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Study of combinatorial cell therapy and neuroprotective agents for the treatment of spinal cord injury in experimental models

PhD. THESIS

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"STUDY OF COMBINATORIAL CELL THERAPY AND NEUROPROTECTIVE AGENTS FOR THE TREATMENT OF SPINAL CORD INJURY IN EXPERIMENTAL MODELS"

has been developed by Pablo Bonilla Villamil under their supervision in the Centro de Investigación Príncipe Felipe, as a Thesis Project to obtain a PhD degree in Biotechnology from the Universitat Politècnica de València.

Valencia, December 2022

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“Quise ser valiente y aprendí a estudiar”

“I wanted to be brave and I learned to study”

-Antonio Escohotado

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RESUMEN

La terapia celular se ha convertido en uno de los enfoques más prometedores en el ámbito de la medicina regenerativa, demostrando su eficacia y seguridad en ensayos clínicos realizados en sujetos con lesión medular. Entre las terapias empleadas, las células madre neurales (NSCs/NPCs) son especialmente prometedoras pues tienen la capacidad de diferenciarse a cualquiera de los linajes neurales. Las estrategias traslacionales actuales incluyen NSCs derivadas de células madre embrionarias (ESCs), de células madre pluripotentes inducidas (iPSCs) o de líneas inmortalizadas de NPCs fetales, en todos los casos, sometidas a una manipulación significativa, lo que aumenta el potencial tumorogénico de dichas terapias. Asimismo, a pesar de los beneficios descritos en modelos preclínicos y en ensayos clínicos, la aplicación única del trasplante celular ha mostrado una limitada recuperación funcional. En esta tesis doctoral, se han estudiado distintas estrategias de combinación con la terapia celular, como el uso de fármacos y biomateriales, con el objetivo de mejorar y potenciar el efecto de la terapia celular en el rescate de la actividad neuronal, en la modulación del proceso inflamatorio, en la supervivencia del trasplante, así como en su integración y diferenciación en el tejido hospedador.

En el capítulo 1 evaluamos una terapia combinada de células madre neurales humanas derivadas de células madre pluripotentes inducidas (iPSC-NSCs) MSCs y un nanoconjugado de curcumina (PA-C). Nuestro estudio *in vivo* demostró que el trasplante de iPSC-NSCs + MSC + PA-C en un modelo subagudo de lesion medular incrementa la preservación de fibras neurales y reduce el tamaño de la cicatriz fibrótica. Adicionalmente, el tratamiento local con PA-C estimula la polarizacion de la microglía activada tras la lesión a un perfil M2 antiinflamatorio, asociado con un efecto pro-regenerador. Así, el tratamiento combinado con PA-C confiere mayor neuroprotección en comparación con los tratamientos individuales. Sin embargo, no encontramos mejoraras funcionales significativas en ninguna de las combinaciones estudiadas.

En el capítulo 2 desarrollamos una estrategia novedosa para el transplante de iPSC-NSCs combinado con PA-C mediante el uso de un andamio de ácido hialurónico en forma de semi-luna, que permite realizar el trasplante celular y el tratamiento farmacológico de forma mínimamente invasiva. Además, la estructura biohibrida generada con el material de ácido

hialurónico, las iPSC-NSC y la PA-C puede incluir fibras recubiertas de polipirrol (PPY) para el guiado axonal. El hidrogel autoensemblable, denonimado comercialmente Puramatrix (PM), se emplea para embeber las iPSC-NSCs y la PA-C en el andamio para su posterior liberación e incorporación en el tejido medular. Una semana después de inducir lesión medular se realizaron los implantes y una semana después del implante se analizaron los tejidos. Cabe destacar que el implante *per sé* ha demostrado no causar un daño adicional en la médula espinal durante la implantación. Además, los análisis histológicos demostraron que el tratamiento combinado de iPSC-NSCs y la PA-C presentaban una mayor preservación de fibras neuronales en la zona de la lesión y una reducción de la extensión de la cicatriz fibrosa.

Por último, en el capítulo 3 se describe una estrategia terapéutica que se aproxima más a la traslación clínica mediante el trasplante de progenitores neurales fetales humanos (hfNPCs). Estos precursores son obtenidos de medulas espinales de muestra fetal de estadios tardíos en la gestación, obtenidos tras interrupciones legalmente inducidas entre la semana de gestación 19,0-21,6. Se describe además un nuevo procedimiento de condicionamiento del transplante por tratamiento previo con la forma conjugada del inhibidor de Rho/Rock fasudil (PGA-SS-FAS). En un modelo de lesión medular aguda, en ratones inmunodeficientes (NU(NCr)-Foxn1nu), el trasnplante de hfNPCs condicionadas previamente con PGA-SS-FAS, favorece una mayor distribución del transplante de dorsal a vental, en la zona de la lesión, aumenta la preservación de las interneuronas somatosensoriales Lbx1 inhibitorias y las Tlx3 excitatorias, e incrementa la activación neuronal alrededor del epicentro de la lesión en comparación con el grupo control no transplantado.

Así pues, en la presente tesis doctoral se estudia y describe el beneficio aportado por hasta tres estrategias combinatorias de terapia celular, incrementando los efectos neuroprotectores sobre el transplante celular individualizado en el tratamiento de lesiones medulares agudas y sub-agudas.

RESUM

La teràpia cel·lular s'ha convertit en un dels enfocaments més prometedors en l'àmbit de la medicina regenerativa, i ha demostrat la seva eficàcia i seguretat en assaigs clínics realitzats en subjectes amb lesió medul·lar. Entre les teràpies emprades, les cèl·lules mare neurals (NSCs/NPCs) són especialment prometedores ja que tenen la capacitat de diferenciar-se a qualsevol dels llinatges neurals. Les estratègies translacionals actuals inclouen NSCs derivades de cèl·lules mare embrionàries (ESCs), de cèl·lules mare pluripotents induïdes (iPSCs) o de línies immortalitzades de NPCs fetals, en tots els casos, sotmeses a una manipulació significativa, cosa que augmenta el potencial tumorogènic d'aquestes teràpies. Així mateix, malgrat els beneficis descrits en models preclínics i en assaigs clínics, l'aplicació única del trasplantament cel·lular ha mostrat una recuperació funcional limitada. En aquesta tesi doctoral, s'han estudiat diferents estratègies de combinació amb la teràpia cel·lular, com l'ús de fàrmacs i biomaterials, amb l'objectiu de millorar i potenciar l'efecte de la teràpia cel·lular en el rescat de l'activitat neuronal, en la modulació del procés inflamatori, en la supervivència del trasplantament, així com en la seva integració i diferenciació al teixit hostatjador.

Al capítol 1 avaluem una teràpia combinada de cèl·lules mare neurals humanes derivades de cèl·lules mare pluripotents induïdes (iPSC-NSCs) MSCs i un nanoconjugat de curcumina (PA-C). El nostre estudi in vivo va demostrar que el trasplantament d'iPSC-NSCs+MSC+PA-C en un model subagut de lesió medul·lar incrementa la preservació de fibres neurals i redueix la mida de la cicatriu fibròtica. Addicionalment, el tractament local amb PA-C estimula la polarització de la microglia activada després de la lesió a un perfil M2 antiinflamatori, associat amb un efecte pro-regenerador. Així, el tractament combinat amb PA-C confereix més neuroprotecció en comparació dels tractaments individuals. No obstant això, no trobem millores funcionals significatives en cap de les combinacions estudiades.

Al capítol 2 desenvolupem una estratègia nova per al trasplantament d'iPSC-NSCs combinat amb PA-C mitjançant l'ús d'una bastida d'àcid hialurònic en forma de semilluna, que permet realitzar el trasplantament cel·lular i el tractament farmacològic de manera mínimament invasiva. A més, l'estructura biohibrida generada amb el material d'àcid hialurònic, les iPSC-NSC i la PA-C poden incloure fibres recobertes de polipirrol (PPY) per al

guiatge axonal. L'hidrogel autoassemblable, denonimat comercialment Puramatrix (PM), s'empra per embeber les iPSC-NSCs i la PA-C a la bastida per al seu posterior alliberament i incorporació al teixit medul·lar. Una setmana després d'induir lesió medul·lar es van fer els implants i una setmana després de l'implant es van analitzar els teixits. Cal destacar que l'implant per sé ha demostrat no causar un dany addicional a la medul·la espinal durant la implantació. A més, les anàlisis histològiques van demostrar que el tractament combinat d'iPSC-NSCs i la PA-C presentaven més preservació de fibres neuronals a la zona de la lesió i una reducció de l'extensió de la cicatriu fibrosa.

Finalment, al capítol 3 es descriu una estratègia terapèutica que s'aproxima més a la translació clínica mitjançant el trasplantament de progenitors neurals fetals humans (hfNPCs). Aquests precursors són obtinguts de medul·les espinals de mostra fetal d'estadis tardans a la gestació, obtinguts després d'interrupcions legalment induïdes entre la setmana de gestació 19,0-21,6. A més, es descriu un nou procediment de condicionament del trasplantament per tractament previ amb la forma conjugada de l'inhibidor de Rho/Rock fasudil (PGA-SS-FAS). En un model de lesió medul·lar aguda, en ratolins immunodeficients (NU(NCr)-Foxn1nu), el trasplantament de hfNPCs condicionades prèviament amb PGA-SS-FAS, afavoreix una major distribució del trasplantament de dorsal a ventral, a la zona de la lesió , augmenta la preservació de les interneurones somatosensorials Lbx1 inhibitòries i les Tlx3 excitatòries, i incrementa l'activació neuronal al voltant de l'epicentre de la lesió en comparació del grup control no transplantat.

Així doncs, en aquesta tesi doctoral s'estudia i descriu el benefici aportat per fins a tres estratègies combinatòries de teràpia cel·lular, incrementant els efectes neuroprotectors sobre el transplantament cel·lular individualitzat en el tractament de lesions medul·lars agudes i sub-agudes.

SUMMARY

Cell therapy has become one of the most promising approaches in the area of regenerative medicine, demonstrating its efficacy and safety in clinical trials in subjects with spinal cord injury (SCI). Among the therapies employed, neural stem cells (NSCs/NPCs) are especially promising as they have the ability to differentiate into any of the neural lineages. Current translational strategies include NSCs derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or immortalized lines of fetal NPCs, in all cases, subjected to significant manipulation, which increases the tumorigenic potential of such therapies. Furthermore, despite the benefits described in preclinical models and clinical trials, the single application of cell transplantation has shown limited functional recovery. In this doctoral thesis, we have studied different combination strategies with NSCs/NPCs transplantation, such as the use of drugs and biomaterials, with the aim of improving and enhancing the effect of cell therapy in the rescue of neuronal activity, in the modulation of the inflammatory process, in the survival of the transplant, as well as in its integration and differentiation in the host tissue.

In Chapter 1 we evaluated a combination therapy of induced pluripotent stem cell-derived human neural stem cells (iPSC-NSCs), mesenchymal stem cells (MSCs) and a curcumin nanoconjugate (PA-C). Our in vivo study demonstrated that transplantation of iPSC-NSCs + MSCs + PA-C in a subacute model of spinal cord injury increases neural fiber preservation and reduces fibrotic scar size. Additionally, local treatment with PA-C stimulates the polarization of activated microglia after injury to an anti-inflammatory M2 profile, associated with a pro-regenerating effect. Thus, combined treatment with PA-C confers greater neuroprotection compared to individual treatments. However, we did not find significant functional improvements in any of the combinations studied.

In Chapter 2, we developed a novel strategy for iPSC-NSCs transplantation combined with PA-C by using a demilune hyaluronic acid scaffold, developing a minimally invasive transplantation strategy. In addition, the biohybrid structure generated with the hyaluronic acid material can include polypyrrole (PPY)-coated fibers for axonal guidance. The commercially denatured Puramatrix (PM) hydrogel is used to embed iPSC-NSCs and PA-C within the scaffold for subsequent release and incorporation into the spinal tissue. One week

after inducing SCI the implants were performed and one week after implantation the tissues were analyzed. Notably, the implant *per se* was shown to cause no additional damage to the spinal cord during implantation. Furthermore, histological analyses demonstrated that the combined treatment of iPSC-NSCs and PA-C showed a greater preservation of neuronal fibers in the lesion area and a reduction in the extent of fibrous scarring.

Finally, Chapter 3 describes a therapeutic strategy that is closer to clinical translation by transplanting human fetal neural progenitors (hfNPCs). These progenitors are obtained from fetal sample spinal cords of late gestational stages, obtained after legally induced terminations between 19.0-21.6 weeks of gestation. A new procedure of transplantation conditioning by pretreatment with the conjugated form of Rho/Rock fasudil inhibitor (PGA-SS-FAS) is also described. In a model of acute spinal cord injury in immunodeficient mice (NU(NCr)-Foxn1nu), transplantation of hfNPCs preconditioned with PGA-SS-FAS favors a greater dorsal-to-ventral transplant distribution in the area of injury, increases the preservation of inhibitory Lbx1 and excitatory Tlx3 somatosensory interneurons, and increases neuronal activation around the lesion epicenter compared to the non-transplanted control group.

Thus, in the present doctoral thesis we study and describe the benefit provided by up to three combinatorial strategies of cell therapy, increasing the neuroprotective effects on individualized cell transplantation in the treatment of acute and sub-acute SCI.

ACRONYMS

AIS: ASIA Impairment Scale

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASIA: American Spinal Injury Association

BDNF: Brain-derived growth factor

bFGF: Basic fibroblast growth factor

CCL: Chemokine (C-C motif) ligand

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

CSPGs: Chondroitin sulfate proteoglycans

CXCL: C-X-C motif chemokine ligand

E14: Embryonic day 14

ECM: Extracellular matrix

EES: Epidural electrical stimulation

EGF: Epidermal growth factor

ESCs: Embryonic stem cells

FSC: Fetal spinal cord

GBD: Global Burden of Diseases, Injuries, and Risk Factors

G-CSF: Granulocyte colony-stimulating factor

GDNF: Glial-derived growth factor

HA: Hyaluronic acid

hfNPCs: Human fetal neural progenitor cells

HGF: Hepatocyte growth factor

IGF: Insulin-like growth factor

IL: Interleukin

iPSC-NSCs: induced NSCs

iPSCs: Induced pluripotent stem cells

ISNCSCI: Neurological Classification of Spinal Cord Injury

Klf4: Krüppel-like factor

MAG: myelin-associated glycoprotein

MSCs: Mesenchymal stem cells

NGF: nerve growth factor

NMDA: ionotropic N-methyl-D-aspartate

Nogo: Neurite outgrowth inhibitor

NPCs: Neural progenitor cells

NSCs: Neural stem cells

NT: Neurotrophin

Oct3/4: octamer-binding transcription factor 3/4

OMgp: Oligodendrocyte-myelin glycoprotein

OPCs: Oligodendrocyte progenitor cells

PCL: Poly-ε-caprolactone

PDGF: Platelet-derived growth factor

PEG: Polyethylene glycol

PLGA: Poly-D,L-lactic-coglycolic acid

PLGA: Poly-D,L-lactic-co-glycolic acid

PLLA: Poly-L-lactic acid

PLLA: Poly-L-lactic acid

PPY: Polypyrrole

RGMa: Repulsive guidance Molecule A

RNS: Reactive nitrogen species

ROCK: Rho-associated protein kinase

ROM: Full range of movement

ROS: Reactive oxygen species

SCI: Spinal cord injury

SCs: Schwann Cells

Sox2: SRY-box 2

TGF: Transforming growth factor

TNF-α: Tumor necrosis factor α

VEGF: Vascular endothelial growth factor

WHO: World Health Organization

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INTRODUCTION

1. INTRODUCTION

1.1. Spinal cord injury

Traumatic spinal cord injury (SCI) is a disabling neurological condition that causes deficits in motor, sensory and autonomic neuronal functions and is characterized by a wide a range of symptoms including paralysis, paresthesia, spasticity, pain, and cardiovascular, bowel, bladder, or sexual dysfunction (Hayta and Elden 2018). The grade of disability depends on the severity and the level of the lesion and may include partial or complete loss of sensory and/or motor functions below the injury level. SCI most often affects the cervical levels (50% of the lesions) causing tetraplegia and affecting the respiratory function. Other injuries include the thoracic level (35%) and lumbar region (11%) although the distribution varies according to the geographic location of the studies. (Hachem, Ahuja, and Fehlings 2017). It is important to note that the life expectancy of SCI patients is highly dependent on the level of injury and, therefore, the affected functions in patients, with neurogenic shock being one of the leading causes of death after traumatic SCI (Majdan et al. 2017). The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) developed by the American Spinal Injury Association (ASIA) and categorizes the severity of the injury on the ASIA Impairment Scale (AIS) (Quadri et al. 2020). AIS, is used as an international gold standard for evaluation of SCI injuries and standardizes injury assessment and treatment by motor examination, sensory examination and anorectal examination (Roberts, Leonard, and Cepela 2017) classifying patients into different grades of severity (Table 1).

Grade	Degree of spinal cord dysfunction
A	Complete lesion ● No motor or sensory function is preserved in the sacral segments S4–S5.
B	Incomplete lesion ● Sensory function preserved but not motor function is preserved below the neurological level and includes the sacral segments S4–S5.
C	Incomplete lesion ● Motor function is preserved below the neurological level. ● Less than 50% loss of key muscles below the neurological level cannot make full range of movement (ROM) against gravity.
D	Incomplete lesion ● Motor function is preserved below the neurological level. ● At leats a 50% of key muscles below the neurological level can make full

	ROM against gravity.
E	Normal ● Motor and sensory function are normal.

Table 1. The American Spinal Injury Association (ASIA) Impairment Scale adapted from (Roberts, Leonard, and Cepela 2017)

A recent study carried out by the Global Burden of Diseases, Injuries, and Risk Factors (GBD) provided a rigorous and comprehensive estimation in 2016 being the annual global incidence of SCI 0,93 million and prevalence 27,04 millions, occurring 2,6 million cases in the United States. Furthermore, these results also showed that there was no significant increase in the prevalence or incidence rates between 1990 and 2016 (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators 2019). In detail within the prevalent population, more severe injuries were described in young individuals being the average age estimated to be 42,6 years of age with males accounting for 80,7% of new cases (Merritt et al. 2019).

Since SCI mainly affects young people and functional impairments persist throughout the patient's life, it is a major cause of physical disability and carries an enormous economic burden on society (Thuret, Moon, and Gage 2006). A recent update estimated the average cost of SCI patients in the United Kingdom at £1.12 million, being higher for women (£1.15 million) versus men (£1.11 million) (McDaid et al. 2019). Another recent review regarding the economic impact of SCI in the United States, estimated the average cost of the initial injury and recovery phase around $142,366, but may increase depending on whether the patient is rehospitalized or long-term complications. In the case of military veterans the average cost was $606,349 within the first year and $92,454 annually for long-term care (Furlan, Gulasingam, and Craven 2017). Furthermore, only 35% of patients return to the labor market (Ottomanelli and Lind 2009), and about 25% declare bankruptcy after 5 years (Relyea-Chew et al. 2009).

1.1.1. Pathophysiology of spinal cord injury

It is well established that the pathophysiology of traumatic SCI occurs in two distinct stages, the initial mechanical impact followed by a secondary phase, characterized by self-propagating biochemical changes that occur after the initial insult. The primary mechanical injury may be caused by a variety of mechanisms including compression, contusion,

transection and shearing (Alizadeh, Dyck, and Karimi-Abdolrezaee 2019). Distinctive features of the primary lesion are local hemorrhage, edema and ischemia that progress and trigger the secondary phase. Disruption of vascular structures at the site of the insult leads to localized hemorrhages and edemas, increasing local pressures within the spinal cord tissue, and enhancing ischemia so that the spinal cord tissue is deprived of oxygen and nutrients. In addition, it increases the permeability of the blood-spinal barrier allowing the passage of vasogenic and cytotoxic factors which contribute to the injury edema (Popa et al. 2010). On the other hand, primary insult directly destroys spinal cord tissue, disrupting descending and ascending spinal pathways causing paralysis and temporary loss of neuronal functions (Dumont et al. 2001). Since pharmacological approaches are not available to treat the primary damage, procedures performed at the time of injury, in conjunction with post-injury body stabilization, are capable of diminishing the severity of potential primary and secondary damage. In theoretical terms, gray matter is irreversibly damaged within the first hour after injury, but white matter can survive injury for up to 72 hours after injury (Mortazavi et al. 2015), offering, thus, a time window for preventive treatment.

The secondary phase begins within minutes following the initial primary injury and continues during weeks or months. It involves a series of propagating cellular, molecular and biochemical phenomena causing progressive damage in the surrounding healthy tissue (Oyinbo 2011) and hindering neurological recovery (Fehlings, Vaccaro, and Boakye 2012). This cascade of molecular, cellular and biochemical phenomena can be divided into three stages: acute, subacute and chronic phases, although some events overlap and continue into the next phase.

1.1.1.1. Acute phase

The acute phase comprise the first 48 hours after SCI and is a direct result of the primary insult that cause local events including spinal shock, vascular dysfunction and edema, membrane compromise and ionic imbalance, calcium influx, neurotransmitter accumulation (excitotoxicity), free radical formation, lipid peroxidation, and necrotic and apoptotic cell death (von Leden et al. 2017; Fehlings, Vaccaro, and Boakye 2012; Oyinbo 2011). Spinal shock is a temporary physiological disturbance of function and reflexes as a result of the initial injury and is characterized by events such as sensory deficit, flaccid paralysis, absence of reflexes

and thermoregulatory dysfunction below the level of the lesion (Quadri et al. 2020). Together with systemic hypotension and local hemorrhage, microcirculatory loss significantly reduces blood flow at the lesion site leading to tissue ischemia that can become progressively worse over the first few hours of insult (Popa et al. 2010). Furthermore, the formation of highly reactive oxygen species (ROS) and reactive nitrogen species (RNS) released during the period of ischemia and early reperfusion causes additional endothelial damage, vascular permeability and edema that are intimately related to secondary spinal cord injury (Oyinbo 2011). Traumatic insult disrupts cell plasma membrane integrity leading to the hyperpermeability for the ions being Ca^{2+} an especially important element in secondary injury progression. The rapid influx and excessive intracellular accumulation of calcium, which is an essential secondary messenger, lead to mitochondrial dysfunction, cytoskeleton destruction, free radical production, axonal degeneration, glutamate discharge, and eventually, activation of apoptotic or necrotic pathways. Moreover, sodium and potassium accumulation cause acidosis, and potentiate calcium influx through Na/Ca exchangers (Borgens, Ben Borgens, and Liu-Snyder 2012). Additionally, extracellular levels of glutamate accumulate to neurotoxic levels around the injury site due to excessive release and impaired uptake increasing calcium influx which in turn feeds back into glutamate leakage (Young 1992). The increase in intracellular Ca^{2+} is also caused by the release of intracellular Ca^{2+} that is stored within the mitochondria, endoplasmic reticulum (Stirling et al. 2014) activating glutamate ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Faden et al. 1989). These receptors are present especially on oligodendrocytes and astrocytes, making them particularly vulnerable to glutamate excitotoxicity. In addition, ionic homeostasis disequilibrium caused by glutamate excitotoxicity impairs the proper functioning of mitochondria, leading to the production of free radicals and subsequent lipid peroxidation. Finally, the cascade of events leads to axonal demyelination leaving axons directly exposed to free radicals and inflammatory cytokines, which in turn leads to loss of neurons around the site of injury and results in significant motor and sensory deficits.

On another note, the role of the immune system and inflammatory reaction following SCI remains controversial, although there is much evidence suggesting a neuroprotective role (Xiangyu Liu et al. 2021). Upon initial mechanical injury a highly coordinated immune response

that contributes to the progression of secondary injury is triggered. Microglia, the resident immune cells of the CNS, in their physiologic state have low immune activity and cytokine production and play a supervisory role (Milich, Ryan, and Lee 2019). However, after SCI microglia respond first to injury signals as well as paracrine signaling from astrocytes and neurons (Hanisch 2002) and within minutes take a proinflammatory state which clear cell and myelin debris and recruit immune cells by releasing cytokines (interleukin (IL)-1, tumor necrosis factor α (TNF-α), and IL-6) and chemokines (including the C-X-C motif chemokine ligand (CXCL) 2, CXCL10, Chemokine (C-C motif) ligand (CCL) 2, and CCL3) (Pineau and Lacroix 2007). Neutrophils are the first blood-derived cells to infiltrate and migrate to the injury site within the first 3–6 hours, get activated and reach a peak of activity within 24 hours after SCI (Quadri et al. 2020). Neutrophils have been described to show both detrimental and beneficial effects after SCI (Zivkovic et al. 2021). Activated neutrophils have a prolonged lifespan and produce and secrete ROS, proteases and chemokines contributing to secondary damage and enhancing a progressive inflammatory response. Nevertheless, there are positive aspects of neutrophil recruitment as they initiate debris clearance and secrete proinflammatory signals and recruit other immune cells that eliminate leftover debris and contribute to tissue healing (B. Zhang and Gensel 2014).

1.1.1.2. Subacute phase

If after 24 hours the situation is not resolved, the subacute phase begins lasting up to 2 weeks after SCI. During this phase, the area of trauma increases and presents a continuation of the processes that occurred in the acute phase with the addition of other mechanisms. As a consequence of increased intracellular calcium levels and mitochondrial dysfunction, ROS and RNS are formed causing lipid peroxidation. Lipid peroxidation constitutes a self-propagating process that can spread to nearby healthy cells damaging cellular structures such as plasma membrane, cytoskeleton and organelles, leading to neuronal loss (Hall et al. 2016). Furthermore, excitotoxicity caused by the release and excessive accumulation of glutamate triggers the death of oligodendrocytes at the injury epicenter within hours and continue to undergo apoptosis in rostral and caudal white matter for many weeks after SCI (Grossman, Rosenberg, and Wrathall 2001). Demyelination leaves axons directly exposed to free radicals and inflammatory cytokines, causing increased neuronal death and inducing a well-regulated

and self-destructive process of axonal degeneration in the injured axons, known as Wallerian degeneration (K. Zhang, Jiang, and Fang 2021). Wallerian degeneration is characterized by fragmentation and disintegration of the injured axons from the distal parts, close to the lesion, to the proximal axon or neuronal soma, followed by removal of the debris by glia and immune cells (K. Zhang, Jiang, and Fang 2021). Thus, neuronal degeneration continues, resulting in further loss of neurological function during the subacute stage.

Under physiological conditions astrocytes surround neurons in the entire central nervous system (CNS) constituting specialized supporting cells, which exert multiple crucial, complex functions in the CNS and are five times more in number than neurons (Sofroniew and Vinters 2010). Nevertheless, astrocytes proliferate within the perilesional zone approximately 2 days after SCI (and continuing until around 7-10 days) (Wanner et al. 2013), intertwine and organize around the edges of damaged and unsalvageable tissue to form the fibrotic scar and surrounding astroglial scar border. Astrocytes, thus, restrict inflammation and separate the core of the non-neuronal lesion from adjacent functioning neuronal tissue (O'Shea, Burda, and Sofroniew 2017). The contribution of astrocytes has been widely debated as scar-forming astrocytes have been historically thought to be the primary cause of axonal regeneration failure (Silver, Edwards, and Levitt 1993). However, recent studies show that astrocytic scars may play a neuroprotective role after SCI securing the borders of lesions by limiting the spread of inflammatory cells throughout the spared tissue (Sofroniew 2015) and aid axonal regeneration (Anderson et al. 2016). Despite the beneficial effects associated with glial scar formation, some components of the fibrotic scar limit and compromise axonal regeneration as will be explained in detail later.

In addition, 2-3 days after neutrophil invasion, microglia and monocyte-derived macrophages are recruited and migrate to the site of injury where they can reside for several months playing a critical role in the degeneration and regeneration after SCI. Depending on the microenvironment signals, their phenotype and activation can be polarized to either pro-inflammatory (M1) or anti-inflammatory pro-regenerative (M2) phenotype and accordingly contribute to injury or repair processes after SCI. The SCI environment drives M1 polarization due to increased levels of proinflammatory cytokines and TNF-α (Anderson et al. 2016). M1 polarized microglia/macrophages show a reduced phagocytosis, a critical process for tissue

repair and also secretes cytokines and chemokines activating and regulating the adaptive immune response and contributing to further progression of secondary injury (S. David, Greenhalgh, and Kroner 2015). On the other hand, M2 microglia/macrophages are polarized by IL-4 and IL-3 and show an increased phagocytic activity (Dyck et al. 2018) and promote axonal regeneration (Samuel David and Kroner 2011). Nevertheless, excessive M2 polarization also promotes fibrotic scar formation, hence a balance between M1/M2 responses is beneficial for the repair of the tissue after SCI (Brennan and Popovich 2018). Although M1 and M2 microglia/macrophages coexist at the lesion epicenter during the initial week, for unknown reasons expression of M2 associated genes is only temporary (Bastien and Lacroix 2014) and only the M1 microglia/macrophages persist and can reside up to a month post injury (Quadri et al. 2020).

1.1.1.3. Chronic phase

The chronic phase extends over the entire life of SCI patients and is characterized by progressive axonal die-back, cystic cavities formation and maturation of the fibrotic scar (Tran, Warren, and Silver 2018). During this period the fibrotic scar finishes forming and stabilizes, constituting one of the most important barriers to tissue regeneration. The process of Wallerian degeneration remains active for many years to eliminate the severed axons and respective cell bodies (Ehlers 2004), although it is considered that within 1-2 years the injury and associated functional deficits have mostly stabilized (Couillard-Despres, Bieler, and Vogl 2017). Furthermore, cell death and tissue degeneration results in the formation of cystic cavities, containing extracellular fluid, connective tissue and macrophages, which eventually coalesce, forming large cysts that reduce tissue volume and act as a barrier preventing regeneration (Milhorat et al. 1995). In addition, after cyst formation, approximately one third of patients develop syringomyelia. Syrinx formation may continue to cause pain in patients, tissue degeneration and loss of neurological functions that were initially preserved (Krebs et al. 2016). Little is currently known about the pathophysiologic processes involved in syrinx formation, making it difficult to develop preventive intervention. However, changes in

cerebrospinal fluid pressure caused by spinal cord injury are thought to be involved in syrinx formation and expansion (Klekamp 2012).

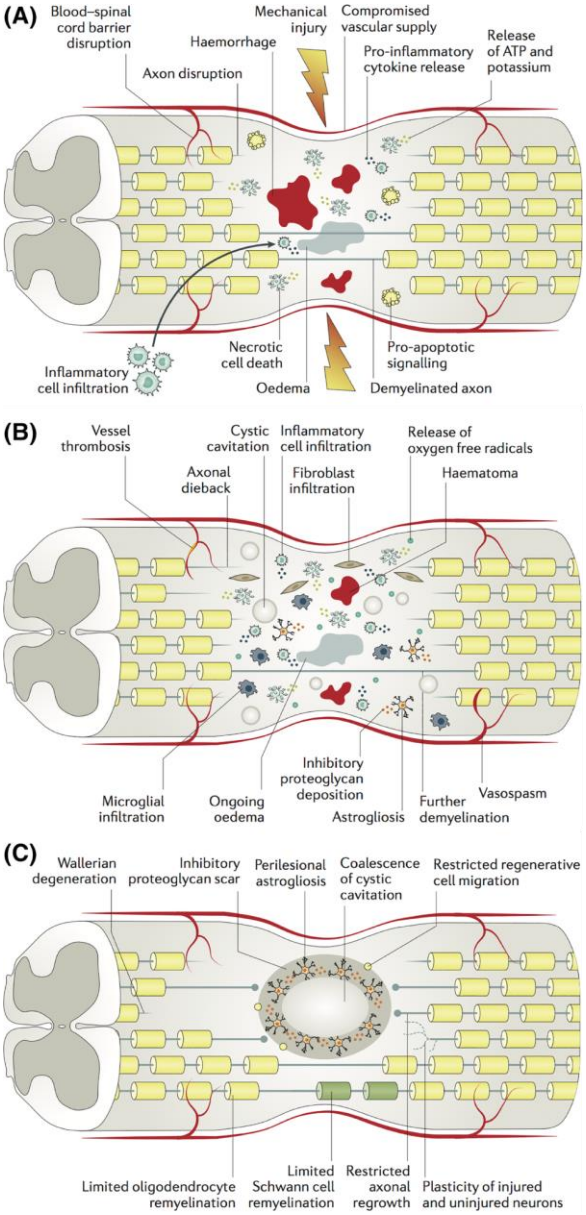

Figure 1. Pathophysiology of SCI. (A) The initial mechanical insult triggers a secondary injury cascade, which in the acute phase (0–48 hours after injury) is characterized by oedema, hemorrhage, ischaemia, inflammatory cell infiltration, release of cytotoxic products and cell death. This secondary injury leads to necrosis and/or apoptosis of neurons and glial cells, such as oligodendrocytes, which can lead to demyelination and the loss of neural circuits. **(B)** In the subacute phase (2–4 days after injury), further ischaemia occurs owing to ongoing oedema, thrombosis and vasospasm. Persistent inflammatory cell infiltration causes further cell death and formation of cystic microcavities. Furthermore, astrocytes proliferate and deposit extracellular matrix molecules into the perilesional area. **(C)** In the chronic phase (2 weeks to 6 months), axons continue degenerating and the astroglial scar matures. Cystic cavities coalesce to further restrict axonal regrowth and cell migration. Adapted from (Ahuja et al. 2020)

1.1.2. Barriers to tissue regeneration

Contrary to the historical perspective, endogenous mechanisms exist to achieve at least partial regeneration after injury. These mechanisms include neuronal synaptic plasticity, which could contribute to continued functional recovery for years after injury (Lynskey, Belanger, and Jung 2008). In rodents there are NPCs pools even in adulthood, which are mainly found in the ependymal central canal and can differentiate into neurons, oligodendrocytes and astrocytes (Barnabé-Heider et al. 2010). However, the central canal in humans is obliterated and the ependymal niche loses the capacity to proliferate any more

(Torrillas de la Cal et al. 2021). In addition, adult axons do not regenerate spontaneously after SCI even after inflammation has subsided due to both, the intrinsic limitation of the axonal regenerative machinery for axon regrowth and the extrinsic limitations since the injured area is not naturally fulfilled by tissues endowed with regenerative capacity (Sofroniew 2018).

Although SCI lesions may vary in cause, size, severity or complexity, SCI lesions are commonly divided into three compartments which differ in their structure, cell biology and molecular mechanisms having entirely different roles in repair and regeneration (Sofroniew 2018) and, therefore influencing axon regeneration in different ways (O'Shea, Burda, and Sofroniew 2017). The non-neural lesion core is composed by stromal or mesenchymal cells, including perivascular fibroblasts, meningeal fibroblasts and pericytes, which proliferate and intermingle with extravasated blood-borne cells such as fibrocytes and diverse immune cells. As the initial debris is cleared and the inflammatory infiltration regresses, the lesion core becomes composed by non-neural stromal cells and extracellular matrix molecules (Burda and Sofroniew 2014) such as chondroitin sulfate proteoglycan (CSPG). In addition, the fragmentation of damaged axons caused by Wallerian degeneration generates debris, resulting in the extracellular deposition of myelin-related molecules including neurite outgrowth inhibitor (Nogo), myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), semaphorins, and plexins activate Rho GTPase leading to the production of RhoA and activation of Rho-associated protein kinase (ROCK) (Forgione and Fehlings 2014). ROCK, is one of the vital molecules involved in the inhibition of axonal regrowth and therefore being closely associated with growth cone collapse (J. Liu, Gao, and Wang 2015) and axonal regeneration failure in adult CNS (Hilton and Bradke 2017). RhoA, affects actin dynamics preventing microtubule protrusion and thus inhibiting axon regrowth. Recently, neuron-specific genetic RhoA ablation promoted axon regeneration after CNS injury (Stern et al. 2021) and previous results showed that the inhibition Rho-activation blocked the transmission of inhibitory signals, thus improving locomotor recovery (Hayta and Elden 2018). The repulsive guidance Molecule A (RGMa) is another potent neurite growth inhibitor which exerts its repulsive activity by binding to the Neogenin receptor and is also upregulated in the spinal cord after SCI (Mothe et al. 2017).

1.2. Cell therapy for spinal cord injury

At present, SCI is treated with palliative approaches in the first hours after injury including surgical decompression, methylprednisolone treatment, hyperbaric oxygen therapy, and later with physical rehabilitation, although in all cases with suboptimal outcomes (Venkatesh et al. 2019). SCI is considered a non-curable disability, requiring new and effective methods for its treatment. During the last decades, stem cell therapy has gradually become a new research hotspot for the treatment of SCI and other neurological disorders.

Historically, cell transplantation has been used to try to repair lesions in the CNS, with different strategies. In the brain there have been many attempts, transplanting brain tissue from adult cats to dogs ("SUCCESSFUL BRAIN GRAFTING" 1890) and from neonatal rats to their littermates (Dunn 1917). From the 1960's onwards a number of studies demonstrated the effectiveness of fetal neural tissues to treat CNS injuries and disorders such as Parkinson disease, in which the donor neurons formed synaptic connections within the host tissue (Bjo¨rklund and Stenevi 1979; Perlow et al. 1979) (further reviewed in (Ishii and Eto 2014). In SCI the origin of the experimental studies testing the ability of neural tissue implants to facilitate spinal cord repair date from the early 1900s. At that time, the most widespread experimental approach was instraspinal graft of peripheral nerve segments with controversial results concluding that spinal cord regeneration was unlikely and tissue grafts were ineffective (Ramón y Cajal, DeFelipe, and Jones 1991; Sugar 1940; Brown and McCOUCH 1947; Barnard and Carpenter 1950). Nevertheless, years later, in the decade of the 80's, studies led by Richardson showed that autologous sciatic nerve grafts enhanced outgrowth of instraspinal fibers (Richardson, McGuinness, and Aguayo 1980, 1982; Richardson, Issa, and Aguayo 1984). Besides, many investigations regarding the transplant of embryonic mammalian spinal cord tissue reported that donor spinal cord could provide axonal repair by bridging the injury and/or forming new neural relays (Paul J. Reier 1985). Subsequent studies showed that the efficacy of transplantation depended on factors such as the origin of the tissue as well as the stage of development. Thus, it was found that embryonic day 14 (E14) fetal spinal cord (FSC) transplantation survived and integrated with the host tissue (Houlé and Reier 1988; P. J. Reier et al. 1988) and even showed neurotransmitter characteristics of the adult spinal cord (Jakeman et al. 1989). Further studies using neural tracing experiments showed that host axons regenerated into the FSC transplants (Tessler et al. 1988) and the FSC

grafts were extending axons up to 5 mm in length into the host spinal cord (Jakeman and Reier 1991).

Subsequently, the characterization of cell types accompanied by advances of *in vitro* culture procedures allowed the identification of distinct neural progenitors and development of specific stem cell therapies, opening up a new horizon in regenerative medicine. Cell transplantation therapies, especially stem cell transplantation, are highly promising due to their ability to provide multiple benefits at the molecular, cellular and circuit levels being ideal candidates to treat the multifaceted pathophysiology of SCI. Currently, stem cell therapies can be classified into two categories, non-neural stem cells and NSCs (stem cells that can generate neural cells).

1.2.1. Non-neural Stem Cells

Non-neural stem cells include mesenchymal stem cells (MSCs) obtained from different sources such as bone marrow, umbilical cord and adipose tissue (Cofano et al. 2019). The use of MSCs stands out due to their easy isolation from different sources and preservation, raising no ethical concerns and the limited risk of tumorigenesis (L.-L. Lu et al. 2006). MSCs show a remarkable autocrine and paracrine activity secreting a variety of molecules in the SCI environment (Vawda et al. 2020). Thus, MSCs show immunomodulatory and anti-inflammatory properties secreting a variety of anti-inflammatory cytokines including Transforming growth factor (TGF)-β1, IL-13, IL-18 and IL-10 (Vizoso et al. 2017); neurotrophic and neuroprotective properties releasing neurotrophic factors such as brain-derived growth factor (BDNF), glial-derived growth factor (GDNF), nerve growth factor (NGF), neurotrophin (NT)-1, NT-3, ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) (Vizoso et al. 2017; Teixeira et al. 2015; Kolar et al. 2017); and angiogenic secreting the tissue inhibitor of metalloproteinase-1, vascular endothelial growth factor, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), IL-6, and IL-8, which production is particularly important for supporting the wound healing processes (De Luca et al. 2011; Zanotti et al. 2016).

Given its advantage in the regulatory processes, as well as the beneficial effects derived from their use, MSCs constitute the main stem cell used in clinical trials in the field of SCI (Cheng et al. 2014; Dai et al. 2013; El-Kheir et al. 2014; Karamouzian et al. 2012; Mendonça

et al. 2014; Vaquero et al. 2018; Oh et al. 2016; Vaquero et al. 2017; Ra et al. 2011; Vaquero et al. 2016; Phedy et al. 2019) and many other ongoing further reviewed in (Cofano et al. 2019). Nevertheless, despite the beneficial effects, MSCs show limited differentiation into neuronal cells and, therefore, scarce regeneration after SCI (Shinozaki et al. 2021).

1.2.2. Neural Stem Cells

The second category encompasses neural stem cells (NSCs) and neural progenitor cells (NPCs), self-renewing and multipotent cells with the capacity to generate all neuronal lineages, although NPCs are considered to be more lineage-restricted with a reduced self-renewing capacity (Oikari et al. 2016). NSCs can be obtained from adult, embryonic and fetal sources harvested from the brain and the spinal cord (Lien, Tuszynski, and Lu 2019; P. Lu et al. 2017). In adult rodents, NSCs are found in the ependymal central canal of the spinal cord (Weiss et al. 1996) and in the dentate gyrus (Altman and Das 1965) and lateral ventricle (Altman 1969) in the brain showing a very restricted proliferative and regenerative activity. In humans, besides, are unable to generate novel neurons (Garcia-Ovejero et al. 2015). Given the limited regenerative capacity of the adult nervous system, transplantation of neurons from an exogenous source for cell replacement seems a reasonable approach for SCI. However, given the vulnerability to the SCI milieu that affects neuronal survival, and the inability of neurons to proliferate, an alternative is transplanting NSCs from early developmental stages and inducing their differentiation to the neuronal lineage as a more suitable therapy for the treatment.

Fetal NSCs are thought to be advantageous over pluripotent embryonic stem cells because they have been demonstrated to be nontumorigenic (Levi et al. 2018), although their use raises both immunological and ethical concerns. In addition, NSCs can be derived from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), obtained with reprogramming factors such as octamer-binding transcription factor 3/4 (Oct3/4), SRY-box 2 (Sox2), Krüppel-like factor (Klf4), and c-Myc (Nori et al. 2011), in which case receive the name of induced NSCs (iPSC-NSCs). The advantages of iPSCs for autologous grafting made them candidates for clinical trials (Tsuji et al. 2019) since iPSC-NSCs bypass many of the ethical and immunological considerations, although may add the additional hurdle of tumorigenicity (Fujimoto et al. 2012). Dr. Okano, an expert in the development and application of iNSCs, is

the leading exponent of the use of iPSCs for the treatment of SCI. Thirteen years ago they developed a method to obtain NSCs derived from mouse iPSCs (Miura et al. 2009) and subsequently from human iPSCs with promising results in SCI models (Nori et al. 2011). These studies showed that transplantation of iNSCs had neuroprotective and regenerative effects and the results guaranteed the safety of the treatment.

ESCs are multipotent stem cells derived from the inner cell mass of the early blastocyst, with capacity of unlimited self-renewal and the ability to differentiate into any cell lineage including NSCs (Martello and Smith 2014). Previous studies have shown the therapeutic potential of ESCs-derived NSCs for SCI treatment. ESCs-NSCs expressed myelin basic protein and displayed myelination after SCI (Salewski et al. 2015; Iwai et al. 2015). Furthermore, a clinical study in2014 demonstrated safety of ESCs-derived oligodendrocyte progenitor cells (OPCs) in SCI patients (C. T. Scott and Magnus 2014) and another study in 2016 showed that human derived ESCs restored neurological functions without causing harmful effects (Shroff 2016). Even so, the use of hESCs raises ethical dilemmas and the risk of potential tumorigenesis due to their proliferation ability still raises safety concerns (Nussbaum et al. 2007).

Potential of NSCs and NPCs lies in the fact that they are multipotent progenitors with the potential to differentiate into any of the three neuronal lineages, giving rise to astrocytes, oligodendrocyte precursor cells, oligodendrocytes and neurons (Shimada et al. 2012). Besides, following transplantation they mediate functional improvements by promoting neuroprotection (Karova et al. 2019), immunomodulation (Rong et al. 2019), remyelination (D. H. Hwang et al. 2009) and modulation of astrogliosis (Kadoya et al. 2016). In this regard, the optimal transplantation window is in the acute and subacute phases, when cell death and inflammation are maximal. NSCs/NPCs, thus, secrete neurotrophic factors such as BDNF, NT-3, NGF, FGF, and GDNF which have continuous positive effects on neurons and astrocytes and maintain tissue around the injury reactivatable, which will function stably due to long-term potentiation (Shinozaki et al. 2021). In addition, neurotrophic factors are also expected to enhance regeneration of functional circuits, rewiring (Lovett-Barr et al. 2012) and modulate synaptic activity (Garraway and Huie 2016; Mendell, Munson, and Arvanian 2001) which promotes axon regeneration (Assinck et al. 2017).

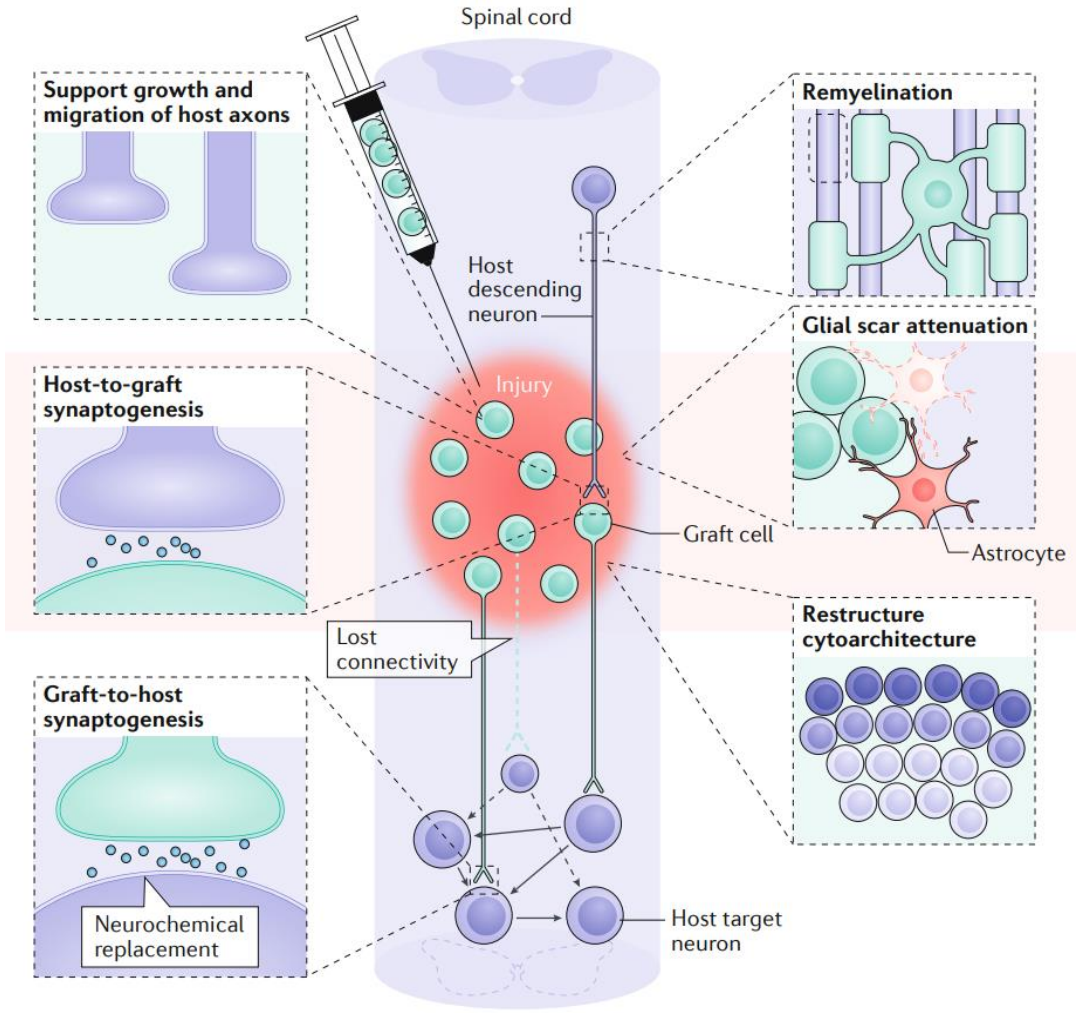

Figure 2. Potential therapeutic effects of NSCs/NPCs transplantation after SCI. The illustration shows the potential therapeutic effects of NSCs/NPCs transplants in the host injured tissue including remyelination, support of host axon growth, glial scar attenuation, synaptogenesis and the restructuring of spinal cord cytoarchitecture. Adapted from (Fischer, Dulin, and Lane 2020).

On the other hand, there are other methods by which cell transplantation directly stimulates endogenous neuronal activity. The most well-established mechanism is through the formation of bridges across the lesion site, structures formed by the transplanted cells that traverse the lesion site providing a substrate on which host descending and ascending axons can regrow. An alternative and promising approach is through the formation of neuronal relays, although being the most difficult to implement (Shinozaki et al. 2021; P. Lu et al. 2012; Kadoya et al. 2016; Bonner et al. 2011). When the transplanted cells are able to survive, differentiate into neurons, elongate axons and form synapses with the host neurons, a relay circuit can form between the descending axons and the newly differentiated

transplanted neurons that will form a connection with the caudal neurons, maintaining the integrity of the spinal circuits. Therefore, relay formation has been considered to establish novel connections to bridge and restore damaged circuits after SCI as NSCs/NPCs have the potential to integrate into the host tissue and differentiate into neurons with the ability to fire action potentials and make synaptic connections with host neurons and other transplanted cells (Lee et al. 2014). Nevertheless, due to the complex network of connections present in the CNS, it is a difficult task to guide the desired connections between host and graft neurons to build relays which form the appropriate connections.

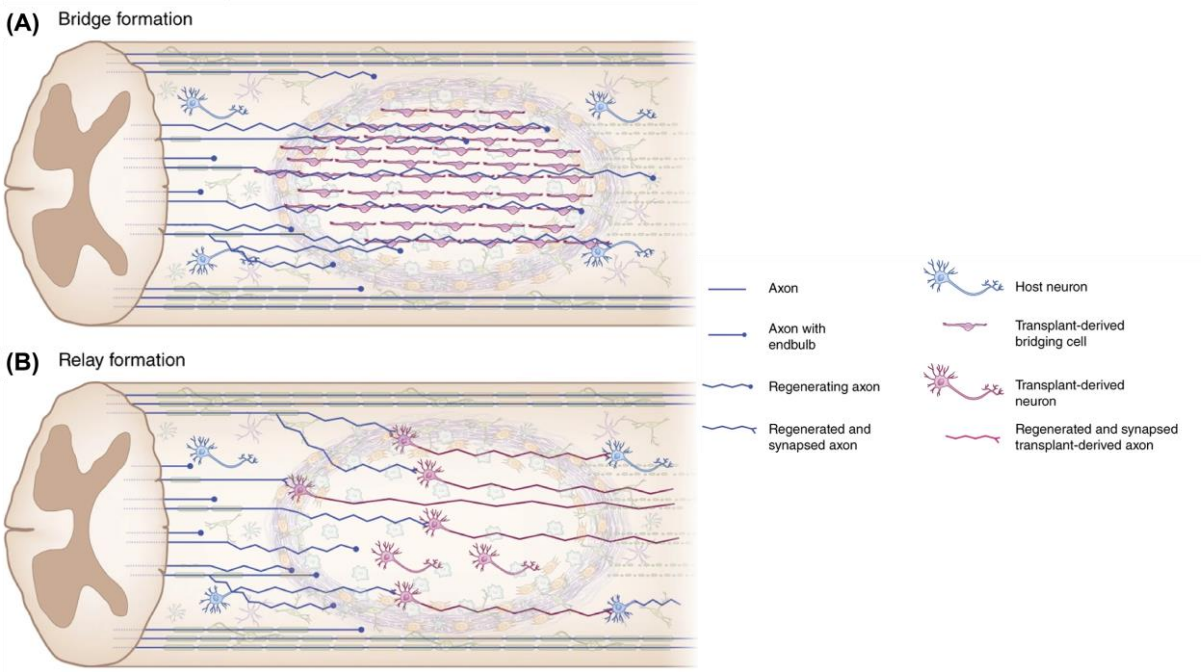

Figure 3. Re-establishment of neuronal connectivity after NSCs/NPCs transplantation. Cell transplantation may restore neuronal connectivity through the formation of a bridge or a relay. **(A)** The transplanted cells provide a bridge through the lesion site that may support axonal growth through the lesion and then re-enter into the host tissue. **(B)** Transplanted cells can enhance connectivity by forming a relay. In these relays, host axons synapse with transplant-derived neurons, which have the ability to extend axons and synapse with host neurons beyond the lesion. Adapted from (Assinck et al. 2017).

Dr Tuszynski, a pioneer in the neural stem cell transplantation after SCI, has succeeded in several studies in forming relays and cell bridges, stimulating both endogenous and transplantation axonal growth to previously unseen levels in the corticospinal tract which is the most important motor system of voluntary movement in humans. They demonstrated

that spinal cord replacement by transplanting spinal cord NPCs, enhanced robust corticospinal axon regeneration beyond the lesion, synapsis formation and functional improvement in murine models (Kadoya et al. 2016). Moreover, it was demonstrated that host inputs activate sets of NSCs derived-neurons and are able to activate host neurons below the lesion, establishing a neuronal relay and restoring synaptic connectivity after SCI (Ceto et al. 2020). Another study showed that NPCs specifically obtained from the dorsal part of rat spinal cord gave rise to specific dorsal horn-like domains when transplanted after SCI and stimulated regeneration of host sensory axons which specifically innervated appropriate neuronal clusters. Demonstrating, thus, that injured axons recognise specific targets within the NPCs grafts (Dulin et al. 2018). Besides, transplanted NSCs also adopted spinal motor and sensory fates forming cell clusters present in the intact spinal cord, and corticospinal axons regenerated and innervated specific motor domains of the graft without additional guidance (Kumamaru et al. 2019). These studies, thus, demonstrate the feasibility of cell replacement with phenotypically specific neurons within the graft, which allow the correct relay formation in specific tracts.

In addition, Dr Lane's group demonstrated that NPCs transplantation into the phrenic circuit network resulted in extensive synaptic integration between donor neurons, between host spinal and brainstem host neurons and donor neurons and between donor and spinal phrenic neurons. Moreover, synaptic integration of the transplanted NPCs also coincided with enhanced plasticity and improved respiratory activity (Zholudeva et al. 2018).

Nevertheless, it is important to consider that NSCs integration in the spinal circuitry and axonal growth are not always associated with enhanced locomotor function. Cell therapy may face and overcome many challenges including survival and integration of NSCs into the host tissue, extension of long distance axonal projections and proper innervation to form functional connections (Charsar, Urban, and Lepore 2017).

On another note, given the relevance of the origin of NSCs, human NSC lines with spinal features have been studied in preclinical models of SCI. A study showed that human fetal NSCs obtained from the cervical and thoracic spinal cord of 8-week-old fetuses were transplanted in non-human primates with cervical SCI. Transplants survived 9 months and extended hundreds of thousands of human axons, formed synapsis and improved forelimb

function (Rosenzweig et al. 2018). In another study, spinal cord NSCs were differentiated from human pluripotent stem cells and transplanted into rats with cervical injury. Spinal NSCs differentiated into diverse populations of propriospinal neurons which extended axons over long distances, integrated in the spinal cord parenchyma, enabled corticospinal tract regeneration and enhanced locomotor recovery (Kumamaru et al. 2018). In a recent study, human NSCs were obtained from the spinal cord of 5.5-7 week old fetuses. Human NSCs were expanded *in vitro* and transplanted, preventing cyst expansion in a model of SCI and post-traumatic syringomyelia (Xu et al. 2021).

1.2.2.1. NSCs in clinical trials

Several efforts have been made to translate NSCs therapies into clinics for the treatment of SCI patients. A small-scale clinical study was initiated to test the feasibility of transplanting fetal spinal cord-derived NSCs into SCI patients with post-traumatic syringomyelia suggesting the safety of transplantation (Falci et al. 1997; Thompson et al. 2001; Wirth et al. 2001). In 2009, the biopharmaceutical company Geron started to transplant human embryonic stem cell-derived oligodendrocyte progenitor cells (AST-OPC 1) into phase 1/2 clinical trials in people with subacute thoracic SCI (Manley et al. 2017) becoming in the first pluripotent stem cell-derived cells to be approved for clinical trials. Later in 2014, the biotechnology company Asterias Biotherapeutics Inc. continued and expanded the trial, including cervical-level injuries getting increased efficacy (Manley et al. 2017) and approval to begin a phase 2 trial (NCT02302157).

NSCs derived from the human fetal telencephalon were transplanted in patients with cervical SCI, and 1 year follow-up confirmed safety of the transplant, despite showing modest neurological benefit (Shin et al. 2015). Later, human CNS-derived NSCs (huCNS-SC®; Stemcells, Inc) were used in a phase I clinical trial, in patients with chronic cervical SCI, demonstrating safety and feasibility and benefits in terms of functional recovery (Ghobrial et al. 2017). In addition, free-hand intramedullary perilesional transplantation of huCNS-SC® cells were performed in thoracic and cervical SCI subjects. Despite showing some adverse effects, these were not related to the therapy, which did not cause motor decrease or neuropathic pain and showed slight functional improvements (Levi et al. 2018). Moreover, the StemCells 'Pathway' trial (phase I/II) also performed with huCNS-SC® reporting some

functional gain (Levi et al. 2019). Unfortunately, the clinical trial had to be stopped due to lack of financial support. A recent report, involving huCNS-SC® transplantation into the thoracic SCI subjects demonstrated short and long-term clinical safety and six-year follow-up showed preliminary reliable sensory improvements (Curt et al. 2020).

On the other hand, human spinal NSCs specifically derived from the fetal spinal cord have also been tested. Thus, in 2014, Neuralstem initiated a phase I trial on the use of human spinal cord-derived NSCs (HSSC) in subjects with ALS (Goutman et al. 2018) and now they are recruiting subjects with thoracic and cervical SCI to study the effects of the HSSC in SCI (NCT01772810). Furthermore, Seneca biofarma developed a human spinal-cord-derived NSCs line (NSI-566) which was tested afterwards in patients of thoracic SCI. All subjects tolerated the procedures and the therapy had no adverse effects throughout the study and two patients showed neurological improvement (Curtis et al. 2018). Therefore, NSI-566 transplantation showed signs of potential efficacy, although further studies must be performed to obtain conclusive statistical results.

On the other hand, advantages in iPSCs technology have led to the development of several clinical trials using iPSCs-derived NSCs (Tsuji et al. 2019; Okano and Sipp 2020). Although autologous transplants would be the ideal case to prevent rejection, they require enormous cost and time to produce the iPSCs lines from the patient, so allogeneic transplantation is the most feasible to have therapies ready at the required time. As a solution, there has been recently established an iPSCs cellbank of “human leukocyte antigen (HLA) super-donors” which are clinical-grade allogeneic clones of human iPSCs-derived NSCs (hiPSC-NSC) adequate for their use in clinical trials (Nagoshi and Okano 2018). Previous experiments suggested safety and effectiveness of this HLA-compatible NSCs and a first-in-human clinical study of hiPSC-NSC transplantation is going to be initiated to study the safety and impact on neurological function in subjects with cervical and thoracic subacute SCI (Sugai et al. 2021).

Author	Trial phase	Cell source	NSCs type	Injury level	Administration route	Number of patients	Time from Injury	Follow-up	Outcomes	Status of the trial
(Shin et al. 2015)	Phase I/II	Allogeneic (Human fetal brain)	Neural Stem Cells	Cervical (C3-C8)	Intramedullary	19	Subacute	1 year	Safe, well tolerated; modest neurological benefit	Complete
(Ghobrial et al. 2017)	Phase II	Allogeneic (Stemcells Inc.)	huCNS-SC®	Cervical (C5-C7)	Intramedullary	5	Subacute	12 months	functional recovery	Complete
(Curtis et al. 2018)	Phase I	Allogeneic (Seneca Biopharma)	NSI-566®	Thoracic	Intramedullary	4	Chronic	60 months	Improvement in motor scores. No serious adverse effects.	Complete
(Levi et al. 2018)	Phase I/II	Allogeneic (Stemcells Inc.)	huCNS-SC®	Cervical and thoracic	Intramedullary	29 (cervical n=17; thoracic n=12)	Chronic	Up to 56 months	Improvement in motor scores. 15 serious adverse effects in cervical group and 4 in toracic	Complete
("Website," n.d.)	Phase I	Allogeneic	human spinal cord-derived NSCs	Cervical and thoracic	-	8	Chronic	54 months	-	ongoing recruitment

(Levi et al. 2019)	Phase I/II	Allogeneic (Stemcells Inc.)	huCNS-SC®	Cervical (C5–C7)	Intramedullary	17	Subacute or chronic	3–12 months	Safe, feasible	Early termination
(Curt et al. 2020)	Phase I/IIa	Allogeneic (Stemcells Inc.)	huCNS-SC®	-	Intramedullary	12	Subacute or chronic	6 years	Sensory improvements	Complete
(Sugai et al. 2021)	Phase I	Allogeneic	hiPSCs-derived NSCs	Cervical (C3/4) and thoracic (Th10)	Intramedullary	4	Subacute	12 months	-	Ongoing

Table 2. Selection of the most relevant clinical trials using NSCs transplantation in SCI from 2015 to date.

1.3. New strategies for NSCs therapy improvement

The milieu generated following SCI limits the potential of cell therapy by reducing survival, integration and proper differentiation (Stern and Knöll 2014). Despite the promising results obtained in preclinical studies, clinical studies continue to show limited benefits, the most important barrier being the functional improvement in SCI patients. After many years of study it has become evident that given the multifactorial nature intrinsic to SCI single therapeutic approaches will not be able to tackle the multiple features of the pathophysiology and so the development of combination therapies is being pursued to provide a greater yield on clinical trials. Thus, therapeutic adjuncts, such as cell coupling strategies, scaffolds, biomaterials, growth factors and neuroprotective agents have a great potential to promote regeneration by enhancing stem cell survival and engraftment, drive NPCs differentiation toward a particular neural lineage, as well as providing immunomodulatory and/or neuroprotective properties, all of which are crucial aspects in the treatment of SCI.

1.3.1. Pharmacological therapies

To support graft survival, growth factors (eg, PDGF, epidermal growth factor (EGF), and insulin-like growth factor (IGF-1)), neurotrophins (eg, BDNF, NT3, NGF), and anti-inflammatory agents (eg, minocycline) have all been successfully delivered (Karimi-Abdolrezaee et al. 2010; Namjoo et al. 2018; Führmann et al. 2018; Sarveazad et al. 2017; Robinson and Lu 2017). The use of these pharmacological agents seek to reduce secondary events such as inflammation or excitotoxicity to protect endogenous tissue and prevent additional degeneration. In addition, these interventions during cell transplantation modulate spinal milieu to create a suitable environment for cell transplantation, which increases the viability of the transplant and stimulates grafting, thereby increasing the effects of the transplant on endogenous tissue and the therapeutic benefits. Pharmacological agents that suppress the immune system or inhibit key signaling pathways involved in inflammation were the first major strategies applied to patients. Minocycline is an antibiotic that inhibits microglial activation, decreases inflammatory cell infiltration and downregulates inflammatory response. Minocyclin has shown synergistic effects with cell transplantation by enhancing the anti-inflammatory properties of MSCs (Chen et al. 2015). Although additional work is needed to optimize the combinatory treatment. Propofol has also been used in

combination with MSCs improving neuroprotection effects promoting locomotor function (Y.-X. Wang et al. 2015). Growth factors are also commonly used to enhance survival and promote a guided differentiation of cell-based therapies towards specific phenotypes (P. Lu et al. 2019; Kumamaru et al. 2019). However, the use of growth factors has a number of drawbacks, such as stability and the time they remain active in the tissue. For this reason, other alternatives are being pursued, such as *in vitro* genetic modification of cell transplants or *in vivo* transfection of endogenous cells to secrete specific growth factors, as well as the use of biomaterials capable of storing and slowly releasing growth factors directly into the environment.

1.3.2. Cell-coupling strategies

Cell-coupling strategies have been carried out seeking a synergistic effect of the therapies in order to enhance the individual effects. Generally, cell-coupling strategies have been based on the use of MSCs and Schwann Cells (SCs), since they are able to modulate SCI milieu improving survival, engraftment, proliferation and differentiation of co-transplanted NSCs. Acute transplant of human MSCs intravenously accompanied by intraspinal injections of human fetal NSCs (hfNSCs) subacutely enhancing functional recovery (Park et al. 2013). Moreover, co-transplant of NSCs and genetically engineered MSCs overexpressing the chemokine stromal-derived factor-1 (SDF-1) improved locomotor outcomes and increased axonal preservation surrounding the lesion, despite not reporting any effect on white matter sparing and finding some tumor formation when cell therapies were combined, highlighting the need to develop safer therapies (Stewart et al. 2017). In another study, concomitant therapy of MSCs injected into the tail vein 1 day after SCI and NSCs transplanted by intraspinal injection 3 days after SCI, provided better histological outcomes as well as locomotor recovery in comparison with individual cell therapies (Hosseini et al. 2018). A more recent study, human MSCs and human fetal brain derived NSCs were transplanted intramedullary one day after SCI and were followed up within 8 weeks. Thus, co-transplantation enhanced graft survival 2 weeks post-transplantation. Although, individual and combinatorial therapies increased BDNF expression levels, only the combinatory treatment increased white matter sparing and enhanced locomotor recovery (Sun et al. 2019). Overall, studies demonstrate that the combination of cell therapies shows synergy, providing increased benefits of NSCs

therapy for the treatment of spinal cord injury. Therefore, future studies will focus on the development and optimization of combinatorial therapies, including combining them with the use of biomaterials or drugs.

1.3.3. Cell and gene therapy

A popular combinatorial approach has been to combine cell and gene therapy by engineering cells that overexpress neurotrophic factors to increase differentiation to neurons and enhance graft-host integration. Thus, (BDNF)-expressing NPCs increases differentiation into neurons and oligodendrocytes (Butenschön et al. 2016) and also improves corticospinal tract (CST) recovery at the site of injury (He et al. 2013) and improved functional recovery (Chang et al. 2021). Likewise, NT-3 overexpression exhibits preferential differentiation to neurons and axotomized regeneration (D. H. Hwang et al. 2011). GDNF-enriched NSCs grafts reduce glial scar formation, promote neurite outgrowth and migration (K. Hwang et al. 2019), promote a motor neuron-like phenotype (Abdanipour, Tiraihi, and Taheri 2014) and can partially overcome the activated Notch signaling and improve functional recovery following SCI (Khazaei et al. 2020). NPCs expressing CNTF differentiate mostly in OPCs enhancing remyelination and functional recovery after thoracic SCI (Q. Cao et al. 2010); granulocyte colony-stimulating factor (G-CSF) increased proliferation of NPCs in the injured area and enhanced regeneration and functional outcomes (Pan et al. 2008).

Another option has been to transplant the NSCs combined with growth factor cocktails to promote survival (Kadoya et al. 2016). Furthermore, after complete spinal cord transection, NPCs formed neural relays across the lesion site promoting electrophysiological and functional recovery (P. Lu et al. 2012; P. Lu, Woodruff, et al. 2014). In addition, in a more recent study the number of NTFs was reduced to a four growth factor cocktail consisting in BDNF, bFGF, Vascular endothelial growth factor (VEGF) and MDL281 to simplify the formula with a view to adapting it for use in clinical trials (Robinson and Lu 2017). Furthermore, combination of NPCs transplantation with chondroitinase ABC and transient infusion of growth factors such as EGF, bFGF, PDGF enhanced functional recovery in chronic SCI, preserving integrity and promoting plasticity of the corticospinal tract and serotonergic pathways (Karimi-Abdolrezaee et al. 2010). Recently, MASH-1 overexpression increased

neuronal differentiation of NSCs and transplantation of NSCs overexpressing MASH-1 enhanced functional recovery after SCI (M. Deng et al. 2021).

1.3.4. Biomaterials

Tissue engineering advocates combining stem cell therapy with the use of biomaterials to provide a three-dimensional scaffold which could bridge the lesion, replace damaged extracellular matrix, and integrate the transplanted cells into the host tissue harboring the cells to enhance survival, engraftment and direct differentiation towards desired cell types (Jin et al. 2019). Furthermore, biomaterials also serve as carriers for controlled release of bioactive molecules in the desired tissue (Shoichet 2010). To optimize therapy, biomaterials must meet some essential features such as being biocompatible, biodegradable, have adaptable mechanical properties, as well as guarantee cell viability and guiding axon regrowth (Perale et al. 2011).

The first biomaterials used, had a natural origin therefore generating less rejection and promoting excellent cell adhesion and growth. This group includes generic mixtures of extracellular matrix (ECM) (Bellamkonda et al. 1995) but also scaffolds of purified ECM molecules like collagen (Sherman, Yang, and Meyers 2015), gelatin (Naseri-Nosar, Salehi, and Hojjati-Emami 2017), hyaluronic acid (HA) (Liang, Walczak, and Bulte 2013), chitosan (S. Wang et al. 2017) and alginate (Sitoci-Ficici et al. 2018). The main advantages of using these natural biomaterials are that they are biodegradable, biocompatible, non-toxic and have good mechanical properties. However, they also have some disadvantages such as biodegrading too quickly (Doblado, Martínez-Ramos, and Pradas 2021).

On the other hand, we find biomaterials of synthetic origin such as poly-ε-caprolactone (PCL) (Aurand, Lampe, and Bjugstad 2012), poly-L-lactic acid (PLLA) (Li et al., n.d.), poly-D,L-lactic-coglycolic acid (PLGA) (Kim et al. 2018), among others (Srinivasan et al. 2015). Furthermore, there are conductive polymers with electrons present in their structure, which enables them to conduct electricity. Some common conductive polymers used are polypyrrole (PPY) and carbon nanotubes (Doblado, Martínez-Ramos, and Pradas 2021). PPy is an organic polymer formed by the polymerization of pyrrole monomer which has been used in combination with other biodegradable non-natural polymers such as PLA (Shu et al. 2019)

enhancing axonal regeneration after SCI. Moreover, carbon nanotubes (CNTs) are carbon allotropes with a cylindrical structure that, in combination with other polymers such as Polyethylene glycol (PEG), promote axon guidance of regenerative axons (Xifeng Liu et al. 2018). Synthetic materials arose from the need to overcome the limitations of natural biomaterials, since they can be manufactured using a variety of techniques and architectures adapted to the type of tissue to be regenerated (Banigo, Iwuji, and Iheaturu, n.d.) and by choosing the right polymer composition, biodegradability can be controlled. Nevertheless, they also have certain limitations, such as an increased risk of rejection after implantation.

Despite the aforementioned complexity and limitations of NSC regeneration some strategies have been designed such as directly injected gels (Austin et al. 2012), porous three-dimensional scaffolds (F. Zhang et al. 2011), meshes with regular pore geometry (Suri et al. 2011), elongated and oriented microfilaments (Hurtado et al. 2011). Hydrogels are gaining importance since they are soft matter, able to be injected or implanted directly to fill the cavities (Jin et al. 2019). After SCI and the disruptions of ascending and descending pathways, the spatial organization must be maintained by the implanted biomaterials, allowing axons to find their appropriate targets beyond the site of injury. Thus, biomaterials with oriented channels or guides have been developed such as HA conduits with PLLA fibers in the lumen (Martínez-Ramos et al. 2019), scaffolds with aligned conduits of fibrin (J. B. Scott et al. 2011) and PLLA (Hurtado et al. 2011) as well as PLGA channel bridges (Tuinstra et al. 2012), which permit the aligned regrowth and regeneration of axons in a directional manner so that they can cross over and establish connectivity in the post-injury area, avoiding the growth inhibitory signals intrinsic to the lesion.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

The development of treatments for clinical application capable of providing recovery of lost neurological functions with motor and sensory recovery after SCI remains a challenge. In pre-clinical studies, among others, the use of neural stem or progenitor cells (NSCs or NPCs) has been shown to provide a promising solution for SCI treatment since provides neuronal replacement, neuroprotection and regenerative support when are ectopically transplanted in the injured tissue. Nevertheless, despite showing promising results in preclinical studies, stem cell-based single therapies have shown limited benefits in clinical trials.

In the present thesis we aimed to evaluate novel combinatorial approaches hypothesizing that the tested combination would enhance the NSCs-based therapy capabilities. The new tested combinatorial approaches have been included: 1) the co-transplantation with mesenchymal stem cells (MSCs) and the local delivery of a curcumin nanoconjugate (Chapter 1), both with known neuroprotective effects; 2) by employing a biocompatible scaffold for a minimally invasive way of cell transplantation (Chapter 2); 3) the generation of new NPC lines from human fetal sample primed with a a Rho/Rock inhibitor in a conjugated form (PGA-SS-FAS) to increase survival and cell differentiation after *in vivo* transplantation (Chapter 3).

Every combinatorial approach included specific experimental objetives as described in every chapter of this Thesis:

Chapter 1, entitled: ***"Human-Induced Neural and Mesenchymal Stem Cell Therapy Combined with a Curcumin Nanoconjugate as a Spinal Cord Injury Treatment"*** includes the following specific objectives:

1.1: *In vitro* study of the effect of a polymeric conjugated form of curcumin (PA-C) on induced neural stem cell cultures (iPSC-NSCs), evaluating the:

- Cell viability preservation in the presence of oxidative damage
- Axonal elongation capacity in neuronal induced cells after spontaneous differentiated iPSC-NSCs

- The immunomodulatory properties of PA-C alone or in co-culture with mesenchymal stem cells (MSC), alone or in the presence of lipopolysaccharide (LPS) mimicking part of the inflammatory milieu found in the injured spinal cord

1.2: *In vivo* evaluation of the effects of the co-transplantation of iPSC-NSC and MSCs with local delivery of PA-C in a model of traumatic SCI by:

- A comparative functional locomotor analysis.
- The histological evaluation of the injury site, the formed scar and the preserved or degenerated neural fibers, sensory and motor neurons.
- The study of the inflammatory response mediated by the infiltrated macrophages and the resident microglia.

Chapter 2, entitled: ***A Hyaluronic Acid Demilune Scaffold and Polypyrrole-Coated Fibers Carrying Embedded Human Neural Precursor Cells and Curcumin for Surface Capping of Spinal Cord Injuries,*** includes the following objectives:

2.1: *In vitro* characterization of the iPSC-iNSCs encapsulation in the self-assembled hydrogel puramatrix (PM) in the presence of PA-C, by evaluating:

- Cell viability and cell differentiation.

2.2: *In vivo* evaluation of of iPSC-NSCs transplantation encapsulated in Puramatrix (PM), loaded with PA-C within a demilune hyaluronic acid-based scaffold in a model of SCI by:

- Histological analysis of the iPSC-NSCs engraftment.
- Studying the injured area, the extension of the scar and the preservation of neural fibers.
- Studying the inflammatory response.

Chapter 3, entitled: ***Transplantation of Human Fetal Spinal cord-derived NPCs Primed with a Polyglutamate-conjugated Rho/Rock Inhibitor as a Therapy for Acute Spinal Cord Injury,*** includes the following objectives:

3.1: Isolation and *in vitro* expansion and culture establishment of human fetal neural progenitor cells (hfNPCs) obtained from the spinal cord of elective abortions of 19.0-21.6 weeks of gestation for cell banking and further allogenic cell transplantation. *In vivo* and *in*

vitro characterization of the neural precursors cells from the human fetal spinal cord were performed by:

- Histological analysis of the neural precursor cell niches in the fixed human fetal spinal cords at 19.0-21.6 weeks of gestation.
- Evaluating the self-renewal and proliferation capabilities as well as the heterogeneicity of the *in vitro* expanded population.

3.2: Generation of a new priming procedure to enhance de hfNPC capabilities in cell survival and cell differentiation. To induce this priming process the hfNPCs treated with a polyglutamate-conjugated form of a Rho/Rock inhibitor (Fasudil; PGA-SS-FAS) for the evaluation of:

- *In vitro* cell differentiation
- *In vitro* analysis of neurite outgrowth

3.3: *In vivo* evaluation of survival, engraftment and neuroprotective capabilities of the primed hfNPCs after transplantation in an acute model of SCI by:

- Histological analysis of cell number, distribution, cell fate and cell activation markers analysis of GFP expressing hfNPCs on the injured spinal cord.
- Immunohistochemical evaluation of the activation and preservation of neuronal subpopulations subrounding the hfNPC transplantation at the injured spinal cord.

RESULTS

3. RESULTS

3.1. CHAPTER 1: Bonilla, P., Hernandez, J., Giraldo, E., González-Pérez, M. A., Alastrue-Agudo, A., Elkhenany, H., ... & Moreno-Manzano, V. (2021). Human-Induced Neural and Mesenchymal Stem Cell Therapy Combined with a Curcumin Nanoconjugate as a Spinal Cord Injury Treatment. International journal of molecular sciences, 22(11), 5966.

My contribution to the paper consisted in the design and development of the in vivo and in vitro experiments, as well as the analysis and discussion of the data. I was also the main author of the manuscript, writing the first version, the figures, as well as making the subsequent corrections prior to publication.

Human-Induced Neural and Mesenchymal Stem Cell Therapy Combined with a Curcumin Nanoconjugate as a Spinal Cord Injury Treatment

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Abstract: We currently lack effective treatments for the devastating loss of neural function associated with spinal cord injury (SCI). In this study, we evaluated a combination therapy comprising human neural stem cells derived from induced pluripotent stem cells (iPSC-NSC), human mesenchymal stem cells (MSC), and a pH-responsive polyacetal–curcumin nanoconjugate (PA-C) that allows the sustained release of curcumin. *In vitro* analysis demonstrated that PA-C treatment protected iPSCNSC from oxidative damage in vitro, while MSC co-culture prevented lipopolysaccharide-induced activation of nuclear factor-κB (NF-κB)

in iPSC-NSC. Then, we evaluated the combination of PA-C delivery into the intrathecal space in a rat model of contusive SCI with stem cell transplantation. While we failed to observe significant improvements in locomotor function (BBB scale) in treated animals, histological analysis revealed that PA-C-treated or PA-C and iPSC-NSC + MSC-treated animals displayed significantly smaller scars, while PA-C and iPSC-NSC + MSC treatment induced the preservation of β-III Tubulin-positive axons. iPSC-NSC + MSC transplantation fostered the preservation of motoneurons and myelinated tracts, while PA-C treatment polarized microglia into an anti-inflammatory phenotype. Overall, the combination of stem cell transplantation and PA-C treatment confers higher neuroprotective effects compared to individual treatments.

Keywords: spinal cord injury; stem cells; curcumin; neuroprotection; polymer–drug conjugate

1. Introduction

The physiopathology of spinal cord injury (SCI) involves the disruption of spinal pathways and a primary succession of processes initiated immediately after trauma that prompt rapid and massive cell death within the nervous tissue and the concomitant invasion of ectopic immune and fibrotic cells [1]. During a secondary injury phase, neuronal death expands to neighboring segments, and neuroinflammation and the associated nonpermissive microenvironment [2] limit the capacity of adult neurons to spontaneously regenerate axons [3]. Therefore, a multifactorial approach to SCI treatment addressing the broad range of pathological factors involved may have better success than conventional monotherapeutic advances that focus on a single factor.

Stem cell transplantation, particularly the use of neural stem cells (NSC) [4], represents a potentially effective treatment approach for SCI. Pre-clinical assays have demonstrated that NSC can replace lost host cells, bridge lesions [5], recover myelin sheaths to reconstitute neuronal circuitry and lost connectivity [6], provide trophic support [7], and modulate neuroinflammation [8]. The US Food and Drug Administration (FDA) approved an immortalized NSC line (NSI-566), derived from human early fetal spinal cord tissue, to treat chronic SCI in phase I clinical trials reporting no serious adverse effects [9]. The administration of NSI-566 in several pre-clinical models of SCI (rodents [10] and non-human primates [11])

prompted significant improvements to neurological function and suppressed spasticity by supporting extensive axonal sprouting and the development of synaptic contacts with host neurons. However, NSI-566 transplantation requires immunosuppression, which is a disadvantage that could be avoided via the use of derivatives of autologous or immune-compatible induced pluripotent stem cells (iPSC).

The induced differentiation of human iPSC into NSC (iPSC-NSC) provides a proliferative and broadly expandable *in vitro* cell source with glial [12] and neuronal differentiation potential [13,14]. In 2019, Okano et al. described the first licensed trial for the clinical evaluation of iPSC-NSC in chronic SCI treatment in Japan [15]. Okano's group had previously employed rodent [13] and primate [16] models to demonstrate how iPSC-NSC significantly improved locomotion after severe to moderate traumatic SCI [17]. Despite the beneficial effects of iPSC-NSC, including their autologous nature that avoids immune rejection [18], their potential tumorigenicity remains a significant impediment to their clinical use [19]. In this regard, we recently generated genetically stable human iPSC using a modified reprogramming procedure employing the transfection of synthetic mRNAs coding for CYCLIN D1 and the OSKL reprogramming factors (OCT3/4, Sex determining Region Y-box 2 (SOX2), Kruppel Like Factor 4 (KLF4), homolog lin-28 (LIN28)), thereby avoiding the use of C-MYC [20], under clinically compatible conditions. iPSC-NSC subsequently derived from these newly generated iPSC displayed reduced genetic instability, reduced cell proliferation in teratoma assays, and efficient survival, engraftment, and differentiation in a hostile SCI microenvironment [20].

Numerous related approaches have also explored the safety and efficacy of mesenchymal stem cell (MSC)-based therapies for SCI [21–25] and highlighted MSC transplantation as an interesting means to locomotor recovery in animal models [26]. Our recent studies indicated that the transplantation of allogeneic MSC combined with immunosuppression supports the survival of engrafted cells, which improves functional and morphological outcomes after SCI [27]. The therapeutic benefits of MSC primarily relate to the secretion of paracrine acting factors [28] that provide neural support and promote remyelination [29]. In addition, MSCs also display immunomodulatory [30], anti-apoptotic [31], and angiogenic [32] abilities but lack tumor-initiating potential [33]. Furthermore, given their accessibility and feasible isolation, MSC can be used in an autologous manner.

Given the pathological heterogeneity of SCI, multifaceted combinatorial approaches have been developed that induce improvements regarding cell grafting and survival [11,34], neural differentiation [35], axonal regeneration [35], and the prevention of secondary damage [36]. We previously described a successful combination therapy comprising NSC transplantation and treatment with a water-soluble pH-responsive polyacetal–curcumin nanomedicine incorporating curcumin in the polyacetal mainchain (PA-Curcumin or PA-C), which supports the local sustained release of the active compound curcumin [37]. This synergistic combination led to increased levels of neuroprotection and induced functional recovery in a severe model of chronic SCI in adult rats [37].

In the current study, we report on the development of an enhanced synergistic combination therapy comprising PA-C and iPSC-NSC + MSC treatment as a treatment option for severe sub-acute traumatic SCI. This advanced approach combines the induction of neuroprotection and neuroregeneration to target the secondary pathological mechanisms that occur after SCI.

2. Results

2.1. Cultured iPSC-NSC Express Neuronal, Astroglial, and Oligodendrocyte-Specific Lineage Markers

We first characterized the heterogeneous iPSC-NSC derived from cyclin D-reprogrammed iPSC [20]. Immunofluorescent evaluation of the three neural lineages performed under proliferative culture conditions (representative images shown in Figure 1A) demonstrated a significant percentage of cells positive for early neural precursor markers, including SOX2 (75.39 ± 3.24% of total cells), NESTIN (93.05 ± 2.69% of total cells), Paired box protein (PAX6) (45.98 ± 5.01% of total cells), and neurogenic locus notch homolog protein 1 (Notch1) (39.76 ± 5.63% of total cells), which confirmed their neural lineage [38,39] (Figure 1B). Furthermore, we discovered that around one-third of iPSC-NSC expressed Forkhead Box J1 (FOXJ1), which is a marker for ciliated cells that include the neuroepithelial cells lining the ependymal canal of the spinal cord [40]. iPSC-NSC also expressed markers of neurons (β-III Tubulin—37.05 ± 4.06% of total cells, and doublecortin (DCX)—54.8 ± 4.23% of total cells), glia (glial fibrillary acidic protein (GFAP)—48.35 ± 5.97% of total cells), and oligodendrocytes

(O4—39.97 ± 8.01% of total cells) (Figure 1A,B). We previously showed comparable neuronal and glial cell identity in iPSC-NSC cultures upon neural induction medium conditions, with about 60% of β-III Tubulin-positive cells and about 34% of GFAP-positive cells [14].

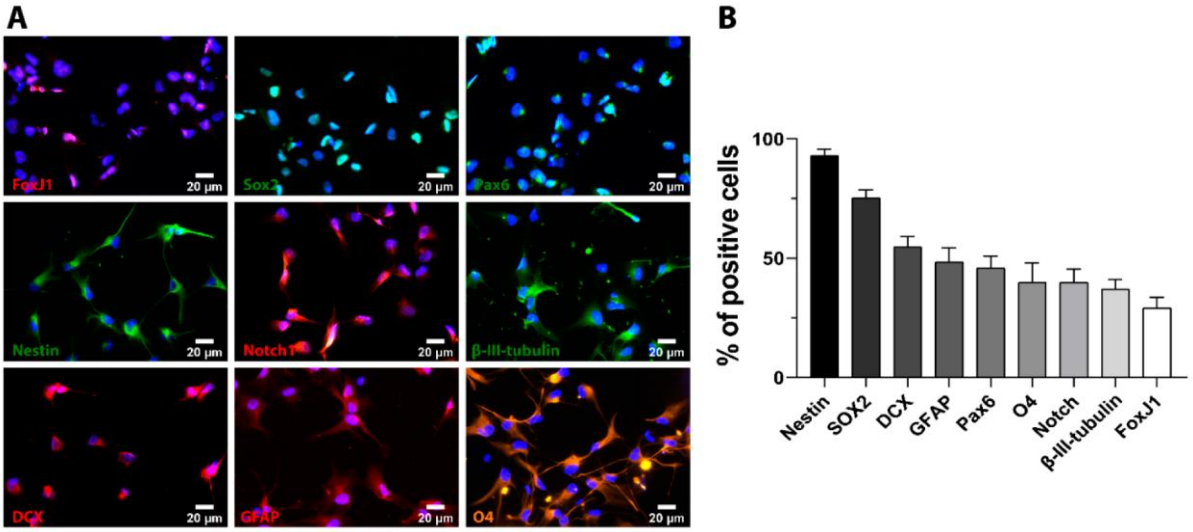


Figure 1. *In vitro* characterization of cultured iPSC-NSC. (**A**). Representative immunofluorescence staining images for the indicated cell markers. DAPI (blue) employed for nuclei counterstaining. Scale bar = 20 µm. (**B**). Quantitative analysis of the percentage of positive cells for each cell marker. Data expressed as mean ± S.E.M. from three independent cell culture experiments.

2.2. PA-C Treatment Increases iPSC-NSC Viability, Enhances Neurite Elongation, but Fails to Induce Neural and Glial Differentiation

We previously described that polyacetal–curcumin conjugate (PA-C, curcumin loading 3.8% w/v) displayed significantly lower cytotoxicity than free curcumin (C) in primary cultures of adult rat spinal cord NSC [37]. To elucidate whether PA-C influences the viability of iPSC-NSC, we performed an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay at 24 h after treatment with increasing concentrations (up to 20 µM) of PA-C at curcumin equivalent concentrations. We discovered that the evaluated concentrations of PA-C failed to induce cytotoxicity in iPSCs-NSC; moreover PA-C treatment significantly increased iPSC-NSC metabolic activity in a dose-dependent manner at concentrations higher than 10 µM (Figure 2A).

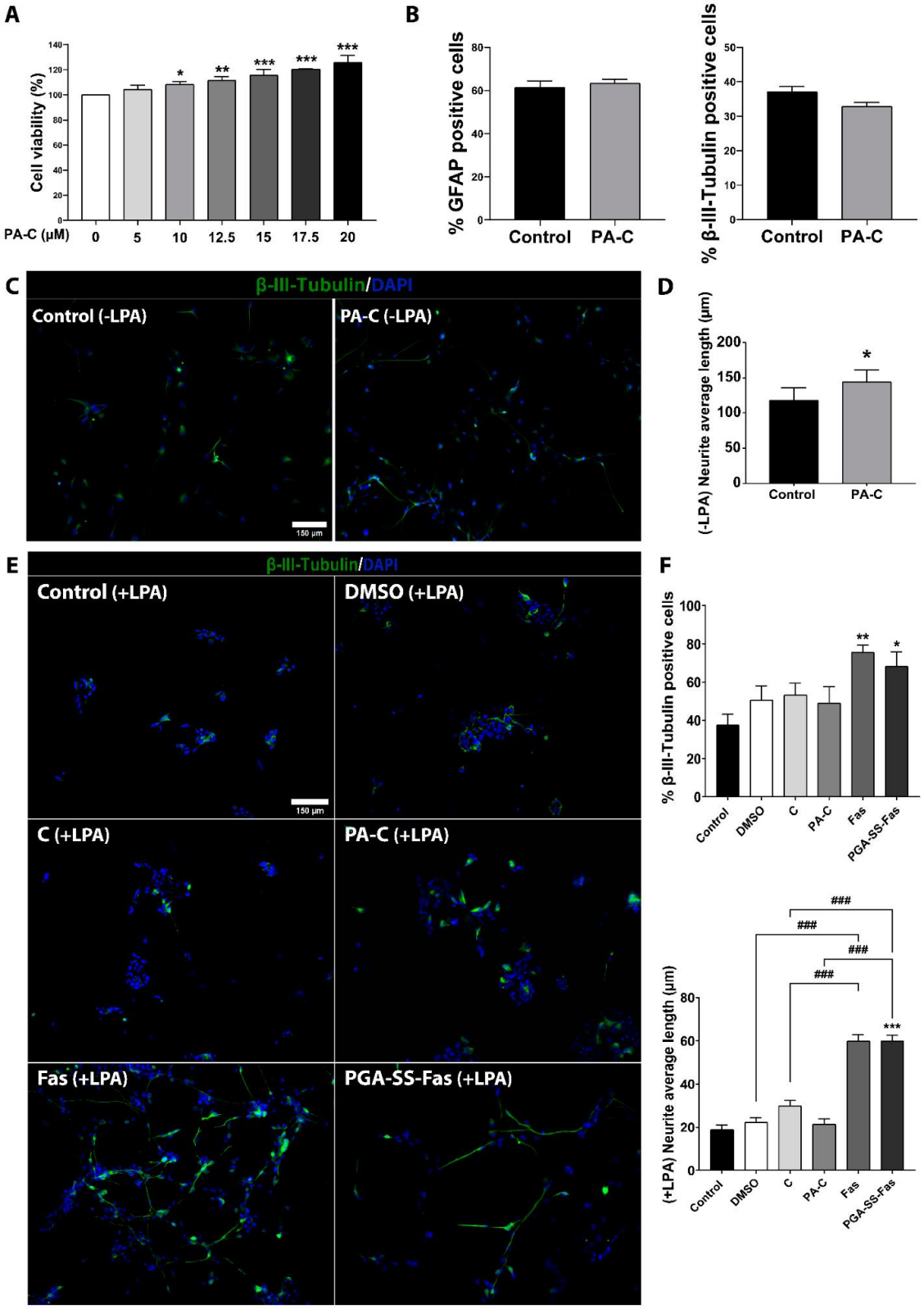


Figure 2. (A). Effect of PA-C treatment on iPSC-NSC viability and neurite outgrowth in vitro. (A). Cell viability evaluations of iPSC-NSC treated with increasing concentrations (5, 10, 12.5, 15, 17.5, and 20 μM) of PA-C for 24 h. Values represented as a percentage relative to vehicle-treated control iPSC-NSC.

Data expressed as mean ± S.E.M. of three independent experiments determined by one-way ANOVA with Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control). (B). Immunofluorescence quantification of the percentage of iPSC-NSC positive for astroglial (GFAP; left panel) and neuronal (β-III-Tubulin; right panel) markers after treatment with 10 μM PA-C for 24 h. (C). Representative images of immunofluorescence staining of β-III-Tubulin (green) and nuclear DAPI staining (blue) (scale bar = 150 μM). (D). Quantitative analysis of neurite elongation in iPSC-NSC treated with 10 μM PA-C for 24 h. Data represented as average neurite length (μm) and expressed as mean ± S.E.M. of three independent experiments determined by unpaired Student's t-test (* $p < 0.05$ vs. Control). (E). Representative images of immunofluorescence staining of β-III-Tubulin (green) and nuclear DAPI staining (blue) (scale bar = 150 μm). (F). Quantitative analysis of neurite elongation in iPSC-NSC pre-treated with 10 μM PA-C or 50 μM PGA-SS-Fas for 24 h and then treated with LPA for a further 24 h. Data represented as average neurite length (μm). Data expressed as mean ± S.E.M. of three independent experiments determined by one-way ANOVA with Tukey's multiple comparison test and unpaired Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control; ### $p < 0.001$ as indicated).

Next, we studied whether a 24 h treatment with 10 μM PA-C influenced iPSC-NSC fate determination. Staining for cells positive for GFAP or β-III-Tubulin indicated that PA-C treatment failed to alter the proportion of astrocytes and neurons, respectively, compared to control vehicle-treated iPSC-NSC (Figure 2B). We also analyzed gene expression profiles using an RT2 Profiler PCR Array that includes a set of genes related to neural differentiation such as Brain-Derived Neurotrophic Factor (BDNF), Oligodendrocyte transcription factor (Olig2), SOX2, Achaete-Scute Family BHLH Transcription Factor 1 (ASCL1), Dopamine receptor D2 (DRD2), and apoptosis-related genes such as Adenosine A1 receptor (ADORA1) and BCL2 (Table S1). PA-C treatment failed to influence the expression of genes involved in apoptosis, which agrees with the results of the cytotoxicity assays (Figure 2A). Furthermore, from this select group of genes, only DRD2 expression became significantly downregulated after treatment with PA-C (* $p < 0.05$ compared to control vehicle-treated iPSC-NSC; Table S1), indicating that PA-C treatment did not significantly induce neural differentiation of iPSC-NSC. Previous studies established that PA-C treatment induced neurite outgrowth [37]. While the incubation of iPSC-NSC with 10 μM PA-C for 24 h failed to induce significant neural differentiation, we found that PA-C treatment significantly induced neurite elongation in β-III Tubulin-positive cells (Figure 2C,D). We also evaluated the functional relevance of induced

neurite elongation by PA-C treatment in an inhibitory environment by treating iPSC-NSC with lysophosphatidic acid (LPA), which activates the Rho/ROCK pathway and induces growth cone retraction and neurite collapse [41,42]. We employed free fasudil (Fas) [43], a well-known Rho kinase inhibitor, and a nano-conjugated form of fasudil (PGA-SS-Fas) [44] as positive controls for neurite elongation. In a similar manner to curcumin, we previously reported that polymer conjugation enhanced the stability of fasudil and supported sustained release, which improved the neuroprotective and regenerative activity of fasudil in SCI models [44]. While treatment with PGA-SS-Fas or Fas (Figure 2E,F) permitted a significant increase in axonal elongation in the presence of LPA, we found no differences following the treatment of iPSC-NSC with PA-C (Figure 2D) or C (Figure 2E,F). Representative images of β-III-Tubulin immunostaining in the absence of LPA treatment show neurite elongation following PA-C treatment (Figure 2C), abundant and long neural extensions following PGA-SS-Fas treatment (Figure 2E,F), and the absence of morphological changes in PA-C-treated iPSC-NSC in the presence of LPA in comparison with vehicle-treated control iPSC-NSC (Figure 2E,F).

2.3. PA-C Protects iPSC-NSC against Hydrogen Peroxide-Induced Toxicity

The excessive production and release of reactive oxygen species following SCI contribute to the secondary injury phase by exacerbating acute damage to spinal cord-resident cells [45,46] and generating a hostile microenvironment for cell-based therapies. Anti-oxidant treatments have emerged as an effective approach to ameliorate cell death/secondary damage. We evaluated PA-C pre-treatment (5, 10, 12.5, 15, 17.5, or 20 µM) in the prevention of oxidative cell damage following the exposure of iPSC-NSC to high doses of hydrogen peroxide (H2O2) (75 or 100 µM) for 24 h. We determined H2O2-induced cytotoxicity by MTS viability assay, discovering that PA-C treatment in the range of 5 to 15 µM prevented 75 µM H2O2-induced cytotoxicity close to the control non-treated condition. Representative phase-contrast images evidenced a cell survival-inducing effect of 10 µM of PA-C in the presence of 75 µM H2O2 (Figure 3A); however, PA-C pre-treatment failed to protect iPSC-NSC treated with 100 µM H2O2 (Figure S1).

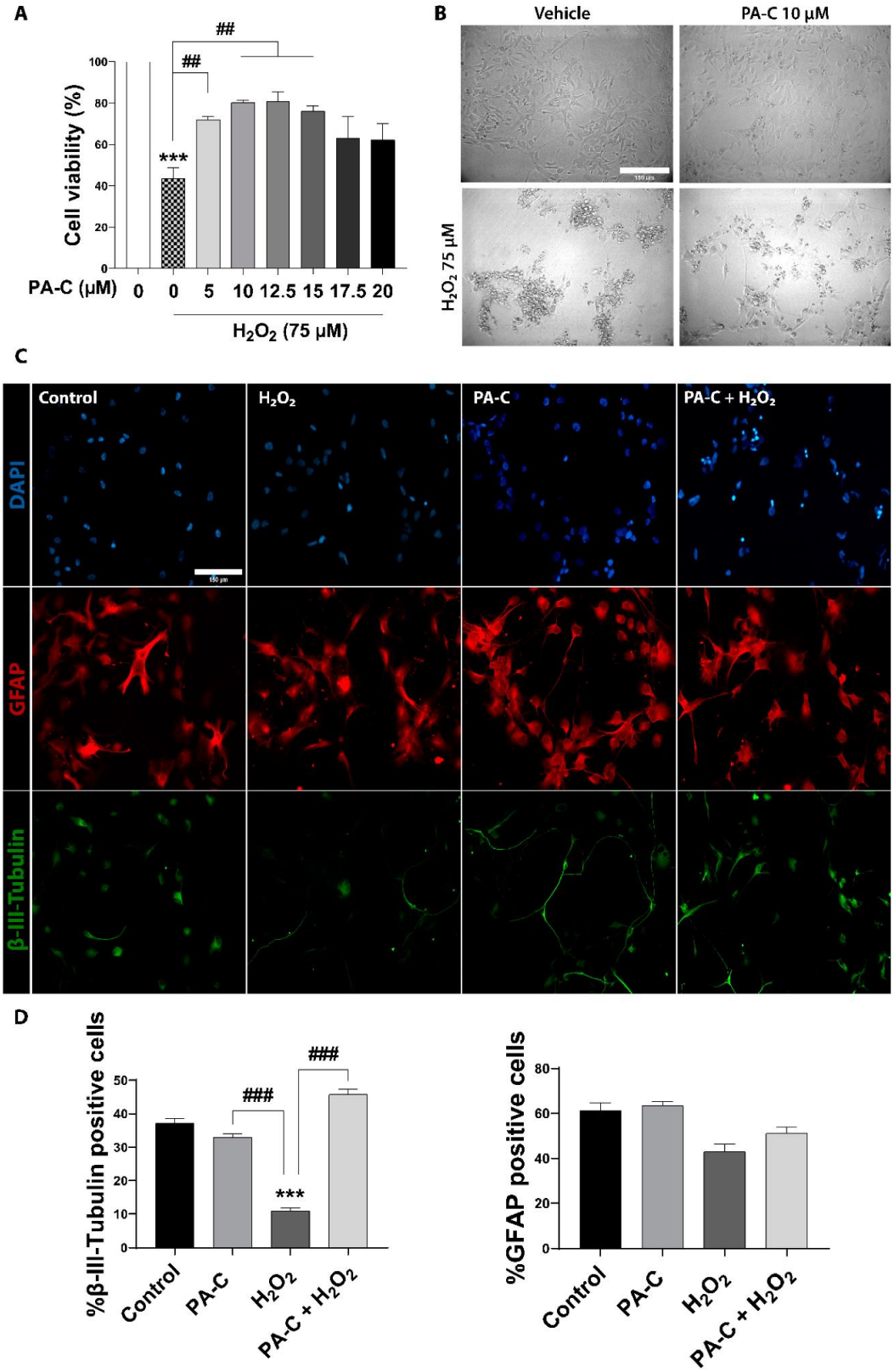


Figure 3. Neuroprotective effect of PA-C pre-treatment following peroxide-induced cytotoxicity in iPSC-NSC. (A). Effect of PA-C pre-treatment (5, 10, 12.5, 15, 17.5, and 20 μM) on iPSC-NSC viability following a 24 h incubation with 75 μM H2O2 . MTS viability values represented as a percentage of

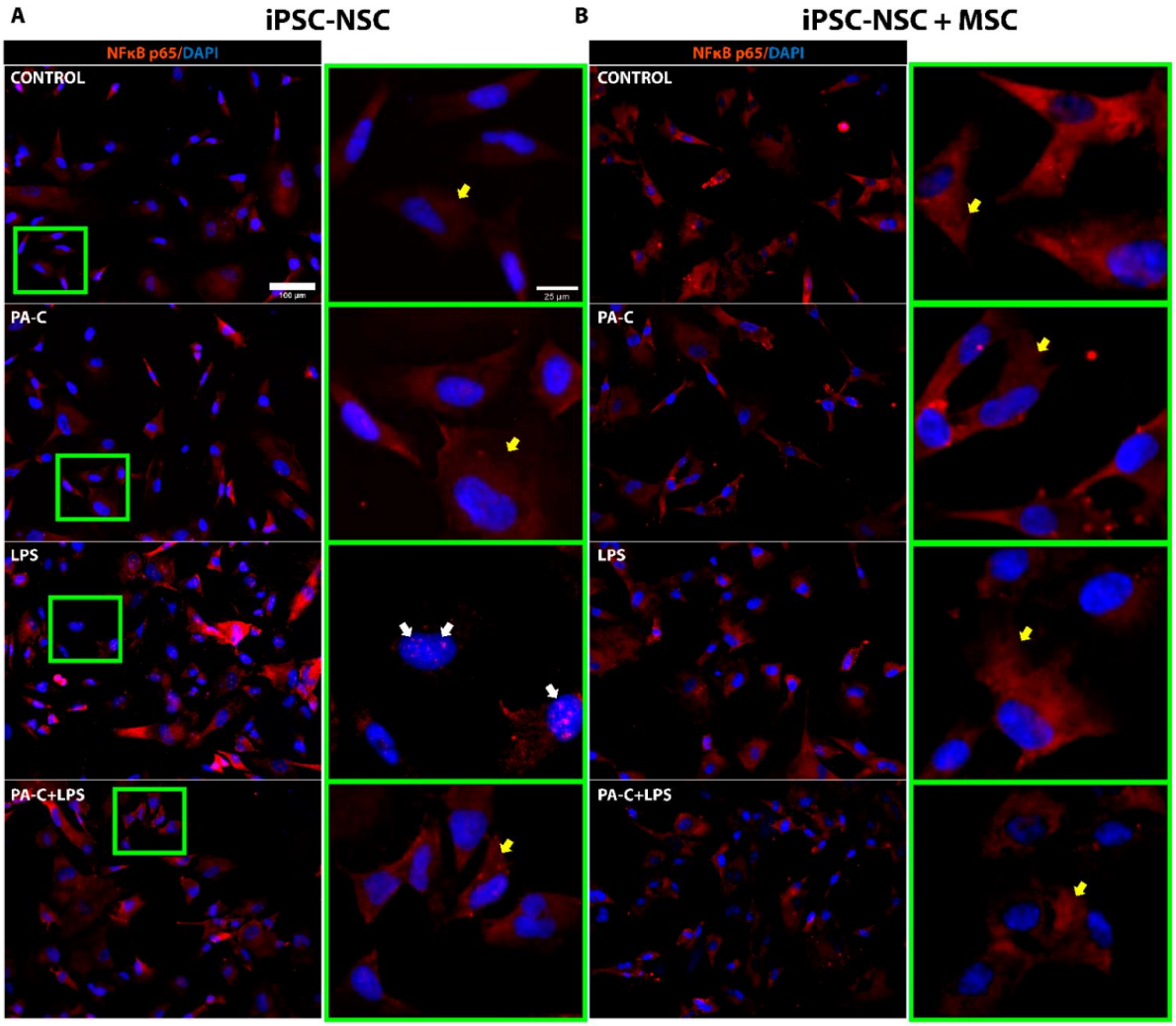
vehicle-treated control cells. Data expressed as mean ± S.E.M. of three independent experiments determined by one-way ANOVA multiple comparison tests (*** $p < 0.0001$ vs. Control; ## $p < 0.01$ as indicated). (B). Representative bright-field images of iPSC-NSC morphology after PA-C pre-treatment and H_2O_2 treatment (scale bar = 150 µm). (C). Representative immunofluorescence staining of β-III-Tubulin (green), GFAP (red), and DAPI (blue) of iPSC-NSC pre-treated for 30 min with 10 µM PA-C and then incubated with 75 µM H_2O_2 for 24 h. Scale bar = 150 µm. (D). Quantitative analysis of the ratio of β-III-Tubulin cells and GFAP cells shown as a percentage of total DAPI from immunostaining. Data expressed as mean ± S.E.M. from three independent experiments as determined by one-way ANOVA with Tukey's multiple comparison test (*** $p < 0.001$ vs. Control; ### $p < 0.001$ as indicated).

Due to the heterogeneous nature of cultured iPSC-NSC (Figure 1), we evaluated the potential neuronal/glial selectivity of PA-C treatment using a 30 min 10 µM PA-C pretreatment (or vehicle treatment in control) followed by a 24 h treatment of 75 µM H_2O_2. We discovered that H_2O_2 significantly inhibited the survival of neuronal progenitors, as shown by a decrease in the levels of β-III Tubulin-positive neural cells, but it had no effect on glial progenitor survival, as shown by the unchanging proportion of GFAP-positive glial cells (Figure 3B shows presentative images and the quantification of β-III Tubulin and GFAP-positive cells). Furthermore, 10 µm PA-C pre-treatment significantly protected neuronal progenitor cells from H_2O_2-induced cytotoxicity (Figure 3B).

2.4. PA-C Treatment and MSC Prevent NF-κB Translocation to the Nucleus of LPS-Treated iPSC-NSC

We assessed the anti-inflammatory effect of a 24 h pre-treatment of 10 µM PA-C on iPSC-NSC co-cultured with MSC subsequently treated with 1 µg/mL LPS (lipopolysaccharides, a bacterial endotoxin) for 24 h to induce the nuclear translocation and activation of NF-κB, a critical mediator of pro-inflammation following SCI [47]. As shown by immunostaining images (Figure 4A) and quantification (Figure 4B), PA-C treatment did not significantly affect NF-κB translocation in iPSC-NSC monocultures or iPSC-NSC + MSC co-cultures. While LPS-induced inflammatory stress significantly increased nuclear NF-κB translocation (Figure 4A; white arrows) and activation in iPSC-NSC monoculture, LPS treatment in iPSC-NSC + MSC co-culture did not induce NF-κB translocation. Furthermore, pre-treatment with PA-C significantly reduced LPS-induced NF-κB translocation in iPSCNSC monocultures. However, no additional effect of PA-C is observed in the co-cultures, as MSCs alone inhibit NF-κB translocation. (Figure

4A; yellow arrows). Overall, these findings provide evidence that PA-C pre-treatment and MSC co-culture inhibit the activity of inflammatory mediator NF-κB in iPSC-NSC cultured under pro-inflammatory stimulus conditions.



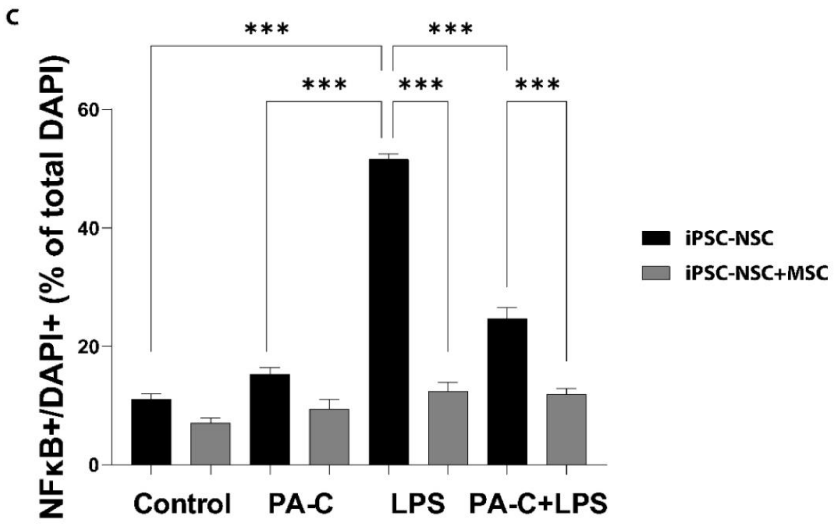


Figure 4. PA-C pre-treatment and MSC co-culture prevent NF-κB translocation/activation in iPSC-NSC following proinflammatory LPS insult. (A,B). Representative immunostaining images of the NF-κB inflammatory marker (orange) and nuclear staining DAPI (blue) in iPSC-NSC monoculture (iPSC-NSC) (A) and MSC co-culture (iPSC-NSC + MSC) (B) incubated for 24 h with 10 μM PA-C or vehicle and then incubated with LPS (1 μg/mL) or vehicle for 24 h (scale bar = 100 μm). Green squares show higher magnification of the nuclei (scale bar = 20 μm), with white and yellow arrows indicating nuclear and cytosolic NF-κB, respectively. (C). Quantification of NF-κB translocation by quantifying iPSC-NSC with nuclear NF-κB (NF-κB+/DAPI+) expressed as a percentage of the total number of iPSC-NSC by DAPI. Data expressed as mean ± S.E.M. (at least eight random fields from three independent experiments) determined by one-way ANOVA with Tukey's multiple comparison test (*** $p < 0.001$ as indicated).

2.5. Functional Locomotor Recovery after SCI in Response to Individual Treatments and Combination Therapies

In vivo comparisons employed adult female rats subjected to traumatic SCI by severe contusion of 200 kdynes at the thoracic T8 level. We subjected all animals to a second surgery after one week to transplant iPSC-NSC, MSC, or iPSC-NSC and MSC. We evaluated PA-C treatment by intrathecal delivery for one week in control SCI rats that received no cell therapies and experimental rats that received the iPSC-NSC + MSC treatment. We evaluated rats using the Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale test [48] for nine weeks after SCI to assess functional recovery (Figure 5A). Two weeks after SCI (one week after cell transplantation), we found statistical differences between the iPSC-NSC and the PA-C treated group (* $p < 0.05$) and between the iPSC-NSC and the combinatory cell transplant (iPSC-NSC + MSC; # $p < 0.01$). However, we found no significant differences in any of the experimental groups compared to control SCI rats. Nevertheless, BBB analysis after treatment with iPSC-NSC or MSC led to better overall functionality resulting in faster improvement after the first week of treatment with higher slopes in the BBB curves (Figure 5A, green and dark blue lines); however, animals receiving cell transplantation (iPSC-NSC, MSC, or iPSC-NSC and MSC) reached a plateau in their BBB curves four weeks after injury. PA-C-treated animals displayed continuous improvements over time (Figure 5A, orange line).

To better visualize these improvements, we subdivided the BBB score [48] (Figure 5B) into three different ranges: from 0 to 8 (indicating locomotion without supporting body weight), from 9 to 10 (indicating steps supporting body weight without coordination), and

from 11 to 21 (indicating coordinated stepping). This separate analysis indicates the percentage of animals receiving each treatment that reach the locomotion skills established at each BBB range. As shown in Figure 5B, almost 80% of animals treated with MSC or PA-C reached a BBB range of 11–21. The number of animals receiving iPSC-NSC, MSC, or PA-C treatment reaching the highest BBB range was more significant than the number of animals receiving the iPSC-NSC + MSC and iPSC-NSC + MSC + PA-C treatments (Figure 5B, lower panel). The control SCI group had a higher percentage of animals in the 0–8 range, with only 50% reaching the 11–21 range.

Locomotor function analysis using Catwalk Gait at nine weeks post-SCI (Figure S2) highlighted significant results in iPSC-NSC-treated SCI rats. These animals displayed an increased maximum contact in seconds (registered maximum contact time within the hind paws during free and straight walking) compared to control SCI animals.

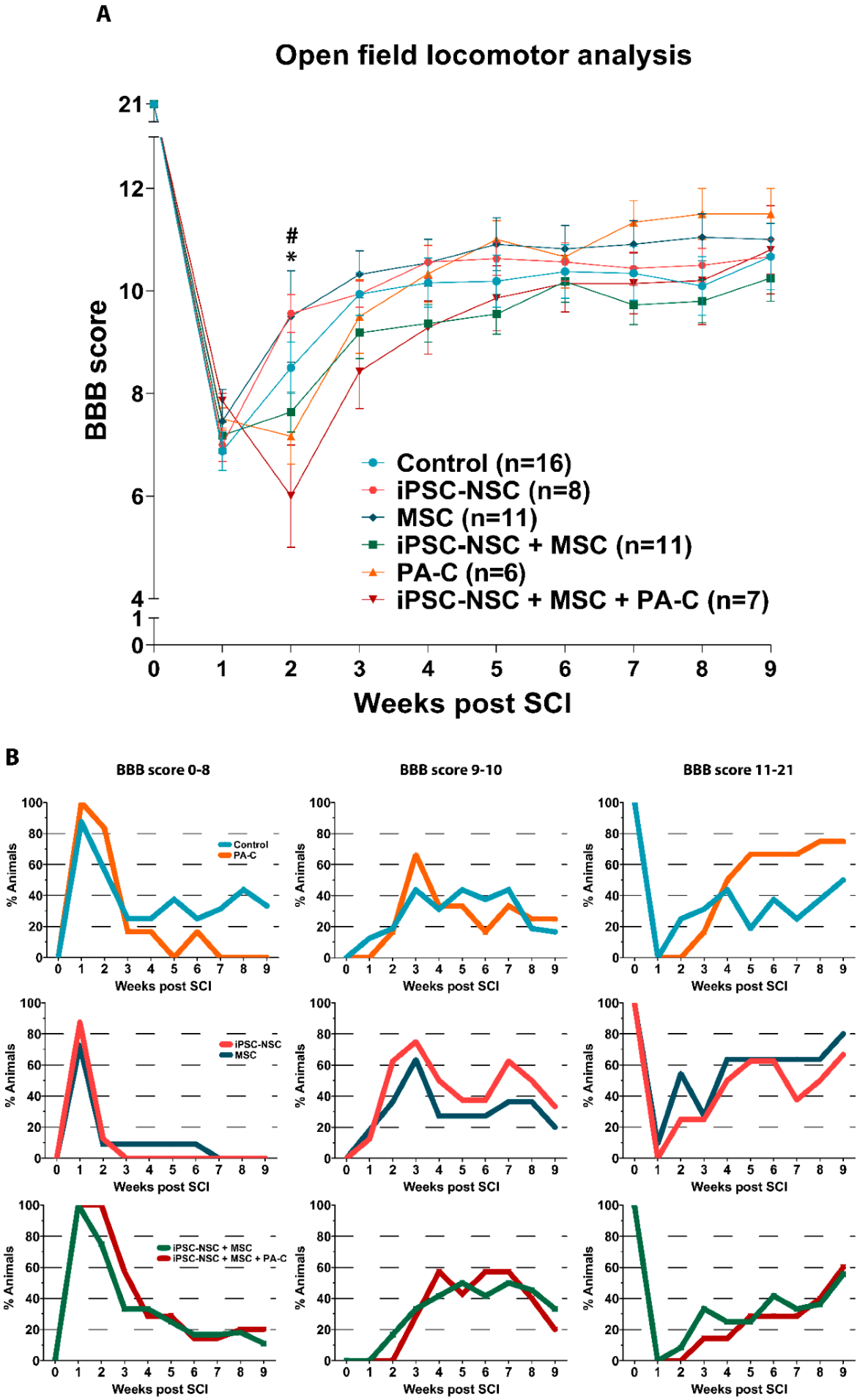

A
Open field locomotor analysis
BBB score
Weeks post SCI
Control (n=16)
iPSC-NSC (n=8)
MSC (n=11)
iPSC-NSC + MSC (n=11)
PA-C (n=6)
iPSC-NSC + MSC + PA-C (n=7)
B
BBB score 0-8
BBB score 9-10
BBB score 11-21
% Animals
Weeks post SCI
Control
PA-C
iPSC-NSC
MSC
iPSC-NSC + MSC
iPSC-NSC + MSC + PA-C

Figure 5. Locomotor recovery after SCI following single treatments and combination therapy. (A). Time course locomotor evaluation by open-field BBB scale over nine weeks post-SCI. (B). BBB locomotor evaluation subdivisions (0–8, 9–10, and 11–21) showing the percentage of animals in each score range. Treatments compared at all post-injury time points. Data expressed as mean ± S.E.M. (control n = 16; iPSC-NSC n = 8; MSC n = 11; iPSC-NSC + MSC n = 11; PA-C n = 6; iPSC-NSC + MSC + PA-C n = 7) determined by two-way mixed model ANOVA with Tukey's multiple comparison test (* $p < 0.05$ iPSC-NSC vs. PA-C; # $p < 0.01$ iPSC-NSC vs. iNSC + MSC).

2.6. PA-C and PA-C Combined with iPSC-NSC + MSC Transplantation Preserve β-III-Tubulin Positive Fibers, Limit Inhibitory Scar Size, and Increase Functional Synapse Number after SCI

We analyzed longitudinal spinal cord sections, including the epicenter of the injury and rostral and caudal segments to the injury, by double immunostaining with β-III-Tubulin (representative images shown in Figure 6A; left panels, green) and GFAP (right panels, red) to quantify the nerve fiber preservation. The quantification of β-III-Tubulin staining (Figure 6B; top panel) revealed a significantly larger positive area present in animals receiving iPSC-NSC + MSC + PA-C treatment than control animals or those receiving iPSCNSC and MSC but not in comparison with PA-C treatment. PA-C-treatment prompted a significantly larger β-III Tubulin-positive area than iPSC-NSC or MSC treatments. Of note, we observed a significant reduction in the number of neuronal fibers following iPSC-NSC or MSC treatment compared to the control or iPSC-NSC + MSC + PA-C treatment.

We also evaluated the size of the inhibitory scar by quantifying the lack of GFAP staining, expressing this value as a percentage of the total quantified tissue area (Figure 6A right panel, dashed line; Figure 6B-bottom panel). PA-C and iPSC-NSC + MSC + PAC treatments significantly reduced scar area compared to other treatments. iPSC-NSC treatment resulted in an increased scar area compared to control; however, iPSC-NSC + MSC or MSC treatments failed to induce any significant alterations.

To evaluate the preservation of functional synapses, we quantified the synaptic bottoms at neuronal somas by co-localizing synaptophysin and NeuN for those treatments providing better outcomes in the previous neuronal fiber preservation analysis (Figure 6C shows representative images of each of the tested staining's from the control group). Analysis of preserved NeuN-positive cells, expressed as the percentage of NeuN-positive area,

demonstrated a significant increase following PA-C treatment compared to control (Figure 6D; left panel). Furthermore, the quantification of colocalization (synaptophysin/NeuN+) normalized to the total NeuN-positive area demonstrated a significant increase following PA-C or iPSC-NSC + MSC + PA-C treatment when compared to control (Figure 6D; right panel).

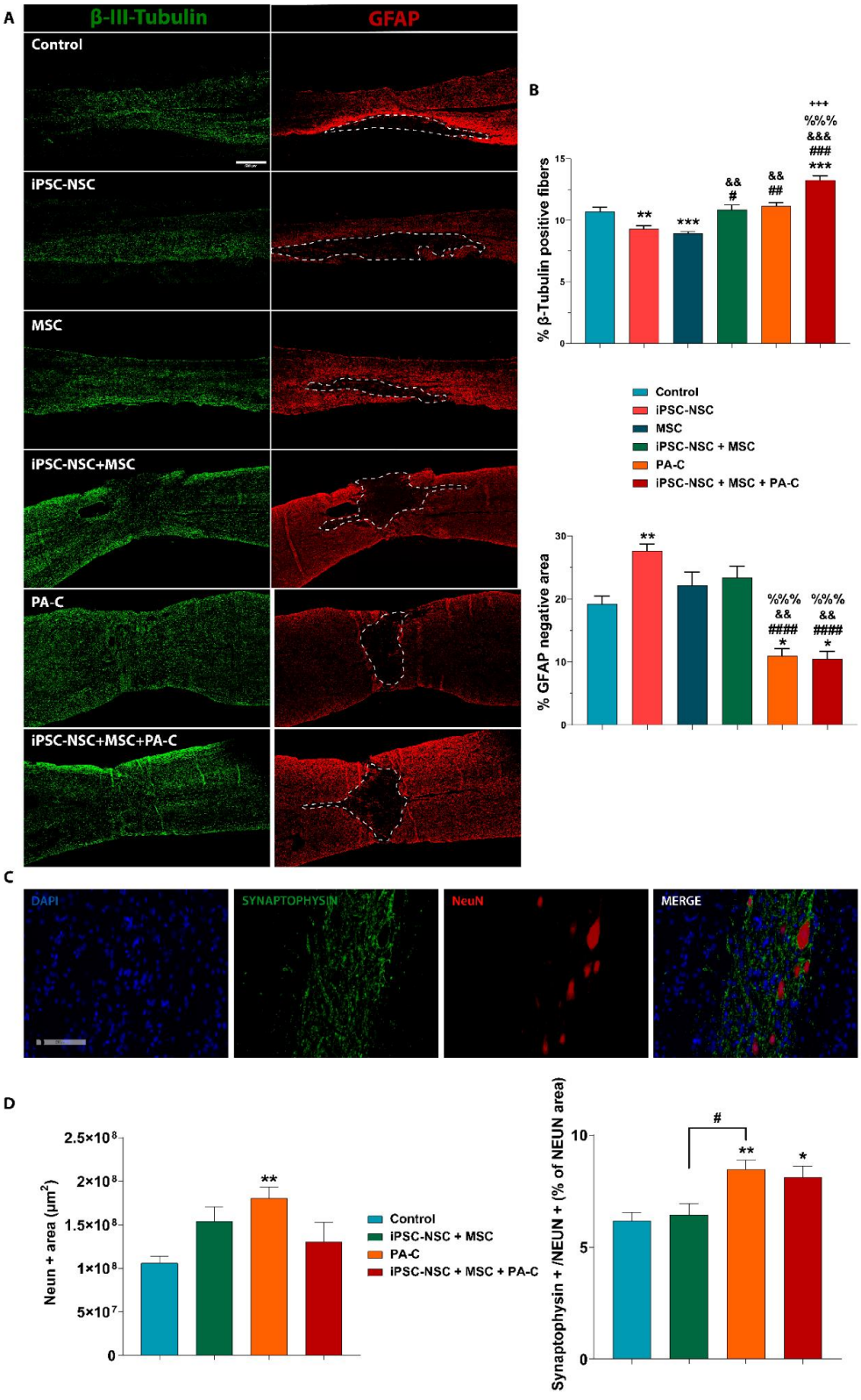


Figure 6. PA-C and PA-C combined with iPSC-NSC + MSC transplantation preserves β-III-Tubulin fibers and synapses and reduces scar size. (**A**) Representative immunofluorescence images (left panel) of β-III-Tubulin (green) and GFAP (red) of longitudinal spinal cord sections, including the injured area nine weeks after SCI (scale bar = 500 μm). Dotted lines delimit the GFAP-negative area. (B). Quantification of β-III-Tubulin-positive fibers (upper panel) and GFAP-negative scar area (lower panel) represented as a percentage of the total analyzed area and expressed as mean ± S.E.M. determined by one-way ANOVA with Tukey's multiple comparison test for β-III-Tubulin and Kruskal–Wallis one-way ANOVA with Dunn's method for GFAP (control, n = 8; iPSC-NSC + MSC, n = 8; n = 4 for iPSC-NSC, MSC, PA-C, iPSC-NSC + MSC + PA-C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs. iPSC-NSC; && $p < 0.01$, &&& $p < 0.001$, vs. MSC, %%% $p < 0.001$ vs. iPSC-NSC + MSC, +++ $p < 0.001$ vs. PA-C. (C). Representative immunofluorescence images of longitudinal spinal cord sections of synaptophysin (green), NeuN (red), and DAPI (blue) staining (scale bar = 200 μ). (D). Quantification of NeuN-positive area (left panel) and functional synapses by analyzing the co-localization of synaptophysin and NeuN (right panel), represented as a percentage of the NeuN-positive area. Quantitative data expressed as mean ± S.E.M determined by one-way ANOVA with Tukey's multiple comparison test (Control n = 4; iPSC-NSC + MSC n = 3; PA-C n = 3; iPSC-NSC + MSC + PA-C n = 3) (* $p < 0.05$, ** $p < 0.01$ vs. control; # $p < 0.05$ as indicated).

2.7. PA-C Combined with iPSC-NSC + MSC Transplantation Induces White Matter Sparing after SCI

We determined white matter sparing by Luxol fast blue (LFB) staining—LFB binds to the myelin sheath lipoproteins and allows quantification of the remaining myelinated areas [49]. LFB analysis (Figure 7A,B) demonstrated that iPSC-NSC and MSC treatments increased spinal cord white matter sparing compared to control and PA-C treatment. Furthermore, iPSC-NSC + MSC + PA-C treatment also increased white matter preservation compared to control and PA-C treatment.

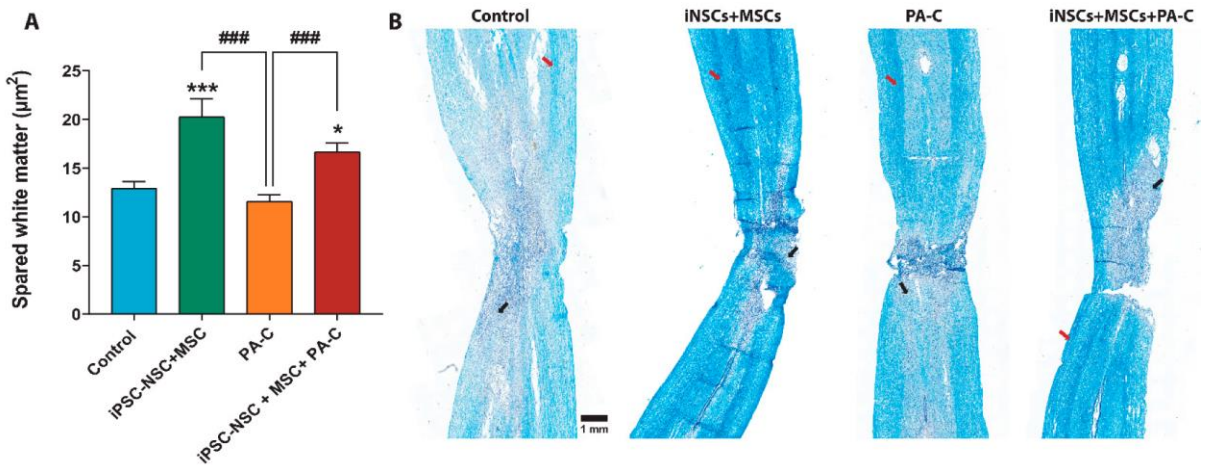


Figure 7. PA-C combined with iPSC-NSC + MSC transplantation increases white matter sparing. (**A**). White matter sparing analysis expressed as mean ± S.E.M determined by one-way ANOVA with Tukey's multiple comparison test (n = 3). * $p < 0.05$, *** $p < 0.001$ vs. control; ### $p < 0.001$ as indicated. (**B**). Representative images of spinal cord longitudinal sections (right panel; scale bar = 1 mm); Black arrows and red arrows indicate demyelinated areas and myelinated areas, respectively.

We also quantified the number of motoneurons (MN) at the epicenter of the lesion (T8), both rostrally (T7) and caudally (T9) (expressed as MN/mm^2), as indicated in the scheme in Figure S4A. The segmented analysis (Figure S4B) demonstrated that iPSC-NSC, MSC, or iPSC-NSC + MSC treatment significantly prevented motoneuron loss compared to the control, PA-C treatment, and iPSC-NSC + MSC + PA-C treatment at the rostral segments (T7). Quantification of motoneurons in caudal segments to the lesion failed to demonstrate significant differences in preservation for any treatment compared to control; however, iPSC-NSC, MSC, or iPSC-NSC + MSC treatment led to more significant levels of motoneuron preservation compared to PA-C treatment. Furthermore, iPSC-NSC treatment increased motoneuron preservation compared to iPSC-NSC + MSC + PA-C treatment (Figure S4C).

2.8. PA-C and PA-C Combined with iPSC-NSC + MSC Transplantation Promotes Microglia Polarization toward an Anti-Inflammatory Profile

We next evaluated the potential *in vivo* anti-inflammatory activity of PA-C or iPSC-NSC + MSC + PA-C by studying the presence of activated microglia with an anti-inflammatory profile by the co-localization of IBA1 expression (Microglia Marker) and Arginase-1 (Arg1) (Figure 8A,B). after quantifying the percentage of cells expressing IBA1, Arg1, or IBA1 and Arg1

(Figure 8C), we found that both treatments (PA-C or iPSC-NSC + MSC + PA-C) increased the presence of anti-inflammatory microglia (IBA1 + Arg1) in the spinal cord nine weeks post-sci.

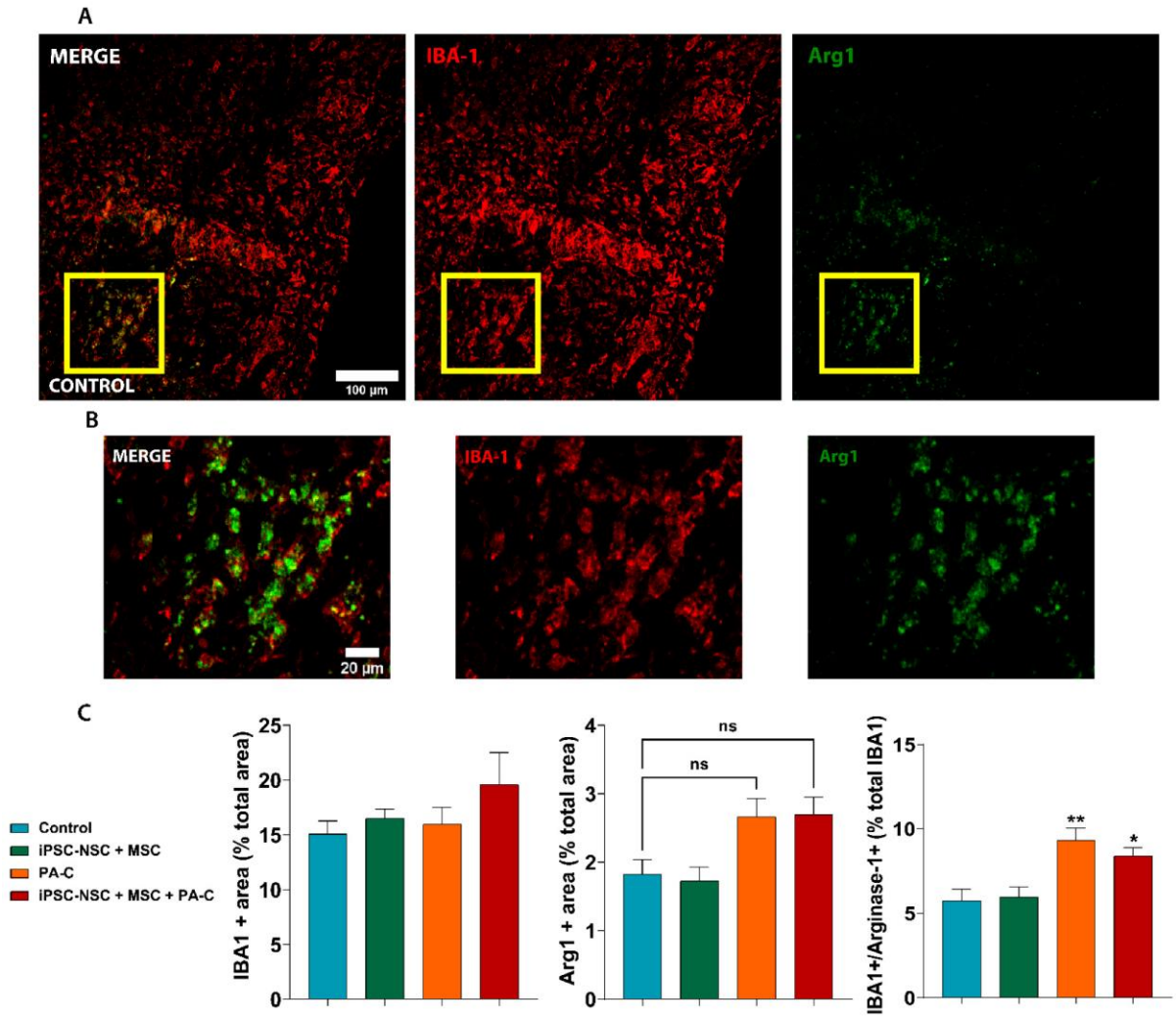


Figure 8. PA-C and PA-C combined with iPSC-NSC + MSC transplantation prompt microglial polarization towards an anti-inflammatory profile. (**A**). Representative immunostaining images of IBA1 (microglia marker; red) and Arg1 (anti-inflammatory marker; green) (scale bar = 100 µm). (**B**). Magnified images of the indicated area in A with a yellow square for IBA1 and Arg1 staining (scale bar = 20 µm) (**C**). Quantification of total microglia, positive for IBA1 (left panel) and the total number of cells positive for Arg1 (central panel), which are represented as the percentage of positive cells of the tissue area analyzed; Right panel represents the percentage of activated microglia, co-expressing IBA1 and Arg1, represented as a percentage of the total microglia (positive for IBA1). Quantitative data expressed as mean ± S.E.M determined by one-way ANOVA with Tukey's multiple comparison test (n = 3). * $p < 0.05$, ** $p < 0.01$ vs. control; ns (non-significant) as indicated.

3. Discussion

Combination therapies have emerged as a promising strategy to inhibit or slow the highly complex and heterogeneous cascade of pathological events following SCI. Combinations of stem cell therapies, including MSC and NSC, have been reported for SCI treatment of SCI, although with contradictory results [50–52]. Park et al. demonstrated that subacute transplant of human NSC led to significant functional recovery; in contrast, human MSC failed to provide any effect alone or in combination with NSC [50]. In another study, the co-transplantation of MSC and NSC enhanced motor function and axon density surrounding the lesion; however, stem cell co-transplantation failed to prompt white matter sparing and induced tumor formation [51]. Finally, Sun et al. demonstrated that the subacute co-transplantation of human NSC and MSC enhanced stem cell survival compared to individual treatments, increased the number of myelinated axons, and improved locomotor function [52]. The use of MSC secretome, alone or in combination with NSC, would provide a successful alternative to cell therapy, since researchers have ascribed paracrine effects to exosomes in the treatment of spinal cord injuries including anti-inflammatory [53], angiogenic [54] and regenerative effects [55], as well as functional recovery [56].

We previously reported that neonatal rat NPCs transplantation combined with local delivery of PA-C, but not the individual treatments, significantly rescued voluntary locomotion in severe and chronic SCI models, increased neuronal preservation, and reduced scarring at the injury site [37,57]. This study aimed to use a clinically relevant combination therapy approach comprising the transplantation of iPSC-NSC and MSC and PA-C treatment. We discovered that this combination therapy confers certain benefits compared with the single treatments, such as preserving neuronal fibers and reducing scar tissue. Unfortunately, these significant improvements failed to translate into functional improvements.

iPSC-NSC tolerated PA-C well in vitro, inducing cell proliferation and neurite elongation, as had been previously demonstrated in rat NSC by Rho/Rock kinase inhibition [37]. Furthermore, curcumin also displayed neurite elongation inducing activity in mouse neural crest-derived cells through inhibition of the proteasome [58], and in cortical neurons, by the concentration-dependent regulation of the Reggie-1 and ERK1/2 pathways [59]. In our *in vitro* model, PA-C induced neurite outgrowth in iPSC-NSC under proliferative conditions; however, PA-C failed to counteract LPA-induced neurite retraction or induce *in vitro*

neurogenesis of iPSC-NSC, as had been previously described in embryonal carcinoma stem cells through the activation of autophagy [60].

After the primary trauma, secondary events cause mitochondrial dysfunction and augment reactive oxygen species formation, leading to cell death [46] and neuronal damage [45,61]. We hypothesized that PA-C pre-treatment might protect iPSC-NSC exposed to cytotoxic doses of hydrogen peroxide, given the previously attributed anti-oxidant and immunomodulatory properties of curcumin [62]. Overall, PA-C counteracted the damaging effects of hydrogen peroxide and increased iPSC-NSC viability, which is a finding supported by a previous study of curcumin treatment in rat NSC [63].

Given the fundamental nature of NF-κB signaling pathway activation to neuroinflammation and SCI pathophysiology [64–66], the modulation of NF-κB signaling could ameliorate inflammation, reduce the impact of the secondary damage stage, and preserve neuronal function. A previous study reported that curcumin reduced neuroinflammation after SCI by specifically suppressing the TLR4/NF-κB signaling pathway [67]. In this study, we established the effect of PA-C on the inflammatory mediators by demonstrating that this pH-responsive nanoconjugate inhibited the LPS-mediated induction of NF-κB signaling and translocation to the nucleus in iPSC-NSC. We also established the potent immunomodulatory properties of MSC *in vitro* by showing how co-culture inhibited NF-κB activation in iPSC-NSC. Gene profiling analysis revealed a significant downregulation of DRD2 in iPSC-NSC after PA-C S treatment in vitro. As H2O2 exposure up-regulates DRD2 in the human SH-SY5Y neuroblastoma cell line through NF-κB nuclear translocation [68], PA-C treatment may downregulate DRD2 expression by inhibiting the NF-κB signaling pathway.

The combination of cellular therapies with or without the addition of PA-C failed to significantly improve locomotor skills after traumatic SCI; however, we did observe noticeable differences in individual parameters. Following the subdivision of the BBB scale, we observed a more rapid recovery following iPSC-NSC or MSC treatments; however, animals soon reached a plateau after the initial functional recovery with regard to functional improvements. Interestingly, animals treated with PA-C alone and PA-C combined with MSC and iPSC-NSC displayed non-significant but increasing improvements until the experimental endpoint. The different severity of the injury may account for the inconsistent result compared to previous

co-transplant studies, in which combination treatments enhanced locomotor recovery [52]. Despite the absence of significant functional locomotion recovery, the histological analysis revealed a relevant benefit of the combination strategy.

Treatment with PA-C combined with MSC and iPSC-NSC significantly preserved β-IIITubulin positive fibers and synaptic buttons and reduced the glial scar area. Notably, PA-C treatment alone also significantly reduced the scar area and increased both the preservation of neurons and the number of synaptic buttons on intact neuronal somas surrounding the injured area compared to control, indicating a significant contribution of PA-C to the neuroprotective effect found in the combinatorial approach.

Previous studies in an Alzheimer's disease model also demonstrated an increase in functional synapses following curcumin administration [69–71]. In this study, we believe that PA-C supported a similar neuroprotective effect after SCI by preventing axonal degeneration and maintaining the synaptic integrity of the neuronal circuits. PA-C may also contribute to the prevention of synaptic dysfunction and degeneration and the formation of novel synaptic connections.

Interestingly, iPSC-NSC or MSC treatment increased motoneuron preservation (in agreement with a previous study [72]), although combined iPSC-NSC and MSC treatment failed to provide significant preservation levels. While a previous study employing ex vivo spinal cord slices demonstrated how curcumin reduced motoneuron apoptosis [73], PA-Ctreatment failed to protect motoneurons. As PA-C treatment increased the preservation of NeuN-positive cells (as previously demonstrated [74,75]), the neuroprotective effect may be attributed to a specific subset of neurons, as previously shown by Seo et al. in a model of Alzheimer's disease, which cannot be identified in the quantification performed here using non-specific neural cell fate identification [76].

We established a significant reduction in demyelination, which occurs due to the progressive oligodendrocyte cell death after SCI [77], following treatment with either iPSCNSC or MSC, but not PA-C. Previous studies found that NSC [78] and iPSC-NSC [79] undergo oligodendrocytic differentiation and remyelinate axons in vivo. Thus, transplanted iPSC-NSC may contribute to the remyelination by differentiating into oligodendrocytes, although MSC

may induce endogenous oligodendrocytic differentiation by secreting factors such as neurotrophins. Previous studies describing the transplantation of NSC and bone-marrow MSC in SCI models support this hypothesis [80,81].

Prolonged inflammation and a lack of injury resolution represent some of the significant impediments to functional regeneration after SCI [82]. Hence, we investigated whether the neuroprotective effects following our various treatment strategies may derive from reduced astrogliosis [83] or altered microglia polarization [84]. After SCI, astrocytes undergo cellular, molecular, and functional changes; they also contribute to the release of inhibitory extracellular matrix components that impair axonal regrowth [85]. While we found that stem cell transplantation had no significant inhibitory effect on glial scar formation (indeed, iPSC-NSC may have contributed to an increase in glial scar area) (Figure 6A), PA-C treatment alone or in combination with iPSC-NSC and MSC transplantation significantly reduced the glial scar area following SCI.

Several signal transduction pathways are involved in astrogliosis, including STAT and NF-κB [86–88], and a recent study demonstrated that curcumin might reduce astrogliosis and glial scar formation after SCI through the inhibition of STAT3 [89] and NF-κB [90] pathways. Therefore, the anti-inflammatory activity of PA-C may contribute to a reduction in glial reactivity through the inhibition of NF-κB signaling, as shown in our *in vitro* models.

Histological analysis revealed that PA-C treatment or PA-C treatment in combination with iPSC-NSC and MSC transplantation prompted an increase in Arg1 expression levels, which is an M2 microglia marker associated with an anti-inflammatory polarization profile [91], among the total microglial cells found within the injury site [92,93]. LPS stimulation *in vitro* induces M1 phenotype polarization; however, curcumin can reverse this polarization and increase the expression of M2-associated biomarkers through the TLR4– NF-κB signaling pathway [94]. These previous studies agree with our *in vitro* LPS experiment in which PA-C reduced the translocation and activation of NF-κB. Thus, PA-C may drive microglial polarization by modulating the NF-κB pathway. While MSC also drive M2 polarization after acute SCI [30], the transplantation of iPSC-NSC and MSC failed to increase M2-polarized microglia post-SCI, with only the addition of PA-C affecting microglia polarization.

The complex pathological nature of SCI requires the implementation of a multifaceted and versatile therapeutic perspective regarding the development of treatments. A combined treatment comprising PA-C, iPSC-NSC, and MSC provides immunomodulatory and neuroprotective effects to prevent axonal degeneration, neuronal death, and loss of neuronal connectivity. Furthermore, this combinatorial strategy reduced the injury area and prevented the expansion of the glial scar in chronic stages, thereby providing a versatile and clinically relevant approach to treating acute SCI.

4. Materials and Methods

4.1. Human iPSC-NSC and MSC Culture Conditions

Human iPSC-NSC were generated as previously described [20]. Briefly, human iPSC were generated using synthetic mRNA transfection of CYCLIN D1 plus base reprogramming factors (OCT3/4, SOX2, KLF4, LIN28), which results in a significantly improved genetically stable footprint in human iPSC. This strategy enables the more accurate and reliable generation of human iPSC for disease modeling and future clinical applications. Ectoderm-like differentiation for iPSC-NSC generation used the PSC Neural Induction Medium kit (A1647801, Life Technologies, Carlsbad, CA, USA) and Geltrex-coated plates (A1413201, Life Technologies). After neural induction, iPSC-NSC were maintained in growth medium: STEMdiff™ Neural Progenitor Medium (05835, STEMCELL™, Vancouver, BC, Canada) supplemented with 200 U/mL penicillin (09-757F, LONZA, Basel, Switzerland) and 200 µg/mL streptomycin (09-757F, LONZA) in Geltrex-coated plates in standard cell incubation conditions. For iPSC-NSC expansion and sub-culture, cells were detached by enzymatic digestion with TrypLETM Select (12563-011, Thermo Fisher Scientific, Waltham, MA, USA) when cultures reached 80% confluence. Human adipose-derived MSC were obtained from surplus suprapatellar fat as previously described [95] and ≈ 6500 cells/cm2 were cultured at 37 ◦C in High Glucose DMEM (SH30022.01, Cytiva HyCloneTM, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (F7524, Sigma-Aldrich, Darmstadt, Germany), 2 mM L-glutamine (H3BE17-605E, LONZA), 0.3 g/mL glucose (G7021, Sigma-Aldrich), 200 U/mL penicillin, and 200 µg/mL streptomycin. MSC were dissociated with trypsin after reaching 80% confluence and expanded for up to ten passages.

4.2. Analysis of Cell Viability, Differentiation, and Neurite Outgrowth in iPSC-NSC

iPSC-NSC were characterized for neural feature markers after seeding on Geltrexcoated coverslips at a cell density of 20,000 cells per well and allowed to expand for 24 h. The expression of neural development markers was analyzed using conventional immunocytochemical staining protocol as described in Section 4.4.

PA-C toxicity and iPSC-NSC tolerance evaluated employed a dose–response experiment, and the MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used to evaluate mitochondrial activity as an indirect measure of cell metabolic activity and thus used as a cell viability assay following the manufacturer's instructions (ab197010, Abcam plc, Cambridge, UK). iPSC-NSC seeded at a density of 1.5 × 104 cells/well on a Geltrex-coated 96-well plate with growth medium were allowed to attach and expand for 24 h and then treated with increasing concentrations of PA-C (3.8 w/w [37]), 5, 10, 12.5, 15, 17.5, and 20 µM for 24 h. Optical density was measured at 490 nm using a microplate reader Victor 2 (Perkin Elmer Inc., Waltham, MA, USA).

The effect of PA-C and C (B3347, TCI Europe, Zwijndrecht, Belgium) on neurite elongation in iPSC-NSC were also examined. Fas (F-4660, LC Laboratories, Woburn, MA, USA) and PGA-SS-Fas (Patent n◦ WO/2020/193802) were also used as positive controls for neurite elongation [96]. iPSC-NSC were seeded at a density of 6 × 104 cells/well on Geltrex-coated coverslips 24 h before treatment with 10 µM C or PA-C and 50 µM Fas or PGA-SS-Fas. Neurite retraction was induced by adding 10 µM of LPA (L726, Sigma-Aldrich) and incubating the cultures for an additional 24 h. Neurite outgrowth was quantified from at least six fields (10×) of three independent experiments from β-III-Tubulin positive cells using the NeuriteJ plug-in from ImageJ as previously described [97], and the average neurite length is represented.

Neuronal differentiation was evaluated by immunostaining and PCR, using the RT2 Profiler PCR Array for human neurogenesis (PAHS-404ZA, Qiagen, Hilden, Germany). iPSC-NSC were seeded on Geltrex coated coverslips in P24 plates at a density of 6 × 104 cells/well in growth medium for immunostaining and in P6 plates at a density of 6 × 105 cells/well in growth medium for RNA extraction. Cells were incubated overnight before treatment with 10 µM PA-C or vehicle (PBS) for an additional 24 h.

The capacity of PA-C to prevent oxidative damage was evaluated using a toxic concentration of hydrogen peroxide to partially mimic the hostile environment generated at the injury site after SCI. NSC were seeded at a density of 1.5 × 104 cells/well on a Geltrex-coated 96-well plate in growth medium and allowed to attach and expand for 24 h. Then, iPSC-NSC were treated with increased concentrations of PA-C (5, 10, 12.5, 15, 17.5, and 20 µM) for 30 min before exposure to 75 or 100 µM of H2O2 and incubated for an additional 24 h before MTS was performed. To elucidate the neuroprotective effect of PA-C on the survival of neural lineages, iPSC-NSC were seeded on Geltrex-coated coverslips in P24 plates at a density of 6 × 104 cells/well with growth medium and allowed to attach and expand for 24 h and then pre-treated with 10 µM PA-C for 30 min before exposure to 75 µM of H2O2 and incubated for an additional 24 h.

4.3. NF-κB Activation Assay in iPSC-NSC + MSC Co-Cultures Treated with PA-C

To assess the potential immunomodulatory properties of PA-C, iPSC-NSC were incubated alone on Geltrex-coated coverslips in P24 plates at a density of 6 × 104 or in co-culture with 2 × 104 MSC, seeded on Matrigel-coated ThinCert cell culture inserts (662640, Greiner Bio-One, Madrid, Spain) with 0.4 µm of pore size at a density of 2 × 104 in growth medium. Cell cultures were treated with 10 µM PA-C or vehicle for 24 h and then stimulated with 1 µg/mL LPS (L8274, Sigma-Aldrich) for an additional 24 h to activate NF-κB and nuclear translocation was evaluated by NF-κB immunostaining.

4.4. Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde (PFA) for 15 min, washed three times with phosphate buffer solution, and blocked with 5% normal goat serum (Thermo Fisher) and 0.1% Triton X-100 (9036-19-5, Merck Millipore, Darmstadt, Germany). Cells were incubated with primary antibodies overnight at 4 ◦C in a humidified chamber. The primary antibodies used were mouse anti-β-III-Tubulin (1:400; MO15013, Neuromics, Edina, MN, USA), chicken anti-GFAP (1:1000; PA1-10004,Thermo Fisher), mouse antiNFκB (p65) (1:400; sc-8008, Santa Cruz, Dallas, TX, USA), guinea pig anti-DCX (1:400ab5910, Chemicon, Temecula, CA, United States), anti-Pax-6 (PAX6) (1:400; PRB-278P, Biolegend, San Diego, CA, USA), rabbit anti-SOX2 (1:400; MAB5326, Abcam), mouse antiNestin (1:400; MAB5326, Sigma

Aldrich), mouse anti-Notch-1 (1:400; AF1057, R&D System, Minneapolis, MN, USA), mouse anti-O4 (1:200; MAB345, Sigma-Aldrich), and mouse antiFOXJ1 (1:300; 14-9965-82, Thermo Fisher). For secondary antibodies, AlexaFluor 488, 555, and 647 (Invitrogen, Carlsbad, CA, USA) conjugated with respective IgGs were used at a dilution of 1:400 and incubated for 1 h at room temperature. Samples were counterstained with DAPI (1:1000) for 5 min and finally mounted using FluorSaveTM reagent (EMD Millipore, Burlington, MA, USA). Immunofluorescence images were captured from *in vitro* experiments using Zeiss ApoTome microscope (Carl Zeiss) and analyzed using ImageJ.

4.5. RNA Extraction, cDNA Synthesis, and RT2 Profiler PCR Human Neurogenesis Array

For transcriptomic analyses, total RNA was extracted using TRIzol® reagent (15596026, Thermo Fisher Scientific) according to the manufacturer's instructions. RNA concentrations were determined with a NanoDrop spectrophotometer (ND1000, NanoDrop Technologies, Wilmington, DE, USA) and reverse transcribed using the high-capacity RNA-to-cDNA™ kit (4368814, Applied Biosystems, Foster City, CA, USA) with 2.5 µg of total RNA, in a reaction volume of 20 µL, through incubation at 37 ◦C during 120 min using random hexamer primers.

PCR was performed using the RT2 Profiler PCR Array for human neurogenesis (PAHS-404ZA, Qiagen, Hilden, Germany). Following the manufacturer's instructions, we used 27.17 ng of cDNA for quantitative PCR in a total volume of 25 µL using AceQ SYBR qPCR Master Mix (Q111-02, VazSyme Biotech Co., Nanjing, China) on a LightCycler 480 Instrument (Roche, Basel, Switzerland). The reaction sequence included 10 min of incubation at 95 ◦C, followed by 40 cycles of 20 s at 95 ◦C, 20 s at 60 ◦C, and 20 s at 72 ◦C. The manufacturer's template was used for gene expression analysis of genes involved in the neural cell fate determination, differentiation, and apoptosis. Relative gene expression levels were calculated using the ΔΔCt method, and the results were expressed with reference to the arithmetic mean of three reference housekeeping genes (Actin Beta (ACTB), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and Hypoxanthine Phosphoribosyltransferase 1 (HPRT1)). Fold change was calculated by normalizing the expression rates of the treatment to the control sample. Genes with aberrant melting curves were discarded. Real-time PCR arrays were performed from at least three independent *in vitro* experiments. Paired Student's t-tests were

used to compare differences in gene expression between control iPSC-NSC and PA-C-treated iPSC-NSC.

4.6. Contusive Spinal Cord Injury, Cell Transplantation, and PA-C Delivery In Vivo

Female Sprague–Dawley rats weighing ≈300g were housed under controlled temperature under a 12 h light/dark cycle with ad libitum access to food and water. Animals were treated with morphine (2.5 mg/kg b.w s.c.) 30 min before surgery. Deep anesthesia was induced with 3% isoflurane and then maintained at 1.5–2% during surgery. Laminectomies were conducted at thoracic segments T7–T9 to perform a moderate contusion at T8 by applying 200 kdyn in all animals (n = 72) using the Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation, LLC) as previously described [98]. Animals were randomly distributed into the following groups: control (intramedullary injection of cell vehicle cell culture medium; and intrathecal administration of saline 0.9%), iPSC-NSC (intramedullary injection), MSC (intramedullary injection), iPSC-NSC + MSC (intramedullary injection of both iPSC-NSC and MSC), PA-C (intrathecal administration of PA-C), and iPSC-NSC + MSC + PA-C (intramedullary injection of both iPSC-NSC and MSC combined with intrathecal administration of PA-C). Stem cell transplants and/or PA-C treatment were performed seven days post-SCI. iPSC-NSC and/or MSC were collected and suspended in 14 µL of DMEM/F12 (11320033, Gibco, Grand Island, NY, USA) at a concentration of 130,000 cells/µL, or mixed 1:1 for co-transplantation, for a total of 1.8 × 106 cells per animal. Control and PA-C treated animals received culture medium injections (DMEM/F12-14 µL) instead. Cell suspensions or vehicle were intramedullary injected at the epicenter of the lesion (6 µL), and 2 mm rostral (4 µL) and caudal (4 µL) was administered to the lesion site at a rate of 2 µL/min using a siliconized pulled glass needle (100 µm internal diameter) coupled to a 10 µL Hamilton syringe controlled by an automatic injector. Immediately after transplantation, a catheter connected to an osmotic pump (model 1007D, Alzet®, Cupertino, CA, USA) was introduced into the intrathecal space from the fifth lumbar segment to guarantee sustained local delivery of PA-C. The pump delivered 1 µL/h for seven days and was filled with 100 µL of 4 mM PA-C diluted in 0.9% saline. After seven days, pumps and catheters were removed.

As previously described, all animals were subjected to post-surgery care, including passive and active rehabilitation protocols [98]. To prevent immune rejection of the

allogeneic cell grafts, animals received daily subcutaneous injections of the immunosuppressant FK506 (1 mg/kg) starting one day before transplantation, which was maintained for one month.

4.7. Functional *In Vivo* Locomotion Analysis

Locomotor recovery was evaluated using the open-field BBB locomotor scale [48] and the video-based system for automated gait analysis CatWalk® (Noldus, Asheville, NC, USA). Animals were individually videotaped for 4 min, and two unbiased observers blindly scored the results. CatWalk analysis was performed in the ninth week. Paw contact was quantified by counting high-intensity pixels as the mean of at least three rounds per analysis [99].

4.8. Histological Studies of Spinal Cords

At week nine after SCI, animals were irreversibly anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and fentanyl (0.05 mg/kg) and transcardially perfused with 0.9% saline immediately followed 4% PFA in 0.1 M phosphate buffer (pH 7.4). Spinal cords were dissected and post-fixed in 4% PFA for 5 h and then conserved in 0.1 M phosphate buffer containing 0.01% sodium azide. Thoracic segments, including T6 to T10, were either cryopreserved, immersed in 30% sucrose before inclusion in Tissue-Teck OCT (Sakura Finetek Europe BV, Flemingweg, Netherlands), and stored at −80 ◦C until cryo-sectioning or dehydrated and included in paraffin, placed in histology cassettes, and processed on a Leica ASP 300 tissue processor (Leica Microsystems, Nussloch, Germany). Then, 8 µm thick longitudinal sections in the horizontal plane were cut on a cryostat or microtome and mounted on gelatin-coated slides, with six series collected.

One complete series was employed to perform hematoxylin/eosin (H/E) staining using an automated station (Autostainer XL Leica, Leica Biosystems, Wetzlar, Germany), scanned on the Aperio Versa scanner (Leica Biosystems), and analyzed using the Aperio ImageScope software (Leica Biosystems). H/E staining was used to determine the anatomical structure and quantify preserved motoneurons nine weeks post-SCI. Spinal cord topography previously described by Waibl [100] was employed to identify the epicenter of the lesion. The center of the T8 thoracic segment was identified as the lesion epicenter, with T7 as the rostral site to the lesion and T9 as the caudal site to the lesion. T7 and T9 were further subdivided into 1

mm segments (as shown in Figure 6A). Motoneurons were quantified considering previously described criteria: polygonal cells with a diameter of ≥20 μm located at the ventral horns (extensively reviewed at [101]). The absolute number of motoneurons was determined along the thoracic segments T7, T8, and T9, measured every 1 mm, and normalized to the area analyzed (data expressed as MN/mm2).

The analysis of the amount of spared white matter and the quantification of myelin preservation used LFB (IW-3005, IHC World, LLC, Ellicott City, MD, USA) staining on longitudinal spinal cord sections. Spinal preparations were scanned on the Aperio Versa scanner (Leica Biosystems). For analysis, images were adjusted to threshold by color, saturation, and brightness to select the signal determined by myelin axons in the white matter and analyzed using the “analyze particles” function of ImageJ. The signal-positive area was normalized to the total area of spinal cord tissue.

The glial scar area was analyzed by quantifying the lack of GFAP staining and expressed as the percentage of the total spinal cord area analyzed (establishing a fixed area analyzed in all samples; 8 mm length). For the quantification of preserved neuronal β-IIITubulin fibers, the background signal was first subtracted; then, images were adjusted to threshold by color, saturation, and brightness to select the specific signal and analyzed using the “analyze particles” function of ImageJ. The signal-positive area was normalized to the total area of spinal cord tissue analyzed.

To evaluate the functional synapsis, the co-localization of Synaptophysin and NeuNpositive signal (NeuN+/Synaptophysin+) was quantified at the epicenter of the injury analyzed using the “analyze particles” function of ImageJ. The positive NeuN positive area was used to estimate the preservation of neurons in the injured spinal cord.

To assess the grade of microglia polarization at the injury area, double staining of IBA1 (a marker of microglia) and Arg1 (an anti-inflammatory marker) (IBA1+/Arg1+) was quantified using the “analyze particles” function of ImageJ and positive area was normalized to the total area of spinal cord tissue analyzed.

For the immunohistochemistry analysis, background signal was first subtracted, and images were adjusted to a threshold by color, saturation, and brightness to select the specific signal prior to each analysis.

The long-term survival of iPSC-NSC and MSC in the spinal cord was evaluated at week nine by immune detection of human mitochondria for iPSC-NSC and detection of human mitochondria and the specific mesenchymal cell marker CD-105 for MSC (Figure S3).

Immunofluorescence for paraffin-embedded spinal cord sections required prior dewaxing, rehydration, and antigen retrieval (immersion in tris-EDTA buffer (10 mM Tris, pH 9.0) for 25 min at 97 ◦C) steps. Longitudinal sections were incubated with blocking solution (5% horse serum, 10% fetal bovine serum in phosphate buffer solution with 0.1% Triton X-100) for 1 h at room temperature and incubated with primary antibodies overnight in a humidified chamber. The following primary antibodies and the indicated dilutions were used: mouse anti-β-III-Tubulin (1:400; MO15013, Neuromics), rabbit anti-GFAP (1:1000; PA1-10004, Dako Denmark A/S, Glostrub, Denmark), chicken anti-NeuN (1:400; ABN91, EMD Millipore), rabbit anti-IBA-1 (1:500; 019-19741, Wako Chemicals USA Inc., Bellwood, VA, USA), mouse anti-Arg1 1 (1:400; sc-271430, Santa Cruz), mouse anti-synaptophysin (1:400; sc-17750, Santa Cruz), mouse anti-human mitochondria (1:400; MAB1273, Chemicon International Inc., Temecula, CA, USA), and anti-CD-105 (1:200 ab53321, Abcam). AlexaFluor 488, 555, and 647 (1:400; Invitrogen) conjugated with secondary antibodies against the respective IgG were used. We stained nuclei with DAPI (1:1000) and mounted samples using FluorSaveTM Reagent (EMD Millipore). H/E, LFB, and immunofluorescence sections were scanned using the Aperio Versa scanner (Leica Biosystems) and analyzed using the Aperio ImageScope software (Leica Biosystems) or ImageJ.

4.9. Ethical Statement

All animal experiments were undertaken in accordance with guidelines established by the European Communities Council Directive (210/63/EU) and the Spanish regulation 1201/2005. All experimental procedures were approved by the Animal Care and Use Committee of the Research Institute Prince Felipe (2021/VSC/PEA/0032) and the Ethics

Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (procedure #10121). All animals were managed by professionally trained staff.

The generation of iPSC and their implementation in this study were approved by the Dirección General de Investigación y Alta Inspección Sanitaria (Generalitat Valenciana, Valencia, Spain) with reference number 4/2020.

Human adipose tissue was obtained from surplus fat tissue during knee prosthesis surgery under sterile conditions. The human samples were anonymized, and the experimental procedure was previously evaluated and accepted by the Regional Ethics Committee for Clinical Research with Medicines and Health Products following the Code of Practice 2014/01. As exclusion criteria, no samples were collected from patients with a history of cancer or infectious diseases at the time of the surgery (viral or bacterial). All human patients voluntarily signed an informed consent document for the use of the adipose samples.

4.10. Statistical Analyses

Statistical analyses were performed using Graph Pad Prism Software (GraphPad Software, San Diego, CA, USA). Outliers were identified and removed by the ROUT method provided by Graph Pad Prism Software, and normality was assessed (ShapiroWilk, Kolmogorov–Smirnov, D'Agostino and Pearson, and Anderson–Darling tests) before performing the corresponding statistical analyses. p-value less than 0.05 was considered significant, and was indicated as followed: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/ijms22115966/s1, Figure S1: PA-C protects against H2O2 -induced cytotoxicity in iPSCNSC, Figure S2: Catwalk gait analysis, Figure S3: Long-term survival of stem cells transplants in the injured spinal cord, Figure S4: Individual iPSC-NSC and MSC transplantation increases motoneuron preservation, Table S1: *In vitro* effects of 24 h 10 µM PA-C treatment on genes involved in neurogenesis, cell cycle, and apoptosis in iPSC-NSC.

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and M.J.V.; data curation, P.B., J.H., E.G., M.A.G.-P., J.H., H.E.; writing—original draft preparation: P.B. and V.M.-M.; draft review and editing analysis P.B., E.G., J.H., H.E., X.N. and V.M.-M.; funding acquisition, V.M.-M., X.N. and M.E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines established by the European Communities Council Directive (210/63/EU) and the Spanish regulation 1201/2005. All experimental procedures were approved by the Animal Care and Use Committee of the Research Institute Prince Felipe (2021/VSC/PEA/0032) and the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (procedure #10121).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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3.2. CHAPTER 2: Elkhenany, H., Bonilla, P., Giraldo, E., Alastrue Agudo, A., Edel, M. J., Vicent, M. J., ... & Manzano, V. M. (2021). A Hyaluronic Acid Demilune Scaffold and Polypyrrole-Coated Fibers Carrying Embedded Human Neural Precursor Cells and Curcumin for Surface Capping of Spinal Cord Injuries. Biomedicines, 9(12), 1928.

My contribution to the manuscript of which I am co-author consisted of histological studies and analysis of the spinal cord using immunohistochemical techniques. With this, I determined the number of surviving cells after transplantation, the migration of transplanted cells from the biomaterial to the tissue, the preservation of fibers in the spinal cord parenchyma and the size of the glial scar. In addition, I had a very relevant contribution in the writing and elaboration of the scientific manuscript and in the preparation of the figures, as well as in the subsequent corrections prior to publication.

A Hyaluronic Acid Demilune Scaffold and Polypyrrole-coated Fibers Carrying Embedded Human Neural Pre-cursor Cells and Curcumin for Surface Capping of Spinal Cord Injuries

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Abstract: Tissue engineering, including cell transplantation and the application of biomaterials and bioactive molecules, has provided a promising approach for spinal cord injury (SCI) regeneration. In this study, we designed a combinatorial tissue-engineered approach to the minimally-invasive treatment of SCI - a hyaluronic acid (HA)-based scaffold containing polypyrrole-coated fibers (PPY) and the RAD16-I self-assembling peptide (Corning® PuraMatrix™ Peptide Hydrogel [PM]) containing human-induced neural progenitor cells (iNPCs) and a curcumin nanoconjugate (CURC). In vitro experiments reveal that PM alone or

in combination with CURC preserves iNPC viability and reduces apoptosis after five days in culture compared to non-embedded iNPCs. Furthermore, the combination of PM and CURC enhances the outgrowth of Nes-tin-positive neurites from iNPCs. Treatment of spinal cord organotypic cultures demonstrates that CURC enhances cell migration and prompts a neuron-like mor-phology of embedded iNPCs implanted over the tissue slices. Following sub-acute SCI by traumatic contusion in a rat model, the implantation of PM-embedded iNPCs and CURC with PPY fibers supported by a significant increase in neuro-preservation (as measured by greater βIII-tubulin staining of neuronal fibers) and decrease in the in-jured area (as measured by the lack of GFAP staining). This combination therapy also restricted platelet-derived growth factor expression, indicating fibrotic pericyte inva-sion. Overall, these findings support PM-embedded iNPCs with CURC embedded within the HA-PPY scaffold as a minimally-invasive combination-based alternative to cell transplantation alone.

Keywords: spinal cord injury; PuraMatrix hydrogel; hyaluronic acid; biomaterials; in-duced neural progenitor cells; curcumin

1. Introduction

Spinal cord injury (SCI) treatment remains a significant challenge due to the complexity and dynamic nature of the intrinsic pathological cascades that occur immediately after the primary lesion and progress to the permanent loss of neuronal activity and the creation of anatomical impediments to treatment application. Pre-clinical studies of complementary neuroprotective and neuroregenerative interventions have provided evidence that such approaches can limit the progression and amplification of the initial damage and rescue neurological deficits (as reviewed by Ahuja and Fehlings [1]). Secondary injury damage beginning soon after the initial trauma results in massive neuronal and glial cell death (including oligodendrocytes), prompting demyelination and expanding the loss of efficient neuronal connectivity to additional neuronal tracts. Persistent inflammatory cell infiltration enhanced by the loss of selective vascular permeability also promotes the continual formation of toxic cystic microcavities. Overall, the early interruption of this second wave of damage may reduce injury severity and, therefore, prompt better functional preservation and prognosis.

The formation of a permissive platform bridging the extrinsic inhibitory microenvironment characteristic of the injured spinal area via cell transplantation could afford collateral neuroplasticity and neuronal regeneration [1]. The transplantation of neural progenitor cells derived from human induced pluripotent stem cells (iNPCs) [2] has provided promising results in terms of repair and neuronal regeneration in rodent [3,4] and primate [5,6] models; however, the limited functional and anatomical improvements obtained in rat models (with poor cell survival being a significant contributor) suggest that iNPC transplantation alone would fail to provide sufficient gain of function. Therefore, the ongoing clinical development of cell transplantation approaches requires additional supportive complementary therapeutic strategies.

The harsh host immune response faced by transplanted cells, which limits survival and engraftment in the host tissue, represents another significant therapeutic obstacle; however, embedding or encapsulation using protective agents may minimize this impact [7]. The biodegradable hydrogel PuraMatrix (PM), the commercial name for the RAD16-I self-assembling peptide hydrogel, represents a candidate for nervous tissue engineering given its ability to support cell proliferation and differentiation [8–10] and its biocompatible, biodegradable, and conductive nature [10,11]. PM also provides a suitable three-dimensional (3D) microenvironment for iNPCs to promote differentiation into neurons and astrocytes, nerve regeneration, myelination, and axon regrowth across the SCI lesion [12]. Recently, transplantation studies with PM-embedded human NPCs demonstrated enhanced cell survival and differentiation, reduced lesion volume, and improved neurological functions in a model of SCI [13].

Our recent studies demonstrated the synergism of iNPC transplantation with a nano conjugated form of the anti-inflammatory molecule curcumin (CURC), which provided long-term drug release and an increase in tissue bioavailability thanks to conjugation to a polyacetal polymer via a pH-responsive linker [14]. The intrathecal delivery of CURC and the intramedullary transplantation of iNPCs and human mesenchymal stem cells polarized microglia towards an anti-inflammatory profile, reduced the extent of the injured area, and increased neuronal fiber preservation, thereby providing a more versatile approach for acute SCI treatment [14].

The intramedullary administration of cell therapies has been intensely debated in terms of clinical application [15]. The spinal cord architecture, surrounded by the vertebrae body and the meningeal layers, confers a critical intrinsic anatomical limitation to local treatment. Additionally, invasive interventions in the soft tissue of the spinal cord can lead to additional tissue damage and neurological dysfunction [16]. Thus, the clinical application of therapeutics requires the development of minimally invasive approaches that reduce tissue manipulation.

Surface capping of SCI has been proposed as a minimally invasive cell and drug delivery approach [17]. This technique requires the integration of biocompatible, non-toxic, and biodegradable biomaterials to safely deliver and transfer cells compatible with the spinal cord soft tissue anatomy. Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, meets the biocompatibility requirements for soft tissue [18–21]. We previously discovered that a highly porous HA demilune (crescent-moon-shaped) scaffold containing polylactic acid (PLA) fibers in the internal lumen seeded with NPCs derived from neonatal rat spinal cord tissue successfully preserved neural tissue with minimal cyst and scar formation [17]. The greater hydrophobicity of PLA fibers than HA allows the adsorption of extracellular matrix proteins, which supports integrin-mediated adhesion of cells and the formation of focal adhesions that promote cell survival. Integrins directly activate survival pathways via the phosphoinositide 3-kinase and mitogen-activated protein kinase pathways [22]. Hence, the characteristics of HA and PLA robustly influence cell morphology and adhesive response [23]. As we recently demonstrated, HA demilune scaffolds filled with PLA fibers coated with polypyrrole (PPY) favor axonal growth and the guidance of dorsal root ganglia explants [21]. Consequently, PPY's application is relevant to biomedical applications, especially in nerve tissue engineering, thanks to its biocompatibility, high electrical conductivity, long-term environmental stability, low cost, and accessible synthesis by chemical or electrochemical polymerization [24–27]. These features qualify PPY as a potentially crucial factor in neural regeneration approaches [28,29]. It is of note that PPY is an intractable solid with very low mechanical processability, which limits direct application as a substrate with a specific topography [25]; therefore, many approaches employ PPY as a surface coating for polymers with better mechanical processability, thereby exploiting the electrical conductivity of PPY and the mechanical properties of a support polymer [30,31]. In this study, we chose PLA as a support polymer, as

its easy mechanical processability allows the production of biocompatible and biodegradable microfibers [32,33].

The present study characterized a novel combinatory cell therapy approach by embedding iNPCs and CURC in a PM hydrogel seeded into a biocompatible capping-like HA scaffold containing PPY-coated PLA guiding fibers. This biocomposite supported minimal invasiveness during implantation and is supported in the acutely injured spinal cord.

2. Materials and Methods

2.1. Embedding iNPCs and CURC within PM

The iNPCs were generated as previously described [34]. Briefly, human iPSCs were reprogrammed using reprogramming factors (OCT3/4, SOX2, KLF4, LIN28) and a synthetic mRNA coding for CYCLIN D1, which supports genetic stability during the reprogramming process. For iNPC generation, a PSC Neural Induction Medium kit (Life Technologies, Framingham, MA, USA) was employed. After neural induction, iNPC cultures were maintained in growth medium (GM) STEMdiff™ Neural Progenitor Medium (STEMCELL™, Vancouver, BC, Canada) supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin (Lonza, Basel, Switzerland) in standard cell incubation conditions. For the iNPC sub-culture, cells at 80% confluence were detached using TrypLETM Select (Thermo Fisher Scientific, Agawam, MA, USA). A total of 1 × 103 iNPCs/µL were infected with a lentiviral vector containing GFP (PLL-eGFP) at a multiplicity of infection of 10 for 1 h and then plated with fresh GM. After 72 h of proliferation, the iNPCs were assessed for positive lentiviral transduction. The PM hydrogel used for embedding iNPCs was prepared at 0.3% by mixing 30 µL pure PM, 50 µL 20% sucrose, and 20 µL dH2O and then sonicated for 30 min. A total of 5 µL of 0.3% PM hydrogel was mixed with 5 × 104 iNPCs in 5 µL of sucrose (final PM concentration 0.15%) and pipetted into 96-well plate containing 200 µL of growth medium (97.5% DMEM/F-12 (Gibco, Waltham, MA, USA), 2.1% NeuroCult including GM supplements, and 0.4% 100 mM penicillin/streptomycin) delivered as a viscous drop. CURC (5 µM) was added to the PM in a dH2O mix.

2.2. Cell Viability and Apoptosis Assays

iNPC viability was evaluated using the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) following the manufacturer's instructions (Promega Biotech Ibérica S.L., Madrid, Spain) after one and five days of in vitro culture. Non-embedded iNPCs were cultured in growth medium in the presence of 5 µM of CURC or the corresponding volume of phosphate buffer solution (PBS; as the vehicle) as controls. Optical density was measured at 490 nm using a Victor2 microplate reader (Perkin Elmer Inc., Waltham, MA, USA) 4 h after adding the MTS reagents. Apoptosis was evaluated using the Apotox-BIOTM Triplex assay kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, Caspase-Glo® 3/7 Reagent was added and incubated for 30 min; then, bioluminescence was measured using a Victor2 microplate reader.

2.3. Phenotypic Characterization of Embedded iNPCs

PM-embedded iNPCs in the presence or absence of CURC were fixed with 4% paraformaldehyde (PFA) for 15 min, washed three times with PBS, blocked, and then permeabilized with 5% normal goat serum (Thermo Fisher) and 0.1% Triton X-100 (9036- 19-5, Merck Millipore, Darmstadt, Germany) for 1 h. The cells were then incubated with primary antibodies overnight at 4 ◦C in a humidified chamber. The primary antibodies used were Ki-67, proliferation marker (1:400, ab15580, Abcam, Cambridge, UK), Nestin (1:400, ab6142, Abcam), and MAP2 (1:500, ab5392, Abcam), along with Alexa488 and Alexa647 dye-conjugated secondary antibodies. After washing with PBS three times to remove excess primary antibody, samples were incubated for 1 h with dye-conjugated secondary antibodies in a 1:400 dilution in blocking solution (α-mouse Alexa-488, α-rabbit Alexa-647). All samples were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, Waltham, MA, USA) for 5 min at room temperature. Cell debris and autofluorescent aggregates found either in the scaffolds or the spinal cord not overlapping with the DAPI signal were excluded from the cell quantifications. The samples were examined by confocal microscopy (confocal microscope Leica TCS-SP2-AOBS).

2.4. Organotypic Longitudinal Spinal Cord Slice Culture

Spinal cord slices were obtained from five-day-old Sprague–Dawley rats and processed for culture as previously described [14,35]. The spinal cord was dissected and

cleaned from the meningeal layers and immersed in ice-cold Hank's balanced salt solution (HBSS). The spinal cord was then cut using a McIlwain tissue chopper into 350 µm thick slices in the parasagittal longitudinal plane. The slices were then cultured on Millicell cell culture inserts (Millipore) preequilibrated with 1.7 mL of culture medium (50% minimum essential medium, 25% HBSS, 25% horse serum, 2 mM GlutaMAX, 1 mM NAC, 0.5% NaHCO3, and 1% penicillin/streptomycin). To mimic an ex vivo model of SCI, a complete transverse section was performed using a scalpel blade on day five. After transection, both PM_iNPC and PM_CURC_iNPC (3 × 105 , GFP-overexpressing iNPCs in this case) were seeded above the created gap and cultured for another five days. The samples were then fixed with 4% PFA for 30 min for immunohistochemical analysis.

2.5. Preparation of HA Demilune with PLA Fibers for In Vitro Experiments

The synthesis of the demilune HA scaffold as well as the characterization of its mechanical properties was carried out as previously described [17]. Briefly, poly-ε-caprolactone (PolySciences, Warrington, PA, USA; Mw = 40 kDa) fibers of 400 µm were extruded in a Hater Minilab. A thin polytetrafluoroethylene block with 2.5 mm-wide grooves and a single poly-ε-caprolactone fiber of 1 mm diameter were used as a mold to obtain conduits. HA (5% (w/v)) was dissolved for 24 h in sodium hydroxide 0.2 M (Scharlab, Barcelona, Spain). Then, HA was crosslinked with divinyl sulfone (Sigma-Aldrich; Darmstadt, Germany; 10:9 monomeric unit molar ratio), mixed, injected into a mold, and then lyophilized for 24 h (Lyoquest-85, Telstar, Bensalem, PA, USA) to generate microporous HA cylinders. Longitudinal cuts generated the demilune scaffolds. Finally, the HA demilune scaffolds were hydrated in distilled water for 2 h, the poly-ε-caprolactone fibers extracted, and the conduits cut to the desired length of 4 mm.

2.6. Preparation, Combination with HA Demilune Scaffolds, and Sanitization of PPY-Coated Microfibers

Highly aligned microfiber bundles (AITEX Textile Research Institute, Alcoy, Spain) formed by 300 PLA microfibers with a diameter of 10 µm were coated with PPY via in situ polymerization [30,36]. Briefly, the microfibers were introduced into a polypropylene tube containing an aqueous solution of 14 mM pyrrole monomer (Py, Sigma-Aldrich 131709) and

14 mM sodium para-toluene sulfonate (pTS, Sigma-Aldrich, 152536). Ultrasonication for 1 min allowed the microfibers to become saturated with the Py/pTS solution. The microfibers were then incubated with shaking at 4 ◦C for 1 h. Next, an aqueous solution of 38 mM ferric chloride (Sigma-Aldrich 157740) was added and incubated with shaking at 4 ◦C for 24 h for the polymerization and deposition of PPY onto the PLA microfibers. The PPY-coated microfibers were washed three times with deionized water with agitation for 10 min and then ultrasonicated for 30 min in deionized water three times. Finally, the microfibers were dried in a desiccator with a fixed vacuum at 40 ◦C for 48 h. Once the PPY coating was completed, one microfiber bundle was introduced into the lumen of the HA scaffold. HA droplets were added at both ends of the PPY-coated microfiber bundle to fix the HA demilune scaffold. In the following text, 'PPY fibers' is used as shorthand for 'PPY-coated PLA fibers' for the sake of brevity. Before cell seeding, the HA demilune scaffolds with PPY-coated fibers were sanitized for 2 h with 70% ethanol (Scharlab) and then 10 min with 50% ethanol, 30% ethanol, and then distilled water. The conduits were incubated with culture medium overnight.

2.7. Scanning Electron Microscopy

The surface morphology of the biomaterials was characterized using scanning electron microscopy (SEM; ULTRA 55, ZEISS Oxford Instruments, Pleasanton, CA, USA). The samples were desiccated under vacuum conditions 24 h prior to evaluation to avoid interference due to evaporated water. The samples were placed on carbon tape, and a carbon bridge was introduced between the sample and the carbon tape. Finally, the samples were coated with a thin layer of platinum, and the images were taken using a voltage of 2 kV. SEM was also used to evaluate the attachment/morphology of PM-embedded iNPCs seeded within HA and HA-PPY conduit scaffolds after three days of culture. This mode of SEM was performed as described previously [17]. Briefly, tissues were placed into prewarmed 3.5% (v/v) glutaraldehyde (Sigma) and 2.5% (v/v) PFA (Sigma) and then left to incubate overnight at 4 ◦C. The constructs were then coated with a conductive layer using 2% w/v osmium tetroxide for 1 h. This step was followed by dehydration using a serial dilution of ethanol (30%, 50%, 70%, 96%, and 100%) and hexamethyldisilane treatment. The scaffolds were then air-dried at room temperature overnight. Images from different spots were examined under a transmission electron microscope (FEI Tecnai G2 Spirit BioTwin, Thermo Fisher Scientific

company, Waltham, Massachusetts, USA) using a Morada digital camera (EMSIS GmbH, Münster, Germany).

2.8. *In Vivo* SCI Model and Biomaterial Implantation

SCI was induced in female Sprague–Dawley rats (weighing ~200 gm) as previously described [37]. Briefly, the animals were treated with subcutaneous morphine (2.5 mg/kg) 30 min before surgery and then deep anesthetized with 3% isoflurane, which was maintained at 1.5–2% during surgery for SCI induction. Laminectomies were conducted at thoracic segments T7–T9 for a moderate contusion at T8 by applying 200 kdyn in all animals using the Infinite Horizon spinal cord impactor (Precision Systems and Instrumentation LLC, Brimstone, VA, USA). One week after SCI, animals were randomly distributed into the following groups (n = 3) (See Table 1 for details): HA_PM_CURC, HA_PM_CURC_iNPC, HA_PM_iNPC, HA_PPY_PM_CURC, HA_PPY_PM_CURC_iNPC, and HA_PPY_PM_iNPC. The iNPCs (1×10^6) were embedded in 10 µL of PM (0.15%). The SCI area was reassessed, a slit was made in the dura mater with a 27G needle, and the Biomedicines 2021, 9, 1928 6 of 19 biomaterial was placed over the spinal cord, as previously described [17]. A laminectomy was performed without applying the injury in the control groups: HA_PPY_PM_iNPC (non-injured) and HA_PM_iNPC (non-injured). After surgery, all animals were subjected to post-operative care. To prevent immune rejection of allogeneic cell grafts, animals received daily subcutaneous injections of the immunosuppressant tacrolimus (1 mg/kg) starting one day before transplantation, until sacrifice one week after implantation.

Groups	Induction of SCI	Treatment
HA_PM_iNPC (non-injured)	No	Hyaluronic scaffold PM-embedded iNPC
HA_PPY_PM_iNPC (non-injured)	No	Hyaluronic scaffold with PPY fibers and PM-embedded iNPC
HA_PM_CURC	Yes	Hyaluronic scaffold PM-embedded CURC
HA_PPY_PM_CURC	Yes	Hyaluronic scaffold with PPY fibers and PM-embedded CURC

HA_PM_iNPC	Yes	Hyaluronic scaffold PM-embedded iNPC
HA_PPY_PM_iNPC	Yes	Hyaluronic scaffold with PPY fibers and PM-embedded iNPC
HA_PM_CURC_iNPC	Yes	Hyaluronic scaffold PM-embedded iNPC and CURC
HA_PPY_PM_CURC_iNPC	Yes	Hyaluronic scaffold with PPY fibers and PM-embedded iNPC and CURC

Table 1: Experimental groups. Abbreviations: HA, hyaluronic acid; PM, PuraMatrix; PPY, Polypyrrole fibers; iNPC, human-induced Neural Progenitor Cells; CURC, Conjugated Curcumin with PA-PEG polymer.

2.9. Histological Studies

One week after scaffold implantation and SCI, animals were irreversibly anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and fentanyl (0.05 mg/kg) and transcardially perfused with 0.9% saline immediately followed by 4% PFA in 0.1 M phosphate buffer (pH 7.4). Spinal cords were dissected and post-fixed in 4% PFA for 5 h and then conserved in 0.1 M phosphate buffer containing 0.01% sodium azide. Thoracic segments, including T6 to T10, were dehydrated and included in paraffin, placed in histology cassettes, and processed on a Leica ASP 300 tissue processor (Leica Microsystems, Nussloch, Germany). Then, 8 mm thick sagittal sections were cut on a microtome and mounted on gelatin-coated slides. For histological analysis, spinal cord tissue sagittal sections were permeabilized with PBS containing 0.5% Triton and 2% goat serum (blocking solution). The primary antibodies used were β-Tubulin III (1:400, MO15052 Neuromics, Edina, MN, USA), GFAP (1:500, Z0334 DAKO, Santa Clara, CA, USA), GFP (1:750, ab13970, Abcam), ED1 CD68 (1:400, MAB1435 Chemicon-Fisher Scientific, Madrid, Spain), Iba-1 (1:400, 019-19741 DAKO, Santa Clara, CA, USA), NeuN (1:600, ABN91 Sigma-Aldrich), Fibronectin (1:50, SC6953, Santa Cruz, USA), Neurofilament (1:750, ab24575, Abcam), and PDGF (1:400, ab32570, Abcam). AlexaFluor-488, -555, or -647 (1:400 Invitrogen) conjugated with secondary antibodies were used. All sections were counterstained with DAPI and mounted using FluorSave TM Reagent (EMD, Millipore).

Mounted sections were scanned by an Aperio Versa scanner (Leica Biosystems, Germany) and analyzed using the ImageJ (Bethesda, MD, USA).

2.10. Ethical statement

All experimental procedures were approved by the Animal Care and Use Committee of the Research Institute Prince Felipe (2021/VSC/PEA/0032) in accordance with the guidelines established by the European Communities Council Directive (210/63/EU) and Spanish regulation 1201/2005.

2.11. Statistical Analysis

In vitro experimental data were collected from three independent experiments, and the results were reported as the mean ± standard deviation (SD). For the comparisons between two groups of values, the statistical analysis of the results was performed using the student's t-test for normally distributed data. Two-way ANOVA with appropriate corrections (such as Tukey's post hoc test) was used to compare groups in viability, apoptosis, and MTS assays. To compare immunofluorescent expression in histological specimens in distinct groups, one-way ANOVA was used; however, the comparison of surface marker expression of Nestin, Ki-67, and MAP2 between PM_iNPC and PM_CURC_iNPC in vitro used a paired t-test. Statistical analyses were performed using GraphPad software (La Jolla, CA, USA). Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results

3.1. PuraMatrix-Embedded iNPCs Increase Long-Term Survival

To evaluate the viability of iNPCs embedded in the PM hydrogel and 3D in vitro culture in the presence or absence of CURC, we first performed an MTS assay to evaluate cell metabolic activity after one and five days of culture (Figure 1A). Interestingly, embedding cells in PM in the presence or absence of CURC (PM_CURC_iNPC and PM_iNPC, respectively) significantly reduced the metabolic activity of iNPCs during the first day of culture compared with iNPCs cultured under traditional conditions (iNPC or CURC_iNPC). However, we found the opposite profile at day five, with increased metabolic activity in PM-embedded iNPCs

compared to traditional 2D iNPC cultures (Figure 1A). Overall, this suggests that the 3D culture conditions in PM support the long-term viability of iNPCs.

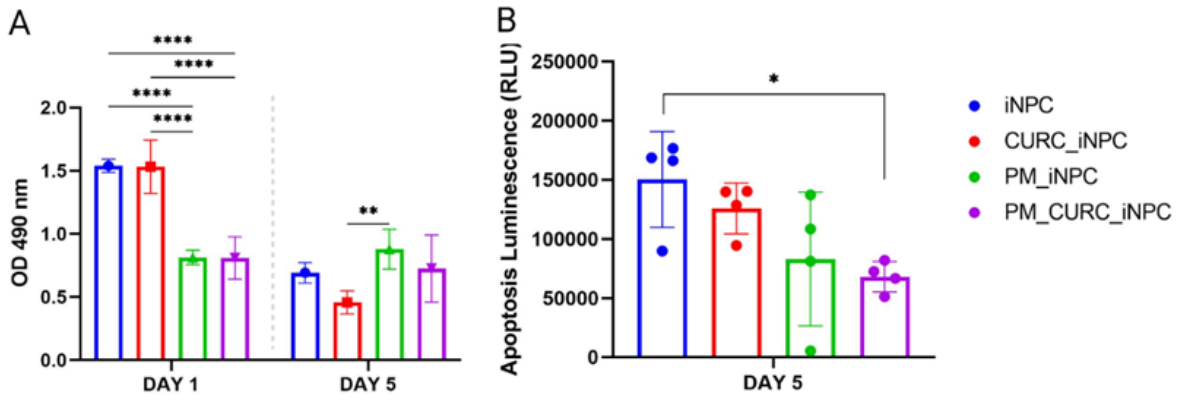


Figure 1. Evaluation of iNPC viability and apoptosis. (**A**) Cell metabolic activity/cell viability evaluated by MTS assay and (**B**) apoptosis evaluated using the Apotox-BIOTM Triplex assay of iNPCs in vitro cultured in traditional 2D or PM-embedded (0.15%) 3D conditions in the presence or absence of CURC (5 µM) at one and five days. Data represented as the mean ± SD (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$).

In this 2D/3D comparison, we also evaluated cell death/apoptosis using the Apotox-BIO^{TM} Triplex assay, which employs a pro-luminescent caspase-3/7 substrate (Figure 1B). The presence of CURC (PM_CURC_iNPCs) compared to non-embedded iNPCs. Overall, these findings suggest that PM and CURC act synergistically to reduce iNPCs apoptosis in long-term culture.

3.2. CURC Induces a Neurogenic-Like Phenotype of PM-Embedded iNPCs

We next evaluated the neurogenic phenotype of PM-embedded iNPCs by assessing cells with an elongated morphology, the expression of the neural stem cell intermediate filament protein Nestin, and cell proliferation (via Ki-67 staining) (Figure 2A). Overall, PM-embedded iNPCs in the presence of CURC (PM_CURC_iNPC) displayed a 2.2-fold greater proportion of elongated Nestin-positive cells (red) than in the absence of CURC (PM_iNPC). However, we failed to encounter any significant differences between PM_iNPC and PM_CURC_iNPC in terms of cell proliferation (Ki-67—green staining) (Figure 2B). Interestingly, we discovered that CURC increased the number of MAP2-positive cells in 3D culture, indicating a potential effect on neuronal cell fate maturation (Figure S1)

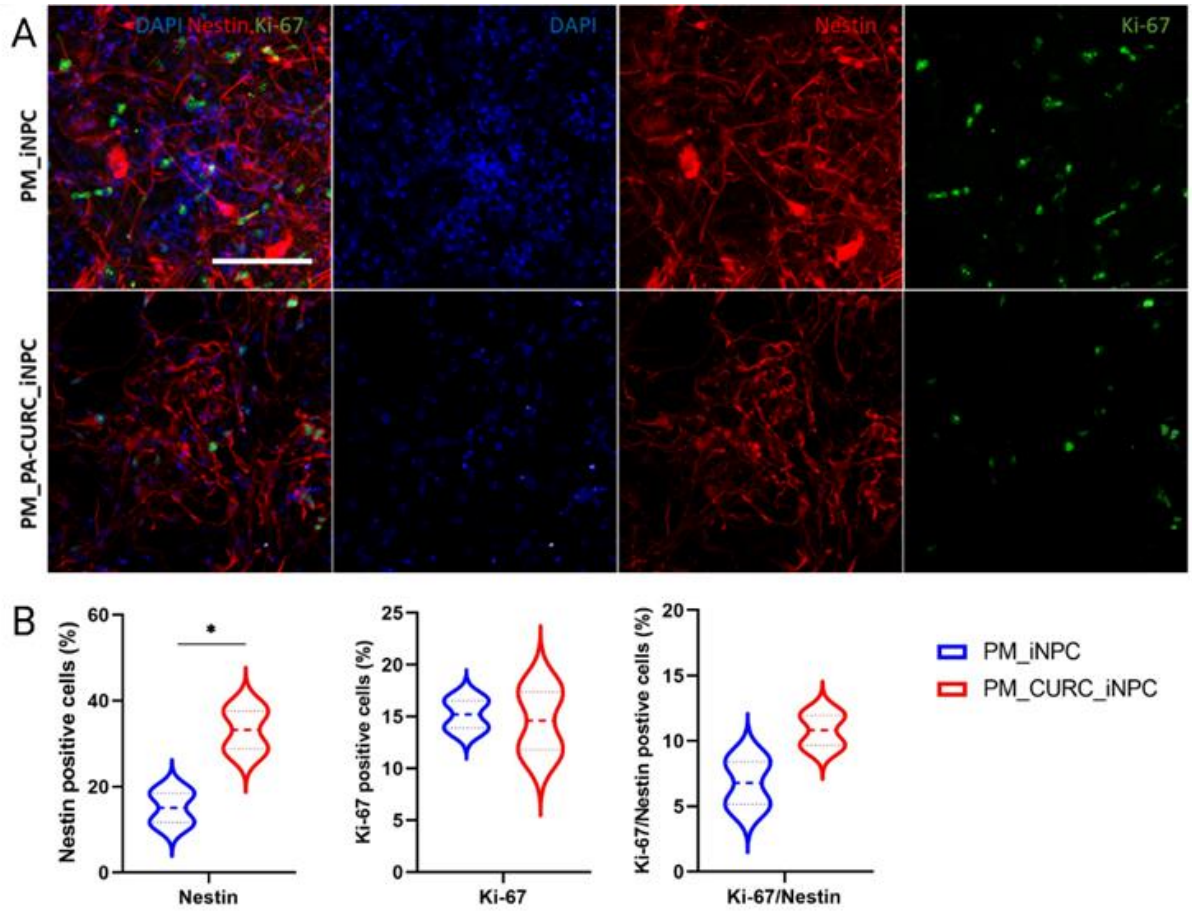


Figure 2. Quantitative and qualitative analysis of the neurogenic-like phenotype in iNPCs in 3D culture. (**A**) Confocal immunofluorescence images for Nestin (red) and Ki-67 (green) in PM-embedded iNPCs in the presence or absence of 5 µM CURC. Nuclei stained with DAPI (blue). White scale Figure 1. Evaluation of iNPC viability and apoptosis. (A) Cell metabolic activity/cell viability evaluated by MTS assay and (**B**) apoptosis evaluated using the Apotox-BIOTM Triplex assay of iNPCs in vitro cultured in traditional 2D or PM-embedded (0.15%) 3D conditions in the presence or absence of CURC (5 µM) at one and five days. Data represented as the mean ± SD (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$).

We next co-cultured GFP-labeled iNPCs with organotypic spinal cord slices to mimic the process of cell transplantation in an ex vivo model of SCI. As shown in Figure 3A, we transversally transected longitudinal slices of neonatal spinal cords and deposited PM-embedded iNPCs in the presence or absence of CURC (PM_CURC_GFP_iNPC and PM_CURC_iNPC) into the gap. After five days of culture, and in agreement with our previous findings, we found that CURC promoted iNPCs to take on a neuron-like morphology. The iNPCs also displayed long neurite-like processes with a parallel orientation in tight association with endogenous neuronal fibers stained with βIII-tubulin (Figure 3B, red, white arrows). In the

absence of CURC, iNPCs took on a wider morphology and a random organization (Figure 3B, yellow arrow). The quantification of the GFP-iNPCs with neurite-like processes demonstrated a significantly increased number following treatment with CURC (Figure 3C).

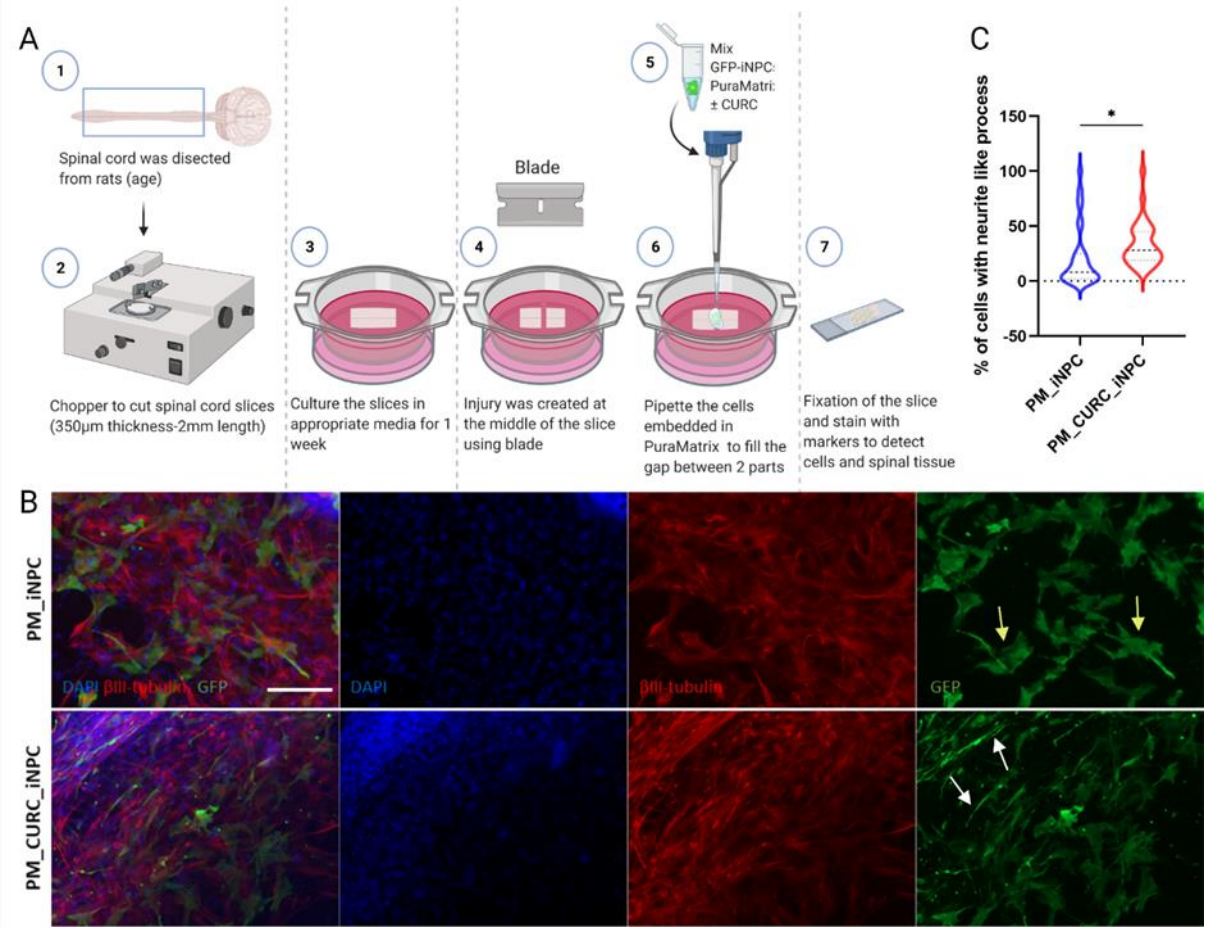


Figure 3. Assessment of iNPC morphology when cocultured with organotypic spinal cord slices. (**A**) Schematic diagram of the experiment. (**B**) Representative immunofluorescence images showing spinal cord fixed tissue labeled with βIII-tubulin (red) and GFP-labeled iNPCs (green). Nuclei stained with DAPI (blue), white scale bar: 100 µm (all images were acquired at the same magnification). (**C**) Quantitative analysis of neurite outgrowth elongation from iNPCs. Data represented as mean ± SD from three independent experiments (*p<0.05).

3.3. Ha Demilune Scaffolds Containing Ppy-Coated Fibers Convey Pm-Embedded iNPCs for Spinal Cord Implantation

As shown in Figure 4A, we obtained HA demilune scaffolds with the proper dimensions for implantation within the lesioned rat spinal cord. The lumen of the HA scaffold contained a bundle of highly aligned PPY microfibers. Figure 4B provides evidence of the high porosity

of the HA scaffold, while Figure 4C demonstrates the parallel alignment of the PPY microfibers. Figure 4D shows the surface morphology of the PM adsorbed onto the fiber surface, while Figure 4E depicts the grainy topography of the PPY fibers. We performed additional SEM analyses to assess construct microarchitecture and evaluate the cell distribution within the HA scaffold and PPY fibers. Figure 4F provides a schematic diagram of scaffolds (HA alone and HA with PPY fibers) seeded with PM-loaded iNPCs. The SEM images in Figure 4G,H depict iNPCs embedded within the PM hydrogel covering the HA scaffold internal surface (Figure 4I, white arrow) and iNPCs attached to PPY fibers (Figure 4J, black arrow).

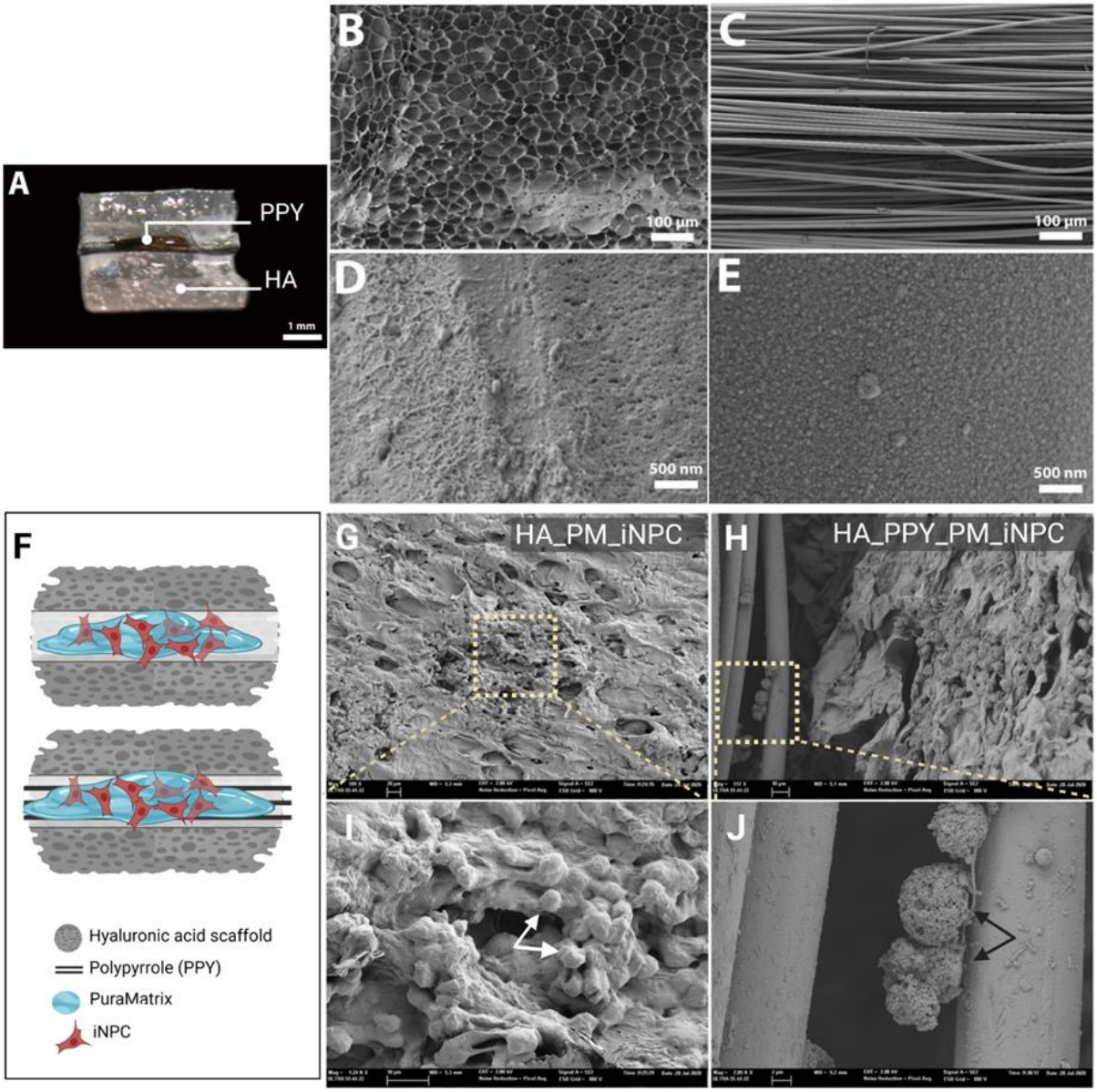


Figure 4. Morphological analysis of iNPCs and scaffold components. (**A**) Macroscopic image of the HA demilune scaffold containing PPY microfibers in the lumen (hydrated state), scale bar: 1 mm. (**B**) SEM image detailing HA scaffold porosity. (**C**) SEM image showing the parallel alignment of PPY microfibers. (**B,C**) scale bar: 100 µm. (**D**) SEM image of PM on the PPY microfiber surface. (**E**) SEM image showing the PPY coating of the PLA microfiber surface. (B,C) scale bar: 500 nm. (**F**) Schematic diagram of scaffolds (HA alone and HA with PPY fibers) seeded with PM-embedded iNPCs. (**G,H**) SEM images of PM-embedded iNPCs seeded on HA (**G**) and HA-PPY (**H**) after three days in culture, scale bar: 50 µm. (**I,J**) show a higher magnification of the indicated area in (**G,H**) respectively, scale bar: 10 µm.

3.4. CURC Supports iNPC Migration within the Injured Spinal Cord

We implanted PPY fiber-functionalized HA scaffolds carrying PM-embedded GFPiNPCs in the presence or absence of CURC to cover the damaged segments of the spinal cord one week after injury, mimicking the most favorable clinical intervention at the sub-acute phase (Figure 5A). To evaluate the damage induced by scaffold implantation in healthy tissue, we also implanted the various scaffolds in non-injured animals and evaluated their impact after one week. We stained sagittal sections with hematoxylin and eosin (H&E) to analyze macroscopic anatomical alterations. An overall view of the spinal cord revealed the formation of a fibrous-like tissue pad under the implantation site in all cases (Figure 5B, white arrow); however, we failed to observe any structural pathological alterations (e.g., cysts or infiltrated cells) at this stage in the injured groups (Figure 5B).

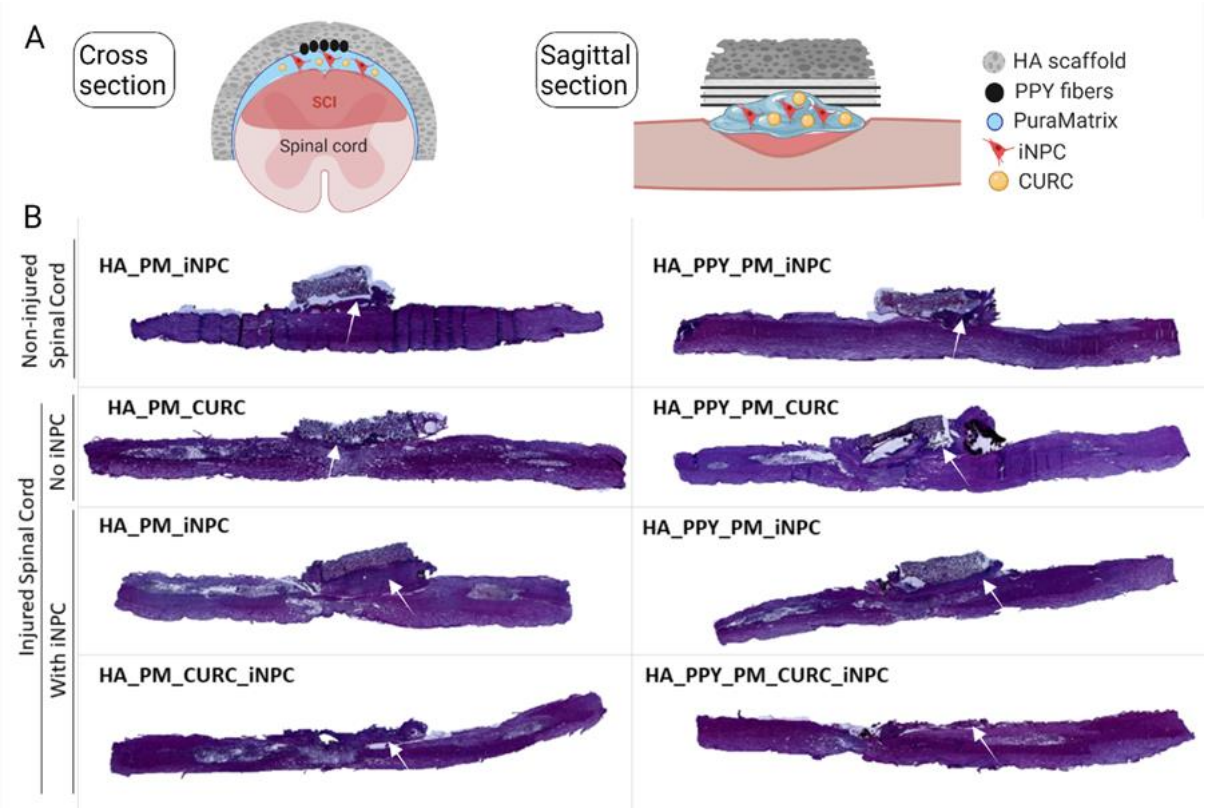
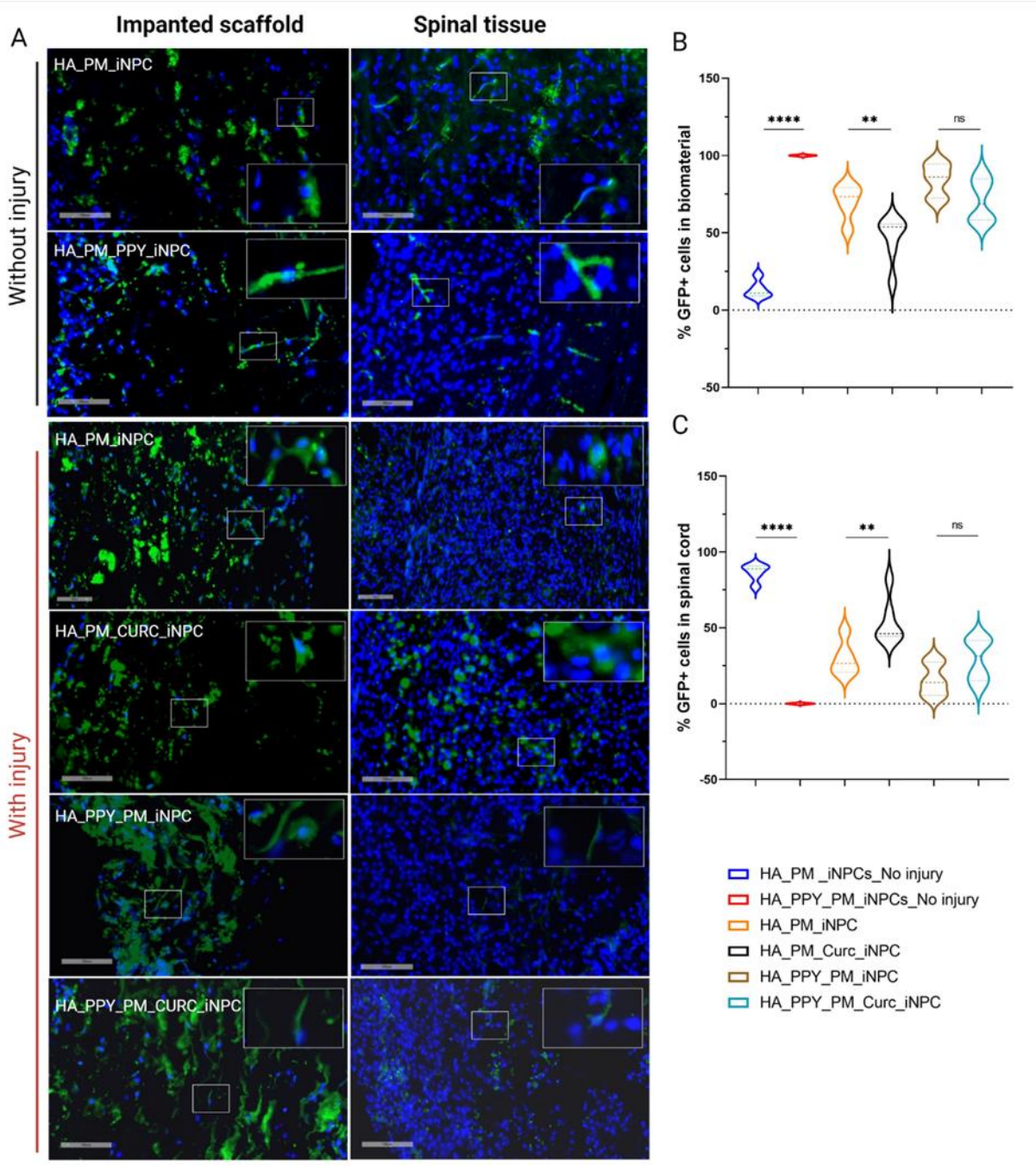


Figure 5. Implantation of demilune scaffolds to cap the SCI. (**A**) Schematic diagram showing the cross and sagittal sections of the SCI and various scaffolds capping the injured area. (**B**) Representative images of H&E staining of sagittal spinal cord sections from one animal per group one week after injury. The white arrow indicates the formation of a fibrous-like tissue pad. Scale bar: 5 mm.

To evaluate the migration of GFP-iNPCs after implantation, we tracked GFP-positive cells within the remaining implanted biomaterial (Figure 6A, left panels) and in the spinal cord tissue (Figure 6A, right panels). One week after injury and implantation, we detected most GFP-iNPCs within the remaining biomaterial (Figure 6B, upper panel). While the global iNPC survival rate was lower in the implanted injured groups compared with non-injured group (data not shown), we hypothesized that the cells that migrated into the injured samples died faster than those migrating into the non-injured tissue and, in addition, the cells remaining in the scaffold were able to survive for a longer period. Nevertheless, we discovered an inverse correlation between the percentage of GFP-positive iNPCs in the biomaterial vs. GFP-iNPCs detected in the spinal cord tissue. It is of note that the presence of CURC prompted iNPC migration from the biomaterial to the spinal cord tissue (Figure 6B,C, black), while the presence of PPY delayed migration (Figure 6B,C, light blue).

We also observed that the few GFP-iNPCs that integrated into the host tissue displayed co-localization with βIII-tubulin expression in all of the groups, indicating a general lack of neuronal differentiation after implantation (Figure 6D); however, most GFP-positive iNPCs co-localized with GFAP staining in all evaluated conditions, which indicates preferential astroglial differentiation (Figure 6E).



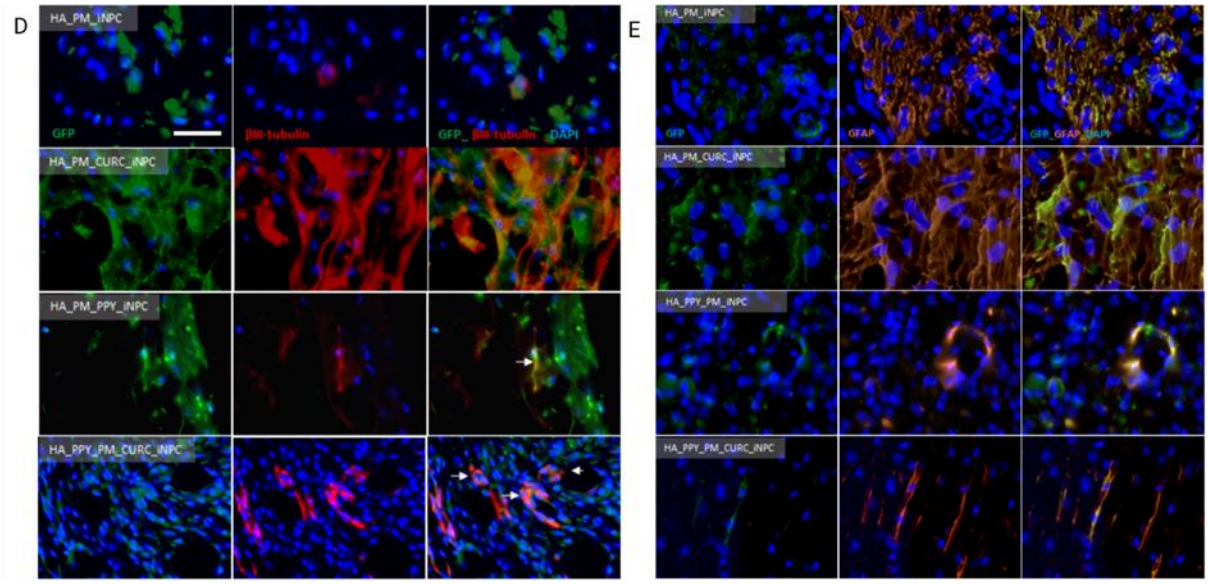


Figure 6. Quantitative and qualitative analysis of iNPC biodistribution and differentiation potential after implantation into the spinal cord. (**A**) Representative images showing the distribution of GFP-iNPCs (green) within the biomaterial scaffold and spinal cord tissue. Cell nuclei stained with DAPI (blue). Scale bar: 100 µm. (**B**) Quantitative analysis of GFP-iNPCs in the biomaterial scaffold and (**C**) in the spinal cord. Data represented as mean ± SD, n = 3 (ns $p > 0.05$, ** $p < 0.01$, **** $p < 0.00001$); representative images of GFP-iNPCs (green) expressing (D) β-III-tubulin (red) and (E) GFAP (orange). Scale bar: 50 µm.

3.5. A Fully Functionalized HA Demilune Scaffold Preserves Neuronal Fibers and Reduces Glial Scarring but Does Not Reduce Neuroinflammation Early after SCI

Assessments of neuronal preservation after SCI by evaluating the expression of neuronal filaments with βIII-tubulin staining (Figure 7A, red) demonstrated that only the fully functionalized scaffold (PM-embedded GFP-iNPCs with PPY fibers and CURC) allowed for the preservation of β-III-tubulin-positive neuronal fibers after implantation (Figure 7B, light blue), as all other approaches led to a significant decrease in fiber preservation compared to the control. Analysis of scar-forming tissue (as delimited by the GFAP-negative area; Figure 7A, white dash line) revealed that the group of animals implanted with the fully functionalized scaffold showed a smaller scar area, in agreement with the above-described neuronal preservation effect (Figure 7C, light blue).

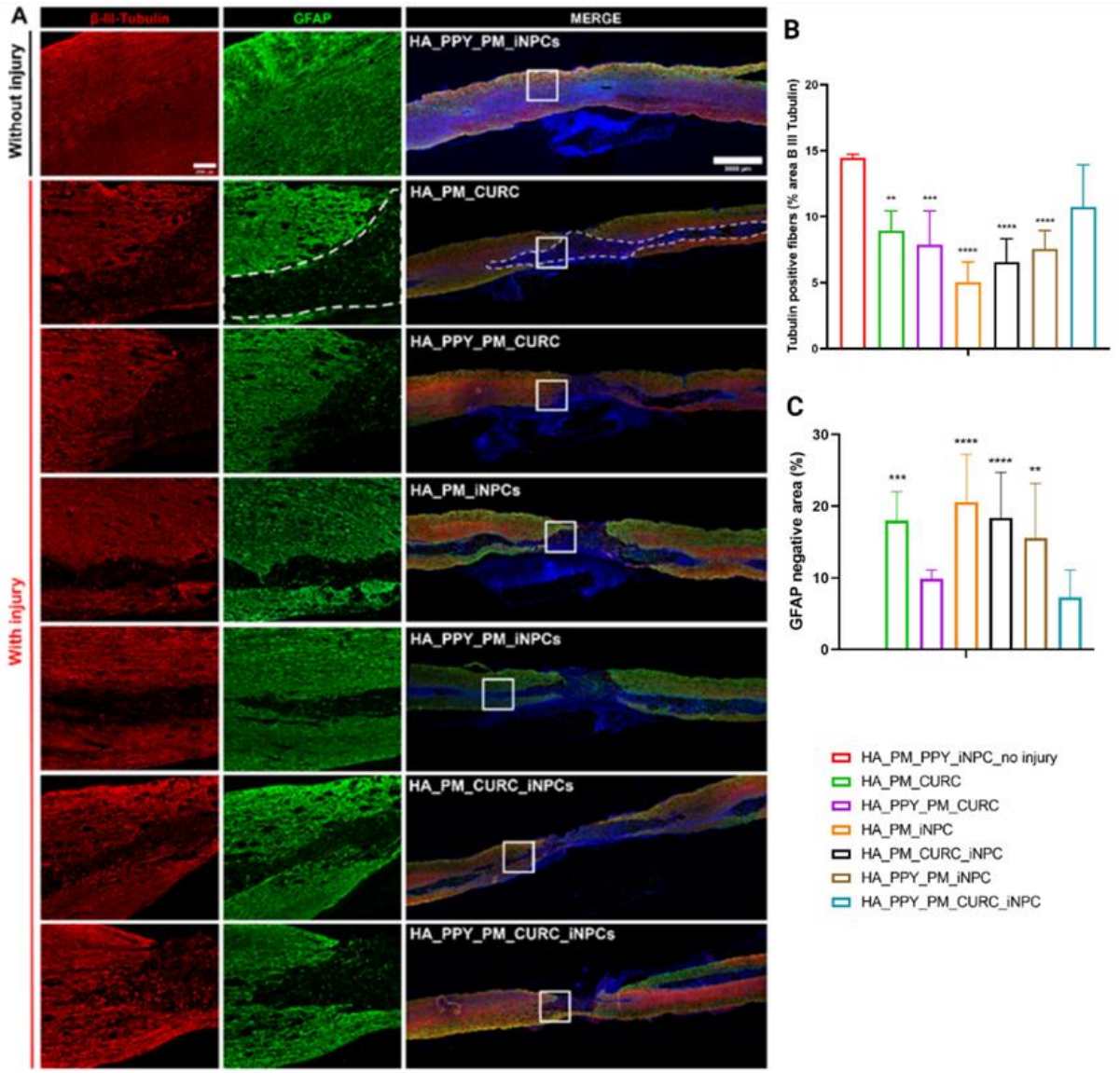


Figure 7. Potential of biomaterial and iNPCs to preserve neuronal fibers and reduce scar formation within the SCI. (**A**) Immunofluorescent staining of β-III-tubulin (neuronal marker, red) and GFAP (astroglial marker, green). (**B**) Quantification of β-III-tubulin expression. (**C**) GFAP negative area. Data represented as means ± SD n = 3, (** p < 0.01, *** p < 0.001, **** p < 0.0001 vs. PM_PPY_iNPC_no injury). Scale bars: 250 µm; 3000 µm.

We next evaluated both endogenous microglial activation using Iba1 immunoreactivity, describing activated and resting microglia as displaying a rounded or stellated morphology, respectively (Figure 8A) [38], and macrophage infiltration by ED1 immune reactivity (Figure 8B) [39]. Quantification demonstrated a significant reduction in resting microglia and increased activated microglia in all injured groups compared to the non-injured group, with no significant difference observed between groups (Figure 8C). The

analysis of infiltrated macrophages revealed a similar result; we failed to find significant differences between the treatment approaches (Figure 8). Overall, these data suggest that the implants did not show any significant immunomodulatory effect one week after implantation.

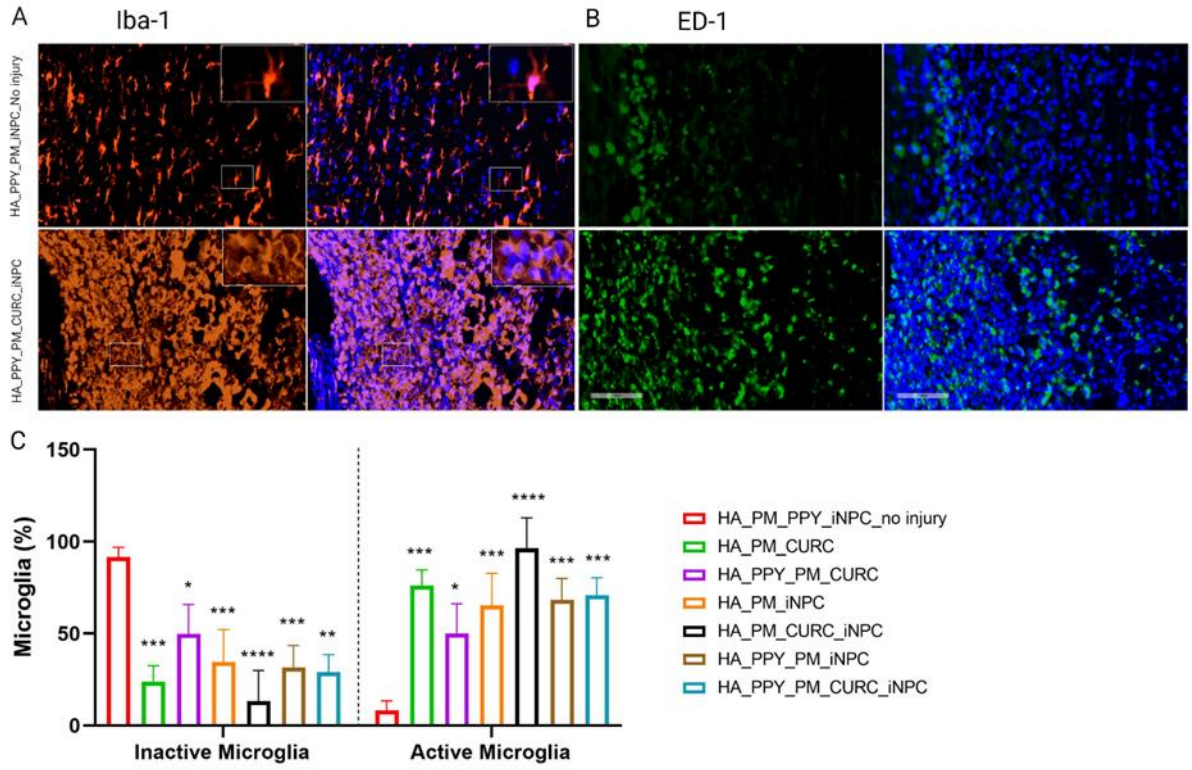


Figure 8. Evaluation of the neuroinflammatory reaction at the early stages after SCI. (**A**) Immunofluorescent staining for Iba-1 (microglial marker, orange) and (**B**) ED-1 (macrophage marker, green). Scale bar: 100 µm. (**C**) Quantification of inactive and active microglia identified by the morphological appearance of Iba-1 expressing cells. Data represented as mean ± SD, n = 3 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.00001$).

Finally, we evaluated the fibrotic formed tissue at the injury site by evaluating the expression of platelet-derived growth factor (PDGF), a pericyte marker involved in fibrotic scar tissue generation [40]. We found that the fully functionalized scaffold induced the lowest PDGF expression (Figure 9A,B). Nonetheless, we failed to find differences in the spared neuronal area among the different injured groups (as confirmed by NeuN expression), indicating a lack of early spinal neuronal preservation after scaffold implantation (Figure 9C,D).

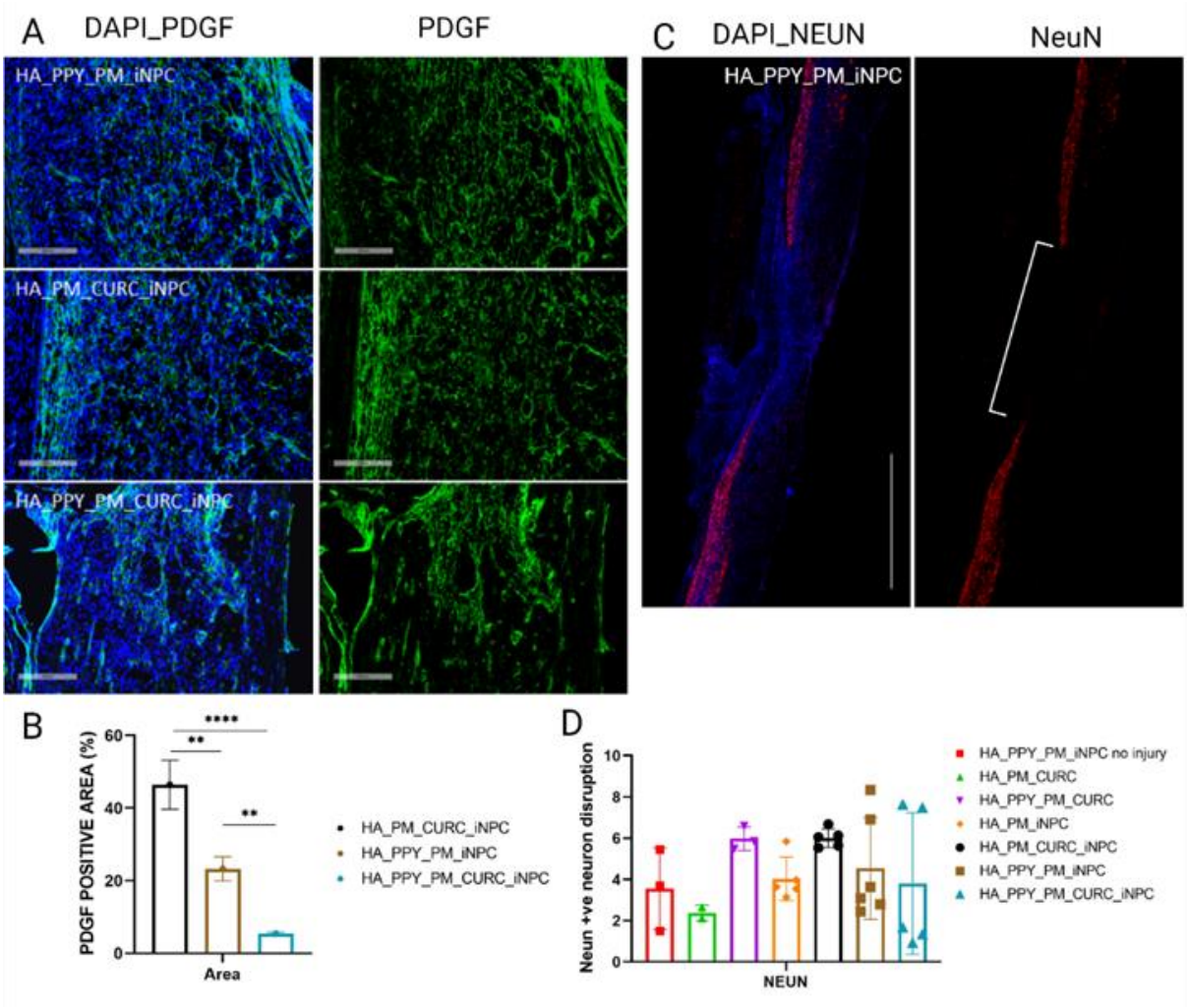


Figure 9. Quantitative and qualitative analysis of PDGF and NeuN expression at the site of injury. (**A**) Immunofluorescent staining for PDGF (green) at low magnification (left panels, including merged images with DAPI staining), Scale bar: 2 mm and a selected area at high magnification (right panel), Scale bar: 200 µm. (**B**) Quantification of PDGF positive expression area. Data represented as mean ± SD by one-way ANOVA with Tukey's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$). (**C**) Representative image of NeuN (red) and DAPI staining. Scale bar: 2 mm. (**D**) Quantification of the disruption of the NeuN-positive neuron area at the injury site. Data represented as mean ± SD by one-way ANOVA with Tukey's multiple comparison test, n = 3.

4. Discussion

In the present study, we report that iNPCs embedded into a PM hydrogel at a concentration of 0.15% containing 5 µM of CURC display improved cell survival compared to a long-term monolayer culture. CURC within the PM hydrogel prompts a higher number of neuronal-like cells with elongated projections. When implanted in the injured rat spinal cord

(capping the injured area), the combination of PM-embedded iNPCs with CURC prompted the preservation of neuronal fibers.

The pH-dependent biodegradable polyacetal's functionalization by the conjugation with curcumin improved its properties in comparison with the free form, including increased water solubility and stability, reduced cell toxicity, and enhanced pH-mediated controlled release in the acidic pH of the lesion site as we previously described [14]. We previously demonstrated the neuroprotective effects of CURC treatment in vitro and in vivo [14,41]. In 2D iNPC culture, CURC treatment induced higher preservation of neuronal-like iNPCs positive for β-III-tubulin when exposed to toxic doses of H2O2. CURC treatment alone or in combination with NPC transplantation in a rat model of chronic stage SCI improved neuronal fiber preservation within the injured area. In the present study, and in agreement with our previous results, we found that the fully functionalized scaffold containing CURC markedly preserved β-III-tubulin neuronal fibers, evaluated at an earlier stage of one week after implantation and two weeks after injury.

Previous studies had suggested that PPY stimulated the release of neurotransmitters and trophic factors [42], although our previous in vitro studies underscored that PPY fibers failed to alter brain-derived neurotrophic factor secretion from Schwann cells [36]. Nevertheless, while only the fully functionalized scaffold that included PPY fibers demonstrated significant neuronal fiber preservation, we hypothesize that PPY fibers may synergize with CURC within the iNPC transplant. Overall, both embedded iNPCs that remained in the hydrogel and those that migrated to the spinal cord parenchyma could enhance endogenous trophic support. We previously demonstrated that CURC reduced the glial scar when intrathecally administered [14], possibly by reducing astrogliosis and glial scar formation by inhibiting the signal transducer and activator of transcription 3 (STAT3) and the nuclear factor-kappa B (NF-κB) pathways [43,44]. Interestingly, the fully functionalized scaffold also contributed to reducing the glial scar.

We also previously demonstrated that CURC reduced macrophage infiltration [41] and promoted the anti-inflammatory polarization of microglia [14] at two months after injury/treatment. This study failed to find a difference in microglial activation or macrophage infiltration one week after treatment (implantation). Further experimentation evaluating

microglia activation at chronic stages will be required to fully assess the potential anti-inflammatory effect of the CURC containing scaffolds. A study on traumatic brain injury revealed that the transplantation of PM-embedded rat NPCs alone or in combination with free curcumin decreased microglial activation and macrophage infiltration when evaluated at one month after treatment [45,46].

We found that the scaffolds containing CURC induced a lower level of fibrosis between the scaffold and the spinal cord tissue, as evaluated by a macroscopic analysis of the H&E staining (data not shown). A recent study developed in a laminectomy model in which curcumin prevented peridural fibrotic tissue formation supports this finding [47]. The anti-fibrotic properties could be attributed to the capability of curcumin to suppress collagen production through down-regulation of transforming growth factor-beta 1 [48,49]. Additionally, pericytes have been determined as a target to reduce fibrotic scarring and improve tissue recovery [50,51], a finding that has prompted the development of PDGF inhibitors as a therapeutic approach to systemic sclerosis [52]. We monitored the expression of PDGF to evaluate the potential modulation of the fibrotic tissue within the SCI by the different scaffolds. We discovered that a fully functionalized scaffold reduced scar formation and increased neuronal preservation, supporting neuronal regeneration.

Curcumin also enhances neural differentiation of pluripotent embryonic carcinoma cells and induced NeuroD, TUJ1, and PAX6 expression through the Notch signaling pathway [53]. In vitro, we found more iNPCs expressing MAP2, a mature neuronal marker, in the presence of CURC (Supplementary Figure S1); however, very few implanted cells showed reactivity to βIII-tubulin in vivo, and we failed to observe significant differences induced by CURC. Nevertheless, Woodruff and collaborators Lu, et al. [54] reported that transplanted iNPCs expressed β-III-tubulin after three months post-transplantation, although these cells rarely displayed mature neuronal markers. Exploring the neuronal phenotype of iNPCs one week after implantation may be too early to induce neuronal in vivo differentiation. Interestingly, most GFP-iNPCs located surrounding the injured area displayed positive staining for GFAP, indicating a preferential glial fate rather than neuronal differentiation at this stage.

The HA demilune scaffolds containing a bundle of highly aligned PPY fibers in their lumen increased neural cell adhesion up to 95% and favored cell alignment and elongation

[30,55]. The PPY coating of the PLA fibers confers a surface nanoroughness that enhances the adsorption of extracellular matrix proteins and thus makes these surfaces more adherent [30,36]. This mechanism may explain the low migration of transplanted cells to the spinal cord, which remains more firmly attached to the fibers. The rigidity of the PPY fibers could also contribute to the lower performance observed when employing non-pre-seeded microfibers. Reducing the number of implanted microfibers and/or using a less rigid support material like silk fibroin may improve this result.

CURC enhanced iNPC migration and homing into spinal cord tissue, which agrees with a previous study [56] in which CURC enhances cell migration through c-Src and protein kinase C phosphorylation and activation. Nevertheless, when CURC-containing scaffolds included PPY, we found no significant differences in iNPC migration. Therefore, the adhesion of iNPCs to PPY fibers may inhibit CURC-stimulated migration.

5. Conclusions

A complementary combination of CURC and iNPCs supported by the HA demilune scaffold containing PPY fibers prevented neuronal degeneration soon after severe SCI by preserving neuronal fibers and reducing glial scar formation without significantly additionally damaging the preserved tissue. Nevertheless, further studies are needed to evaluate the consequences for long-term transplantation of the scaffolds employed in this study.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: MAP2 positive staining for evaluation of neuronal maturation rates in iNPC-PM embedded with or without CURC

Author Contributions: Conceptualization, V.M.M. and H.E.; methodology, validation and formal analysis H.E., P.B., E.G., A.A.A, C.M., F.G, L.D.; resources, V.M.M., M.E., M.J.V., M.M.P.; data curation, H.E., P.B., E.G., M.M.P., F.G.; writing—original draft preparation: H.E. and V.M.M.; draft review and editing analysis H.E., P.B., E.G., M.M.P. and V.M.M.; funding acquisition, V.M.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of CENTRO DE INVESTIGACION PRINCIPE FELIPE (protocol code (2021/VSC/PEA/0032), approved in January of 2021).

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3.3. CHAPTER 3: Giraldo, E., Bonilla, P., Mellado, M., Garcia-Manau, P., Rodo, C., Alastrue, A., ... & Moreno Manzano, V. (2022). Transplantation of Human-Fetal-Spinal-Cord-Derived NPCs Primed with a Polyglutamate-Conjugated Rho/Rock Inhibitor in Acute Spinal Cord Injury. Cells, 11(20), 3304.

I contributed to the article of which I am co-author mainly on the histological studies of the spinal cord by means of immunohistochemical techniques. I studied the organization of ependymal cells at the central canal of the spinal cord by transmission electron microscopy and the distribution of neural precursors in the human fetal spinal cords by immunohistochemistry. Moreover, I studed the distribution of the transplants in the spinal cord parenchyma and their invasiveness in other tissues and their activation in the tissue after transplantation. Furthermore, I studied the effect of the transplant with human neural precursors in the host neuronal preservation and activation, and in the preservation of specific sensory neuronal subpopulations located in the dorsal horns of the spinal cord. In addition, I had relevant contribution in the elaboration of the manuscript and in the preparation of the figures, as well as in the subsequent corrections prior to publication.

Transplantation of Human-Fetal-Spinal-Cord-Derived NPCs Primed with a Polyglutamate-Conjugated Rho/Rock Inhibitor in Acute Spinal Cord Injury

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Abstract: Neural precursor cell (NPC) transplantation represents a promising therapy for treating spinal cord injuries (SCIs); however, despite successful results obtained in preclinical models, the clinical translation of this approach remains challenging due, in part, to the lack of consensus on an optimal cell source for human neuronal cells. Depending on the cell source, additional limita-tions to NPC-based therapies include high tumorigenic potential, alongside poor graft survival and engraftment into host spinal tissue. We previously demonstrated that NPCs derived from rat fetal spinal cords primed with a polyglutamate

(PGA)-conjugated form of the Rho/Rock inhibitor fasudil (PGA-SS-FAS) displayed enhanced neuronal differentiation and graft survival when com-pared to non-primed NPCs. We now conducted a similar study of human-fetal-spinal-cord-derived NPCs (hfNPCs) from legal gestational interruptions at the late gestational stage, at 19–21.6 weeks. *In vitro*, expanded hfNPCs retained neural features, multipotency, and self-renewal, which supported the development of a cell banking strategy. Before transplantation, we estab-lished a simple procedure to prime hfNPCs by overnight incubation with PGA-SS-FAS (at 50 µM FAS equiv.), which improved neuronal differentiation and overcame neurite-like retraction after lysophosphatidic-acid-induced Rho/Rock activation. The transplantation of primed hfNPCs into immune-deficient mice (NU(NCr)-Foxn1nu) immediately after the eighth thoracic segment com-pression prompted enhanced migration of grafted cells from the dorsal to the ventral spinal cord, increased preservation of GABAergic inhibitory Lbx1-expressing and glutamatergic excitatory Tlx3-expressing somatosensory interneurons, and elevated the numbers of preserved, c-Fos-expressing, activated neurons surrounding the injury epicenter, all in a low percentage. Overall, the priming procedure using PGA-SS-FAS could represent an alternative methodology to improve the capabilities of the hfNPC lines for a translational approach for acute SCI treatment.

Keywords: human fetal neural precursor; NPC transplantation; Rho/ROCK kinase inhibition; cell priming; spinal cord injury

1. Introduction

Spinal cord injury (SCI) following severe physical trauma triggers a series of complex multicellular and molecular responses, resulting in a diverse degree of permanent motor, sensory, and/or autonomic dysfunctions. Despite the promising results obtained from intense research efforts in preclinical models over the past decade, no effective therapy has been efficiently translated into the clinic. Cell transplantation and advanced cell engineering have provided hope for treatment strategies with translational potential [1,2]. Neural progenitor cell (NPC) transplantation prompts neuroprotection and induces neuroregeneration by providing neurotrophic support, attenuating secondary damage, supplying a permissive substrate for axon regrowth [3,4], replacing lost neurons and establishing novel synaptic connections with host axons [5,6], and enhancing remyelination [7,8], which ultimately promote functional recovery in rodents [9,10]. The features and origin of NPCs represent

crucial aspects of developing translatable cell therapies. Primary self-renewing NPCs isolated from fetal tissue dis-play *in vitro* expansion potential, thereby providing an optimal cell source, which avoids inherent variations associated with the use of different fetal donors and the cell heterogeneity, which can elevate the risk of immune rejection and/or tissue contamination.

Standard procedures have been proposed to obtain “clinical-grade” NPCs from fetal neural tissue with minimal manipulation and in compliance with pharmaceutical good manufacturing practice (GMP) guidelines to ensure the production of advanced therapies for safe clinical use [11]. The transplantation of *in vitro*-expanded human fetal NPCs (hfNPCs) obtained from the lower cervical and upper thoracic spinal cord of an eight-week-old fetus prevented cyst expansion in an adult rat model of chronic SCI and post-traumatic syringomyelia [12]; however, current clinical trials with fetal tissue have employed immortalized cell lines derived from early fetal spinal cord [13] or brain tissue [14,15]. Alternative clinical approaches have employed NPCs derived from pluripotent stem cells; for example, Kumamaru et al. developed a xeno-free methodology to culture human spinal neural stem cells (NSCs) differentiated from embryonic stem cells (ESCs), thereby providing a scalable source for clinical translation [16]. These NSCs integrated into host tissue, induced the regeneration of the injured corticospinal tract, and enabled the extension of prolonged and persistent axonal projections, resulting in improved motor function in nude rats [16]. In non-human primates, these NSCs survived for nine months after transplantation and extended hundreds of thousands of human axons through monkey white matter, which established synapses and improved forelimb function, overcoming the immune graft rejection of the exogenous human cells [9]. Sugai et al. recently reported on the first clinical trial using NPCs derived from human induced pluripotent stem cells (iPSCs) as an SCI treatment [17]. Of note, while immortalized cell lines and pluripotent-cell-derived NPCs provide an inexhaustible source of cells, their application entails a high risk of tumorigenesis [18].

Here, we propose *in vitro*-amplified hfNPCs derived from the spinal cords of electively aborted fetuses at 19–21.6 weeks of gestation as an innovative approach for SCI treatment. As we recently demonstrated that priming fetal rat NPCs with PGA-SS-FAS, a polyglutamic-acid (PGA)-conjugated form of the Rho/Rock inhibitor fasudil (FAS), which provides improved

stability and controlled release, enhanced graft sur-vival and improved cell migration through the injured spinal cord [19], we also evaluated the impact of PGA-SS-FAS priming on hfNPCs. Transplantation of PGA-SS-FAS-primed hfNPCs in the acute stage of compressive SCI promoted host neuron preserva-tion and the increased expression of c-fos, a hallmark for cell activation, without any sign of tumorigenesis.

2. Materials and Methods

2.1. Isolation and Expansion of hfNPCs

hfNPCs were isolated and expanded from human fetal spinal cord tissue obtained from five fetuses from legally elective abortions induced by vaginal Misoprostol ad-ministration after being diagnosed with severe congenital malformations at 19.0–21.6 gestational weeks of gestation at the maternal–fetal medicine department at Vall d'Hebron Hospital Campus (Barcelona, Spain). The experimental procedures were evaluated and accepted by the clinical ethical committee at the Vall d'Hebron Hospital with the approved protocol PR(AMI)120/2017. Written informed consent for anonymized tissue collection was signed by each donor. Samples with an identified central nervous system-associated anomaly during the ultrasound examination were excluded. Negative serology for hepatitis and AIDS from donors was confirmed.

Human fetal spinal cords were dissected with sterile gloves and dissecting tools during the first hour after delivery. Each spinal cord, from cervical to lumbar segments, was transferred and maintained in 4 °C Hibernate™-E CTS™ Medium (Gibco™, Waltham, MA, USA) supplemented with 2% CTS™ B-27™ Supplement (Gibco™) in a hermetic sterile tube on ice for up to 5 h. In biosafe flow hoods, the spinal cords were washed twice with fresh hibernation media, and the meninges, dura, and pia mater were carefully removed, cut into ~1 mm3 pieces, and mechanically dissociated by repeated pipetting. The obtained cell suspension was centrifuge, and the cell pellet plated on ultra-low attachment plates in proliferation culture media—CTS™ Neurobasal™ Medium (Gibco™) supplemented with CTS™ GlutaMAX™-I Supple-ment (Gibco™), CTS™ B-27™ XenoFree Supplement, 100 µg/mL penicillin–streptomycin (Sigma-Aldrich; Darmstadt, Germany), 0.7 U/mL heparin (Sigma-Aldrich), 20 ng/mL human recombinant epidermal growth factor (hEGF; Peprotech, London, U.K.), 20

ng/mL basic human recombinant fibroblast growth factor (hbFGF; Peprotech), and 10 ng/mL human recombinant leukemia inhibitory factor (hLIF; Peprotech). Plates were incubated in 5% CO2 at 37 °C for two to three days until neuro-spheres formed. hfNPCs effectively formed neurospheres, which supported selection, clonal division, and cell proliferation. Neurospheres were enzymatically and mechanically dissociated using Accutase (STEMCELL™, Vancouver, Canada), and individualized cells were seeded at a density of 2.6 × 104 cells/cm2 in six-well culture plates coated with human recombinant Laminin 521 (BioLamina, Sundbyberg, Sweden). Subsequential sub-cultivation and expansion were performed for up to an additional nine passages. hfNPC cryopreservation as neurospheres or adherent growing cultures was performed using CryoStor® (Merck, Darmstadt, Germany). hfNPCs were then stored in liquid nitrogen.

2.2. Priming hfNPCs with PGA-SS-FAS Prior to Transplantation

Passage 5–7 hfNPCs were thawed and cultured in neurosphere-forming conditions in ultra-low attachment plates for 24 h and then incubated with PGA-SS-FAS (50 µM FAS-equiv.) for an additional 24 h in 5% CO2 and 37 °C. PGA-SS-FAS was synthe-sized and fully characterized as previously reported [19] following ICH guidelines to achieve an endotoxin-free nanoconjugate with a reliable impurity profile. The guidelines for good manufacturing practices (GMP) manufacturers were provided by PTS S.L. (Valencia, Spain). In general, PGA-SS-FAS was highly pure, with no significant impurities of residual solvents or ionic impurities. Before transplantation, hfNPCs were harvested and centrifuged at 200× g for 5 min and washed twice with culture media. The cell media discarded at every wash step was collected for inspection of the extracellular content of fasudil by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sample preparation and LC-MS/MS method for fasudil quantification were previously developed and described in Giraldo et al. [19]. Nevertheless, the developed LC-MS/MS method and extraction protocol were re-evaluated for linearity, the limit of quantification (LOQ), the limit of detection (LOD), recovery, and matrix effects to ensure its status as fit for purpose. Linearity was evaluated by constructing a calibration curve obtained by the internal standard method (ranitidine as an internal standard). LOD and LOQ values were calculated from the LINEST function. At the same time, matrix effects were evaluated by analyzing two different sample types: (i) blank cell medium spiked after sample

preparation with fasudil as three quality control (QC) samples (low, medium, and upper) and with 1 ng/mL of ranitidine and (ii) water solution of fasudil and ranitidine in the same concentration as spiked samples. Recovery was assessed at the three QC levels by comparing the mass of extracted analyte and analyte in the sample (plasma spiked after the sample preparation) and represented as a percentage. Fasudil was not encountered in any cell washing media, suggesting that hfNPCs did not secrete fasudil or PGA-SS-FAS during washing and confirming that the cell preparation will not deliver fasudil or PGA-SS-FAS to the extra-cellular space or surrounding cells after transplantation into the spinal cord tissue.

2.3. hfNPC Proliferation Assay

The doubling time, in hours, of hfNPCs was studied by subculturing passage 1 cells in adherent conditions at a density of 1.5 × 104 cells/cm2 until confluency, according to the following formula, t = t × log2/(logNt − logN0), where t is the culture time in hours, N0 is the initial cell number, and Nt is the harvested cell number.

The number of individual proliferating cells in the cell population was assayed by adding 10 µM 5′-Br-2′-deoxyuridine (BrdU, Sigma-Aldrich) 24 h before analysis. Cells were fixed with 2% paraformaldehyde (PFA) for 10 min, washed with phosphate buff-er solution (PBS), treated with 2M HCl for 20 min at room temperature, and then stopped with 0.1 M $Na_2B_4O_7$ for 30 min. The expression of Ki67 was assayed by immunocytochemistry in the same cultures to evaluate the number of cells undergoing the G1 to mitosis transition. Cells were blocked for 1 h in Tris-buffered saline (TBS) containing 0.2% Triton-X-100 and 5% normal goat serum and then incubated with anti-BrdU (1:400; Sigma-Aldrich) and anti-Ki67 (1:400; GTX16667, GeneTex) antibodies diluted in blocking solution overnight at 4 °C. Cells were subsequently incubated with Alexa-Fluor-conjugated antibodies for 2 h at room temperature, washed twice with PBS, and finally, incubated with DAPI for nuclear staining.

2.4. Spontaneous hfNPC Differentiation Assay

Neurosphere hfNPC cultures at passage 1 were dissociated with Accutase and seeded onto human recombinant Laminin 521-coated (BioLamina, Sundbyberg, Sweden) coverslips in DMEM/F12 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 5 mM HEPES buffer, 0.125% NaHCO3, 0.6% glucose, 0.025 mg/mL insulin, 80

µg/mL apotransferrin, 16 nM progesterone, 60 µM putrescine, 24 nM sodium selenite, and 2% of human serum for seven days. hfNPCs were then treated with 50 µM of PGA-SS-FAS on Day 3 to determine any effect of PGA-SS-FAS on cell differentiation. Cells were fixed on Day 7, and the neuronal, astrocytic, and oligodendroglia identity of the cells were evaluated by immunostaining with beta-III tubulin, GFAP, and OLIG2 antibodies, respectively.

2.5. Neurite Elongation Assays

hfNPCs in growth media were pre-treated with or without 50 µM of PGA-SS-FAS for 24 h. 10 µM lysophosphatidic acid, a pharmacological activator of the Rho/ROCK pathway (LPA; Sigma-Aldrich), was added for an additional 24 h to induce neurite re-traction. Cultures were then fixed and immunolabeled with Nestin, and neurite out-growth was quantified using the NeuronJ plug-in from ImageJ v1.48 [20].

2.6. Immunostaining

Cells were fixed with 4% PFA in PBS for 10 min, then permeabilized and blocked with 5% normal goat serum (NGS; Thermo Fisher) and 0.2% Triton X-100 (Sigma) and incubated overnight at 4 °C with primary antibodies. Fixed spinal cord tissues were first cryoprotected in 30% sucrose overnight at 4 °C before inclusion in Tissue-Teck OCT (Sakura Finetek Europe BV, Flemingweg, Netherlands) and then cryo-sectioned to provide 20 µm-thick sections. Tissue sections were permeabilized and blocked in PBS containing 0.1% Triton X-100, 5% horse serum, and 10% fetal bovine serum for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies.

The employed primary antibodies were: chicken anti-GFAP (1:1000; PA1-10004,Thermo Fisher), guinea pig anti-DCX (1:400; ab5910, Chemicon, Temecula, California, United States), anti-PAX6 (1:400; PRB-278P, Biolegend, San Diego, California, USA), rabbit anti-SOX2 (1:400; MAB5326, Abcam), rabbit anti-Ki67 (1:400; GTX16667, GeneTex), mouse anti-Nestin (1:400; MAB5326, Sigma Aldrich), mouse anti-Notch-1 (1:400; AF1057, R&D System, Minneapolis, Minnesota, USA), mouse anti-Olig2 (1:400; AB9610, Sigma-Aldrich), mouse anti-FOXJ1 (1:300; 14-9965-82, Thermo Fisher), mouse anti-Neurogenin1 (1:400; sc-100332, Santa Cruz), chicken anti-NeuN (1:400; ABN91, Sigma-Aldrich), rabbit anti-Tlx3

(1:5.000 gift of C. Birchmeier), guinea pig anti-Lbx1 (1:10,000 gift of C. Birchmeier), rabbit anti-c-Fos (1:400; ab190289, Abcam), and chick-en anti-GFP (1:400; ab13970, Abcam).

After three washes with PBS, cells or tissues were incubated with AlexaFluor-488, -555, or -647 (1:400, Invitrogen) secondary antibodies for 2 h at room temperature. Nuclei staining was performed by incubation with DAPI (1:1000, Sigma).

Fluorescent images were acquired using an apotome fluorescent microscope (Zeiss) or confocal microscope SP8 (Leica) as indicated. Consistent exposures were applied, and images were visualized and quantified with ImageJ/Fiji software v1.48.

Grafted cells found along the spinal cord were enumerated to analyze hfNPC survival, with the figure normalized to the microns of tissue thickness analyzed. The quantification of activated hfNPCs (double-positive for GFP and c-Fos) and the percentage of hfNPCs positive for c-Fos used a similar procedure. The neuroprotective properties of cell grafts were determined by analyzing neuronal preservation and activation 2 mm rostral and caudal to the lesion site and the lesion site (corresponding to the transplanted and injured area). Neural preservation was measured by enumerating the number of NeuN+ neurons, while neuronal activation used the number of neurons double-positive for NeuN and c-Fos normalized to the total analyzed area. All stainings and cell quantifications were performed from every fifth horizontal section of 20 mm in thickness in 3 animals per group. Cell number quantifications were then normalized to the analyzed summed tissue thickness (from ventral to dorsal sections) and expressed in mm^2.

Further analysis of neuroprotective effects on specific dorsal horn somatosensory interneuron populations measured the preservation of GABAergic Ladybird homeobox 1 (Lbx1)-expressing inhibitory and glutamatergic T cell leukemia homeobox 3 (Tlx3)-expressing excitatory interneurons 2 mm rostral and 2 mm caudal to the lesion site and in the lesion site normalized to the total analyzed area.

2.7. Spinal Cord Injury, hfNPC transplantation, and tissue processing

All experimental procedures were approved by the Animal Care Committee of the Research Institute Principe Felipe (Valencia, 2020/VSC/PEA/0119, Spain) in accordance with the National Guide to the Care and Use of Experimental Animals (Real Decreto 53/2013).

Immune-deficient female NU (NCr)-Foxn1nu mice (Charles River, France) weighing ≈20 g were housed under controlled light and temperature conditions. For surgical interventions, mice received subcutaneous morphine (5 mg/kg) 30 min prior to surgery and were anesthetized with 2% isoflurane in a continuous oxygen flow of 1 L/min. Laminectomy was performed at the T8-T9 level, exposing the dorsal surface of the du-ra matter. Using Bonn Micro Forceps (11083-07, Fine Science Tool, Heidelberg, Germa-ny) (0.3 mm wide), a one-second compressive spinal cord injury was performed at the T8 thoracic level, as previously described [21]. Immediately after compression, 2 µL containing 2.5 × 105 hfNPCs (previously infected with pll3.1-eGFP lentivirus for eGFP ectopic expression) primed with PGA-SS-Fas (hfNPCs + PGA-SS-FAS group), vehicle (hfNPCs group), or culture medium (control group) were intramedullary injected at the epicenter of the lesion in one single point to a 1 mm depth from the dorsal side. All animals were subjected to post-surgery care consisting of manual drainage of bladders twice a day until vesical reflex was recovered and subcutaneous administration of 5 mg/kg of enrofloxacin for seven days and 0.1 mg/kg of buprenorphine twice a day for four days. One month after injury and transplantation, animals were overdosed with an intraperitoneal administration of pentobarbital and transcardially perfused with PBS, followed by 4% of PFA in 0.1 M phosphate buffer (PB, pH = 7.4). Spinal cords were collected and maintained in 0.1M PB for further cryopreservation and histological analysis.

Hind limb motor function was evaluated for up to four weeks using the Basso Mouse Scale (BMS) locomotor rating scale [22] by videotaping the animals in an open field twice a week using a high-definition camera. Two individuals blinded to the treatment of the mice then examined and scored motor function. We did not find significant differences at any analyzed point between the three compared groups.

In addition, 2.5 × 105 hfNPCs primed with PGA-SS-Fas or vehicle were transplanted into non-injured mice at the T8 segment to evaluate tumorigenic/invasive potential one month after transplantation. hfNPCs were monitored by semiquantitative PCR for the eGFP gene expressed by transplanted hfNPCs in brain, heart, and liver samples using specific primers (fw_AAGTCGTGCTGCTTCATGTG; rv_GACGTAAAC-GGCCACAAGTT). Total RNA was isolated using TRIzolTM Reagent (Invitrogen), and cDNA was synthesized from 1 µg RNA using the high-capacity RNA-to-cDNA™ kit (4368814, Applied Biosystems) following the manufacturer's instructions. For PCR reactions, 50 ng of cDNA was amplified using GoTaq® DNA Polymerase (Promega, Mad-ison, Wisconsin, EEUU), using 55 °C for primer annealing. Mouse GAPDH amplifica-tion was used as a housekeeping gene (fw_CGGTGCTGAGTATGTCGTGGAGT; rv_CGTGGTTCACACCCATCACAAA).

2.8. Transmission Electron Microscopy

For electron microscopy studies, human spinal cords were fixed in 4% PFA over-night at 4ºC. After washing steps in 0.1 M PB, 200 µm coronal sections were cut on a Leica VT-1000 vibratome (Leica, Heidelberg, Germany). Sections were post-fixed with 2% osmium, rinsed, dehydrated, and embedded in Durcupan resin (Fluka, Sigma-Aldrich, St. Louis, USA). Semithin sections (1.5 µm) were cut with an Ultracut UC-6 (Leica microsystems, Wetzlar, Germany) and stained lightly with 1% toluidine blue. Finally, ultrathin sections (70–90 nm) were cut with a diamond knife, stained with lead citrate (Reynolds solution), and examined under an FEI Tecnai G2 Spirit BioTwin transmission electron microscope (ThermoFisher Scientific, Oregon, USA) using a Morada digital camera (Olympus Soft Image Solutions GmbH, Münster, Germany).

2.9. Statistical Analysis

Data are graphically represented as the mean ± standard error mean (SEM) and analyzed using Graph Pad Prism software. The Shapiro–Wilk normality test was performed to evaluate each dataset's Gaussian distribution. For comparisons between the two groups, a one-tailed t-test with a confidence level of 95% was used. If normality was not met, the non-parametric Mann–Whitney rank sum test was used.

3. Results

3.1 Human Fetal Spinal Cord NPCs Reside in the Ependymal Central Canal and the Spinal Parenchyma

Rodents maintain proliferative NSCs in the ependymal central canal, representing an endogenous source of cells for the repair of lesions [23–25]; however, we currently lack a detailed study of the human fetal spinal cord canal. Transmission electron mi-croscopy (TEM) images (**Figure 1A**) demonstrated the organization of ependymal cells at a gestational stage corresponding to 20 weeks—a tight alignment of ependymal cells with a radial morphology organized as a pseudostratified epithelium forming the central canal. We observed features previously described in fetal mice spinal cord [26]—highly polarized and multiciliated ependymal cells with a large number of cilia in the apical zone facing the lumen of the central canal (white arrow), nuclei with condensed chromatin (*), and long large junction complexes (white arrowheads).

We explored the expression of sex determining region Y (SRY)-box 2 (SOX2), the earliest transcription factor expressed in neural stem/progenitor cells [27], which controls the specification of early neural lineages and brain and spinal cord development [28] as a means to identify spinal-cord-resident NPCs in fetal samples at the late gestational stage of 19–21.6 weeks. We found abundant positive nuclear staining for SOX2 in the spinal cord parenchyma and the cells of the central canal (**Figure 1B**). We also observed double-SOX2-/Ki67-positive cells in the central canal and distributed throughout the spinal cord parenchyma contributing to the active proliferative NPC population [29]. As the cell constituting the central canal migrate towards the dorsal and ventral regions of the spinal cord, they differentiate, specialize, and determine the spinal cord cytoarchitecture. In our samples, we identified elongating processes from both NESTIN- and GFAP-expressing cells from the roof plate of the central canal (**Figure 1B**), which may help to determine the dorso-ventral regionalization of the spinal cord, as previously reported in rodents [30,31]. We also observed neurons, oligodendrocytic precursors, and astrocytes positive for NEUN (red), OLIG2 (yellow), or GFAP (red), respectively, distributed through the spinal cord parenchyma, allowing the de-termination of dorso-ventral regionalization (**Figure 1B**).

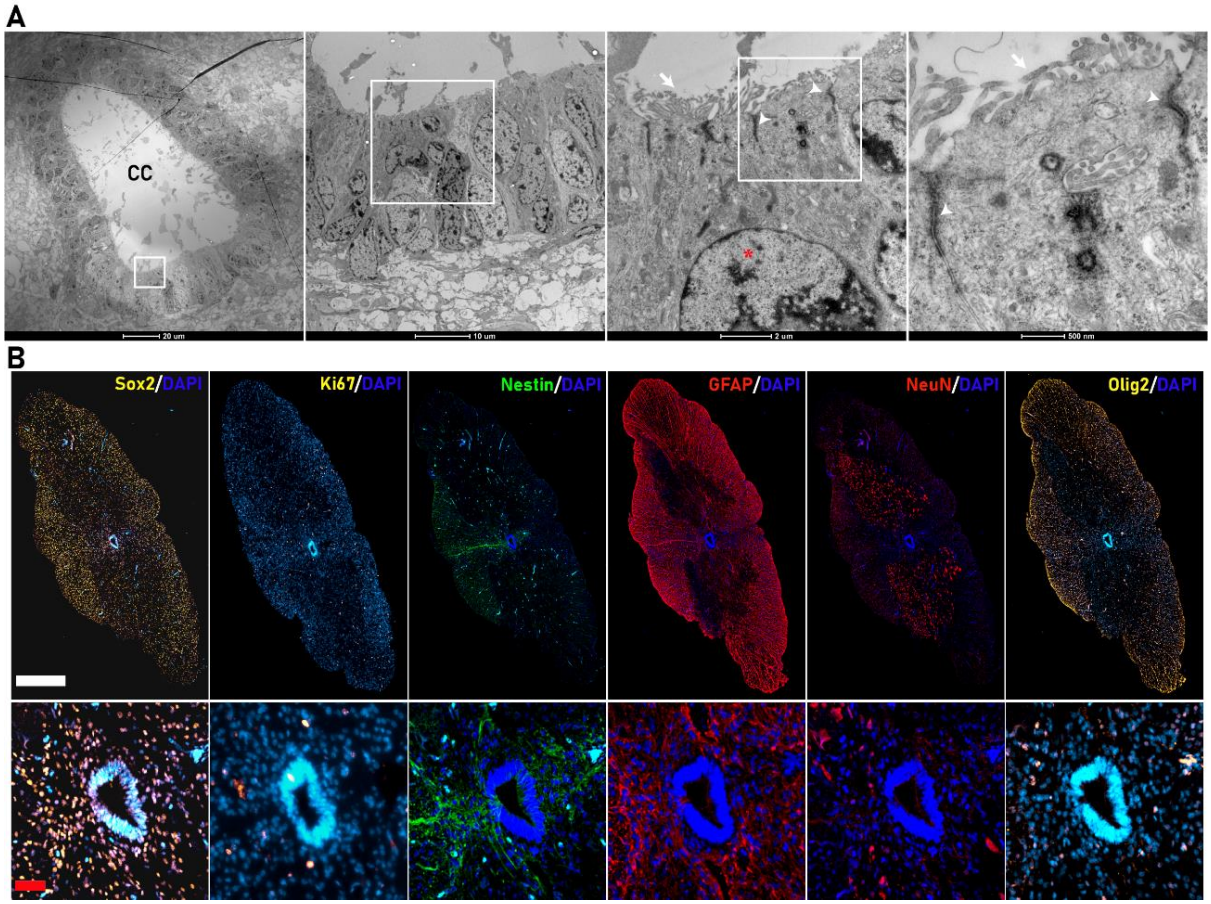

Figure 1. Cytoarchitecture of the human fetal spinal cord. **(A)** Transmission electron microscopy (TEM) images of the central canal of the human fetal spinal cord (scale bar (from left to right) = 20 µm, 10 µm, 2 µm, and 500 nm). The central canal is formed by ependymal cells organized as a pseudo-stratified epithelium with a large number of cilia in the apical zone (white arrow), nuclei with condensed chromatin (*), and large apical junction complexes (white arrowheads). **(B)** Representative coronal immunostaining images (complete coronal sections in upper panels and magnified view of the corresponding CC area in the lower panels) of human fetal spinal cords for SOX2 (yellow), Ki67 (yellow), Nestin (green), Gfap (red), NeuN (red), and Olig2 (yellow) (white bar scale= 500 µm; red bar scale = 50 µm).

3.2. hfNPCs Proliferate and Express Canonical Neural Markers in vitro

NPCs self-renew, proliferate, and differentiate into the three neural lineages [32]; therefore, we evaluated the isolation and expansion of NPCs from whole human fetal spinal cord homogenates in neurosphere-like-forming cultures that support the self-renewal and clonal-like growth of NPCs [33]. Dissociation and culture of a human fetal spinal cord supported the efficient generation of neurospheres in free-floating conditions after two days

of *in vitro* culture in the presence of the human recombinant mi-totic factors bFGF, EGF, and LIF. After three days of culture, we observed an enrichment of primary (white arrow) and secondary (black arrow) neurospheres (**Figure 2A**).

After three days of culture, we expanded hfNPCs in adherent conditions in laminin-coated wells, retaining their morphology during the long-term expansion of sub-cultures (**Figure 2B**) as previously described for adult human NPCs [34]. Following subculture and reaching 80% confluency, we calculated the cell doubling time in hours (t) for fhNPCs for four passages (Figure 2C, black line). The t of rat NPCs derived from E15.5 spinal cords was also evaluated as a reference sample (**Figure 2C**, red line). Although we observed more significant heterogeneity in the results during the first two passages, cell doubling times during four passages did not significantly differ, ranging from 75.68 ± 10.87 to 103.1 ± 16.23 h, more than three times the time needed for the rat fetal cells to duplicate in culture (**Figure 2C**). Nevertheless, hfNPCs at passage 4 con-tinued to proliferate, with 21.4 ± 3.3% of cells incorporating BrdU at the S phase and 37.5 ± 4.7% displaying positive staining for the Ki67 mitotic marker (**Figure 2E**).

Previous studies of the subventricular zone of adult brain tissue revealed a pinwheel structure, which corresponds to the adult neurogenic niche [35]; furthermore, this structure also represents a hallmark of stemness in mouse-spinal-cord-derived neurospheres [36]. To study if hfNPCs from late gestational stages also form neurogenic niche-like structures, we immunostained for γ-tubulin to indicate microtubule-organizing centers, centrosomes, basal bodies, and β-catenin to delineate cell borders for the identification of pinwheel structures. For the first time, we revealed that *in vitro* cultures of spinal cord hfNPC-derived neurospheres adopt a pinwheel structure and display the neural features of fetal developmental stages (**Figure 2D**).

To ascertain whether amplified hfNPCs expressed canonical neural markers at passages 2 to 3, we immunoassayed samples for neurogenic locus notch homolog protein 1 (NOTCH1), paired-box protein 6 (PAX6), NESTIN, SOX2, Forkhead Box J1 (FoxJ1), doublecortin (DCX), and Neurogenin1 (**Figure 2F**). hfNPCs expressed high levels of nuclear NOTCH1 and PAX6 (90.5 ± 13.8% and 91.7 ± 4.4% positive cells, respec-tively)—activated NOTCH translocates to the nucleus to support stemness [37], while Pax6 regulates NPC proliferation

and self-renewal [30]. hfNPCs also stained positive for NESTIN (83.4 ± 2% positive cells) and SOX2 (79.7 ± 2.2% positive cells), which are expressed in late NPCs [38,39]. Additionally, 65 ± 6.4% of hfNPCs stained positive for FOXJ1, which is expressed in ciliated cells such as the ependymal cells [40], while hfNPCs also expressed DCX (56.4 ± 7.4%) and Neurogenin1 (45.8 ± 9.5%), with both proteins involved in neurogenesis [41,42] (**Figure 2F**).

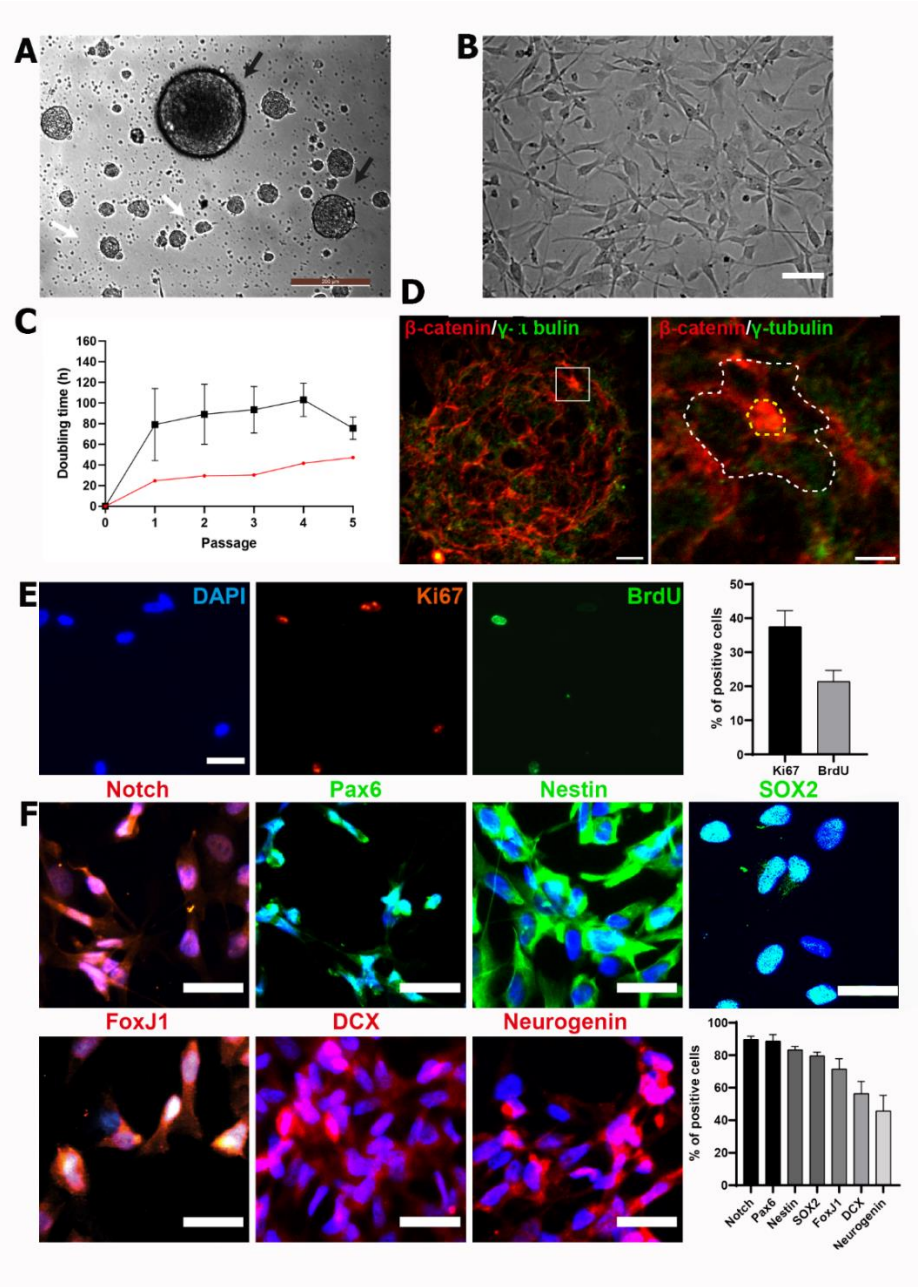

Figure 2. hfNPCs display proliferative potential and express canonical NPC markers *in vitro*. **(A)** Phase contrast image from hfNPCs as neurospheres-like cultures forming primary (black arrows) and secondary (white arrows) neurospheres as a hallmark of self-renewal in the presence of mitogens at passage 1. Scale bar = 200 µm. **(B)** Representative phase contrast image of hfNPC culture in adherent

conditions in laminin-coated wells when cultured at passages 2–5 (Scale bar = 20 µm). **(C)** Doubling time (t) analysis of hfNPCs (black line; data presented as the mean ± SEM (n = 5 samples) and one rat fetal NPC sample (red line) growing in adherent conditions over five passages. **(D)** The pinwheel cytoarchitecture (indicated with a white dotted line) of hfNPC at passage 1 from neurosphere-like cultures is highlighted after β-catenin (red; delimiting the cell perimeter) and γ-tubulin (green; for cilia detection) staining (scale bar = 20 µm (left panel) and 10 µm (right panel)). **(E)** Representative images from nuclear staining of DAPI (blue), Ki67 (red), and BrdU (green) of hfNPCs in adherent conditions (scale bar = 10 µm). Right panel: Quantification of the percentage of positive cells for BrdU and Ki67. Data presented as the mean ± SEM (n = 3 samples). **(F)** Left panel: Representative images of immunofluorescent staining for Notch, Pax6, Nestin, Sox2, FoxJ1, DCX, and neurogenin (scale bar = 20 µm). Right panel: Quantification of the percentage of positive cells for each canonical neural progenitor cell marker. Data presented as the mean ± SEM (n = 5 samples).

3.3. PGA-SS-FAS Priming Enhances the Neuronal and Oligodendroglial Differentiation of hfNPCs

We next assessed the multipotent differentiation potential of hfNPCs after mitogen withdrawal and serum supplementation in the absence and presence of PGA-SS-FAS priming via immunofluorescence analysis for β-III-tubulin (pan-neuronal marker; green), GFAP (astrocyte marker; red), OLIG2 (oligodendrocyte marker, green), and NEUN (mature neuronal marker, red) (**Figure 3A**). Immunofluorescence quantitative analysis demonstrated that hfNPCs gave rise to neurons (9.85 ± 1.91 of cells positive for β-III-tubulin), astrocytes (17.78 ± 3.49% of cells positive for GFAP), and oligodendrocytes (15.54 ± 2.54% of cells positive for OLIG2) (**Figure 3B**), revealing the multipotency of the hfNPCs after *in vitro* amplification. Interestingly, PGA-SS-FAS priming significantly increased neuronal differentiation (up to four-times the number of β-III-tubulin-positive neurons in comparison with non-primed control hfNPCs; 43.61 ± 4.92% vs. 9.85 ± 1.91) and promoted mature neuronal differentiation (4.75 ± 1.11% NeuN-positive cells compared to non-primed hfNPCs, which lacked mature neurons) (**Figure 3B**). PGA-SS-FAS priming also significantly increased oligodendrocyte differentiation (30.11 ± 3.78% OLIG2-positive cells compared to non-treated hfNPCs), alt-hough we observed no differences regarding astrocyte differentiation (**Figure 3B**).

SCI activates the Rho/ROCK pathway, constituting one of axonal regrowth's most significant inhibitory signals [43]. We activated the Rho/ROCK pathway by culturing hfNPCs in

the presence of 10 µM LPA, a potent mitogen that induces neurite collapse [44], for 24 h, mimicking the intrinsic mechanism that blocks axon regeneration following SCI (**Figure 3C,D**). LPA induced a significant retraction of neurite-like processes in hfNPCs compared to control non-primed hfNPCs (28.45 ± 4.41% vs. 72.07 ± 8.96; **Figure 3C**, red arrows); however, PGA-SS-FAS priming of hfNPCs efficiently inhibited neurite-like retraction after LPA exposure (120.8 ± 8.16 vs. 28.45 ± 4.41%; **Figure 3D**, yellow arrows). These data suggest that priming with PGA-SS-FAS would prevent neuronal-like retraction of hfNPCs at the injury site following Rho/ROCK signaling activation.

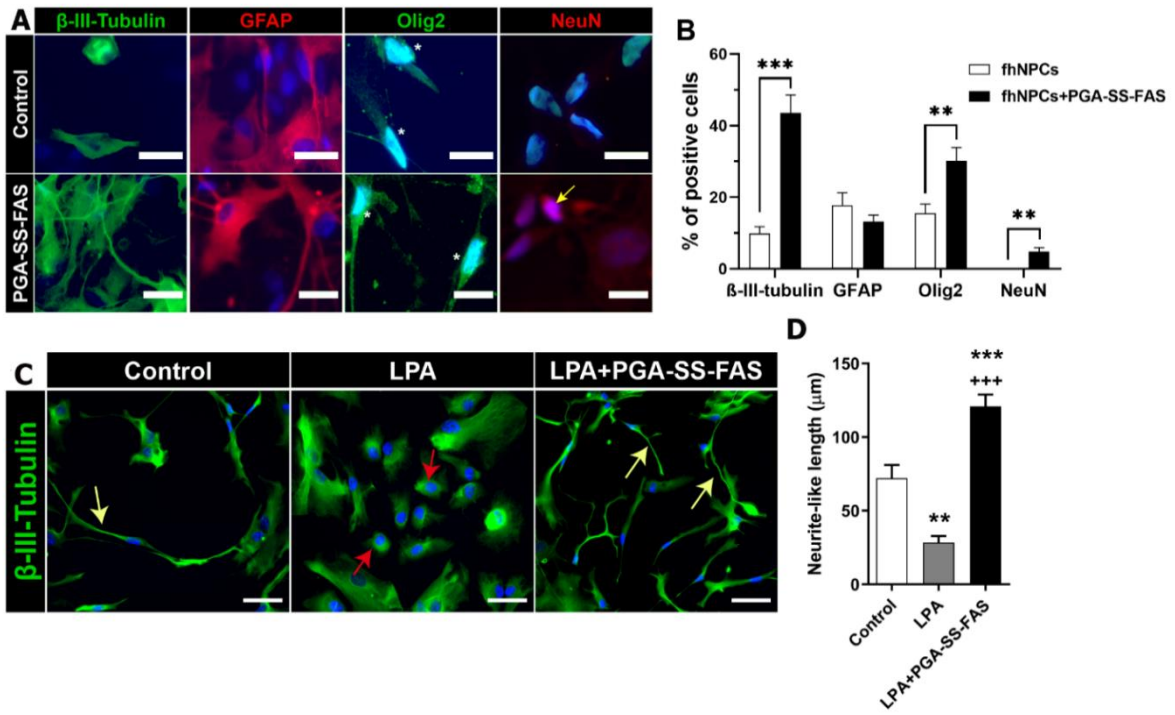

Figure 3. PGA-SS-FAS priming process enhances neuronal and oligodendrocyte differentiation and induces neurite outgrowth in a model of neurite retraction. **(A)** Representative immunofluorescence images of hfNPCs using β-III-tubulin for immature neurons (green), GFAP for astrocytes (red), Olig2 for oligodendrocytes (green), NeuN for mature neurons (red), and DAPI (blue) (scale bar = 20 µm) (positive nuclear staining is indicated with an arrow) in the presence of the PGA-SS-FAS com-pound for inducing the priming process (PGA-SS-FAS) or its vehicle (control). **(B)** Quantification of the percentage of positive cells for each of the indicated cell markers with (black bars) or without (white bars) 24 h PGA-SS-FAS priming. Data presented as the mean ± SEM determined by Student's unpaired t-test (n = 3). ** $p < 0.01$, *** $p < 0.001$ vs. hfNPCs. **(C)** Representative immuno-fluorescence images of β-III-tubulin (green) and DAPI (blue) staining during the *in vitro* neurite retraction assay induced by lysophosphatidic acid (LPA) treatment during 24 h in hfNPC adherent cultures. Yellow arrows indicate

neurite-like processes emanating from neuronal progenitors in the control condition (in the absence of LPA) and in co-treated cultures (LPA + PGA-SS-FAS); red arrows indicate rounded cells 24 h after LPA treatment and induced neurite-like retraction (scale bar = 50 µm). **(D)** Neurite length quantification presented as the mean ± SEM determined by one-way ANOVA with the Tukey multiple comparison test (n = 3). ** $p < 0.01$, *** $p < 0.001$ vs. control; +++ $p < 0.001$ vs. control.

3.4. PGA-SS-FAS Priming Enhances the Ventral Engraftment of hfNPCs, Endogenous Neuronal Activation, and Neuronal Survival After Transplantation into the Injured Spinal Cord

We next performed an intramedullary transplant of hfNPCs primed with PGA-SS-FAS (hfNPCs + PGA-SS-FAS) or vehicle (hfNPCs) immediately after compressive injury to the eighth thoracic vertebrae segments in nude mice to evaluate their therapeutic potential (**Figure 4**). We did not observe significant differences in the total number of surviving hfNPCs when comparing hfNPCs and hfNPCs + PGA-SS-FAS (**Figure 4A–C**), in both cases with a low survival rate (with an estimated percentage for the to-tal cells of 2,7±1,1 for hfNPCs + PGA-SS-FAS and 2,6 ± 0,5 for the hfNPC group), as shown in the representative images of GFP-positive grafted cells (**Figure 4A**). hfNPCs primed with PGA-SS-FAS (hfNPCs + PGA-SS-FAS) possessed enhanced grafting and migratory capacities and were encountered from the ventral to the dorsal areas of the spinal cord, while non-primed cells were restricted to the dorsal and injected areas (**Figure 4D**). In addition, hfNPCs + PGA-SS-FAS grafts possessed increased expression of c-Fos (**Figure 4E,F**) (a marker associated with neuronal activity [45]) when compared to non-primed hfNPCs, thereby suggesting that PGA-SS-FAS priming enhances the activation of transplanted hfNPCs. To determine the safety of the hfNPC transplantation and the priming process, we evaluated the potential invasiveness of grafted cells for both groups outside the spinal cord. Four weeks after transplantation, we evaluated the expression of eGFP by transplanted hfNPCs in the brain, heart, and liver by semiquantitative PCR. We failed to detect GFP expression in any of the analyzed tissues (data not shown). GABAergic inhibitory Lbx1 and glutamatergic excitatory Tlx3 are transcription factors involved in neuronal fate determination of somatosenso-ry interneuron populations located in the dorsal horns of the spinal cord, which modulate and integrate peripheral somatosensory inputs [46]. A low percentage of hfNPCs displayed Lbx1 and Tlx3 expression in the grafts, while priming with

PGA-SS-FAS did not influence this cell fate determination, as we failed to find any significant differ-ences between the groups (**Figure 4G–I**).

Quantifying neuron survival at the injury site demonstrated no significant differences between primed and non-primed hfNPCs rostral or caudal to the injury and at the lesion epicenter (**Figure 4J–L**). Nevertheless, PGA-SS-FAS-primed hfNPCs showed a modest, but significant number of c-Fos+/NeuN+ cells at the injury site (**Figure 4M**), indicating a potential effect of the primed grafts on the subrounded neuronal activation. Furthermore, a modest increase in Lbx1 and Tlx3 interneurons surrounding the graft was found in the hfNPCs + PGA-SS-FAS group at the dorsal horn compared with the hfNPC group (**Figure 4N–P**).

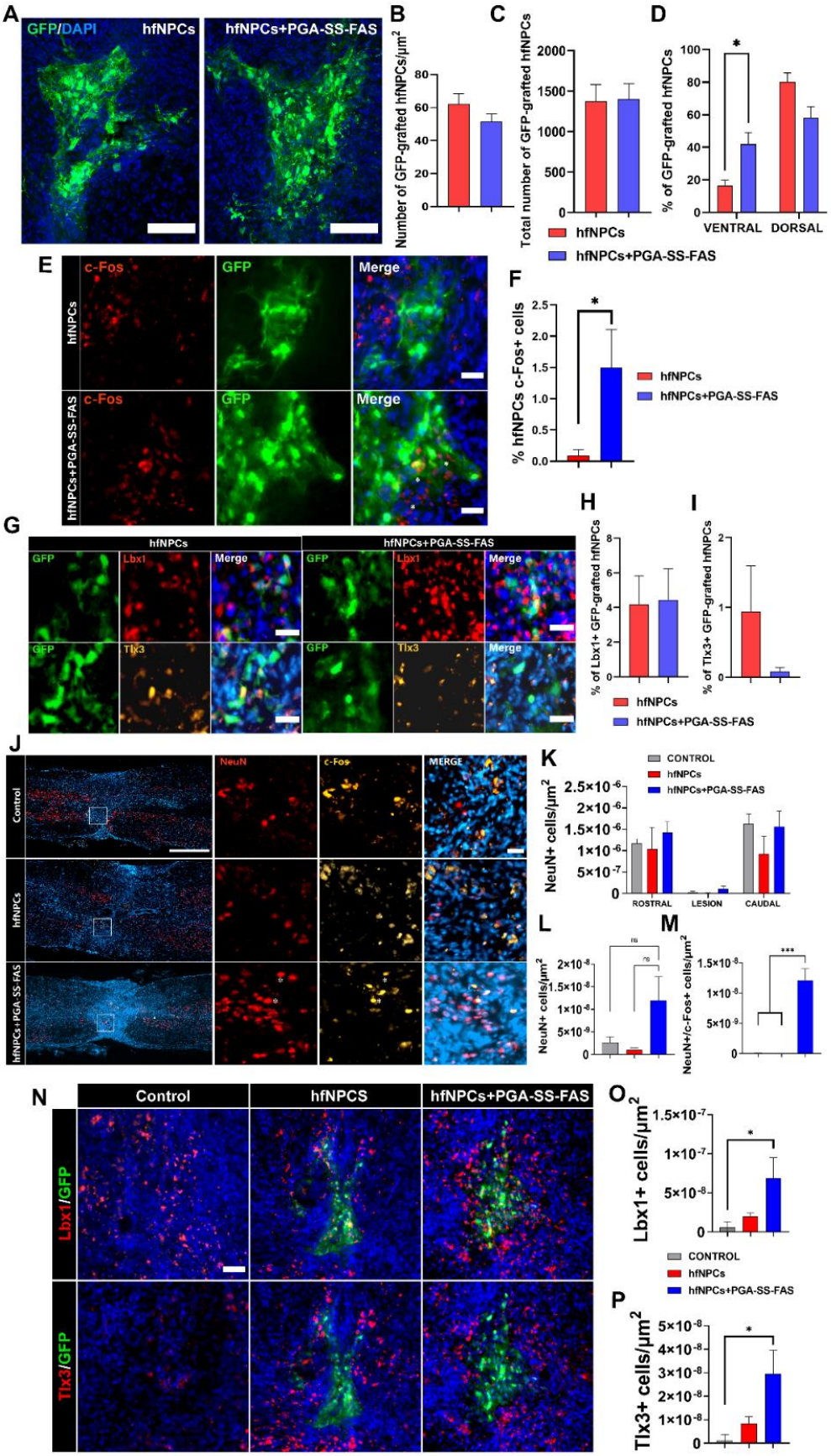
A
GFP/DAPI
hfNPCs
hfNPCs+PGA-SS-FAS
B
Number of GFP-grafted hfNPCs/µm²
C
Total number of GFP-grafted hfNPCs
hfNPCs
hfNPCs+PGA-SS-FAS
D
% of GFP-grafted hfNPCs
VENTRAL
DORSAL
E
c-Fos
GFP
Merge
F
% hfNPCs c-Fos+ cells
G
Lbx1
Tlx3
H
% of Lbx1+ GFP-grafted hfNPCs
I
% of Tlx3+ GFP-grafted hfNPCs
J
Control
NeuN
c-Fos
MERGE
K
NeuN+ cells/µm²
CONTROL
ROSTRAL
LESION
CAUDAL
L
M
NeuN+/c-Fos+ cells/µm²
N
Control
hfNPCS
Lbx1/GFP
Tlx3/GFP
O
Lbx1+ cells/µm²
P
Tlx3+ cells/µm²

Figure 4. Quantification, distribution, and phenotypic characterization of non-primed and primed hfNPCs post-transplantation. **(A)** Representative images of GFP staining for hfNPC-grafted cells in spinal cord horizontal sections for the indicated groups (green) (scale bar = 50 µm). **(B)** Quantification of GFP-positive transplanted cells normalized to the total measured thickness in μm^2. **(C)** quantification of GFP-positive transplanted cells in absolute numbers every 5th 20 mm section. **(D)** Quantification of the dorso-ventral distribution of the GFP-positive cells expressed in percentage of the total number of quantified cells. **(E)** Representative images of c-Fos+ (red) and GFP (green) and the merged image including DAPI (blue) -positive immunostaining of grafted GFP-positive hfNPCs. **(F)** Quantification of the percentage of c-Fos-positive hfNPCs in the primed or non-primed groups, presented as the mean ± SEM and determined by Student's unpaired t-test (n = 3). (* $p < 0.05$). **(G)** Representative images of immunofluorescent staining for GFP- (green), Lbx1- (red), and Tlx3- (yellow) positive grafted cells and merged images with DAPI (blue) of both experimental groups (scale bar = 25 µm). **(H)** Quantification of the percentage of Lbx1. **(I)** Tlx3-positive hfNPCs presented as the mean ± SEM and determined by Student's unpaired t-test (n = 3). **(J)** Representative immunostainings of c-Fos (yellow), NeuN (red), and DAPI (blue) (white scale bar = 500 µm; red scale bar = 25 µm). **(K)** Quantification of NeuN-positive cells in the rostral, lesion normalized to the total measured thickness in µm2, and caudal sites. **(L)** Quantification of NeuN-positive cells in the lesion site; data presented as the mean ± SEM determined by one-way ANOVA with Tukey's multiple comparison test (n = 3). **(M)** Quantification of NeuN/c-Fos-double-positive cells in the lesion site normalized to the total measured thickness in μm^2. Data presented as the mean ± SEM determined by one-way ANOVA with Tukey's multiple comparison test (n = 3). **(N)** Representative immunostainings of (top panel) Lbx1 (red), GFP (green), and DAPI (blue) and (bottom panel) Tlx3 (red), GFP (green), and DAPI (blue) (scale bar = 50 µm) for the indicated experimental groups. **(O)** Quantification of Lbx1. **(P)** Tlx3-positive cells in the area surrounding the graft and le-sion normalized to the total measured thickness in μm^2. Data presented as the mean ± SEM and determined by one-way ANOVA with Tukey's multiple comparison test (n = 3) (* $p < 0.05$, *** $p < 0.001$ (* $p < 0.05$, *** $p < 0.001$, ns = non-significant).

4. Discussion

The inflammatory and secondary damage milieu of SCI, which causes poor cell survival and grafting and improper differentiation, has been attributed as the factor limiting the therapeutic potential of cell therapy [47]. Survival rates and sufficient engraftment and integration into spinal cord circuits represent critical factors for suc-cessful cell transplantation and functional improvements [2]. In addition, aberrant neuronal connections induced by SCI can prompt allodynia, among other undesired effects [48]. Despite their

multipotency, NPC transplants lack consistent neuronal motor differentiation [9,49], with differentiation generally driven towards the generation of glia by the SCI milieu. Combination therapies with varying levels of success have been developed to overcome these limitations—as reviewed by Griffin and Bradke [50]—with strategies including the combination of Notch inhibitors, to induce neuronal dif-ferentiation [51], with biocompatible matrices [52] or exogenous neurotrophic factors [53,54].

The origin and properties of NPCs have enormous significance in regenerating damaged spinal circuits [53]. Kadoya et al. recently demonstrated that rat fetal NPCs with spinal cord features (and not brain-derived NPCs) promoted regeneration of the corticospinal tract and functional motor improvement [53]. Moreover, Dulin et al. revealed that NPCs retain their features and differentiate towards specific phenotypes after transplantation into homologous regions of the host tissue, depending on their dorsal or ventral origin. [55].

Currently, translational strategies under investigation employ ESC- [16] or iPSC- [17] derived NSCs or immortalized fetal NPC lines [13] that have undergone significant manipulation prior to transplantation (e.g., reprogramming, long differentiation processes, or cell cycle manipulation for cell immortalization). We wanted to propose an alternative source for NPCs with minimal manipulation in the present study. To adhere to clinical-grade conditions, we isolated and *in vitro* expanded hfNPCs from 19.0- 21.6 weeks of gestation human fetal spinal cords under xeno-free conditions. We found that the isolated hfNPCs retained canonical neural features and multipotency *in vitro*, making them suitable as a clinical translational approach for SCI. In addition, we evaluated a combinatorial strategy by priming hfNPCs with an in-house-developed polymer-conjugate of the Rho/ROCK inhibitor fasudil (PGA-SS-FAS), which previously demonstrated neurogenerative properties in an immunocompetent rat model of SCI [19]. This approach allowed for the standardized culture of spinal-cord-derived hfNPCs and the generation of a procedure for improved combinatorial therapy suita-ble for cell replacement therapy in the injured spinal cord.

We encountered SOX2-expressing proliferative NPCs densely packed within the central canal, but also occupying most of the spinal cord, giving rise to a heterogeneous population of progenitors at the primary culture expanded from the whole fetal spinal cord. Nevertheless, the heterogeneous hfNPC population possesses a homogeneous-like cell

morphology in culture with a consistent cell fate profile when comparing different samples. hfNPCs retained stemness, with more than 80% of cells expressing SOX2 over several passages *in vitro*; however, we uncovered an important barrier for cell banking: hfNPCs possessed a low amplification efficiency, with a PD time more than three-times the time needed for cell duplication when compared with other cell populations derived from earlier gestational stages [12]. Although fhNPCs retained their proliferative capacity under the evaluated conditions, the employed growth-factor-enriched formulation (supplemented with bFGF, EGF, and LIF) requires further improvement to promote higher *in vitro* proliferative rates and cell expansion to make cell banking more feasible since rat-derived NPCs, also from late gestational stages, displayed a significant lower doubling time in the same cell culture conditions. Direct comparative analyses between different gestational ages for NPC isolation, expansion, and banking production will be required.

In vitro priming with PGA-SS-FAS promoted faster cell maturation, favoring neuronal and oligodendroglial differentiation without interference with astroglial differentiation within 24 h of incubation. Poor neuronal differentiation has been reported as a significant limitation of NPC transplantation approaches; we now report a one-step priming procedure to partially overcome this problem, avoid *in vivo* application of PGA-SS-FAS, and reduce the potential side effects on host tissue. The convergence RhoA/ROCK pathway becomes activated by inhibitory molecules within the SCI milieu and plays a central role in inflammation, apoptosis, neuronal degeneration, and axon retraction [56]. Given the importance of these processes to SCI pathogenesis and the impairment of functional recovery, several pharmacological strategies have been developed to prevent cell death and promote axonal regeneration and functional recov-ery after SCI [57,58]. We previously described that NPCs derived from fetal rat spinal cords displayed improved neurite regrowth *in vitro* following treatment with PGA-SS-FAS, which also enhanced engraftment, induced a neuronal-like morphology, and elongated neurons from NPCs *in vitro* and *in vivo* [19]. Conversely, Stern et al. report-ed that RhoA inhibition had opposing roles in neurons and astrocytes, with RhoA activation limiting astrogliosis and RhoA ablation enhancing axon regeneration in neurons [59]. Considering these results, selective cell-specific approaches will be required to avoid side effects in host tissues.

Following SCI, axonal disruption and neuron death cause irreversible functional losses; therefore, preserving neuronal circuits, replacing lost neurons, and providing a regenerating environment to enhance plasticity after injury represent critical objectives for SCI therapeutics. We found that primed hfNPCs significantly preserved Tlx3- and Lbx1-expressing neuronal cells, which could be supported by the higher percent-age of oligodendrocyte precursors induced by the treatment with PGA-SS-FAS prior to transplantation demonstrated *in vitro*. The Tlx3 homeobox gene functions as a developmental regulator of excitatory neurons, promoting glutamatergic excitatory specification and suppressing GABAergic specification in dorsal spinal cord neurons [60]. Lbx1 is required for the correct specification of early dorsal interneuron populations and plays a critical role in developing the spinal cord sensory pathways that transmit pain and touch [61]. Furthermore, grafted hfNPCs without the influence of the PGA-SS-FAS priming differentiated into Tlx3 and Lbx1 interneurons, showing phenotypically appropriate host target regions in the dorsal area of the spinal cord.

PGA-SS-FAS-primed hfNPCs induced the *in vivo* expression of c-Fos, a classical marker of neuronal activity [62], which is also related to synaptic plasticity and learning [63]. We observed a double effect: First, PGA-SS-FAS priming activated grafted hfNPCs, which could influence the differential migratory profile encountered compared with the non-primed hfNPCs. Fasudil-induced migration has been previously described via activation of the MAPK signaling pathway in mesenchymal stem cells [64] and the ERK signaling pathway in microglia [65]. Further analysis of the mechanism of action involved in hfNPC migration needs further investigation. Second, the transplantation of PGA-SS-FAS-primed hfNPCs induced the increased activation of endogenous neurons surrounding the graft (measured by the increased expression of c-Fos in host neurons), which has been related to synaptic plasticity and learning in oth-er systems [63]. Nonetheless, despite the identified histological signs showing modest, but significant, improved neural preservation capabilities on the hfNPCs + PGA-SS-FAS group, we did not find differences on the locomotion recovery by the BMS test weekly analysis (data not shown).

5. Conclusions

Overall, our results provide evidence that PGA-SS-FAS-primed hfNPCs exert modest, but significant improved neuroprotective and a more migratory engraftment capability with

an increased activation of surrounding endogenous neurons, which could provide a new combinatorial approach in a single formulation, which may serve as an improved cell therapy for SCI. However, since the differences reported here, em-ploying PGA-SS-FAS-primed hfNPCs, did not improve graft survival and did not improve functional regeneration, we expect that PGA-SS-FAS-primed hfNPCs from earlier fetal stages would provide better results. In addition, since an immune-deprived mouse model was employed hosting the exogenously transplanted human cells, further evaluation in an immune competent model will be needed to address the important limitation of the immune rejection prior to clinical application.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from sample donors to publish this paper.

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DISCUSSION

4. DISCUSSION

In recent decades the work done in various fields of new generation biomedicine including regenerative medicine, nanomedicine (Abbas et al. 2020), tissue engineering (Saremi et al. 2022) and genetic engineering (Allahdadi et al. 2019), rehabilitation strategies with electrical stimulation implants for the control of the brain and spinal cord (Flett, Garcia, and Cowley 2022) as well as the use of innovative neuroprosthesis (Kilgore, Anderson, and Peckham 2020) has allowed to question the dogma established at the beginning of the XX century by Ramon y Cajal of the impossibility of the adult central nervous system in mammals to regenerate stating: “In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (Ramon y Cajal 1928).

Although prosthetic and implant therapies are strong candidates to achieve functional recovery, stem cell therapy, especially NSC therapy, is the strongest candidate to obtain not only functional improvement, but also to promote tissue regeneration so that the tissue recovers its original organization or an equivalent one. Currently, allogeneic transplantation is the most realistic option for the translation of cell therapies to clinical trials, although it has some drawbacks such as committing the patient to immunosuppressive treatment, increasing the risk of infection or malignancy. Thus, iPSCs technology presents some advantages over other cellular sources such as ease of sample collection and virtually unlimited availability, allowing for autologous transplants, and avoiding ethical concerns. Nevertheless, the cost and complexity of the procedures, the need to guarantee genomic stability and the risk of tumorigenicity which is associated with the use of tumor-inducing reprogramming factors and residual undifferentiated cells (J. Deng et al. 2018; Lund, Närvä, and Lahesmaa 2012) are still limitations for its implementation in the clinic.

With the aim to prevent undesired outcomes and develop a therapy potentially transferable to clinical practice, in chapter 1 and 2 we transplanted a novel “clinical grade” iPSC-NSCs line. It is described that the reprogramming factor c-Myc is related to induction of tumorigenesis (Okita, Ichisaka, and Yamanaka 2007), therefore our iPSC-NSCs have been reprogrammed using a synthetic mRNA transfection replacing c-Myc by cyclin-D1 resulting in significantly improved genetic stability and successfully engrafted and differentiated showing no tumor formation in the SCI model (Alvarez-Palomo et al. 2021). Data obtained in chapter

1 and chapter 2, demonstrated that the application of the cell therapy showed no signs of tissue damage or malformation and no tumor formation throughout the experiments, therefore our treatments were not inducing tumorigenic effects. Thus, our “clinical grade” iPSC-NSCs has so far proven its safety and beneficial effects in our *in vivo* models and thus constitute a candidate therapy for the treatment of spinal cord injury. Nevertheless, further studies should be developed to provide more data about grafting, differentiation and proliferation and the consequences of long-term transplantation in the tissue.

Furthermore, translating advanced therapies, such as cell-based therapy, to clinical trials is not a straightforward path and for the development of “clinical grade” cell lines we must follow some regulatory requirements. First, it is required to establish a cell bank for allogeneic products to culture and expand therapeutic cell lines under good manufacturing practice (GMP) conditions (Han et al. 2020). Moreover, during the process of cell culture and proliferation it is necessary to characterize and define by standardized tests their identity, potential, genetic stability and viability to guarantee that they retain their original features unaltered before initiating complete translation programs (Heathman et al. 2015). Therefore, before we can perform clinical trials with our new cell line, in addition to long-term studies, we should establish a cell bank and perform these cell characterization studies prior to translation to clinical assays. In chapter 3 we first isolated hfNPCs from late gestational stages, providing a minimally manipulated source of NPCs, although low in vitro proliferative rates limited the establishment of a cell bank. Unlike the other two chapters, the advantage of the combination used in chapter 3 lies in the use of a single formulation combination therapy. hfNPCs have also shown to be safe in immunosuppressed mice and have shown short-term neuroprotective effects, although it would also be necessary to evaluate these outcomes in the long term. Moreover, we also would need to improve the proliferation *in* vitro of the hfNPCs in order to establish a cell bank before considering its use in the clinic.

SCI patients are subjected to complex surgical processes that carry the risk of damaging vital structures located in the spinal cord parenchyma, so the search for minimally invasive approaches that allow delivery of cells and/or drugs are particularly attractive for preserving tissue integrity. Biomaterials are particularly interesting for these approaches since they are suitable for cell embedding and nanoparticles carrying, but the success of the

minimally invasive approaches depends on the ability of the cells to survive in the cerebrospinal fluid (CSF) and migrate into the spinal cord parenchyma (Oliveira et al. 2018). In chapter 2 we placed the demilune scaffold directly on the spinal cord, reducing the direct damage caused during cell transplantation. Thus, we confirmed that both the biomaterial and the implantation procedure represent a safe therapy for transplantation in our short-term study. Furthermore, the biomaterial did not impede the migration of the cell transplants to the lesion site, similar to previous studies in which cell therapy was transplanted by intrathecal injections in preclinical models (T.-T. Cao et al. 2022; Vives et al. 2022). However, long-term studies should be carried out to study the long-term safety and outcomes of this non-invasive approach.

Apart from the described benefits provided by our therapies, we have also found a number of limitations in our studies. Firstly, the low survival of transplants poses a challenge, since it is of vital importance to maintain transplants in a suitable environment to enhance survival and differentiation that allow to obtain beneficial effects of therapy. Even so, we have described a number of therapeutic benefits at the histological level which, unfortunately, have not been translated into terms of functional improvement. Restoration of function relies on enhanced neuroplasticity to enhance the regeneration of spared and injured axons, to strengthen remnant connections, and to stimulate new connections and circuit formation. Current clinical trials have described the restoration of sensory function, but locomotor recovery remains one of the main limitations and one of the main goals to be achieved (Silvestro et al. 2020). It is known that providing neuroplasticity and regeneration does not always translate into functional improvement, and therapies should be more targeted to stimulate axonal regrowth as well as circuit reorganization by establishing the right connectivities in specific spinal circuits (Sofroniew 2018) since formation of aberrant connectivities may have detrimental consequences such as neuropathic pain (Basbaum et al. 2009). Recovery of locomotor function has been accomplished in a number of preclinical studies (Kadoya et al. 2016; Brock et al. 2018), and the molecular mechanisms underlying intrinsic axonal regeneration have also been described (van Niekerk et al. 2016). However, there is a gap in knowledge about the molecular mechanisms activated by cell transplantation that stimulate regeneration and the formation of functional connections rather than the formation of aberrant or malfunctioning connections, that actually lead to functional

improvement and explain the differences found *in vivo*. It was recently reviewed the intrinsic molecular mechanisms involved in axonal regeneration in the dorsal column and corticospinal tract after SCI, in which it is discussed the necessity to reveal the specific molecular mechanisms that regulate axonal regrowth for the development of potent therapeutic targets (Noristani 2022).

Differences between species may also be considered when translating the strategies into SCI subjects since the evolutionary differences in the location and function of the descending motor tracts also play a role in the functional recovery found in different species. Quadrupedal animals such as rodents and bipedal animals such as non-human primates and humans present important differences in the distribution and involvement of descending tracts in locomotor function. Thus, while in rats it is found effective redirectioning around the lesion, the enlargement of the CST in primates, together with the reduction of the rubrospinal tract in humans, may influence plasticity in humans (Filipp et al. 2019). Despite the neuroanatomical differences, most studies are performed in rodents, so it is necessary to perform studies in non-human primates to obtain a closer view of the regenerative processes that occur in humans (Noristani 2022). It is, thus, possible that the limited functional improvement obtained in clinical trials is partly due to the limitations of the preclinical models we currently use, and models closer to the human such as primates should be used before starting clinical studies to ensure the effectiveness of the therapy in humans.

In the last few years different approaches are being developed to improve and optimize current therapies for SCI. Tuszynski's group, use a cocktail of trophic factors when transplanting NSCs, increasing the viability of the cells in the spinal cord parenchyma and enhancing the differentiation to mature post-mitotic neurons allowing the formation of axonal relays by forming connections between the host injured axons and the grafted cells which also extend axons below the lesion (Kumamaru et al. 2018; P. Lu et al. 2019). Thus, in our case, we could increase the efficiency of transplantation and overcome one of the main limitations mentioned above, which is the viability of the cells. Likewise, if we were able to direct the differentiation of NSCs to neurons and enhance axon formation and tissue connections, it is also very likely that we would achieve a more evident functional improvement.

Tissue engineering and the combination of biomaterials and cell transplantation constitutes another option. Recently a synthetic bioscaffold containing two peptidic sequences that promotes nerve regeneration, blood vessel formation and reduces glial scarring have been recently developed and tested in the context of SCI increasing axonal regrowth myelination survival of motor neurons and improving functional recovery (Álvarez et al. 2021). The use of this scaffold in conjunction with our combination therapy would represent a very promising approach, as it could increase the viability of transplants and could also act as a carrier maintaining the stability of the curcumin conjugate within the tissue.

On the other hand, Dr Courtine stands out in the field of spinal cord injury for the development of electronic devices to restore neurological function. Its approach is totally different and complementary to the use of cell therapy, since its objective is not so much tissue regeneration, but focuses directly on the recovery of neurological functions, including locomotor function. Using epidural electrical stimulation (EES) their group restored trunk and leg motor functions within 1 day in three individuals with complete paralysis (Rowald et al. 2022). However, it is described that rehabilitation by EES is much more efficient after incomplete SCI (Wenger et al. 2016) and residual pathways also play a role in promoting recovery. Furthermore, it has been discussed the critical importance of combining biological approaches in order to enhance neurorehabilitation supported by EES (Courtine and Sofroniew 2019). Therefore, cell therapy and neurorehabilitation with EES are complementary and synergistic, so the future treatments may consist in the combination of both approaches.

Single therapies based on the transplantation of NSCs/NPCs that are effective in rodents have not shown the same grade of neuroplasticity and recovery in human subjects and given the multifaceted nature of SCI, combinatorial strategies are likely to be required. From this point of view, the ideal combination should synergize to provide clinically relevant improvements, especially in terms of functional recovery which is the main limitation in clinical trials. Overall, in the present thesis, our results show the advantages of the application of combined therapies over individual treatments, making them a more appealing approach to tackle the cascade of events occurring after SCI. In our studies, the inclusion of drugs in the treatment increased the benefits the cell therapy, and the combination with the scaffold

allowed us to treat SCI in a less invasive way for the recipient tissue, while ensuring a more suitable environment for transplantation. Despite the described benefits of our therapies in terms of fiber preservation, reduction of fibrotic scarring and immunomodulatory effects, survival, engraftment and differentiation of the transplant are still the major limitations in our studies. Furthermore, we believe that the limited functional improvement must be related to these limitations. Therefore, our future steps should be aimed at improving transplant survival and differentiation, by transplanting the cells with a cocktail of trophic factors, similar to the one used in Tuszynski's group. In this way, we could direct differentiation towards a neuronal profile, which in turn could increase integration of the transplant in the host axonal circuitry and estimulate the formation of new conections, in order to obtain a robust functional improvement. Therefore, there is still a long way to go before we can translate our therapies to the clinic, and we need to continue working to improve the performance of cell transplants to obtain safer and more effective therapies for the treatment of SCI.

CONCLUSIONS

5. CONCLUSIONS

This doctoral thesis provides relevant contributions to the cell therapy approaches for the treatment of SCI beyond the state of the art, adding to the previous knowledge with the experimental validation up to three new cell-based therapeutic formulations described in each of its three chapers:

CHAPTER 1

1. *In vitro* evaluation of tolerated doses of the poly-acetilated polymeric conjugation of Curcumin (PA-C) in induced neural stem cell (iPSC-NSCs) cultures demonstrates its capabilities to increase cell viability against hydrogen peroxide-induced toxicity.
2. *In vitro* treatment of PA-C in iPSC-NSC cultures demostrates its potential to induce neurite elongation, however, it does not overcome the neurite retraction induced by the activation of the Rho/Rock signaling by ectopic administration of lisophophatidic acid.
3. *In vitro* treatment of iPSC-NSC with PA-C alone or in co-culture with MSCs prevents NF-κB activation induced by the treatment with lipopolysacharid, indicatinig its potential immunomodulatory properties.
4. The treatment with PA-C by local administration, in combination with iPSC-NSCs and MSC transplantation at the injury site one week after SCI significantly preserves β-III-tubulin positive neuronal fibers, reduce the fibrotic scar dimensions, increases the number of synaptic bottons and promotes microglia polarization towards an anti-inflammatory profile.
5. Cell transplantation one week after SCI of a combination of both stem cell types, iPCS-NSCs and MSC, enhances white matter sparing.

CHAPTER 2

1. PuraMatrix-Embedded iPSC-NSCs increases long-term metabolic activity and PA-C reduces apoptosis tested *in vitro*.
2. PA-C induces iPSC-NSCs long neurite-like processes with a guided/parallel orientation in tight association with endogenous neuronal fibers after *in vivo* implantation.
3. PA-C prompted iPSC-NSC migration from the hyaluronic acid demilune scaffold into the injured spinal cord tissue while polypyrrole coated fibers delayed migration.

4. IPSC-NSCs migrated into the host inured spinal cord tissue preferentially differentiate into astroglial lineage.
5. The fully functionalized hyaluronic acid demilune scaffold containing polypirrole coated fibers and PA-C increased the preservation of neural fibers, reduces fibrotic scar formation and the fibrotic like PDGF-positive cells invasion into the injury, indicating a limited fibrotic-like tissue formation.
6. At the short time of evaluation, any of the tested implants do not have any impact on the microglial activation or macrophage infiltration after SCI.

CHAPTER 3

1. Human fetal NPCs can be isolated and expanded *in vitro* from 19.0- 21.6 weeks of gestational stage spinal cords.
2. Human fetal NPCs from late gestational stages retaine canonical neural features, stemness and multipotency.
3. Althought the NPCs isolated from fetal spinal cords can be expanded for at least 9 passages, the amplification efficiency is significantly lower to NPCs isolated from earlier gestational stages.
4. *In vitro* priming of human NPCs with PGA-SS-FAS promotes faster cell maturation, favoring neuronal and oligodendroglial differentiation and enhances neurite outgrowth.
5. *In vitro* priming procedure of hNPCs with PGA-SS-FAS prevents neurite retraction induced by the activation of the Rho/Rock signaling by the treatment with lysophosphatidic acid.
6. *In vivo* transplant of primed hfNPCs with PGA-SS-FAS induces c-fos expression indicating induced activation of the grafted cells.
7. Priming procedure of hfNPC do not promote preferential neural differentation *in vivo.*
8. Transplantation of PGA-SS-FAS-primed hfNPCs increases activation and neuronal preservation of endogenous neurons surrounding the graft.

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6. REFERENCES

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