The secretome of Mesenchymal Stem Cells as a cell-free based therapy for Spinal Cord Injury
Rita Catarina Assunção Ribeiro Silva
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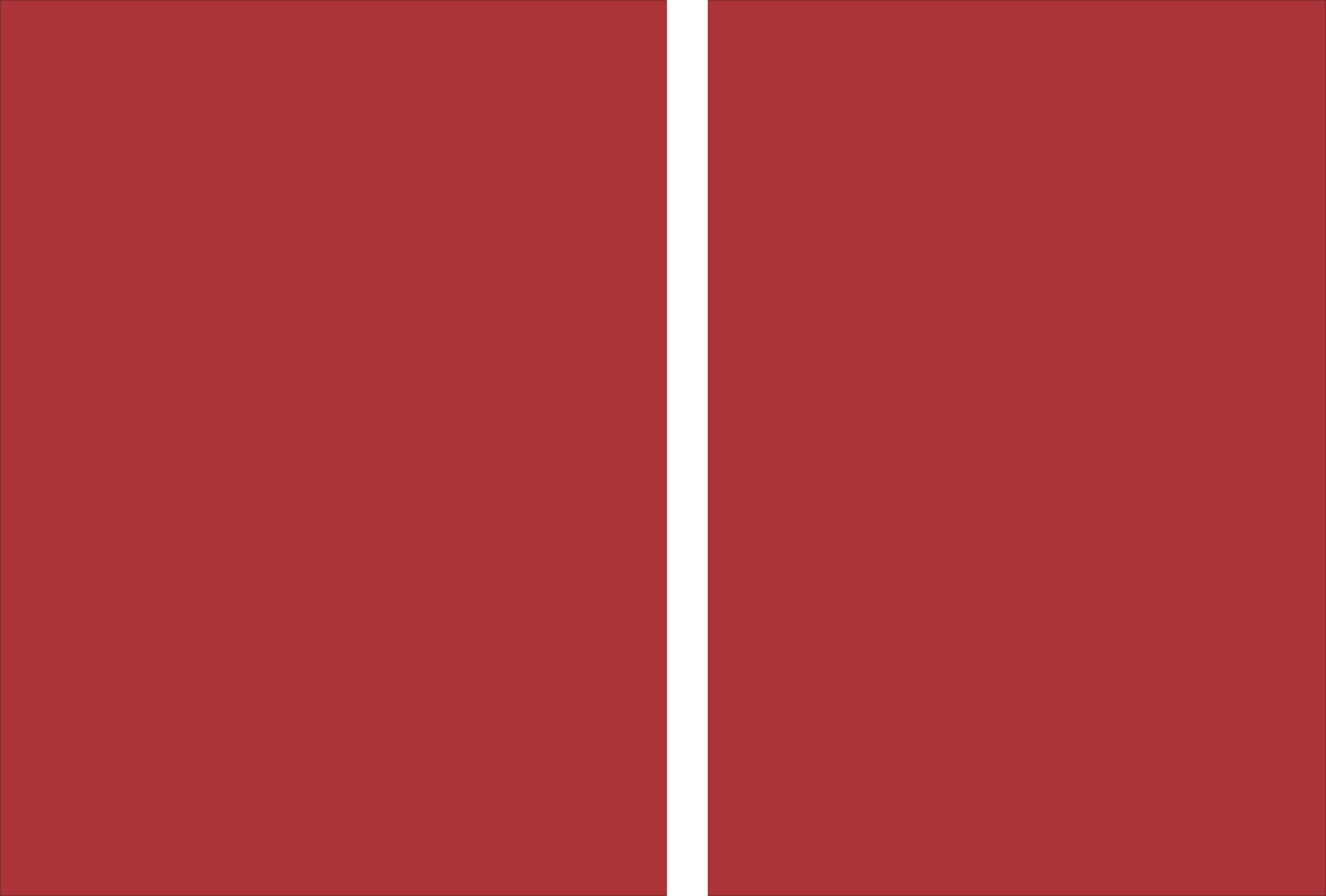

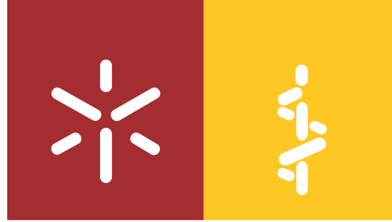

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The secretome of Mesenchymal Stem Cells as a cell-free based therapy for Spinal Cord Injury

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Doutor António Salgado
e da
Doutora Luísa Pinto

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STATEMENT OF INTEGRITY

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TÍTULO: O SECRETOMA DE CÉLULAS ESTAMINAIS MESENQUIMATOSAS COMO UMA TERAPIA PARA LESÕES VERTEBROMEDULARES

RESUMO

As lesões vertebro-medulares (LVM) resultam de um trauma na espinal medula, seguido da perda parcial ou completa da função motora e sensorial abaixo do nível de lesão. A transplantação celular tem estado na vanguarda de muitas estratégias, mas apresentam uma taxa de implantação e sobrevivência baixas perante o ambiente de lesão nocivo. Como alternativa, estratégias baseadas no uso do secretoma de células estaminais têm sido muito exploradas. O secretoma de células estaminais mesenquimatosas (MSCs) têm mostrado efeitos benéficos através de citoquinas anti-inflamatórias e fatores de crescimento e regenerativos. Uma expressão diferencial destas moléculas por MSCs derivadas do tecido adiposo (ASCs), medula óssea (BMSCs) e do cordão umbilical (HUCPVCs) sugerem um impacto terapêutico distinto. Esta hipótese foi confirmada por experiências *in vitro*, onde o secretoma de ASCs promoveu níveis de crescimento axonal superiores aos das outras populações celulares. De seguida, o potencial terapêutico do secretoma de ASCs foi avaliado num modelo de LVM em *Xenopus Laevis*. A sua administração na espinal medula dos *Xenopus* após transeção completa aumentou a regeneração axonal e crescimento neuronal no local de lesão. Os animais tratados apresentaram formação de uma ponte axonal na zona lesionada, assim como uma melhoria da função motora. Finalmente, o potencial terapêutico do secretoma de ASCs foi avaliado num modelo de LVM em ratinho. O secretoma foi administrado por via intravenosa após transeção completa torácica da espinal medula. Os animais tratados demonstraram melhorias das funções motoras e sensoriais, acompanhado por uma redução evidente do número de células inflamatórias no local de lesão, o que sugere uma ação anti-inflamatória do secretoma das ASCs. Foi também observado crescimento e regeneração axonal após tratamento, assim como uma diminuição da cavidade de lesão.

Em resumo, os resultados aqui apresentados providenciam evidências do potencial terapêutico do secretoma de ASCs após LVM, considerando os seus efeitos positivos ao nível da inflamação neuronal e crescimento e regeneração axonal, observado nos modelos *in vitro* e *in vivo* aqui estudados, e que estão associados à recuperação motora dos *Xenopus Laevis* e do ratinho.

Palavras-chave: Células estaminais mesenquimatosas, crescimento axonal, lesões vertebro-medulares, neuroinflamação, secretoma.

TITLE: THE SECRETOME OF MESENCHYMAL STEM CELLS AS A CELL-FREE BASED THERAPY FOR SPINAL CORD INJURY

ABSTRACT

Spinal cord injury (SCI) results from a mechanical trauma to the spinal cord, followed by partial or complete loss of motor and sensory function below the level of injury. Cell transplantation has been in the forefront of regenerative medicine strategies, but often presents low engraftment and survival rate within the aggressive environment of SCI. Alternatively, cell-free based strategies using the secretome of stem cells has been highly explored. Mesenchymal stem cells (MSCs) secretome has been showing beneficial effects through anti-inflammatory cytokines and regenerating- and growth-permissive factors. A differential expression of these molecules by adipose tissue (ASCs)-, bone-marrow (BMSCs)- and umbilical cord (HUCPVCs)-derived MSCs suggested a distinct therapeutic outcome. Indeed, this was confirmed in *in vitro* experiments, where ASCs secretome promoted significantly higher levels of axonal growth, when compared to the other cell populations. Following this, the therapeutic potential of ASC secretome was evaluated in a *Xenopus laevis* model of SCI. ASC secretome delivery into a transected *Xenopus* spinal cord increased axonal regeneration and neuronal regrowth at the lesion site. Treated animals showed ablation gap closure and axonal bridge formation between the two spinal cord stumps, as well as an improved motor function. Finally, the therapeutic potential of ASC secretome was evaluated in a mice model of SCI. ASC secretome was intravenously administered into mice spinal cord after complete thoracic transection. Treated animals showed improved motor and sensorial function, accompanied by a marked reduction on the number of inflammatory cells at the lesion site, suggesting an anti-inflammatory action of ASC secretome. Axonal outgrowth and regeneration through the injury was also observed upon ASC secretome treatment, as well as decreased lesion cavities.

Altogether, these results provide evidences of the therapeutic potential of ASC secretome after SCI, supported by indications on the positive effects exerted on neuroinflammation, and axonal outgrowth and regeneration, observed for *in vitro* and *in vivo* models herein studied, and that were associated to locomotor recovery to both *Xenopus laevis* and mice.

Keywords: Axonal Outgrowth; Mesenchymal Stem Cell Secretome; Neuroinflammation; Regeneration; Spinal Cord injury.

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ABBREVIATIONS LIST

α - Alfa

β - Beta

% - Percent

v/v - Volume per volume

cm^2 - Centimeter square

°C - Celsius degrees

U/ml - Units per mililiter

mg/ml - Micrograms per mililiter

nm - Nanometer

μl - Microliter

μL/s - Microliter per second

w/v - Weight per volume

A

ASIA - American Spinal Injury Association

ASCs - Adipose Tissue-derived Mesenchymal Stem Cells

ANOVA - Analysis of Variance

α-MEM - alfa-Minimum essential medium

B

BDNF - Brain Derived Neurotrophic Factor

bFGF - basic Fibroblast Growth Factor

BM-MSCs - Bone Marrow-Derived Mesenchymal Stem Cells

β4Gal-T1 - Beta-1,4-galactosyltransferase 1

BMS - Basso Mouse Scale

BSA - Bovine Serum Albumin

C

CNS - Central Nervous System

CSPGs - Chondroitin Sulfate Proteoglycan

cAMP - cyclic Adenosine Monophosphate

CST - Corticospinal Tract

CNTF - Ciliary Neurotrophic Factor

CDH - Cadherins

CLUS - Clusterin

CM - Conditioned Media

Ca^{2+} - Calcium

$CaCl_2$ - Calcium Chloride

CO_2 - Carbon Dioxide

D

DRG - Dorsal Root Ganglion

DCN - Decorin

DAPI - 4’,6-diamidino-2-phenylindole

E

ECM - Extracellular Matrix

EMG - Electromyography

ESCs - Embryonic Stem Cells

EDTA - Ethylenediamine Tetraacetic Acid

F

FGF - Fibroblast Growth Factor

FBS - Fetal Bovine Serum

FITC - Fluorescein Isothiocyanate

G

GAP-43 - Growth Associated Protein 43

GDN - Glia-Derived Nexin

GDNF - Glial-Derived Neurotrophic Growth Factor

GFAP - Glial fibrillary acidic protein

H

HUC-MSCs - Human Umbilical Cord Mesenchymal Stem cells

HGF - Hepatocyte Growth Factor

HCG - Human Chorionic Gonadotropin

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H_2O - Water

I

IGF-1 - Insulin Growth Factor

iPSCs - Induced Pluripotent Stem Cells

IL - Interleukin

IHC - Immunohistochemisty

IV - Intravenous

IT - Intrathecal

K

kDA - kilodalton

KCl - Potassium Chloride

KHz - Quilo hertz

L

LIF - Leukemia Inhibitory Factor

LC - Lesion Core

M

MP - Methylprednisolone

MSCs - Mesenchymal Stem Cells

MBS - Modified Barth Buffer solution

Mg^{2+} - Magnesium

$MgSO^4x7H_2O$ - Magnesium Sulfate

MS222 - Tricaine Methanesulfonate

N

NT - Neurotrophin

NGF - Nerve Growth Factor

NB - Neurobasal-A medium

NSCs - Neural Stem Cells

NSPCs - Neural Stem/Progenitor Cells

NaCl - Sodium Chloride

$NaHCO_3$ - Sodium Bicarbonate

NAP-2 - Neutrophil-Activating Protein-2

O

OECs - Olfactory Ensheathing Cells

OPCs - Oligodendrocyte Progenitor Cells

P

PNS - Peripheral Nervous System

PEDF - Pigment Epithelium-Derived Factor

P/S - Penicilin-Streptomycin

PFA - Paraformaldehyde

PBS - Phosphate Buffered Saline

R

RT - Room Temperature

ROI - Region of Interest

S

SEM - Semaphorin

SCI - Spinal Cord Injury

SCs - Schwann Cells

SCF - Stem Cell Factor

SH - Sham

SEM - Standar Error of Mean

T

TFG-β - Transforming Growth Factor-beta

TNF- α - Tumor Necrosis Factor alfa

U

USVs - Ultrasonic Vocalizations

UC-MSCs - Umbilical Cord-derived Mesenchymal Stem Cells

V

VEGF - Vascular Endothelial Growth Factor

FIGURES & TABLES LIST

CHAPTER I. INTRODUCTION

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CHAPTER V. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Figure 1. The regenerative potential of MSC secretome for SCI treatment.

THESIS AIMS AND LAYOUT

In this thesis, we pursued to explore the potential of the secretome of mesenchymal stem cells (MSCs) as a cell-free based therapy for Spinal Cord Injury (SCI). For this purpose, the impact of MSC secretome on neuronal differentiation and axonal outgrowth, was initially evaluated *in vitro*. Furthermore, we explored how the secretome of MSCs derived from adipose tissue (ASCs) would impact the regeneration of both *Xenopus Laevis* and mice models of SCI after spinal cord complete transection.

The thesis will present the following layout:

Chapter 1 presents a general overview covering fundamental aspects of SCI pathophysiology and the clinical management strategies currently accessible for SCI patients. Furthermore, a detailed description of the animal models used for fundamental studies on SCI research is provided, along with the main cellular-based therapeutic strategies available for SCI treatment in both pre- and clinical setting.

Chapter 2 comprises research work focused on disclosing the impact of the secretome of MSCs derived from the adipose tissue, bone marrow and umbilical cord on neuronal differentiation and axonal growth, *in vitro*. This chapter is presented as an original paper published in Biochimie, in 2018 (Assunção-Silva et al., 2018).

Chapter 3 addresses the use of a naturally regenerating animal model – the *Xenopus laevis* – to study the regenerative role of ASC secretome after a complete thoracic transection of *Xenopus*' spinal cord. Behavioral and histological assessments are presented.

Chapter 4 presents the work in which a spinal cord thoracic complete transection mice model was used to further explore the potential of ASC secretome in promoting functional and sensorial recovery in mammals. Moreover, the impact of ASC secretome on neuroinflammation, and axonal regeneration and outgrowth after SCI was also studied.

Chapter 5 encompasses a general discussion incorporating all the findings of the work performed on the scope of this thesis. Moreover, the limitations of this work, along with future perspectives are also debated.

CHAPTER I

INTRODUCTION

1. Spinal Cord injury

1.1 Pathophysiology

Spinal cord injury (SCI) is commonly initiated by a mechanical trauma of the tissues surrounding the spinal column, after which the spinal cord suffers an extensive disruption. This initial mechanical event sets the primary injury at which several post-injury events are immediately triggered within the lesion site (Fig. 1). These include massive cell death, bone, muscle and vasculature damage, as well as the formation of an edema [1, 2]. Several other biomechanical and biological mechanisms follow and contribute to the progression to a secondary injury (Fig. 1), a concept first introduced by Allen and colleagues in 1991 [3]. The secondary injury comprises three phases: acute, sub-acute and chronic, as described by Alizadeh et al. [2]. From days to weeks or months, spinal cord parenchyma is invaded by inflammatory cells, and a local release of chondroitin sulfate proteoglycans (CSPGs), glutamate, and other axonal growth inhibitory molecules establishes an excitotoxicity environment [2, 4, 5]. Focal demyelination of both injured and surviving axons is also of great magnitude [4, 6]. Finally, a fluid-filled cyst is formed at the lesion site, surrounded by a glial scar mainly composed of reactive astrocytes and fibroblasts, which contributes to a progressive axonal dead and constitutes a physical barrier to axonal outgrowth and regeneration through the lesion [5, 7]. This axonal growth-restrictive character of the SCI sets a chronic injury. The location and severity of the injury determines the overall neurological deficits, and usually lead to chronic pain syndromes and hypersensitivity of the patients [2].

1.2 Clinical Management

The clinical management of traumatic SCI encompasses a rapid clinical assessment and classification of the injury, according to the American Spinal Injury Association (ASIA) grade [2], and further stabilization of the patients to minimize the primary injury and prevent the secondary injury [1]. Along with the neurological impairments, neurogenic and spinal shock are also other SCI-associated conditions, that together with respiratory, circulatory, cardiovascular, urinary and bowel complications are the main causes of morbidity and mortality of the SCI patients [8-10]. Therefore, it is extremely important that patients' vital signs are stabilized and eventual hemorrhages are controlled in the acute phase of injury upon their entrance in the healthcare unit [9, 11]. Respiratory support, namely tracheostomy and mechanical ventilation, should be immediately provided to the patients in cases of potential airway and breathing obstruction [12], especially for cervical and thoracic SCI, that affects the spinal nerves innervating the respiratory muscles [13, 14]. Further surgical decompression and/or stabilization of the spinal cord is a standard procedure [9, 15] to restore spinal alignment and stability. Actually, surgical

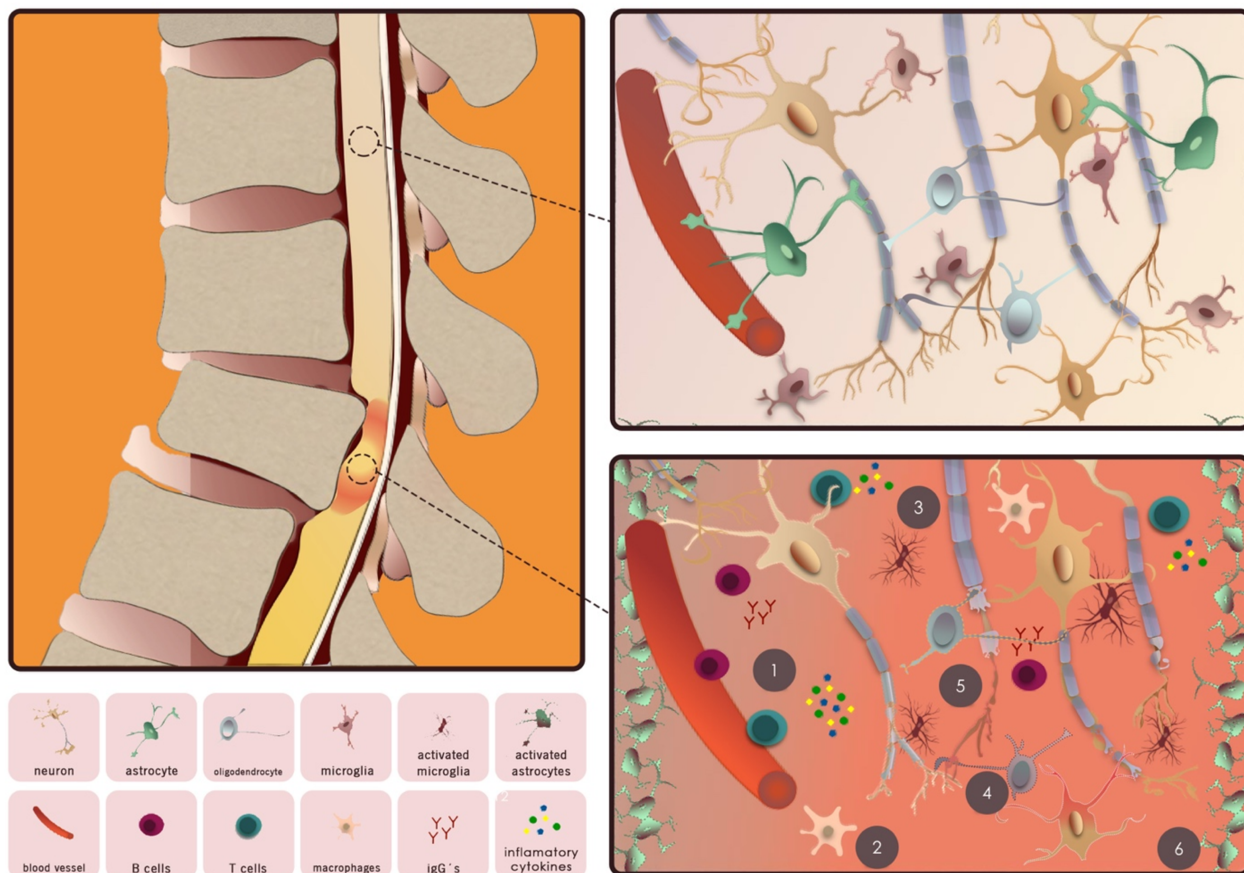

Figure 1. Pathophysiology of SCI. Within an injured spinal cord, both healthy (upper right) and injured (bottom right) environments can be found. The pathophysiology of SCI comprises different events, namely the infiltration of peripheral T and B cells (1) and macrophages (2) releasing inflammatory cytokines and axonal growth-inhibitory molecules, activation of resident microglia and astrocytes (3), oligodendrocyte damage and consequent axonal demyelination (4), axonal damage and retraction (5), and formation of a glial scar composed of activated astrocytes surrounding the lesion site (6). The astrocytic scar surrounds a fluid-filled cystic cavity and constitutes a physical barrier for axons to cross the lesion site, thus impairing the re-establishment of the neural circuitry and the regeneration and repair of the spinal cord. [1, 2, 4]

intervention is the most crucial step in the management of SCI patients, after the control over the life-threatening complications. Early spinal decompression was correlated to improved clinical and neurological outcomes following injury and reduced health care costs [15, 16]. However, it is more likely that surgical decompression cannot completely cease the secondary injury [17]. For that purpose, pharmacological treatment using steroids is the current treatment option [18, 19]. Within these, the most common is Methylprednisolone (MP). The effect of MP in the neurological function of SCI patients was shown for the first time in 1984, and later in 1990 and 1997 by the American National Acute Spinal Cord

Injury Study – phases I-III [20-23]. The findings of these studies provided a standard protocol for the administration of high dosage of MP within 8h post-injury, which showed significant sensory and motor improvements of SCI patients one year following SCI. MP is believed to act upon specific secondary injury-related mechanisms such as inflammation and ischemia [24]. However, the first studies were severely criticized regarding the methodology used, as well as its scientific and statistical reliability. For example, a very small size of sample population showing beneficial effects were considered, and statistical differences could only be found upon stratification of the data. Moreover, the clinical relevance of the statistical functional improvements was not well defined [25-28]. In addition, severe side effects were presented by the patients upon MP treatment, such as respiratory complications that resulted in death, pneumonia and wound infections [22, 23]. In a scenario where the positive outcomes of MP treatment may not compensate the hazardous effects, new Guidelines for the Management of Acute Cervical Spine and Spinal Cord Injury were released, in which the use of MP treatment for acute SCI was not recommended [29]. The controversy around MP treatment for SCI still exists nowadays, and currently there is no pharmacological alternative. Nonetheless, several pre-clinical and clinical tests are currently running with other neuroprotective drugs, such as riluzole, minocycline, and glutamate antagonists [21, 30].

The therapeutic window for intervention in the primary phase of injury is usually difficult to tackle, and the exacerbating secondary injury hamper specialized intervention [4]. The above-mentioned clinical complications that follow the mechanical trauma contributes for this delay in the management of SCI. Therefore, deeper knowledge over the secondary mechanisms is needed to tailor post-traumatic therapeutic strategies that counteract the current clinical failure in the treatment SCI.

1.3 Research on SCI: fundamental studies in animal models

Conceptually, animal models are *in vivo* systems that provide investigational methods that are used to assess specific events under controlled conditions. They are extremely valuable to investigate a given human disease or condition. However, to be reliable and reproductive enough to further translate these investigations to clinics, *in vivo* animal studies must mirror all aspects of the disease or condition, not only in terms of the cause but also concerning its physiological and pathological repercussions.

Given the complexity of the pathophysiology of SCI, finding a strategy that completely promotes the regeneration of the spinal cord after lesion is inevitably dependent on innumerous factors, and the efforts in the clinics are yet not enough. The wide variability in human SCI accounts for this. The inhibitory nature of the biological mechanisms following SCI to axonal regeneration in mammals contribute to the failure

of human SCI to regenerate [2, 5, 7]. But there are other factors to consider. For example, the anatomical level of the trauma can determine the severity of the injury [10]. The time-window and dosage response at which a therapy is given to the patient is also crucial to avoid the progression of the damage [15, 31]. Moreover, some biomechanical factors associated to the injury, such as the mechanism, velocity, and compression level may also affect the treatment outcomes [5]. Therefore, an effective therapy should ideally tackle all these SCI-related aspects, which is probably the most puzzling issue faced by researchers and clinicians in the field. So, before that, determining what features should be addressed in such therapies and how to overcome them is crucial to tailor the most suitable approach that could hold its efficacy at the different stages of one lesion.

1.3.1 Basic experimental models of SCI

Under the premise that robust and fully axonal regrowth must be achieved to promote complete functional regeneration after injury, the common aim of any strategy is to represent the pathophysiology of human SCI as close as possible. As no *in vitro* system is able to mimic the complex environment after injury, different animal models, with varying degrees of complexity have been established throughout the years (Fig. 2).

Amphibians

Amphibians are a group of animals that include anurans, such as frogs, and urodeles, such as salamanders. This group of animals have been used to study embryonic development since the 1900s. After Spallanzani demonstrated that frogs tadpoles and salamanders regenerate their tails, limbs and eye lens [32], a new window of research upon the natural regenerative ability of these animals was opened. The two classes of animals differ in their regenerative process throughout life, although they share closely related biology [33]. The main difference between them relies on the fact that salamanders can regenerate throughout all stages of life, while regeneration in frogs is restricted to early stages of development.

Salamander

Urodeles, such as the Salamanders possess the ability to regenerate tail, limbs, and the lens of the eye, throughout any stage of development. Life cycle of salamanders is very similar to the *Xenopus laevis*, but unlike these, salamanders rarely goes through metamorphosis. In fact, they are neotenous, thus never complete metamorphosis. Nevertheless, salamanders that undergo this process, either spontaneously or hormonally induced, did not present any effect on their regenerative capacity [34, 35]. Only subtle

changes were observed in the immune system upon induced metamorphosis [36], in comparison to the extreme changes observed in *Xenopus laevis* [33], highlighting the impact that an unbalanced immune system might have in the regeneration.

Salamanders are the only amphibians able to regenerate the spinal cord in any stage of development, including embryonic, larval, juvenile and adult. However, using urodeles as a model of SCI and regeneration is not as simple as it might seem. There are different patterns of regeneration among the different developmental stages, with embryonic and larvae animals showing higher numbers of new and qualitatively different neurons than adults [37-39], as well as differences on the time course of regeneration, with adult urodeles taking longer to regenerate than larval and juvenile after trunk transection [40]. The injury models used in salamanders may also influence the overall regenerative processes. The two major models of SCI in salamanders are spinal cord transection and tail amputation. Both share common features, and spinal cord regeneration is easy to follow, where the interactions between growing axons with ependymal cells is very clear [41]. Crush injuries had also been used in salamanders since it is the most studied in mammals, however this model presents an extensive cell death and debris that do not allow a clean view over the regenerative process [42].

It has been established that the regeneration process of urodele's spinal cord after injury is divided in three main parts: ependymal outgrowth, formation of an ependymal tube and channels that facilitate tissue regeneration, and axonal regrowth [43]. Following SCI, a controlled reorganization of ependymal radial glia cells occurs to form an ependymoglial tube in the continuity of the spinal cord's central canal [44]. An interaction of epithelial and mesenchyme occurs to bridge the gap between the two stumps of the spinal cord [43]. Simultaneously, GFAP-expressing ependymoglial cells along with infiltrating macrophages migrate to the lesion gap and clear cell debris and extracellular matrix (ECM) [45, 46]. During salamander spinal cord regeneration, neuronal repopulation of the lesion gap is extremely important to obtain a fully functional spinal cord. Cell tracking studies suggested the ependymoglial cells that are mobilized into the injury site as progenitor cells that generate several cell types, including neurons and glia [47, 48]. How these progenitor cells were committed to differentiate into neural cells was later reported by Schnapp et al. [49] to be due to signaling of healthy portions of the spinal cord through the expression of specific transcription factors such as Msx1, PAX7 and PAX6. Other studies also suggests that at least some embryonic patterning and differentiative processes are required to fully regenerate the spinal cord and restore its function [50, 51]. This was in fact confirmed by Clarke et al. [52], showing that these cells retained the expression of a transcriptional factor code from the embryonic development, which may be related to the continued neurogenesis observed in the salamanders spinal cord throughout

life. Later on, the spinal cord resident Sox2^{+} progenitor cells were found to establish a gene expression program where downregulation of pro-neural genes and upregulation of proliferation-promoting genes was observed [53]. Additionally, they switched from a neurogenic to a proliferative state, supporting spinal cord outgrowth [54]. This goes in accordance to previous reports showing that spinal cord cells did not proliferate after tail amputation in Sox2^{+} progenitor cells knockdown models. This resulted in the total absence of spinal cord in the regenerating tail [55], therefore confirming the role of Sox2-dependent progenitor cells to reconstitute different spinal cord cell types in salamanders after SCI [56]. Others have also shown that the regenerating spinal cord cells activate the expression of some genes associated with Wnt and PCP signaling pathways [53, 57], and that also express factors related to tight junctions proteins and to cell-cycle such as MARCKS protein [58]. Successful spinal cord regeneration in salamanders involves not only stem cell growth and neural replacement but also axonal regrowth into the spinal cord lesion. The features of salamander's neural-ependymal cells contribute to a directed outgrowth of the newly formed neurons. In addition, descending axons from the healthy spinal cord were found to extend into the lesion site, also contributing to the initial formation of newly spinal cord [59]. This pro-regenerative response of the healthy axons in salamanders might be explained by a miRNA array-based analysis performed by Diaz Quiroz et al. [60]. In this analysis, miR-125b was found to be highly expressed in the spinal cord of these animals after transection, and associated to the downregulation of the axon-repulsive gene semaphoring (SEM)-4D. Additionally, increased levels of miR-125 were correlated with better functional outcome of the transected animals [60].

In summary, salamanders' ependymal cells with radial glia phenotype seem to be the key cell type for a successful regeneration. But whether this is enough to identify and counteract to what is missing in the mammals and go towards their injury environment is still to be clarified.

Xenopus laevis

Frog's ability to regenerate after injury started to be reported around 1915, when Hooker and colleagues showed a physiological and anatomical regeneration of frog larvae following spinal cord transection [61]. Interestingly, Piatt and Piatt later revealed that the same was not observed for adult frogs after spinal cord transection [62]. In light of these observations, the recovery and regeneration of spinal cord after transection in frogs in all stages of development became a matter of study. It is now well recognized that the regenerative capacity of anuran amphibians is highly dependent on metamorphosis [63]. A good example of this class of amphibians is the *Xenopus laevis* frog. The development of this specie comprises 65 anatomically identifiable stages, according to Nieuwkoop and Faber's table [64]. In fact, *Xenopus*

larvae was shown by many to progressively decrease their ability to regenerate their tail (containing the spinal cord, the notochord, and the segmented muscles, surrounded by connective tissue and epidermis) and limbs with age. In fact, limb amputation or spinal cord transection before metamorphosis climax (stage 57-60) results in a perfect regeneration of both limbs and spinal cord in 10-14 days after damage [65, 66]. In contrast, post-metamorphic *Xenopus* froglets lost their ability to fully regenerate their tail and limbs [33], demonstrating instead a deficient pattern or incomplete regeneration, along with the appearance of fibrotic wound healing in older frogs [67]. The gradual loss of regenerative ability of these animals may be related to specific signaling cues related with the Bmp and Notch [68-70], Wnt and Fibroblast growth factor (FGF) [71, 72], and Transforming growth factor-beta (TGF-β) signaling pathways [73]. Likewise, genes associated with these signaling pathways were also found to be similar during development and regeneration of the tail and limbs of *Xenopus* [74, 75], such as genes associated with Notch signaling and Msx genes [75]. Moreover, other genes such as HoxA13, msx1, shh, Hox2 and fgf-2 and -8 were also identified to be involved in correct limb regeneration [76], and in the regeneration of the tail, including the spinal cord, after amputation [77]. This latter study provided by Zhang et al. [77] suggested the involvement of the spinal cord in the normal regeneration of *Xenopus* tail and goes in accordance to the suggestion that normal tail and limb regeneration is nerve-dependent [76]. In fact, this was recently supported by Lee-Liu et al. [78], which found clear differences in the gene expression profiles in injured spinal cords from regenerative (tadpoles) and non-regenerative (post-metamorphic froglet) stages. Main changes were associated with stress response, metabolism, cell cycle, development, inflammation, and neurogenesis. Additionally, the expression level of the genes in the regenerated spinal cords was significantly different from those found in the non-regenerative spinal cords [78]. The involvement of the spinal cord in the regenerative process was also reported by Holtzer et al. [79] and, later, by Taniguchi et al. [80], which demonstrated that the complete ablation of the spinal cord of *Xenopus laevis* tadpoles inhibited tail regeneration completely. While spinal cord-ablated tadpoles showed a twisted and smaller regenerated tail, the spinal cord-containing tadpoles did not, confirming this hypothesis. This deficient tail regeneration was further attributed to a decrease of notochord cell proliferation and differentiation, possibly due to a loss of FGF-2 and -10 signaling molecules upon spinal cord removal [80]. Shh signaling pathway may also account for this, as the notochord was reported to be a source of Shh to the cells lying near the amputation region [66]. In fact, at the cellular level, a question that has been risen is at what extent nerve cells or other cellular elements, such as the ependymal cells in the tail, can play a role in the regenerative process of *Xenopus laevis*. In similarity to the ependymal response to SCI in salamanders, the first hallmark of tail and spinal cord regeneration in *Xenopus laevis*

is the migration and rearrangement of ependymal cells from the central canal to the lesion area, where they proliferate to fill the gap between both stumps [81-83]. These data supported previous evidence that axonal elongation beyond the cut ends of the spinal cord of Xenopus tadpoles was found to be properly guided by pre-existing ependymal cellular processes or channels in the lesion gap [81]. Later on, the reestablishment of the spinal cord after tail amputation and spinal cord transection was found to be dependent on $Sox2/3^{+}$ cells lining in the ependymal canal which migrate and proliferate into the lesion gap [84]. Interestingly, $Sox2/3^{+}$ neural progenitor cells were found to generate neurons in response to injury, and Sox2/3 protein levels increase correlated with functional recovery of the regenerating animals [85]. This Sox2/3 progenitor cell response to injury was not observed in non-regenerating froglets though [85], thus proving evidences of their contribution for proper spinal cord and tail regeneration.

Another interesting paradigm around *Xenopus laevis* regeneration is the relationship between that and the maturation of their immune system. This indication came from the observation that *Xenopus laevis* transiently lose their capacity to regenerate between stages 45 and 47 [74, 75]. This period is called the refractory period, and tadpoles whose tails were partially removed at this stage did not regenerate at all, instead solely forming skin across the wound [75]. Interestingly, as soon as tadpoles reached stage 48, their regenerative capacity increased to 75%, reaching up to 100% at stage 49. The reason of this loss has been mainly attributed to the maturation of the immune system of these animals, as it overlapped with the development of immune cells [86]. Experiments using immunosuppressant drugs or immune cell depletion in this period restored tadpole's regeneration [86]. Moreover, the restoration of their regenerative capacity coincides with the emergence of T-regulatory cells [33], suggesting that changes in the immunity may be the cause of the refractory period. These immunity changes during this period mirrors what happens in metamorphic and post-metamorphic *Xenopus* where immune signaling is considered the major regulator of the gradual loss of regeneration [33]. Another explanation might be that the refractory period concurs with a major change in metabolism, as tadpoles start their feeding behavior at this stage. The consequent physiological transition in the nutrition and digestion may therefore impact the regenerative process [87]. Other differences found between the refractory and the regenerative period of *Xenopus* tadpoles were the quite lower levels of Bmp transcripts after injury [88], a higher rate of apoptosis [89], and a failure of the wound epithelial membrane to repolarize normally [90].

The cellular and molecular responses to the regeneration of *Xenopus laevis* after SCI in the pre- versus the post-metamorphic stages are clear by now. On the other hand, the inactivation and activation of the regeneration program during the refractory period of this specie and the altered potential of the injured tissues to regenerate is not yet fully understood. Though the evidences mainly attribute the maturation of

the immunity of these animals to this shift in the regenerative capacity, the mechanisms that underlie the selective retention or loss within this specie needs to be further explored. Only then the overall information obtained from this model should be considered to translate to mammals and human context.

Fish

Lamprey

Sea lamprey is the most basal extant vertebrate and, to date, was the only specie to demonstrate fully functional recovery, according to NIH criteria, following spinal cord hemissection [91, 92], complete transection [93, 94], and even after re-transection [95]. Functional recovery was accompanied by axonal regeneration of reticulospinal neurons through the injury site. However, only approximately half of the axotomized axons actually regenerate [96]. The reason of the failure of the other half to regenerate may be explained by increased levels of neuronal apoptosis on those neurons after spinal cord transection [97]. It could also be caused by the action of myelin inhibitory-molecules, in similarity to what happens in mammals' CNS. However lamprey CNS lacks myelin [98], thus contradicting this assumption. Instead, the downregulation and/or upregulation of molecules repulsive to axonal growth after SCI was a more valid hypothesis. For example, the downregulation of netrin and SEM4D and upregulation of SEM3 mRNA in the spinal cord of transected lamprey were possibly restricting axonal regeneration, as reported by Shifman and Selzer et al. [99]. Moreover, changes in the expression of the repulsive guidance molecule together with its receptor neogenin, was also found to be a player in the restriction of the "bad regenerator" neurons to regenerate in the transected lamprey [100].

Another interesting aspect of lamprey regeneration is that axonal regrowth after SCI is directionally specific [101], which goes in accordance with other studies showing that regenerating axons grew across a lesion site after spinal cord hemissection, when they could rather grow along uninjured tissue [92, 102]. Furthermore, selective synapse formation occurred between neurons near the transection site and distal to the lesion [101, 102]. This data strongly suggests the presence of axon guidance cues in the regenerative lamprey. For example, the role of cyclic Adenosine Monophosphate (cAMP) in post-injury axon growth and regeneration has been well established in these animals. After spinal cord transection, the injection of a cAMP analog to injured animals increased axonal regrowth across the lesion site and prevented axonal retraction and abnormal growth patterns [103, 104]. In addition, the number of axomatized neurons that survived and regenerated were increased [104]. cAMP also led to increased numbers of microglial/macrophages, which was believed to improve the repair of the lesion site [104]. This makes sense, as it appears that glial cells are required for normal axonal regeneration after SCI in

lamprey, similar to their involvement in other vertebrates' regenerative processes. In fact, microglia/macrophages numbers increased after lamprey SCI and were shown to contribute to the expression of the above-mentioned repulsive and guidance molecules at the lesion environment [97, 100, 104]. Moreover, they also played a role in the proper guidance of axonal regrowth, by orienting and extending their own glial processes across the lesion site to form the ependymal bridge for the regenerating or resident axons to grow [104-106]. As observed in mammals, lamprey microglial cells show no features of reactive gliosis, which may also contribute to its ability to regenerate [105].

Besides lampreys' spontaneous axonal regrowth and functional recovery after injury, previous studies suggested that this might also be accompanied by neurogenesis. In an attempt to clarify this, Zhang and colleagues [107] recently tried to determine whether cell proliferation and neurogenesis occurred after lamprey spinal cord transection. Interestingly, higher percentage of proliferative cells were found in the transected spinal cord, expressing markers of glia and neurons, thus indicating that both post-injury gliogenesis and neurogenesis occurred. The newly formed neurons were restricted to the ependymal zone only, as there were no signs of neurogenesis in other regions [107]. Whether these neurons extended their axons away from the ependymal zone and contributed to axonal regrowth was not explored by the authors. Likewise, if this restricted neurogenesis or other mechanisms are having any impact in restoring the function of the SCI in lamprey is still unknown. In fact, studies concerning the molecular and cellular signature for lamprey regeneration are still lacking. But the recent discovery of lamprey genome [108] has increased the possibility to pursue with further studies.

Zebrafish

Fish, like Zebrafish, are another vertebrate that show spontaneous ability to regenerate after injury to the adult CNS. First evidences of axonal regeneration came from behavioral observations of Zebrafish swimming recovery few weeks after spinal cord transection [109, 110]. The same was observed, later on, on adult Zebrafish after crush injury and complete spinal cord transection [111, 112]. In the former study, zebrafish response following SCI was compared to mammalian injury, in the sense that the same cellular and molecular entities were involved. However, while there is prevalence of inhibitory molecules expression and establishment of a non-permissive environment for axonal growth in the lesioned mammalian central nervous system (CNS), Zebrafish shows a more conducive response. A good example of this is the expression of some myelin-associated inhibitory molecules in both models, such as Nogo-A [113]. Nogo-A is a myelin-associated inhibitory molecule in the injury response of mammals [114]. But zebrafish CNS lacks the N-terminus neurite inhibitory domain of Nogo-A, and the other domain (Nogo66)

is growth-permissive to zebrafish axons, in contrast to mammals [113]. The inexistence a glial scar after injury in Zebrafish is another good example. In both models, there is an activation of astrocytes and microglia cells. In mammals, these cells are responsible to form a glial scar composed of ECM components such as CSPGs, that contribute to the environment excitotoxicity [114]. There are however any signs of CSPGs in the glial scar of the zebrafish after optic nerve lesion, although the regenerating axons are repelled by these molecules [115]. On the other hand, glial cells of zebrafish CNS are mainly present in their spinal cord and belong to a population of ependymo-radial glial cells which also have astroglial functions, and that are involved in the early regenerative response following SCI. In similarity to the above described ependymal response of *Xenopus laevis* and Salamanders to SCI, also Zebrafish regenerative processes initiate at the spinal cord stumps by ependymo-radial glial cells [116]. These cells are also generators of neuronal cell types after injury, thus contributing to the re-population of the lesion site and to axonal regrowth of the newly formed neurons throughout the ependymal tube [117, 118]. Resident neurons also contribute to axonal regrowth after SCI in Zebrafish. There are 20 brainstem nuclei axons that project into the spinal cord of zebrafish [109]. Many of them regenerate across and caudally into the injured spinal cord, supporting the anatomical restoration of the spinal cord and leading to functional recovery of the injured animals [111]. Interestingly, not all of them seem to have the ability to regenerate. In fact, Becker and colleagues have been reporting differences in the ability of neurons to regenerate after SCI [119]. In 1998, 84-92% of neurons that display high regenerative brainstem nuclei express GAP-43 following SCI, while only 49-63% GAP-43 expressing neurons were found in low regenerative brain stem nuclei [119]. The varied neuronal regenerative capacity in Zebrafish was later confirmed in other study in which the same author proposed that the locomotor recovery on spinal-transected adult zebrafish was influenced by the regrowth of descending axons from the brainstem and to the rearrangement of intraspinal circuitry, rather than by ascending axons [120]. The different expression of cell recognition molecules among diverse axotomized neuronal populations was again observed, as the expression of growth-related genes, such as GAP-43 and L1.1, was increased in the descending axons, and decreased in the ascending ones. The functional importance of other regeneration/growth-associated molecules for axon regrowth has been widely reviewed by Vajn et al. [112] and Becker et al. [121]. Other evidences suggest that the molecular response to injury in Zebrafish may be more similar to mammals than previously thought, in the sense that some signaling pathways are upregulated during axonal regeneration in the zebrafish after axonal lesion, that inhibit axon regeneration in mammals. For example, the upregulation of a strong neuron-intrinsic inhibitor of axon regeneration in Zebrafish, the socs3, clearly hindered axonal regeneration after an optic nerve lesion

[122]. On the other hand increased levels of cAMP in a specific class of neurons, the Mauthner neurons, increased and directed axonal growth, and correlated to functional recovery, which also occurs in mammalian neurons [123]. Nevertheless, the environment found within Zebrafish CNS after SCI is undoubtedly more conducive to regeneration than in mammals. This lead us to one drawback of zebrafish as a model of SCI, related to the inability to perform studies in a non-regenerative environment that could be comparable to mammals. Still, it is an important model to identify and understand the complex events that regulate regeneration, especially due to the reported similarities between Zebrafish and mammal's systems, therefore guiding future studies on species that do not regenerate at all, such as humans.

VERTEBRATES

Spontaneous regeneration and functional recovery after SCI

salamander

xenopus laevis

lamprey

zebrafish

salamander

- Regenerate tail, spinal cord, limbs and eye's lens throughout life.

xenopus laevis

- Regenerate tail, including spinal cord, the notochord, and the segmented muscles, connective tissue and epidermis;
- Regenerative capacity highly dependent on metamorphosis;
- Temporary loss of regenerative ability.

lamprey

- Complete regeneration after SCI with fully functional recovery and axonal outgrowth.

zebrafish

- Similar molecular and cellular post-injury events with mammals, but that are conductive for regeneration;
- Inexistence of glial scar after injury.

HALLMARKS OF REGENERATIVE PROCESS

salamander | xenopus laevis | lamprey | zebrafish

Tube formation at the lesion site by ependymo-radial glial cells

- Clearance of cell debris and ECM by GFAP-expressing ependymo-radial glial cells and macrophages (salamander)

Neural replacement by gliogenesis and neurogeneris

- Ependymo-radial glial cells are SOX2+/Sox3+ neural progenitor cells (salamander, xenopus laevis)
- Spinal cord-containing notochord cell proliferation and differentiation (xenopus laevis)

Directed axonal regrowth

Interaction between ependymo-radial glial cells and host and newly growing axons

Involvement of growth- and guidance-related molecules and specific signaling pathways

- Only half of axotomized axons regenerate
  Selective synapse formation (lamprey)
- Locomotor recovery supported by descending axons from the brainstem and rearrangement of intraspinal circuitry (zebrafish)

OUTCOMES

- Biomolecular and neuromorphological signature of SCI events
- Environmental and neuron-intrinsic and -extrinsic factors involved in the regenerative process
- Do not address the heterogeneity of SCI pathology

MAMMALS

No regeneration and recovery after SCI

rodents

- Similar genome and SCI pathophysiology to humans
- No cystic cavity in mice
- Depressive/anxiety-like behavior after SCI
- Differences to humans
  - Size of the spinal cord
  - Heterogeneity of the injury
  - Less complexity of nervous system
  - CST mainly dorsal

canine/ feline

- Injury mechanisms, classification and functional monitoring similar to humans
- SCI pathophysiology similar to humans
- Spinal walking" motor behavior

porcine

- SCI pathophysiology similar to humans
- Validated post-operative care and physical rehabilitation
- Neurological evaluation techniques
- Eletrophysiological and electromyographical studies
- Scale for hindlimb motor function assessement
- Porcine Thoracic Injury Behavior Scale for thoracic SCI contusion models

non-human primates

- Anatomical organization and control of motor system closest to humans
- Cognition and behavior similar to humans
- SCI pathophysiology:
  - Spontaneous neuroplasticity of CST after SCI
  - Motor function recovery
  - Axonal sprouting and remyelination;
  - Oligodendrocyte and astrocyte replacement in the injury site by endogenous neurogenesis;
  - Axonal remyelination by newly formed oligodendrocytes
- Electromyography studies and kinematics

OUTCOMES

- Extensive characterization of molecular, biochemical and histological signature of SCI
- Standardization of procedures for interventions and assessments.
- Ideal to study the efficacy of certain therapies and its reliability to move towards the clinics

Figure 2. Vertebrate and Mammal models of research on SCI. Fundamental studies on SCI can be performed in basic models such as vertebrates and more complex models like mammals. These animal models provide important insights on the signature of SCI, and allow to identify potential targets for new therapies. In addition, new treatment methodologies can be tested in these models within a variety of environmental challenges aiming to represent the pathophysiology of SCI as close as possible. [33, 40, 74, 94, 110, 124-129]

1.3.2 Mammal models of SCI

Non-mammalian regenerating models can give us insights on the biomolecular and neuromorphological signature of SCI events, as well as on the environmental and neuron-intrinsic and -extrinsic factors involved in the regenerative process. By doing so, potential targets for new therapies can be identified and encourage the development of new treatment approaches. However, it is worth to note that these smaller animal models do not allow to address the heterogeneity of SCI pathology. Therefore, it is still of the utmost importance to keep SCI studies in non-human mammals that share many similarities with human spinal cord. Several animal models are available, including rodents, dogs/cats, pigs and non-human primates. Moreover, different environmental challenges can be addressed by using different patterns of injury (spinal cord contusion, compression, transection, hemissection, dislocation and chemical) [124, 125]. The contusion or compression injuries are preferred [125, 126], because most of human SCI happen due to a blunt trauma to the spinal cord. Contusion, for example, is the underlying SCI cause in 49% of paraplegic patients [127]. While contusion allows to better investigate the pathophysiology of SCI [3, 128] and may also be more appropriate for acute management strategies [129], compression models enables to study post-traumatic spinal cord decompression techniques or neuroprotective treatment [130]. Transection is also a preferred pattern of injury in SCI models. These models provide a clean injury environment, where both ascending and descending spinal tracks are totally interrupted and there is no issue of axon sparing. However, they are rarely encountered in clinics. Nevertheless, transection models are useful in studying processes of axon degeneration and regeneration [131], and neuroplasticity [132-134], and are the most suitable for the implementation of a tissue engineering related strategies, in which scaffolds can be easily implanted within the injury site [129].

All of these models can also be used in less or more severe context, by performing partial (incomplete) or complete lesions, respectively [134]. Different levels of trauma (cervical, thoracic, lumbar) [126], and treatment's time-window, route and frequency of administration, can also be other variants differently

tested among the current SCI models. It is also important to establish the time period between the injury itself and the intervention or treatment to be performed. For that, it is necessary to distinguish the acute phase of injury [135, 136] – within hours or days post-injury – from the chronic phase [137, 138].

Rodents

Rodents SCI models include both rat and mice. Similar histopathological, behavioral and neurophysiological outcomes after SCI can be found between both species [129, 139], besides sharing a similar genome to human. Nevertheless, rat models are still the most widely used [125, 126], due to their similarity to human SCI pathophysiology, such as the development of large cystic cavities at the lesion site [6, 140]. Rats are, however, quadrupeds and their corticospinal tract is mainly dorsal [141], unlike the biped's of humans. On the other hand, mice do not exhibit a cystic cavity in the lesion site, but instead a densely packed cellular mass that gradually decreases in size [142, 143]. Moreover, these animals offer the possibility to easily manipulate their genome and thus create transgenic models than can be useful to study cellular and molecular aspects of SCI [140]. The differences between rat and mice regarding the pathophysiology of SCI goes beyond the formation of the cystic cavity in the lesion site. In fact, main differences have been correlated to species-specific variances in the distribution, magnitude and composition of the inflammatory response after injury [144]. After spinal contusion in rats and mice, microglia/macrophages reactions were similar between the two species, but lymphocytes and leucocytes infiltration was different [145]. In addition, mice presented a unique fibroblast-like cells in the injured spinal cord, which was previously thought to be involved in the wound repair by activating T-cells and release neuroprotective cytokines to the injured milieu [145]. Interestingly, reactive astrocytes were also found to be important players in the protection of spinal cord tissue and motor function preservation in transgenic mice with reactive astrocytes ablation after crush injury [146]. The absence of these cells aggravated tissue disruption, cellular degeneration, wound spread and severe motor deficits in the injured animals [146, 147]. This goes in contrast to the reported implication of the reactive astrocytes in the formation of the glial scar in rats after SCI, which has long thought to be detrimental to axonal regeneration and functional improvement [148, 149]. However, this is still a matter of debate, as several evidences attribute a rather tissue-protective effects of this cell population in SCI response [150]. Infiltrating macrophages were also reported to play an anti-inflammatory role in the recovery of SCI in mice, which was correlated with motor improvements of the animals [151].

So far, not only the inflammatory response after SCI in rodents has been a matter of study. Functional

analysis techniques as indicator of recovery are also well-established [152], such as behavioral and electrophysiological assessments [124, 153-155]. Evaluation of other biomolecular and cellular events have also been extensively reviewed. Cell necrosis and apoptosis in response to SCI is a good example of that. Crowe et al. [156] and Shuman et al. [157] showed that post-traumatic neural necrosis and oligodendrocyte apoptosis mediated secondary axonal degeneration and chronic demyelination, as a consequence of microglial activation. Other dimensions such as depression and/or anxiety, and pain recently came into light in what regards SCI animal models [158]. It is worth to note that the development of depression and chronic pain are very common among human SCI patients [159, 160], thus requiring appropriate attention to be given to this particular behavior in animal models of SCI. Therefore, the establishment of tools to assess depressive/anxiety-like phenotypes, as well as pain behaviors in both rat and mice has been increasing [158, 161].

In light of the above reported findings, rodents are probably the most suitable animal models for SCI research. The more evident difference between rodents and humans are the size of the spinal cord. The heterogeneity of injury, and the less complex functional organization of rodent nervous system should also be considered [162]. Therefore, the direct translation from rodents to clinics remains under debate. While some believe in the accuracy of rodent models by itself, others keep requiring further investigation in upper, larger animal models, as well as in non-human primates [163-165].

Larger animals and non-human primates

Large animal models such as dogs/cats and pigs, and non-human primates are the closest to human researchers can get in pre-clinical setting. However, unlike rodents, that are easy of handle, with a relatively low cost handling [126], the use of larger animals usually involves practical and ethical concerns, so that they are rarely used [133].

Canine/Feline

Canine SCI model has been mostly considered for SCI research due its similarities in the mechanisms of injury, classification and functional monitoring to those of human patients. In fact, dogs usually suffer from contusive-compressive injuries as result of road traffic accidents or disc degeneration, at both cervical and thoracolumbar levels [166-168]. Whenever naturally injured dogs are not used, balloon compression injuries are the most preferred injury pattern to use due to it reproducibility [169, 170]. The pathophysiology of canine SCI is also very similar to rodents and humans [171]. In addition, scales to

determine canine SCI severity has been established and are very similar to the ASIA scale used for human SCI patients [172]. Finally, functional assessment of dogs has also been established. In this regard, electrophysiological studies have been performed in both acute and chronic SCI dogs [170, 173]. Following severe SCI, dogs usually loose pelvic limb sensation but regain their ambulation, resulting in a phenomenon known as "spinal walking". Recent electrophysiological recordings tried to correlate the incidence of this phenomenon and pelvic limb sensation with changes in somatosensory and motor evoked potentials, but no apparent association between them was found, suggesting that other factors may be leading to the motor recovery of these animals [173]. Cats also present this phenomenon of spinal walking, even after complete SC transection at the cervical level, if they receive appropriate physical training [174, 175]. The existence of central pattern generators in the spinal cord has been linked to the occurrence of this phenomenon. Contrarily to this, non-human primates and humans are unable to walk after spinal cord complete transection [176, 177] .

Alike dogs, cats also frequently suffer from SCI as result of road traffic accident or falls. Most of them occurs at the thoracolumbar and sacrococcygeal level and are usually accompanied by concomitant injuries [178, 179]. Cats rarely die from SCI, and the primary clinical care encompasses spinal and systemic stabilization [180], to avoid the progression to secondary injury, following the procedures that are taken with human SCI patients. Neurological assessment follows with the assessment of multiple spinal lesions and the presence of nociception, to determine the prognosis of the animal [181, 182]. From a more histological point of view, cellular response to cat SCI has been explored since the early 60's. Bunge et al. [183] was the first to show remyelination events on the adult mammalian central nervous system. However, the author used an experimental injury based on the manipulation of cerebrospinal fluid by withdrawal and reinjection in cat spinal cord, so a limited demyelinating lesion could be produced. Following injury, the demyelinated axons remained intact, and the first evidences of newly formed myelin appeared 19 days post-injury, with all axons showing myelination by 64 days post-injury. This follows the surrounding of axons by glial cells, resembling the mechanism of myelinogenesis [183]. However, these data do not go in agreement with the present evidences on the mechanisms of demyelination and remyelination of mammalian systems. Additionally, the experimental injury model used by this author has nothing to do with the SCI that is usually used in a pre-clinical setting with SCI animal models and observed in clinics. In fact, later in 1983, Blight et al. [184] showed that a contusion injury at mid-thoracic spinal cord of female cats provoked severe neuronal death in the damaged spinal cord at a chronic stage, accompanied by marked and sustained axonal demyelination and selective elimination

of large fibers. Most of the animals did not recover their locomotion. For those who did, the recovery of effective locomotion was associated with the maintenance of 5-10% of neuronal population in the lesion site. No spontaneous axonal remyelination was, however, observed.

Porcine

Adult pig SCI models have been developed over the last years, aiming to provide an intermediate valid model between rodents and humans. Recently, the corticospinal tract in pigs was found to be anatomically similar to human, suggesting the importance of the porcine as a pre-clinical model. One of the first studies on pigs subjected to SCI was reported in 1991 by Blight et al. [185]. In this study, guinea pigs were moderately injured by compression of the spinal cord at the thoracic level, and comparable to contusion injuries in cats regarding the type and distribution of axonal damage. At the chronic stage of injury, some degree of functional recovery was obtained, which correlated with the number of surviving axons in the lesion. Following the time course of the injury, a delayed secondary response to injury was observed, which was suggested by the authors to be due to a slower phagocytic activity of macrophages during the inflammatory response at the injury site [185]. More recently, a sacrocaudal SCI porcine model was developed using minipigs [186]. After sacrocaudal spinal cord transection, marked neurological deficits restricted to animals' tail was observed. The aim of this study was to use this model for cell transplantation in an acute SCI phase, which at the end proved valid for that effect [186]. In a more severe injury context, Zahra et al. [187] demonstrated that a complete cervical SCI in piglets provoked acute hemodynamic alterations and withdrawal of sympathetic tone. The establishment of more chronic injuries in porcine models has also been described. For example, a chronic complete paraplegia porcine model was well-established by Zurita and colleagues [162]. Several procedures of postoperative care in both acute and chronic phase of injury were validated, as well as the treatment of possible complications. Neurological evaluations of the animals were also optimized and established and enabled to assess the development of neuropathic pain and autonomic dysreflexia. Importantly, a new scale for hindlimb motor function assessment was developed based on a modification of the previously reported Tarlov scale [188]. Physical rehabilitation of the hindlimbs and other locomotion-based training were also applied, such as kinesitherapy and daily gait training in a treadmill [189]. Electrophysiological studies were also performed in these animals to assess the integrity of ascending and descending spinal cord tracts, which in the future can be used to confirm the potential of the tested therapeutic approaches in this model [162]. Eletrophysiological and electromyographical studies in several porcine models of SCI had been already

described by others [190]. More recently, a new reliable Porcine Thoracic Injury Behavior Scale was developed in order to assess locomotor recovery of minipigs following thoracic SCI contusion [191]. In this study, varied degrees of injury severity were induced, and the locomotor recovery of the animals differed accordingly, and correlated with the extent of SC white and gray matter sparing, as well as neurofilament expression at the epicenter of the lesion [191].

Non-human Primates

The need to demonstrate a therapy's efficacy in non-human primate SCI models before moving with a clinical trial is still under intense debate [163]. Nevertheless, non-human primates are undoubtedly the closest to human that researchers can get in the pre-clinical setting. The anatomical organization of their motor system and the contribution of the descending cortical and subcortical spinal cord pathways in controlling their movement in an injury context corresponds to that found in the other species, such as cats, and humans [192]. This may explain the remarkably spontaneous neuroplasticity of the corticospinal projections in the primate spinal cord after cervical hemissection SCI, which was found to reconstitute 60% of the pre-lesion axon density in the injury site [193]. Motor function recovery of muscle recruitment, hand function and locomotion was correlated with this improved anatomical recovery. The motor recovery in these animals was evaluated by electromyography (EMG) recordings and kinematics during the performance of fine motor tasks and treadmill training [193]. The use of EMG as a measure of impairment and recovery after traumatic injury had also been used by others [194], which demonstrated to be a feasible and safe method to use.

The reported improvement on motor recovery of mammals has been associated with mechanisms of spontaneous recovery, either by the use of the spared systems after injury [195], and axonal sprouting or remyelination [132]. In primates, endogenous neurogenesis was also a mechanism suggested to contribute to this, by replacing oligodendrocytes and astrocytes within the lesion area [196]. The newly formed olygodendrocytes were seen to ensheath and remyelinate the host axons. This goes in accordance to other studies, also highlighting the importance of neurogenesis as a compensatory mechanism after injury in mammals through cell replacement [196].

The main goal of SCI research in animal models is, in fact, to extensively characterize all the available SCI animal models and standardize procedures so several interventions and assessments can be comparable among studies, as well as their correlations with molecular, biochemical and histological

evidences. Only then the insights given by this understanding over a pathology will enable to move towards the development of strategies that can be further used to control or revert the consequences of that pathology in the clinics.

The proximity with humans makes non-human primates excellent models to study the effectivity of certain therapies and its reliability for the clinics. Moreover, the differences between rodents and larger animals such as dogs and cats, and monkeys are significant. As well reviewed by Courtine et al. [197], major differences concern the evolution of the neuroanatomy throughout the evolution of the species, namely the motor cortex and its corticospinal tract (CST) projections. While CST lesions had almost no effect on stepping in rodents, suggesting a poor motor cortex influence in sustaining locomotion in rat and mice [198], monkeys showed marked deficits in stepping after CST damage [199], and humans were unable to walk independently after that [200]. Likewise, different inflammatory and immune responses after SCI were found between rodents and monkeys [201], which could largely influence the cellular and molecular events characteristic of the SCI pathophysiology. These and other differences among species may result in divergent anatomical and functional outcomes. Nonetheless, there are some constrains regarding the use of larger animals, namely monkeys. For instance, proper housing and post-operative care of these animals are under extreme regulation control. Plus, it is not always easy to apply and maintain urinary catheters or incontinence pads on these animals to control the incontinency consequent of the injury [162]. Regarding monkeys, the difficulty in maintaining them in their upper limbs limits the rehabilitation care to passive techniques, specially in cases of complete paraplegia, instead of using active rehabilitation and physiotherapy such as that applied to SCI human patients [202]. On the other hand, the relatively large size of monkeys is obviously more comparable to human than to lower animals, which is not only reflected in the neuroanatomy and physiology of the monkeys, but in other dimensions such as cognition and behavior [197].

In conclusion, all animal models have advantages as well as caveats. The choice on the best model to use highly depends on the specific aims of the study and which outcomes are expected from there, which normally goes from determining the effect of a therapeutic approach in terms of spinal axonal growth, neuronal cell recovery, remyelination and regeneration, and the inflammatory responses after injury and/or treatment, but also to assessment of functional recovery. Fundamental studies on rodents and larger animals should be continued, especially if never-tested therapies are being explored. After that, and before moving to human clinical trials, non-human primates are most likely the unique models that provide ways to truly understand the safety and efficacy of that therapy, and examine their effects on

multiple variables.

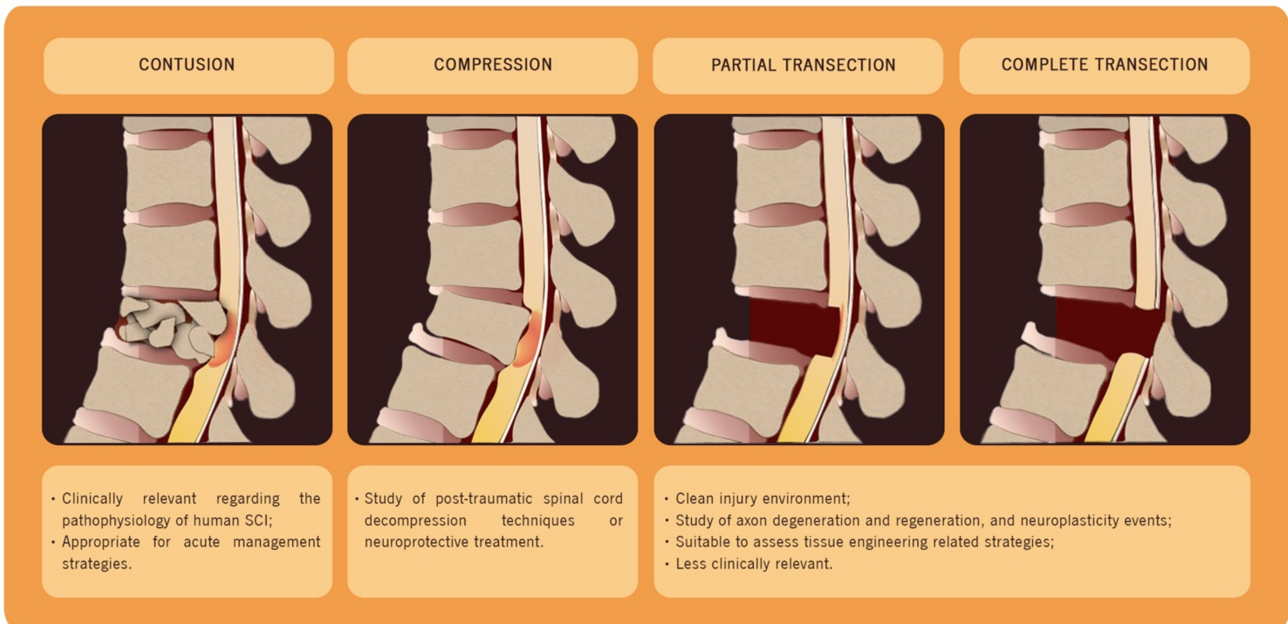

Figure 3. Injury models of SCI. Contusion-, compression-, and transection-based models are the main injury models available to study the pathophysiology of SCI and to test new therapeutic approaches [131, 132].

2. Cell transplantation: A relevant regenerative approach for SCI treatment

The above referred established animal models have been of great importance not only to understand the basic pathophysiology of SCI, but also for the possible development of therapeutic strategies. Indeed, the establishment of therapies for SCI patients that lead to functional improvements is still a priority for researchers and clinicians. The challenge relies in obtaining robust pre-clinical evidence from animal model research before moving to clinics.

At the moment, various cellular, molecular and tissue engineering strategies are being studied under different conceptual rationales. Cell therapies have been in the forefront of most SCI regenerative strategies, due to their neuroprotective and/or neuroregenerative potential [203]. In the context of SCI, cells can have the ability to replace the lost ones, promote and guide axonal growth and myelinate newly and resident neurons [203]. Going beyond these functions, many cells secrete an array of trophic factors which give support to the resident cells and modulate the local inflammatory response as well as potentiate plasticity in the spared spinal cord [204-207]. With this plethora of potential mechanisms, a

variety of cells from several tissue sources have been investigated for SCI, namely pluripotent and adult stem/progenitor cells, and adult myelinating cells. The vast majority of studies have been conducted in rodent models, but also some in larger mammals or primates. Along this sub-chapter, the most recent and promising pre-clinical studies using cell transplantation on animal SCI models will be addressed. As some of these strategies have reached clinical trials, their translation to human SCI will also be tackled.

2.1 Adult Myelinating Cells

Schwann Cells

SCs are the myelinating cells of the peripheral nervous system. As so, they can be valuable to use in CNS regenerative strategies. In fact, these cells have been extensively used in SCI animal models, with the first studies concerning SC transplantation being presented by Duncan and colleagues, who showed the ability of these cells to remyelinate demyelinated host axons in both the brain and spinal cord models of demyelination [208, 209]. Since then, several animal models have been used for SC transplantation studies. In a rat SCI model of contusion, the transplantation of SC suspensions supported the extension of sensory and spinal axons into SC grafts, with some of them showing evidence of remyelination, accompanied by improved motor recovery after transplantation [210]. Similar results had also been previously shown by Pinzon et al. [211] in a thoracic transection SCI in rats, in which the transplantation of matrigel channels-containing SCs increased axonal myelination and regeneration through SC grafts, with improved measurable electrophysiological conduction. More recently, SC grafts were shown to support axonal growth, both descending and ascending fibers, in rats with chronic thoracic SCI, correlated with improved motor function of the animals [212]. Interestingly, the genetic modification of SCs to express neurotrophins, namely Brain derived neurotrophic factor (BDNF) and Neurotrophin (NT)-3 and further transplantation into thoracic transected rat spinal cord, was shown to increase axonal regrowth and regeneration [213, 214].

Due to the positive results obtained in animal models, some of this work has been translated to the clinics. For instance, Saberi and colleagues used autologous transplantation of SCs in patients with chronic thoracic SCI. However, there were no beneficial neither adverse effects following treatment [215]. Later in 2016, the Miami Project to Cure Paralysis initiated a phase I clinical trial to evaluate the safety of autologous human SC transplantation in patients with subacute complete SCI (NCT01739023) [216]. No adverse events were found upon SC usage up to one year post-transplantation, proving the safety of both the administration procedure and treatment. No additional spinal cord damage or formation of mass

lesions were also observed [216]. After this, the same group is looking for the safety of human autologous SC transplantation in chronic SCI patients receiving rehabilitation (NCT02354625). This trial is currently ongoing, and results are expected by the end of 2019.

Olfactory Ensheathing Cells

Olfactory Ensheathing Cells (OECs) can be found in the nerve fiber layer of the olfactory bulb and in the nasal olfactory mucosa [217, 218]. These cells are specialized glial cells, recognized by their pro-regenerative potential in the olfactory system as they support the continuous growth of olfactory neurons located in the olfactory bulb following damage [219]. This regenerative capacity is reported to be mediated by the secretion of lipid vesicles, neurotrophic factors and ECM molecules, among others [220]. The application of this cell-type for SCI was firstly reported by Li et al. [221]. Following unilateral cervical SCI in rats, OECs transplantation was found to improve CST fibers regeneration, with further motor improvements [221]. Similar observations were done by Ramon-Cueto and colleagues in 2000 [222]. When combined with treadmill training, OECs transplantation into fully transected SCI rats showed further improved motor function [223]. However, the authors revealed no regeneration of serotonergic fibers bellow the injury site [223]. Others have also found some difficulties in achieving robust axonal regeneration upon OECs transplantation. For example, no CST regeneration was observed after primate-derived OECs transplantation into nude rats. In this study, only a modest regeneration of 5-HT fibers was achieved [224]. Similar findings showed no rubrospinal or CST axonal regeneration in adult rats after OECs transplantation [225, 226]. Despite this controversy around OECs positive effects, some clinical trials were conducted using this cell population in SCI patients. From a safety phase I/IIa clinical trial using autologous transplantation of OECs into chronic SCI patients, no adverse events were reported up to three years [227]. Moreover, no significant motor improvements were observed in any patient [228], possibly due to the use of a cohort with complete thoracic SCI, which might be too aggressive, thus limiting the observation of any OECs positive effects. More recently, autologous transplantation of OECs in three SCI patients with complete chronic thoracic paraplegia was reported to induce functional improvements in two of the patients, which upgraded from ASIA A to C and B, correlated with the restitution of the continuity of some white matter tracts. The third patient showed enhanced motor and sensory function bellow the injury level, although no changes occurred in ASIA scale [229]. Despite these works demonstrating the safety and feasibility of OECs transplantation in SCI patients, there is still a lack of studies evaluating OECs efficacy and their possible mechanisms of action.

2.2 Stem cells

Pluripotent Stem Cells

Embryonic Stem Cells (ESCs) are pluripotent stem cells isolated from the inner cell mass of blastocysts that have the ability to self-renew indefinitely and differentiate into any cell type [230]. The interest around these cells for SCI relies mostly on the possibility to pre-differentiate them into neural and glial precursor cells, either by *in vitro* cell culture conditioning or using genetic tools [231-233]. For instance, the transplantation of neuron- and glial-restricted precursor cells derived from ESCs into rats' spinal cord after contusion, led to improvements of motor and bladder function in injured animals. Interestingly, sprouting of descending nerve pathways was observed, and attributed to the ability of the transplanted cells to differentiate into neurons- and glial-like cells [234]. Neuronal differentiation of ESCs-derived neural progenitor cells (NPCs) after transplantation in a rat model of SCI was also reported by McDonnald et al. [235]. Moreover, motor recovery of the transplanted animals was significantly better than that of the control group. In another study, oligodendrocyte precursor cells (OPCs) derived from human ESCs were found to remyelinate injured host axons after transplantation into demyelinated rat spinal cords, which was associated with improved motor recovery [236]. These promising pre-clinical studies encouraged the use of ESCs in SCI patients in a phase I clinical trial by Geron Corporation in 2009. Safety studies were conducted using human ESC-derived OPCs in complete subacute thoracic SCI patients. No signs of positive effects in patients receiving the transplants were observed. The lack of positive outcomes, together with financial constraints, resulted in the Geron's program to be discontinued later in 2011 [237]. More recently, a safety study also using ESC-derived OPCs, but this time in patients with subacute cervical SCI, was conducted in a phase I/IIa clinical trial by Asterias Biotherapeutics (NCT02302157). The study was finished in December 2018, but safety results are still to be provided. Nevertheless, there are many constraints regarding the use of human ESCs. One is the formation of teratomas following ESCs transplantation, although there are attempts to decrease this phenomena by applying specific protocols of differentiation or genetic manipulation before transplantation [238, 239]. Other concern is how and from where ESCs are acquired, because usually they are obtained from human oocytes and embryos that normally die along the experimental process [240]. A fact that is not accepted by many in the field of cell transplantation, although there are published guidelines for ethical development of ESC-based therapies [241, 242].

The ethical concerns related to ESCs can be counteracted by the use of induced pluripotent stem cells (iPSCs). Through the introduction of four transcription factors, namely OCT4, SOX2, KLF4 and c-MYC, into adult somatic cells, it is possible to revert the phenotype of differentiated cells into an ESC-like state [243-246]. This iPSCs technology was pioneered by Yamanaka's lab in 2006 [247]. Further on, the production of iPSCs comparable to ESCs was achieved, mostly in terms of pluripotency, self-renewal capacity and gene expression, although not so effective in differentiating into neural cells [248, 249].
Despite this lower neural differentiation ratio, iPSCs have been used for CNS applications. For example, NPSCs were successfully derived from iPSCs through induction of NOTCH signaling pathway [250, 251]. When transplanted into mice with thoracic SCI, iPSCs-derived NSPCs improved functional recovery of treated animals, by remyelination of the damaged axons [252]. The transplantation of iPSC-derived OPCs in a rat model of mild contusion was also shown to remyelinate the host axons following SCI [253]. Synaptic contacts throughout the spinal cord, and enhancement of serotonergic innervations, was also observed. As a result, motor and electrophysiological recovery was achieved [253]. Similar results were observed in a rat model of mild contusion, in which the acute iPSCs-derived OPCs transplantation led to increased number of myelinated axons, reduction of cavity size and glial scar, together with improved motor function [253]. In a more complex model, Kobayashi et al. [254] transplanted both murine and human iPSC-derived NPCs into a non-human primate, following cervical contusive SCI. The grafted cells survived and differentiated into neurons, astrocytes and oligodendrocytes. In addition, enhanced axonal sparing/regrowth and angiogenesis were observed at the lesion site. Finally, functional recovery was also evident, namely in open field, bar grip strength and cage climbing tests. In contrast to these studies, others have reported issues in grafting survival or transplantation timing of human iPSC-derived NPCs [255, 256], and even reduced animals' functional recovery [257]. A tumorigenic character is also reported for iPSCs, which should be further tested [258, 259]. This has, so far, hindered their translation into clinical trials. Nevertheless, the first-in-human clinical trial using iPSCs for SCI is approved in Japan, and is expected to start later this year [260].

Neural Stem Cells/NPCs

Neural Stem Cells (NSCs) and NPCs are multipotent cells that have the capacity to differentiate into the three main neural lineages and their beneficial effects include mostly cell replacement and remyelination [261]. This was in fact shown by transplanting adult NPCs into an adult transgenic mice model of dysmyelination, in which the cells differentiated into oligodendrocyte-like cells that promoted robust axonal

ensheathment, myelination and improved axonal conduction [261]. Similar results were found in rat demyelinating and dysmyelinating models, where NSCs derived from adult spinal cord of rats were able to generate OEC- and SC-like cells, with myelinating activities [262]. Moreover, NPCs transplantation into a larger model has also been reported. Iwanami et al. established a non-human primate model of cervical contusion and transplanted NPCs into the spinal cord [263]. The transplanted cells survived within the lesion site and differentiated into the three neural lineages. In addition, decreased injury cavities was observed, as well as improved motor activity of the transplanted animals [263]. Several evidences also show that NPCs are able to provide trophic support to the resident cells. Increased levels of neurotrophic and neuroprotective factors, such as BDNF, basic bFGF, nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), insulin growth factor (IGF-1), Leukemia inhibitory factor (LIF), among others were found in the spinal cord of rats after transplantation of NPCs, following cervical dorsal column injury [204], and thoracic compression [264]. In addition, NSCs genetically modified to produce NT-3, increased NSC effects on host axons [204]. Modulation of the inflammatory local response after injury may also be mediated by these factors and other produced molecules, such as cytokines [265]. All this promising pre-clinical evidence led to two phase II clinical trials using NSCs (NCT02163876; NCT01321333) by the company Stem Cells Inc. In these, the safety and preliminary efficacy of purified human fetal NSCs transplantation was evaluated in incomplete and complete thoracic SCI patients at a chronic stage of injury [266]. Patients were temporarily immunosuppressed before NSCs transplantation. After a single administration of NSCs, some positive sensory improvements were reported, however many disappeared after immunosuppressive treatment was removed [266]. The trial was interrupted 5 years later though, due to financial restraints. Currently, Neural-stem Inc. is recruiting patients for a phase I safety study of human spinal cord-derived NSCs in both cervical and thoracic chronic SCI (NCT01772810). Results of this clinical trial are expected in 2022.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are mesodermal lineage multipotent stem cells capable of differentiating into osteoblasts, adipocytes and chondrocytes [267, 268]. Moreover, MSCs were defined as cells that are plastic-adherent and express specific clusters of differentiation (CD), namely CD105, CD73 and CD90, lacking the expression of CD45, CD34, CD14 or CD11b, CD79-α or CD19 and human leukocyte antigen DR isotype surface markers [269].

MSCs were originally identified in bone marrow [270, 271], but they can be found in several other adult tissue sources, including adipose tissue [272], umbilical cord and umbilical cord blood [273], placenta [274], dental pulp [275], skeletal muscle [276], endometrium [277], synovial membrane [278], among others. In addition, MSCs isolation can be easily performed [279], without rising any ethical or political issues. This makes MSCs widely accessible for autologous transplantation. Moreover, the transplantation of adult stem cells such as MSCs dodges all the ethical and practical concerns related to ESCs and embryonic-derived NSCs/NPCs, enabling the use of autologous transplants which should not elicit immune rejection from the host [280].

Despite all the available alternative sources, bone marrow-derived MSCs (BM-MSCs), adipose tissue-derived stem/stromal cells (ASCs) and MSCs derived from the umbilical cord (UC-MSCs) are the most studied in what regards their potential application to SCI. MSC's suitability for CNS applications was for many years considered to be related to a transdifferentiation capacity of these cells into neural phenotypes, which would enable the replacement of damaged neurons and glial cells in the context of injury. This was in fact studied both *in vitro*, where BM-MSCs were found to putatively differentiate into neuron-like cells and glial cells [281], and *in vivo*, showing the migration of these cells across the blood-brain barrier (BBB) and differentiation into microglial-like cells [282]. Despite these findings, MSCs are more likely to provide support and protection to endogenous cells, rather than differentiating into fully functional neural cells. There is clear evidence of improved axonal growth and sprouting, remyelination and axonal conduction following BM-MSCs transplantation into the rat spinal cord after contusive injuries [283-285], going up to one year following transplantation [188]. BM-MSC-mediated histological improvements were correlated with functional recovery for both rats [285, 286], and pigs [188]. Similar findings were reported using blood UC-MSC transplantation. Saporta et al. [287] was one of the first transplanting human UC-MSCs (HUC-MSCs) acutely and subacutely, in SCI rats. When transplanted in the subacute stage (five days post-injury), HUC-MSCs were found to significantly improve animals' locomotor function, but not in the acute stage (one day post-injury). In addition, transplanted HUC-MSCs were able to migrate to the damaged tissue [287]. The therapeutic potential of UC-MSCs has also been studied in larger SCI models such as dogs. The ability of blood-derived UC-MSCs to promote axonal remyelination of dog's spinal cord after injury was accompanied by a significant improvement in nerve conduction [288, 289]. In addition, 4 out of 5 HUC-MSCs transplanted dogs presented significant hindlimb recovery, which was sustained up to three years following transplantation [289]. Functional improvements following ASCs transplantation into SCI rodent models have also been reported [290],

together with evidence of homing capacity of the transplanted cells towards the injury site [291, 292], decreased levels of apoptosis and astrogliosis [290, 293], and tissue preservation and axonal regeneration [294]. ASCs transplantation has also been shown to positively impact the locomotion and EMG activity of dogs with lumbar compression-induced SCI [295-298].

Following on this, these numerous clinical trials have demonstrated the biosafety and therapeutic potential of MSC transplantation into SCI patients. BM-MSCs transplantation into subacute and chronically injured SCI patients contributed to improvements of AIS grades and decreased spasticity levels following transplantation. Better quality of life (Barthel Index) and improved bladder function were also observed [299]. Interestingly, multiple routes of administration of BM-MSCs were used, namely directly into the spinal cord, directly into the spinal canal, and intravenous. Any patient have shown tumor formations, infection or increased pain, therefore demonstrating that the administration of BM-MSCs through multiple routes is feasible and safe [299]. Currently, Pharmicell Co. is evaluating BM-MSCs safety and efficacy in a phase II/III trial (NCT01676441) in patients with chronic cervical SCI. Results are expected in 2020. On the other hand, clinical application of HUC-MSCs from the wharton's jelly was effective in thoracolumbar SCI patients SCI, promoting improved motion and activity of the patients, as well as tactile sensation and algesia, and bladder and bowel function [300, 301]. Functional recovery of the patients was also reported. Although no adverse events were reported in any of the clinical studies, the safety of HUC-MSCs transplantation into SCI patients was not actually evaluated. More recently, some phase I/II clinical trials were undertaken using UC-MSCs for both subacute and chronic SCI (NCT03521323; NCT03505034; NCT02481440). Finally, ASCs have also been tested in two clinical trials, where the transplantation of these cells either intravenously or intrathecally to SCI patients have promoted slight functional improvements [302, 303]. No adverse effects were seen in any of the trials. Finally, ASCs are being assessed in a phase I clinical trial, by the Mayo Clinic. Chronic SCI patients have been enrolled in the Adipose Stem Cells for Traumatic Spinal Cord Injury (CELLTOP) study (NCT03308565) to receive autologous MSCs into the cerebrospinal fluid. The results of this study are estimated to be available in 2023.

3. MSC secretome: A cell-free based therapy for SCI regeneration

3.1 MSC Paracrine Activity: Secretome vs Transplantation

Over the years, MSCs contribution to the repair and regeneration of damaged tissues following SCI has been confirmed in several studies, by supporting host axonal remyelination and outgrowth [304-306] and contributing to overall tissue protection and preservation [307, 308]. Furthermore, these cells present anti-inflammatory, anti-scarring, anti-apoptotic and pro-angiogenic properties in response to SCI [309-312]. However, the percentage of surviving MSCs upon transplantation is surprisingly low [276, 313, 314], so long-term engraftment and survival of these cells is very unlikely. This strongly suggests that their neuroprotective and neuroregenerative potential relies mostly on a trophic activity of these cells. In fact, MSCs secrete a broad spectrum of bioactive molecules and/or microvesicles, known as secretome, which is believed to mediate their regenerative properties [315-317]. Therefore, a detailed discrimination of which factors MSCs secrete, and under which conditions, is the key to understand the innate capacity of MSCs to contribute to the injury response. Protein analysis performed on the BM-MSC secretome revealed the presence of IGF-1, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and TGF-β1, which supported hippocampal neuronal survival and axonal outgrowth in culture [318]. Our group found similar results using the secretome of ASCs and UC perivascular cells (HUCPVCs), which were related to the presence of NGF, VEGF, HGF and stem cell factor (SCF) in their secretome [319]. Likewise, the viability, proliferation and densities of cortical and cerebellar neuronal cultures were found to be increased by the secretome of HUCPVCs [320]. In another study, the secretome of ASCs was reported to protect cortical neurons from inflammation *in vitro* [321], by increasing TGF-β1 and interleukin (IL)-10 while decreasing TNF-alfa (α), nitric oxide or prostaglandin E2 in culture. Many *in vivo* studies, although not applying the secretome itself, have explored gene and protein expression in the transplanted cells following SCI. For instance, in a rat SCI model, the expression of human neutrophil-activating protein-2 (NAP-2), NT-3, bFGF, glucocorticoid induced tumor necrosis factor receptor (GITR) and VEGF-receptor 3 (VEGFR-3) by Wharton's Jelly UC-MSCs was related to an overall improvement of injured animals [322]. Another evidence was shown after the transplantation of HUC-MSCs in rats after SCI, where the neurotrophic factors GDNF and VEGF were detected in animals' spinal cord, possibly explaining the improvements observed in neurological functions [323]. NAP-2, NT-3, bFGF, GITR and VEGF were also detected in the spinal cord of completely transected rats following Wharton's Jelly UC-MSCs, accompanied by axonal growth and regeneration, and locomotor improvements of the treated animals [324].

Altogether, these data indicate that MSCs might engage in a paracrine profile, providing a favorable environment for neuroregeneration after SCI. In recent years, the progress in discriminating the composition of MSC secretome triggered the idea of using the secretome of MSCs therapeutically, in alternative to cellular transplantation. In line with this, pre-clinical studies concerning the use of MSCs secretome were first shown by Cantineaux et al. [325] with the intrathecal delivery of BM-MSCs secretome into the spinal cord of rats after SCI contusion. The secretome presented a pro-angiogenic character, promoting a reduction in cystic cavities and leading to functional recovery. No effect on axonal regrowth was observed though, neither on modulation of the inflammatory response after injury. These results were confirmed by exploring the properties of BMSC secretome *in vitro*, performed simultaneously by the authors [325]. Similar results were later reported by others [326, 327]. Kanekiyo et al. [326] and Cizkova et al. [327] have shown that repeated intrathecal injections of BMSC secretome in rats after SCI contusion induced interesting levels of functional recovery. This was correlated with increased regeneration of the damaged tissues, indicated by higher axonal densities throughout the injury site, spared spinal cord tissue, axonal remyelination and enhanced GAP-43 expression [326, 327]. In contrary to Cantinieaux et al. [325], Cizkova and colleagues reported attenuated inflammation following BMSC secretome treatment, further confirmed by decreased levels of pro-inflammatory markers, namely IL-6 and TNF-α [327]. All these promising data suggest that the secretome of MSCs by itself is able to promote neurological improvements after SCI and provide means for spinal cord repair after injury. Therefore, the therapeutic application of MSC secretome seems to provide a safe and viable alternative to cell transplantation for SCI treatment. Nevertheless, the extensive characterization of MSC secretome pointed to significant distinct secretory profiles of MSCs [328, 329], which seems to vary according to the experimental conditions being used [330-332], as well as to the intrinsic properties of the cells, and to the different available donor and tissue-sources [333, 334]. These variances may have impact on their efficacy, therefore should be carefully considered.

3.2 MSC secretory profile from a mechanistic point of view

Differences among MSC populations go beyond donor and experimental disparity [333], and include tissue-source variations in respect to cell-surface markers expression, yield and expandability, differentiation patterns, and epitope profile [292, 335, 336]. Thus, it would be expected that different tissue-source MSCs have distinguishable secretory patterns as well as different therapeutic profiles. This has been in fact confirmed over the years. Hsiao and colleagues provided a comparative analysis on the

expression of several factors in human BM-MSCs, ASCs and Dermal tissue-derived MSCs [329]. VEGF-A, angiogenin, bFGF, and NGF were expressed by all the MSC populations at comparable levels, whereas IGF-1, VEGF-D, and IL-8 were preferentially expressed by ASCs, which contributed to a better performance of this cell population on angiogenesis [329]. More recently, we have also found different patterns and composition on the secretome of BM-MSCs, ASCs, and HUCPVCs [337], which might explain differential levels of axonal growth on DRGs observed among the three cell populations [338]. These data were consistent with previous work from our group showing that different sets of growth factors were being secreted by HUCPVCs and ASCs under the same culture conditions, which acted differently on neuronal cell density and metabolic activity [339]. Interestingly, when triggered by inflammatory stimuli, UCB-MSCs showed higher anti-inflammatory effect than ASCs and BM-MSCs. Besides variations in the secretory profile among MSC populations, the same population can also alter the production of factors when exposed to different agents. For instance, ASCs were found to increase the levels of VEGF, HGF and IGF-1 in response to an inflammatory stimulus [340]. Hypoxia also triggered prominent production of VEGF when compared to normoxia conditions, providing a more angiogenic and anti-apoptotic activity of MSC secretome in a mice model of ischemia [341].

The differences among MSC populations should not be considered a problem upon the use of MSC secretome for SCI therapeutic applications. On the contrary, the progress made in the determining the exact content of MSC secretome enables to discriminate the corresponding therapeutic impact along with the putative mechanisms being activated or controlled by it. These are important insights, as it might give the opportunity to achieve the most suitable cocktail of paracrine factors for a specific context of injury in future studies. Plus, it should be emphasized that the response of MSCs to the environment offer ways to manipulate and control the features of the secretome, an advantage not provided by cellular transplantation strategies.

Table 1. Studies of stem cell-based regenerative strategies for SCI repair and regeneration.

	SCI model	Injury	Strategy	Administration route	Administration Frequency & Timing	Main achievements	Ref
Schwann Cells	Rat	Contusion - T9	Transplantation of SCs suspensions	Intraspinal injection - epicenter	$2x10^6$ cells; once, 7 dpi	Sensory and spinal axons outgrowth and remyelination; Motor function improvement	[342]
		Transection - T8	Implantation of SCs-containing matrigel channels in SCI site	Local application within a guidance channel	N/A; once, immediately post-injury	Axonal myelination and regeneration; improved axonal electrophysiological conduction	[211]
		Chronic contusion - T9	SCs transplantation	Intraspinal injection - epicenter	$2x10^6$ cells; once, 8 wpi	Axonal growth of descending and ascending fibers; motor function improvement	[212]
		Transection - T8	Transplantation of genetically modified SCs to express BDNF and NT-3	Local application and intraspinal caudal injection	$1x10^6$ cells; once, immediately post-injury	Axonal regrowth and regeneration	[343]
	Patients	Chronic mid-thoracic injury	Autologous SCs transplantation	Intraspinal injections - syrinx	3 to $4.5x10^6$ cells; once, > 6 mopi	No beneficial nor adverse effects	[215]
		Subacute complete	Autologous SCs transplantation - Phase I clinical trial for safety studies (MIAMI Project)	Intraspinal injection - epicenter	5 to $15x10^6$ cells; once, 4-7 wpi	No adverse effects up to 1-year post-transplantation; No additional spinal cord trauma; No formation mass lesions. Safe administration procedure and treatment	[216]
		Chronic thoracic and cervical	Autologous SCs transplantation - Phase I clinical trial for safety studies	N/A	N/A	Ongoing	N/A
Olfactory Ensheathing Cells	Rat	Hemissection - C1/C2	OECs transplantation	Intraspinal injection - epicenter	N/A; once, immediately post-injury	CST fibers regeneration; motor function improvement	[221]
		Complete transection - T9	OECs transplantation + treadmill training	Intraspinal injection – rostral/caudal to lesion	$4x10^5$ cells; once, immediately post-injury	Motor improvement; no regeneration of serotonergic fibers bellow SCI site	[223]
		Transection - T9/T10	Primate-derived OECs transplantation	Intraspinal injection – rostral/caudal to lesion	$4x10^5$ cells; once, immediately post-injury	Modest regeneration of 5-HT fibers; no CST regeneration	[224]
		Dorsal hemissection - T11/T12	OECs transplantation	Local application and intraspinal rostral/caudal injections	$4x10^5$ cells; once, 4 wpi	No rubrospinal nor CST axonal regeneration	[225]

	Patients	Chronic complete injury - T4-T7	Autologous OECs transplantation - Phase I/IIa clinical trial	Intraspinal injections	Once, 18-32 mopi	No adverse effects up to 3 years post-transplantation; No motor improvements	[228, 344]
		Chronic complete transection/compression – T3-T11	Autologous OECs transplantation in three patients + neurorehabilitation	Intraspinal microinjection – rostral/caudal to lesion; around lesion site	$3x10^4$-$20x10^4$ cells; 120-212 injections; 1.3-5 ypi	Functional improvement in 2 out of 3 patients - upgraded ASIA scale from A to B and C; Motor and sensory amelioration in 1 of 3 patients with no upgraded ASIA scale.	[229]
Embryonic Stem Cells	Rat	Contusion – T8/T9	Transplantation of neuron- and glial-restricted precursor cells derived from ESCs + single dose of MP (10 min post-injury; Immunosuppression with cyclosporine A (3dpi and up to 2wpi)	Intraspinal injections – rostral/epicenter/caudal	$1x10^5$ cells; once; 9dpi	Motor improvement; Bladder function improvement; descending axonal outgrowth; ESCs differentiation into neuron- and glial-like cells	[234]
		Contusion – T9-T10	ESCs-derived NPCs transplantation	Intraspinal injections	$1x10^6$ cells; once; 9dpi	ESCs-derived NPCs survival and differentiation into neural lineages; Motor function improvement	[345]
		Induced demyelination – T10	ESCs-derived OPCs transplantation	Intraspinal injections – dorsal column white matter	$1,25x10^5$ cells; once; 3dpi	Axonal remyelination; motor function improvement	[346]
	Patients	Subacute complete thoracic	ESC-derived OPCs transplantation - safety studies (Geron Corporation)	N/A	$2x10^6$ cells; once; 7-14dpi	No observations of positive effects; discontinuity of the program	[237]
		Subacute cervical	ESC-derived OPCs transplantation - Phase I/Iia clinical trial for safety studies (Asterias Biotherapeuthics)	N/A	N/A	Results to be provided	N/A
Induced Pluripotent Stem Cells	Mice	Compression – T6	Transplantation of iPSCs-derived NSPCs; Immunosuppression with cyclosporine A	Intraspinal injections – rostral/caudal to lesion	$5x10^4$ cells; once; 7dpi	Axonal remyelination; motor function improvement	[252]
	Rat	Mild-contusion – T10	Acute transplantation of iPSCs-derived OPCs	Intraspinal injections - epicenter	$5x10^5$ cells; once; 9dpi	Axonal remyelination; serotonergic innervations; synaptic contacts	[253]

						establishment; cavity size and glial scar reduction; motor and electrophysiological improvement	
	Non-human primate	Contusion – C5	Transplantation of murine and human iPSCs-derived NPCs;	Intraspinal injections - epicenter	$1x10^6$ cells; once; 9dpi	Grafted cells survival and differentiation into neural cells; Axonal outgrowth; angiogenesis; Motor function improvement	[254]
Neural Stem/Progenitor Cells	Mice	Dysmyelination	Adult NSPCs transplantation into transgenic mice + delivery of bFGF/EGF/PDGF-AA	Intraspinal injections for cell transplant – epicenter; Intrathecal injections for growth factors – lesion site	$5x10^4$ cells; twice; immediately post-injury	NPCs differentiation into oligodendrocyte-like cells; robust axonal ensheathment and myelination; Improved axonal conduction	[261]
	Rat	Demyelination and dysmyelination – T8/T9	Transplantation of Spinal cord-derived NSCs	Intraspinal injections – lesion site	$1x10^5$cells; once; 3dpi	NSCs differentiation into myelinating OEC- and SC-like cells	[262]
		Compression – T7-T8	Adult spinal cord-derived NPCs transplantation + Immunosuppression with cyclosporine A (daily)	Intraspinal Injections – midline of spinal cord/rostral/epicenter/caudal	$2x10^5$cells; once; 1 wpi	Modest change in trophin expression in the spinal cord of NGF, LIF, IGF-1, TGF-β1; Co-infusion of trophins led to increased levels of trophic factors: BDNF, bFGF, NGF, CNTF, IGF-1, LIF	[264]
			Adult brain-derived NPCs + delivery of EGF/bFGF/PDGF-A + Immunosuppression with cyclosporine A (daily) + minocycline (10 days)	Intraspinal injections for NPCs – 2 midlines/rostral/epicenter/caudal; intrathecal injections for growth factors	$4x10^5$ cells; once; 2 wpi		
	Non-human primate	Contusion – C5	NPCs transplantation into the spinal cord + immunosuppression with cyclosporine (daily, up to 8wpi)	Intraspinal injection - epicenter	$1x10^6$cells; once; 9dpi	NPCs survival and differentiation into neural cells; injury cavities decrease; motor function improvement	[263]
	Patients	Chronic incomplete and complete cervical and thoracic	NSCs transplantation - Two Phase II clinical trial for safety and preliminary efficacy (Stem Cells Inc.); Patients temporary immunosuppression	Intramedullary perilesional injections	Thoracic cohort: Up to $4x10^6$ cells; once Cervical cohort: $2x10^6$ cells; once	Positive sensory improvement; Benefits of cell transplantation disappeared upon immunosuppression withdrawal	[347]
		Chronic cervical and thoracic	Human spinal cord-derived NSCs (Neural Stem Inc.)	N/A	N/A	Recruiting; Results expected by 2020	N/A

Mesenchymal Stem Cells	Rat	Hemicompression – T8/T9	Acute and sub-acute human UC-MSCs transplantation + immunosuppression with cyclosporine (daily)	Intravenous injection	1x10^{6} cells; once; 1 dpi or 5 dpi	UC-MSCs migrated to the lesion site; motor function improvement in the acute phase	[287]
		Compression	ASCs transplantation + Immunosuppression with cyclosporine A (24h prior-injury and daily post-injury)	Intraspinal injections – rostral central caudal part of lesion site	5x10^{5} cells; once; 7dpi	Motor function improvement; apoptosis and astrogliosis decrease	[290]
		Contusion – T10	ASCs transplantation	Intravenous injection	2.5x10^{6} cells; once; 8dpi	Motor function improvement; migration of ASCs to injury site (homing); reduced lesion cavity; CINC-1 secreted by ASCs contributed to cell survival and functional recovery.	[291]
		Hemissection – C3/C4	ASCs transplantation (ASC incubation with bFGF prior transplantation) + Immunosuppression with cyclosporine A (daily)	Intraspinal injection – rostral/caudal to lesion	5x10^{4} cells; once; immediately post-injury	Expression of BDNF, VEGF and FGF-2; 5HT axonal outgrowth and sprouting throughout the spinal cord; apoptosis and astrogliosis decrease	[348]
		Complete transection	Transplantation of UC-MSCs from wharton's jelly	Intraspinal injection – rostral/caudal to lesion	5x10^{5} cells; once; immediately post-injury	Axonal growth and regeneration and motor improvements mediated by NAP-2, NT-3, bFGF, GITR and VEGFR-3;	[322]
		Contusion – T9	Transplantation of BM-MSCs overexpressing BDNF	Intraspinal injection - epicenter	3x10^{5} cells; once; 7dpi	Reduced lesion cavity; increased spared white matter; axonal outgrowth; Increased levels of trophic factors: BDNF and GDNF	[323]
		Contusion – T8-T10	BMSCs Secretome administration	Intrathecal injection	BMSC secretome: 10μl; once; immediately post-injury	Angiogenesis; cystic cavities reduction; functional recovery; no axonal regrowth	[325]
					BMSC secretome: 10μl/h for 2 weeks	Axonal densities increase; astrocyte-devoid lesion site; Motor function improvements	[326]
					BMSC secretome: 30μl at 1, 5, 9 and 13 dpi.	pared spinal cord tissue; enhanced GAP-43 expression; attenuated inflammation (decreased IL-2 and -6 and TNF-α, and increased CNTF and VEGF); Motor function improvements	[327]
	Pig	Compression – T12-T13	Autologous BM-MSCs transplantation + rehabilitation (daily)	Intraspinal injections – lesion site and adjacent subarachnoid space	15x10^{6}; twice; 3mopi	Axonal outgrowth in neo-formed tissue; axonal conduction recovery; motor function improvement	[188]

	Dog	Compression – L4	Transplantation of canine ASCs transplantation + MPSS	Intravenous injections of ASCs and MP	ASCs: $10x10^6$ cells; Daily for 3 days; 6hpi MPSS: 5.4mg/kg/hour; 47h; 6hdpi	Migration of ASCs to the lung, spleen and injured spinal cord; Motor function improvement; Antioxidative and anti-inflammatory molecules increased; Several side effects of MPP;	[294]
		Compression - L4	Transplantation of canine ASCs overexpressing Heme oxygenase-1	Intraspinal injections – rostral/epicenter/caudal	$10x10^6$ cells; once; 1wpi	Decreased fibrosis and microglial cell infiltration; motor function improvement	[295]
		Compression – L4	Transplantation of canine ASCs and chondroitinase ABC (chABC)	Intraspinal injections – rostral/epicenter/caudal	ASCs: $10x10^6$ cells; once; 3wpi chABC: 5U/mL	Increased expression of digested CSPGs, βIII-tubulin and neurofilament; Increased levels of COX2 and TNF-α; Significant motor function improvement	[296]
		Thoracolumbar	ASCs transplantation + Electrotherapy	ASC injection by lumbar puncture; Subcutaneous electrical stimulation	ASCs: $1x10^7$ cells; once; 6-24 mopi. Electrotherapy: voltage 30mV, frequencies 5/10/15/20Hz; 5min/Frequency	Motor function improvement	[298]
	Patients	Subacute and chronic	BM-MSCs transplantation - Clinical trial for safety studies; Physical therapy	Intraspinal – in and around lesion epicenter; directly into spinal canal; intravenous injections	$4x10^8$ cells; once; minimum 1ypi	AIS grades, Barthel index and bladder function improvements; decreased spasticity; no tumor formation and infection; no increased pain. Multiple routes of administration feasible and safe	[299]
		Chronic cervical	BM-MSCs transplantation - Phase II/III clinical trial for safety and efficacy studies (Pharmicell Co.)	intramedullary and intrathecal	$1.6x10^7$ cells (intramedullary route); $3.2x10^7$ cells (intrathecal route); once; immediately post-injury	Results expected in 2020	N/A
		Thoracolumbar – T10-L1	Transplantation of human UC-MSCs from wharton's jelly + rehabilitation - clinical application	Intraspinal injections through lumbar puncture	$4x10^7$ cells; once; 21mopi	Motor and activity improvement; tactile sensation and algesia amelioration; bladder and bowel function improvement; no adverse effects	[300] [301]
		Subacute, and early and late chronic	Human UC-MSCs transplantation - phase I/II clinical trials for safety and efficacy studies	Intrathecal	Monthly, for 4 months	Results expected in 2021	N/A
		chronic	ASCs transplantation - Phase I clinical trial for safety studies (CELLTOP study, Mayo Clinic)	Intrathecal in cerebrospinal fluid	Single dose	Results expected in 2023	N/A

4. Concluding Remarks

The management of SCI and it devastating effects on motor and sensorial control in patients concerns a huge challenge in neuroscience research. Preclinical and clinical investigation has been trying to address this, but there is still no effective treatment showing functional recovery of patients. This is mostly due to the complexity of the central nervous system, which contains the spinal cord, regarding their anatomy and physiology, cell population content and neural circuitry. Therefore, injury to the spinal cord of humans has acute outcomes, followed by secondary pathophysiological events that lead to the progression of the lesion until complete and permanent loss of sensation and motor function that occurs bellow the injury level, establishing an irreversible chronic lesion. It is widely known that the spinal cord of humans lacks the capacity to regenerate after injury, in contrast to that of vertebrates, such as the amphibians and fish, which presents an extraordinary ability to fully regenerate. Thus, the investigation on the regenerative capacity of those vertebrates allow us to identify and understand the mechanisms and features of spinal cord regeneration that lack in human and that may be translatable to clinics. On the other hand, mammalian models that share the inability to regenerate with humans, such as rodents, pigs and non-human primates, are of the utmost importance to study the regeneration of the spinal cord along with testing the therapeutic efficacy of a given therapy in a loss-of-function paradigm. While the insights given by the more basic models may be directed to the understanding of the pathophysiology of SCI and thus lead to more preventive strategies, larger models provide means to follow the cure of SCI in more applied and translatable systems. Ideally, the key to develop new therapies for SCI patients with functional improvements would be to focus in combining both resources. However, robust pre-clinical evidences should first be obtained so that it can be translated to clinics, which remains a challenge. Nevertheless, the application of stem cells to SCI have been shown promising in many studies. Their therapeutic role has been mostly attributed to a neuroprotective and neuroregenerative activity towards the repair of damaged tissues. Among the variety of cell populations available, MSCs have been shown interesting. These cells have an innate capacity to respond to injury by stimulating the endogenous repair of spinal cord. The therapeutic role of these cells for SCI are believed to be mediated by autocrine and paracrine activities through their secretome, which by itself has been shown to provide means for spinal cord repair after injury. Considering the many issues related to cell transplantation, the potential of solely use of MSC secretome as a therapy for SCI seems promising.

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CHAPTER II

EXPLOITING THE IMPACT OF THE SECRETOME OF MSCS ISOLATED FROM DIFFERENT TISSUE SOURCES ON NEURONAL DIFFERENTIATION AND AXONAL GROWTH

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ABSTRACT

Cell transplantation using Mesenchymal stem cell (MSC) secretome have recently been presented as a possible free-based therapy for CNS related disorders. MSC secretome is rich in several bio-factors that act synergically towards the repair of damaged tissues, thus making it an ideal candidate for regenerative applications. Great effort is currently being made to map the molecules that compose the MSC secretome. Previous proteomic characterization of the secretome (in the form of conditioned media - CM) of MSCs derived from adipose tissue (ASC), bone-marrow (BMSC) and umbilical cord (HUCPVC) was performed by our group, where proteins relevant for neuroprotection, neurogenic, neurodifferentiation, axon guidance and growth functions were identified. Moreover, we have found significant differences among the expression of several molecules, which may indicate that their therapeutic outcome might be distinct. Having this in mind, in the present study, the neuroregulatory potential of ASC, BMSC and HUCPVC CM in promoting neurodifferentiation and axonal outgrowth was tested *in vitro*, using human telencephalon neuroprogenitor cells and dorsal root ganglion explants, respectively. The CM from the three MSC populations induced neuronal differentiation from human neural progenitor cells, as well as neurite outgrowth from dorsal root ganglion explants. Moreover, all the MSC populations promoted the same extent of neurodifferentiation, while ASC CM demonstrated higher potential in promoting axonal growth.

Keywords

Mesenchymal Stem Cells, Secretome, Neuroregulatory Factors, Neurodifferentiation, Axonal outgrowth, Cell-free based CNS therapy

1. INTRODUCTION

An extensive body of literature suggests that Mesenchymal stem cell (MSC)-mediated paracrine activity plays a role in promoting tissue repair. MSCs were firstly identified by Friedenstein as multipotent stem cells characterized by the capacity to self-renew, to adhere to plastic and colonize, and to differentiate into three mesodermal cell lineages [1]. However, the real interest around MSCs is their contribution towards regeneration of tissues upon injury. In fact MSC-based therapies have been used in the context of several neurodegenerative diseases, where neuronal survival has been reported in animal models of stroke [2] and traumatic brain injury (TBI) [3]. Additionally, the recovery of motor function of induced models of Parkinson's disease (PD) [4] and SCI, with an observed remyelination and reconnection of the neural circuitry [5, 6], has also been observed upon MSCs administration. Direct evidences attribute the regenerative potential of MSCs to their ability to secrete several biomolecules and trophic factors, namely neurotrophic growth factors, chemokines, cytokines, and extracellular matrix proteins, as well as extracellular vesicles, that might be relevant in a clinical setting [7-10]. In the context of neuroregeneration, pre-clinical and clinical findings show that these molecules can directly stimulate the recruitment, proliferation and differentiation of the endogenous cells [11, 12]. Additionally, they can regulate local mechanisms such as apoptosis, scarring and revascularization, as well as modulate immune and inflammatory responses [9, 13], thus contributing to reduce tissue damage.

Recent insights on the effective therapeutic role of MSC-secreted bio-factors, especially given the fact that MSCs have a limited engraftment and survival rate when delivered into a damaged tissue [14], opens the possibility of using MSC secretome as a cell-transplantation free based regenerative therapy. In fact, the therapeutic application of the secreted molecules in replacement of stem cells presents enormous advantages as it should minimize stem cell-related ethical and immune-compatibility issues [15], as well as allow a precise dosing and localized delivery to the damaged tissues in a minimally invasive manner [16].

The perspective of safer and more effective strategies motivated further investigation towards the identification of the molecules composing the cell secretome, rather than only looking to its overall regenerative effects. In this line, proteomic profiling of MSC conditioned media (CM) became intensively explored in recent years [17-21].

While valuable tools for MSC secretome characterization were being used, several studies highlighted significant differences between different tissue-sources of MSCs. In fact, the heterogeneity of MSCs residing in different tissues has been reported some time ago [10]. So far, the best characterized and the most studied sources of adult MSCs are obtained from the bone-marrow (BMSCs), adipose tissue (ASCs)

and umbilical cord. Several studies provided us with distinct characteristics of each population, that goes from different expression of cell surface markers [22], to specific differentiation processes [23] and immunomodulatory functions [24]. A relevant point of interest that arises from a collective analysis on this heterogeneity studies is the fact that different tissue sources of MSCs are likely to have different secretion profiles [19, 25]. For instance, we have previously shown that the exposure of primary cultures of hippocampal neurons to the CM of ASCs and Human Umbilical Cord Perivascular Cells (HUCPVCs) had different effects on cell proliferation and metabolic activity [26]. Hsieh and colleagues also found that MSCs isolated from Wharton's jelly secreted more factors related to angiogenesis and neurogenesis than BMSCs, which improved neural differentiation and migration and decreased cell apoptosis in an *in vitro* model of acute ischemic stroke [27]. Considering the existence of such differences on the secretome composition of MSCs obtained from different tissue-sources, the choice of the best MSC population for a particular application must be determined according to their characteristics and secretory profile. Thereafter, our group found that it is crucial to perform a detailed mapping of the CM obtained from BMSCs, ASCs and HUCPVCs. In a recently published proteomic analysis, we have shown that all these populations were able to secret important factors known to be involved in processes of several CNS disorders/injuries [28]. Moreover, the pattern and composition of ASCs, BMSCs and HUCPVCs CM differed, a fact that could indicate a certain degree of specificity towards different CNS related conditions. After this, it remains unclear if these different secretion profiles could activate distinct mechanisms by which the repair and regeneration of tissues from the nervous system may be regulated. Based on these results, the present study proposes to further explore the previously analyzed MSC CM potential in promoting the differentiation and axonal growth of neural populations *in vitro*.

2. MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Human bone marrow mesenchymal stem cells (BMSCs), *adipose tissue derived stem cells* (ASCs), and *Human umbilical cord perivascular cells (HUCPVCs)*

BMSCs (Stem Cell Technologies, Grenoble France) were thaw and expanded according to protocol established in our lab [29]; ASCs were kindly provided by Professor Gimble (Pennington Biomedical Research Center/Tulane University, USA); and HUCPVCs were kindly provided by Professor Davies (University of Toronto, Canada). ASCs and HUCPVCs were isolated as previously described [30, 31].

Cells were cultured and maintained in α-MEM medium (Invitrogen, USA) supplemented with sodium bicarbonate ($NaHCO_3$; Merck, USA), 10% of fetal bovine serum (FBS; Biochrom, Germany) and 1%

Penicilin-Streptomycin antibiotic (P/S; Invitrogen, USA). Upon confluence, cells were enzymatically dissociated with 0.05% trypsin/EDTA (Invitrogen, USA), re-plated at a density of 4000 cells/cm^2 and maintained at 37°C, 5% humidified CO_2, 95% air and 90% relative humidity.

2.1.2 Human Telencephalon Neural Progenitor Cells (htNPCs)

htNPCs were isolated from 10-week human fetus telencephalon region, as previously described [31]. Ethical consent was approved by the Conjoint Health Research Ethics Board (CHREB), University of Calgary (ID: E-18786). htNPCs were thaw and plated in Nunc T-25 flask containing 5 mL of a serum-free medium PPRF-h2, described in detail by Baghbaderani et al. [32]. Cells were maintained in culture for 48h, during which aggregated into neurospheres. After this time, htNPCs were mechanically dissociated and re-plated into fresh medium. Every 4 days, 40% of the medium was replaced by fresh.

2.2 Conditioned media (CM) collection

The CM of ASCs, HUCPVCs and BMSCs was collected from cells in passage 5. For that, cells were plated at a density of 4000 cells/cm^2, and allowed to grow for 72 hours. After this, cells were washed 5 times with PBS without Ca^{2+} and Mg^{2+} (Invitrogen, USA), and once with the conditioning medium.

For neurodifferentiation experiments, Neurobasal A medium (Invitrogen, USA) supplemented with 1% Kanamycin (Invitrogen, USA) was added to the cells. For axonal growth assays, Neurobasal medium (Invitrogen, USA) supplemented with 1% Pen-Strep was used. After 24h of conditioning period, the CM was collected and frozen at -80°C until used.

2.3 htNPCs culture with MSC CM

For neurodifferentiation experiments, htNPCs were enzymatically dissociated with 0.05% trypsin-EDTA, and plated onto glass coverslips pre-coated with poly-D-lysine hydrobromide (100 μg/mL; Sigma) and laminin (10 μg/mL; Sigma) at a density of 5.5 x 10^4 cells. Cells were maintained in culture for 5 days with the CM collected from the three MSC populations, at 37°C, 5% CO_2, 95% air and 90% relative humidity. htNPCs culture with Neurobasal-A medium supplemented with 1% of kanamycin was used as control.

2.4 Isolation and culture of dorsal root ganglion (DRG) explants with MSC CM

Dorsal root ganglion explants were used for axonal growth experiments. For that, DRGs from 5 days-old neonatal Wistar-Han rat pups were dissected as previously described [33]. Briefly, DRGs from cervical

and thoracic regions of the spine of neonatal rat pups (P5) were dissected and the remnants of peripheral nerve processes were cleaned. The explants were placed on top of collagen hydrogels, prepared as previously described [34] and incubated with MSC CM for 7 days. DRG cultures in collagen gels in Neurobasal Medium supplemented with B27, L-glutamine, glucose and 1% of P/S was used as control.

2.5 Immunostaining

htNPCs. htNPCs were fixed, washed, and blocked as previously described [4]. The following primary antibodies were then used: rabbit anti-doublecortin (DCX; 1:500, Abcam, Cambridge, MA, USA) to detect immature neurons, and mouse anti-rat microtubule associated protein-2 (MAP-2; 1:500, Sigma) to detect the mature ones, for 1h at 37°C. After washing, samples were incubated with the secondary antibodies Alexa Fluor 488 *goat anti-rabbit* immunoglobulin G (IgG, Life Technologies) and Alexa Fluor 594 *goat anti-mouse* immunoglobulin G (IgG, Life Technologies) for 1h at 37°C. Further incubation with *4-6-diamidino-2-ph enylindole-dihydrochloride* (DAPI; Life Technologies) was performed for 10 min at Room Temperature (RT). Samples were then observed under an Olympus BX-61 Fluorescence Microscope (Olympus, Hamburg, Germany).

DRGs. For the immunocytochemistry (ICC) of DRGs, the following antibodies were used: Mouse monoclonal anti-human neurofilament 200 kDa (Millipore) as the primary antibody and Alexa fluor 488 goat anti- mouse IgG (Invitrogen) as the secondary antibody. DRGs were fixed with 4% paraformaldehyde (PFA) in PBS for 45 min at RT and washed with PBS. A further incubation 0.3% Triton X-100 (Sigma, USA) for 10 min at RT was used for cell permeabilization, and washing with PBS. Samples were then incubated with a blocking buffer solution [PBS containing 10% fetal bovine serum (FBS)] for 90 min at RT, after which they were incubated with the primary antibody (diluted 1:200 in PBS solution with 10% FBS) for 48 h at 4 °C. After washed with a PBS solution containing 0.5% FBS, samples were incubated with the secondary antibody (diluted 1:1000 in PBS/0.5%FBS solution) overnight at 4°C. After PBS washing, DAPI (1 μg ml^{-1}; Invitrogen) was added to the samples for 10 min to stain cell nuclei. Samples were finally washed and maintained hydrated in PBS for fluorescence microscopy analysis (Olympus BX-61 Fluorescence Microscope, Olympus, Hamburg, Germany).

2.6 Neurodifferentiation Assessment

Neurodifferentiation of htNPCs was inferred by qRT-PCR for NeuroD1 (ND1) and βIII-Tubulin (βIII-Tub), which protocol is described in the next sub-section, and by the number of Doublecortin (DCX) and

Microtubule associated protein (MAP-2) expressing cells. For this purpose, three coverslips and ten representative fields per condition were chosen and imaged using a fluorescence microscope as referred above. To normalize the data between the different experiments, the results are presented in percentage (%) of cells. This was calculated by counting the number of cells with positive staining for DCX and MAP-2 markers, and dividing this value by the total number of cells/field (DAPI-positive cells; n=3).

2.7 Protein Association Network analysis and quantitative Real time PCR

For neurodifferentiation experiments, the mRNA expression levels of selected genes of interest (Table 1) were measured by quantitative real time polymerase chain reaction (qRT-PCR), after htNPCs incubation with MSC CM. These genes were selected based on protein-protein interaction networks from the previously identified biomolecules in the MSC CM [28] using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) bioinformatics tool. The neurodifferentiation markers NeuroD1 and Tubulin beta 3 Class III (TUBB3) were also used to assess the neurodifferentiation stage after incubation of htNPCs with MSC CM. The oligonucleotide primers for the target genes were designed using Primer-BLAST software (NCBI). The real time reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences). Target gene expression levels were normalized against the housekeeping gene Beta2-microglobulin (B2M), and presented as fold-change of mRNA levels compared to the Control group. The $2^{-\Delta\Delta CT}$ method was used to calculate fold-change levels.

Table 1. Forward and reverse sequences of oligonucleotide primers used in the qRT-PCR, and the respective gene symbol, name and product size.

Gene symbol	Gene name	Primer sequence 5'–3' forward=reverse	Product Size (bp)
TUBB3	Tubulin beta 3 class III	**Fw** GGC CTC TTC TCA CAA GTA CG **Rv** CCA CTC TGA CCA AAG ATG AAA	317
NeuroD1	Neuronal differentiation 1	**Fw** CCG TCC GCC GAG TTT G **Rv** GCG GTG CCT GAG AAG ATT G	173
FLT1	Fms-related tyrosine kinase-1	**Fw** CTG GGC AGC AGA CAA ATC CT **Rv** AAA AGT CAC ACC TTG CTT CGG	113
NRP1	Neuropilin 1	**Fw** CGC AAG GCG AAG TCT TTT GA **Rv** TGT GAG CTG GAA GTC ATC ACC	265

TGFBR1	Transforming growth factor beta receptor 1	**Fw** TCC AAC TAC TGG TTT ACC ATT GC **Rv** TTC TTC TCC CCG CCA CTT TC	123
SMAD2	SMAD family member 2	**Fw** GGC CTT TAC AGC TTC TCT GAA CA **Rv** ACTGGAGGCAAAACTGGTGTC	240
STAT3	Signal transducer and activator of transcription 3	**Fw** GGA GAA GGA CAT CAG CGG TAA **Rv** GCT CTC TGG CCG ACA ATA CT	205
B2M	Beta2-microglobulin (Reference gene)	**Fw** GAT AGT TAA GTG GGA TCG AG **Rv** GCA AGC AAG CAG AAT TTG GA	95

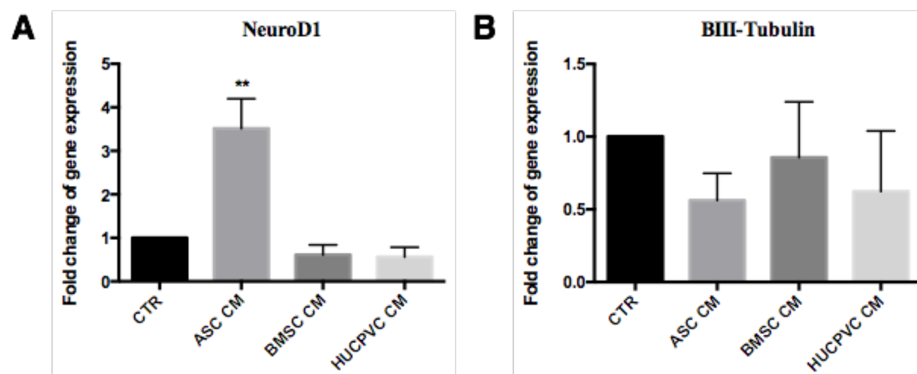

Figure 1. Gene expression of neuronal markers in htNPCs after incubation with MSCs CM. The gene expression levels of (**A**) NeuroD1 (for immature neurons) and (**B**) BIII-Tubulin (for mature neurons) was assessed in cells incubated for 5 days with regular media, denoted as CTR, or with either ASC-, BMSC- and HUCPVC-CM. Results are presented as Mean ± SD; n=3 per condition; **p<0.01

2.8 Axonal outgrowth/migration quantification

As previously described [34], DRG axonal growth was inferred by the quantification of the area occupied by the neurites within the collagen gels. For that, after confocal imaging, samples (n=8/condition) were analyzed by Image J software. The image scale was first set and converted to 8 bit and binary, after which the body of the DRG itself was excluded. Thereafter, the area occupied by the neurites were automatically calculated considering the image black background as contrast. The area of neurite outgrowth is presented as μm^2.

2.9 Statistical analysis

Statistical evaluation was performed using GraphPad Prism (version 5.0; GraphPad Software, USA). Differences among groups were assessed using Student's t-test or One-way ANOVA test. A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance (*).

3. RESULTS AND DISCUSSION

3.1 MSC CM induced htNPCs neuronal differentiation

To assess the potential of MSC secretome in promoting neuronal differentiation, htNPCs were cultured with ASC, BMSC and HUCPVC CM. After 5 days in culture, gene expression analysis of NeuroD1, a neuronal cell-fate and early differentiation marker, and Tubulin beta III (βIII-Tubulin), a marker of mature neurons, was performed in htNPCs to assess the differentiation stage of these cells (Fig. 1).

While NeuroD1 transcriptional expression was particularly overexpressed in htNPCs incubated with ASC CM (Fig. 1A), BIII-tubulin was similarly expressed in all the experimental groups, with no statistically significant differences comparing with the control (Fig. 1B).

The differentiation of htNPCs was further confirmed by immunocytochemistry analysis for DCX and MAP-2, staining for immature- and early stage mature neurons, respectively. Higher levels of differentiation into a neuronal phenotype were revealed for all the CM (Fig. 2C-H). In fact, quantified as the percentage of DCX^+ and $MAP\text{-}2^+$ cells present in culture, and denoted in figure 2 (I-K), htNPCs differentiation into immature neurons (DCX^+ cells; Fig. 2I) was found to be promoted by the CM of all MSC populations (ASCs: 72,98±13,75 %; BMSCs: 84,20±2,913 %; and HUCPVCs: 72,88±6,490 %). Regarding the control condition (Ctr: 47,49±2,208 %), significant differences were found when compared to all MSC CM. No significant differences were found between the three populations though.

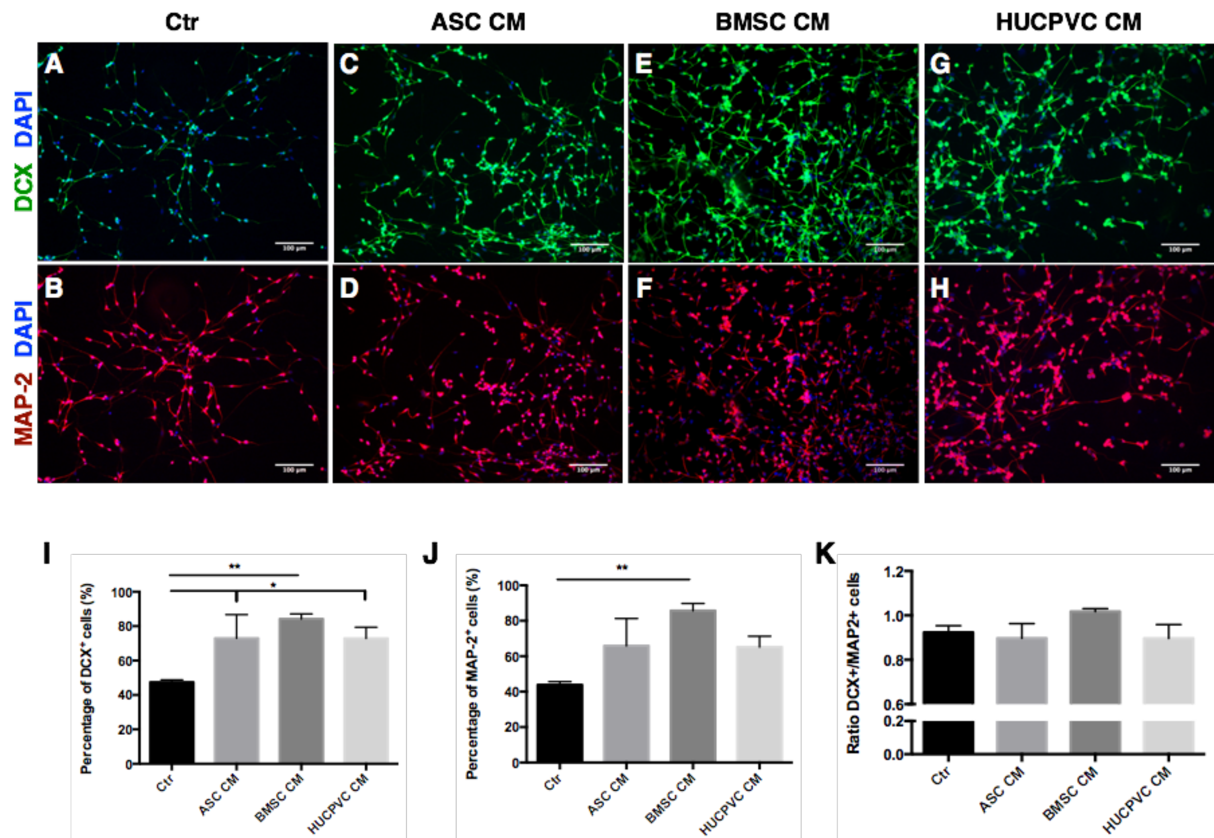

Figure 2. htNPCs neuronal differentiation induced by MSC CM. Cells were incubated with (**A,B**) regular media, denoted as Ctr; (**C,D**) ASC CM, (**E,F**) BMSC CM, and (G,H) HUCPVC CM for 5 days, and stained for immature (DCX; upper panel) and mature (MAP-2, lower panel) neurons. htNPCs differentiation after incubation with the CM of ASCs, BMSCs and HUCPVCs, was calculated as the percentage of **I)** DCX^+ and **B)** MAP-2^+ cells, in comparison to control condition (Ctr). **K)** The ratio of immature (DCX^+) to mature (MAP-2^+) cells was also calculated. Results are presented as Mean ± SD; n=3 per condition; *$p<0.05$; **$p<0.01$.

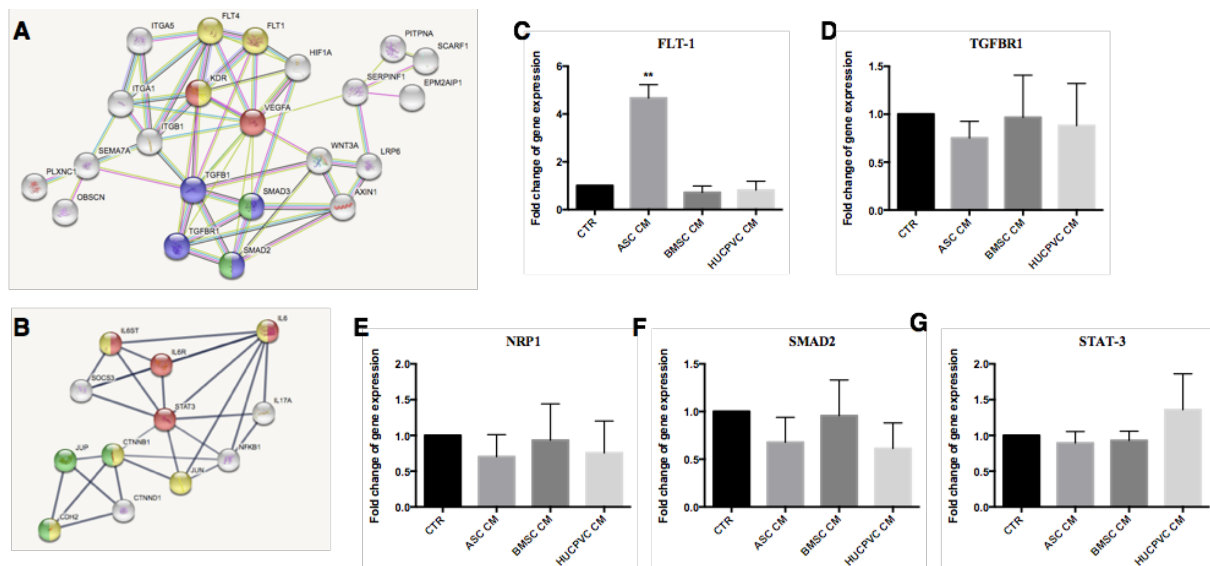

Figure 3: Gene expression of receptors and signaling molecules in htNPCs after incubation with MSCs CM. Representative networks of the receptors and signaling molecules identified by the convergence of proteins present in the MSC CM, using STRINGS bioinformatics tool (**A,B**). Gene expression levels of receptors putatively involved in the response to MSC CM (**C,D**) and of the corresponding signaling molecules (**E, F, G**) was assessed in cells incubated for 5 days with regular media, denoted as CTR, or with either ASC-, BMSC- and HUCPVC-CM. Results are presented as Mean ± SD; n=3 per condition; **p<0.01.

Similar results were obtained for the differentiation of htNPCs into mature neurons (MAP-2$^+$ cells; Fig. 2J). All MSC CM induced neuronal cell differentiation (ASCs: 65,88±15,38 %; BMSCs: 85,67±4,088 %; HUCPVCs: 65,24±6,063 %), with no significant differences between them. Moreover, only BMSC CM was found to significantly increase the percentage of MAP-2$^+$ cells in comparison to control (Ctr: 43,84±3,240 %). Finally, the ratio of immature (DCX$^+$) to mature (MAP-2$^+$) cells was found to be similar between all the experimental groups (Fig. 2K).

To investigate whether the CM effects on htNPCs neurodifferentiation may result from the presence of proteins that have been previously identified in the CM of these MSC populations, namely the Pigment epithelium-derived factor (PEDF, also known as SERPIN1), Semaphorin 7A (SEM7A), Cadherin2 (CHD2) and Interleukin-6 (IL-6) [28], we analyzed the gene expression for some of these factors' receptors in htNPCs after incubation with CM (Fig. 3). In order to identify converging receptors and signaling molecules that could be involved in the molecular response to these proteins we used STRINGs bioinformatics tool, and identified two protein-protein interaction-based networks, one that includes SERPIN1 and SEM7A (Fig.3A), and another including CDH2 and IL-6 (Fig.3B). In the first network, VEGF and TGFB were

identified as common effector molecules. Thus, the gene expression of FLT-1 (Fig. 3D) and TGFBR1 (Fig. 3E), the receptors for VEGF and TGFB, respectively, was assessed as well as the related-signaling molecules NRP1 (Fig. 3F) and SMAD2 (Fig. 3G). The signaling molecule STAT3 was also assessed as it responds to both IL-6 and CDH2.

Gene expression results show that cells from all experimental groups express the receptors necessary to respond to the factors present in the MSC CM. The expression of FLT-1 was significantly increased in htNPCs after incubation with ASC CM, when compared to Ctr, BM and HUCPVC CM. However, no variations were found in the expression of TGFBR1, NRP1, SMAD2, and STAT3 between the experimental groups.

3.2 Axonal Growth promoted by MSC CM

The MSC CM-mediated axonal growth was herein studied using a DRG-based neurite outgrowth *in vitro* model. DRG explants were placed on top of collagen matrices, and the CM collected from ASCs, BMSCs and HUCPVCs were added to the culture for 7 days. The neurites growing from the explants were stained for Neurofilament and analyzed by fluorescence microscopy, as represented in figure 4.

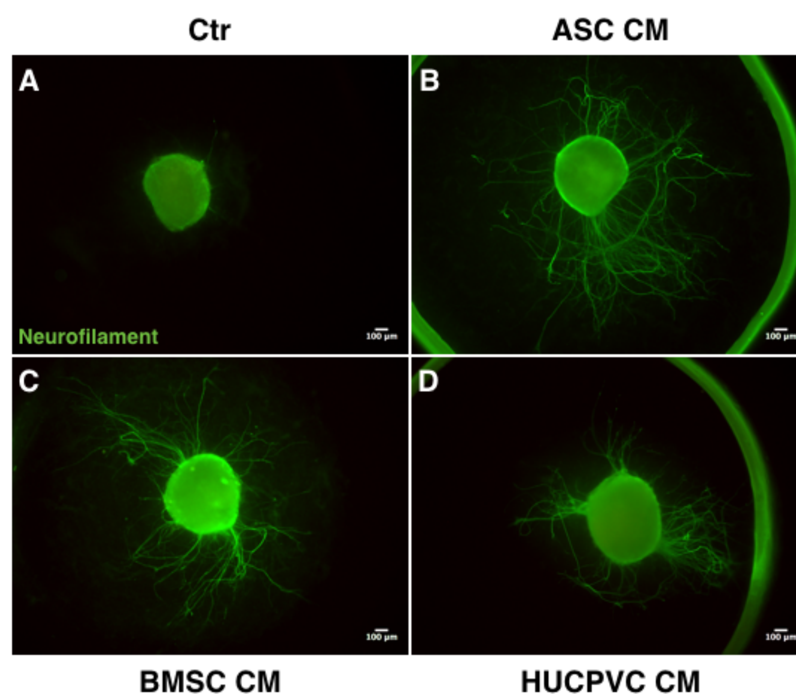

Figure 4. Neurite outgrowth from DRG explants incubated with MSC CM. DRG staining against neurofilament was performed after culture with CM collected from **B)** ASCs (ASC CM), **C)** BMSCs (BMSC CM) and **D)** HUCPVCs (HUCPVC CM). Control conditions (**A**), denoted as Ctr, regards to DRGs incubated with supplemented Neurobasal medium (please see M&M section). Scale bar: 100μm.

DRGs were found to be able to extend long neurites in the presence of ASC (Fig. 4A), BMSC (Fig. 4C) and HUCPVC CM (Fig. 4D). The same was not found for the control condition (Ctr; Fig. 4A), as almost no neurite growth was observed.

This qualitative analysis was confirmed by quantifying the area of neurite outgrowth (denoted as μm^2) in the explants for each referred condition (Fig. 5).

Axonal growth was significantly increased in the presence of ASC CM (520952±228401 μm^2), when compared to control (Ctr; 5589 ± 9979 μm^2). In addition, ASC CM induced significantly higher axonal growth than BMSC and HUCPVC CM (194926±139336 μm^2; 196718±129509 μm^2, respectively). This suggests that the ASC secretome provides a more robust and adequate environment for DRG axons to grow and migrate.

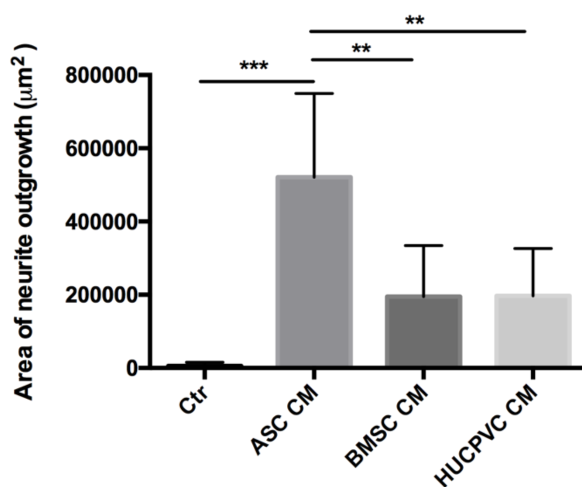

Figure 5. Area of neurite outgrowth promoted by ASC, BMSC and HUCPVC CM, in comparison to control (Ctr). The mean area occupied by neurites (μm^2) was calculated using NeuriteJ plugin for ImageJ (NIH) software. Results presented as Mean ± SD; n=8 per condition; **p<0.01; ***p<0.001.

4. DISCUSSION

Mapping the molecules that compose the secretome of MSCs intends to determine the factors upregulated by these cells in response to specific triggers, and thereafter finding their role in mediating one or more mechanisms of repair. Until recently, many studies focused on identifying only a subset of factors released by MSCs at high levels, therefore providing only a very superficial knowledge on the composition of its secretome. Contrary to this, a comparative analysis of the proteins secreted by ASCs, BMSCs and HUCPVCs was recently performed by our group using a more integrated proteomic approach, named Liquid chromatography tandem-mass spectrometry (LC-MS/MS) [28]. In that study, a vast panel

of proteins with neuroprotection, neurogenic, neurodifferentiation, and axon guidance and growth functions was identified. The three MSC populations differed in their secretion profile, posing the question of whether their neuroregulatory action may differ accordingly. Therefore, the study herein presented aimed at evaluating the role of MSC secretome in mediating neurodifferentiation and axonal growth as a function of tissue source. For that purpose, htNPCs and DRGs were incubated with the CM of ASCs, BMSCs and HUCPVCs, for neurodifferentiation and axonal growth assessment, respectively.

htNPCs are normally expanded as neurospheres in the presence of a serum-free medium PPRF-h2, as demonstrated by Teixeira et al [4]. Upon removal of the expansion medium, these cells are described to spontaneously differentiate into neural phenotypes. For that reason, this cell population was used in this study to test the neurodifferentiation potential of MSC CM. The incubation of htNPCs with the MSC CM induced significantly higher levels of differentiation into neuronal phenotypes in comparison to control conditions, as shown by the expression of DCX (immature neurons) and MAP-2 (mature neurons) markers. On the other hand, no differences were observed in BIII-tubulin levels, another marker of fully mature neurons, as assessed by gene expression (Fig.1 and 2). Moreover, no differences were observed between the MSC populations, suggesting that the different CM had the same differentiation potential.

Regarding the effects of MSC CM on axonal growth, the well described DRG-based *in vitro* model of neurite outgrowth [35] was used. After incubation with MSC CM, we observed that neurite extension from DRGs was promoted by the CM of all populations, in comparison to controls (Fig 4). Moreover, we verified that ASC CM induced more neurite extension from the explants, with significant differences regarding BMSC and HUCPVC CM, and control (Fig.5).

The results herein presented showing the neuroregulatory potential of MSC secretome in neural cultures goes in accordance to some previous studies. For example, our group has shown that the CM of both ASCs and HUCPVCs promoted both proliferation and metabolic activity of hippocampal neurons [26]. We have also observed that BMSC CM improved both neuronal and glial cell survival. In that study, different CM collection times were tested – 24h and 96h. While the former increased the survival of neurons, the later was more prone to improve glial cell survival [36]. High *in vitro* neuronal differentiation [4, 37] and *in vivo* cell proliferation in the dentate gyrus (DG) of adult rat hippocampus [4] was also found using HUCPVC CM. Interestingly, proteomic analysis on these CM revealed differences in proteins related with neural cell viability, proliferation and differentiation, namely 14-3-3, Ubiquitin C-Terminal Hydrolase L1 (UCHL1), Heat shock protein (hsp) 70 and Peroxiredoxin-6 (PRDX6), which may explain the above-referred results [36]. Others observed the neurotrophic factors Brain-derived neurotrophic factor (BDNF) and Beta-nerve growth factor (β-NGF) to be correlated with the ability of undifferentiated MSCs to induce the survival

and neurite outgrowth of neuroblastoma cells and DRGs, respectively [7]. Some other factors not related to MSC so far but that were recently shown to have regenerative and neurotrophic functions are the ASC-secreted Macrophage-colony stimulating factor (MCSF), Matrix metalloproteases (MMPS), Follistatin (FST)-like 1, Mesencephalic astrocyte-derived neurotrophic factor (MANF), and Neuron derived neurotrophic factor (NDNF) [38]. Others like Semaphorins (SEM), Galectins (Gal), Platelet-derived growth factor (PDGF) and Transforming growth factor-beta (TGF-β) were found in the secretome of BMSCs by Cizkova et al. [39]. Altogether, these results suggest the existence of tissue-source based differences, as recently evidenced by Pires et al [28]. In this proteomic data, the expression of some factors related to neuronal differentiation, namely of PEDF, SEM7A, CDH2 and IL-6 varies in the CM of ASC, BMSCs and HUCPVCs, which would indicate that neurodifferentiation and axonal growth processes mediated by the MSC CM would be distinct. Still, the role of these factors in the CNS is robustly proved. PEDF, for instance, was shown to induce a neuronal phenotype in cultured human retinoblastoma cells *in vitro* [40], as well as to contribute to the survival and differentiation of embryonic chick spinal cord motor neurons [41]. CDH2 was also found to be essential for the neural differentiation of mouse induced pluripotent stem cells [42], and to regulate the pattern of neurodifferentiation in P19 carcinoma cells [43, 44]. Similarly, IL-6 was recently demonstrated to promote neural differentiation of pluripotent stem cells upon treatment with an immunosuppressive drug [45]. However, the presence of these factors in the CM of MSCs did not translate into a fully maturation into neurons at least using this period of CM exposure. Nonetheless, we have shown that htNPCs express some of the receptors that have been implicated in the promotion of neurogenesis, neurodifferentiation and neuronal migration, namely FLT-1 [46] and TGFBR1 [47], suggesting they might be responsive to the factors present in the MSC CM and that longer culturing periods may potentiate further their differentiation into fully mature neurons (Fig.3). The putative downstream signaling molecules involved in neuronal differentiation signaling cascades upon activation of these receptors, such as NRP1 [48], SMAD2 [49] and STAT3 [50, 51], were not differentially expressed in the cells from the different experimental groups (Fig3). In fact, signaling molecules display a very quick turnover, which may have accounted for the lack of significant differences in their expression between groups. Facing these observations, the absence of differences of htNPCs neurodifferentiation herein observed can therefore suggest that the protein levels presented may not be within the optimal dose to fully exert their effects, opening up the possibility to explore new approaches that may promote the secretion of higher doses of these molecules by MSC. Yet, the existence of innumerous distinct protocols among the different studies evaluating the differentiation of neural cultures may also account for different outcomes. Another possible explanation to be considered is the existence of multidirectional function of

most of neuroregulatory factors. For example, the upregulation of PEDF in ASC CM does not necessarily mean it should promote higher levels of neurodifferentiation of htNPCs. On the contrary, this factor is also related to axonal growth [52], which supports the pronounced effects of ASC CM in the levels of neurite outgrowth from DRG explants, depicted in figure 5. So, in this case, PEDF appears to be mediating axonal growth over neurodifferentiation. A similar phenomenon might be happening for IL-6. Indeed, this factor was shown to have a role in both neurodifferentiation and axonal growth [53]. But there is a clear upregulation of this factor in HUCPVCs CM in the proteomic analysis [28]. However, the effect of MSC secretome on axonal growth is clearly provided by ASC population (Fig 5), and not by HUCPVCs, suggesting a poor contribution of IL-6 in that effect. This is supported by the upregulation levels of SEM7A and Glial-derived nexin (GDN) in the ASC CM, which suggests that it is more likely that these two factors are responsible for mediating axonal growth, rather than IL-6. The potential of SEM7A and GDN in CNS has been in fact shown. The SEM7A-mediated axonal guidance has been proven to be required for proper axon tract formation during embryonic development [54], and to promote spreading and dendricity in human melanocytes [55]. On the other hand, the action of GDN has been also reported several years ago to improve chick sympathetic neurons [56] and hippocampal pyramidal cell [57] neurite extension *in vitro*. Based on these results, we believe that ASC-mediated neurite outgrowth of DRG explants is mostly based on the combined action of PEDF, SEM7A and GDN. Adding to this, Beta-1,4-galactosyltransferase 1 (β4Gal-T1) protein was found only in the ASC CM [28]. β4Gal-T1 was suggested to regulate the neurite outgrowth on PC12 cells [58] and DRG when co-cultured with Schwann Cells [59]. Moreover, the overexpression of this protein in the lesion site after sciatic nerve crush suggest it involvement in the regeneration of the injured tissue [60]. Therefore, the exclusive presence of this molecule on ASC CM may have indeed improved their impact in the *in vitro* DRG model of neurite outgrowth herein used. Another multi-functional protein relevant for axonal growth guidance is the extracellular chaperone Clusterin (CLUS), found highly expressed by ASC CM in comparison to BMSC and HUCPVC CM [28]. It is mainly involved in modulating toxic protein deposition in CNS disorders such as Alzheimer's disease [61]. However, studies have also found this protein to contribute to neurite outgrowth of PC12 cells [62] and to induce sensory nerve outgrowth after sciatic nerve transection [63]. Finally, Decorin (DCN), a leucine proteoglycan protein, was also found to be upregulated in ASC CM in our proteomic analysis [28]. This anti-scarring molecule was elsewhere reported to promote robust neurite outgrowth across SCI lesion sites, by reducing the expression of chondroitin sulfate proteoglycan (CPSGs) [64, 65].

5. CONCLUSIONS

This study revealed that the incubation of ASC, BMSC and HUCPVC CM in htNPCs induced their differentiation towards the neuronal phenotype although with no significant differences among them. Moreover, all MSC CM improved neurite/axonal outgrowth in an *in vitro* model of axonal regeneration based on DRG explants. However, ASC CM provided higher extent of axonal growth when compared to BMSC and HUCPVC. The presence of important neuroregulatory factors in the secretome of MSCs, namely PEDF, CADH2, IL-6, SEM7A and GDN, may explain the observed results. Specifically, the upregulation of PEDF, SEM7A and GDN on ASC CM may be responsible for the higher levels of axonal growth observed with this population. Therefore, these results suggest two mechanisms underlying MSC secretome therapeutic action, namely neurodifferentiation and axonal growth. However, its potential is not limited to these two mechanisms. The modulation of other events such as excitotoxicity, apoptosis, inflammation, should be elucidated to understand their relevance in recovering the normal function of the CNS. The molecular and cellular pathways implicated on them may also be clarified to envisage the future application of MSC secretome in a clinical setting.

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CONFLIT OF INTERESTS

The authors declare no conflict of interests associated with this publication.

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CHAPTER III

XENOPUS LAEVIS –
BEYOND A BASIC MODEL OF REGENERATION

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XENOPUS LAEVIS – BEYOND A BASIC MODEL OF REGENERATION

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ABSTRACT

Non-mammalian regenerating species, such as the amphibian *Xenopus laevis*, are good models to understand tissue regeneration, as they are capable of perfectly and fully regenerate their tail and spinal cord after injury. However, this capacity is restricted to early stages of development, due to a gradual shift on the regenerative potential from a regenerative to a non-regenerative stage as they go through metamorphosis. Interestingly, a transient loss of this capacity also occurs within the regenerative stage of *Xenopus* tadpoles, called the refractory period, along with animal's immune system maturation. These aspects are exclusive to *Xenopus laevis*, which make them the ideal basic models among other regenerating vertebrates to be used in experiments testing potential targets for new therapies for spinal cord injury (SCI).

Cell-free based therapies based on the use of stem cell secretome has been one of the most explored regenerative approaches for the treatment of SCI. Mesenchymal Stem Cell secretome is nowadays well characterized to be composed of several central nervous system-related neurotrophic and neuroprotective biomolecules. The secretome of Adipose-derived mesenchymal Stem Cells (ASCs) was previously shown to have a predominant role in promoting axonal growth of dorsal root ganglion explants *in vitro*, supporting further studies on the regenerative impact of ASC secretome *in vivo*. Therefore, the potential of ASC secretome in promoting neuronal growth and functional recovery of both refractory and regenerative *Xenopus laevis* tadpoles after complete spinal cord transection was herein tested. The group treated with ASC secretome showed increased anti-acetylated tubulin and growth associated protein (GAP)-43 expression at the lesion site indicating enhanced neuronal regrowth when compared to untreated group. Early ablation gap closure and robust axonal bridge formation was also observed for the ASC secretome-treated group. This was accompanied by significantly improved animal's motor function following ASC secretome treatment. These data suggest ASC secretome as a good candidate for the development of new cell-free based therapeutic strategies for SCI, as well as a possible alternative to replace direct cell transplantation approaches.

1. INTRODUCTION

Human spinal cord lacks the ability to regenerate after injury, leading to permanent loss of sensorial and motor function below the level of trauma. In contrast, amphibians, such as the salamander and *xenopus*, or vertebrate fish, namely zebrafish and lamprey, have a remarkable capacity to fully regenerate [1]. Therefore, using these species as models for Spinal Cord Injury (SCI) study can unveil the features and mechanisms by which a natural regeneration process occurs. Among these different naturally-regenerating species, *Xenopus laevis* is probably the most interesting model as it presents a stage-dependent regeneration [2] in which their entrance in metamorphosis initiates a progressive loss of their regenerative capacity, outlining the transition from a regenerative to a non-regenerative stage. Interestingly, these animals also display a particular developmental stage in which they transiently lose this capacity, the so called refractory period. The alteration of the regeneration program on *Xenopus laevis* during this period is not dependent on metamorphosis but rather on other mechanisms, such as immune [3, 4] and/or metabolic [2] alterations, and other cellular and transcriptomic changes [5-8]. Therefore, *Xenopus laevis* stands between the fully regenerative salamanders, fish and lampreys, and the non-regenerating mammals, such as rodents and humans, providing a system in which both loss and gain of function experiments can be directed at understanding the cellular and molecular processes that inhibit or stimulate regeneration. Going beyond this mechanistic point-of-view, *Xenopus laevis* can be used to explore the therapeutic effect of a given regenerative strategy, at both regenerating and non-regenerating stages, giving emphasis on the mechanisms that can initiate regeneration. So far, SCI-related regenerative strategies have been only tested in pre-clinical mammalian models but no successful treatments have been set in the clinics so far. Meanwhile, the insights that a basic model of regeneration such as the *Xenopus laevis* can provide to the field are reasonable enough to use them for this purpose.

In the last decade, cell free-based approaches using mesenchymal stem cells (MSCs) secretome have been showing promising as a therapeutic strategy for SCI. Many reports on the therapeutic effects of MSCs in SCI have been indicating a paracrine action of these cells on the injured tissues upon MSC transplantation, through the secretion of protective and trophic factors [9-15]. In line with this, we have previously shown that Adipose tissue-derived mesenchymal Stem Cells (ASCs) have a predominant role in promoting neuronal differentiation of human neural progenitor cells and axonal growth of dorsal root ganglion explants *in vitro* [16], through the solely use of the secretome of these cells in the referred cell cultures. These outcomes have been further attributed

to the composition of ASC secretome in several identified central nervous system (CNS)-related neuroregulatory factors, namely Pigment epithelium-derived factor (PEDF), Semaphorins (SEM), Cadherins (CDH), Interleukin (IL)-6, Glial-derived nexin (GDN), Clusterin (CLUS), Decorin (DCN) and Beta-1,4- galactosyltransferase 1 (β4Gal-T1) [16, 17]. These molecules were elsewhere shown to be important for neurogenesis, neuronal differentiation and proliferation [18-21], and axonal growth and migration [22-27], thus supporting our evidences. We thus decided to propose ASC secretome as a cell-free based therapy for SCI to better elucidate it potential in promoting axonal growth and functional recovery *in vivo*.

MSC secretome-based strategies have been prevalently tested in rodents, namely rats, most likely due to their similarity to humans concerning the existence of a mammalian non-regenerative environment. This allows to study the efficacy of given therapy and more easily translate it to larger animals or to the clinics. However, the recent emphasis on dissecting the molecular mechanisms associated with SCI, along with the therapeutic effect of a given regenerative strategy, poses the need to use more basic models of regeneration. With this in mind, the regenerative potential of ASC secretome was herein evaluated in *Xenopus laevis* tadpoles, in both refractory (non-regenerative) and regenerative stages, after complete transection of the spinal cord. Animal's functional recovery after treatment with ASC secretome was assessed, as well as axonal regeneration and growth at the injury site.

2. METHODS

2.1 Adipose tissue-derived mesenchymal stem cells

2.1.1. ASC culture

ASCs were kindly provided by professor Gimble (Lacell, USA). After thawing, cells were cultured in alfa-minimum essential medium (α-mem, invitrogen, usa) supplemented with sodium bicarbonate ($NaHCO_3$; Merck, USA), 10% (v/v) of fetal bovine serum (FBS; Biochrom, Germany) and 1% (v/v) penicilin-streptomycin antibiotic (p/s; Invitrogen, USA). When confluent, cells were enzymatically dissociated with 0.05% (v/v) trypsin/EDTA (Invitrogen, USA), re-plated at a density of 4000 cells/cm^2 and maintained at 37°C, 5% humidified CO_2, 95% air and 90% relative humidity.

2.1.2 Conditioning and secretome collection

The secretome, denoted as conditioned media (CM), was collected from cells in passage 5, as previously described [16]. Cells were plated at a density of 4000cells/cm^2 and maintained in

culture for 72 hours. Cells were then washed 5 times with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Invitrogen, USA), and 1 time with the conditioning medium – Neurobasal A Medium supplemented with 1% (v/v) Kanamycin (Invitrogen, USA). After 24 hours of conditioning period in supplemented Neurobasal-A Medium, the secretome was collected and centrifuged to remove cell debris. The collected secretome was concentrated 100x using a Vivaspin 20 centrifugal concentrators (MWCO 5kDa, Sartorius™ Vivaspin™ 20, Germany) at 3000g, and frozen at -80°C until further required.

2.2 Xenopus laevis in vitro fertilization, eggs maintenance and follow-up

2.2.1 In vitro fertilization

Female frogs were primed with human chorionic gonadotropin (HCG) 5-14 days before the experiments. 500-800 units of HCG was injected in the female frog's lymph sac approximately 12 hours before the eggs were needed. In the fertilization day, female frogs were placed for egg laying into 1x Modified Barth Buffer solution (MBS, 10x MBS salts [NaCl, KCl, $MgSO_4x7H_20$, HEPES, $NaHCO_3$], 0.1M $CaCl_2$ and 5M NaCl in destilated H_2O). Eggs were carefully collected regularly into 1x MBS into 100mm petri-dishes. Meanwhile, male frogs were anesthetized in 0.1% (w/v) MS222 for 15-30'minutes until slow or no heartbeat was found. Male's tests were excised and kept on ice. Small pieces of testis were macerated and mixed with the collected eggs into 0.1x MSB (10 x MBS salts [NaCl, KCl, $MgSO_4x7H_20$, HEPES, $NaHCO_3$] and 0.1M $CaCl_2$ in destilated H_2O), for 20 minutes at room temperature (RT). A cysteine treatment was then performed to remove egg's jelly coat by incubating them with 2% (v/v) Cysteine solution in 0.1x MBS for 5-7 minutes at RT with gentle rock. Eggs were washed for 5-10 minutes with 0.1x MBS and transferred to a cold plate at 14°C, or at RT for approximately 1 hour until the first division occurs and then transferred to 14°C.

2.2.2 Eggs maintenance and follow-up.

Dead eggs were removed from the petri dished twice a day using a pipette, and 0.1x MBS was replaced. The developmental stages of the animals were followed every day, using the developmental data from Nieuwkoop and Faber [28]. Garland Publishing Inc, New York ISBN 0-8153-1896-0] and the digital images provided by XeNBase, as reference.

2.3 Spinal Cord Injury and post-operative care

Tadpoles in the stages 45-47 (refractory period) and 50-54 (regenerative period) were used in this work. For that, animals were closely check daily for their developmental stages. When the desired stage was reached [29], animals were carefully collected and used for the experiments. A complete transection of the spinal cord of *Xenopus laevis* was the injury model used in this work, for both stage 45 and 50 tadpoles. To inflict the transection into the animal's spinal cord, the protocol described by Edwards-Faret et al. [30] was used as reference. Briefly, tadpoles were anesthetized with 2% (w/v) of freshly prepared tricaine methanesulfonate (MS222) by immersing the animals in the solution for 1-2 minutes. Animals were then carefully immobilized on their abdominal area using forceps under a dissecting microscope. A small incision on the skin and dorsal muscles perpendicular to the body's axis were made at the mid-thoracic level of the animals, at the central level of the gut. The meningeal layer was then removed using forceps, to completely expose the spinal cord [30]. At this stage, animals were grouped according to the procedure/treatment to receive: 1) non-injured tadpoles, injected with saline (SH group; n=12); 2) tadpoles subjected to SCI, injected with Neurobasal-A medium (NB group, n=12); and 2) tadpoles subjected to SCI, injected with ASC secretome (CM group, n=12). To fully transect the spinal cord of the tadpoles, a tip of a 30-gauge needle was used to make a clean cut at the thoracic level, perpendicular to the spinal cord. A successful spinal cord transection was confirmed by checking the presence of a dark line between rostral and caudal stumps of the spinal cord [30]. After surgery, all animals were transferred to small contents containing 1x MBS and antibiotics [penicillin (5,000 U/ml)-streptomycin (5 mg/ml) solution (Sigma-Aldrich, Germany) and gentamycin (1.25 mg/ml, Fisher Scientific, UK)], defined as 0,1x MBS + 3A, and kept at 20-21 °C until they recover from anesthesia. The post-operative care of the animals with the antibiotics were maintained for 3 days, with the 0,1x MBS + 3A solution being replaced twice a day.

2.4 Secretome injection in the Xenopus laevis tadpole's spinal cord after injury

2.4.1 FITC-labelling of the secretome.

To visualize that the secretome was being correctly administered in the ependymal canal of the spinal cord of *Xenopus laevis* tadpoles, and that it was not being washed out by the time the animal has been placed back in the water, the *secretome* was labelled with Fluorescein isothiocyanate (FITC) prior to injection. Using the FluoReporter FITC Protein Labelling Kit (Invitrogen, USA), one has the possibility to bind a FITC dye to free amines of proteins larger than 30kDa in a certain

solution. As so, a dye-protein conjugate displaying a fluorescent signal (Em: 580nm, Ex: 488nm) was formed, providing means to label the desired proteins of the secretome herein used. The labelled secretome was imaged in real time using a fluorescent microscope (Olympus IX-53), immediately, 1 and 2 days after injection.

2.4.2 Local injection of ASC secretome.

The secretome of ASCs was injected locally in the rostral stump of the spinal cord, immediately after injury. For that, a Pneumatic Pico Pump System (Narishige Group, Tokyo, Japan) was used. First, a pulled-glass capillary needle was filled with 2μl of the labelled secretome. The needle was then set in the needle holder, and the tip of the needle was carefully placed on the ependymal canal, rostral to the spinal cord, with the help of the micromanipulator under the dissection microscope. The secretome was administered at a rate of 0,05μl/s. NB group animals were injected Neurobasal-A medium.

2.5 Swimming Behavior of Xenopus Laevis tadpoles

To evaluate the effect of ASC secretome on the functional recovery of the animals after injury, their free-swimming behavior was analyzed at 2, 3 and 5 days post-injury. Animal's swimming trajectory was evaluated using a custom-made optimized vibrating six-well plate along with a video-tracking system (DanioVision, Noldus, Netherlands). For that, animals were individually placed in wells of a six-well plate containing vibrating motors attached to their walls, and their movement was tracked and recorded by a camera inside the DanioVision chamber. The parameters of the test were set using an Ethovision software. The animals were left to acclimatize the environment without disturbance for 10 minutes before testing. Once the vibration mode was set to ON, animals were subjected to cycles of 4 seconds of vibration, followed by 12 seconds of resting period (no vibration), to a total of 80 seconds of test. The recording data of the animals was acquired by the EthioVision software.

2.6 Histological preparation of the animals

Tadpoles were sacrificed by deeply anesthetize by immersion into 2% (w/v) of MS222 for 15 minutes, and placed in 4% (w/v) of paraformaldehyde (PFA) solution for 1 hour. Animals were washed 3 times with 1x PBS, and placed on a solution of saccharose at 30% (w/v). After 24-48 hours, animals were carefully immersed in section medium (Neg-50, Thermo Scientific, USA),

frozen in liquid nitrogen, and stored at -20°C. Later on, longitudinal cross sections of 20μm thickness were taken using Leica CM1900 cryostat and kept at -20°C until required for immunohistochemistry.

2.7 Immunohistochemisty (IHC)

Tadpole's spinal cord sections were immunostained for axonal growth and regeneration [βIII-Tubulin and growth associated protein (GAP)-43, respectively]. For that, sections were first washed with 0.1% (v/v) of Triton-X 100 (Sigma) in 1x PBS (PBS-T), 3 times, for 5 minutes to remove the excess of frozen section medium. Sections were then incubated with 3% (v/v) of PBS-T for 10 minutes for permeabilization of the tissue. After that, tissue sections were blocked with a solution of 3% (w/v) bovine serum albumin in PBS-T for 1 hour at room temperature (RT) to avoid unspecific binding of the antibodies. Next, sections were incubated for 1 hour with the following antibodies: mouse monoclonal Acetylated Anti-Tubulin (1:500, Sigma) and rabbit anti-GAP-43 (1:500, Abcam). Sections were then exposed for 1 hour at RT to the respective secondary antibodies: Alexa Fluor 488 rabbit anti-mouse and Alexa Fluor 594 goat anti-rabbit (1:500; Invitrogen). Finally, all sections were counterstained with DAPI (4',6'-diamino-2-fenil-indol; 1mg/ml, Invitrogen) for 5 minutes at RT. 3 washes with 1x PBS were performed between steps. The sections were mounted in Immu-Mount (Thermo Scientific, USA) and imaging of the tissue was performed using a confocal point-scanning microscope (Olympus FV1000) at 20x magnification. All images were treated and analyzed using Image J software.

2.8 Tissue Histological Analysis

Quantification of βIII-Tubulin and GAP-43 positivity was performed on epicenter segment of the spinal cord, in both injured secretome-treated (CM group) and non-treated (NB group) animals, for stage 45 and 50. Using the Image J software, all acquired images were converted into monochrome 8-bit images. Fluorescent particles appear as black pixels, and background as white pixels. The region of interest (ROI) to analyze was determined using the free-hand drawing tool. The expression of immunofluorescence within the ROI was evaluated automatically by the software. Mean values within each group were calculated as the percentage of βIII-tubulin- or GAP-43-expressing axons.

2.9 Statistical Analysis

The data obtained from behavioral analysis and tissue fluorescence quantification analysis were reported as Mean±SEM. Statistical differences among groups were assessed by One-way ANOVA and Tukey's post hoc tests using GraphPad PRISM software (version 5.00). A p-value of ≤ 0.05 (95% confidence level) was set as criteria for statistical significance. Significant values were denoted with * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

3. RESULTS

In this study, the potential of ASC secretome in promoting *Xenopus laevis* spinal cord regeneration after injury was evaluated in both refractory and regenerative periods. For that, tadpoles in stage 45-47 and 50-54 were grouped in refractory and regenerative period groups, respectively, and SCI was performed by completely transecting their spinal cord. Immediately after injury, animals received a single injection of ASC secretome (CM group) through the ependymal canal, rostral to the injury site. Animals inflicted with SCI and injected with Neurobasal-A medium (NB group), or not subjected to SCI and injected with saline solution (SH group) were used as control groups.

ASC secretome distributed well in the lesion site and was retained in spinal cord tissue

The secretome of ASCs was labelled with FITC fluorochrome prior to injection in *Xenopus laevis* animals with the purpose to follow its distribution and retention within the spinal cord tissue. Immediately after injection, the labelled molecules were clearly observed surrounding the injury site (Fig. 1 – Day 0; arrow heads). 1 and 2 days after injection, some of the labelled molecules were seen in the spinal cord tissue, both at the lesion site and in the proximities (Fig. 1 – Day 1; Fig. 1 Day 2; arrow heads). These data suggest that the secretome of ASCs was correctly injected in the ependymal canal of the spinal cord, and that it was not washed away by the time that animals were placed back in the water, after the surgical procedure was finished. In addition, the retention of secretome's molecules in the spinal cord tissue for at least 2 days after injection may indicate that some may still be exerting their effect upon the lesioned animals for some days after treatment.

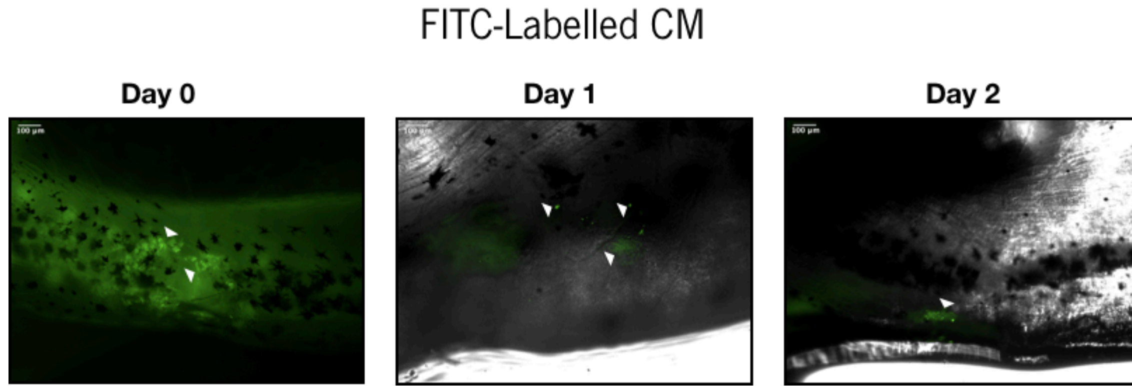


Figure 1. FITC-labelled secretome at the injury site of *Xenopus laevis* tadpoles. Real time representative fluorescent microscopy of the Xenopus laevis immediately (Day 0), 1 (Day 1) and 2 (Day 2) days following injection of FITC-labelled secretome in the spinal cord after transection. The secretome was adequately injected in the ependymal canal of the spinal cord, and was well distributed and retained up to 2 days following injection.

ASC secretome improves functional recovery of Xenopus laevis tadpoles after SCI

Tadpoles motor recovery in response to treatment was assessed by monitoring animal's free-swimming ability using a motion capturing software, for both the refractive period at 2, 3 and 5 days post-injury, and the regenerative period, at 3 and 5 days post-injury. Paralysis of all animals in both periods was observed during the two initial days post-treatment. On the following days, the ASC secretome-treated group in the refractive period showed a swimming pattern very similar to healthy animals (SH group), in opposition to NB-treated animals (Fig. 2A). Significant differences in the swimming distances between the ASC secretome-treated groups and the NB-treated group, 5 days post-treatment confirmed these observations (* $p<0.05$; Fig. 2B).

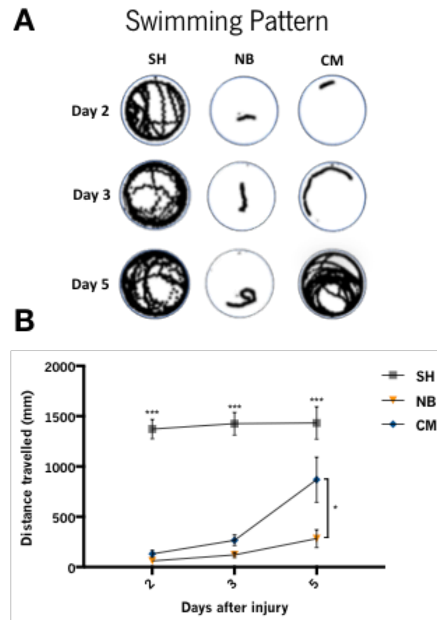
Regarding the regenerative period, clear differences in the swimming pattern between the ASC secretome- and NB-treated groups were observed (Fig. 2E), with the first group showing a pattern very similar to the SH group. However, no significant improvements of animal's motor function were found throughout time between the two treated groups (Fig. 2F). Additionally, although the secretome-treated group show similar swimming pattern to the SH group (Fig. 2E), 5 days after treatment, this was not reflected in the distance travelled during the swimming recording (Fig. 2F).

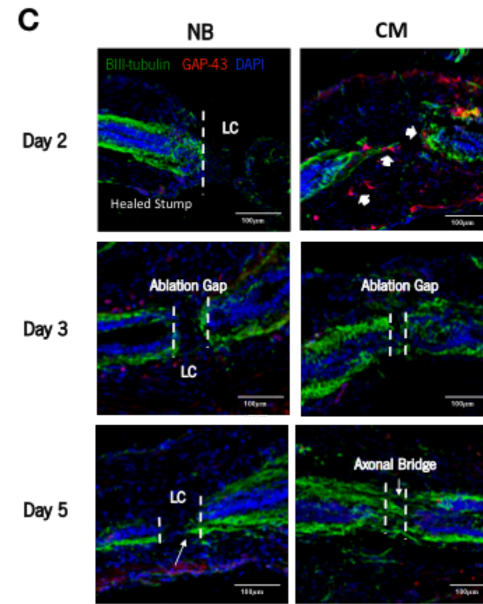
ASC secretome favors axonal sprouting and regeneration in Xenopus laevis tadpoles after SCI

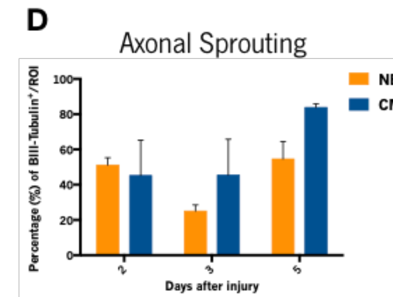
Neuronal regrowth and regeneration after treatment was assessed by performing anti-acetylated tubulin and anti-GAP-43 immunostaining, respectively, at 2, 3, and 5 days post-injury for refractive period animals, and 3 and 5 days post-injury for regenerative period. Substantial GAP-43 expressing cells in the lesion core (LC) and both rostral and caudal ends of the spinal cord was observed in the ASC secretome-treated group from the refractive period (Fig. 2C), 2 days after treatment, but few were observed for the NB-treated group (Fig. 2C, arrow heads). This was confirmed by significant differences in the mean percentage of GAP-43^{+} cells between the ASC secretome-treated group and the NB-treated group (**$p<0.01$, Fig. 2D). Considerable ablation gap closure and a robust axonal bridge formation was observed in the secretome-treated animals, 3 and 5 days after treatment, respectively (Fig. 2C). Furthermore, increased expression of βIII-tubulin in the LC, 5 days post-treatment, indicated neuronal regrowth throughout the injury site (Fig. 2E), though no statistical differences were found between groups.

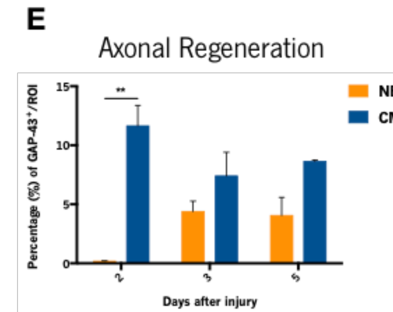
In the regenerative period, GAP-43 staining was mostly found rostral and caudally to the LC in the secretome-treated group (Fig. 2G), confirmed by the elevated, but not statistically significant, mean percentage of GAP-43^{+} cells in this group, in comparison to the NB-treated group (Fig. 2H), 3 and 5 days after treatment. Also, complete ablation gap closure with formation of βII-tubulin positive axonal bridge was observed in the secretome-treated group, 3 days post-treatment (Fig. 2G). However, no statistical differences were found in the mean percentage of βIII-tubulin expression between secretome- and NB-treated groups (Fig. 2I), most likely due to the proximity of the two stumps of the spinal cord, not enabling to distinguish the βIII-tubulin expression between the ends of the spinal cord and the axonal bridge itself.

Refractory Period

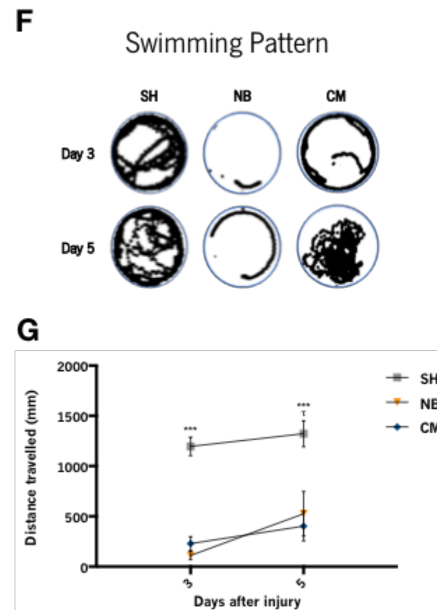


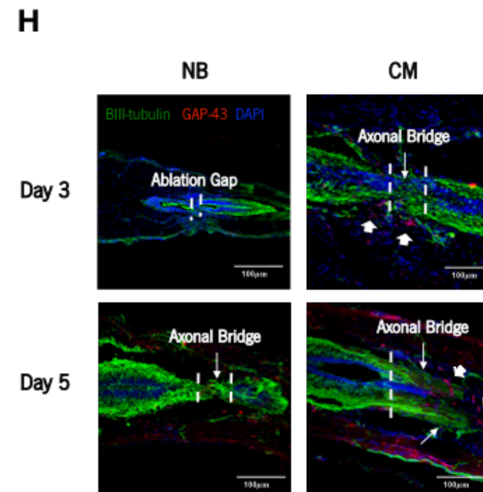


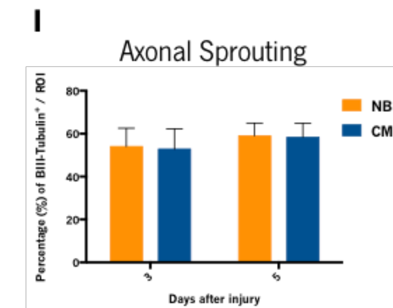




Regenerative Period







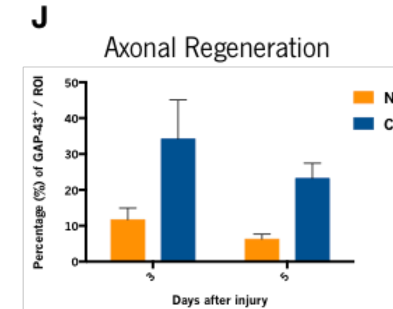


Figure 2. Therapeutic effects of ASC secretome on Xenopus laevis tadpoles after complete transection on swimming recovery, axonal growth and regeneration, in both refractory (A-E) and regenerative (F-J) stages. Swimming pattern and quantification of the distance travelled by the **(A-B)** refractory animals at 2, 3 and 5 days post-treatment, and **(F-G)** regenerative animals 3 and 5 days post-treatment. ASC secretome promoted functional recovery of Xenopus laevis tadpoles after SCI from the refractory period, 5 days post-treatment, when compared to NB-treated animals. On the other hand, no differences were found in the locomotor performance between ASC secretome- and NB-treated animals in the regenerative stage. Representative confocal images of longitudinal crossections of Xenopus laevis spinal cord after immunostaining for βIII-tubulin (axonal sprouting) and GAP-43 (axonal regeneration), at **(C)** refractory and **(H)** regenerative stages. Quantification of the percentage of **(D; I)** βIII-tubulin and **(E; J)** GAP-43 positivity, at both refractory (**D, E**) and regenerative (I, **J**) stages. In the refractory period, ASC secretome group (CM) shows a clear gap closure and the formation of a robust axonal bridge between the two stumps of the spinal cord, 5 days post-treatment. This was confirmed by elevated, but not statistical significant, expression of βIII-tubulin. GAP-43 positive regenerating cells were present in the spinal cord tissue of these animals, with significant differences observed at 2 days post-treatment. In the regenerative period, CM group presented axonal bridge formation and some GAP-43^{+} cells caudally to the injury site, 5 days post-treatment. However, no statistical differences were observed in the percentage of both βIII-tubulin and GAP-43 positivity in this stage. Mean±SEM; n=15 for locomotor assessment; n=5 for histological evaluation *$p < 0.05$; **$p < 0.01$; ***$p<0.001$.

4. DISCUSSION

The secretome of ASCs was previously shown by our group to contain several neurotrophic factors with anti-apoptotic, pro-inflammatory, angiogenic, and neuromodulatory roles in the CNS [17]. When applied to *in vitro* systems, ASC secretome was reported to be effective for neuronal survival and differentiation, and neurite growth [16]. These results motivated further investigation on the effectiveness of ASC secretome in an *in vivo* context. The present study demonstrated that the treatment of *Xenopus laevis* tadpoles with the secretome of ASCs after a complete transection of the spinal cord could promote locomotor improvements and tissue repair after SCI.

So far, these naturally-regenerating species were exclusively used for investigation of the regenerative process, from understanding the pathophysiology of SCI to disclosing the mechanisms responsible for their ability to fully regenerate their tail and spinal cord after lesion. However,

Xenopus laevis is the unique amphibian that present shifts on their potential to regenerate throughout their life from a regenerative to non-regenerative contexts. This particular property makes them ideal to be used in experimental paradigms dealing with the establishment of therapies for SCI regenerative medicine. However, the impact of a regenerative therapy based on a cell-free approach on a *Xenopus laevis* SCI model was herein investigated for the first time.

After complete transection of tadpoles' spinal cord in the refractive period (stage 45-47), NB-treated animals (NB group) showed a low regenerative ability following injury, as shown by the absence of βIII-tubulin^{+} axonal sprouting from both rostral and caudal ends of the spinal cord and GAP-43^{+} regenerating cells in the lesion cavity, from 2 to 5 days post-injury (Fig. 2C). In contrast, tadpole's treatment with ASC secretome at this stage promoted significant increased levels of regenerating cells at 2 days post-treatment (Fig. 2D), and supported extensive axonal sprouting accompanied by the formation of a robust axonal bridge between the two stumps of the spinal cord at 5 days post-treatment (Fig. 2E). Regarding tadpoles in the regenerative stage (stage 50-54), ASC secretome treatment increased GAP-43^{+} regenerating cells and the formation of an axonal bridge in the lesion cavity already at 3 days post-treatment (Fig. 2G), whereas the NB group presented few regenerating cells in the lesion cavity and only discrete axonal sprouting at 5 days post-injury (Fig. 2G). According to Beck et al. [5], refractory period tadpoles never progressed to tissue regeneration after tail amputation, whereas regenerative tadpoles showed regenerated tails, including the spinal cord, within approximately 1 week after injury. In addition, complete cellular bridge formation across the lesion cavity in regenerative tadpoles was only reported at 10 days post injury by Munoz and colleagues [31]. Others have reported similar dynamics of regeneration [32, 33]. Thus, our observations suggest that ASC secretome treatment is restoring the regenerative ability of the refractive tadpoles, and anticipating the regenerative process in the regenerative tadpoles. Additionally, the recovery of the swimming ability was observed for both refractory and regenerative tadpoles after ASC secretome treatment (Fig. 2A-B and E-F, respectively), in contrary to NB-treated animals, which correlated with the observed histological improvements. Interestingly, the temporary loss of tadpoles' regenerative ability in the refractory period has been previously attributed to a suppression of specific molecular pathways [5, 34] and to a transitory imbalanced immune response [3], as well as to a marked decrease of Sox2/3 positive cells recruitment to the lesion site following injury [31, 33], all contributing differently but possibly synergistically to the decreased regenerative capabilities of these animals. Whether ASC secretome may be acting upon these or other mechanisms was not unveiled in this study. In future,

studies directed to explore the mechanisms underlying the effects of ASC secretome on the regeneration of tadpoles after SCI will be of the utmost importance.

CONCLUSION

Following our previous findings concerning the potential of ASC secretome in promoting *in vitro* neuronal differentiation and axonal growth, the present study provides evidences on the impact that the secretome of these cells may have in the regeneration of *Xenopus laevis* tadpole' spinal cord tissue after SCI and in the motor functional recovery of the injured animals treated with this molecular cocktail. So, these promising data support the potential of ASC secretome as a cell-free based strategy for the treatment of SCI, and encourage the use of *Xenopus laevis* to study the efficacy of therapeutic strategies for SCI, expanding the boundaries of their use in research, taking them beyond development studies to, also, regeneration paradigms.

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CHAPTER IV

ADIPOSE-DERIVED MESENCHYMAL STEM CELL THERAPEUTIC EFFECT IN A MICE MODEL OF SPINAL CORD INJURY

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ADIPOSE-DERIVED MESENCHYMAL STEM CELL THERAPEUTIC EFFECT IN A MICE MODEL OF SPINAL CORD INJURY

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ABSTRACT

Spinal cord injury (SCI) in humans results in complete paralysis below the level of injury, thus representing a highly incapacitating condition to SCI patients. Only palliative care is currently available for these patients, therefore posing an urgent need to find other therapeutic strategies. The current treatment paradigm for SCI focus in providing neuroprotection and neuroregeneration to the damaged tissues and cells in order to avoid the exacerbation of the lesion to a chronic and irreversible state. In this line, stem cell-based therapies have been showing as an interesting option, among which mesenchymal stem cells (MSCs) are regarded as one of the most promising cell population due to their paracrine action upon damaged tissues. The neuroregenerative role of this cell population is believed to be related to the contents of their secretome, particularly in anti-inflammatory cytokines, and regenerating- and growth-permissive growth factors, as previously reported by our group. In this study, we propose the use of the secretome of adipose tissue-derived mesenchymal stem cells (ASCs) as a cell-free based therapy for SCI. For that, *in vivo* studies were conducted on a complete SCI transection mice model. Our results revealed significant motor and sensorial recovery of SCI animals after continuous treatment with ASC secretome, when compared with vehicle-treated animals, accompanied by axonal outgrowth and regeneration through the lesion site. Moreover, the number of inflammatory cells recruited after injury were markedly reduced upon ASC secretome treatment, and decreased lesion cavities were also observed. These results provide evidences of the neuroprotective and neuroregenerative potential of ASC secretome after SCI.

1. INTRODUCTION

Human spinal cord inability to regenerate after injury is due to the extremely complex pathophysiology of spinal cord injury (SCI). The currently available clinical intervention to SCI patients is limited to the decompression/stabilization of the spine [1-3] and control over possible clinical complications through cardiovascular, respiratory, and circulatory support [3-5] as well as pharmacological intervention [6]. All of these, aim to minimize the primary injury and prevent the secondary injury known to exacerbate the condition [7]. Despite the progress in these surgical, clinical and pharmacological approaches, they still do not provide significant sensory and motor improvement of SCI patients [8]. The main mechanisms related to the secondary SCI are neuronal death, inflammation, reactive gliosis, axonal demyelination, and cysts formation as shown by animal and human studies [9-16]. Furthermore, the accumulation of inhibitory molecules and loss of trophic support in the lesion site are the major contributors for the limited regeneration of the injured tissues. In spite of the development of several strategies, mostly at a pre-clinical stage, focused in controlling or reverting these SCI-related mechanisms, they are yet to be successful, which poses an urgent need to find new strategies for SCI treatment.

The two main current treatment paradigms for SCI rely in the modulation of the inflammatory response after injury, avoiding the exacerbated secondary injury (neuroprotection), or through the replacement of the damaged neural tissue while stimulating the endogenous neural regeneration (neuroregeneration) [7, 17]. In this context, stem cell therapies have been a key element in restoring the spinal cord function after injury [7, 18]. Relevant advances in pre-clinical experiments with a variety of stem cells have led to some clinical trials, most of them are currently ongoing. Nevertheless, the interest on these cells have now been turned to their paracrine activity on the damaged tissues rather than looking to their local effect [19]. This has been due to the perception that some stem cell populations secrete a cocktail of biomolecules to the external milieu which were considered to be the main contributors for their neuroregenerative actions [20-25]. One of the best example of a stem cell population with a therapeutic impact truly mediated by the neuroprotective and neuroregenerative character of their secretome are mesenchymal stem cells (MSCs). Their secretome is known to contain important proteins, growth factors, chemokines and cytokines that mediate regeneration [19]. Indeed, Nerve growth factor (NGF), Vascular endothelial growth factor (VEGF), Hepatocyte growth factor (HGF), Insulin-like growth factor 1 (IGF-1), Transforming growth factor-beta 1 (TGF-β1), Interleukin (IL)-10, Glial-derived neurotrophic growth factor (GDNF), basic Fibroblast growth factor (bFGF), Pigment epithelium-derived factor (PEDF), Cadherin 2 (CADH2), Semaphorin 7A (SEM7A), and Glial-derived nexin (GDN) are some of the factors that have been pointed out as responsible for MSCs neurotrophic and immunomodulatory effects [23, 26-29]. In line

with this, the *in vivo* application of the secretome of bone-marrow derived MSCs (BM-MSCs) in rats' spinal cord after injury, markedly improved animal's locomotor function and fostered tissue repair, by stimulating axonal outgrowth and regeneration, and attenuating infiltration of astrocytes and inflammatory response, in comparison to vehicle-treated animals [30-32]. BM-MSCs are the most widely MSC population, however the isolation procedure of these cells is too painful and invasive, and is likely to cause generalized infections [33]. There are other available MSC sources, from which the adipose-tissue has emerged as a potential alternative. In contrary to BM-MSCs, Adipose-derived MSCs (ASCs) are of easy access and can be obtained through a simple and less invasive procedure. Moreover, ASC paracrine activity upon damaged tissues has also been attributed to the composition of their secretome, providing them with both neuroprotective and anti-inflammatory roles [34, 35]. In fact, the therapeutic impact of ASCs on SCI has been reported in several SCI animal models. The transplantation of ASCs into rodent and dogs following SCI was reported to support functional improvements, and to contribute to tissue repair and regeneration, mostly by decreasing apoptosis and astrogliosis after injury while supporting axonal growth and regeneration [36-41]. Considering the beneficial effects of ASCs shown in animal models, this cell population has been put forward to clinical trials, where some preliminary improvements were found in some patients, with no adverse effects associated with ASC transplantation [42, 43]. Currently, a phase I clinical trial using autologous ASCs in chronic SCI patients is ongoing at the Mayo Clinic, with expected outcomes to be reported in 2023. Recent evidences attribute the therapeutic effects of ASCs to the biomolecules presented in their secretome. In fact, previous studies from our group reported that the presence of NGF, VEGF, HGF and stem cell factor (SCF) in ASC secretome supported hippocampal neuronal survival in culture [27]. Moreover, cortical neurons were found to be protected from inflammation *in vitro*, due to an increase of TGF-β1 and IL-10 and decrease of Tumor necrosis factor-alfa (TNF-α), nitric oxide or prostaglandin E2 in culture by ASC secretome [29]. More recently, we have shown that ASC secretome was able to promote axonal regeneration of dorsal root ganglion explants and neurodifferentiation of neural progenitor cells [44]. Considering such promising data, ASCs seems to be a good alternative to BM-MSCs. Thus, ASC secretome is herein proposed as a cell-free based therapy for SCI to better elucidate it potential in promoting axonal growth and functional recovery *in vivo*.

2. METHODS

2.1 Adipose tissue-derived mesenchymal Stem Cells (ASCs)

2.1.1 ASC culture

ASCs were kindly provided by Professor Gimble (Pennington Biomedical Research Center/Tulane University, USA). After thaw, cells were cultured in alfa-Minimum Essential Medium (α-MEM, Invitrogen, USA) supplemented with sodium bicarbonate (NaHCO3; Merck, USA), 10% (v/v) of fetal bovine serum (FBS; Biochrom, Germany) and 1% (v/v) Penicilin-Streptomycin antibiotic (P/S; Invitrogen, USA). When confluent, cells were enzymatically dissociated with 0.05% (v/v) trypsin/EDTA (Invitrogen, USA), re-plated at a density of 4000 cells/cm^2 and maintained at 37°C, 5% humidified CO_2, 95% air and 90% relative humidity.

2.1.2 Conditioning and secretome collection.

The secretome, denoted as conditioned media (CM), was collected from cells in passage 5, as previously described [44]. Cells were plated at a density of 4000cells/cm^2 and maintained in culture for 72 hours. Cells were then washed 5 times with PBS without Ca^{2+} and Mg^{2+} (Invitrogen, USA), and 1 time with the conditioning medium – Neurobasal A Medium supplemented with 1% (v/v) Kanamycin (Invitrogen, USA). After 24 hours of conditioning period in supplemented Neurobasal-A medium, the secretome was collected and centrifuged to remove cell debris. The collected secretome was concentrated 100x using a Vivaspin 20 centrifugal concentrators (MWCO 5kDa, Sartorius™ Vivaspin™ 20, Germany) at 3000g, and frozen at -80°C until further required.

2.2 Spinal cord injury and post-operative care

Eight weeks-old female C5Bl/6 mice (Charles River, France), were used in this *in vivo* study. Animals were group housed - 5 per cage, on corncob bedding with access to food and water ad libitum, and holding rooms were maintained on a 12-hour light/dark cycle. A complete transection of the spinal cord was the injury model herein used. Briefly, animals were anesthetized with a mixture of 1.5x Imalgene and 1x Dorbene. When no reaction to pinch was observed, animals were considered ready for surgery. First, animals were placed under a dissecting microscope. An incision on the skin and dorsal muscles was performed from T2-T10 and the muscles retracted. A laminectomy was performed at the T8 level, and the spinal cord exposed. At this stage, animals were grouped according to the procedure and/or treatment to receive: 1) mice subjected to sham operations - laminectomy but no SCI, injected with Neurobasal-A medium (SH group, n=8); 2) mice subjected to SCI, injected with Neurobasal-A medium (NB group, n=7);

and 3) mice subjected to SCI, injected with ASC secretome (CM group, n=9). The spinal cord of NB and CM group animals was totally cut using a microdissection scissor. The complete separation of both ends of the spinal cord was confirmed under the microscope using forceps. Animals were finally closed with Vicryl sutures (Johnson and Johnson, USA). After the surgical procedure, anesthesia effect was reverted by a single subcutaneous administration of atipamezole (5mg/ml, Antisedan/Pfizer, USA). Post-operative care consisting in subcutaneous administration of the analgesic butorphanol (10mg/ml, Butamidor, Richter Pharma AG, Austria), the antibiotic enrofloxacin (5mg/ml, Baytril/Bayer, Germany), 0.9% (v/v) NaCl and vitamins (Dulphalyte, Pfizer) was then given to every animal. Animals were then kept under heat lamps until recover from anesthesia. Post-operative care was maintained twice a day for 1 week post-injury. Manual bladder evacuation was performed twice a day until animals recover their bladder control completely. The general health of the animals was carefully checked every day for sins of illness and weight loss of the animals, during the time of post-surgery recovery and treatment.

2.3 ASC secretome administration to mice after SCI

Animals from the CM group were intravenously administrated with ASC secretome. The treatment was given systemically in the three 24 hours post-injury, the first immediately after SCI, and then weekly until 6 weeks post-injury (100μl per injection). SH and NB group animals were administered with 100μl of Neurobasal-A medium.

2.4 Motor and emotional behavior of mice after SCI

2.4.1 BMS score.

Basso Mouse Scale (BMS) scoring was used to assess the locomotor recovery of secretome-treated mice after SCI [45]. The first BMS evaluation was performed 2 days after injury in order to confirm hindlimbs paraplegia in all animals. Scores of 0 were selected for the experiment. BMS scoring for locomotion evaluation was then performed weekly until the end of the experiment (6 weeks post-injury). Mice were allowed to explore an open field arena for 5 minutes, while their locomotion was being recorded by video-camera. Two blinded researchers were evaluating mice locomotion during all time of the trial.

2.4.2 Von-Frey Test and Ultrasonic Vocalizations'.

The ability of secretome-treated SCI mice to respond to a mechanical stimulus at the hindlimb paws was assessed by the Von Frey test [46]. For that, mice were individually placed in a clear glass in an elevated grid and the plantar surface of the hind paws was poked with Von Frey filaments of varying forces – 2g,

1.4g, 1g, 0.6g, 0.4g, 0.16g, 0.07g, 0.04g, 0.02g, and 0.008g. The trial started using the middle force filament (0.16g) and went further up the higher force filaments in case of no reaction (=0), or down to the lower ones in case a reaction occurred (=X), in a total of 6 measurements [47]. If no response was obtained up to the maximal filament (2g), or if a positive response occurs down to the minimal (0.008g), the 1.4 and 0.008 values were assumed for the measurement of that animal, respectively. Positive reactions considered included paw withdrawal, licking, shaking or extension of the paw, either during application of the stimuli or immediately after. The presence of nociception or hypersensitivity on the hindpaws was indicated by an exaggerated reaction to the lower diameter filaments. The response to Von Frey filaments was deduced as the 50% response threshold, calculated using the formula $50\%g_{threshold} = \frac{10^{Xf+K.\delta}}{10000}$, where X is the value corresponding to the final Von Frey filament tested (in log units); k is the tabular value concerning the pattern of positive (=X) and/or negative (=0) responses, and δ is the mean difference between stimuli (in log units) [48]. Low threshold is indicative of hypersensitivity, while high threshold indicates normal sensitivity to the mechanical stimuli, usually found among healthy individuals [46].

Another possible indicator of animal's sensitivity is the presence of ultrasonic vocalizations (USVs) by the animals at the time of the stimuli [49]. As so, ultrasound vocalizations were recorded during the Von Frey trial. For that, ultrasound Microphones (CM16/CMPA, Avisoft Bioacoustics) sensitive to frequencies of 10-200 KHz were used, next to the Von Frey apparatus. Vocalizations were recorded using the Avisoft-Recorder (version 5.1.04), and identified by automatic data processing on the software. Vocalizations were analyzed by the DeepSqueak software [50] and the total number and duration of the vocalizations per animal were the considered parameters. Vocalizations of 22Hz, the so called "negative vocalizations", were the only considered in this assessment, as they are related to a reaction of the animals to an unpleasant or noxious stimulus [51], as is the case of the Von Frey. According to Portfors et al. [51], 22Hz vocalizations typically present a frequency between 18 and 35KHz, therefore the data obtained was analyzed following this parameter.

2.5 Histological preparation of the animals

Six weeks after SCI, mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (200mg/ml, Eutasil, Ceva Saúde Animal, Portugal), and transcardially perfused with 0.9% NaCl followed by cold 4% (w/v) PFA. The spine and the spinal cord were dissected and incubated with PFA for 24 hours, at 4°C. The spinal cord was then carefully dissected and placed on a solution of 30% (w/v) of saccharose for 24 hours at 4°C. After that, 3cm length of spinal cord tissues were cut having the lesion site at the

middle point, carefully immersed in section medium (Neg-50, Thermo Scientific, USA), frozen in liquid nitrogen, and stored at -20°C. Longitudinal cross sections of 20μm thickness were then taken using Leica CM1900 cryostat and kept at -20°C until required for immunohistochemistry.

2.6 Immunohistochemisty (IHC)

Mouse spinal cord sections were immunostained for axonal growth and regeneration [βIII-Tubulin and growth associated protein (GAP)-43], de/re-myelinization (Fluoromyelin) and neuroinflammation [Ionized calcium binding adaptor molecule 1 (Iba-1)]. For that, sections were permeabilized with 0.2% (v/v) of PBS-T for 10 minutes, and washed 3 times with PBS 1x. All sections were incubated with a blocking solution of 5% (v/v) fetal calf serum in 0,2% (v/v) PBS-T for 30 minutes at RT, and incubated overnight at RT with the following antibodies: rabbit anti-beta III tubulin (1:1000, Abcam), mouse anti-GAP-43 (1:1000, Abcam), rabbit anti-Iba1 (1:750, Wako). Sections were then incubated for 1 hour at RT with the following respective secondary antibodies: Alexa Fluor 488 rabbit anti-mouse, and Alexa Fluor 594 goat anti-rabbit and rabbit anti-mouse (1:1000; Invitrogen). Cell nuclei was counterstained with DAPI (4',6'-diamino-2-fenil-indol) for 10 minutes. For Fluoromyelin staining, sections were incubated with the FluoroMyelin™ Green Fluorescent Myelin Stain (1:300, ThermoFisher), along with DAPI, for 10 minutes at RT. 3 washes were performed between steps. The sections were mounted in Immu-Mount (ThermoFisher Scientific, USA) and kept at 4°C until imaged. βIII-Tubulin, GAP-43 and Fluoromyelin were imaged by fluorescence microscope (Olympus BX61), and Iba-1 immunostained sections by confocal point-scanning microscope (Olympus FV1000).

2.7 Tissue Histological Analysis

βIII-tubulin, GAP-43, Fluoromyelin and Iba-1 positivity was quantified on rostral, epicenter and caudal segments of the spinal cord, for both CM and NB groups. The process of imaging analysis was performed using Image J software. Thus, mean values of immunofluorescence within the region of interest (ROI) defined were calculated as the percentage of βIII-tubulin^{+} or GAP-43^{+} axons, per group. Fluoromyelin staining was used to quantify the lesion areas of the spinal cord, for both CM and NB groups. For that, the ROI to analyze was determined using the free-hand drawing tool and the corresponding area was automatically calculated by the software. Finally, Iba-1 positivity was used to compare the area occupied by activated and deactivated inflammatory cells throughout the spinal cord tissue of the CM and NB groups. For that, ROI was determined by the free-hand drawing tool and the mean values of

immunofluorescence within the ROI was calculated as the percentage of Iba-1 positivity. βIII-tubulin, GAP-43 and Iba-1 measures were normalized to the total segment of the spinal cord analyzed.

2.8 Statistical Analysis

The data obtained from behavioral analysis and tissue fluorescence quantification analysis were reported as Mean±SEM. Statistical differences among groups were assessed by One-way ANOVA and Tukey's post hoc tests using GraphPad PRISM software (version 5.00). A p-value of ≤ 0.05 (95% confidence level) was set as criteria for statistical significance. Significant values were denoted with * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and **** for $p<0.0001$.

3. RESULTS

The impact of ASC secretome treatment of mice after spinal cord complete transection was herein evaluated. The secretome of ASCs was intravenously administered through animal's tail vein after injury, immediately, 24, 48 and 72 hours post-injury, and then weekly for a total of 6 weeks. Control animals were administered with Neurobasal-A media after laminectomy (SH group) or SCI (NB group).

ASC secretome improves motor function and sensitivity recovery of mice after SCI

Animal's functional recovery after treatment was weekly assessed using the BMS score for a total of 6-weeks, as depicted in Figure 1A. Two days post-injury, all SCI animals presented complete paralysis of the hindlimbs, when compared to laminectomy animals (SH group). On the following weeks, ASC secretome-treated animals show a gradual recovery of the hindlimbs movement up to 6 weeks, while the NB-treated animals only showed limited and slight movement of one or two joints. The CM-group improvement was found to be significantly higher already at 2 weeks post-treatment in comparison to NB-group (*** $p<0.01$), and persisted to increase from 3 to 6 weeks (**** $p<0.001$). At 6 weeks post-treatment, secretome-treated animals showed the ability to frequently or consistently perform plantar stepping, accompanied by some degree of coordination.

The motor function improvements of the secretome-treated SCI mice were accompanied by a sensorial recovery of the hindlimbs. The ability of SCI mice to respond to a mechanical stimulus at the hindlimb paws was assessed by the Von Frey test at 2 and 6-weeks post-treatment (Fig. 1B). NB-treated animals presented lower magnitude of response to the Von Frey filaments than secretome-treated animals, at both time points. Healthy individuals usually present high threshold of response, indicating normal sensitivity to the mechanical stimuli, while low threshold is indicative of hypersensitivity [46]. Thus, the

higher level of response of the CM-group to the Von Frey filaments, suggest a recovery of the sensitivity of this group when compared to NB group. At the time of the mechanical stimulus, animal's vocalizations were recorded, also as indicator of animal's sensitivity. 22Hz vocalizations were assessed as indicative of a reaction to a noxious or discomfort stimulus [51], provoked by the Von Frey filaments. The number of 22Hz vocalizations of the CM group was slightly lower than for the NB group, although no statistical differences were found (Fig. 1C), indicating that the mechanical stimuli with the Von Frey filaments had provoked less noxious sensations in the secretome-treated animals. Moreover, the duration of the vocalizations (Fig. 1D) was slightly higher for the CM group when compared to NB group, again with no statistical differences found.

Altogether, these results suggest an important role of ASC secretome in both motor and sensorial recovery after SCI.

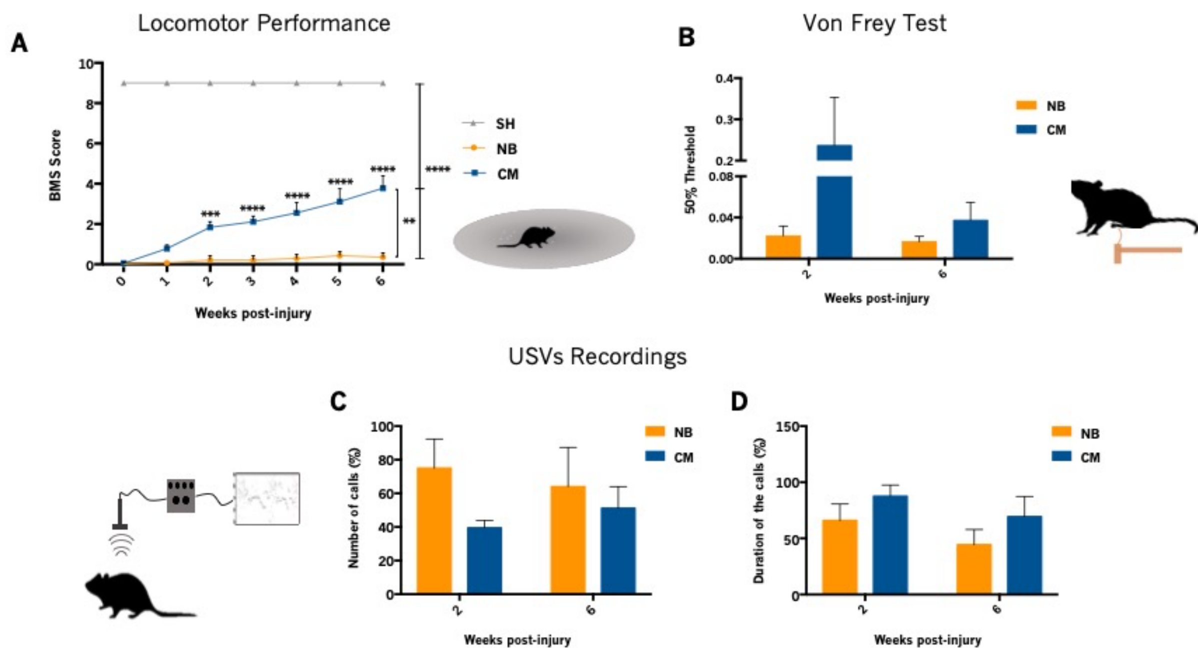


Figure 1. Recovery of motor and sensorial function of mice with complete spinal cord transection after ASC secretome treatment. (A) BMS test was performed up to 6 weeks after treatment. ASC secretome treatment significantly improved the locomotor function of the transected animals, when compared to NB treatment. Animals with no SCI treated with NB medium (SH group) showed completely normal locomotor performance. **(B)** Von-Frey Trial was performed at 2 and 6-weeks after ASC secretome or NB treatment as measure of sensitivity regain. Although no statistical differences were found between groups at both time-points, CM group show a trend of recovery of the sensorial function, when compared to NB group. **(C-D)** Recordings of USVs from mice during Von Frey trial was performed at 2 and 6 weeks after ASC secretome treatment. Mice treated with ASC secretome was shown to vocalize less **(C)** but for longer

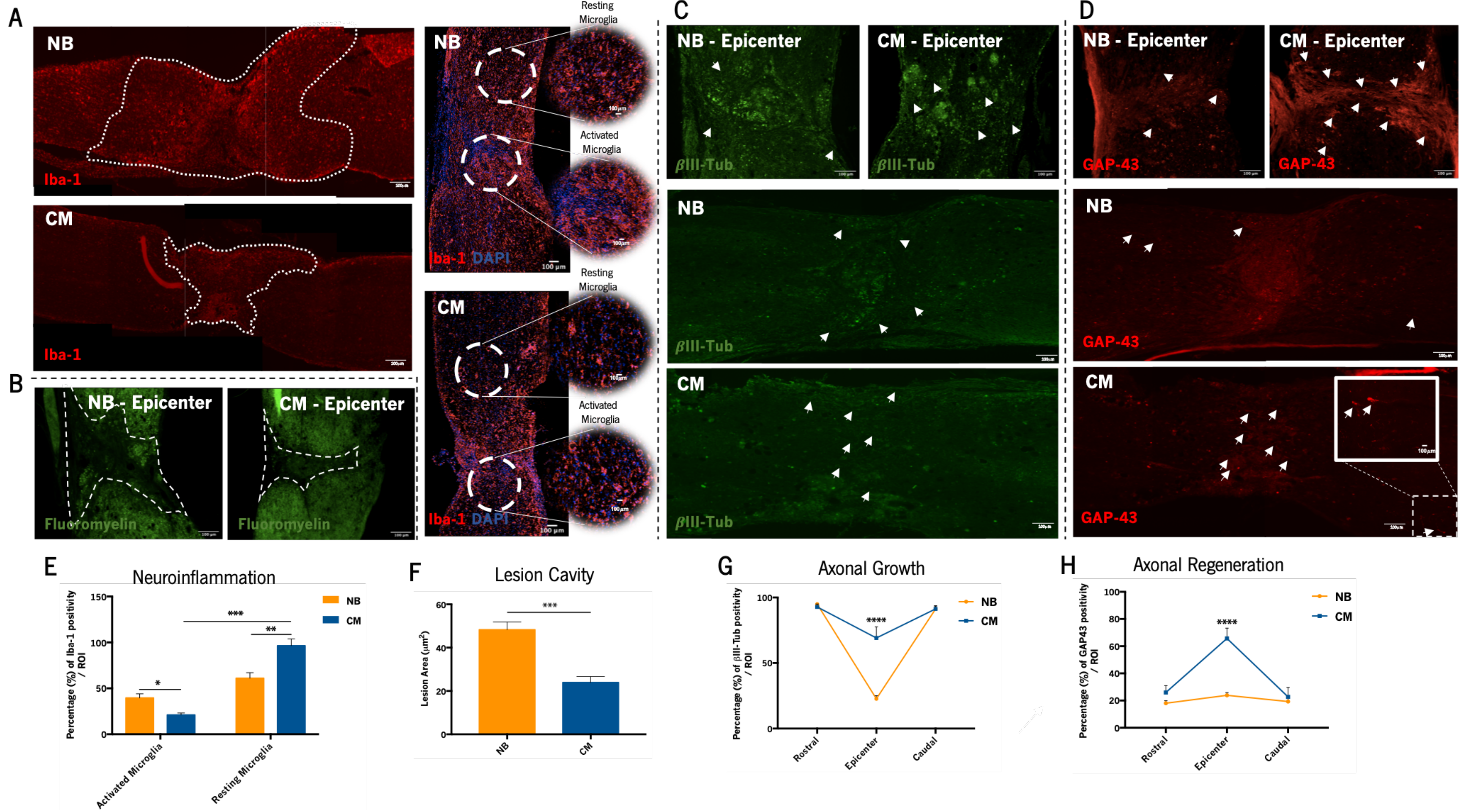
periods of time (**D**), when compared to NB group, although no statistical differences were obtained. Data is presented as Mean ± SEM; n=8 (SH), n=7 (NB), n=9 (CM); $*p < 0.05$; $**p < 0.01$; $***p<0.001$; $****p<0.0001$

ASC secretome modulated neuroinflammation in mice after SCI

The inflammatory response following injury was clearly different between the CM and NB groups, as suggested by the Iba-1 staining between the two groups throughout the spinal cord tissue (Fig. 2A). Different distribution of resting and activated inflammatory cells was also shown in the two groups, with prominent round-shape reactive cell accumulation beyond the lesion site for the NB group, as outlined in Figure 2A (dashed lines). Quantification of the percentage of Iba-1 positivity confirmed the significantly higher levels of activated inflammatory cells (Fig. 2E; * $p<0.05$) and lower resting ones (Fig. 2E; ** $p<0.01$) in the NB and CM groups, respectively.

ASC secretome reduces lesion cavity in mice after SCI

The border of the lesion area, shown in Figure 2B (dashed line), was clearly outlined by fluoromyelin staining. Larger cavities were found for the NB group (Fig. 2B, NB), when compared to the CM group (Fig. 2B, CM), further confirmed by the quantification of the area of the lesion cavity (Fig. 2F) 6 weeks after treatment, which was significantly higher in the former group (*** $p<0.001$).


A
NB
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Percentage (%) of GAP43 positivity / ROI
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CM
Rostral
Epicenter
Caudal

Figure 2. Therapeutic effects of ASC secretome in mice spinal cord 6 weeks after complete transection on neuroinflammation, lesion cavity, axonal growth and regeneration. (A-D) Representative confocal images of longitudinal crossections of mice spinal cord after immunostaining for Iba-1 (A, neuroinflammation), fluoromyelin (B, lesion cavity), βIII-tubulin (C; axonal growth) and GAP-43 (D, axonal regeneration). Quantification of the **(E)** percentage of Iba-1 positivity, **(F)** area of lesion cavity, **(G)** percentage of βIII-tubulin and **(H)** percentage of GAP-43. CM group presented decreased inflammatory response of microglial cells and significant reduced area of the lesion cavity. Moreover, axonal outgrowth and regeneration at the lesion site, as measured by a significant increase of βIII-tubulin and GAP-43 expression, respectively, were observed for CM group, in comparison to NB group. Data is presented as Mean±SEM; n=5; *p < 0.05; **p < 0.01; ***p<0.001; ****p<0.0001.

ASC secretome promotes neurite regeneration and sprouting in mice spinal cord after injury

Axonal sprouting and regeneration were evaluated 6 weeks after treatment by βIII-tubulin and GAP-43 positivity in mice spinal cord tissue, respectively. Immunohistochemistry analysis of the spinal cord tissue of ASC secretome treated animals showed βIII-tubulin$^+$ axons sprouting from the stumps of the spinal cord into the lesion area (epicenter) (Fig. 2C, CM-epicenter; white arrows). In contrast, NB group presented only few βIII-tubulin$^+$ axons in the proximities of the epicenter, but did not go through the lesion site (Fig. 2C, NB-epicenter; white arrows). These observations were further confirmed by the significant differences on the percentage of βIII-tubulin positivity in the epicenter between the two groups (Fig. 2G; ****p<0.0001). Moreover, regenerating GAP-43$^+$ axons were found extending longitudinally through the lesion area in the ASC secretome treated animals (Fig. 2D, CM-epicenter), while the NB-treated animals only presented few of them surrounding the lesion site (Fig. 2D, NB-epicenter). Some GAP-43$^+$ axons were also found rostral and caudally to the lesion area in the CM-group (Fig. 2D, CM), but few were seen in the NB-group (Fig. 2D, NB). Accordingly, significant differences were obtained in the percentage of GAP-43 positivity in the epicenter between groups (Fig. 2H; ****p<0.0001).

4. DISCUSSION

In this study, the *in vivo* therapeutic potential of ASC secretome was explored in a mice model of SCI after complete spinal cord transection.

The delivery of MSC secretome in rodents after SCI has been shown by Cantinieaux et al. [30], and later by Cizkova et al. [52] and Kanekyo et al. [32]. These authors used the secretome of bone-marrow MSCs (BM-MSCs) delivered intrathecally in contusive SCI rats. The secretome-treated animals demonstrated marked locomotor improvements, correlated with increased axonal regeneration, sprouting and remyelination, and attenuated inflammatory response following injury. So far, only BM-MSCs have been used as a cell-free based therapy for SCI. However, there are other available sources of MSCs, such as the adipose tissue (ASCs), which have also been shown to be a promising alternative to tackle SCI. In fact, ASCs have been associated to some therapeutic effects on SCI following transplantation, in both animals and patients [37-39, 41-43]. Interestingly, these effects have been attributed to specific molecules present in their secretome, as previously shown by our group and others [27, 29]. Recently, we have reported some *in vitro* data suggesting the neuroregenerative potential of the secretome of this and other MSC populations, specifically on axonal outgrowth from DRG explants [44], which supported the use of ASC secretome for the purpose of the work herein developed. To ensure a sufficient supply of trophic molecules to animals during both acute and chronic phase of injury, we decided to systemically deliver a concentrated formulation of ASC secretome, in the first three 24h post-injury and then weekly for 6 weeks.

All the transected mice presented complete paraplegia of both hindlimbs 2 days after injury, confirming the complete transection of the spinal cord. Locomotor analysis of mice treated with ASC secretome showed a significant clear and progressive motor recovery, beginning at 2 weeks post-treatment, which did not plateau until the end of the experiment, 6 weeks post-treatment. At this time-point, the CM group presented the ability to perform coordinated plantar stepping, while the NB-treated group only presented slight movement of ankles (Fig. 1A), according to the BMS score [45]. The locomotor improvements of the secretome-treated animals were accompanied by a regain of sensitivity from 2- to 6-weeks post-treatment, implied by the higher magnitude of animal's response to Von Frey filaments in the CM-group (Fig. 1B). Interestingly, no pain or discomfort was associated with Von Frey stimuli, measured by the lower number (Fig. 1C) and higher duration (Fig. 1D) of negative vocalizations (22Hz) during the time of the mechanical stimulus to these animals (Fig. 1D). 22Hz vocalizations are usually emitted by rodents in response to aversive behavioral situations or distress events, such as exposure to predators, pain, startling noises [51]. The basis of Von Frey trial is to present the animals with a pinch in the paws that only brings

discomfort to animals presenting hypersensitivity of the limbs [46]. As so, higher responses to Von Frey filaments accompanied by few 22Hz vocalizations indicated a recovery of sensitivity of secretome-treated animals.

An important factor that influences the successful regeneration of a tissue after injury is the inflammatory response that follows. Inflammatory cells, namely microglia and macrophages are the major contributors to the post-injury inflammatory response [53]. These cells are described to rapidly activate upon injury, changing their morphology and phenotype from an activated form or classically activated microglia (M1) - a round and enlarged cell soma with retracted processes, to an alternatively activated or acquired deactivated microglia (M2) - a ramified morphology with extended thin processes [54, 55]. The production of cytokines and growth factors by these cells provide them with either neurotoxic (M1 state), or neuroprotective (M2 state) character [54, 56-58], although their role in the injured spinal cord is still controversial [59, 60]. Therefore, the magnitude of an inflammatory response might be determined by the activation/deactivation state of the inflammatory cells along the rostro-caudal axis of the spinal cord after injury. In this study, the identification of inflammatory cells was performed by Iba-1 positivity in the spinal cord tissue. Clear differences in Iba-1 expression were found between the secretome- and NB-treated animals at 6 weeks post-treatment (Fig. 2A), with the CM group revealing significant decreased Iba-1^{+} activated inflammatory cells in the spinal cord than the NB group, and significant increased Iba-1^{+} resting cells (Fig. 2A, 2E), respectively. This data suggests a cytotoxic impact of the inflammatory cells on the injured animals, which was attenuated following ASC secretome treatment, possibly partially accounting for the observed improvements of locomotion and tissue regeneration. In similarity to what we have found here, the transplantation of MSCs directly into lesion site led to increased numbers of M2 macrophages, and decreased numbers of the M1-type in a contusion model of SCI [61]. Interestingly, these changes were associated with motor function improvements, accompanied by axonal preservation, less scar tissue formation and increased myelin sparing. The benefic effects of MSCs were attributed to increased levels of IL-4 and IL-13, and reduced levels of TNF-α and IL-6, supporting an immune modulation of MSCs through paracrine actions [61]. In this line, the anti-inflammatory role of MSC secretome after SCI has been accomplished either by the secretion of anti-inflammatory molecules, or activation of pathways that modulate the inflammatory process. In fact, the regulation of microglia cytotoxicity by cytokines and growth factors is currently well described. For example, NGF, BDNF or NT-3 were shown to drive immune deactivation [62], and the upregulation of TGF-β, IL-4 or IL-10 downregulated microglial cytotoxicity [63, 64]. In the context of traumatic SCI, the suppression or depletion of microglia activation led to significant locomotor improvements and overall tissue integrity [65-67]. More recently,

the attenuation of inflammatory response after SCI was clearly shown on the above-referred Cizkova's study using a contusion rat model of SCI. This was correlated with the modulation of pro- and anti-inflammatory molecules by the secretome of BM-MSCs, namely by decreasing the levels of IL-6 and TNF-α, and increased levels of VEGF and Ciliary neurotrophic factor (CNTF) [52]. Regarding the immunomodulatory character of ASC secretome, there are numerous studies showing the ability of ASCs to secrete anti-inflammatory biomolecules. The detection of TGF-β, HGF, prostaglandin E2 (PGE2) and IL-10 on the secretome of ASCs suggested that the immunosuppressive effect of these cells might be mediated by secreted cytokines [68]. Moreover, ASCs were found to respond to inflammatory stimuli by adjusting the secretion of hematopoietic factors such as colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GMCSF), macrophage colony stimulating factor (M-CSF) and monocyte chemoattractant protein 1 (MCP-1), pro-inflammatory cytokines such as IL-6, IL-8, IL-7, IL-11 and TNF-a [69], and angiogenic factors such as VEGF, HGF and IGF-1 [70]. The secretion of these molecules was associated with decreased proliferation of peripheral blood mononuclear cells [71], and increased monocyte migration to inflammation sites [72, 73]. The presence of such factors in ASC secretome is also indicative of a neuroprotective character of this cell population. Evidences on this were reported in a study in which ASC secretome was found to protect a PC12 cell line from excitotoxicity, through the secretion of BDNF, VEGF and HGF [74]. Secreted IGF-1 and BDNF were also indicated as mediators of protection and recovery in a rat model of brain ischemia [75]. In addition, our group have recently revealed the presence of proteins involved in the regulation of inflammation, neuronal differentiation and axonal outgrowth, through a proteomic analysis on MSC secretome [28]. In this study, Decorin (DCN) was one of the proteins found to be expressed in ASC CM. This is an anti-scarring molecule that has been shown to be involved in the process of fibrosis, by direct interaction with pro-inflammatory factors [76]. For example, DCN was reported to neutralize and repress the pro-inflammatory TGF-β, thus reducing fibrotic scar [77]. In addition, DCN was found to reduce astrogliosis and to decrease the levels of scar-related elements, which further supported axonal regeneration after lesion [78]. From the proteomic analysis, plasma protease C1 inhibitor (C1-Inh) was another molecule identified in ASC CM. This molecule has been described to play an important role in the suppression of inflammation in a variety of inflammatory diseases, but the true effect in the CNS is still not known neither the mechanisms upon which it acts [79, 80]. Finally, proteins like SEM7A and clusterin (CLUS) have also been identified in ASC secretome. We have previously considered these two proteins to be supportive of DRG axonal outgrowth *in vitro*, while others have associated them with an immune role [81], which might indicate that they can be involved in different mechanisms, all ultimately contributing to tissue regeneration. The fact is that the

modulation of neuroinflammation in SCI mice after ASC secretome treatment observed in this study was accompanied by axonal elongation and regeneration in the transected spinal cord, at 6 weeks post-treatment, as indicated by βIII-tubulin and GAP-43 positivity, respectively (Fig. 2C-2G; 2D-2H). Considering the previously discussed data, it might be that axonal outgrowth and regeneration is a result of the observed modulation of the inflammatory response following injury, in which a synergistic effect of several proteins and growth factors might be responsible for protecting the spinal cord tissue from further damage at early stages post-injury (neuroprotection), therefore facilitating the outgrowth of host axons along the injury site contributing to neuroregeneration. Moreover, it is worth to note that the regeneration of an adult tissue is normally due in short periods of time after the beginning of the regenerative process [82, 83]. Therefore, the presence of GAP-43^{+} axons at the end of this experiment would not be expected. However, our data seems to suggest that the secretome of ASCs extended the regenerative process up to 6 weeks post-injury, which goes in accordance with previous indications that the exogenous supply of trophic support can stimulate GAP-43+ axons over longer periods [83, 84]. Along with axonal sprouting and regeneration, decreased lesion cavities were also observed for the secretome-treated animals, in comparison to the NB-treated animals (Fig. 2B, 2F).

Considering this promising data, we strongly believe that the anti-inflammatory cytokines and regenerating- and growth-permissive growth factors provided by the continuous administration of secretome of ASCs to the SCI mice contributed to the overall tissue repair, adding to the observed improvements in locomotor function of these animals, as well as to the regain of their hindlimbs sensitivity. To confirm our expectations on the ability of ASC secretome in providing an anti-inflammatory enriched environment after injury that may explain the attenuated inflammatory response in the secretome -treated animals observed in this study, future studies should focus on fully understand by which means was the secretome of ASCs targeting the inflammatory cell reaction following injury.

5. CONCLUSION

The use of MSC secretome as a cell-free based therapy for SCI has been shown promising in pre-clinical research in the last years.

From this work, we show that the treatment of mice after spinal cord transection with the secretome of ASCs supports the recovery of animal's motor and sensorial function. Moreover, animals continuously treated with ASC secretome showed a decreased inflammatory response after injury, accompanied by axonal sprouting and regeneration at the lesion site, and smaller lesion cavities. In conclusion, our results

suggest the therapeutic potential of ASC secretome for the treatment of SCI, encouraging further investigation towards the clinical setting for SCI patients.

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CHAPTER V

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The axonal growth-restrictive character of traumatic spinal cord injury (SCI) makes finding a therapeutic strategy a very demanding task. The post-injury events that follow the injury are impeditive to spontaneous axonal outgrowth and regeneration. In addition, the failure of clinical management of SCI in patients at the onset of the injury contributes to its progression and other clinical complications. Considering the complexity of SCI pathophysiology, it has been suggested that an effective therapy should tackle all the SCI-related aspects and provide sensory and motor improvement to SCI patients. For that, the current aim of any therapeutic approach for SCI relies in providing neuroprotection by modulating the inflammatory response and avoid the exacerbation of the injury, and support neuroregeneration by replacing damaged neural tissues and cells and stimulating axonal outgrowth and endogenous repair. Acknowledging the current SCI treatment paradigm, cell transplantation is one of the most explored approaches for SCI, aiming to provide an adequate growth-promoting and regenerative environment to the injured spinal cord. Among the innumerous available cell populations, mesenchymal stem cells (MSCs) have been in the forefront of many of these approaches. Studies showing the beneficial effects of MSC transplantation after SCI have been proposing a paracrine action of these cells on the injured tissues, through the secretion of protective and trophic factors, rather than attributing it to the action of cells itself [1-5]. Thus, the work herein developed have specifically focused on the potential neuroregenerative effect of the secretome of MSCs as a cell-free based therapy for SCI. The main challenge of any strategy proposed for SCI treatment relies in obtaining robust pre-clinical evidences from *in vitro* and *in vivo* models, before moving to the clinics. So, the regenerative potential of MSC secretome was herein explored in two *in vitro* models of regeneration and in two *in vivo* models of SCI (Fig. 1).

In vitro (chapter 2), the secretome of bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue-derived MSCs (ASCs) and Human Umbilical Cord Perivascular Cells (HUCPVCs) induced neuronal differentiation from human neural progenitor cells (hNPCs), and neurite outgrowth from dorsal root ganglion (DRGs), suggesting a mediation of their neuroregulatory actions through these two mechanisms of repair. The three MSC populations seems to share equal differentiation profiles, but acted differently on axonal outgrowth, with ASC secretome revealing higher induction of DRG neurite outgrowth. This indicated that distinct processes of regeneration were being mediated by the MSCs as a function of tissue source, going in accordance with previous reports [6-9]. Under this assumption, our group and others have been putting effort in mapping the molecules secreted by these cells under specific conditions, aiming to determine which factors are upregulated and disclose which mechanisms of repair underlie their action. A varied expression of factors on the secretome of ASCs, BMSCs and HUCPVCs that are

related to neuronal differentiation and axonal outgrowth have been in fact previously shown [3, 7, 10-13]. These evidences support our hypothesis that the neurodifferentiation and axonal growth processes mediated by the MSC secretome in this study is more likely to translate the differences in the secretome composition among the three cell populations. In the end, the absence of differences among the three MSC populations in promoting neurodifferentiation was not expected, as some of the identified proteins in their secretome, namely IL-6, PEDF, CDH2, elsewhere reported to promote neuronal differentiation [14-17], presented different levels of upregulation. In addition, the receptors necessary to respond to these factors were present in hNPCs when incubated with all MSC secretome, even though signaling molecules involved in neuronal differentiation signaling cascades upon activation of these receptors were not differentially expressed. These results may be explained by protein levels below the optimal dose to exert some effect, a quick turnover of the molecules, or even a multidirectional function of the factors identified with their action directed to axonal outgrowth over neurodifferentiation. Therefore, this study would have benefited from a clarification on this. The usage of more concentrated version of secretome, different times of cell culture, or more frequent renewal of the secretome in culture would be of interest to test. Other *in vitro* models of neuronal differentiation could also have been tackled to further understand the results obtained. Likewise, other neuronal populations, such as motor neurons, could have been used to further address axonal outgrowth. Nevertheless, from all the MSC populations tested, ASC secretome demonstrated higher potential in this study regarding axonal outgrowth, thus being selected for further studies in the scope of this work.

Moving forward to an *in vivo* context, ASC secretome have demonstrated positive results in two SCI models – non-mammalian regenerating *Xenopus Laevis* and mammalian non-regenerating mice (Fig. 1).
In the former model (chapter 3), the treatment of *Xenopus laevis* tadpoles with ASC secretome after spinal cord complete transection favored axonal sprouting and regeneration and improved functional recovery after injury. At first, the use of a naturally regenerating model in the scope of this work seems irrelevant, since we were proposing to study a condition that results from the inability of the spinal cord to regenerate and recover after injury in mammals. In fact, *Xenopus laevis* was the only non-mammalian regenerating model that could be used to test the effects of therapeutic strategy for SCI, as it exclusively presents shifts from a regenerative to non-regenerative context throughout life, providing a system where loss and gain of function experiments can be directed at understanding what exactly inhibit or stimulate regeneration. Therefore, the regenerative potential of ASC secretome was herein evaluated in *Xenopus Laevis* tadpoles, in both refractory (non-regenerative) and regenerative stages, after complete transection of the spinal

cord. In the refractory period, a single injection of ASC secretome was sufficient to restore the regenerative ability of the tadpoles, indicated by the recovery of locomotor function of the treated animals. The notable recovery could be attributed to significant increased levels of regenerating cells and extensive axonal sprouting at the lesion site. Refractory tadpoles were reported to present no signs of tissue repair after tail amputation [18], therefore indicating that the recovery of the transected animals in this study is due to the effect of ASC secretome. Regarding the regenerating period, it is more likely that the treatment of ASC secretome was able to anticipate the regeneration of the injured spinal cord, as locomotor function recovery occurred earlier than for non-treated animals, accompanied by increased regeneration and axonal sprouting. The mechanisms upon which the secretome of ASCs may be acting was not elucidated, but studies directed to explore this question would be of great importance. Nevertheless, these promising data support the potential of ASC secretome as a cell-free based strategy for SCI treatment. Moreover, the use of *Xenopus laevis* is strongly encouraged as a model for SCI, opening the way to be used as novel, simple and low-cost tool for SCI research field, which provides a clear regeneration environment to test the therapeutic effect of new strategies.

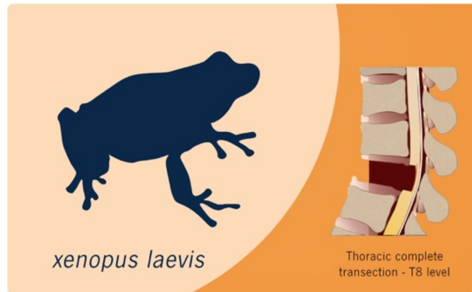
Following the positive outcomes obtained from the *Xenopus laevis* SCI model, we hypothesized whether ASC secretome would have a therapeutic effect in a larger animal, such as mice, using the same type of lesion (chapter 4; Fig. 1). The application of ASC secretome to lesioned animals revealed impressive in promoting motor and sensorial recovery of the treated animals, accompanied by significant axonal elongation and regeneration in the lesion site, suggesting a neuroprotective and neuroregenerative effect of the secretome. In addition, an attenuated inflammatory response after injury was observed, which is indicative of the anti-inflammatory action of the secretome of these cells. The complete transection of the spinal cord is not the most relevant in the clinics, as contusion or compression injury models are, since it does not reflect the SCI pathophysiology in humans [19, 20]. Besides, it is a very aggressive model of injury which could have been impeditive of observing any type of therapeutic effect of the strategy herein proposed. Nonetheless, transection is advantageous in providing a clean injury environment for studying processes of regeneration after lesion, therefore having shown valuable to assess our hypothesis on whether ASC secretome could promote axonal elongation and regeneration throughout the lesion site after SCI. Still, the therapeutic effect of ASC secretome in other lesion models such as contusion could have been addressed. Although mice share many similarities with human SCI pathophysiology, there are slightly differences mostly related to the absence of cystic cavity formation and to the inflammatory response following the injury [21, 22]. Thus, going up to larger animals with this strategy, namely dogs,

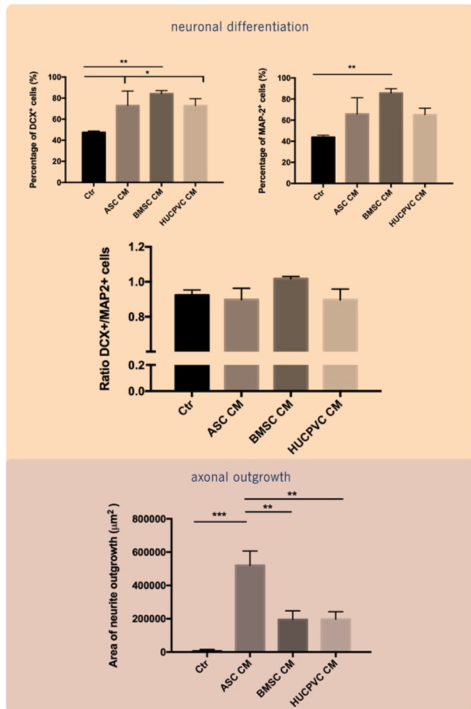
pigs and primates, would be of great interest to take us closer to the human SCI pathology. Another important aspect of this work is the route and frequency used to administer the secretome of ASCs. The secretome has been intravenously delivered to the animals through their tail vein (IV route). A concentrated formulation of ASC secretome has been injected once a day in the first three 24 hours post-injury and then weekly for 6 weeks, under the rationale that a sufficient supply of the molecules composing the secretome should be available during both acute and chronic phases of injury to increase the chances of having a measurable therapeutic effect. The continuous supply of ASC secretome to transected animals might explain the behavioral and histological outcomes of this study, and may have accounted for the prolonged regenerative effect observed 6-weeks post-injury, which is normally due in shorter periods of time [23-25], as well as to the marked modulation of microglia and macrophages recruitment to the injury site. These evidences are promising to envisage a SCI therapeutic strategy based on the use of ASC secretome. Although the IV route has been successfully used for the administration MSCs into rats and dogs after SCI [26, 27], this kind of administration poses some constraints to the animals, that have to be injected several times in short periods of time, which may add to some pain and stress associated to the injections. Also, it requires a very precise technique and practice of the animal's handler not only for the sake of the animals' well-being but also to avoid other issues such as the loss of secretome associated with less precise injections. The use of other routes of administration should therefore be considered in the future, namely through intrathecal catheters, intraspinally or subcutaneously, as successfully applied by others for the administration of either MSCs or MSC secretome [28-34].

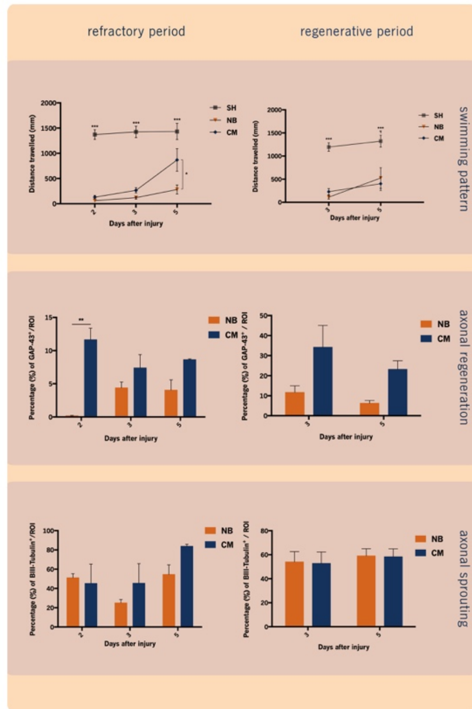
Altogether, the work of this thesis demonstrates that the secretome of ASCs is able to promote the desired effects in stimulating the repair and regeneration of the spinal cord after lesion, highlighting the benefits of using the secretome of MSCs in alternative to cell transplantation. In fact, the transplantation of cells into the injury site would have some advantages, such as the possibility to provide a continuous secretion of biomolecules, adjusted to the needs of the lesion microenvironment, especially given that these cells have the capacity to respond to the stimuli of the surroundings, migrate into the injured tissues and adjust their activity accordingly [35-37]. However, cell transplantation approaches are very time and money consuming, and it requires high number of cells [38]. Plus, the survival rate of the transplanted cells is too low [39, 40]. The use of the secretome not only avoids these limitations, but can still be provided in a continuous way, depending on the route and frequency of administration used, as above discussed. Overall, the findings of this work are indicative of the positive effects exerted by the secretome of ASCs on axonal outgrowth and regeneration, and neuroinflammation, observed for the three models herein

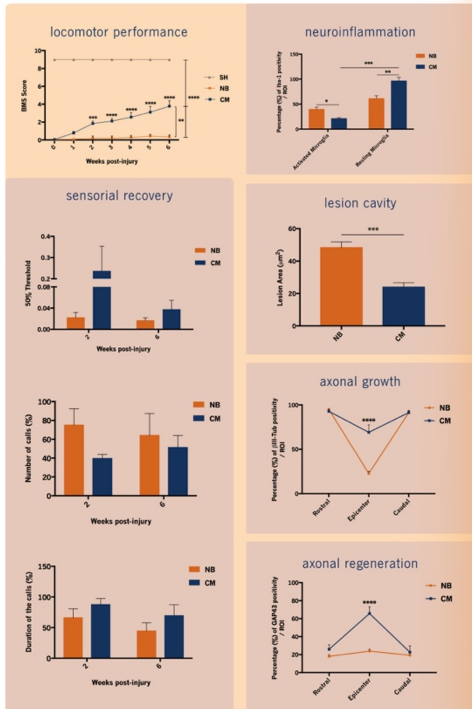
studied, and that were associated to locomotor recovery to both *Xenopus laevis* and mice. It should be emphasized that the impact of ASC secretome in neuroinflamation is of extreme importance, as the inflammatory response after injury is one of the main processes that contributes to the exacerbation of the lesion. Likewise, axonal outgrowth/regeneration is also very important to restore the normal SC function [41]. The amelioration of the inflammation and support of axonal growth has been previously correlated with significant improvements of locomotor function after SCI [4, 5, 29, 30, 42-44], which could support the remarkable recovery observed in this study. Finally, our *in vitro* data gave us insights on some possible molecules related to neurite outgrowth stimulation. In line with this, we could assume that these molecules could also be responsible for the effects observed in both *Xenopus Laevis* and mice model. To validate this, future studies should focus on a detailed screening of the cytokines and factors that may be responsible for the observed effect, in order to elucidate on the mechanisms that are targeted by the molecules composing it and disclose the downstream processes being modulated.

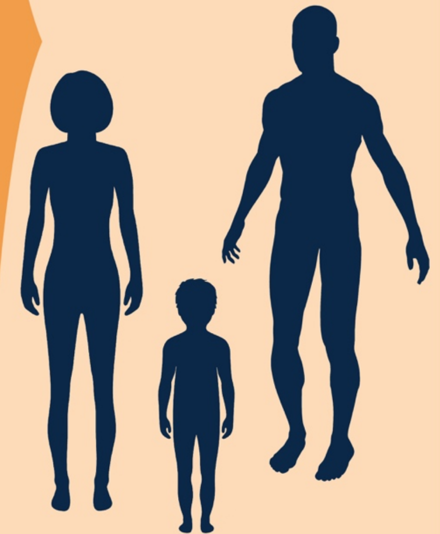
When it comes to research, there is always room for further testing with the aspiration of increasing the value of our work and validate our findings. However, the work herein presented can add valuable insights to the SCI regenerative field, involving a novel model to study therapeutic approaches for this condition, and a therapeutic strategy that is able to restore the spinal cord function after an extremely traumatic injury based on a cell-free based approach.











- ASCs, BMSCs and HUCPVCs secretome stimulated similar levels of neuronal differentiation.
- ASC secretome induced higher levels of DRG axonal outgrowth, in comparison to BMSCs and HUCPVC secretome.
- The presence of PEDF, SEM7A, CDH2, IL-6, GDN, beta4Gal-T1 and CLUS on ASCs, BMSCs and HUCPVCs secretome might be responsible for the observed outcomes.

- ASC secretome improves functional recovery of completely transected Xenopus laevis tadpoles.
- ASC secretome favors axonal sprouting and regeneration in Xenopus laevis tadpoles after SCI.
- ASC secretome restored regenerative capacity of refractory period tadpoles, and anticipated spinal cord regeneration in regenerative tadpoles.

- ASC secretome support motor and sensorial recovery of mice after spinal cord complete transection.
- ASC secretome attenuated neuroinflammation, reduced lesion cavity, and promoted axonal regeneration and sprouting after SCI.

Figure 1. The regenerative potential of MSC secretome for SCI treatment. Schematic representation of the *in vitro* models of neuronal differentiation and axonal outgrowth, and *in vivo* models of SCI used to test MSC secretome, particularly from the adipose tissue (ASCs), as a cell-free based approach. In this work, the positive effects of ASC secretome were related to the attenuation of neuroinflammation and axonal outgrowth and regeneration, for the three models used. These effects were associated to locomotor recovery of both *Xenopus laevis* and mice. These findings suggest ASC secretome as therapeutic strategy that could restore spinal cord function after traumatic SCI.

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