantation of OECs in humans has also already been tested [28, 29]. The use of these cells was shown to be safe and in some cases patients presented improvements in motor and light touch scores [30].

In addition to the beneficial properties above described, ASCs and OECs have the advantage of being good candidates for autologous transplantation, avoiding ethical concerns. Moreover, previous work from our group showed that ASCs and OECs present positive paracrine interactions, with improved proliferation and metabolic activity of both cells when seeded in an indirect co-culture system [31]. In this work, we aimed to assess the regenerative potential of the combined application of ASCs and OECs either in an *in vitro* model of axonal regeneration as well as in an *in vivo* model of SCI.

Materials and Methods

Cell isolation and culture

Human ASCs were isolated according to Dubois et al. (2008) [32] with a collaboration with LaCell LLC. The culture of these cells is described in supporting information.

OECs were harvested and cultured according to a previously described protocol [33]. Details about the culture of these cells are described in supporting information.

Secretome collection

For the secretome used in *in vitro* experiments, ASCs at sixth passage (P6) were seeded at 4 000 cells/cm^2 density in cell culture flasks, with their normal growth medium (supp. info). Seventy-two hours after seeding, the media was removed and cells washed three times with PBS without Ca^{2+}/Mg^{2+} (Invitrogen, USA), followed by neurobasal medium (Invitrogen, USA) with 1 % penicillin/streptomycin (pen/strep, Invitrogen, USA). Then, fresh neurobasal media (with 1 %

pen/strep) was added to the cells. Twenty-four hours later, this media was collected [now called conditioned media (CM)], filtered (0.2 µm pore diameter) and frozen in liquid nitrogen for later application.

OECs isolated and purified as above-mentioned were seeded at 40 000 cells/cm^2 density in fibronectin-coated cell culture flasks, with their normal growth medium (supp. info). Then, the same conditioning protocol was followed as for ASCs, in order to collect OECs' secretome.

As a control, flasks without cells but with neurobasal media (with 1 % pen/strep) were kept for 24 h at the same conditions, before being filtered and frozen in liquid nitrogen.

Proteomic data analysis

Proteomic data of ASCs' secretome, previously obtained from Pires et al. (2016) [34] and newly obtained OECs proteomic data were analyzed using two distinct approaches. In the first approach, the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://pantherdb.org) was used to identify the signaling pathways related with ASCs and OECs secreted proteins. Then, the main hits obtained were grouped according to their main function and represented in a pie chart. In the second approach, a PANTHER overrepresentation test was performed to highlight the most representative

Reactome pathways. This test was performed using the following criteria: entire human database as background list; applying a Binomial distribution test with Bonferroni correction for multiple comparisons and a cut-off of 0.05 p-value.

Dorsal Root Ganglia (DRG) isolation and culture with ASCs/OECs

OECs were firstly isolated and purified as above-mentioned. They were seeded at 20 000 cells/cm^2 density on fibronectin-coated 24 well plates, with their normal growth medium. Seventy-two hours after seeding, ASCs were seeded on top of OECs cultures (in co-culture group) or on fibronectin-coated 24 well plates, at 7 500 cells/cm^2 density. The growth medium of OECs was used for this step, since previous experiments (non-published data) showed that ASCs proliferation and metabolic viability were not altered in this medium. Twenty-four hours later, DRG explants were isolated according to Allodi et al. (2011) [35] and placed on top of all experimental groups (ASCs and OECs alone, or co-culture). Thoracic DRG explants were isolated from P5-P7

rats and cleaned to remove peripheral processes. Then they were placed over the experimental conditions under study. The medium was changed to DRGs normal growth medium: neurobasal medium supplemented with 6 mg/ml D-glucose (Sigma, USA), 1 % pen/strep, 2 % B27 (Invitrogen, USA) and 2 mM L-Glutamine (Invitrogen, USA). Some DRG explants were additionally placed on a control group without cells, cultured with normal growth medium. The cultures were kept for four days, with daily medium exchanges. After fixation, an immunocytochemistry (ICC) for neurofilament (NF) was performed and samples imaged with an Olympus IX81 fluorescence microscope. The analysis was made using the software ImageJ (NIH).

DRG isolation and culture with ASCs/OECs secretome

Collagen hydrogel droplets were used as a matrix for DRGs adhesion and growth. The matrix was prepared by mixing rat tail collagen type I [3.61 mg/ml; 89.6 % (v/v); BD Biosciences, USA] with DMEM concentrated medium [10×; 10 % (v/v); Invitrogen, USA] and a solution of $NaHCO_3$ [7.5 % (w/v); 0.4 % (v/v)]. After mixing, collagen droplets were incubated for 2 h at 37 °C, 5 % CO_2 (v/v). Then, DRGs were isolated and placed on top of previously prepared collagen droplets with their normal growth medium. Twenty-four hours later, this medium was replaced with the CM from ASCs, OECs or the control group. In the moment of media addition, both secretomes, as well as the control, were further supplemented with B27, L-Glutamine and Glucose. DRGs were kept in culture for five days and media were replaced once, at day three. After five days, samples were fixed and subjected to an ICC for NF. Images were obtained through confocal microscopy (Olympus FV1000).

Immunocytochemistry

ICC protocols were used to identify DRG neurite projections in culture, applying the mouse anti-NF 200 kDa antibody (1:200, Millipore, USA). Details can be found in supporting information.

DRG outgrowth analysis

To calculate the area occupied by the neurites, Neurite J plugin for ImageJ was applied [36]. Initially, the body area of the explant was defined and then the threshold contrast was

adjusted to highlight the neurites formed from the DRG body. After determining the scale, the plugin automatically converted the images to 8 bits. Finally, using the menu "analyze particles" the software automatically calculated the areas occupied by the neurites, using the dark background as contrast. In order to calculate the number of intersections Neurite J plugin was used.

In vivo Proof of Concept

Animals and groups

Eight weeks old female Wistar rats (Charles River, France), housed in light and temperature controlled rooms and fed with standard diet, were used in the *in vivo* studies. Handling was performed for three days before the surgeries. Animals were divided in three distinct groups according to the respective treatment/procedure: 1) Animals subjected to SCI with no treatment (SCI, n=5); 2) SCI animals treated with a transplantation of ASCs and OECs (Cells, n=5); 3) Animals with laminectomy only, without SCI (Sham, n=5). In the cells-treated group, a total of 40 000 OECs and 40 000 ASCs were injected per animal, divided by two injections, 2 mm rostral and 2 mm caudal to the lesion, using a Hamilton syringe.

Spinal Cord Injury surgery

All animals were anesthetized by intraperitoneal injection of a mixture (1.5:1) of ketamine (100 mg/ml, Imalgene/Merial, France) and medetomidine hydrochloride (1 mg/ml, Domitor/Pfizer, USA). Once anesthetized, fur was shaved from the surgical site and the skin disinfected with ethanol 70 % and chlorohexidine. Then a dorsal midline incision was made from T7-T13 and the paravertebral muscles retracted. A laminectomy was performed at T10 level, in which the spinous processes were removed and the spinal cord exposed. A unilateral defect (hemisection) on the left side of the spinal cord was done, removing 2-3 mm of nervous tissue. After the respective treatment, paravertebral muscles and skin were closed with Vicryl sutures (Johnson and Johnson, USA). The incision of SCI control animals was closed after injury without treatment. Post-operative care was given to all SCI rats (protocol described in supporting information).

Behavioral analysis

BBB test

To evaluate motor behavior the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [37] was employed every week, starting exactly one week post-injury, up to a total of eight weeks. Locomotion of the affected hindlimb is rated by two blinded observers in a four-minutes test.

Activity box test

General locomotion activity was measured by assessing the number of rearings and distance travelled in a closed arena, five and eight weeks post-injury [38]. The arena (43.2 cm × 43.2 cm) has transparent acrylic walls (Med Associates Inc., USA) and is placed in a brightly illuminated room. Animals started the test at the arena's center and were given 5 min to explore it.

Swimming Test

Eight weeks after the injury, spontaneous motility was also measured through a swimming test. Each animal was placed at the border of a pool with 170 cm of diameter, having a central platform with 12 cm of diameter. Each animal had three trials to find the platform, with each trial having a maximum of 2 min. The average velocity of these animals was determined through an infra-red camera, associated to the VideoTrack software (Viewpoint, France).

Immunohistochemistry (IHC)

After obtaining the spinal cord sections (detailed in supporting information) IHC protocols were performed. The following primary antibodies were used: i) mouse anti-CD11b/c (Pharmingen, USA); ii) rabbit anti-rat GFAP (Dako, Denmark); iii) mouse anti-NF (Millipore, USA); iv) rabbit anti-tyrosine hydroxylase (TH, Millipore, USA) and v) mouse anti-nuclei antibody (HuNu, Millipore, USA). IHC and staining quantification protocols can be found in supporting information.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by: 1) one way ANOVA test

in results presented in figures 1B-C, 3B, 5C, and 6; 2) two-way ANOVA in results presented in figures 1D, 3C, 4 and 5A-B. One or two-way ANOVA tests were followed by the Bonferroni post-hoc test. A p-value of ≤ 0.05 was set as the criteria for statistical significance.

Results

Effects of direct co-culture of ASCs/OECs with DRGs

In order to assess the effects of both cell types on neuritogenesis, we started by performing direct cultures of both cells (alone or in combination) with DRG explants. After four days of culture, it was visible a clear effect of OECs (or co-culture of OECs with ASCs) on neurite outgrowth from the explants (Fig. 2.1A). The area occupied by the neurites cultured with OECs ($4.1 \pm 0.8 \times 10^6$ μm^2) was similar to the area of the neurites from the co-culture group ($4.3 \pm 0.6 \times 10^6$ μm^2), but significantly higher in comparison to ASCs group ($1.2 \pm 0.3 \times 10^6$ μm^2) or the control group ($0.4 \pm 0.1 \times 10^6$ μm^2) (Fig. 2.1B). After analyzing the arborization pattern of each experimental group it was possible to observe that co-culture and OECs groups present significantly more intersections (Fig. 2.1D), which is a synonym of higher ramification of the neurites formed. In addition, the neurites formed in these groups extended to longer distances than ASCs group (OECs: 3 433 ± 400 μm; Co-culture: 3 093 ± 239 μm; ASCs: 2 446 ± 274 μm) or the control (1 231 ± 161 μm) (Fig. 2.1C).

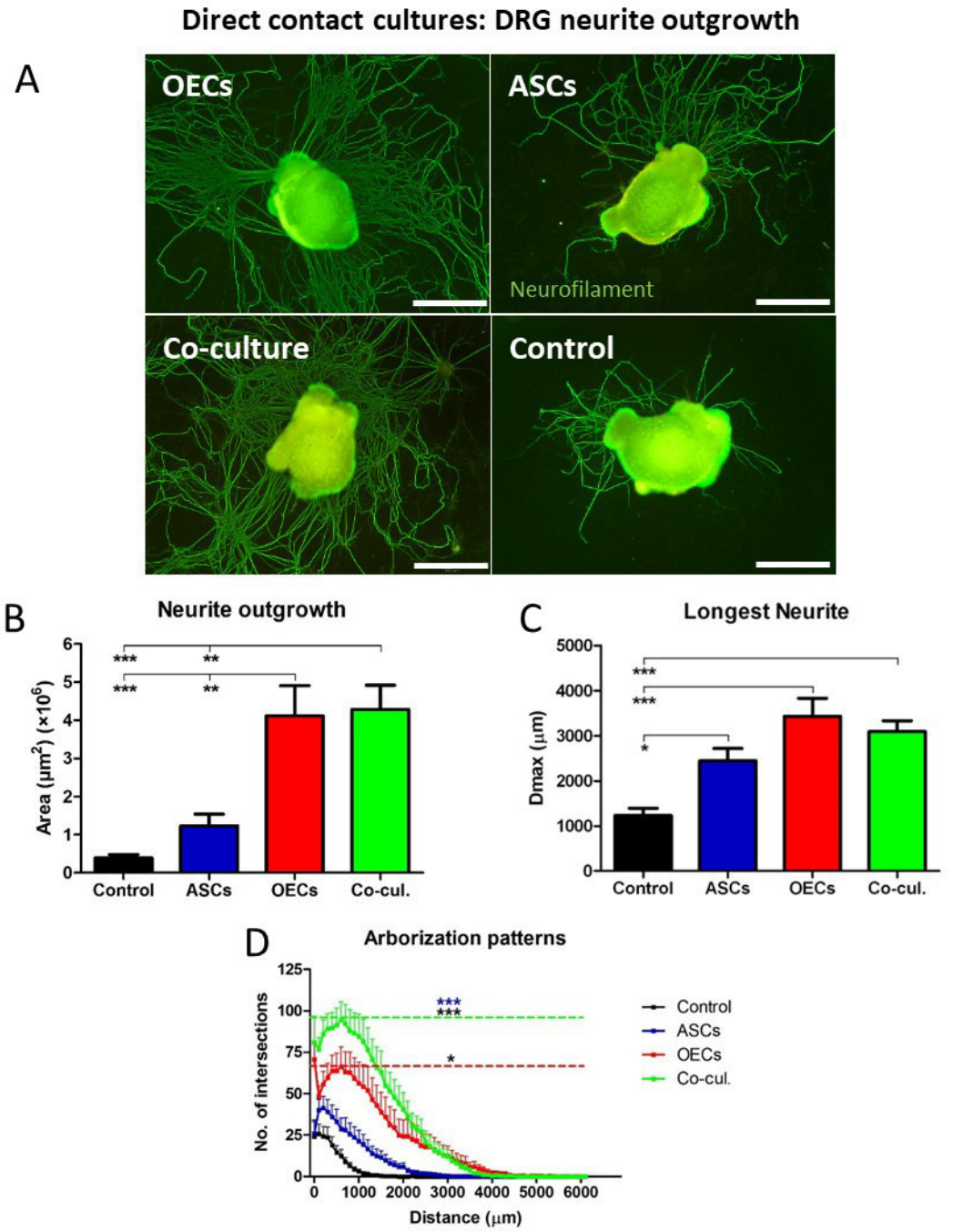


Figure 2.1 – 2D direct contact cultures of ASCs, OECs, or both with DRG explants. In direct culture conditions, OECs have a higher impact on neurite outgrowth in comparison to ASCs. (A) – Representative fluorescence microscopy images of DRG explants and their neurites stained with neurofilament (in green), for each of the groups under evaluation. (B) – Quantification of the neurite area with ImageJ software. (C) – Quantification of the distance of the longest neurite (Dmax) with Neurite J plugin. (D) – Analysis of the arborization patterns produced by each experimental group. Scale: 1 mm; Values are shown as mean ± SEM (n=3 independent experiments); *p≤0.05, **p≤0.01, ***p≤0.001. In graph D, the differences between groups are highlighted according to their group colors.

After a more detailed analysis to the cellular disposition and neurite formation it becomes clear that newly formed neurites followed very closely OECs direction and orientation, which was not so evident in cultures with ASCs (Fig. 2.2). These results might indicate that the influence of OECs on neurite outgrowth, under a direct contact paradigm, is higher in comparison to ASCs. In particular, OECs seem to have a guidance capacity, favoring the growth of neurites towards OECs-enriched regions.

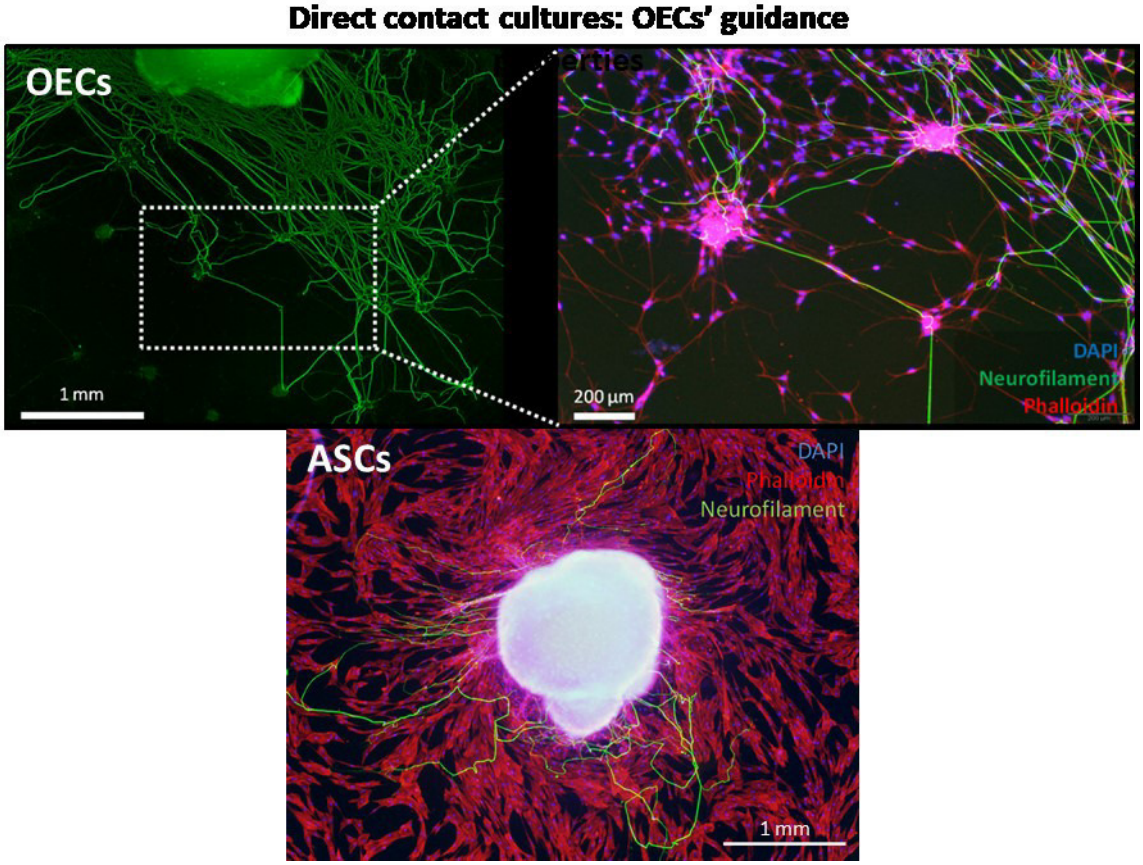


Figure 2.2 – Culture of DRG explants with OECs or ASCs. Neurites (in green) formed by the DRG follow the orientation of OECs (in red, upper right panel). In the case of ASCs (lower panel), it seems that the neurites formed do not follow the direction and orientation of cells as clearly as in OECs cultures.

Effects of ASCs/OECs secretome on DRGs neurite formation

Using the same *in vitro* model, we cultured DRG explants without the presence of the cells, but in contact with the factors secreted by ASCs or OECs. In this context, and after five days in contact with ASCs or OECs secretome, the only group that presented relevant neurite formation was the CM of ASCs (total area: 32 675 ± 11 869 µm^2). OECs secretome and the

control did not induce any significant neurite extension (Fig. 2.3A-C). In order to characterize and explore the role of ASCs' and OECs' secretome on the effects observed, a set of proteomic data previously obtained from ASCs, was re-analyzed [34] and a new proteomic analysis was performed for OECs secretome (supp. tables 2 and 3). The proteins identified in the secretome of ASCs may be involved in several different pathways (Fig. 2.3D), from which we highlight the VEGF and Cadherin signaling pathways, as well as the PI3 kinase pathway, which are responsible for a myriad of cellular processes, including neurite outgrowth and neuronal protection. Proteins involved in neuronal communication pathways are also present in ASCs' secretome. On the other hand, even though OECs secretome also contains proteins related to neurite outgrowth pathways, these seem less relevant (fig. 2.3D). For example, the VEGF signaling pathway is absent of the main hits analysis for signaling pathways. A complementary analysis, based on a PANTHER overrepresentation test, also highlights the importance of reactome pathways associated with neurite outgrowth (supp. table 1).

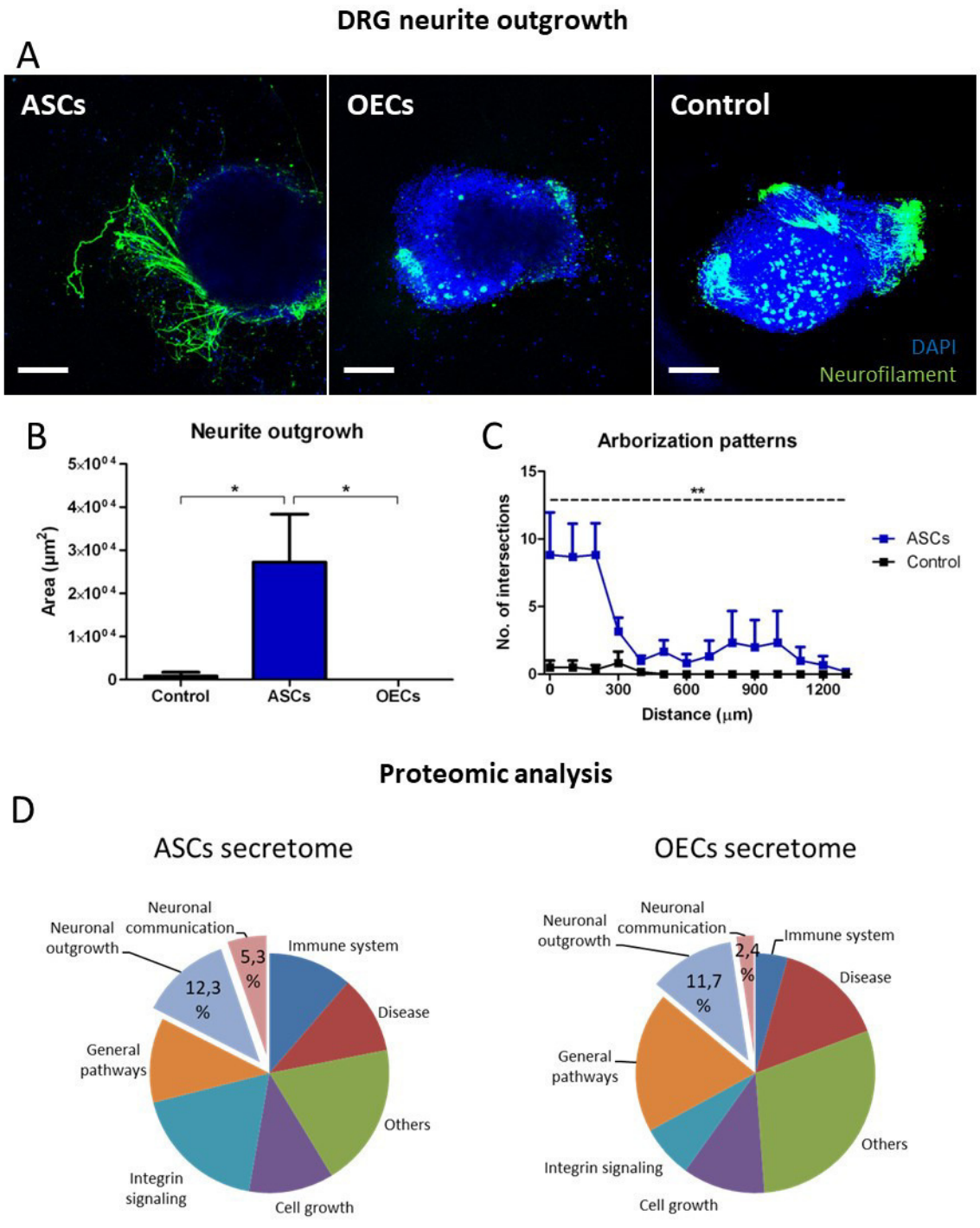


Figure 2.3 – Cultures of DRG explants with the secretome derived from different cell sources and proteomic analysis. (A) – Representative confocal images of DRG explants and their neurites stained in green. (B) – Quantification of the neurite area with ImageJ software. (C) – Analysis of the arborization patterns produced by each experimental group. Only ASCs' secretome was able to induce neurite outgrowth on DRG explants, while OECs secretome and the control did not present a significant neurite formation. (D) – Proteomic analysis performed for ASCs' and OECs' secretome. The proteins identified are

involved in several different pathways, some of which are responsible for processes such as neuronal outgrowth and neuroprotection. Scale: 100 μm; Values are shown as mean ± SEM (n=6 replicates); *p≤0.05, **p≤0.01.

ASCs and OECs treatment leads to an improvement of locomotion in SCI rats

Taking into account the results obtained *in vitro*, we decided to move to an *in vivo* proof of concept, by transplanting both cell types into SCI animals. We divided the animals in three different groups: SCI rats without treatment (hemisection – HS group); SCI rats transplanted with ASCs/OECs (Cells group); and non-injured rats (laminectomy only – Sham group).

After eight weeks, the BBB analysis revealed that animals treated with cells displayed improved motor outcomes, when comparing to non-treated animals (Fig. 2.4B-C).

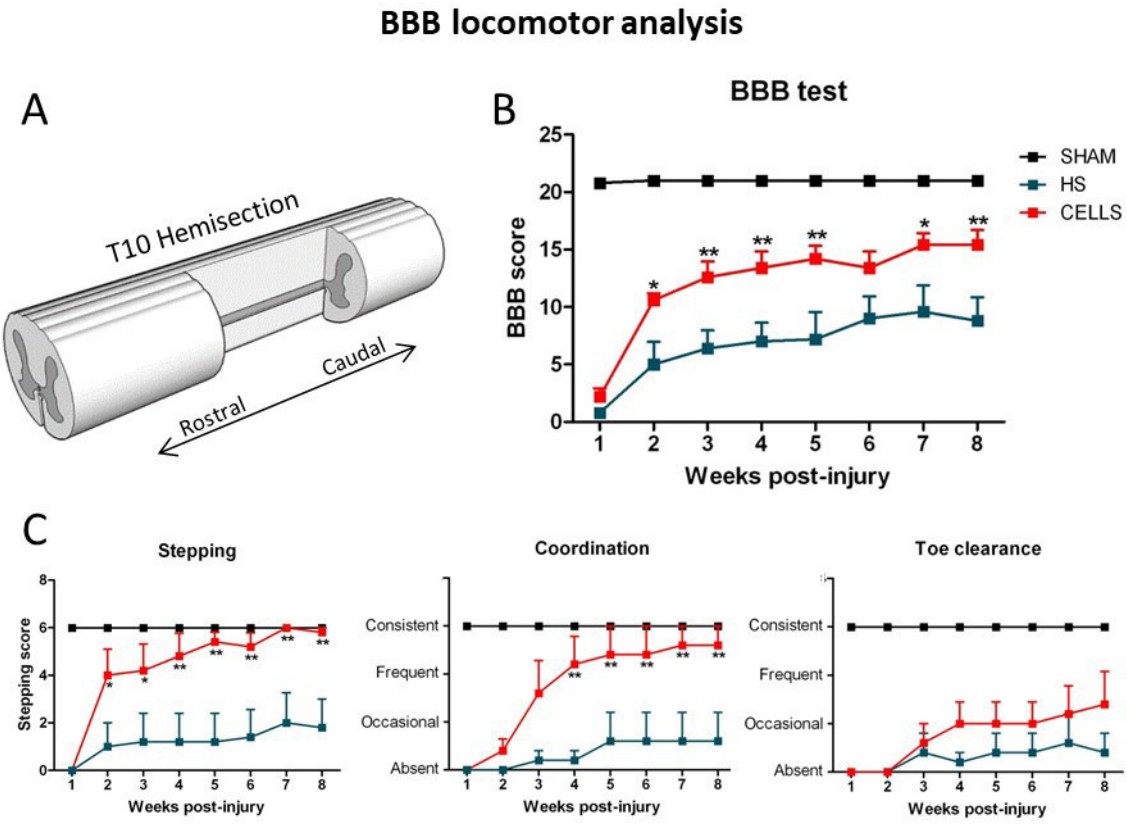


Figure 2.4 – Locomotor recovery assessed by the BBB test, during eight weeks of follow-up. (A) – Schematic representation of the T10 left hemisection injury induced. (B) – Mean BBB score of the different experimental groups. (C) – Evaluation of three different parameters used in the BBB subscore: stepping, coordination and toe clearance. Cells-treated animals presented significant improvements of

BBB score, in different time points, in comparison to nontreated (HS) animals. Values are shown as mean ± SEM (n=5); *p≤0.05, **p≤0.01.

In particular, the mean BBB score was statistically different at 2, 3, 4, 5, 7 and 8 weeks post-injury (Fig. 2.4B). In addition, sham animals did not present motor deficits during this period. After a detailed analysis on the BBB test, we concluded that the main differences between Cells and HS groups were in the stepping capability, coordination and toe clearance (Fig. 2.4C). Transplanted rats presented higher stepping scores, meaning that were capable of doing plantar steps while non-treated rats did mostly dorsal steps. They also showed more frequent coordinated steps and a tendency to perform better in toe clearance. No differences were observed in parameters such as the position of the paw, tail position and trunk instability (data not shown).

No significant differences between cells-treated and non-treated rats were found in the total distance measurements (Fig. 2.5A) and in the number of rearings (Fig. 2.5B), both evaluated at zero and eight weeks post-injury. However, it is interesting to notice that in rearing behavior, sham animals are only significantly different in comparison to the non-treated group (HS), at eight weeks post-injury, a fact that can be interpreted as indicative of the relevance of the ASCs/OECs based therapy.

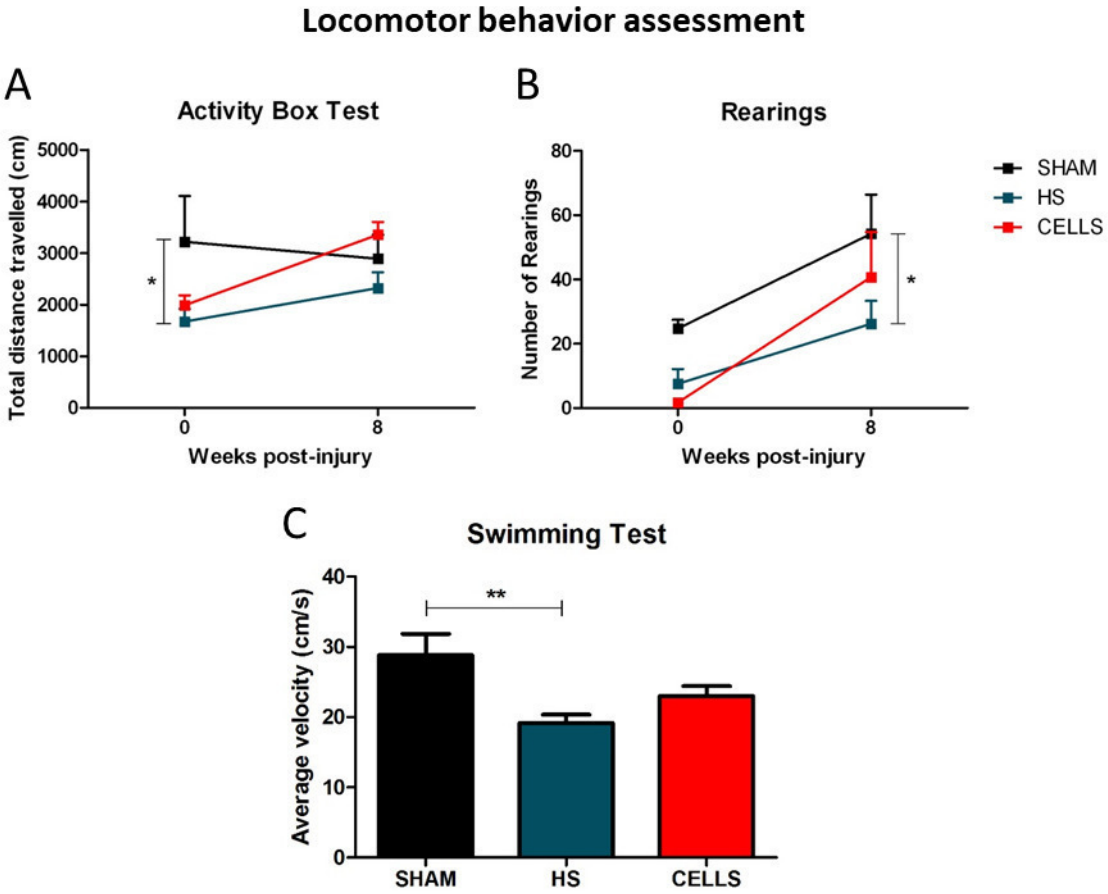


Figure 2.5 – General locomotor activity measured by the activity box test and swimming test. Average distance travelled by each group (A) and total number of rearings (B) in the activity box test, both at zero and eight weeks post-injury. There are no significant differences between cells-treated and non-treated animals. (C) – Average velocity for each experimental group measured in a swimming test, eight weeks post-injury. The transplantation of ASCs and OECs did not lead to an improvement of the average velocity in water of injured animals. Values are shown as mean ± SEM (n=5); *p≤0.05, **p≤0.01.

A similar result was observed in the swimming test (Fig. 2.5C) performed eight weeks post-injury. HS group was not significantly different from Cells' transplanted group; however sham animals presented a significantly higher average velocity in water, only when compared to non-treated animals.

Transplantation of ASCs and OECs reduces the infiltration of inflammatory cells

Eight weeks after the injury, IHC against CD11b/c revealed that the area occupied by inflammatory cells in regions close to the injury site was significantly increased in the HS group (18 765 ± 3 932 μm^2) in comparison to the levels of the sham group (5 590 ± 1 606 μm^2), and to rats co-transplanted with ASCs and OECs (7 548 ± 525 μm^2, Fig. 2.6A-B). This indicates that ASCs/OECs treatment was apparently preventing the infiltration of inflammatory cells, which partially explains the functional results obtained. In the contralateral side to the lesion, there were no differences between groups in the area of CD11b/c (data not shown). IHC against GFAP revealed a similar trend, although there were no significant differences among groups (Fig. 2.6A, 2.6C). Nevertheless, injured rats present higher levels of astrogliosis than sham animals, while treatment with cells provides an evident reduction in those values.

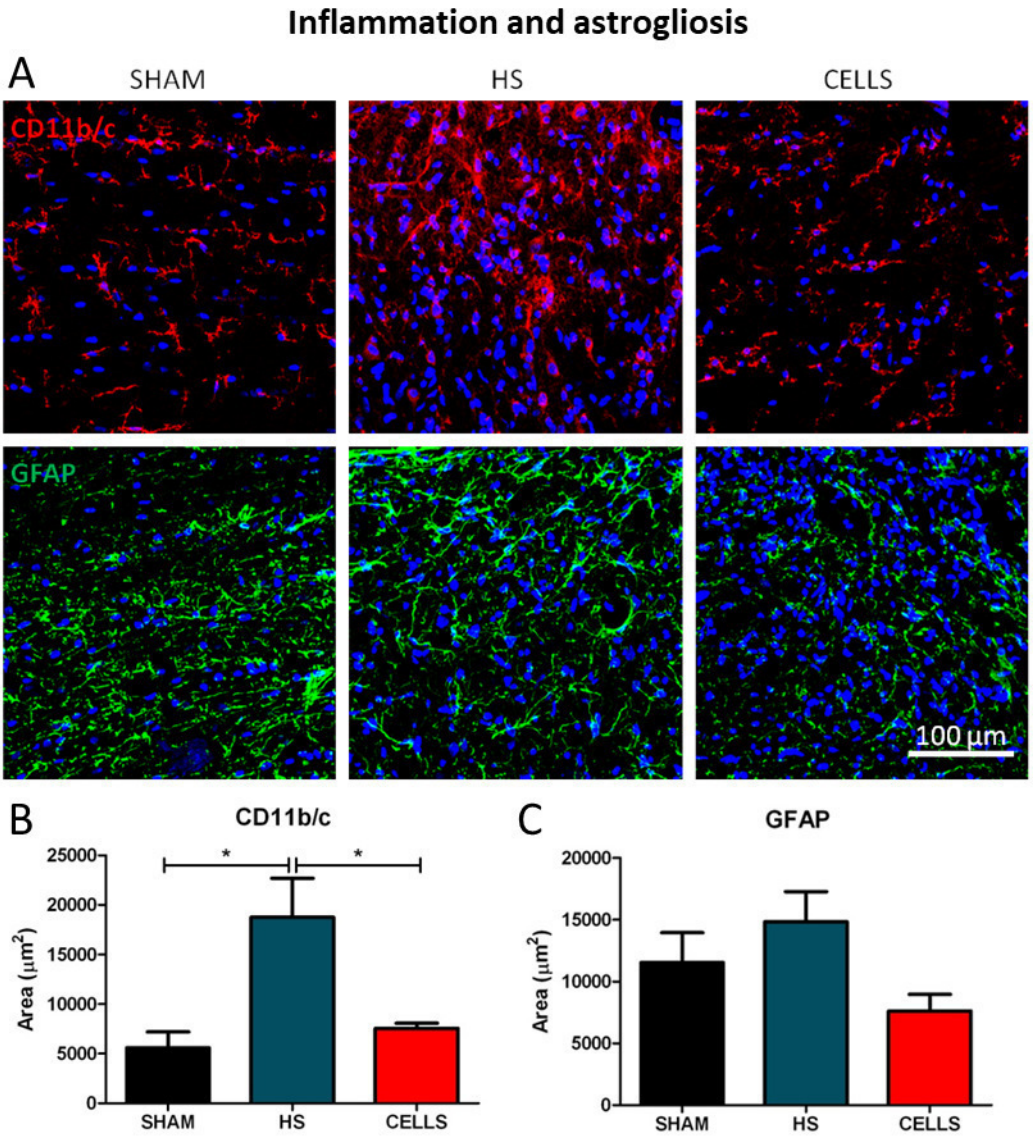


Figure 2.6 – IHC results for CD11b/c and GFAP markers. (A) – Representative confocal images of spinal cord longitudinal sections, immunostained for CD11b/c (upper panel) and GFAP (lower panel). (B) – Quantification of CD11b/c and (C) GFAP markers' area. Injured animals (HS group) have significantly increased levels of CD11b/c, while cells-treated animals present values close to those of sham animals. Values are shown as mean ± SEM (n=5); * p≤0.05.

Transplanted ASCs were present in the spinal cord eight weeks after transplantation

An IHC against HuNu was performed in order to identify transplanted ASCs, since it is a specific marker for human cells. In fact, results showed that ASCs were still present at the spinal

cord, eight weeks post-transplantation (for all transplanted animals). These cells could mostly be found in regions close to the injury site, or where there was high infiltration of cells (Fig. 2.7).

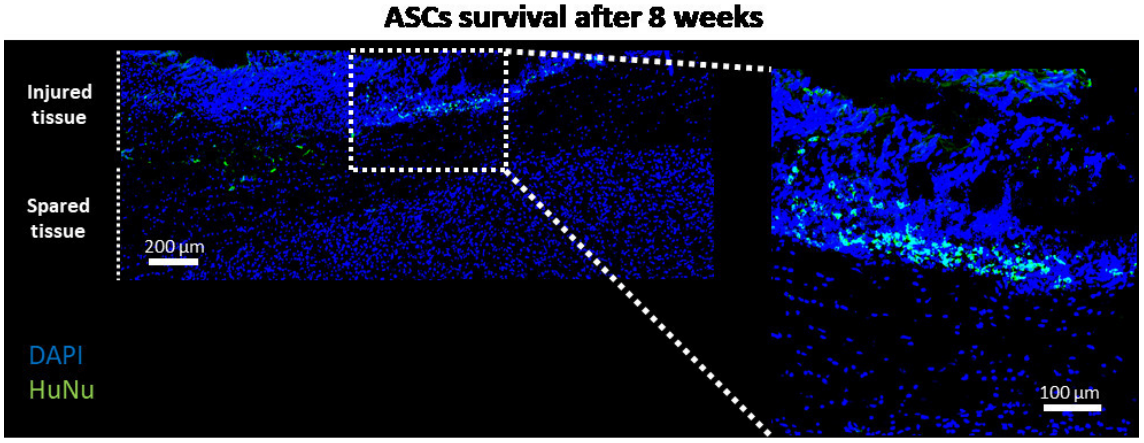


Figure 2.7 – Confocal images depicting ASCs (stained for HuNu, in green) present at the site of injury. ASCs might have migrated from the injection sites to the border of the hemisection injury.

Discussion

The use of cellular therapies in SCI research has been widely explored [39]. Several different types of cells have already been tested [40] and even numerous clinical trials have been done or are still ongoing [14, 41, 42]. The big advantage of the cells used in our therapeutic approach is their accessibility and easy translatability to the clinics. Despite of the promising results demonstrated by the solo transplantation of ASCs or OECs in other works [12, 13, 20, 22], the combined administration of these two specific cell populations has rarely been tested. In addition, the mechanisms by which they act remain elusive. In order to understand ASCs and OECs influence on a specific phenomenon such as neurite outgrowth, we started by using DRG explants as a model of axonal regeneration [35].

From the data obtained, we can conclude that OECs provide better support to guided neurite outgrowth than ASCs, producing massive neurite arborizations (Fig. 2.1 and 2.2). On the other hand, when we used the secretome of the cells, the only one capable of inducing neurite formation was ASCs CM (Fig. 2.3). This could mean that both ASCs and OECs have the potential to induce neurite formation, but their main mechanism of action might be different. For instance, in a study from Leaver et al. [43] Olfactory Ensheathing Glia (OEG) also induced significant

increases in the number and extension of neurites from retinal explants, in that case in comparison to Schwann cells (SCs). This enhanced growth was also suggested to be contact-mediated since CM from OEG did not present the same effects [43]. Another example of contact mediated increase of axonal outgrowth induced by OECs includes DRG axons grown on myelin substrates [44]. In our study the secretome of OECs did not reveal to be a growth promoting substrate for axonal outgrowth of DRG explants, even though there is a substantial body of literature supporting the relevance of OECs' secreted factors in axonal regeneration context [45-47]. The contact-mediated ability of OECs might be partially due to the action of metalloproteinases (MMPs) such as MMP-2, MMP-3 and MT1-MMP, which seem to play a role on cellular motility and on their neurotrophic properties [48]. Meanwhile, the secretome of ASCs has been characterized by our group and others and it is known to contain several axonal-growth factors, such as nerve growth factor (NGF), glia-derived nexin (GDN) or semaphorin 7A, as well as factors related to neuronal differentiation such as pigment epithelium-derived factor (PEDF) [6, 10, 34, 49]. Some of these might be responsible for the effects observed in DRG explants. Furthermore, after exploring our proteomic data [34], we could find in ASCs' secretome proteins associated to several different signaling pathways. From those we highlight the VEGF signaling pathway for instance, which has a key role in angiogenesis [50] but also has been shown to promote adult neurogenesis and neuronal cell migration by stimulating endothelial cells in vascular niches to release cues for neural stem cells [51]. The cell cycle regulator PI3K pathway also plays a crucial role in neuronal cell survival and proliferation being stimulated by growth factors such as NGF or brain-derived neurotrophic factor (BDNF) [52, 53]. Finally, cadherin signaling pathway is involved in central processes such as cell adhesion, neurogenesis and synaptic plasticity [54-56] and importantly it has been demonstrated to promote neurite outgrowth by facilitation of fibroblast growth factor (FGF) receptor signaling [57]. Several other pathways are highly associated with our secretome, namely the hypoxia response via hypoxia-inducible factor (HIF), as well as the endothelial growth factor (EGF) signaling pathway or the FGF signaling pathway, which in general are implicated in neurite outgrowth, neurogenesis, neuroprotection and in many cellular processes that may contribute to SCI regeneration. Proteins involved in neuronal communication pathways, such as the dopamine receptor-mediated signaling pathway, are also present in ASCs secretome. Moreover, by exploring reactome pathways (supp. table 1) we could highlight the possible role of neural cell adhesion molecule 1 (NCAM1) pathway in ASCs mediated-neurite outgrowth. ASCs secretome was enriched in proteins

directly or indirectly related to NCAM1. This molecule modulates neuronal cell adhesion, survival and synaptic plasticity among others. We found 14-3-3 protein which blocks neuronal apoptosis [58]; fibronectin, a main component of the ECM, responsible for cell adhesion through integrin activation [59]; or spectrin, implicated in the normal morphology of neuron cell bodies and neurites [60]. Additionally, the semaphorin receptor protein neuropilin-1 (in its soluble form [61] or released through exossomes [62]), which is involved in axonal guidance mechanisms, was also present in our sample [63]. On the other hand, our proteomic analysis of OECs secretome shows in fact that different proteins were present, and consequently associated to numerous signaling pathways. Nevertheless, none of them showed to be sufficient to induce neurite outgrowth of DRG explants. The absence of the VEGF pathway, together with a reduction of neuronal communication pathways or integrin signaling, could be responsible for the lack of effects observed.

Considering the *in vitro* results herein obtained, we opted to transplant ASCs and OECs together, so we could take advantage from the contact-mediated regenerative capacity of OECs and at the same time, benefit from the neurite outgrowth and neuroprotective properties associated to ASCs secretome. In this study, the acute transplantation of ASCs and OECs lead to functional improvements, most evident in the BBB test. This was associated with a marked decrease of CD11b/c marker and a slight decrease of GFAP in areas close to the lesion. The effect on microglial/inflammatory cells is supported by an *in vitro* experiment in which ASCs and OECs secretome reduced significantly the number of CD11b/c cells in culture, revealing that the factors secreted by both types of cells might mediate this mechanism (supp. Fig 2). We did not see differences in any other markers analyzed, namely neurofilament for axons or tyrosine hydroxylase for specific dopaminergic neurons (supp. fig. 2.1). This excludes a direct effect of OECs on neuronal rewiring, and supports the idea that OECs might have worked as “feeders” supporters for ASCs growth, as previously shown by our group in *in vitro* experiments [31]. Therefore, the functional improvements obtained were not directly connected to preservation or regeneration of the neuronal circuitry, but more linked to modulation of the injury environment. In fact, a reduction of inflammation and astrogliosis has been previously associated with improvements in locomotor function [64, 65].

In the histological analysis, we could detect transplanted ASCs, eight weeks after injury. Their distribution seems to be restricted to regions close to the injury site, or in areas of visible cellular infiltration. In this sense, ASCs were able to migrate from the injection sites to the injury

site and were integrated into the spinal cord tissue. The survival of ASCs two months after transplantation is an important finding because this means that ASCs were able to exert their paracrine-based effect over longer periods of time. As previously mentioned, ASCs secrete immunomodulatory molecules, which associated with their long survival may explain the decrease observed on inflammation. Unfortunately, we could not trace transplanted OECs. These cells share many markers with SCs and it is known that SCs infiltrate the spinal cord after a lesion [66], thus we could not distinguish between these two types of cells. Nevertheless, cultures of OECs before transplantation presented around 90 % purity levels, based on ICC against p75 positive cells (supp. fig. 2.3).

Overall, our results unveil a beneficial effect of transplanting both ASCs and OECs after a SCI. However, many issues remain to be clarified regarding the application of these cell populations in SCI. In the case of OECs, the influence of the specific region from where they are isolated, the purity of the cultures, and the existence of subtypes of OECs [16] are just some examples of the variety existing in the literature. In addition, for both ASCs and OECs it will be important to understand which mechanisms and molecules are mediating their effects, so one can take the maximum advantage of these cells. Finally, it will be interesting to test the transplantation of ASCs and OECs after the establishment of the lesion, in a subchronic or chronic phase, or even combine ASCs/OECs with a biomaterial or scaffold that can enhance their survival in the hostile environment existing after a SCI.

Conclusion

Cellular therapies for SCI treatment are one of the most explored strategies in pre-clinical research. The choice of a cell population suitable for transplantation needs to take into account not only its potential therapeutic effect but also its translatability into a clinical setting. In this context, ASCs and OECs could be an interesting solution.

From this work, it is possible to conclude that both ASCs and OECs have the potential to induce neurite outgrowth in a DRG explant model, even though their main mechanism of action might be different. If on one side, OECs seem to provide physical support and guidance for neurite outgrowth and elongation, ASCs on the other hand may secrete different molecules that potentiate neurite formation and growth, assuming a more paracrine role on neuritogenesis. In

addition, their combined administration into an animal model of SCI resulted in significant improvements of locomotor behavior and a marked reduction of inflammatory cells. Neurite outgrowth was not observed at the lesion site, which may suggest a more supportive role for OECs *in vivo*, instead of a rewiring of the neuronal network. Importantly, ASCs could be detected two months post-transplantation. In summary, the acute transplantation of ASCs and OECs after a SCI is a promising and easily translatable approach that with the proper refinements, could lead to a significant amelioration of patients suffering from SCI.

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Rui Lima, Nadiya Kubasova and Deolinda Santinha for the help with DRG experiments and analysis; Eduarda Oliveira and Natália Vasconcelos for assistance in the *in vivo* experiments.

Disclosure of Potential Conflicts of Interest

Jeff Gimble discloses employment at LaCell LLC; Patents pending to LaCell LLC and Tulane University; Consultant/Advisory role with Obatala Sciences. Dr. Gimble is also a co-owner of LaCell LLC and Obatala Sciences Inc. All other authors have declared that no conflict of interest exists.

Supporting Information

Materials and Methods

Cell isolation and culture

Human ASCs were isolated according to the protocol described by Dubois et al. (2008) [32] from human lipoaspirates obtained from consenting donors under an institutional review board approved protocol at LaCell LLC. These cells were cultured and maintained in α-MEM (Invitrogen, USA), with 10 % Fetal Bovine Serum (FBS, Biochrom AG, Germany) and 1 % antibiotic/antimycotic solution – penicillin/streptomycin (pen/strep; Invitrogen, USA) at 37 °C and 5 % CO_2 (v/v).

The animal care committee of the research institute approved all the animal protocols in accordance with standardized animal care guidelines [67]. OECs were harvested from olfactory bulbs of neonatal (P5-P7) Wistar-Han rats, according to the protocol previously described [33]. Briefly, upon dissection, the meninges and blood vessels were removed and the tissue was digested with collagenase type I (2.5 mg/ml, Sigma, USA) for 30 min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5 ml pipette and centrifuged at 1000 rpm for 5 min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation, cells were resuspended and seeded on uncoated plates for two consecutive periods of 24 h. It is expected that most of the fibroblasts and astrocytes attach in the first and second periods, respectively. After this purification step, the remaining cells were seeded on fibronectin coated surfaces. Coating was done overnight with a 1 mg/ml fibronectin solution (Sigma, USA). Cells were cultured in DMEM/F12 (Invitrogen, USA) with 10 % FBS and 1 % pen/strep solution at 37 °C and 5 % CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5.36 µg/ml, Invitrogen, USA) and Forskolin (1.4 µg/ml, Sigma, USA).

Immunocytochemistry

ICC protocols were used to identify DRG neurite projections in culture. Mouse anti-NF 200 kDa antibody (1:200, Millipore, USA) was used to identify neurite outgrowth from DRG

explants. Controls were made by the omission of the primary antibody. Briefly, samples were fixed with a solution of 4 % paraformaldehyde (PFA, Panreac, Spain) in PBS for 20 min (in 2D cultures) or 45 min (in 3D collagen cultures) at room temperature (RT). Then, after three washes with PBS, cell membranes were permeabilized with 0.3 % Triton X-100 (Sigma, USA) for 5 min (2D) or 10 min (3D). Non-specific binding sites were blocked using a solution of 10 % Fetal Calf Serum (FCS, Biochrom AG, Germany) in PBS for 1 h (2D) or 1 h 30 min (3D). The anti-NF primary antibody was then added for 1 h (2D) or 48 h (3D), after which cells were exposed to the specific secondary antibody for 1 h (2D) or 18 h (3D). Alexa Fluor 488 goat anti-mouse (Invitrogen, USA) was used to label DRG neurites. Cell nuclei were then counterstained with 1 µg/ml of DAPI (4',6-diamidino-2-phenylindole, Invitrogen, USA) and cytoskeleton of cells stained with 0.1 µg/ml phalloidin (Sigma, USA). The staining protocol was performed for 30 min (2D) or 45 min (3D). Imaging was done using fluorescence (2D) and confocal (3D) microscopy.

In vivo Proof of Concept

Post-Operative care

Following SCI surgery, rats were kept under heat lamps and received subcutaneous injections of vitamins (10 ml/Kg, Duphalyte/Pfizer, USA), 0.9 % NaCl, the analgesic butorphanol (10 mg/ml, Butomidor/Richter Pharma AG, Austria) and the antibiotic enrofloxacin (5 mg/ml, Baytril/Bayer, Germany), besides atipamezole (5 mg/ml, Antisedan/Pfizer, USA) a drug used in order to revert anesthesia. Bladder evacuation was done manually twice a day. Then, during the first week post-injury, rats received daily subcutaneous injections of all the above-mentioned components with the exception of atipamezole. Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment. The diet was enriched with mini-treats (Bio-Serv, USA) and the food was presented to the rats in the cage. In case of weight loss superior to 10 %, Nutri-Cal (Vetoquinol, France) was administered orally.

Histological analysis

Rats were deeply anesthetized by an intraperitoneal injection of 200 mg/ml sodium pentobarbital (Eutasil, Ceva Saúde Animal, Portugal) and perfused through the ascending aorta with 0.9 % NaCl followed by 4 % PFA. A rough dissection of the spine and spinal cord was performed, centered on the site of hemisection and the tissues were fixed in 4 % PFA overnight. A

more detailed dissection of the spinal cord was then done and the tissues were carefully placed on a solution of sucrose at 30 % (w/v). After 24 h, 2.5-3 cm length of spinal cord tissues, centered on the lesion, were involved in frozen section medium (Neg-50, Thermo Scientific, USA), frozen with liquid nitrogen and stored at -20 °C. Later, longitudinal sections of 10 µm thickness were performed using a Leica CM1900 cryostat.

Immunohistochemistry (IHC)

Spinal cord longitudinal sections were initially permeabilized with 0.2 % PBS-T for 10 min. Then, the slides were blocked with a solution of 5 % FBS in PBS for 30 min. After that, the samples were incubated overnight with the following primary antibodies: i) mouse anti-CD11b/c (Pharmingen, USA); ii) rabbit anti-rat GFAP (Dako, Denmark); iii) mouse anti-NF (Millipore, USA); iv) rabbit anti-tyrosine hydroxylase (TH, Millipore, USA) and v) mouse anti-nuclei antibody (HuNu, Millipore, USA). On the next day, samples were incubated for 2 h with the respective secondary antibodies: alexa fluor 488 goat anti-rabbit for GFAP, alexa fluor 594 goat anti-mouse for CD11b/c, alexa fluor 488 goat anti-mouse for NF and HuNu and alexa fluor 594 goat anti-rabbit for TH (all from Invitrogen, USA). All samples were counterstained with DAPI (Sigma, USA) for 10 min. Between steps, three to five washes with PBS were performed. Finally, the slides were mounted in Immu-Mount® (Thermo Scientific, USA) and observed at a confocal point-scanning microscope, Olympus FV1000. All images were treated using ImageJ software.

Staining area analysis with ImageJ software

After obtaining micrographs of regions of the spinal cord close to the injury site through confocal microscopy, the photos were opened with the ImageJ software. Before starting the analysis, the scale was determined. 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 each marker, using the dark background as contrast. The mean value of six micrographs per animal was considered for analysis.

Supplementary Figures and Tables

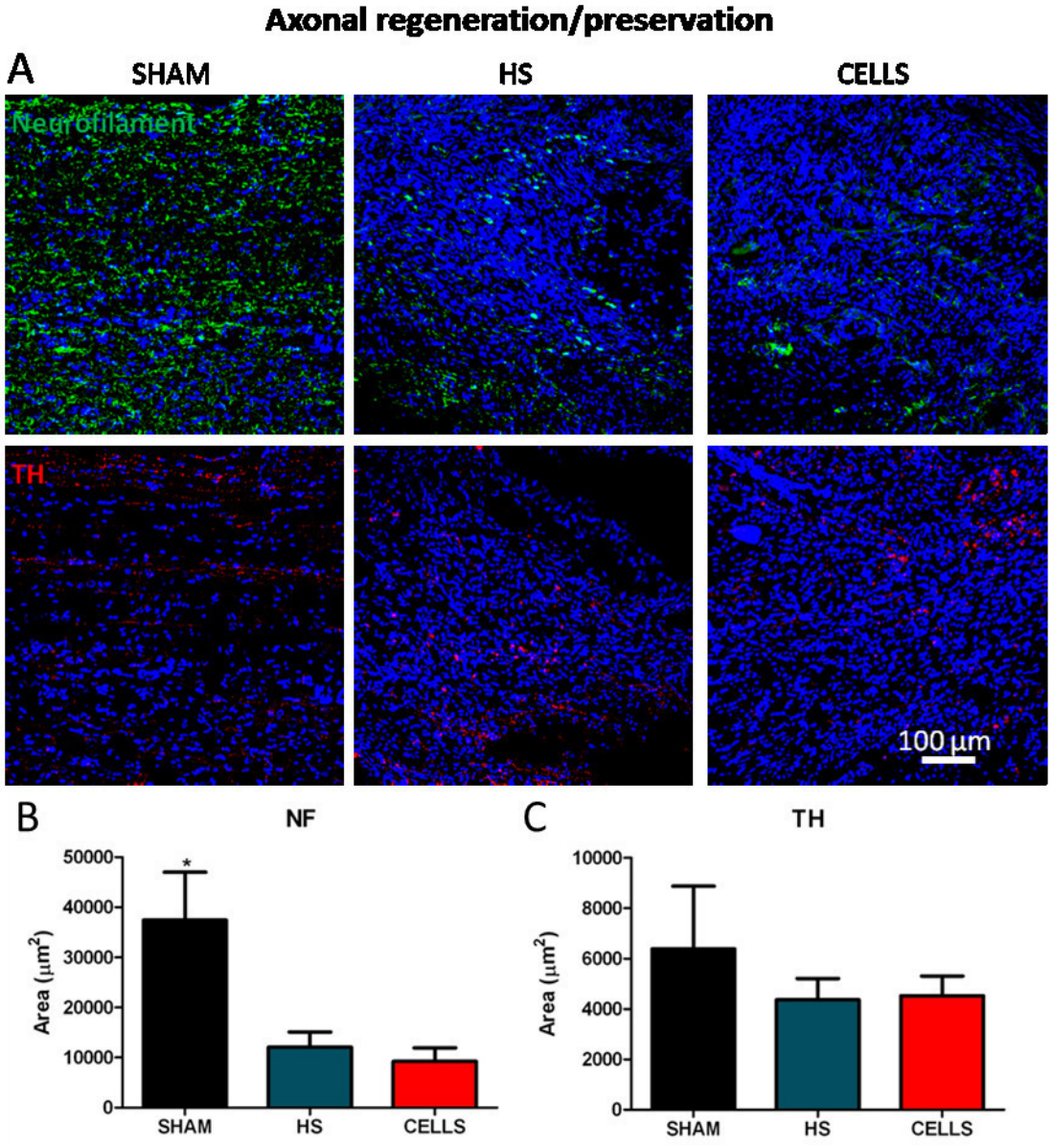

Figure S2.1 – IHC results for NF and TH markers. (A) Representative confocal images of spinal cord longitudinal sections, immunostained for NF (neurofilament, upper panel) and TH (tyrosine hydroxylase, lower panel). (B) Quantification of NF and TH markers' area. Injured animals (HS group) and cells-treated animals have a significant reduction of NF levels; no differences were observed in TH expression. Values are shown as mean ± SEM (n=5); * $p<0.05$.

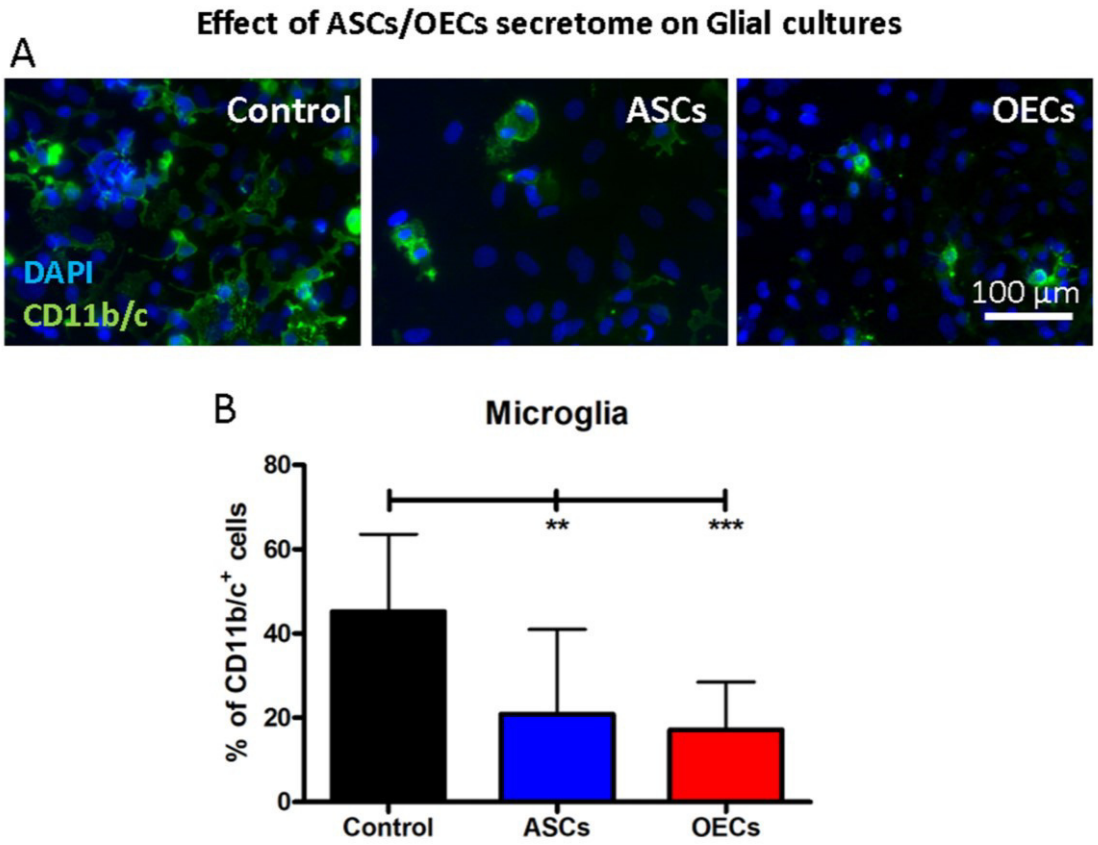

Figure S2.2 – Effect of ASCs' and OECs' secretome on glial cultures. (A) – Representative fluorescence microscopy images of glial cultures stained for CD11b/c (microglia). (B) – Average percentage of CD11b/c positive cells per field for each group: ASCs and OECs secretome and control medium. Values are shown as mean ± SD (n=5 replicates); ** $p<0.01$; *** $p<0.001$.

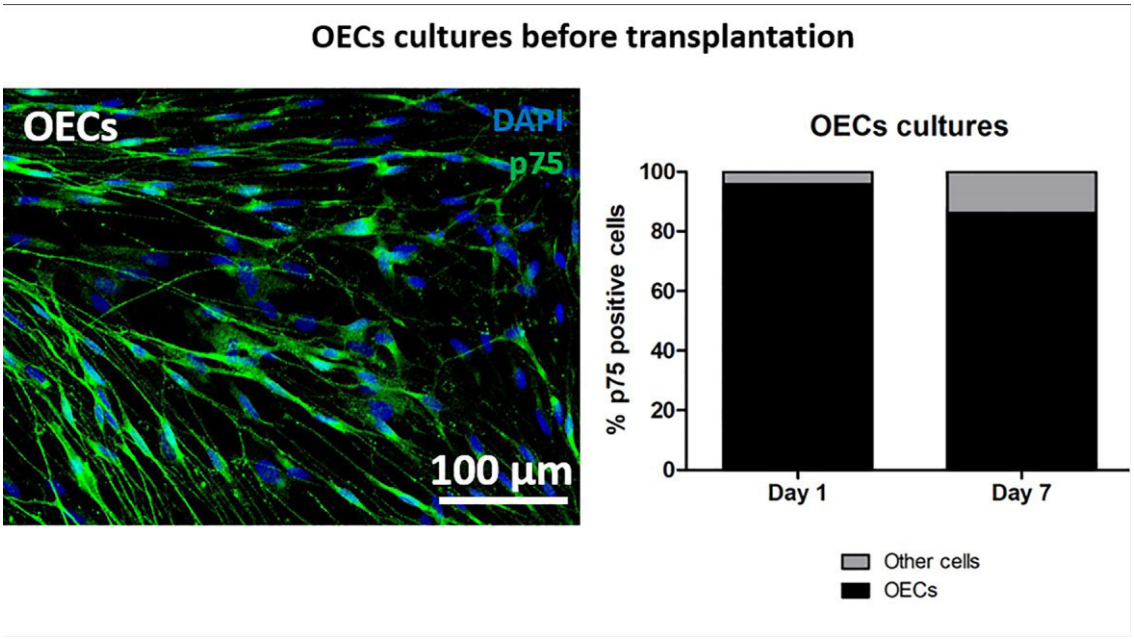

Figure S2.3 – Cultures of OECs after 7 days stained for p75, one of the main markers for OECs. These cultures present high purity levels, above 85% p75 positive cells. Scale: 100 µm.

Supplementary Table 2.1 – PANTHER overrepresentation test focused on the most representative Reactome pathways. PANTHER overrepresentation analysis was performed using all the proteins identified in ASCs secretome (Pires et al., 2016) [34] against the entire human database, by applying a Binomial distribution test with Bonferroni correction for multiple comparisons. Reactome pathways are "hierarchically" clustered and sorted by the Fold enrichment value of the most specific subclass of each cluster (in bolt). For each enriched pathway, it is presented: the name; the number of proteins in the reference list (the human database), in the samples analyzed (ASCs secretome), and the number of expected proteins; the fold enrichment (ASCs secretome/expected) and the respective adjusted p-value. The cluster associated with neurite outgrowth is highlighted. # - indicates the number of the cluster.

#	Reactome pathways	No. proteins				P-value
		Ref.	ASCs Secretome	Expected		
1	**Crosslinking of collagen fibrils (R-HSA-2243919)**	**11**	**5**	**0.16**	**32.1**	**1.15E-03**
	Assembly of collagen fibrils and other multimeric structures (R-HSA-2022090)	54	17	0.76	22.23	1.66E-14
	Collagen formation (R-HSA-1474290)	88	22	1.25	17.65	2.99E-17
	Extracellular matrix organization (R-HSA-1474244)	292	51	4.14	12.33	1.13E-35
2	**Anchoring fibril formation (R-HSA-2214320)**	**15**	**5**	**0.21**	**23.54**	**5.20E-03**
3	Scavenging by Class A Receptors (R-HSA-3000480)	19	6	0.27	22.3	7.10E-04
	Binding and Uptake of Ligands by Scavenger Receptors (R-HSA-2173782)	69	9	0.98	9.21	1.50E-03
	Vesicle-mediated transport (R-HSA-5653656)	598	25	8.47	2.95	3.48E-03
4	**Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs) (R-HSA-381426)**	**21**	**6**	**0.3**	**20.18**	**1.26E-03**
	Metabolism of proteins (R-HSA-392499)	1338	46	18.95	2.43	4.86E-05
5	**RHO GTPases activate PAKs (R-HSA-5627123)**	**21**	**6**	**0.3**	**20.18**	**1.26E-03**
6	**Collagen biosynthesis and modifying enzymes (R-HSA-1650814)**	**67**	**19**	**0.95**	**20.02**	**1.28E-15**
7	**Collagen degradation (R-HSA-1442490)**	**64**	**18**	**0.91**	**19.86**	**1.25E-14**
	Degradation of the extracellular matrix (R-HSA-1474228)	137	30	1.94	15.46	1.17E-22
8	**Smooth Muscle Contraction (R-HSA-445355)**	**33**	**9**	**0.47**	**19.26**	**3.06E-06**
9	**ECM proteoglycans (R-HSA-3000178)**	**75**	**20**	**1.06**	**18.83**	**4.93E-16**

10	**Syndecan interactions (R-HSA-3000170)**	**27**	**7**	**0.38**	**18.31**	**2.83E-04**
	Non-integrin membrane-ECM interactions (R-HSA-3000171)	59	13	0.84	15.56	1.01E-08
11	**Defective B3GALT6 causes EDSP2 and SEMDJL1 (R-HSA-4420332)**	**20**	**5**	**0.28**	**17.65**	**2.07E-02**
	Diseases associated with glycosaminoglycan metabolism (R-HSA-3560782)	39	7	0.55	12.67	3.21E-03
	Diseases of glycosylation (R-HSA-3781865)	139	13	1.97	6.6	2.54E-04
12	**Defective B3GAT3 causes JDSSDHD (R-HSA-3560801)**	**20**	**5**	**0.28**	**17.65**	**2.07E-02**
13	**Defective B4GALT7 causes EDS, progeroid type (R-HSA-3560783)**	**20**	**5**	**0.28**	**17.65**	**2.07E-02**
14	**Detoxification of Reactive Oxygen Species (R-HSA-3299685)**	**32**	**8**	**0.45**	**17.65**	**4.82E-05**
15	**Integrin cell surface interactions (R-HSA-216083)**	**85**	**19**	**1.2**	**15.78**	**9.41E-14**
16	**Platelet degranulation (R-HSA-114608)**	**126**	**28**	**1.78**	**15.69**	**3.61E-21**
	Response to elevated platelet cytosolic Ca2+ (R-HSA-76005)	131	28	1.86	15.09	1.01E-20
	Platelet activation, signaling and aggregation (R-HSA-76002)	275	31	3.89	7.96	2.88E-15
	Hemostasis (R-HSA-109582)	590	35	8.36	4.19	2.84E-09
17	**NCAM1 interactions (R-HSA-419037)**	**42**	**8**	**0.59**	**13.45**	**3.75E-04**
	NCAM signaling for neurite out-growth (R-HSA-375165)	260	18	3.68	4.89	9.55E-05
	Axon guidance (R-HSA-422475)	546	37	7.73	4.79	1.17E-11
	Developmental Biology (R-HSA-1266738)	806	39	11.41	3.42	6.07E-08
18	**Gluconeogenesis (R-HSA-70263)**	**32**	**6**	**0.45**	**13.24**	**1.39E-02**
	Glucose metabolism (R-HSA-70326)	76	8	1.08	7.43	2.85E-02
	Metabolism of carbohydrates (R-HSA-71387)	279	15	3.95	3.8	2.53E-02
19	**Molecules associated with elastic fibers (R-HSA-2129379)**	**38**	**7**	**0.54**	**13.01**	**2.71E-03**
	Elastic fiber formation (R-HSA-1566948)	45	8	0.64	12.55	6.28E-04
20	**Activation of Matrix Metalloproteinases (R-HSA-1592389)**	**33**	**6**	**0.47**	**12.84**	**1.65E-02**
21	**EPH-Ephrin signaling (R-HSA-2682334)**	**93**	**9**	**1.32**	**6.83**	**1.64E-02**
22	**L1CAM interactions (R-HSA-373760)**	**115**	**10**	**1.63**	**6.14**	**1.32E-02**
23	**Signaling by PDGF (R-HSA-186797)**	**354**	**19**	**5.01**	**3.79**	**1.87E-03**

Supplementary Table 2.2 – Complete list of proteins found (total 206 proteins) in ASCs secretome (24h of conditioning period).

Protein name	Accession number
10 kDa heat shock protein, mitochondrial	P61604
14-3-3 protein beta/alpha	P31946
14-3-3 protein zeta/delta	P63104
40S ribosomal protein S28	P62857
45 kDa calcium-binding protein	Q9BRK5
72 kDa type IV collagenase	P08253
78 kDa glucose-regulated protein	P11021
A disintegrin and metalloproteinase with thrombospondin motifs 2	O95450
Actin, cytoplasmic 1	P60709
Actin, cytoplasmic 2	P63261
Acyl-CoA-binding protein	P07108
ADAMTS-like protein 1	Q8N6G6
Adipocyte enhancer-binding protein 1	Q8IUX7
ADM	P35318
ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	Q10588
Alpha-actinin-1	P12814
Alpha-actinin-4	O43707
Alpha-enolase	P06733
Antithrombin-III	P01008
Basement membrane-specific heparan sulfate proteoglycan core protein	P98160
Beta-1,4-galactosyltransferase 1	P15291
Beta-1,4-galactosyltransferase 5	O43286
Beta-2-microglobulin	P61769
Biglycan	P21810
Biotinidase	P43251
Bone morphogenetic protein 1	P13497
Brain acid soluble protein 1	P80723
Cadherin-11	P55287
Cadherin-2	P19022
Caldesmon	Q05682
Calmodulin-1	P62158
Calsyntenin-1	O94985
Calumenin	O43852
Cathepsin B	P07858
Cathepsin Z	Q9UBR2
CD166 antigen	Q13740
Clusterin	P10909
Cofilin-1	P23528
Coiled-coil domain-containing protein 80	Q76M96
Collagen alpha-1(I) chain	P02452
Collagen alpha-1(III) chain	P02461
Collagen alpha-1(IV) chain	P02462

Collagen alpha-1(V) chain	P20908
Collagen alpha-1(VI) chain	P12109
Collagen alpha-1(VIII) chain	P27658
Collagen alpha-1(XI) chain	P12107
Collagen alpha-1(XII) chain	Q99715
Collagen alpha-2(I) chain	P08123
Collagen alpha-2(IV) chain	P08572
Collagen alpha-2(V) chain	P05997
Collagen alpha-2(VI) chain	P12110
Collagen alpha-3(VI) chain	P12111
Collagen triple helix repeat-containing protein 1	Q96CG8
Complement C1r subcomponent	P00736
Complement C1s subcomponent	P09871
Connective tissue growth factor	P29279
C-type lectin domain family 11 member A	Q9Y240
Cystatin-B	P04080
Cystatin-C	P01034
Cytochrome c	P99999
Decorin	P07585
Dermatopontin	Q07507
Dickkopf-related protein 3	Q9UBP4
Dystroglycan	Q14118
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	Q13822
EGF-containing fibulin-like extracellular matrix protein 1	Q12805
EMILIN-1	Q9Y6C2
Endosialin	Q9HCU0
Epididymal secretory protein E1	P61916
Extracellular matrix protein 1	Q16610
Fibrillin-1	P35555
Fibrillin-2	P35556
Fibronectin	P02751
Fibulin-1	P23142
Fibulin-5	Q9UBX5
Filamin-A	P21333
Follistatin-related protein 1	Q12841
Fructose-bisphosphate aldolase A	P04075
Galectin-1	P09382
Galectin-3-binding protein	Q08380
Gamma-glutamyl hydrolase	Q92820
Ganglioside GM2 activator	P17900
Glia-derived nexin	P07093
Heat shock protein beta-1	P04792
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626
Histone H2B type 1-B	P33778
Histone H2B type 1-C/E/F/G/I	P62807
Histone H2B type 1-D	P58876

Histone H2B type 1-H	Q93079
Histone H2B type 1-J	P06899
Histone H2B type 1-K	O60814
Histone H2B type 1-L	Q99880
Histone H2B type 1-M	Q99879
Histone H2B type 1-N	Q99877
Histone H2B type 1-O	P23527
Histone H2B type 2-E	Q16778
Histone H2B type 2-F	Q5QNW6
Histone H2B type 3-B	Q8N257
Histone H2B type F-S	P57053
Hyaluronan and proteoglycan link protein 1	P10915
Hyaluronan and proteoglycan link protein 3	Q96S86
Hyaluronan and proteoglycan link protein 4	Q86UW8
Immunoglobulin superfamily containing leucine-rich repeat protein	O14498
Inhibin beta A chain	P08476
Insulin-like growth factor-binding protein 3	P17936
Insulin-like growth factor-binding protein 4	P22692
Insulin-like growth factor-binding protein 6	P24592
Insulin-like growth factor-binding protein 7	Q16270
Interstitial collagenase	P03956
Katanin p60 ATPase-containing subunit A1	O75449
Keratin, type II cytoskeletal 1	P04264
Laminin subunit gamma-1	P11047
Latent-transforming growth factor beta-binding protein 2	Q14767
LIM and SH3 domain protein 1	Q14847
L-lactate dehydrogenase A chain	P00338
Lumican	P51884
Macrophage migration inhibitory factor	P14174
Mannan-binding lectin serine protease 1	P48740
Matrix metalloproteinase-14	P50281
Matrix remodeling-associated protein 8	Q9BRK3
Metalloproteinase inhibitor 1	P01033
Metalloproteinase inhibitor 2	P16035
Microfibrillar-associated protein 5	Q13361
Moesin	P26038
Myosin light chain 1/3, skeletal muscle isoform	P05976
Myosin light chain 3	P08590
Myosin light polypeptide 6	P60660
Myristoylated alanine-rich C-kinase substrate	P29966
Neural cell adhesion molecule 2	O15394
Neuroblast differentiation-associated protein AHNAK	Q09666
Neuroblastoma suppressor of tumorigenicity 1	P41271
Nucleobindin-1	Q02818
Nucleobindin-2	P80303
Olfactomedin-like protein 3	Q9NRN5

Out at first protein homolog	Q86UD1
Pentraxin-related protein PTX3	P26022
Peptidase inhibitor 16	Q6UXB8
Peptidyl-prolyl cis-trans isomerase A	P62937
Peptidyl-prolyl cis-trans isomerase B	P23284
Periostin	Q15063
Peroxidasin homolog	Q92626
Peroxiredoxin-1	Q06830
Peroxiredoxin-2	P32119
Phosphatidylethanolamine-binding protein 1	P30086
Pigment epithelium-derived factor	P36955
Plasma protease C1 inhibitor	P05155
Plasminogen activator inhibitor 1	P05121
Plectin	Q15149
Polyubiquitin-B	P0CG47
Polyubiquitin-C	P0CG48
Pregnancy-specific beta-1-glycoprotein 4	Q00888
Pregnancy-specific beta-1-glycoprotein 5	Q15238
Procollagen C-endopeptidase enhancer 1	Q15113
Profilin-1	P07737
Protein CutA	O60888
Protein disulfide-isomerase A3	P30101
Protein FAM3C	Q92520
Protein S100-A11	P31949
Protein S100-A4	P26447
Protein/nucleic acid deglycase DJ-1	Q99497
Protein-lysine 6-oxidase	P28300
Putative trypsin-6	Q8NHM4
Reticulocalbin-1	Q15293
Reticulocalbin-3	Q96D15
Retinoic acid receptor responder protein 2	Q99969
Semaphorin-7A	O75326
Serine protease 23	O95084
Serpin H1	P50454
Serum albumin	P02768
SH3 domain-binding glutamic acid-rich-like protein 3	Q9H299
Small ubiquitin-related modifier 2	P61956
Small ubiquitin-related modifier 3	P55854
Small ubiquitin-related modifier 4	Q6EEV6
Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	A1L4H1
SPARC	P09486
Spondin-2	Q9BUD6
Stathmin	P16949
Sulfhydryl oxidase 1	O00391
Superoxide dismutase [Cu-Zn]	P00441
Testican-1	Q08629

Tetranectin	P05452
Thioredoxin	P10599
Thioredoxin domain-containing protein 5	Q8NBS9
Thrombospondin-1	P07996
Transforming growth factor-beta-induced protein ig-h3	Q15582
Transgelin	Q01995
Transgelin-2	P37802
Triosephosphate isomerase	P60174
Tropomyosin alpha-1 chain	P09493
Tropomyosin alpha-3 chain	P06753
Tropomyosin alpha-4 chain	P67936
Tropomyosin beta chain	P07951
Trypsin-1	P07477
Trypsin-2	P07478
Tubulin alpha-1A chain	Q71U36
Tubulin alpha-1B chain	P68363
Tubulin alpha-1C chain	Q9BQE3
Tubulin-specific chaperone A	O75347
Tyrosine-protein kinase receptor UFO	P30530
Ubiquitin carboxyl-terminal hydrolase isozyme L1	P09936
Ubiquitin-40S ribosomal protein S27a	P62979
Ubiquitin-60S ribosomal protein L40	P62987
Vasorin	Q6EMK4
Versican core protein	P13611
Vimentin	P08670
V-type proton ATPase subunit S1	Q15904

Supplementary Table 2.3 – Complete list of proteins found (total 450 proteins) in OECs secretome (24h of conditioning period). Database: 1- Manually annotated proteins; 2- Rat reference proteome.

Protein Name	Accession number	Database
14-3-3 protein beta/alpha	P35213	1
14-3-3 protein eta	P68511	1
14-3-3 protein gamma	P61983	1
14-3-3 protein theta	P68255	1
14-3-3 protein zeta/delta	P63102	1
26S proteasome non-ATPase regulatory subunit 11	F1LMZ8	1
40S ribosomal protein S10	P63326	1
40S ribosomal protein S11	P62282	1
40S ribosomal protein S13	P62278	1
40S ribosomal protein S14	P13471	1
40S ribosomal protein S15	P62845	1
40S ribosomal protein S15a	P62246	1
40S ribosomal protein S16	P62250	1
40S ribosomal protein S17	P04644	1
40S ribosomal protein S18	P62271	1
40S ribosomal protein S19	P17074	1
40S ribosomal protein S2	P27952	1
40S ribosomal protein S25	P62853	1
40S ribosomal protein S28	P62859	1
40S ribosomal protein S3	P62909	1
40S ribosomal protein S3a	P49242	1
40S ribosomal protein S4, X isoform	P62703	1
40S ribosomal protein S7	P62083	1
40S ribosomal protein S8	P62243	1
40S ribosomal protein S9	P29314	1
40S ribosomal protein SA	P38983	1
45 kDa calcium-binding protein	Q91ZS3	1
60S acidic ribosomal protein P0	P19945	1
60S ribosomal protein L10a	P62907	1
60S ribosomal protein L12	P23358	1
60S ribosomal protein L15	P61314	1
60S ribosomal protein L27	P61354	1
60S ribosomal protein L5	P09895	1
60S ribosomal protein L6	P21533	1
6-phosphogluconate dehydrogenase, decarboxylating	P85968	1
72 kDa type IV collagenase	P33436	1
78 kDa glucose-regulated protein	P06761	1
Aa1018 - vitronectin	Q7TQ11	2
Ab2-450	Q7TP34	2
Acid ceramidase	Q6P7S1	1
Acid sphingomyelinase-like phosphodiesterase 3a	Q641Z7	1
Acidic leucine-rich nuclear phosphoprotein 32 family member A	P49911	1

Acidic leucine-rich nuclear phosphoprotein 32 family member B	Q9EST6	1
Aconitate hydratase, mitochondrial	Q9ER34	1
Actin, alpha skeletal muscle	P68136	1
Actin, cytoplasmic 2	P63259	1
Acylamino-acid-releasing enzyme	P13676	1
Acyl-CoA-binding protein	P11030	1
Adapter molecule crk	Q63768	1
Adenylate kinase 2, mitochondrial	P29410	1
Adenylyl cyclase-associated protein 1	Q08163	1
Adipocyte enhancer-binding protein 1	A2RUV9	1
ADP-ribosylation factor 5	P84083	1
AHNAK nucleoprotein	A0A0G2JUA5	2
A-kinase anchor protein 12	Q5QD51	1
Alcohol dehydrogenase [NADP(+)]	P51635	1
Alcohol dehydrogenase class-3	P12711	1
Aldose 1-epimerase	Q66HG4	1
Aldose reductase	P07943	1
Alpha 4 type V collagen	Q9JI04	2
Alpha glucosidase 2 alpha neutral subunit (Predicted)	D3ZAN3	2
Alpha-2 antiplasmin	Q80ZA3	2
Alpha-2-macroglobulin	P06238	1
Alpha-actinin-1	Q9Z1P2	1
Alpha-actinin-4	Q9QXQ0	1
Alpha-centractin	P85515	1
Alpha-enolase	P04764	1
Alpha-mannosidase	Q6P762	2
Alpha-N-acetylgalactosaminidase	Q66H12	1
Aminoacylase-1A	Q6AYS7	1
Aminopeptidase	F1M9V7	2
Aminopeptidase-like 1	D4A3E2	2
Amyloid-beta A4 protein	P08592	1
Angiopoietin-like 2	G3V862	2
Annexin A5	P14668	1
Annexin A6	P48037	1
ARP1 actin-related protein 1 homolog B	B2RYJ7	2
Arylsulfatase A	Q32KK2	2
Arylsulfatase B	P50430	1
Asparaginyl-tRNA synthetase	Q4KLM9	2
Aspartate aminotransferase, cytoplasmic	P13221	1
Aspartate aminotransferase, mitochondrial	P00507	1
Aspartyl aminopeptidase	Q4V8H5	2
Astrocytic phosphoprotein PEA-15	Q5U318	1
ATP synthase subunit beta, mitochondrial	P10719	1
Attractin	Q99J86	1
Axl receptor tyrosine kinase	E9PSY0	2
Beta-2-microglobulin	P07151	1

Beta-galactosidase	D3ZUM4	2
Beta-glucuronidase	P06760	1
Beta-hexosaminidase subunit alpha	Q641X3	1
Beta-hexosaminidase subunit beta	Q6AXR4	1
Beta-mannosidase	Q4FZV0	1
Biglycan	P47853	1
Biliverdin reductase B	B5DF65	2
Bleomycin hydrolase	P70645	1
Branched-chain-amino-acid aminotransferase, cytosolic	P54690	1
Cadherin-2	Q9Z1Y3	1
Calmodulin-3	P0DP31	1
Calponin-3	P37397	1
Calreticulin	P18418	1
Calretinin	P47728	1
Calsyntenin-1	Q6Q0N0	1
Calumenin	O35783	1
Canopy 2 homolog (Zebrafish)	A0JN30	2
Carbonic anhydrase 2	P27139	1
Carbonic anhydrase 3	P14141	1
Carboxypeptidase E	P15087	1
Carboxypeptidase Q	Q6IRK9	1
Catenin beta-1	Q9WU82	1
Cathepsin B	P00787	1
Cathepsin D	P24268	1
Cathepsin L1	P07154	1
CD9 antigen	P40241	1
Cell adhesion molecule L1-like	M0RC17	2
Cell surface glycoprotein MUC18	Q9EPF2	1
Ceruloplasmin	P13635	1
Chloride intracellular channel protein 1	Q6MG61	1
Chloride intracellular channel protein 4	Q9Z0W7	1
Chordin-like 2	F1LW58	2
Citrate synthase, mitochondrial	Q8VHF5	1
Clathrin heavy chain 1	P11442	1
Coactosin-like protein	B0BNA5	1
Coatomer subunit delta	Q66H80	1
Cofilin-1	P45592	1
Collagen alpha-1(I) chain	P02454	1
Collagen alpha-1(III) chain	P13941	1
Collagen alpha-1(V) chain	Q9JI03	1
Collagen alpha-1(XI) chain	P20909	1
Collagen alpha-1(XII) chain	A0A0G2KAJ7	2
Collagen alpha-2(I) chain	P02466	1
Collagen type IV alpha 1 chain	F1MA59	2
Collagen type IV alpha 2 chain	F1M6Q3	2
Collagen type V alpha 2 chain	F1LQ00	2

Collagen type VIII alpha 1 chain	D4AC70	2
Collagen type VIII alpha 2 chain	D4ADG9	2
Collagen type XVIII alpha 1 chain	F1LR02	2
Complement C1q tumor necrosis factor-related protein 5	Q5FVH0	1
Complement component 1 Q subcomponent-binding protein, mitochondrial	O35796	1
Connective tissue growth factor	Q9R1E9	1
COP9 signalosome complex subunit 4	Q68FS2	1
Core histone macro-H2A.1	Q02874	1
Creatine kinase B-type	P07335	1
Crk-like protein	Q5U2U2	1
Cullin-associated NEDD8-dissociated protein 1	P97536	1
Cysteine and glycine-rich protein 1	P47875	1
Cysteine and glycine-rich protein 2	Q62908	1
Cytochrome c, somatic	P62898	1
Cytokine receptor-like factor 1	D3ZIV9	2
Cytoplasmic aconitate hydratase	Q63270	1
Cytosol aminopeptidase	Q68FS4	1
Cytosolic non-specific dipeptidase	Q6Q0N1	1
D-dopachrome decarboxylase	P80254	1
Deoxyribonuclease-2-alpha	Q9QZK8	1
Destrin	Q7M0E3	1
Dihydrofolate reductase	Q920D2	1
Dihydrolipoyl dehydrogenase, mitochondrial	Q6P6R2	1
Dihydropyrimidinase-related protein 2	P47942	1
Dihydropyrimidinase-related protein 3	Q62952	1
Di-N-acetylchitobiase	Q01460	1
Dipeptidyl peptidase 2	Q9EPB1	1
Dipeptidyl peptidase 3	O55096	1
DNA topoisomerase 1	Q9WUL0	1
DNA-(apurinic or apyrimidinic site) lyase	P43138	1
Dynactin subunit 2	Q6AYH5	1
Dystroglycan 1	F1M8K0	2
Ectonucleotide pyrophosphatase/phosphodiesterase family member 5	P84039	1
EF-hand domain-containing protein D2	Q4FZY0	1
Elongation factor 1-alpha 1	P62630	1
Elongation factor 1-gamma	Q68FR6	1
Elongation factor 2	P05197	1
Elongin-B	P62870	1
Endoplasmic reticulum resident protein 29	P52555	1
Endoplasmin	Q66HD0	1
Enolase-phosphatase E1	Q5PPH0	1
Eukaryotic translation elongation factor 1 beta 2	B5DEN5	2
Eukaryotic translation initiation factor 5	Q07205	1
Eukaryotic translation initiation factor 5A-1	Q3T1J1	1
Extracellular matrix protein 1	Q62894	1

Extracellular superoxide dismutase [Cu-Zn]	Q08420	1
Ezrin	P31977	1
F-actin-capping protein subunit beta	Q5XI32	1
Fascin	P85845	1
Fatty acid-binding protein, epidermal	P55053	1
Ferritin heavy chain	P19132	1
Ferritin light chain 1	P02793	1
Fibronectin	P04937	1
Fibulin 2	G3V6X1	2
Filamin A	C0JPT7	2
Filamin-C	D3ZHA0	1
Follistatin-related protein 1	Q62632	1
Fructose-bisphosphate aldolase A	P05065	1
G3BP stress granule assembly factor 1	D3ZYS7	2
Galectin-1	P11762	1
Gamma-enolase	P07323	1
Glia-derived nexin	P07092	1
Glial fibrillary acidic protein	P47819	1
Glucose-6-phosphate isomerase	Q6P6V0	1
Glutamate dehydrogenase 1, mitochondrial	P10860	1
Glutathione reductase (Fragment)	P70619	1
Glutathione S-transferase alpha-4	P14942	1
Glutathione S-transferase Mu 1	P04905	1
Glutathione S-transferase P	P04906	1
Glyceraldehyde-3-phosphate dehydrogenase	P04797	1
Glypican-1	P35053	1
GM2 ganglioside activator	Q6IN37	2
Golgi apparatus protein 1	Q62638	1
Granulin, isoform CRA_c	G3V8V1	2
Growth factor receptor-bound protein 2	P62994	1
Guanine deaminase	Q9WTT6	1
Haloacid dehalogenase-like hydrolase domain-containing protein 2	Q6AYR6	1
Heat shock 70 kDa protein 4	O88600	1
Heat shock cognate 71 kDa protein	P63018	1
Heat shock protein HSP 90-alpha	P82995	1
Heat shock protein HSP 90-beta	P34058	1
Hemoglobin subunit beta-1	P02091	1
Hepatoma-derived growth factor	Q8VHK7	1
Heterogeneous nuclear ribonucleoprotein A/B	Q9QX81	2
Heterogeneous nuclear ribonucleoprotein A1	P04256	1
Heterogeneous nuclear ribonucleoprotein A3	Q6URK4	1
Heterogeneous nuclear ribonucleoprotein C	G3V9R8	1
Heterogeneous nuclear ribonucleoprotein D0	Q9JJ54	1
Heterogeneous nuclear ribonucleoprotein Q	Q7TP47	1
High mobility group protein B1	P63159	1
High mobility group protein B2	P52925	1

Histone H1.4	P15865	1
Histone H2A type 1-F	Q64598	1
Histone H2A.Z	P0C0S7	1
Histone H2B type 1	Q00715	1
Histone H4	P62804	1
Hsc70-interacting protein	P50503	1
Importin 5	D4A781	2
Importin 7	D4AE96	2
Importin subunit beta-1	P52296	1
Inhibin beta A chain	P18331	1
Insulin-like growth factor binding protein 7, isoform CRA_b	F1M9B2	2
Insulin-like growth factor-binding protein 2	P12843	1
Insulin-like growth factor-binding protein 3	P15473	1
Insulin-like growth factor-binding protein 5	P24594	1
Isocitrate dehydrogenase [NADP] cytoplasmic	P41562	1
Lactoylglutathione lyase	Q6P7Q4	1
Lamin A, isoform CRA_b	G3V8L3	2
Lamin-B1	P70615	1
Laminin subunit gamma 1	F1MAA7	2
Latent transforming growth factor beta binding protein 2, isoform CRA_b	F1M7L7	2
Leukotriene A-4 hydrolase	P30349	1
LIM and SH3 domain protein 1	Q99MZ8	1
L-lactate dehydrogenase A chain	P04642	1
Lupus La protein homolog	P38656	1
Lysosomal alpha-glucosidase	Q6P7A9	1
Lysyl oxidase homolog 2	B5DF27	1
Macrophage migration inhibitory factor	P30904	1
Macrophage-capping protein	Q6AYC4	1
Major vault protein	Q62667	1
Malate dehydrogenase, cytoplasmic	O88989	1
Malate dehydrogenase, mitochondrial	P04636	1
Mammalian ependymin-related protein 1	Q5XII0	1
Matrilin 2	F1LXS3	2
Matrix metallopeptidase 19	C0M4B0	2
Matrix remodeling-associated protein 8	Q5XI43	1
Mesencephalic astrocyte-derived neurotrophic factor	P0C5H9	1
Metalloproteinase inhibitor 1	P30120	1
Metalloproteinase inhibitor 2	P30121	1
Meteorin	Q5Q0T9	1
Meteorin-like protein	Q5RJL6	1
Microtubule-associated protein 1B	P15205	1
Microtubule-associated protein 4	Q5M7W5	1
Microtubule-associated protein RP/EB family member 1	Q66HR2	1
Moesin	O35763	1
Myosin light polypeptide 6	Q64119	1

Myosin regulatory light chain 12B	P18666	1
Myosin-9	Q62812	1
N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase	P30919	1
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	1
N-acetylgalactosamine-6-sulfatase	Q32KJ6	1
N-acetylglucosamine-6-sulfatase	Q32KJ5	2
NAD(P)H dehydrogenase [quinone] 1	P05982	1
NAD(P)H-hydrate epimerase	B0BNM1	1
NADP-dependent malic enzyme	P13697	1
Nardilysin	P47245	1
Nestin	P21263	1
Neural cell adhesion molecule 1	P13596	1
Neurocan core protein	P55067	1
Neurofilament medium polypeptide	P12839	1
NHL repeat-containing 3	D4A2F6	2
Nidogen-1 (Fragment)	P08460	1
Non-muscle caldesmon	Q62736	1
NPC intracellular cholesterol transporter 2	F7FJQ3	2
NSFL1 cofactor p47	O35987	1
Nuclear migration protein nudC	Q63525	1
Nuclease-sensitive element-binding protein 1	P62961	1
Nucleobindin-1	Q63083	1
Nucleolin	P13383	1
Nucleophosmin	P13084	1
Nucleoside diphosphate kinase A	Q05982	1
Nucleoside diphosphate kinase B	P19804	1
Nucleosome assembly protein 1-like 1	Q9Z2G8	1
Nucleosome assembly protein 1-like 4	Q5U2Z3	1
Omega-amidase NIT2	Q497B0	1
Out at first protein homolog	Q6AYE5	1
Palmitoyl-protein thioesterase 1	P45479	1
PDZ and LIM domain protein 5	Q62920	1
Peptidyl-prolyl cis-trans isomerase	Q6AYQ9	2
Peptidyl-prolyl cis-trans isomerase A	P10111	1
Peptidyl-prolyl cis-trans isomerase B	P24368	1
Peptidyl-prolyl cis-trans isomerase D	Q6DGG0	1
Peptidyl-prolyl cis-trans isomerase FKBP1A	Q62658	1
Periostin	D3ZAF5	2
Peroxidasin	A0A0G2JWB6	2
Peroxiredoxin-1	Q63716	1
Peroxiredoxin-2	P35704	1
Peroxiredoxin-5, mitochondrial	Q9R063	1
Peroxiredoxin-6	O35244	1
Phosphatidylethanolamine-binding protein 1	P31044	1
Phosphoglycerate kinase 1	P16617	1
Phosphoglycerate mutase 1	P25113	1

Phosphoserine aminotransferase	E9PSV5	2
Plasminogen activator inhibitor 1	P20961	1
Plasminogen activator inhibitor 1 RNA-binding protein	Q6AXS5	1
Platelet-derived growth factor subunit A	P28576	1
Plectin	P30427	1
Pleiotrophin	P63090	1
Polyadenylate-binding protein 1	Q9EPH8	1
Prelamin-A/C	P48679	1
Pro-cathepsin H	P00786	1
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Q63321	1
Profilin-1	P62963	1
Programmed cell death 6-interacting protein	Q9QZA2	1
Prosaposin	P10960	1
Prostaglandin F2 receptor negative regulator	Q62786	1
Prostaglandin reductase 1	P97584	1
Proteasome activator complex subunit 1	Q63797	1
Proteasome subunit alpha type-1	P18420	1
Proteasome subunit alpha type-2	P17220	1
Proteasome subunit alpha type-3	P18422	1
Proteasome subunit alpha type-4	P21670	1
Proteasome subunit alpha type-6	P60901	1
Proteasome subunit alpha type-7	P48004	1
Proteasome subunit beta type-1	P18421	1
Proteasome subunit beta type-2	P40307	1
Proteasome subunit beta type-3	P40112	1
Proteasome subunit beta type-4	P34067	1
Proteasome subunit beta type-5	P28075	1
Proteasome subunit beta type-6	P28073	1
Proteasome subunit beta type-7	Q9JHW0	1
Protein disulfide-isomerase	P04785	1
Protein disulfide-isomerase A3	P11598	1
Protein disulfide-isomerase A4	P38659	1
Protein disulfide-isomerase A6	Q63081	1
Protein kinase C substrate 80K-H	B1WC34	2
Protein S100-A11	Q6B345	1
Protein S100-A4	P05942	1
Protein S100-A6	P05964	1
Protein S100-B	P04631	1
Protein SET	Q63945	1
Protein tyrosine kinase 7	D3ZHG3	2
Protein/nucleic acid deglycase DJ-1	O88767	1
Purine nucleoside phosphorylase	P85973	1
Putative hydroxypyruvate isomerase	F1LZJ4	2
Putative phospholipase B-like 2	Q4QQW8	1
Pyridoxal kinase	O35331	1
Pyruvate kinase PKM	P11980	1

Rab GDP dissociation inhibitor alpha	P50398	1
Rab GDP dissociation inhibitor beta	P50399	1
RAN GTPase-activating protein 1	F1MAA5	2
Ras-related protein Rab-11A	P62494	1
Ras-related protein Rab-1A	Q6NYB7	1
Ras-related protein Rap-1b	Q62636	1
RCG43947	D3ZZC1	2
RCG55135, isoform CRA b	G3V852	2
Rcn3 protein	I6L9G5	2
Receptor of activated protein C kinase 1	P63245	1
Receptor-type tyrosine-protein phosphatase zeta	Q62656	1
Regulator of chromosome condensation 2	F1LVV4	2
Reticulocalbin 1	D3ZUB0	2
Reticulocalbin-2	Q62703	1
Reticulon-4	Q9JK11	1
Rho GDP-dissociation inhibitor 1	Q5XI73	1
RNA-binding motif protein, X chromosome	Q4V898	1
RNA-binding motif protein, X chromosome retrogene-like	P84586	1
RNA-binding protein 3	Q925G0	1
Secernin-1	Q6AY84	1
Secreted frizzled-related protein 1	F1LLX7	2
Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (Semaphorin) 7A (Predicted)	D3ZQP6	2
Semaphorin 3B	D3ZHJ3	2
Serine protease 23	Q6AY61	1
Serine protease HTRA1	Q9QZK5	1
Serpin H1	P29457	1
SH3 domain-binding glutamic acid-rich-like protein	B5DFD8	2
Sorbitol dehydrogenase	P27867	1
SPARC	P16975	1
SPARC-like protein 1	P24054	1
Spectrin alpha chain, non-erythrocytic 1	P16086	1
Spectrin beta chain	G3V6S0	2
Spondin-1	Q9GLX9	1
Stathmin	P13668	1
Stress-induced-phosphoprotein 1	O35814	1
Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	B2GV06	1
Sulfhydryl oxidase 1	Q6IUU3	1
Superoxide dismutase [Cu-Zn]	P07632	1
Superoxide dismutase [Mn], mitochondrial	P07895	1
Syntenin-1	Q9JI92	1
T-complex protein 1 subunit gamma	Q6P502	1
Tenascin C	A0A0G2K1L0	2
Tetraspanin	Q6P9V1	2
Thimet oligopeptidase	P24155	1
Thioredoxin	P11232	1

Thioredoxin domain-containing 17	B0K010	2
Thioredoxin domain-containing protein 12	Q498E0	1
Thioredoxin reductase 1, cytoplasmic	O89049	1
Threonine–tRNA ligase, cytoplasmic	Q5XHY5	1
Tissue-type plasminogen activator	P19637	1
Transaldolase	Q9EQS0	1
Transcobalamin 2, isoform CRA_a	G3V6K1	2
Transcobalamin-2	Q9R0D6	1
Transcriptional activator protein Pur-beta	Q68A21	1
Transforming growth factor, beta-induced	D4A8G5	2
Transgelin	P31232	1
Transgelin-2	Q5XFX0	1
Transitional endoplasmic reticulum ATPase	P46462	1
Transketolase	P50137	1
Translationally-controlled tumor protein	P63029	1
Transmembrane protein 2	D3ZZ19	2
Triosephosphate isomerase	P48500	1
Tripeptidyl-peptidase 1	Q9EQV6	1
Tripeptidyl-peptidase 2	Q64560	1
Tropomyosin 1, alpha, isoform CRA_i	A0A0G2JX64	2
Tropomyosin alpha-1 chain	P04692	1
Tropomyosin alpha-3 chain	Q63610	1
Tropomyosin alpha-4 chain	P09495	1
TROVE domain family, member 2	D3ZRN5	2
Tubulin alpha-1B chain	Q6P9V9	1
Tubulin beta-2B chain	Q3KRE8	1
Tubulin beta-5 chain	P69897	1
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q00981	1
Ubiquitin-60S ribosomal protein L40	P62986	1
Ubiquitin-conjugating enzyme E2 N	Q9EQX9	1
Ubiquitin-conjugating enzyme E2 variant 2	Q7M767	1
Ubiquitin-like modifier-activating enzyme 1	Q5U300	1
Ubiquitinyl hydrolase 1	D3ZVQ0	2
UMP-CMP kinase	Q4KM73	1
UV excision repair protein RAD23 homolog B	Q4KMA2	1
Versican core protein (Fragments)	Q9ERB4	1
Vimentin	P31000	1
Vinculin	P85972	1
Vitamin K-dependent protein S	P53813	1
WD repeat-containing protein 1	Q5RKI0	1
Xaa-Pro aminopeptidase 1	O54975	1
Xaa-Pro dipeptidase	Q5I0D7	1
Zyxin	D4A7U1	2

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Chapter 3

Combination of a Peptide-modified Gellan Gum Hydrogel with Cell Therapy in a lumbar Spinal Cord Injury Animal Model

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Combination of a Peptide-modified Gellan Gum Hydrogel with Cell Therapy in a lumbar Spinal Cord Injury Animal Model

Eduardo D. Gomes,[a,b] Sofia S. Mendes,[a,b] Hugo Leite-Almeida,[a,b]
Jeffrey M. Gimble,[c] Roger Y. Tam,[d,e] Molly S. Shoichet,[d,e] Nuno Sousa,[a,b]
Nuno A. Silva,[a,b] António J. Salgado[a,b,*]

[a] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[b] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

[c] Center for Stem Cell Research and Regenerative Medicine, Tulane University, 1430 Tulane Avenue, SL-99, New Orleans, LA 70112, USA

[d] Department of Chemical Engineering and Applied Chemistry, 200 College St., University of Toronto, Toronto, Ontario M5S 3E5, Canada

[e] Institute of Biomaterials and Biomedical Engineering, 160 College St., University of Toronto, Toronto, Ontario M5S 3E1, Canada

* Corresponding author: A. J. Salgado, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: asalgado@ecsaude.uminho.pt; Tel: +351 253 60 49 47; Fax: +351 253 60 48 20

Abstract

Spinal Cord Injury (SCI) is a highly incapacitating condition for which there is still no cure. Current clinical approaches are mainly based on palliative care, so there is a need to find possible treatments to SCI. Cellular transplantation is regarded with great expectation due to the therapeutic potential of cells such as Adipose tissue-derived Stromal/Stem Cells (ASCs) or Olfactory Ensheathing Cells (OECs). Both are accessible sources and present positive paracrine and cell-to-cell interactions, previously reported by our group. Additionally, biomaterials such as hydrogels have been applied in SCI repair with promising results. We propose to combine a GRGDS-modified gellan gum hydrogel with ASCs and OECs in order to promote SCI regeneration. *In vitro*, ASCs and OECs could be co-cultured within GG-GRGDS hydrogels inducing a more robust neurite outgrowth when compared to controls. *In vivo* experiments in a hemisection SCI rat model revealed that the administration of ASCs and OECs encapsulated in a GG-GRGDS hydrogel led to significant motor improvements when compared to both control (SCI) and hydrogel alone (GG-GRGDS) groups. This was accompanied by a decreased infiltration of inflammatory cells and astrocytes, and by an increased intensity of neurofilament. These results suggest evident gains induced by the encapsulation of ASCs and OECs in GG-GRGDS based hydrogels.

Keywords

Spinal Cord Injury; Regenerative Medicine; Adipose tissue-derived Stromal/Stem Cells; Olfactory Ensheathing Cells; Extracellular Matrix like Hydrogels

1. Introduction

Spinal Cord Injury (SCI) is a highly debilitating condition for which there is still no cure. SCI individuals usually have life-long loss of function and reduced quality of life. Its incidence internationally varies from as low as 2.3 to as high as 83 per million inhabitants every year [1]. The secondary events occurring after the primary injury increase the complexity of the disease, which hinders SCI treatment [2]. The current medical approaches after a spinal cord trauma are limited, consisting of the stabilization of the spine, decompression of the cord and eventually the administration of anti-inflammatory drugs [2]. Therefore, the development of novel therapeutic strategies targeting this condition is imperative.

Amongst the different approaches suggested so far, cellular based therapies have been one of the most frequently explored. From the different sources of cells currently being tested, Adipose tissue-derived Stromal/Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) have shown promising results [3, 4]. For instance, intraspinal transplantation of murine ASCs in a SCI animal model, one week after injury, promoted the protection of denuded axons probably by preventing oligodendrocytes' degeneration and by participating in the regeneration of the myelin sheath [5]. In addition, ASCs transplantation also induced evident gains in motor performance [5]. These beneficial outcomes have been mostly related with the nature of the ASC's secretome, that is, the panel of molecules secreted by these cells to the extracellular milieu. In fact several reports have shown that ASC's secretome contains important neuroregulatory molecules such as Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Glial cell line-derived Neurotrophic Factor (GDNF), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), basic Fibroblast Growth Factor (bFGF), Insulin-like Growth Factor 1 (IGF-1), Transforming Growth Factor Beta 1 (TGF-β1), among others, that are able to modulate neuronal and glial survival and differentiation [6-8]. Moreover, the molecules secreted by ASCs have also been shown to modulate the response of the immune system [7, 9]. Alternatively, OECs are mainly characterized by participating in the growth and guidance of primary olfactory neurons. Their common origin with Schwann cells may explain some similarities observed between these two cell types, namely the capacity of OECs to surround olfactory axons, form fascicular processes and synthesize peripheral-like myelin [10]. The potential OEC transplantation as a therapy for CNS damage has already been explored *in vivo*. About two thirds of the experimental studies using these cells reported improvements in behavior outcome [11]. For instance, murine

OECs were able to remyelinate axons in spinal cord injured rats [12, 13], leading also to functional improvement of electric conduction in previously demyelinated axons [13]. Therefore, it is considered that OECs can create a permissive environment for axonal regeneration, in the usually hostile milieu of the damaged CNS [14]. For all these reasons, autologous transplantation of OECs in SCI patients has already been performed. In one clinical trial, results showed that autologous OECs are safe after three years post-transplantation [15, 16].

ASCs and OECs present themselves as promising candidates for SCI cell therapy, mostly because they are easily accessible (ASCs can be obtained in large quantities from lipoaspirates while OECs can be safely isolated from nasal biopsies) and can be applied in an autologous manner, avoiding ethical concerns and the need for immunosuppression. By combining both, we envision taking advantage of the beneficial properties of each cell type simultaneously; namely, the neuronal regeneration guided and supported by OECs, which can be boosted by the paracrine effects of ASCs. Furthermore, previous work from our group showed that the secretome of rat-derived OECs has a positive effect on MSCs from different sources, but more evidently on human ASCs by increasing their proliferation and metabolic activity. Similarly, the secretome of ASCs also proved to be particularly beneficial for OECs in the same parameters [17]. These results reinforce the potential benefits of the combined use of these cells.

Therefore, the objective of the present work was to assess if the combined delivery of human ASCs and murine OECs into the injury site of a rat lumbar hemisection model, was able to induce motor and histological improvements in the injured rats. For this purpose and in order to efficiently deliver both cell populations, a hydrogel-based biomaterial (gellan gum, chemically grafted with a fibronectin-mimetic GRGDS peptide – GG-GRGDS) was used. Hydrogels are particularly appealing to be used as vehicles for cell transplantation because not only can they enhance cell survival, but they can also be designed to match the mechanical properties and water content of the CNS [18-21]. Our proposed chemically-modified gellan gum hydrogel is a biocompatible and biodegradable natural polysaccharide composed of repeating units of glucose, glucuronic acid and rhamnose [22, 23] and is FDA approved [24]. Its physical properties allow the injection *in situ* and the filling of cavities provoked by the injury, in a minimally invasive manner. Moreover when chemically grafted with the fibronectin-mimetic peptide GRGDS (GG-GRGDS), this biomaterial was shown to have enhanced cell adhesion and proliferation [25, 26], which lead to significant improvements of the metabolic activity and secretome of encapsulated cells [25, 26].

2. Materials and Methods

2.1 Cell isolation and culture

Human Adipose tissue-derived Stromal/Stem Cells (ASCs) were isolated according to the protocol described by Dubois *et al.* [27] from human lipoaspirates obtained from consenting donors under an institutional review board approved protocol at LaCell LLC. These cells were cultured and maintained in α-MEM (Invitrogen, USA), with 10 % Fetal Bovine Serum (FBS, Biochrom AG, Germany) and 1 % antibiotic-antimycotic solution – penicillin-streptomycin (Invitrogen, USA) at 37 °C and 5 % CO_2 (v/v).

The animal care committee of the research institute approved all the animal protocols in accordance with standardized animal care guidelines [28]. Olfactory Ensheathing Cells (OECs) were harvested from olfactory bulbs of neonatal (P5-P7) Wistar-Han rats, according to the protocol previously described [29]. Briefly, upon dissection, the meninges and blood vessels were removed and the tissue was digested with collagenase type I (2.5 mg/ml, Sigma, USA) for 30 min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5 ml pipette and centrifuged at 1000 rpm for 5 min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation, cells were resuspended and seeded on uncoated plates for two consecutive periods of 24 h. It is expected that most of the fibroblasts and astrocytes attach in the first and second periods, respectively. After this purification step, the remaining cells were seeded on fibronectin coated surfaces (for 2D direct co-cultures) or encapsulated in GG-GRGDS hydrogel (for 3D co-cultures). Coating was done overnight with a 1 mg/ml fibronectin solution (Sigma, USA). Cells were cultured in DMEM/F12 (Invitrogen, USA) with 10 % FBS and 1 % antibiotic-antimycotic solution at 37 °C and 5 % CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5.36 µg/ml, Invitrogen, USA) and Forskolin (1.4 µg/ml, Sigma, USA).

2.2 2D direct 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 and purification (described in section **2.1**), OECs were seeded (60 000 cells/cm^2) on fibronectin coated coverslips. 24 h later, 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 showed its suitability for the culture of ASCs (data not shown). Medium was changed once, after three days of culture. Following one and seven days of incubation both cell growth and morphology was assessed by immunocytochemistry (ICC). OECs (60 000 cells/cm^2) and ASCs (10 000 cells/cm^2) monocultures were used as controls. Cell counts were performed by taking ten representative micrographs per sample, and the mean number of cells per field was determined for each sample of each group (n=3).

2.3 Synthesis of GG-GRGDS hydrogel

The synthesis of GG-GRGDS hydrogel was performed according to the protocols described by Silva *et al.*[25]. Briefly, gellan gum (Sigma, USA) was first dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5, Sigma, USA) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Sigma, USA) and furfurylamine (Acros Organics, Belgium) were then added in a 4:1 M ratio (of each reagent relative to the -COOH groups in gellan gum) and stirred at 37 °C for 48 h. The solution was then dialyzed (Mw cutoff 12-14 kDa, Spectrum Labs, USA) alternately against distilled water and PBS (0.1 M, pH 7.2) for five days. Finally, water was removed by lyophilization to obtain furan-modified gellan gum (furan-GG) as a white powder.

Immobilization of maleimide-modified GRGDS peptide (mal-GRGDS, Anaspec, USA) to furan-GG was performed via Diels-Alder chemistry between the maleimide functional group of the peptide with the furan group of the gellan gum [25]. Furan-GG was first dissolved in MES buffer (100 mM, pH 5.5) at 37 °C (1.2 mg/ml). Mal-GRGDS was then added in a 5:1 maleimide:furan molar ratio and vigorously stirred for 48 h. The solution was then dialyzed (Mw cutoff 12-14 kDa) alternately against distilled water and PBS (0.1 M, pH 7.2) for five days. Finally, the water was removed by lyophilization to obtain GRGDS-modified Gellan Gum (GG-GRGDS) as a white powder. The amount of peptide immobilized on the hydrogel was calculated by amino acid analysis. In brief, this method involved acid hydrolysis of the peptide with 6 N HCl for 24 h, followed by derivatization with phenylisothiocyanate (PITC). The derivatized hydrolyzates were then quantified using reverse phase HPLC. As a control, a known amount of mal-GRGDS was incubated with unmodified gellan gum, and the peptide concentration was quantified by amino acid analysis.

2.4 GG-GRGDS 3D hydrogel preparation

GG-GRGDS lyophilized powder was sterilized by exposure to UV lights for 15 min, a method previously used without affecting the material properties [26]. Then, GG-GRGDS was dissolved in ultra pure water, at 1 % (w/v) concentration and heated at 40 °C overnight, in order to obtain a homogenous solution. Before encapsulating the cells, $CaCl_2$ at 0.3 % (w/v) was added [to obtain a final concentration of 0.03 % (w/v) of $CaCl_2$ in solution] to enable the ionic crosslinking of the hydrogel. GG-GRGDS hydrogel used for the *in vitro* 3D cultures was mixed with unmodified GG (1 % w/v) in a 1:1 ratio, while for the *in vivo* studies only the modified hydrogel was used.

2.5 3D cell culture – 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 in a 3D environment. Moreover, this experiment was also crucial to determine if the hydrogel was suitable for the growth of both cell types and a proper vehicle for cellular transplantation.

OECs and ASCs were obtained as described in **2.1** and GG-GRGDS hydrogel was prepared according to **2.4**. Both cell types were resuspended in complete DMEM/F12 medium and encapsulated into the hydrogel, by mixing the pellets with the appropriate volume of hydrogel. A total of 30 000 ASCs and 30 000 OECs were encapsulated in 100 µl of GG-GRGDS and monocultures of each cell type were used as controls, using the same cellular densities as co-cultures. Cells were allowed to grow for seven days, with medium changes every two days. After seven days of incubation, phalloidin and DAPI were used to assess cellular growth and morphology. Immunocytochemistry against human nuclear antigen (HNA) was used to specifically identify ASCs. Samples were analyzed by confocal microscopy using a confocal point-scanning microscope, Olympus FV1000.

2.6 3D cultures – DRG explants in co-culture with GG-GRGDS/ASCs-OECs

Dorsal Root Ganglia (DRG) explant organotypic cultures can be used as a model of axonal regeneration as they are able to produce extensive neurite outgrowth. The explants were

dissected from the cervical and thoracic regions of the spine of neonatal rat pups (P5) and placed in cold HBSS (1x) without Ca^{2+}/Mg^{2+} (Invitrogen, USA). After cleaning the remnants of peripheral nerve processes, DRGs were placed on the top of GG-GRGDS hydrogels with or without encapsulated cells. Four groups were performed: 1) hydrogel alone; 2) hydrogel + ASCs; 3) hydrogel + OECs; and 4) hydrogel + ASCs/OECs. The encapsulation of cells was performed 24 h before DRG isolation and co-culture. A total of 30 000 cells were encapsulated in 50 µl of GG-GRGDS (15 000 ASCs and 15 000 OECs in co-culture group). In the first 24 h cells were maintained in complete DMEM/F12 medium to promote cellular growth. After DRGs isolation and seeding onto GG-GRGDS hydrogels, the medium was changed to neurobasal medium (Invitrogen, USA) supplemented with 6 mg/ml D-glucose (Sigma, USA), 1 % penicillin-streptomycin, 1x B27 (Invitrogen, USA) and 2 mM L-Glutamine (Invitrogen, USA). Medium was changed every two days and cultures were kept in a humidified atmosphere at 37 °C, 5 % CO_2 (v/v). Following seven days of co-culture, DRGs were fixed and subjected to an immunocytochemistry for neurofilament, a major component of axonal cytoskeleton. Imaging was performed using an Olympus IX81 fluorescence microscope and images were analyzed using the ImageJ software (NIH).

2.7 Immunocytochemistry (ICC) and phalloidin/DAPI staining

ICC protocols were used to identify OECs/ASCs in 2D and 3D co-cultures, as well as DRGs neurite projections in 3D co-cultures. Rabbit anti-NGF receptor (p75) antibody (1:100, Millipore, USA) was used to identify OECs and mouse anti-Human Nuclear Antigen antibody (HuNu, 1:100, Millipore, USA) for ASCs. Mouse anti-neurofilament 200 kDa antibody (1:200, Millipore, USA) was used to identify neurite outgrowth from DRG explants. Controls were made by the omission of the appropriate primary antibody. Briefly, samples were fixed with a solution of 4 % paraformaldehyde (PFA, Panreac, Spain) in PBS for 20 min (in 2D cultures) or 45 min (in 3D cultures) at room temperature (RT). Then, after three washes with PBS, cell membranes were permeabilized (with exception of p75 antibody) with 0.3 % Triton X-100 (Sigma, USA) for 5 min (2D) or 10 min (3D). Non-specific binding sites were blocked using a solution of 10 % Fetal Calf Serum (FCS, Biochrom AG, Germany) in PBS for 1 h (2D) or 1 h 30 min (3D). The respective primary antibodies were then added for 1 h (2D) or 48 h (3D), after which cells were exposed to the specific secondary antibody for 1 h (2D) or 18 h (3D). Alexa Fluor 488 goat anti-rabbit was

used for OECs and Alexa Fluor 488 goat anti-mouse for ASCs and DRG neurites (both from Invitrogen, USA). Cell nuclei were then counterstained with 1 μg/ml of DAPI (Invitrogen, USA) for 15 min (2D) or 30 min (3D). Imaging was performed using an Olympus BX61 fluorescence microscope.

Phalloidin and DAPI staining protocol was performed in 3D co-cultures of ASCs and OECs. Cells were subjected to a 45 min period of fixation with 4 % PFA at RT. Then, cell membranes were permeabilized with 0.3 % Triton X-100 for 10 min and the cells were washed three times with PBS. A PBS solution with 0.1 μg/ml phalloidin (Sigma, USA) and 1 μg/ml DAPI (Invitrogen, USA) was then added to the cells for 45 min at RT. Imaging was performed using a confocal point-scanning microscope, Olympus FV1000.

2.8 DRGs neurite extension analysis with Image J software

After obtaining the micrographs of DRG explant cultures through fluorescence microscopy, the pictures were opened with the Image J software. Firstly, the scale was defined and then, with the help of drawing tools, the area occupied by the neurite arborization was shaped, as well as the area occupied by the DRG. After the software automatically calculated both areas, the area occupied by the DRG was subtracted from the total area of the neurite arborization, in order to obtain only the area occupied by the arborization extension. To calculate the length of the longest neurites Neurite-J plugin was used [30]. Briefly, with this plugin we defined the area of the DRG body and then concentric rings with a 25 μm interval were automatically created. The length of the longest neurite was defined as the length of the last ring intersecting neurites (fig. 3.1).

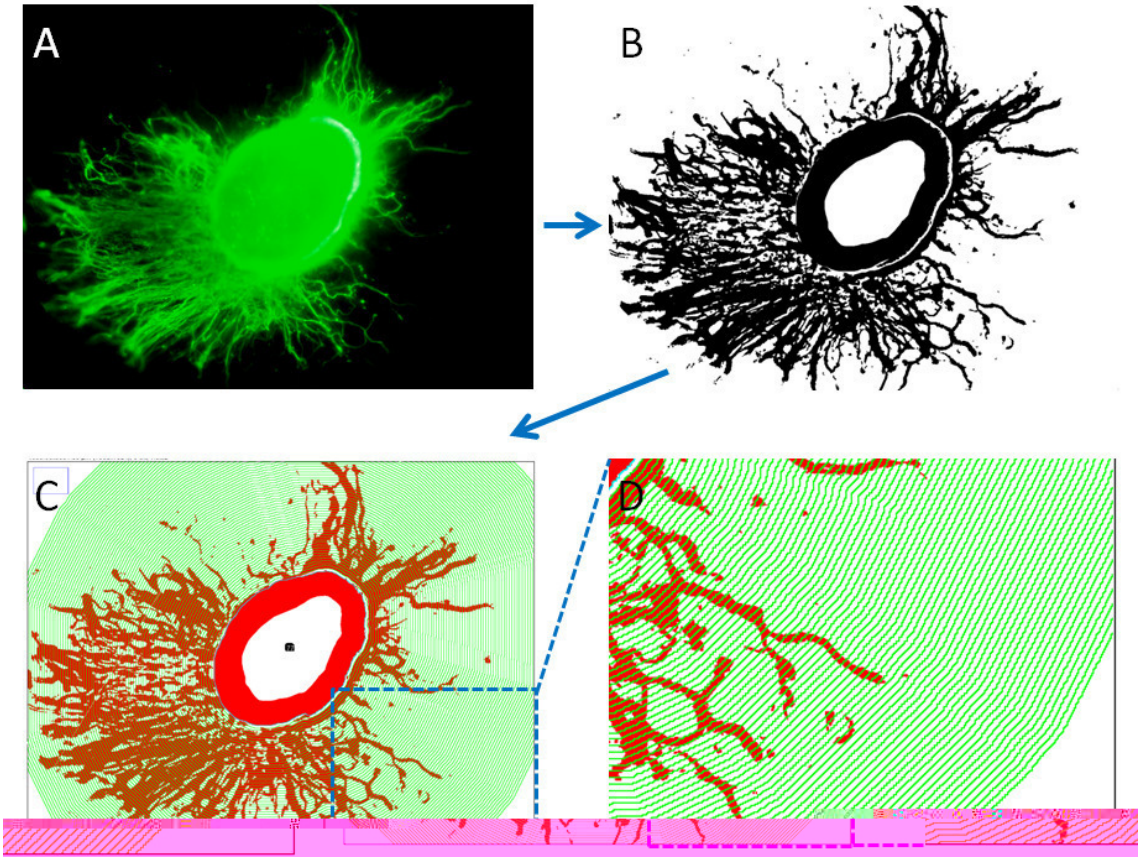


Figure 3.1 – Quantification process used for determining the length of the longest neurite using the Neurite-J plugin [30] for Image J software. Briefly, the micrographs obtained after fluorescence microscopy (A) were transformed into 8-bits images (B). Then, the area occupied by the DRG body as well as the neurites were defined through threshold adjustments. Finally, the software automatically drew concentric rings with 25 µm interval (C-D) and the distance of the last ring intersecting neurites was defined as the length of the longest neurite.

2.9 *In vivo* testing

2.9.1 Animals and groups

Eight weeks old female Wistar rats (Charles River, France), housed in light and temperature controlled rooms and fed with standard diet, were used in the *in vivo* studies. Handling was performed for three days before the surgeries. Animals were divided in five different groups according to the respective treatment/procedure (initial n=5/6): 1) Animals subjected to SCI with no treatment (SCI, n=6); 2) SCI animals treated with GG-GRGDS implanted at the injury site (GG-GRGDS, n=6); 3) SCI animals transplanted with a injection of ASCs/OECs, rostral to the injury site (ASCs/OECs, n=6); 4) SCI animals treated with local administration of

ASCs and OECs encapsulated in GG-GRGDS (GG-GRGDS+ASCs/OECs, n=6); and 5) Animals with laminectomy only, no SCI (Sham, n=5). Due to health complications after the SCI, a total of seven animals died or were sacrificed during the post-operatory period, one from the SCI group (final n=5), three from the ASCs/OECs group (final n=3) and three from GG-GRGDS + ASCs/OECs group (final n=3). In the groups applicable, 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 **2.4**. Both cell types were encapsulated within the hydrogel, as described in section **2.5**. Rats treated with cells received around 60 000 ASCs and 20 000 OECs, either encapsulated in 4 µl of GG-GRGDS placed at the injury site, or through an intraspinal injection 2 mm rostral to the injury site, suspended in 4 µl of DMEM/F12.

2.9.2 Spinal Cord Injury surgery

All animals were anesthetized by intraperitoneal injection of a mixture (1.5:1) of ketamine (100 mg/ml, Imalgene/Merial, France) and medetomidine hydrochloride (1 mg/ml, Domitor/Pfizer, USA). Once anesthetized, fur was shaved from the surgical site and the skin disinfected with ethanol 70 % and chlorohexidine. Then a dorsal midline incision was made from T8-L4 and the paravertebral muscles retracted. A laminectomy was performed at L1 in which the spinous processes were removed and the spinal cord exposed. A unilateral defect (hemisection) on the left side of the spinal cord was done, removing 2-3 mm of nervous tissue. After the respective treatment, spine stabilization was performed as previously described [31]; paravertebral muscles and skin were then closed with Vicryl sutures (Johnson and Johnson, USA). The incision of SCI control animals was closed after injury without treatment.

2.9.3 Post-Operative care

Following SCI surgery, rats were kept under heat lamps and received subcutaneous injections of vitamins (Duphalyte/Pfizer, USA), 0.9 % NaCl, the anti-inflammatory drug carprofen (5 mg/ml, Rimadyl/Pfizer, USA), the analgesic butorphanol (10 mg/ml, Butomidor/Richter Pharma AG, Austria) and the antibiotic enrofloxacin (5 mg/ml, Baytril/Bayer, Germany), besides atipamezole (5 mg/ml, Antisedan/Pfizer, USA) a drug used in order to revert anesthesia. Bladder evacuation was done manually twice a day. 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 three 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 (Mucedola 4RF25, Italy) and the food was presented to the rats in the cage.

2.9.4 Motor Behavior Analysis by BBB Score

The motor behavior of all rats was assessed by the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [32] on day three post-injury and every week, starting exactly one week post-injury, up to four weeks. The test was then repeated eight weeks after injury. The BBB is a 21-point scale designed to assess hindlimb locomotor recovery following spinal cord injury. In summary, animals were placed in an open arena with no obstacles and allowed to move freely. During the 4 min of the test, two observers (blinded to the animal treatment) evaluated the animal locomotion profile, recording and attributing a score to the animal performance.

2.9.5 Activity Box Test

The activity box test was used to assess general locomotor behavior of SCI rats, by measuring the amount of rearing activity and the total distance travelled by the animals, eight weeks after injury [33]. The test was performed in a square arena (43.2 cm × 43.2 cm) with transparent acrylic walls (Med Associates Inc., USA) placed in a brightly illuminated room. Animals started the test at the arena's centre and were given 5 min to explore it. The total distance travelled in the arena and the number of rearings were automatically registered by the equipment sensors.

2.9.6 Histological analysis

Rats were deeply anesthetized by an intraperitoneal injection of 200 mg/ml sodium pentobarbital (Eutasil, Ceva Saúde Animal, Portugal) and perfused through the ascending aorta with 0.9 % NaCl followed by 4 % PFA. A rough dissection of the spine and spinal cord was performed, centered on the site of hemisection and the tissues were fixed in 4 % PFA overnight. A more detailed dissection of the spinal cord was then done and the tissues were carefully placed on a solution of saccharose at 30 % (w/v). After 24 h, 2.5-3 cm length of spinal cord tissues, centered on the lesion, were involved in frozen section medium (Neg-50, Thermo Scientific, USA), frozen with liquid nitrogen and stored at -20 °C. Later on, longitudinal and transversal cross sections of 10 and 20 μm thickness were performed using a Leica CM1900 cryostat.

2.9.7 Immunohistochemistry (IHC)

Spinal cord cross sections were initially permeabilized with 0.2 % TBS-T for 10 min. Then, the slides were blocked with a solution of 5 % FBS in PBS for 30 min. After that, the samples were incubated overnight with the following primary antibodies: i) mouse anti-CD11b/c (Pharmingen, USA); ii) rabbit anti-rat GFAP (Dako, Denmark); iii) mouse anti-neurofilament (Millipore, USA) and iv) mouse anti-nuclei antibody (HuNu, Millipore, USA). On the next day, samples were incubated for 2 h with the respective secondary antibodies, alexa fluor 488 goat anti-rabbit for GFAP, alexa fluor 594 goat anti-mouse for CD11b/c and alexa fluor 488 goat anti-mouse for neurofilament and HuNu (all from Invitrogen, USA). All samples were counterstained with DAPI (Sigma, USA) for 10 min. Between steps, 3-5 washes with PBS were performed. Finally, the slides were mounted in Immu-Mount® (Thermo Scientific, USA) and observed at a confocal point-scanning microscope, Olympus FV1000. All images were treated using ImageJ software.

2.9.8 HuNu positive cells quantification

Spinal cord sections from all animals were screened for the presence of HuNu, a marker for human cells (ASCs). In animals were this marker was detected, three micrographs per section were taken at 400x magnification, in a total of three sections per animal. Then, the total number of HuNu positive cells per image was quantified, and the average number of cells per field was calculated for each section and for each animal.

2.9.9 Antibodies' area analysis with Image J software

Six micrographs per animal were taken at the ipsilateral side of rostral and caudal regions surrounding the 3 mm gap created with the hemisection injury.

After obtaining micrographs through confocal microscopy, the photos were opened with the Image J software. Before starting the analysis, the scale was determined. 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 each marker, using the dark background as contrast. The mean value of the six micrographs per animal was considered for analysis.

2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by: 1) one way ANOVA test in results presented in Figs. 4B-C, 5C-D, 6C and 7B-C; 2) two-way ANOVA in results presented in Figs. 2B and 5A; 3) students' t-test in results from figure 8C. One or two-way ANOVA tests were followed by the Bonferroni post-hoc test. A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance.

3. Results

3.1 2D direct co-culture of Adipose tissue-derived Stromal/Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs)

In order to assess the interactions between ASCs and OECs, direct co-cultures were performed. Immunocytochemistry after seven days revealed that cellular morphology was not affected (fig. 3.2A). In fact, both cell types maintained their typical morphology, despite being in a co-culture system.

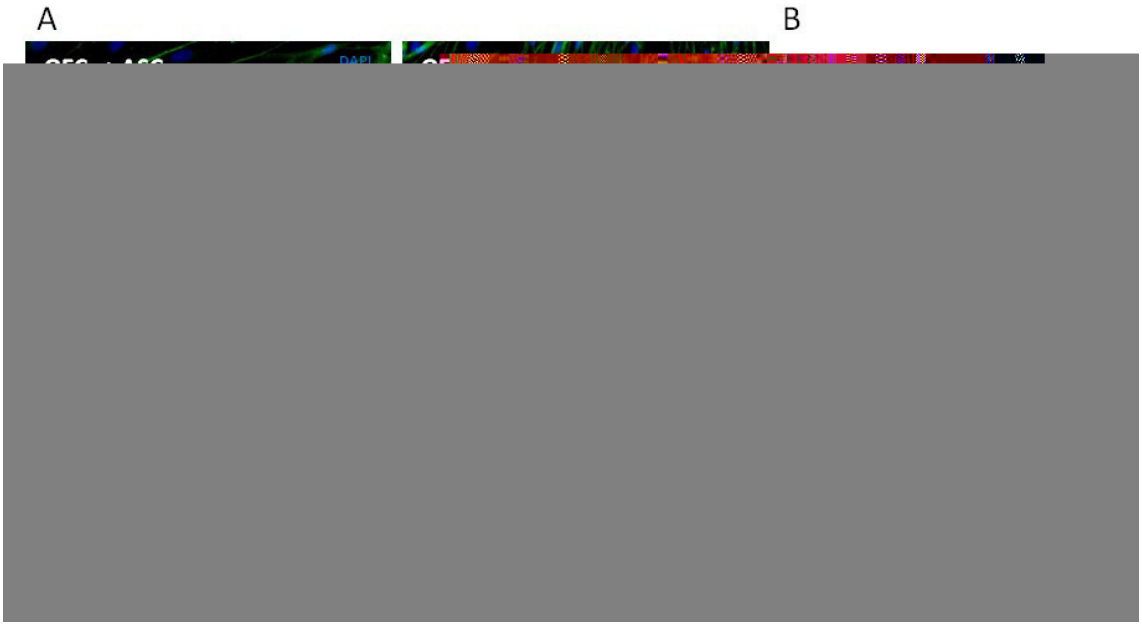


Figure 3.2 – 2D direct co-cultures of ASCs and OECs. Cellular morphology (A) after seven days was not affected by being in a co-culture system. Cell numbers (B) were increased after one day in co-culture but no differences were seen after seven days. Scale bar: 100 μm. Values are shown as mean ± SEM (n=3); *p<0.05.

Regarding cell numbers, in the first 24 h (at day 1) both cell types presented more cells in co-culture, when compared to mono-culture conditions (for ASCs: 9.7 ± 1.9 vs 3.4 ± 0.3 cells/field; for OECs: 18.7 ± 2.4 vs 9.0 ± 3,1 cells/field, p=0.063, fig. 3.2B). However, these differences disappeared after seven days of co-culture and the number of cells was similar in mono- and co-cultures (ASCs: 30.2 ± 5.6 vs 26.1 ± 18.8 cells/field; OECs: 41.0 ± 2.7 vs 48.0 ± 4.3 cells/field, fig. 3.2B). These results indicate that both cell types can grow together and may benefit from cell-cell interactions, particularly in the first hours of co-culture.

3.2 GG-GRGDS synthesis and 3D co-culture of ASCs and OECs

In order to synthesize GG-GRGDS hydrogel, we used Diels-Alder "click" chemistry to immobilize maleimide-containing GRGDS peptides with furan-modified gellan gum. Functionalization of GG with furan was previously performed by our group [25], in which the degree of furan substitution to GG was calculated to be approximately 27 %. Maleimide-modified GRGDS peptide was then reacted with furan-GG in a 5:1 molar ratio. After dialysis to remove

unbound peptide, quantification by amino acid analysis of the immobilized peptide was calculated to be 189 nmol of GRGDS per mg of GG (fig. 3.3A).

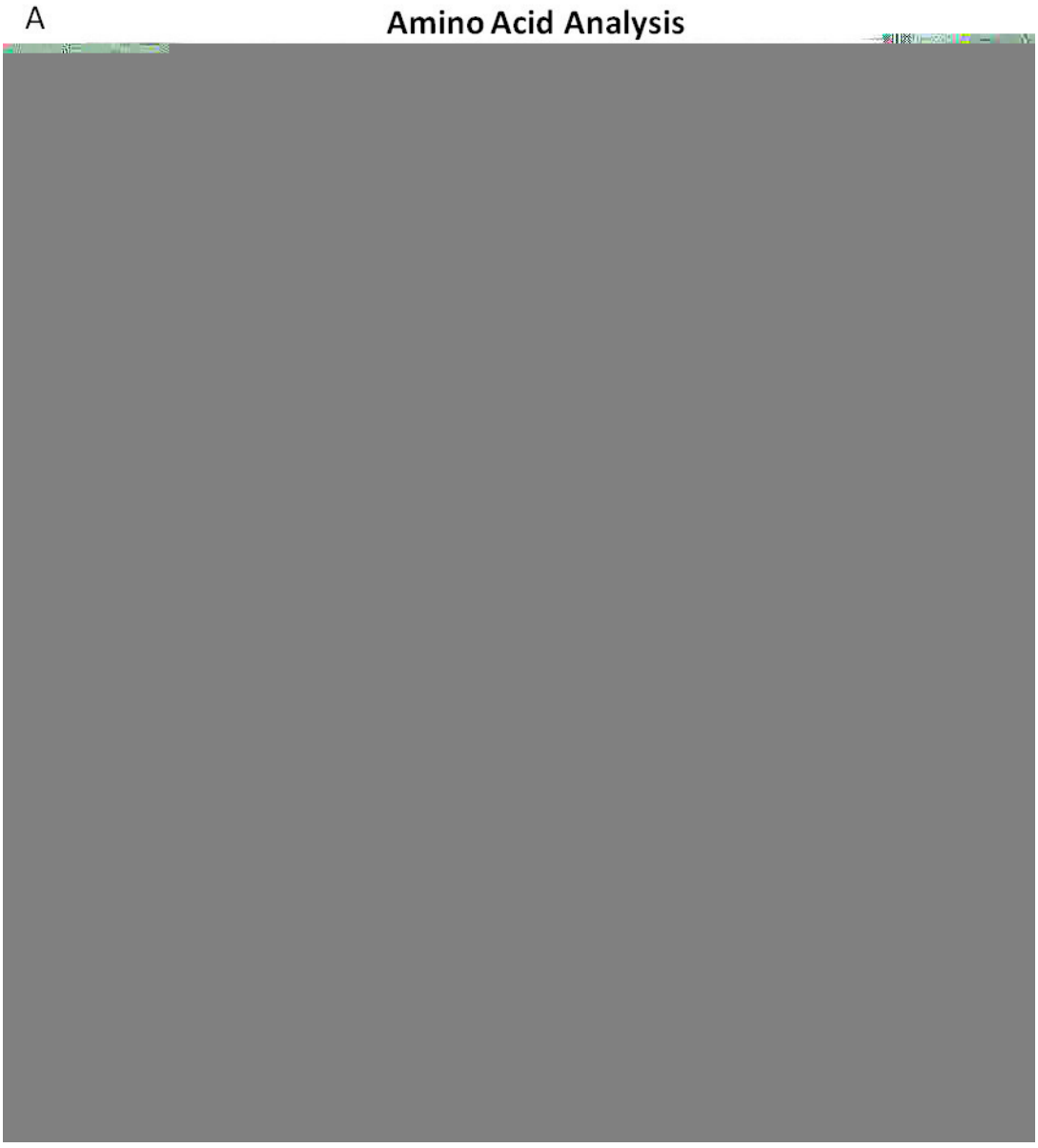


Figure 3.3 - Immobilization of the GRGDS peptide into the GG hydrogels and its effect on the growth of ASCs and OECs. (A) Amino acid analysis was used to quantify the amount of immobilized peptide into GG hydrogels (approximately 189 nmol GRGDS/mg of GG, grey arrows point to each peak). (B-E) Confocal images of ASCs and OECs cultures encapsulated in (B-D) GG-GRGDS hydrogels and (E) unmodified GG. Cells grown in GG-GRGDS presented their typical morphology either in co-cultures. In contrast, cells grown in regular GG do not show cells interacting with the hydrogels. Scale bar: 100 µm. Images are representative of n=3 independent experiments.

The encapsulation of ASCs and OECs inside the GG-GRGDS hydrogel was a critical ste to demonstrate the suitability of this biomaterial as a vehicle for cellular transplantation. In t sense, both cell types were cultured in GG-GRGDS hydrogels, either alone or in co-culture. As i visible on figures 3B-D, ASCs and OECs were able to grow within the hydrogel, extending their processes and presenting their typical morphology. The co-culture of both cell types was also successfully performed as they were evenly distributed in the hydrogel, maintaining their cellular morphology identical to monoculture conditions (fig. 3B-D). Moreover, it was possible to observe the beneficial impact of the GRGDS modification on the cell morphology, as both ASC and OECs encapsulated in regular gellan gum hydrogels were unable to extend any cellular processes (fig. 3E).

3.3 *In vitro* axonal outgrowth assessment through DRG explants

DRG explant organotypic cultures were used to evaluate the degree of axonal/neurite outgrowth promoted by ASCs and OECs when encapsulated in the GG-GRGDS hydrogel. For purpose, ASCs and/or OECs were firstly encapsulated inside GG-GRGDS hydrogels. The following a period of 24 h to induce cellular growth, DRG explants were placed on the top of hydrogels with or without encapsulated cells. After seven days of co-culture, both the DRG total neurite extension area and the length of the longest neurite were significantly increased in group combining hydrogel and ASCs ($4.8 \pm 0.7 \times 10^{6}$ µm² and 1644.4 ± 171.7 µm, respectively), in comparison to the group of hydrogel alone ($2.2 \pm 0.3 \times 10^{6}$ µm² and 1017.5 ± 79.6 µm, fig. 3.4). GG-GRGDS + ASCs group also induced a significant increase in the neurite area in comparison to GG-GRGDS + OECs group (4.8×10^{6} vs $2.7 \pm 1.4 \times 10^{6}$ µm²). The encapsulation of OECs did not translate into a significant increase of the neurite arborization obtained, since there were no differences to the group of hydrogel alone. Despite a positive trend towards an increase of neurite area and length, the group of co-culture of ASCs/OECs did not present significant differences in comparison to hydrogel alone. These results point out to a major role of ASCs on neurite induction, in this particular case of co-culture with hydrogel and DRGs. The importance of peptide engraftment in the gellan gum (GG) hydrogel was once again demonstrated. DRGs cultured on regular GG were unable to extend any neurite (fig. 3.4A). For this reason, the quantification of neurite extension and the longest neurite were not performed in this group.

Figure 3.4 – Effects of GG-GRGDS hydrogel encapsulated with ASCs and/or OECs on neurite outgrowth of DRG explants, after 7 days. (A) Representative confocal images of neurites from DRGs (stained with neurofilament) cultured in the presence or absence of cells. (B) Quantification of neurite extension area and (C) quantification of the longest neurite. GG-GRGDS with ASCs promoted a significant increase of neurite extension area in comparison to hydrogel alone and hydrogel with OECs. Moreover, hydrogel with ASCs also induced a significant increase in the length of the longest neurite, in comparison to hydrogel alone. DRGs were unable to grow on unmodified gellan gum (GG). Scale: 100 μm. Values are shown as mean ± SEM (n=8/10); * $p<0.05$, ** $p<0.01$.

3.4 *In vivo* functional and histological assessment following SCI

A left lumbar hemisection SCI model (L1 level) in eight-weeks-old Wistar female rats was used in order to assess the efficacy of the proposed therapy. Immediately after injury, animals were treated either with a transplantation of ASCs and OECs, a combination of GG-GRGDS hydrogel with ASCs/OECs, or alternatively with the hydrogel alone. Non-treated animals (HS group) or animals without injury (Sham, only subjected to laminectomy) were used as controls. At day 3 post-injury, every week up to four weeks and then one month later at eight weeks, rats were subjected to the BBB test in order to evaluate the locomotor behavior of their hindlimbs. Notably, rats treated with ASCs/OECs encapsulated in the GG-GRGDS presented significant locomotor improvements, either in comparison to non-treated rats (6.3 ± 2.6 vs 1.8 ± 0.8 at the BBB score eight weeks post-injury, for instance) or to GG-GRGDS-treated animals (6.7 ± 1.2 vs 2.8 ± 0.9 at BBB score, in the four weeks time point, fig. 3.5A).

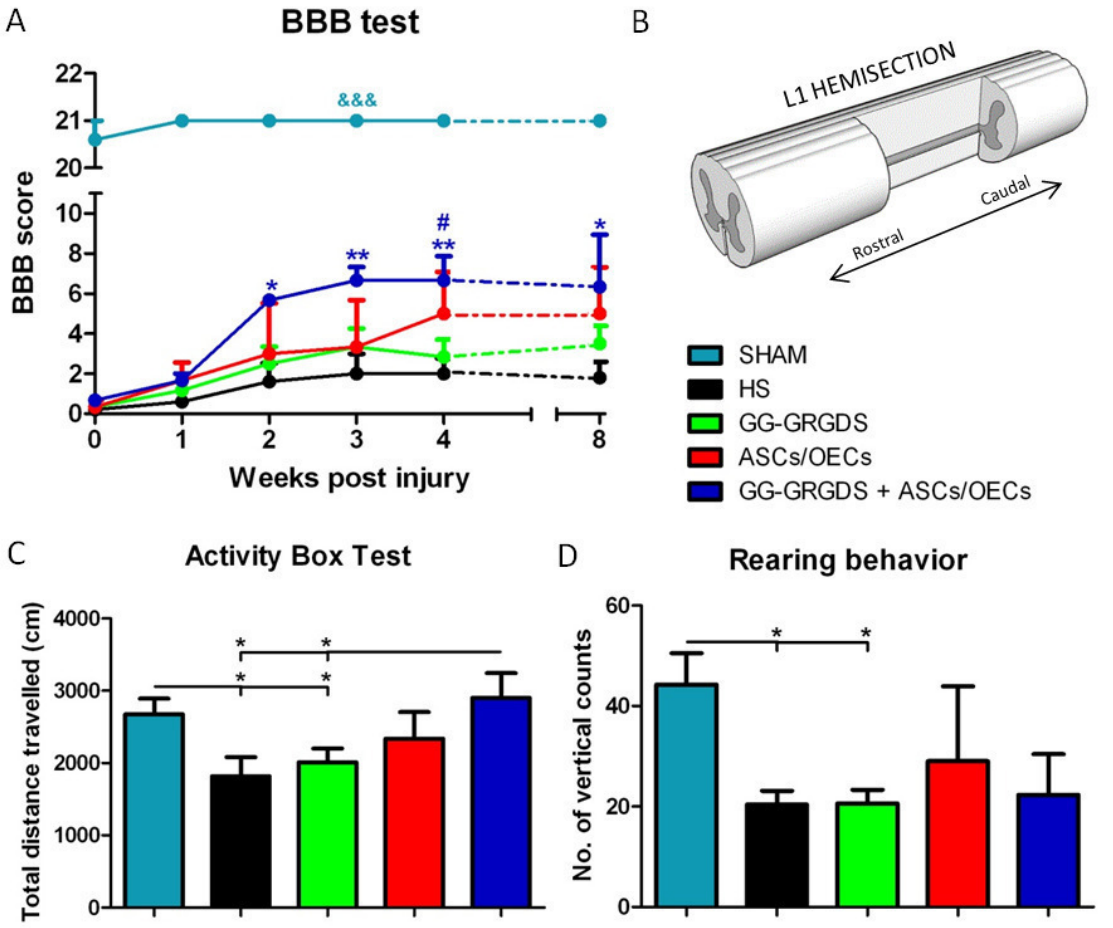


Figure 3.5 – *In vivo* locomotor evaluation of SCI rats. (A) BBB test performed during a follow-up of eight weeks post-injury. (B) Schematic representation of the hemisection injury induced in SCI animals at L1 level. (C) Activity box test and (D) rearing behavior evaluated in the open field arena, eight weeks after the lesion. Animals treated with the combination of GG-GRGDS and ASCs/OECs presented significant motor improvements in comparison to non-treated animals (HS group) and animals treated with GG-GRGDS only, both in the BBB test and in the total distance travelled on the activity box test. Values are shown as mean ± SEM. In graph A, (*) represents differences in comparison to HS group, (#) represents differences to GG-GRGDS group and (&) represents differences between sham group and all the others; one symbol $p<0.05$, two symbols $p<0.01$; three symbols $p<0.001$.

These BBB values mean that on average, rats treated with the combination of hydrogel and cells were able to extensively move two joints and slightly move one joint of the hindlimb, while non-treated animals were only able to extensively move one joint. Significant locomotor improvements were also seen at two, three and four weeks post-injury (fig. 3.5A). At eight weeks, rats were also submitted to a more general test of locomotion in an open field arena. Regarding

the total distance travelled in the activity box test, GG-GRGDS + ASCs/OECs-treated animals travelled significantly higher distances (2901.7 ± 343.8 cm) in comparison to non-treated and GG-GRGDS-treated ones (1817.7 ± 264.8 and 2011.9 ± 190.7 cm, respectively, fig. 3.5C). This group of animals was even able to travel similar distances to sham animals (2675.1 ± 218.0 cm). However, concerning rearing behavior, there were no differences between the four injury groups (fig. 3.5D).

Animals were then sacrificed and their spinal cords processed to perform histological analysis. Levels of axonal regeneration/preservation were estimated through immunostaining for neurofilament (NF). Both GG-GRGDS and GG-GRGDS + ASCs/OECs revealed significantly higher levels of NF expression area at the lesion site ($1.9 \pm 0.3 \times 10^4$ and $2.3 \pm 0.2 \times 10^4$ μm^2, respectively), when compared to non-treated animals ($0.8 \pm 0.03 \times 10^4$ μm^2, fig. 3.6C).

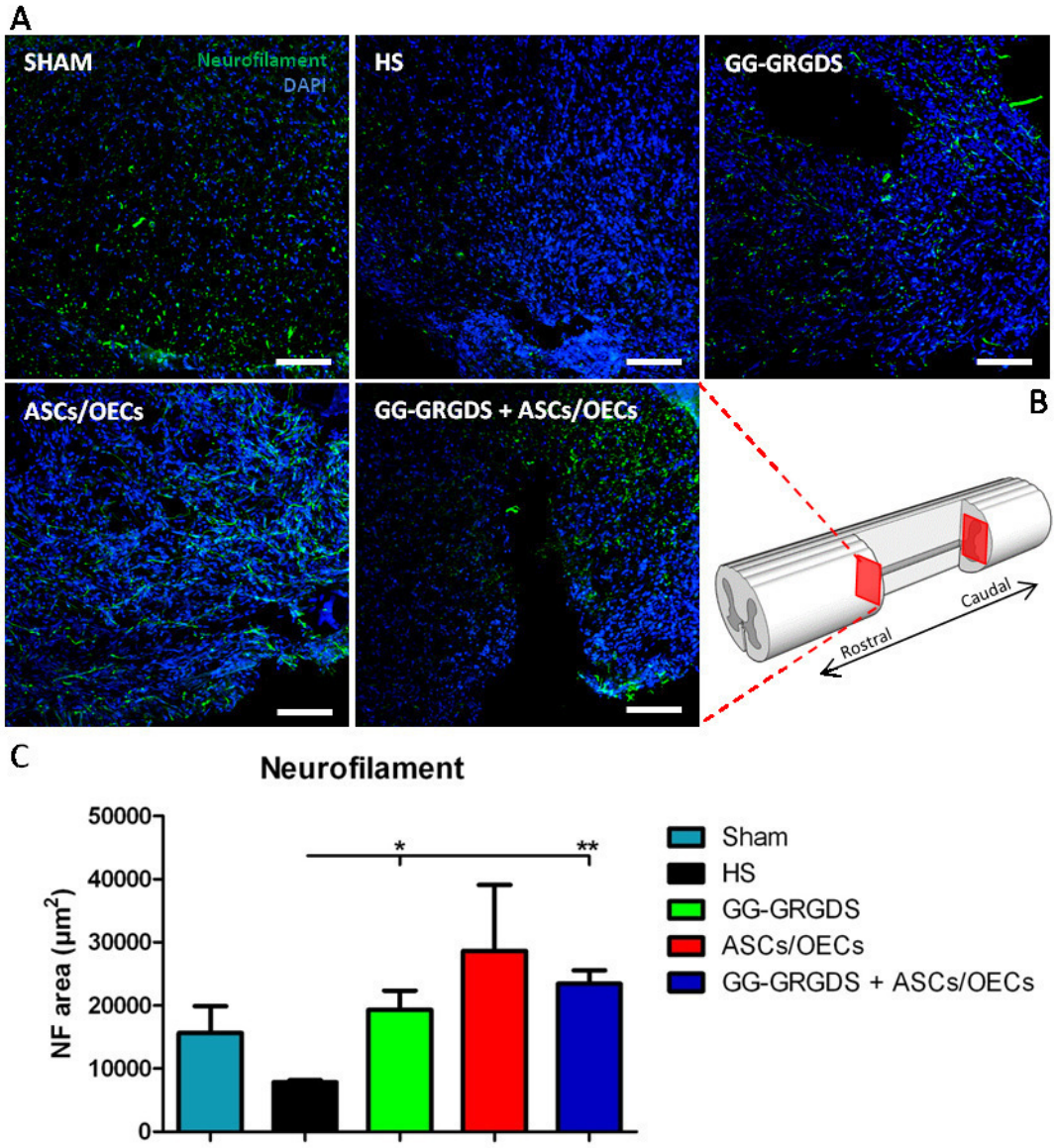
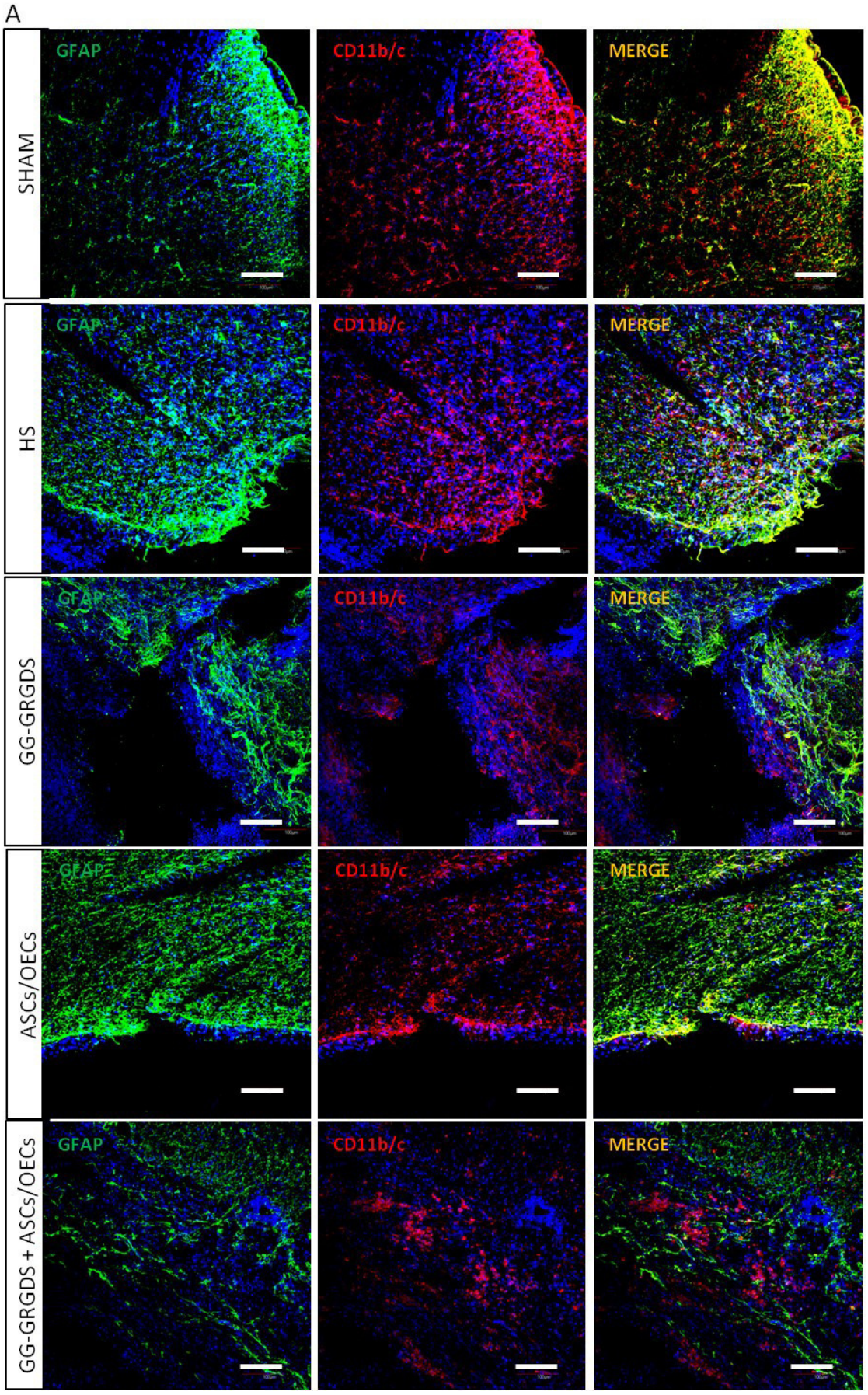


Figure 3.6 – Representative confocal images of the immunostaining for neurofilament; scale: 100 µm. (B) Schematic representation of the regions analyzed by confocal microscopy to determine axonal regeneration/preservation. (C) Quantification of expression areas of neurofilament marker. The treatment with the combination of GG-GRGDS and ASCs/OECs induced a significant increase in neurofilament area. Values are shown as mean ± SEM; *p<0.05, **p<0.01.

After immunohistochemistry for CD11b/c and GFAP (markers of inflammatory cells and astrocytes respectively), it was possible to observe an increase in expression of both markers after injury in the non-treated group, when compared to sham group (HS group: 7.7 ± 3.3 × 10^4 and 11.8 ± 7.4 × 10^4 µm², for CD11b/c and GFAP respectively; Sham group: 0.4 ± 0.1 × 10^4 and 1.0 ± 0.5 × 10^4, for CD11b/c and GFAP respectively, fig. 3.7). Nevertheless, there were not statistically significant differences between all groups after quantification (figs. 3.7B and 3.7C).

Treatment with GG-GRGDS + ASCs/OECs promoted the greatest decrease of CD11b/c and GFAP expression ($1.5 \pm 0.8 \times 10^4$ and $1.1 \pm 0.5 \times 10^4$ μm^2, respectively) reaching levels close to those of Sham animals (fig. 3.7B and 3.7C).


A
SHAM
HS
GG-GRGDS
ASCs/OECs
GG-GRGDS + ASCs/OECs
GFAP
CD11b/c
MERGE

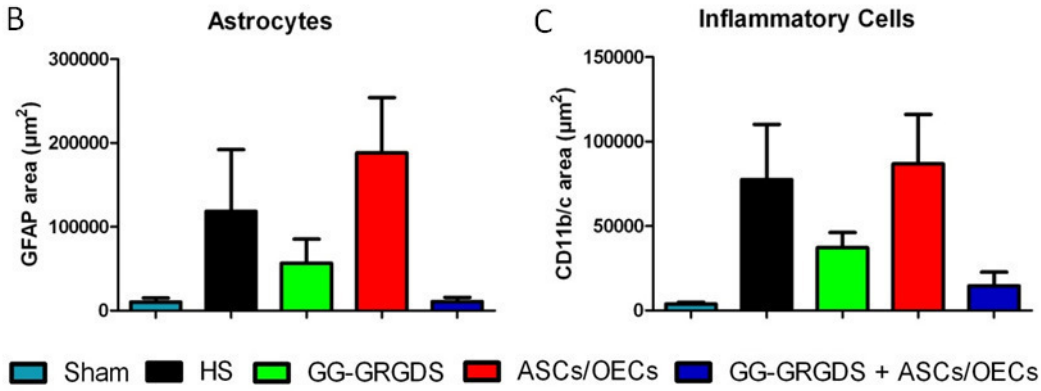


Figure 3.7 – (A) Representative confocal images of the immunostaining for GFAP, CD11b/c and the respective merge; scale: 100 µm. The analysis was performed at the beginning and at end of the gap produced by the hemisection (represented in fig. 3.6B). (B-C) Quantification of the area expressing GFAP (B) and CD11b/c (C) markers. Although there are no significant differences between groups, there is a trend for rats treated with GG-GRGDS + ASCs/OECs presenting less expression areas of both markers in comparison to non-treated animals. Values are shown as mean ± SEM (n=3).

Finally, following an IHC for Anti-Nuclei Antibody (HuNu), it was possible to identify transplanted ASCs in the spinal cord tissue eight weeks after injury (fig. 3.8). ASCs delivered through intraspinal injection, or within GG-GRGDS hydrogel were mainly found in regions close to the injury site. After quantification of the cells positive for the HuNu marker, we could find more ASCs when transplanted within the hydrogel (48.5 ± 7.0 cells per field) than when transplanted intraspinally (32.7 ± 7.0 cells per field), however this difference was not statistically significant.

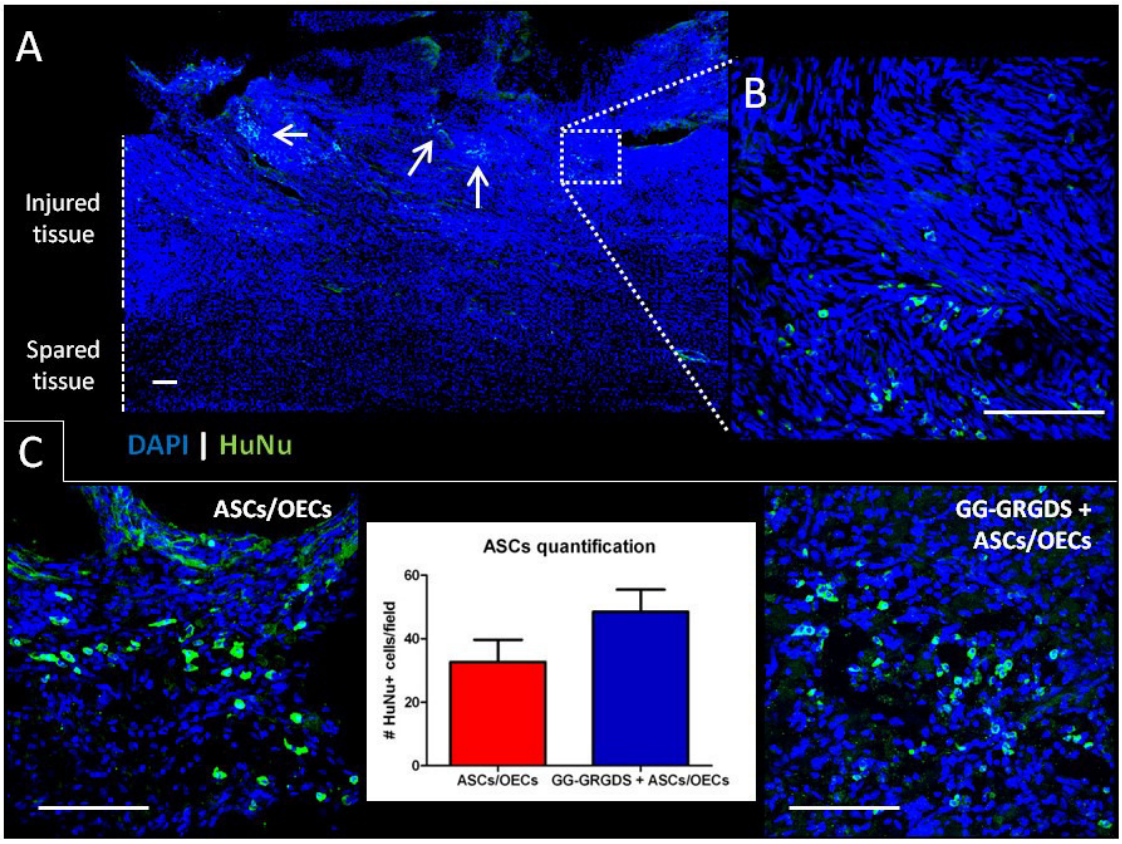


Figure 3.8 – Representative confocal microscopy images from longitudinal sections of spinal cord tissues of rats transplanted with GG-GRGDS with cells or only cells, eight weeks after lesion. (A) ASCs identified with HuNu (specific marker for human cells – represented by the white arrows) were mainly found in regions close to injury site and the adjacent tissues, in both transplanted groups. (B) Magnification detail of HuNu positive cells dispersed in tissue adjacent to the lesion. (C) Quantification of HuNu positive cells per field in each group with transplanted cells. The number of HuNu positive cells is higher in animals where ASCs were transplanted encapsulated in GG-GRGDS hydrogels, although this difference is not significant. Scale bar: 100 μm; values are shown as mean ± SEM (n=3).

4. Discussion

In the present work, we have developed a combinatorial approach to SCI repair based on the use of ASCs and OECs with GG-GRGDS hydrogels. Although the use of this hydrogel in CNS regenerative medicine approaches has been limited, it has been shown to present mechanical, as well as thermal and conduction properties very similar to soft tissues like the spinal cord [34]. Moreover, previous studies from our laboratory have shown that these GRGDS modified GG hydrogels are able to support the growth of multiple cell types including neural stem/progenitor

cells, OECs and MSCs [25, 26]. Before encapsulating the cells, we tested whether both cell types were capable of growing in direct contact cultures. Different cellular densities were used (1:6 ratio ASCs-OECs) because OECs need a minimal number of 60 000 cells/cm^2 to properly grow, while on the other hand, at this density ASCs would reach confluence in less than 48h. After seven days of co-culture, cellular morphology of ASCs and OECs was similar to monoculture conditions, which reveals that direct cell contact did not have a significant impact on the size and morphology of the cells. In addition, both cell types presented more cells in the first 24 h of co-culture in comparison to mono-cultures. This might indicate a beneficial interaction between ASCs and OECs even under direct cell-cell contact. However, this difference was lost after seven days of co-culture, mostly probably because cells reached confluence and could not expand over those numbers. The results herein obtained, together with the beneficial effects previously observed in indirect co-cultures [17] led us to choose these cells to combine with GG-GRGDS hydrogel. As shown in figure 3, ASCs and OECs grew within the 3D hydrogel matrix, extending their processes and presenting their typical morphology. Previous works from our group have already shown the suitability of GG-GRGDS hydrogel for promoting cellular growth and survival of OECs [25], ASCs [35] as well as MSCs from other sources [26]. The presence of GRGDS peptides is essential, as cells encapsulated in regular gellan gum do not present their normal morphology, instead having a round shape (fig. 3.3E). Moreover, DRGs were unable to extend their neurites when cultured on regular GG (fig. 3.4A). Both experiments reinforce the value of our peptide functionalization procedure.

Dorsal Root Ganglia explants have been used as a model of axonal regeneration *in vitro* [36]. To test our therapeutic approach, DRGs were cultured with ASCs and/or OECs encapsulated on the GG-GRGDS hydrogel. In this case the same cellular densities (15 000 cells/50 µl) were used for both cells (1:1) since there is less proliferation in 3D matrices and, at the same time, we could compare more precisely each cell type effect on DRG explants. ASCs induced a significant increase of DRG neurite extension area, as well as an increase in the length of the longest neurite, when compared to hydrogel alone and hydrogel + OECs cultures. From the literature it is known that ASCs have a potent neuroregulatory secretome, expressing growth factors that are able to mediate neurite/axonal outgrowth. From these the most representative are NGF, BDNF, and GDNF [6, 7]. On the other hand, OECs are known to guide axonal migration through a direct interaction with neuronal processes of neighboring axons [10]. This capacity is partially related with the expression of different matrix metalloproteinases (MMPs) by OECs.

Particularly MMP-2, MMP-3 and MT1-MMP seem to contribute to OECs motility across the extracellular matrix and their neurotrophic properties [37]. However, in this experiment most of the cells were not in direct contact with DRG explants, which were seeded on the top of hydrogel droplets. In this sense, the effect of the secretome, that is, the molecules secreted by the encapsulated cells, seemed to be more relevant than direct cell-cell interactions. As we have seen in 2D experiments with DRG explants [38], the secretome of ASCs induces higher neurite arborization levels, while in direct contact cultures, OECs provide a better support for neurite outgrowth than ASCs. Overall these findings are a strong indicator of the positive effects exerted by ASCs (mainly through secreted molecules) and OECs (direct cell-cell contact) on a particular phenomenon, neurite/axonal outgrowth, which is essential for the reestablishment of the functionality of the injured spinal cord.

In this study a left hemisection injury was performed at the L1 level, which results in a very aggressive injury. This happens because the motor central pattern generator (CPG) present at the lumbar region [39, 40] is partially affected, which explains the low motor recovery profile observed in these animals (fig. 3.5A), especially compared to hemisection injuries performed at the thoracic level [31]. Despite the aggressiveness of the injury, the combined treatment with GG-GRGDS and ASCs/OECs was capable of inducing significant motor improvements in the paralyzed hindlimbs of SCI rats, as assessed by the BBB test. The combination of cellular therapy with the hydrogel proved to be important in this recovery, since GG-GRGDS treatment or ASCs/OECs alone did not induce significant improvements in comparison to non-treated animals. Nevertheless, no major differences were found between groups with treatment. This means that our therapeutic strategy can be refined in order to further improve the functional outcomes obtained. Similar results were observed in the activity box test, since animals treated with the combinatorial strategy travelled longer distances than hydrogel- and non-treated groups. No differences between treatments were observed with respect to the number of vertical counts. Rearing is a type of behavior highly dependent on weight-support capacity on the hindlimbs and none of the groups reached the mean score of nine in the BBB scale, which is indicative of weight-support ability. Another study using the same combination of cells, but seeded on a serum-derived albumin scaffold, showed that rats treated with the combinatorial approach also presented improved locomotor skills over untreated animals [41]. Histologically, those rats presented reduced glial scar formation and more cells expressing markers of neurons and axons at the lesion site [41]. Our functional results can be partially explained by the CD11b/c and

GFAP levels observed. Despite not being statistically different between groups, there was a trend for rats treated with GG-GRGDS and ASCs/OECs to have reduced CD11b/c and GFAP expression areas, close to the lesion site. These results point out that the combinatorial treatment resulted in a less prominent inflammatory status and less astrogliosis, two features that have been associated with better motor performances in different studies [42, 43]. The combined treatment also resulted in an increased area of neurofilament expression, close to the lesion site, a fact that could be the result of an improved preservation of the remaining neuronal network and/or the creation of an environment propitious to neuroregeneration. In any case, the combination of these three major histological alterations (i.e. increased neurofilament, decreased astrogliosis and inflammation) can partially account for the functional improvements obtained with the combinatorial strategy herein tested. Eight weeks after injury, we could not find any trace or evidences of the hydrogel presence at the injury site. From previous experiments we know that GG has a slow degradation rate (for a hydrogel) and it can be found at least four weeks following injury [22]. Thus, the present study indicates that its full degradation happens for periods between four-eight weeks. In this case it may have been cleared by body fluids with time; however it must have remained at the injury site long enough, in order to allow cellular integration within the injured tissue (fig. 3.8). Moreover, we have previously demonstrated that this hydrogel integrates very well within the spinal cord tissue and it does not cause any kind of inflammatory reaction [22], which goes along with the anti-inflammatory profile found in GG-GRGDS-only transplanted animals (fig. 3.7C). The lesion epicenter was predominantly filled with connective tissue in all groups and there was no infiltration of blood vessels, astrocytes, axonal ingrowth or any other type of cells. The hydrogel did not bridge the injury epicenter, but was important to prevent the infiltration of cells in the tissues close to the lesion. This effect was even more visible in the group combining hydrogel and cells (fig. 3.7). It is also noteworthy the presence of ASCs two months following injury (fig. 3.8), which indicates that cells were capable of integrating the injured tissue, possibly exerting a long-lasting effect. A higher number of ASCs was transplanted in comparison to OECs (3:1 ratio, ASCs-OECs) due to the results obtained from DRG explants regarding their regenerative potential. Both ASCs transplanted through intraspinal injection or delivered within the hydrogel could be found in the spinal cord tissue. There was no significant difference in the mean number of HuNu positive cells per field between groups with hydrogel + cells and cells only (48.5 ± 7.0 vs 32.7 ± 7.0, respectively). This means that GG-GRGDS had a marginal impact on ASCs survival after transplantation. However, it is interesting to

notice that the BBB score from animals of the hydrogel + cells group was higher in the first time points evaluated, reaching a plateau at the third week post-injury. This fact could be related with the combined effects of cell survival provided by the hydrogel's physical and structural support at earlier time points. Regarding the fate of OECs, we could not identify these cells, since there is no specific marker for them. Most of the markers are shared with Schwann Cells and these cells can easily invade the CNS tissue after an injury [44]. All together, these data support the use of GG-GRGDS with ASCs and OECs as a possible candidate for SCI repair studies. Even though the results are promising, we should be careful in their interpretation, due to the low final number of animals. Nevertheless, one advantage of using a tissue engineering approach like the one herein presented is that it can work as a baseline for further refinements. For instance, GG-GRGDS hydrogel can be tested for additional modifications, such as the engraftment of other peptides also suitable to neuronal migration and differentiation [45]. In addition, the hydrogel may also be used as a depot for neurotrophic factors [46] and drug delivery [47]. By addressing spinal cord injuries with this kind of integrated view, we believe meaningful functional improvements to SCI patients may be obtained in the future.

5. Conclusions

The present work revealed that ASCs and OECs are able to grow in direct contact cultures, either in 2D or 3D surfaces, without significant alterations in their morphology and proliferation. Moreover, GG-GRGDS based hydrogels proved to be suitable for the culture of these two cell types. Additionally the combination of hydrogel and the cell populations herein tested, particularly ASCs, promoted an increased neurite/axonal outgrowth, using an *in vitro* model of axonal regeneration. Finally, combinatorial treatment with GG-GRGDS hydrogel and ASCs/OECs resulted in significant motor and histological improvements of SCI rats. Overall, our results suggest that the conjugation of GG-GRGDS hydrogel and cells with therapeutical potential, such as ASCs and OECs, is a promising therapy for the repair of Spinal Cord Injuries.

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Disclosures

The authors declare no conflict of interest.

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Chapter 4

Combination of a Gellan Gum-based Hydrogel with Cell Therapy for the Treatment of Cervical Spinal Cord Injury

Combination of a Gellan Gum-based Hydrogel with Cell Therapy for the Treatment of Cervical Spinal Cord Injury

Eduardo D. Gomes,[1,2] Rui Lima,[1,2] Mark Urban,[3] Biswarup Ghosh,[3] Miguel Goulão,[1,2,3] Jeffrey M. Gimble,[4] Angelo C. Lepore,[3] Nuno Sousa,[1,2] Nuno A. Silva,[1,2] António J. Salgado[1,2#]

[1] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal

[2] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3] Vickie and Jack Farber Institute for Neuroscience, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, 19107, USA

[4] Center for Stem Cell Research and Regenerative Medicine, Tulane University, 1430 Tulane Avenue, SL-99, New Orleans, LA, 70112, USA

Corresponding author:

A. J. Salgado, Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: asalgado@med.uminho.pt; Tel: +351 253 60 49 47; Fax: +351 253 60 48 20

Abstract

Cervical Spinal Cord Injuries (SCI) represent more than half of the SCI cases worldwide. Respiratory compromise, as well as severe motor deficits are among the main consequences of cervical lesions. A Gellan Gum (GG)-based hydrogel modified with GRGDS peptide, together with Adipose tissue-derived Stem/Stromal Cells (ASCs) and Olfactory Ensheathing Cells (OECs) was used as a therapeutic strategy after a C2 hemisection SCI in rats. Hydrogel or cells alone, and a control group without treatment were also tested. Four weeks after injury, compound muscle action potentials (CMAPs) were performed to assess phrenic motor neurons (PhMN) activity and no differences were observed between groups, confirming that the PhMN pool located between C3 and C5 is not affected by a C2 injury. Five weeks post-injury spontaneous activity of the affected hemidiaphragm was evaluated through EMGs of dorsal, medial and ventral portions. All treatments increased significantly the EMG signal at the ventral portion in comparison to the untreated control, but importantly only the combinatorial group presented increased EMG signal at the medial portion of the hemidiaphragm.

Following three assessments of the staircase test, no differences were found among groups regarding the performance of the affected forelimb. Similar results were observed at the grooming test, meaning no improvements were seen in motor behavior, regardless of the treatment. Interestingly, following the Von Frey test, it was possible to find a significant effect of the group combining hydrogel and cells on hypersensitivity. Rats with a SCI displayed an increased response of the contralateral forelimb to a mechanical stimulus, but after treatment with the combinatorial therapy, this behavior was reverted almost to the levels of uninjured controls.

Key words

Cervical Spinal Cord Injury; Respiratory compromise; Adipose tissue-derived Stem/Stromal Cells; Olfactory Ensheathing Cells; Modified Gellan Gum hydrogels;

Introduction

Cervical spinal cord injuries (SCI) represent more than half of the SCI cases worldwide [1]. Traumatic lesions at cervical level often result in respiratory compromise, due to damage to neural circuits controlling the diaphragm [1, 2]. This circuit comprises phrenic motor neurons (PhMN) located in the mid-cervical region of the spinal cord innervating the diaphragm, while in turn, this population is controlled by the rostral ventral respiratory group (rVRG) of neurons located in the brainstem [1]. Despite the importance of this specific local neural circuitry, and despite the higher frequency of cervical lesions, most pre-clinical studies have focused on thoracic lesions, which encompass different specificities and eventually different responses to treatment.

Among the existing works in traumatic cervical SCI, cellular transplantation strategies have been designed to meet, essentially, the following purposes: replacing or inducing plasticity of neurons involved in the respiratory circuit [3, 4]; replacing glial cell types [5] or local interneurons [6]; providing trophic support [7]; and restoring neurotransmitter signaling [8].

Our group has developed a tissue engineering (TE) strategy for SCI repair, previously tested in a rat model of lumbar injuries [9]. This approach is based on the combination of adipose tissue-derived stem/stromal cells (ASCs) and olfactory ensheathing cells (OECs), together with a modified Gellan Gum (GG)-based hydrogel, which can be used both as a matrix for neural regrowth, and/or as a vehicle for cellular transplantation. ASCs and OECs represent two distinct cellular populations with complementary effects, as already demonstrated by our group [9-11]. Moreover, both cells can be obtained from autologous sources, increasing their potential application to the clinics. The GG hydrogel presents physical properties very similar to the spinal cord tissue and was previously modified with GRGDS motifs to increase cell adhesion [12]. This modification also led to improved morphology, viability and secretome properties of encapsulated cells [13]. In a lumbar SCI model, the combined therapy of hydrogel and cells led to significant locomotor improvements of the paralyzed hindlimbs, associated with a decreased inflammatory and astroglial response [9].

Based on the abovementioned results, we hypothesized if the application of the same therapeutic strategy to a cervical hemisection injury could result in a similar outcome, modulating the local environment and possibly favoring neuronal preservation or inducing axonal regeneration through the injury. In this sense, following a C2 hemisection, ASCs and OECs

encapsulated on the GG hydrogel were transplanted into the spinal cord and functional and histological recovery were assessed.

Materials and Methods

ASCs and OECs cultures

Human ASCs were isolated according to Dubois et al. (2008) [14] in collaboration with LaCell LLC. Briefly, ASCs were isolated from human lipoaspirates obtained from consenting donors under an institutional review board approved protocol at LaCell LLC. These cells were cultured and maintained in α-MEM (Invitrogen, USA), with 10 % Fetal Bovine Serum (FBS, Biochrom AG, Germany) and 1 % antibiotic solution – penicillin/streptomycin (pen/strep; Invitrogen) at 37 °C and 5 % CO_2 (v/v).

OECs were isolated and cultured using a modified version of the protocol described by Cao et al. (2006) [15]. The animal care committee of the research institute approved all the animal protocols in accordance with standardized animal care guidelines [16]. Briefly, OECs were harvested from olfactory bulbs of neonatal (P5-P7) Wistar-Han rats. Upon dissection, the meninges and blood vessels were removed and the tissue was digested with collagenase type I (2.5 mg/ml, Sigma, USA) for 15 min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5 ml pipette and centrifuged at 1000 rpm for 5 min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation step, cells were resuspended and seeded on uncoated plates for two consecutive periods of 24 h. It is expected that most of the fibroblasts and astrocytes attach in the first and second periods, respectively. After this purification step, the remaining cells were seeded on fibronectin coated surfaces. Cells were cultured in DMEM/F12 (Invitrogen) with 10 % FBS and 1 % pen/strep solution at 37 °C and 5 % CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5.36 µg/ml, Invitrogen) and Forskolin (1.4 µg/ml, Sigma).

Hydrogel preparation

The synthesis of GG-GRGDS hydrogel was performed according to the protocols described by Silva et al. (2012) [12]. Briefly, GG (Sigma) was firstly dissolved in 2-(N-

morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5, Sigma) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Sigma) and furfurylamine (Acros Organics, Belgium) were then added in a 4:1 M ratio (of each reagent relative to the -COOH groups in GG) and stirred at 37 °C for 48 h. The solution was then dialyzed (Mw cutoff 12-14 kDa, Spectrum Labs, USA) alternately against distilled water and phosphate buffered saline (PBS, 0.1M, pH 7.2) for five days. Finally, water was removed by lyophilization to obtain furan-modified GG (furan-GG) as a white powder. Immobilization of maleimide-modified GRGDS peptide (mal-GRGDS, Anaspec, USA) to furan-GG was performed via Diels-Alder chemistry between the maleimide functional group of the peptide with the furan group of the GG. Furan-GG was first dissolved in MES buffer at 37 °C (1.2 mg/ml). Mal-GRGDS was then added in a 5:1 maleimide:furan molar ratio and vigorously stirred for 48 h. The solution was then dialyzed (Mw cutoff 12-14 kDa) alternately against distilled water and PBS for five days. Finally, the water was removed by lyophilization to obtain GRGDS-modified GG (GG-GRGDS) as a white powder.

GG-GRGDS 3D hydrogel preparation

GG-GRGDS lyophilized powder was sterilized by exposure to UV lights for 15 min, a method previously used without affecting the material properties [13]. Then, GG-GRGDS was dissolved in ultrapure water, at 1 % (w/v) concentration and heated at 40 °C overnight, in order to obtain a homogenous solution. Before encapsulating the cells, $CaCl_2$ at 0.3 % (w/v) was added [to obtain a final concentration of 0.03 % (w/v) of $CaCl_2$ in solution] to enable the ionic crosslinking of the hydrogel.

C2 hemisection injuries

Animals and groups

Ten weeks old female Wistar-Han rats (Charles River, France), housed in light and temperature-controlled rooms and fed with standard diet, were used in the *in vivo* studies. Handling was performed for three days before the surgeries. Two sets of experiments were designed: the first one to assess respiratory function and the second one dedicated to motor and sensorial evaluation. Within each set, animals were divided into four different groups according to the respective treatment/procedure: 1) animals subjected to SCI with no treatment (SCI, n=6-8); 2) SCI animals treated with GG-GRGDS (GG-GRGDS, n=6-8); 3) SCI animals transplanted with cells (ASCs/OECs, n=6-9); 4) SCI animals treated with ASCs and OECs encapsulated in GG-

GRGDS (GG-GRGDS+ASCs/OECs, n=6-10). Treatments were applied immediately after injury using a Hamilton syringe. A total of 10 µl of hydrogel, cells or hydrogel with cells were injected per animal at the injury site, in the respective groups. Culture medium was used as a vehicle for transplanted cells and injected as a control in non-treated animals. Rats treated with cells received 200 000 cells (1:1 ASCs-OECs), either encapsulated in GG-GRGDS or dissolved in culture media.

Cervical SCI

All animals were anesthetized by intraperitoneal injection of a mixture (1.5:1) of ketamine (100 mg/ml, Imalgene/Merial, France) and medetomidine hydrochloride (1 mg/ml, Domitor/Pfizer, USA). Once anesthetized, the dorsal surface of the skin was shaved and disinfected with a 70 % ethanol solution and topical iodine (Dynarex, Orangeburg, New York). Using a sterile #11 surgical blade (Electron Microscopy Sciences, Hatfield, Pennsylvania), a three-cm midline incision was made on the dorsal surface of skin and muscle, starting from the caudal portion of the occipital bone. Retractors were then used to expose the dorsal surface of the C2 and C3 vertebrae. Using rongeurs (Fine Science Tools, Foster City, California), remaining tissue was removed from the vertebrae and a laminectomy was performed to expose the spinal cord. The C2 and C3 dorsal roots were located, and a hemisection was performed at a location just caudal to the C2 root with a dissecting knife (Fine Science Tools, Foster City, California). To ensure a complete hemisection, a 30-gauge needle (BD Biosciences, San Jose, California) was passed through the injury several times. Following complete hemisection and respective treatments, the dorsal muscle layers were sutured with 4–0 silk sutures (Covidien, Minneapolis, Minnesota), and the skin was stapled closed with surgical staples (Fine Science Tools, Germany). The surface of the skin was treated with a topical iodine solution.

Post-operative care

Following SCI surgery and respective treatment, rats were kept under heat lamps and received subcutaneous injections of a solution containing vitamins (10 ml/Kg, Duphalyte/Pfizer, USA), 0.9 % NaCl, the analgesic butorphanol (10 mg/ml, Butomidor/Richter Pharma AG, Austria) and the antibiotic enrofloxacin (5 mg/ml, Baytril/Bayer, Germany), besides atipamezole (5 mg/ml, Antisedan/Pfizer, USA) a drug used in order to revert anesthesia. Bladder evacuation was done manually twice a day until animals regained control. Then, for five days rats received

daily subcutaneous injections of all the above-mentioned components except for atipamezole. Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment. The diet was enriched and the food was presented to the rats in the cage. In case of weight loss superior to 10 %, Nutri-Cal (Vetoquinol, France) was administered orally.

Compound muscle action potential (CMAP) recordings

Rats were anesthetized with isoflurane (Piramal Healthcare, Bethlehem, Pennsylvania) at a concentration of 3.0-3.5 % diluted in oxygen. Animals were placed supine and the region just below the rib cage was shaved and cleaned with 70 % ethanol. Phrenic nerve conduction studies were performed with stimulation at the neck via near nerve needle electrodes placed along the phrenic nerve. A reference electrode was placed on the shaved surface of the right costal region. The phrenic nerve was stimulated with a single burst at 6 mV (amplitude) for a 0.5 ms duration. Each animal was stimulated between 10 and 20 times to ensure reproducibility, and recordings were averaged for analysis. ADI Powerlab 8/30 stimulator and BioAMPamplifier (ADInstruments, Colorado Springs, CO) were used for both stimulation and recording, and Scope 3.5.6 software (ADInstruments, Colorado Springs, CO) was used for subsequent data analysis.

Electromyography (EMG) recordings

Animals were anesthetized with isoflurane (Piramal Healthcare, Bethlehem, Pennsylvania) at a concentration of 3.0-3.5 % diluted in oxygen. Once deeply anesthetized, a laparotomy was performed to expose the right hemi-diaphragm. Bipolar electrodes spaced 3 mm apart were placed for recording in three separate sub-regions of the hemi-diaphragm: dorsal, medial, and ventral. Recordings were averaged over a five-minute time frame for each animal, and peak amplitude, burst duration and frequency were taken. Using LabChart 7 software (AD Instruments, Colorado Springs, Colorado), the EMG signal was amplified and filtered through a band-pass filter (50–3000 Hz). Following recordings, animals were immediately euthanized with a triple-dose of ketamine/xylazine/acepromazine and tissue was collected.

Motor assessment

Staircase Test

The staircase (also called skilled paw reaching test) was performed with double staircase boxes (Campden Instruments, Lafayette, IN, USA), as previously described [17]. The shape and dimensions of the boxes were similar to the ones described by Montoya et al. (1991) [18]. The apparatus consists of a clear chamber with a hinged lid that was developed to assess the independent forelimb use in skilled reaching and grasping tasks. A narrow compartment, with a central platform running along its length, is connected to this chamber. The removable double staircase with seven steps on each side can be inserted in the space between the platform and the box walls. Five pellets were placed into each well of the double staircase apparatus. On the first day, the rats were familiarized with the test and pellets were feely available at random positions for 10 min. During the test session, animals were kept inside the box and had 15 min to reach, retrieve, and eat food pellets present on the steps. All sessions were performed at the same time of day under food-restriction. After each test interval, animals were removed from the staircase boxes and the uneaten pellets were counted. During the first five test days, rats were presented with pellets on both sides of the staircase, while in the sixth and seventh day, pellets were placed only on one side, alternating sides from one day to the other (forced choice paradigm). The staircase test was performed during the second- and fifth-weeks post-injury. As main outputs, eating scores and precision scores were calculated. The first considers the ratio between the number of pellets eaten and the total number of pellets available; the second is the ratio between the number of pellets eaten and the number of pellets touched by the rat (both eaten and misplaced pellets from the staircase).

Grooming Test

Three weeks after injury grooming behavior was evaluated for all rats. A soft gauze with fresh tap water was applied to the rats' back head to induce grooming. Then the rats were placed in a glass cylinder and their behavior was filmed. After two complete cycles of grooming (starting by licking the paws and ending by cleaning behind the ears) the rats were placed back in their cages. Later, both forelimbs were scored using a scale varying from 0 (unable to touch the nose) up to 5 (reach the back of their ears), adapting a protocol from Bertelli and Mira (1993) [19].

Von Frey

The Von Frey test was performed at four weeks post-injury, as described in Guimarães et al. (2018) [20]. Animals were placed in an elevated grid and left to acclimatized to the experimental conditions for 5 minutes. Mechanical allodynia was then assessed using the up-and-down method [21] as described previously [22]. Briefly, the sural dermatome of the contralateral limbs (fore- and hindimb) was probed with a series of von Frey calibrated monofilaments: 15.0g, 8.0g, 6.0g, 4.0g, 2.0g, 1.0g, 0.6g and 0.4g (North Coast Medical Inc., USA). Starting with the 2.0g filament, the test would advance upward if no response was elicited (=0) or downward if a brisk withdrawn of the limb was produced (=X) until 6 measurements were obtained around the threshold point according to the model developed by Dixon [23]. Paw movements, associated with locomotion or weight shifting, were not counted as a response. The 50 % response threshold was then calculated using the following formula:

$$50\%g\_threshold = \frac{(10^{Xf+K.\delta})}{10000}$$

where X_f= value (in log units) of the final von Frey filament; k= tabular value corresponding to pattern of positive and negative responses (X and 0 sequence; consult [21]); δ = mean difference (in log units) between stimuli (0.224). If no response was obtained up to maximal force (15.0g) or conversely, if all filaments elicited a response down to the minimal force (0.4g), the values 15 and 0.25 were assumed as the 50 % withdrawal threshold, respectively.

Histological characterization

Neuromuscular junctions (NMJs) analysis

Labeled muscles were analyzed for total numbers of NMJs and also intact, partially-denervated or completely denervated NMJs. Whole-mounted diaphragms were imaged on a FluoView FV1000 confocal microscope (Olympus, Center Valley, Pennsylvania). We conducted NMJ analysis on ipsilateral hemi-diaphragm because previous published work showed no denervation or sprouting in contralateral hemi-diaphragm after cervical injury [24, 25].

Statistics

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 Bonferroni post-hoc test. A p-value of ≤ 0.05 (95 % confidence level) was set as the criteria for statistical significance.

Results

Functional and morphological innervation of the ipsilateral hemi-diaphragm

Lesions at the C2 spinal cord level are not expected to directly affect the PhMN pool located between C3 and C5. Therefore, in order to confirm the functional innervation of the ipsilateral hemi-diaphragm, CMAPs were recorded after supramaximal stimulation of the phrenic nerve. No significant alterations were observed among groups regarding CMAP amplitudes (Fig. 4.1A-B), even in comparison to the non-lesioned contralateral hemi-diaphragm (Fig. 4.1B). Moreover, after histological assessment of NMJs in the ipsilateral hemi-diaphragm, no differences were seen in the percentage of intact, partially-denervated or completely-denervated NMJs (Fig 1C). The majority of NMJs analyzed were intact (almost 100 %), demonstrating that the injury did not affect diaphragmatic morphological innervation. In both assessments, the treatments did not influence the results obtained.

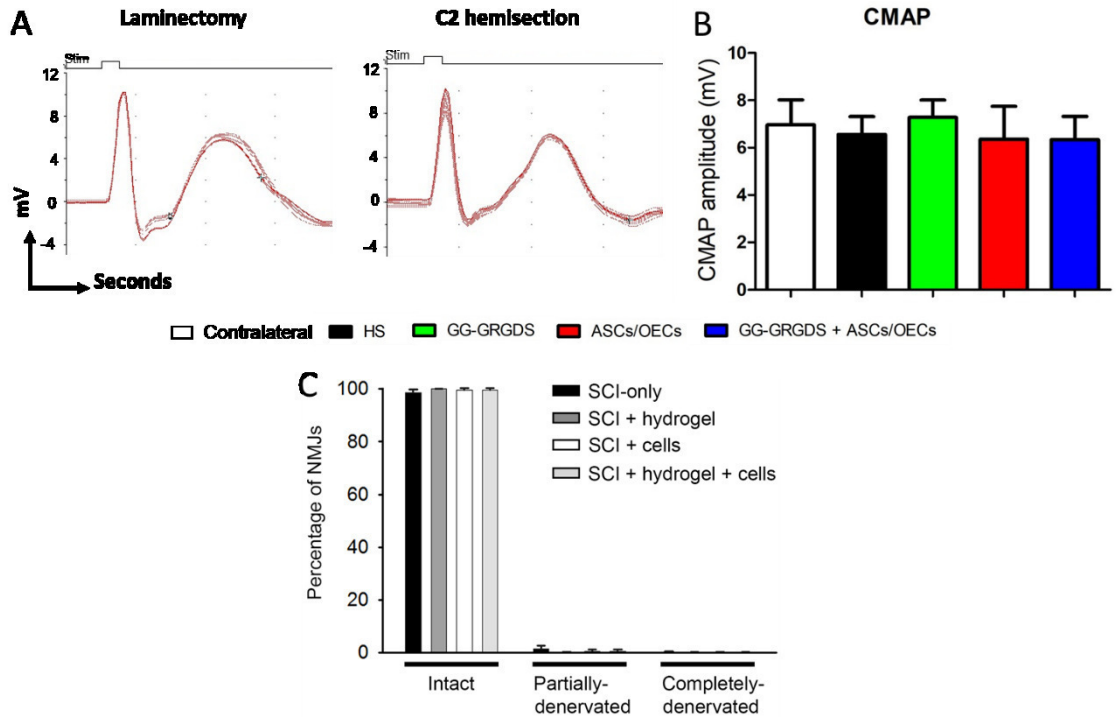

Figure 4.1 – Cervical hemisection injury at C2 level does not affect CMAPs (A-B) neither the morphology of NMJs, both analyzed at the ipsilateral hemi-diaphragm. Data is presented as mean ± SEM (n= 8).

Ipsilateral hemi-diaphragm function assessment

Five weeks after injury, diaphragmatic function was assessed through EMGs analysis. After recordings at three different subregions of the lesioned hemi-diaphragm, it was observed a clear reduction in EMGs amplitudes following SCI (Fig. 4.2A-B). However, at the ventral portion of the hemi-diaphragm, all treatments induced a significant recovery of EMG activity, and at the medial portion, only the combination of hydrogel and cells was capable of promoting a significant improvement (Fig. 4.2B). This result suggests a beneficial effect for the conjugation of hydrogel and cells.

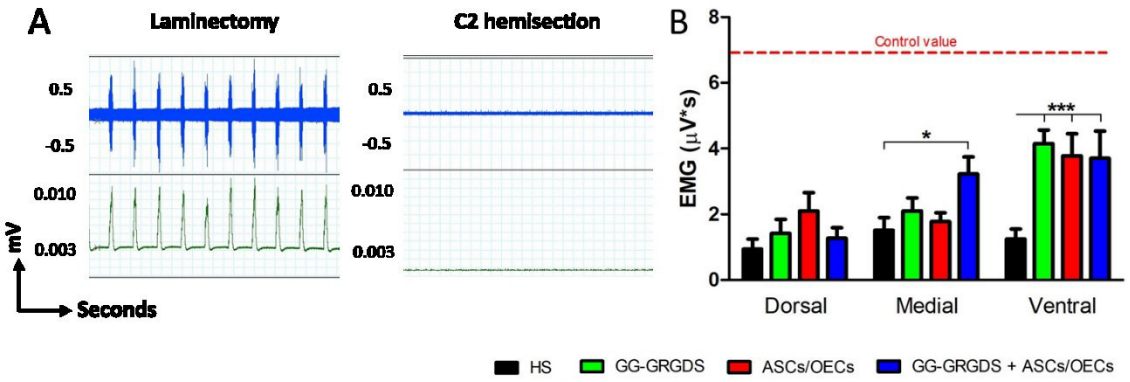

Figure 4.2 – EMGs of the ipsilateral hemi-diaphragm, five weeks after C2 cervical hemisection injury. Non-treated rats present an almost complete silencing of EMG activity [(A) representing dorsal recordings] in comparison to non-lesioned rats (only subjected to a laminectomy). Treatments with hydrogel, cells or both led to significant recovery of EMG signals at the ventral portion of the ipsilateral hemi-diaphragm (B) while in the medial portion only the combinatorial treatment provided significant improvements. No differences were seen among groups at the dorsal portion. Data is presented as mean ± SEM (n= 8).

Forelimb motor evaluation

Motor impairments of the forelimbs is another consequence of cervical SCI. In this case, only the right forelimb was expected to be affected. In order to evaluate the recovery of motor function, two different motor behavior paradigms were performed. The first one was the staircase test, in which rats under food restriction are assessed for their fine motor skills, such as reaching and grasping abilities, to retrieve and eat a sugared pellet. At two and five weeks after injury, rats were subjected to the test, where during the first five days they could retrieve pellets from both right and left sides (Fig. 4.3) and in the last two days, pellets were only available on the left or on the right side (forced choice, Fig. 4.4). In both modalities of the test, no significant differences

were observed among groups, either for the eating score, representing the total percentage of eaten pellets, or for the precision score, which only considers the efficacy of total pellets eaten over the number of pellets that each rat could actually touch.

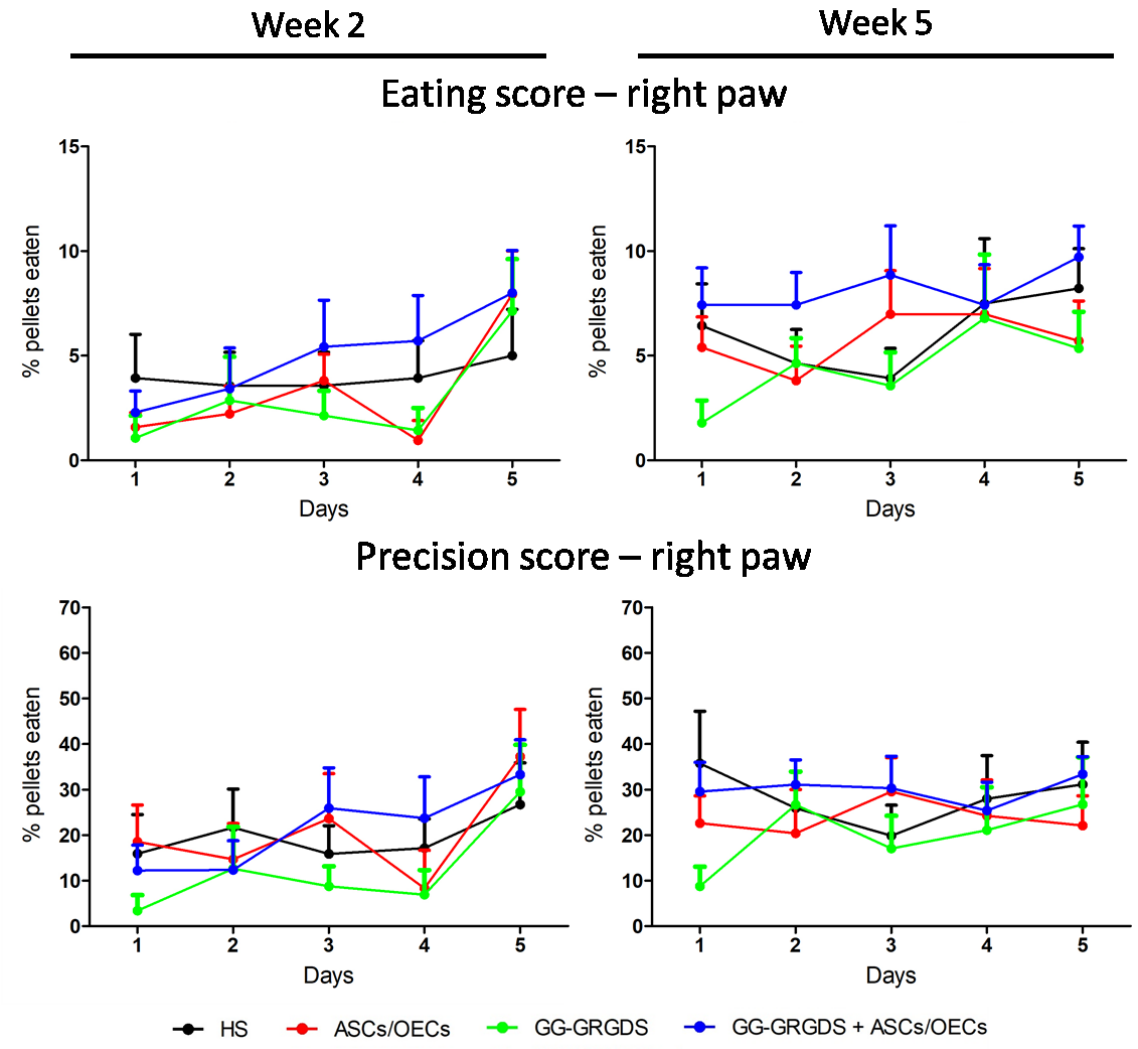

Figure 4.3 – Staircase test for the right paw, performed at two and five weeks after injury. There are no significant differences among groups for both the eating score (upper graphs) and the precision score (lower graphs), in none of the time points evaluated. Data is presented as mean ± SEM (n= 8).

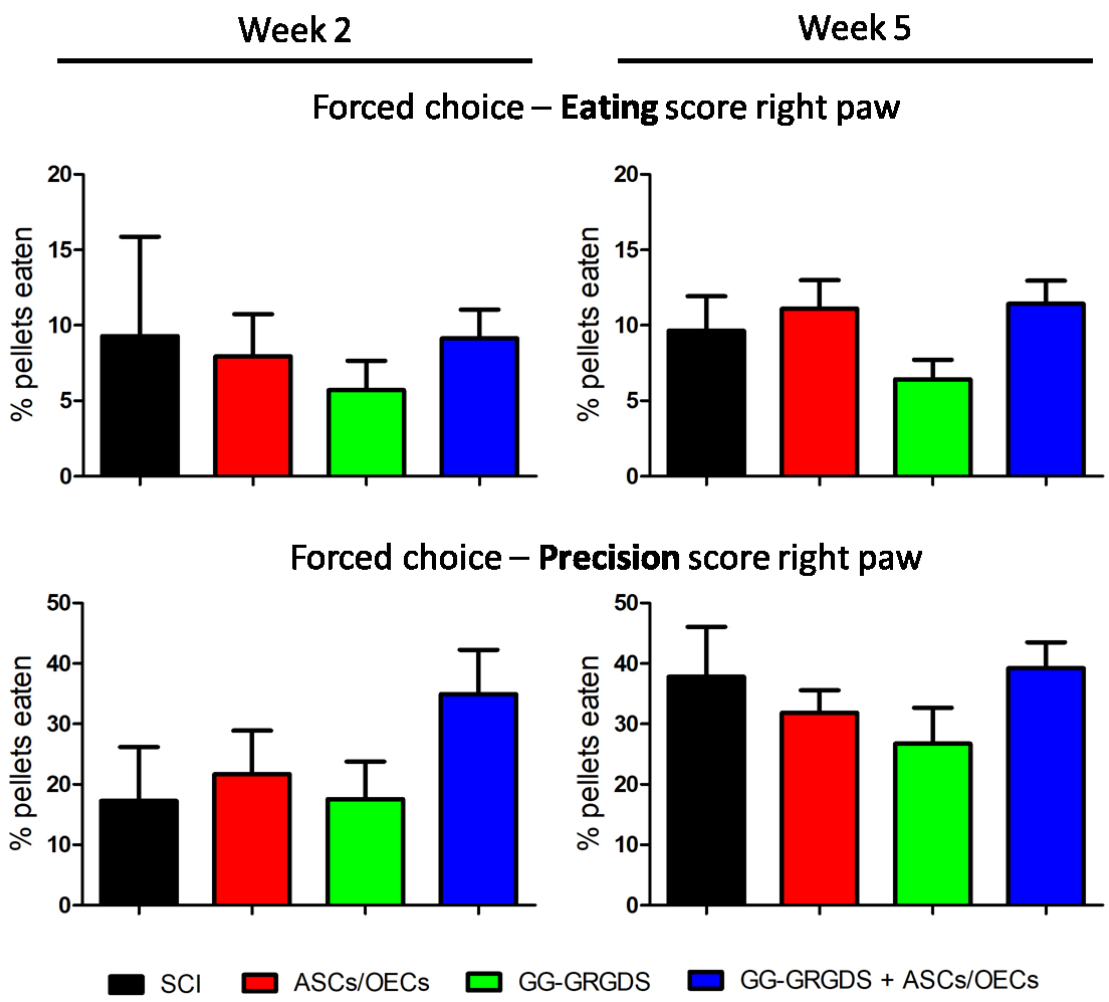

Figure 4.4 – Forced choice paradigm of the staircase test, performed at two- and five-weeks post-injury (right paw). Once again, no differences were observed in the eating score and precision score, at both time points. Data is presented as mean ± SEM (n= 8).

The second motor paradigm used was the grooming test. Grooming is a natural behavior in rodents that can be used to assess forelimb movement capacity. Using a pre-defined scale, where 1 means limited movements, while 5 represents a normal movement with complete grooming cycles, rats were evaluated at three weeks post-injury (Fig. 4.5). The movement capacity of the right forelimb was significantly affected in all rats, however there were no differences among groups. The same was true for the left, contralateral forelimb.

Overall, these motor paradigms reinforce the idea that the injury was correctly performed, but treatments were unable to induce any motor recovery.

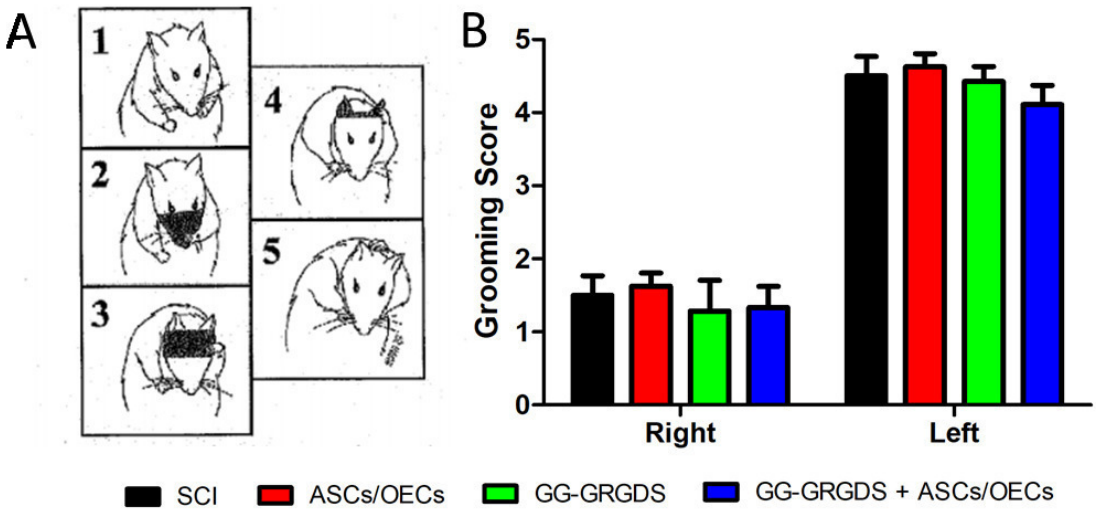

Figure 4.5 – Grooming test performed at three weeks after injury. No differences were seen in grooming capacity among groups, for both right and left paws. As expected, the lesioned right paw presents deficits in grooming ability. Data is presented as mean ± SEM (n= 8).

Sensorial perception after injury

One common denominator following SCI is sensorial perception deficits. With the objective of inferring about sensorial function, rats were subjected to the Von Frey test four weeks post-lesion. Following C2 cervical injury, it was observed a marked increase in sensitivity of the contralateral limbs (Fig. 4.6), reacting to a stimulus usually innocuous to non-injured rats. However, after treatments, all treated groups demonstrated recovery from the hypersensitivity observed at the hindlimb left paw, while very interestingly, in the forelimb paw, only the combinatorial strategy induced significant recovery of sensorial function to levels more similar to healthy controls (Fig. 4.6). The combination of ASCs/OECs with GG-GRGDS hydrogel seems to disclose an important role in sensorial modulation and recovery.

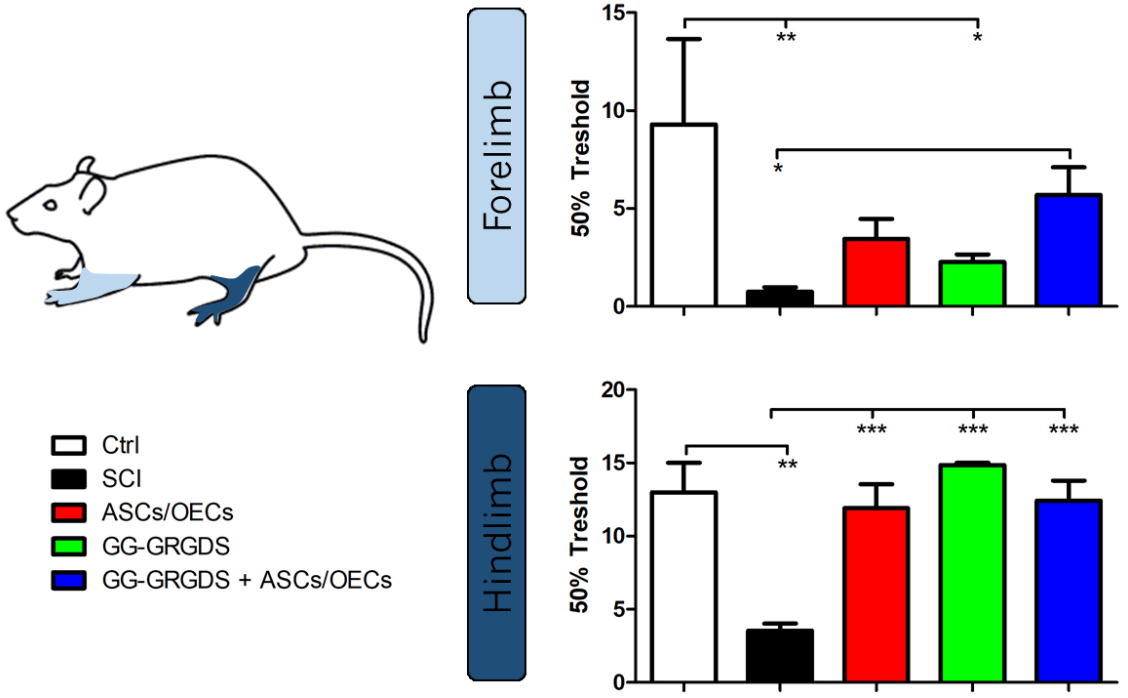

Figure 4.6 – Von Frey test performed at four weeks after injury. Forelimb and hindlimb sensorial responses to Von Frey filaments were assessed and quantified. The combinatorial treatments led to a recovered sensorial phenotype for both contralateral limbs. Data is presented as mean ± SEM (n= 8); *p<0.05, **p<0.01, ***p<0.001.

Discussion

Cervical SCI pre-clinical research still represents a minor percentage of the total number of publications in this field, despite its clinical relevance, accounting for more than half of SCI cases every year [1]. TE approaches to cervical lesions have already been employed with some success. For instance, Ghosh et al. (2018) [26] have recently demonstrated that local BDNF delivery using an engineered hydrogel significantly enhanced diaphragmatic respiratory function.

In our case, the combination of ASCs and OECs with the peptide-grafted GG hydrogel has led to relevant functional and histological improvements in other models of SCI [9]. After a hemisection injury at the thoracic level, the administration of cellular transplants of ASCs/OECs was sufficient to induce a significant recovery of locomotor function, assessed by the BBB test, together with a marked decrease in inflammatory cells close to the lesion site [11]. On the other hand, in a more aggressive hemisection lumbar injury model, the combination of ASCs/OECs with GG-GRGDS hydrogel was the only treatment capable of promoting functional locomotor

improvements, associated with similar decreases in inflammatory cells. In addition, the combined treatment reduced the levels of astrocytes close to the injury and preserved the levels of neurofilament-positive cells. Importantly, the number of ASCs that survived transplantation was higher when cells were transplanted within the hydrogel matrix [9]. Taking these results into account, the objective of this study was to ascertain if the same strategy could induce similar results in a cervical model of SCI.

The model used, C2 unilateral hemisection is not the most clinically relevant, however considering the mortality associated with animal models of cervical contusion, or complete transection, this represents a good alternative to more aggressive models. In addition, it is an appropriate model to understand regeneration mechanisms, since it creates a very precise and restricted injury, allowing a more accurate axonal tracing analysis. C2 hemisection interrupts the input of rVRG neurons from the brainstem, to PhMNs located around C3-C5 segments resulting in paralysis of the ipsilateral hemidiaphragm. In this sense, as a first experiment, the functionality and morphology of diaphragmatic innervation were analyzed (Fig. 4.1). As expected, there were no alterations in both the action potentials of the phrenic nerve when stimulated, and no significant morphological denervation of NMJs of the hemidiaphragm. This was already seen in other recent works using C2 cervical hemisections [27]. This happens because the PhMN pool responsible for diaphragm innervation is not directly affected by the lesion, maintaining a response when activated. However, since they lose supraspinal input from the brainstem (rVRG neurons), they stop controlling diaphragmatic function. This loss of function is then reflected in the EMG recordings of the lesioned hemi-diaphragm (Fig. 4.2). In the present works, different regions were analyzed independently, to dissect more closely the effects observed. Even though no differences were seen among groups at the dorsal portion of the diaphragm, medial and ventral portions registered significant improvements. In the ventral portion all treatments provided significant recovery of function, while in the medial portion, only the combinatorial treatment resulted in a significant increased EMG signal. These results might be related with the differential control of the diaphragm by distinct segments of the spinal cord. The C3 segment's PhMNs are more associated with control of the ventral portion, the C4's control the medial portion and C5's control the dorsal portion of the diaphragm. Even though the histological analysis is still ongoing, we hypothesize that therapeutic effect was greater on the PhMNs nearest to the injection site. This was translated to better diaphragmatic functional recovery of the ventral portion (C3 segment), in opposition to limited or no recovery of medial and dorsal portions (controlled by C4

and C5 segments respectively). The combined treatment was effectively the only one capable of inducing partial recovery of EMG activity at the medial portion of the hemi-diaphragm, indicative of a more potent beneficial effect, in comparison to single treatment of hydrogel or cells. The mechanisms behind this recovery are still elusive, but based on previous results, some possibilities can be exploited. ASCs have a very strong immunomodulatory component, as noted in different models of injury [28]. We have seen that these cells are able to integrate and survive in the spinal cord injured tissue for at least two months [11]. Thus, ASCs-mediated reduction of inflammatory cells is a strong possibility, an effect that is often associated with better neuronal survival and tissue protection [29, 30]. The fact that ASCs transplanted within GG-GRGDS hydrogels present higher survival rates [9] could also explain the higher magnitude of recovery observed in this study. The regenerative properties attributed to OECs might have also played a role. The hydrogel alone also induced partial recovery of function at the ventral portion. In this sense, the physical support provided by GG-GRGDS could also be in part responsible for neuronal regeneration, or, as previously shown by our laboratory even prevents the infiltration of inflammatory cells and reactive astrocytes [9].

Regarding motor function analysis, none of the therapeutic strategies employed seemed to have an impact on motor recovery. The motor behavior paradigms used address general forelimb movements (grooming test) but also skilled and fine detailed movements (staircase test). However, in none of the paradigms there was a significant difference among groups. The specificity of the cervical neuronal circuitry might explain the different results observed between cervical and thoracic/lumbar studies. Forelimb motor function is highly dependent of supraspinal inputs, while thoracic and lumbar-derived movements rely more on local circuitries, such as central pattern generator (CPG) activity [31]. Long-distance CST projections are more difficult to regenerate, so probably that helps to explain the lack of motor recovery. Moreover, even if there was regeneration of fibers, these might not be functional, at least in the time frame that we evaluated (five-weeks experiment). The fact that EMG activity was partially recovered, might also indicate that the therapeutic effect was more specific for a certain type of neuronal populations, such as those from the respiratory circuitry. A thorough histological characterization will help to answer some of these questions.

Conclusions

Cervical spinal cord injuries are misrepresented in SCI pre-clinical research, therefore, there is a need for more studies focusing on traumatic lesions affecting this spinal cord region. TE approaches such as the one presented here are promising, as they can address multiple targets, increasing the chances for functional recovery following an injury. In our work, although no forelimb motor improvements were observed, the combination of GG-GRGDS hydrogel with ASCs/OECs led to an increased diaphragmatic activity together with a reestablishment of sensorial deficits provoked by the lesion. This is of the utmost relevance, as respiratory compromise and chronic pain are two of the main concerns of SCI patients. The specific mechanism by which this therapeutic strategy exerts its effects needs to be further addressed in future studies, however, it opens a window for improvement of a critic condition such as cervical SCI.

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Chapter 5
Discussion and Conclusions

The search of a therapeutic solution for SCI is a demanding task. The limited regenerative capacity of the CNS poses a great challenge because traumatic SCI is associated with massive neuronal damage and death. Moreover, several secondary molecular and cellular alterations continue after injury, leading to further neuronal loss. The SCI pathophysiology is so complex that most likely only combinatorial therapies will lead to relevant improvements in the affected population. In addition, many of the research performed in the field has been focused just on one model of injury. Knowing that the consequences and deficits following a SCI are different according to the spinal cord segments affected, it is important to test treatment strategies in different models.

The combinatory therapeutic approaches herein proposed have demonstrated positive results in three different SCI models (Fig. 5.1).

In the thoracic lesion (chapter 2), a single transplantation of cells (both ASCs and OECs) was sufficient to induce a significant recovery of function, as assessed by the BBB scale. Interestingly, the impressive recovery observed could not be attributed to increased numbers of axons neither specific dopaminergic fibers, at the lesion site. Even though other neuronal populations could have been addressed, such as serotonergic fibers, further analysis of neuronal survival and integrity in regions rostral and caudal to the lesion could have helped to explain the results obtained. Hindlimb locomotion is highly dependent on short-distance, local neuronal circuitries [1], thus a histological assessment of the low-thoracic/high lumbar regions should be of interest. Very importantly, ASCs were detected in the spinal cord tissue, eight weeks after injury. This reveals that ASCs could survive and even migrate from injection sites to the lesion area. Therefore, the significant decrease in inflammatory cells at the lesion (CD11b/c positive cells) could be partially explained by the immuno-modulatory capacity of ASCs [2]. Reduced inflammation has been associated with significant improvements of locomotor function [3].

In a different model, testing the same type of lesion (hemisection) but at the lumbar region, the cellular therapy was further complemented with a biomaterial implantation (chapter 3). The GG-GRGDS hydrogel has been extensively studied *in vitro*, demonstrating to be a suitable biocompatible matrix for the growth of different types of cells [4-7]. In addition, this hydrogel has demonstrated to improve the secretome abilities of different cells and increase the expression of neurotrophic-related genes [5, 7]. Thus, its application to a SCI model held great promise. In comparison to the thoracic injury, the lumbar lesion produced more aggressive locomotor impairments, because several neurons belonging to the CPG area were affected. In turn, this

limits the spontaneous regeneration and recovery of function, as it can be compared by the BBB scores of lesioned non-treated animals (BBB score of 2 vs 9, in lumbar and thoracic lesions respectively, eight weeks post-injury). This could explain the lack of functional improvements observed in cells-only transplanted rats. However, conjugating ASCs/OECs with GG-GRGDS based-hydrogels led to significant recovery of motor function, potentiating the therapeutic effect. The combination of both strategies was indeed vital, since the single implantation of hydrogel was also non-effective regarding motor function. While the cellular transplantation was performed rostrally to the lesion, cells within the hydrogel were implanted directly in the hemisection gap that was created. Despite the aggressive environment associated with the lesion area, ASCs transplanted within the hydrogel survived more than single-cells transplantation, which probably increased their effects. Immunomodulation and even glial scar alterations (less astrocytes) were induced with the combined treatment. In addition, more NF-positive cells were present at the lesion site. The presence of the hydrogel also seemed to have an effect, since hydrogel-only controls also presented increased numbers of NF cells.

Following the successful application of this therapeutic approach to a very aggressive model such as lumbar injury, we hypothesized if the same strategy would be applicable to a cervical model of SCI. Due to its frequency in the SCI population [8], cervical injuries should represent an important concern in the SCI research field. Relevant functions, such as respiratory control, are dependent on cervical spinal cord neuronal populations, and could be severely compromised following an injury. In this sense, we opted to evaluate the acute treatment with GG-GRGDS hydrogel and ASCs/OECs after a C2 cervical hemisection. This model has two main advantages: 1) it creates a very specific and restricted lesion, from which regeneration studies can be easily performed; 2) it allows a greater survival of animals, in comparison to complete transection or contusion models. In this study, the histological analysis is still incomplete, therefore the results obtained so far are mostly functional data. Despite still not knowing any specific mechanisms, the combined application of hydrogel and cells led to significant recovery of respiratory function, as assessed by diaphragmatic electric activity (EMG signals). These improvements were not associated with differences in PhMN activity neither diaphragm innervation, since CMAPs remained unchanged and NMJs were mostly intact, independently of the injury or any treatment. This happens because injuries at C2 level are not supposed to affect directly the PhMN pool located around C3 and C5. So, it is assumed that recovery of function might have been due to protection or regeneration at the level of injury. On the other hand, even

though respiratory function was improved, no differences in motor behavior of the forelimbs were observed. One open possibility is the selective protection/regeneration of specific neuronal circuits, more devoted to diaphragmatic control rather than those regulating forelimb movements. It is known that forelimbs rely more on supraspinal, long-distance inputs, which have been associated with a limited regenerative potential [1]. In contrast, the circuit controlling diaphragm function, receives inputs from the brainstem (rVRG neurons) and some works have shown the regeneration of this circuit [9]. Very interestingly, sensorial perception of the contralateral forelimb was also normalized following the combined treatment. The sensorial fibers are also affected following SCI, with patients frequently developing chronic pain [10]. In this sense, the recovery observed is also very important, for the establishment of therapies that could improve recovery of different systems, often considered more important than locomotion by the patients [11]. The explanation for this recovery is still elusive, although chronic pain and sensorial mechanisms have been associated with inflammation [12], and our strategy has demonstrated immuno-modulatory properties in the other two injury models.

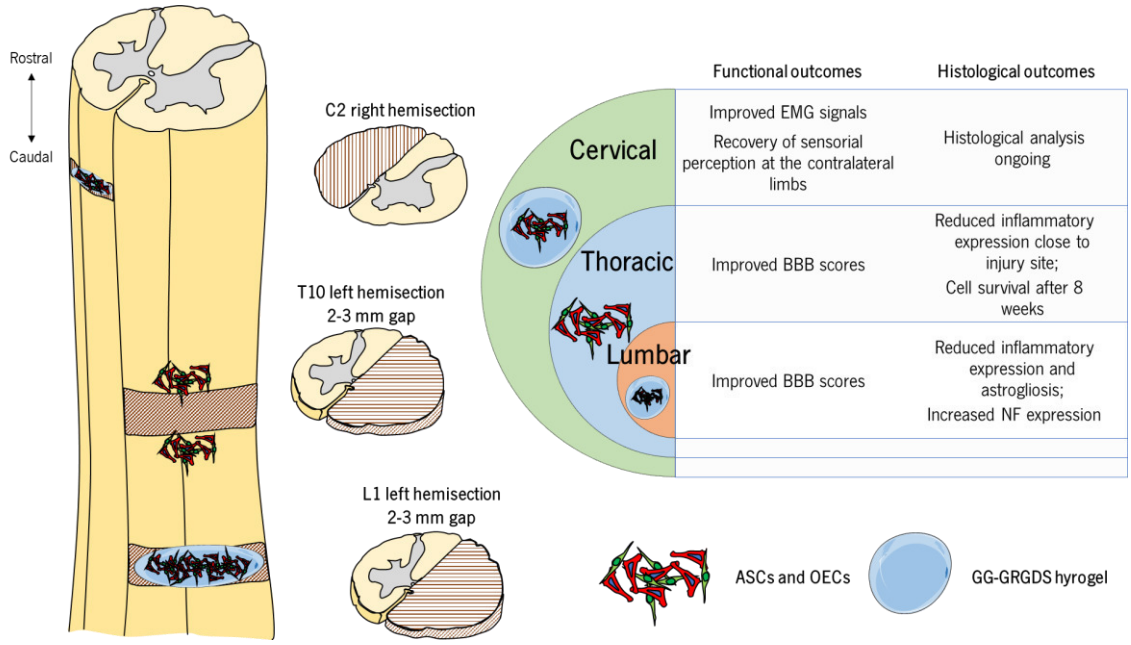

Figure 5.1 – Schematic representation of the different SCI models evaluated and the respective treatment approaches (only cells, in the thoracic hemisection, hydrogel and cells in the cervical and lumbar injuries). The combinatorial treatments led to the best functional and histological outcomes, resulting mainly in improved motor functions and reduced inflammatory and astrogliosis states.

There are limitations in some aspects of this work. One of the main setbacks was the fact the OECs were not traced after transplantation. It is widely reported the capacity of these cells to adhere, grow and even bridge spinal cord lesions [13]. The fact that we could not develop, or obtain, OECs expressing endogenous fluorescence, did not allow us to identify these cells, which share many markers with SCs. In this way, we were not able to determine OECs fate, and therefore, their possible mechanism of action. From the *in vitro* studies (chapter 2), we saw a very close interaction between OECs and neurites from DRG explants. The direct interaction could have occurred *in vivo*, and possibly related with that, in the work from chapter 3, more NF-positive cells were found in areas close to the injury site.

Another important issue is the lack of some control groups. For instance, the single transplantation of ASCs or OECs, separately, could have helped to elucidate the single effects of each cellular population. Or at least, it would clarify if the results observed were only possible due to the combined transplantation of both types of cells. Nevertheless, the *in vitro* data obtained suggested that a combined administration would have beneficial effects, therefore this work focused on the conjugation of both ASCs and OECs.

The SCI research field has reached a decisive point to define its future. Emerging techniques such as epidural stimulation [14] or brain-machine interfaces [15] might change the paradigm of the field. Nevertheless, and despite the great potential of these techniques, there is always room for tissue and molecular therapies, that could even be conjugated with those techniques. In this sense, the work herein described, is of the utmost importance, englobing a strategy easily translatable to humans, and that was tested in different models of SCI, with promising results, increasing its validity. Further testing on bigger animal models, and chronic injury treatments are needed. The work developed during this thesis represents a relevant step in SCI research, where the slightest recovery of function, could be extremely meaningful to patients.

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