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Biohybrids for Spinal Cord Injury Repair

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Abstract

Spinal cord injuries (SCI) result in the loss of sensory and motor functions with massive cell death and axon degeneration. We have previously shown that transplantation of spinal cord-derived ependymal progenitor cells (epSPC) significantly improve the functional recovery after acute and chronic SCI in experimental models, contributing by delivering neurons through their differentiation as well as supporting glial cells with trophic capacity. Here we propose an improved procedure for cell transplantation increasing the effectiveness on the surgical approach, based on transplantation of epSPC in a tubular conduit of hyaluronic acid (HA) containing poly(lactic acid) (PLLA) fibers creating a biohybrid scaffold. In vitro analysis showed that the PLLA fibers included in the conduit induce a preferential neuronal fate of the epSPC rather than glial differentiation, favoring elongation of cellular processes. The safety and efficacy of the biohybrid implantation was evaluated in a complete SCI rat model. The conduits allowed efficient epSPC transfer into the spinal cord, improving the preservation of the neuronal tissue by increasing the presence of neuronal fibers at the injury site and by reducing cavities and cysts formation. The biohybrid-implanted animals presented diminished astrocytic reactivity surrounding the scar area, higher number of preserved neuronal fibers with a horizontal directional pattern, and enhanced co-expression of the growth cone marker GAP43. The biohybrids offer an improved method for cell transplantation with potential capabilities for neuronal tissue regeneration, opening a promising avenue for cell therapies and SCI treatment.

Introduction

Spinal cord injury (SCI) is a devastating and so far incurable disorder. New therapeutic strategies for SCI are required to overcome the complex and progressive degenerative process. Implantation of biomaterial scaffolds, alone, enriched with stem cells or with bioactive molecules, has already shown satisfactory results on several SCI models, evidencing to be promising strategies (Li et al., 2016; Li & Dai, 2018; Wen et al., 2016) . The anatomical impediments to access the spinal cord as well as the surgical complexity with their related risks during spinal cord interventions constitute, in fact, a major challenge for scaffolds implantation (Song et. al, 2015). To mimic the shape and mechanical behaviour of the soft tissue and to achieve medium or long-term biodegradability properties are additional challenges not yet overcome by most of the so far described biomaterials employed in SCI (Assunção-Silva et al., 2015). Hyaluronic acid (HA) injectable hydrogels have shown to protect transplanted cells resulting in improved cell engraftments. However, the integrity of the HA gel has a short lifetime, since it is soluble. This short durability is detrimental for the slow reconstruction of the neural tissue, since it does not match the slow pace of reconstruction of the neural tissue (Liang et al., 2013). When HA is cross-linked to form 3D scaffolds longer lifetimes of the biomaterial can be obtained, retaining the good biocompatibility and therapeutic benefits for neuronal regeneration (Liang et al., 2013). HA can be manufactured as tubular conduits with excellent biocompatibility and with desired controlled degradation (Vilariño-Feltrer et al., 2016). HA constructs can incorporate fibrillary structures of different polyesters such as poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA) and poly(lactide-co-caprolactone) (PLCL) (Raspa et al., 2015) or poly(lactic acid) (PLLA) (Vilariño-Feltrer et al., 2016) with proven supporting properties for axonal growth stimulation and other beneficial paracrine effects alone or in combination with cells (Liu et al., 2015). Tubular complex conduits can contain among other structures artificial channels and are thought to protect the cell transplants and/or to contribute of guiding the

growing axonal processes (Simitzi et al., 2017). It is thought that these structures reduce infiltration of fibrotic tissue, provide a substrate for the diffusion of neurotrophic factors, and allow the diffusion of macromolecules (Ortuño et al, 2016).

Cell transplantation is based on the general hypothesis of the beneficial effects of the additional contribution of cells and its released factors on the regeneration of damaged or degenerated neural structures. Indeed, cell replacement therapies provide the opportunity to reconstitute a tissue bridge containing relay neurons (Bonner & Steward, 2015) by generating a more permissive environment for endogenous axonal sprouting. In recent years different cell therapies have been developed with success in animal models (Ahuja & Fehlings, 2016; Kjell & Olson, 2016) however few ones have already been translated into the clinic (Vismara et al., 2017; Curtis et al., 2018; Theodore et al., 2016) . Both, adult and neuronal neural progenitor cells offer a suitable niche, comprising neurons and glial cells, compatible with the neural microenvironment, avoiding the undesired potential tumorigenic effect of embryonic tissues (Steward et al., 2014). We recently showed that the spinal cord-derived ependymal progenitor cells (epSPC) transplanted immediately after SCI or in chronic injuries, migrated long distances from the rostral and caudal regions to reach the neurofilament-labeled axons in and around the lesion zone, reducing cavity formation and scar area (Alastrue-Agudo et al., 2014; Gómez-Villafuertes et al., 2015; Requejo-Aguilar et al., 2017). However, a poor survival rate and limited neuronal differentiation after cell engraftment limit the cell transplantation potential, especially in chronic injuries which demand additional anatomic reorganization for axon regeneration over long distances (Ahuja CS et al., 2016).

Here we present a new biohybrid construct for epSPC transplantation consisting in a tubular scaffold of cross-linked HA containing aligned (PLLA) fibers, to cover the injured area after SCI and to support reconstruction of the anatomical structure of the neural tissue. Both synthetic components are biodegradable and offer different advantages; while the highly

hydrophilic HA tubular scaffold is intended to protect and to allow the transfer of the cells, the PLLA microfibers represent a cell-friendly substrate onto which the epSPC adhere and differentiate. Moreover, PLLA microfibers are highly aligned and provide a guidance support for axonal, sprouting elongation and facilitating tissue re-organization. Beside all additional benefits supported by the biohybrid, the main hypothesis underlying this experimentation was the demonstration that the epSPC transplanted to the spinal cord within the biohybrid scaffold would survive and integrate at the transplant site, helping to regenerate the damaged tissue structures after complete SCI.

Material and Methods

Preparation and morphological characterization of HA, HA-PLLA conduits: HA conduits were fabricated as previously described (Vilariño-Feltrer et al., 2016). Briefly, 5% (w/v) HA sodium salt (Sigma-Aldrich, 1.5-1.8 MDa) solutions in sodium hydroxide (0.2 M NaOH) were introduced in a polytetrafluoroethylene (PTFE) mould, with a poly-ε-caprolactone (PCL) fiber of 400 μm diameter acting as channel template (miniextruder, HAAKE MiniLab II). The HA was crosslinked with divinyl sulfone (90% of DVS). After 11 min the mold was frozen at -80ºC and the whole structure was lyophilized for 24 h (Lyoquest-85, Telstar) to generate HA microporous matrices. Finally, the PCL fiber was extracted and the conduits were hydrated in distilled water for 2 h and cut 6 mm length. For HA-PLLA conduits generation, 20 PLLA fibers (30 μm diameter each fiber, purchase from Aitex, Spain) were placed inside the channel. Prior to the seeding stage the conduits were sanitized with 70% ethanol for 2 h, and consecutively immersed in 50%, 30% and distilled water previously to be conditioned with culture medium for overnight incubation.

Ependymal progenitor cell isolation and culture: EpSPC or GFP-epSPC were harvested from neonatal (P4-6) female Sprague Dawley-Tg (GFP) 2BalRrrc rats; eGFP+/+ homozygote

rats were used for *in vivo* experiments and eGFP−/− rats for *in vitro* assays. The spinal cords were dissected after complete laminectomy and the overlying meninges and blood vessels removed. The dissected tissue was cut into 1-mm^3 pieces and mechanically homogenize without enzymatic treatment. EpSPCs were isolated and cultured as neurospheres-like form (Figure S1A) with NeuroCult™ Proliferation Medium supplemented with NeuroCult™ Proliferation Supplement (STEMCELL Technologies, USA) including 40 ng/ml epidermal growth factor (EGF, Invitrogen), 40 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 1X P/S, and 2 µg/ml heparin (Sigma). The epSPC cultures in proliferative medium was characterized to test whether express the neural precursor cell markers PDGFR, NG2, Olig1 and 2, NCAM-1, Nkx2.2, Dlx2 and Sox2 assayed by semi-quantitive PCR analysis at the mRNA level (Figure S1B). Glast, Sox2, Doublecortin (DLX) and Nestin progenitor markers were also detected by immunostaining in proliferative cell medium (Figure S1C).

EpSPC were seeded as dissociated cells in the lumen of the conduits using a Hamilton syringe (SGE Analytical Science) with a density of 10^5 cells/sample suspended in 3 µL of growth medium, placed in a 48-well plate and incubated for 60 min in order to optimize the initial cell adhesion at 37°C and 5% CO_2. The culture environment was maintained for 14 days, changing the proliferation medium every 48 h. For spontaneous differentiation process in 2D dimension, the epSPC were seeded on Matrigel® (BD Bioscience, San Jose, CA, USA) coated plates. The heparin and the mitogen factors, epidermal (EGF) and fibroblast growth factors (FGF), were removed from growth medium and bovine serum albumin was replaced by 2% FBS. Forced spontaneous differentiation of epSPC was maintained for 24 h and afterwards the cells were fixed with 2% paraformaldehyde (PFA) and processed for immunostaining evaluation.

Cell viability assay: Indirect cytotoxic analysis was performed according to ISO 10993-5. Briefly, L929 mouse fibroblasts (Sigma Aldrich, Spain) and epSPCs seeded at a density of 10^4 cells into 96-well plates for 24 hours were exposed to a liquid extract obtained after incubating 0.1 g of HA-PLLA into 1 ml of proliferative medium in order to study the possible toxic substances leached from the biomaterial. The MTS (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) assay was performed 24, 48 and 72 h after cell stimulation with the conditioned media or in control conditioned, non-exposed to the biomaterial. Three replicates of three independent experiments were performed. Cell viability was expressed as % of change versus control condition (considered as the 100% viability) for both cell types.

Immunostaining: Samples, epSPC from in vitro 2D cultures alone or seeded into HA or HA-PLLA or fixed spinal cord sections from in vivo experimentation were washed with 0.1M Phosphate Buffered Saline (PBS, pH 7.4) and fixed for 20 min in 4% PFA at room temperature. Permeabilization and blocking steps were performed using PBS 0.1M containing 0.1% triton-X and 10% FBS or 5% of normal goat serum respectively for 1 h at room temperature, and then incubated overnight with mouse monoclonal for neural class III-beta-tubulin (TUJ-1; 1:250, MO15013, Neuromics), mouse neural precursor cell markers such as mouse monoclonal antibody anti-A2B5 (1:400, A8229, Sigma) and mouse monoclonal antibody anti-Nestin (1:400, AB6142, Abcam), the guinea pig polyclonal antibody anti-GLAST (Glutamate transporter) (1:500, AB1783, Chemicon), rabbit polyclonal antibody anti-glial fibrillary acidic protein (GFAP; 1:400, Z0334, Dako Cytomation), guinea pig polyclonal antibody anti-doublecortin (DCX; 1:400, AB5910, EDM Millipore), rabbit polyclonal antibody anti-Sox2 (1:200, AB97959, Abcam), rabbit polyclonal antibody anti-GAP43 (1:250, ab128005, Abcam), rabbit polyclonal antibody anti-Olig2 (1:250, AB9610, Millipore) or the mouse monoclonal antibody anti-ED1/CD68 (1:250, MAB1435, Millipore) overnight at 4ºC in the dark. After washing in 0.1M PBS, cells were incubated with goat anti-mouse Alexa

555 or goat anti-rabbit Alexa 647 (1:200, Jackson Immunoresearch, West Grove, PA for 1 h at room temperature). Finally, nuclei were visualized with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; 1:5000, Sigma). After additional washes, immune-reactivity cell images were obtained and analyzed using the Leica IM 500 4.0 image-processing program (Leica, Bensheim, Germany) and confocal microscopy (Leica). For cell quantification analysis 15 random fields covering the entire scaffolds (0.003 cm^2) per group in the *in vitro* analysis (n=3 samples for each substrate) and one complete series for each spinal cord of all samples of each experimental group were quantified and normalized to the total counted area. The expression of TUJ1, GFAP, GAP43, ED-1 and Olig2 proteins, was evaluated at the entire spinal cord thickness, from dorsal to ventral side, normalized to the total analyzed area (~2,5 cm) using Image J software. The TUJ 1 directionality analysis was performed using ImageJ/Fiji software and Directionality plugin.

RNA isolation and semi-quantitative RT-PCR: Total RNA was extracted by using Rneasy Mini-kit (Qiagen, Germany) accordingly to manufacturer's instructions. One microgram of total RNA was reverse transcribed (RT) in a total reaction volume of 50 μl through incubation at 42°C during 30 min using random hexamer primers. The sequence of specific primers for semiquantitative PCR are described in the Supplementary Table 1. The semi-quantitative PCR was performed in a thermal cycler (Eppendorf, Germany) following the PCR amplification program: 3 min of denaturation at 94°C followed by 30 PCR cycles consisted of 1 min at 94°C, 1 min at each corresponding annealing temperature (indicated in the Supplementary Table 1) and 1 min at 72°C. A negative (without a prior reverse transcription reaction) control was always included. After amplification, 25 μl of each PCR mix was electrophoresed through a 2% (w/v) agarose gel with ethidium bromide (0.5 mg/ml) to be visualized under a uv trans-illuminator (BioRad).

Scanning electron microscopy (SEM): SEM was used as a tool for the observation of the cell morphology on conduits. HA and HA-PLLA conduits scaffold were fixed with 2.5 % (w/v) glutaraldehyde for 1 h at 37ºC after 14 days in culture. Thereafter, samples were post-fixed by immersion in 2% osmium tetroxide in phosphate buffer (PB) 0.1 M for 90 min in dark, washed with distilled water at 4ºC and then dehydrated in gradient concentrations of ethanol (30, 50, 70, 96, and 100 %) at 4°C for 10 min per concentration. After being freeze-dried in vacuum for 2 h, the cellular constructs were coated with silver sputter and observed under the SEm (Hitachi S4800, Japan) at a voltage of 10 kV.

Spinal Cord complete section, HA-PLLA with or without epSPC implantation and functional locomotor analysis: SCI was induced in female Sprague Dawley rats of ~ 200 g as previously described (Erceg et al., 2010). Briefly, a complete section of the spinal cord at thoracic segment T8 was performed by completely cutting the spinal cord with an iridectomy scissor by using an instrumental hook to elevate the cord. In all cases it was checked that both stumps were completely separated by passing the hook thought the cut segment (a representative image before and after complete section is shown in Figure 4A, *left*). Six animals per group were randomly assigned and distributed into control (without conduits); HA-PLLA or HA-PLLA + epSPC ($1x10^5$ dissociated epSPC were seeded onto the HA-PLLA lumen 5 days before implantation). Immediately after complete section the conduits were opened at the base with a scalpel and placed covering the spinal cord at the injured area (a representation is shown in Figure 4A, *right*). The rats were pre-medicated with subcutaneous morphine (2.5 mg/kg) and Baytril (enrofloxacine, 5 mg/kg, Bayer, Germany) and anesthetized with 2% isofluorane in a continuous oxygen flow of 1 L/minute. Animals were daily treated with 10mg/kg (intraperitoneal) of body weight of cyclosporine. Open-field locomotion was evaluated by two blinded observers by using the 21-point BBB locomotion scale after blind visualization of a minimum of 5 minutes of free walking in an open space once a week. Three

weeks after injury and conduits implantation all animals were sacrificed for histological analysis.

Eosin-Hematoxylin histological analysis of neuronal tissue degeneration after SCI: The animals were transcardially perfused with a 0.9% saline solution followed by 4% PFA in PBS, and 2 days incubation time in 30% sucrose before inclusion in Tissue-Teck OCT (Sakura Finetek U.S.A). Sagittal cryosections of 10 μm thickness were used for histology and immunoassays. Every fifth section was collected for eosin-hematoxylin (E&H) staining, scanned in a Pannoramic 250 Flash II scanner (3DHISTECH Ltd.; HUNGARY) and images of approximately 20 mm^2 of the spinal cord (including the epicenter of the lesion) were acquired with the Pannoramic viewer software. To determine the anatomical structure and tissue degeneration, quantification of the area with lost white/grey matter physiological distribution was performed in the injured area of the entire spinal cord thickness, from dorsal to ventral side, normalized to the total analyzed area (~2,5 cm) using Image J software.

Ethical Statement Regarding the Use of Animals: Sprague Dawley rats (weighing ~200 g) from Charles River and SD-Tg (GFP)2BalRrrc from Rrrc (University of Missouri Columbia, Columbia, MO, USA) were bred at the Animal Experimentation Unit of the Research Institute Príncipe Felipe (Valencia, Spain). The experimental protocol was previously approved by the Animal Care Committee of the Research Institute Principe Felipe (Valencia, Spain) in accordance with the National Guide to the Care and Use of Experimental Animals (Real Decreto 1201/2005).

Statistical analysis: All experimental data was collected from at least three independent experiments and results were reported as the mean ± the standard error of the mean (S.E.M.) as indicated at each set of data. For the comparisons between two groups of values, the statistical analysis of the results was performed by the Student's t test in a normal distribution data. For the comparison between groups one-way ANOVA, with appropriate corrections

such as Tukey's post hoc tests was used. Statistical analyses were performed using GraphPad software. Differences were considered significant at *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

HA and HA-PLLA conduits show a highly porous morphology

HA and HA-PLLA soft but stable conduits were obtained as previously described (Vilariño-Feltrer et al., 2016) with external dimensions of 6 mm long,1 mm width and an internal cylindrical channel of 400 µm diameter in the fully hydrated stage. A macroscopic view of both conduits in aqueous solution is shown in Figure 1 (Figure1A and D). SEM images of longitudinal (Figure 1B) and transversal (Figure 1C) cuts of the HA conduits revealed a highly porous structure of the scaffold's walls, with an interconnected honeycomb-like pore structure. However, a compact and continuous layer with a very limited porosity is created at the internal wall (Figure 1B). In the central channel, or lumen, of the conduit 20 PLLA microfibers are laid out parallel to the conduit's axis (Figure 1E). An extensive and thorough description of conduit's morphology as well as the water uptake, degradability and mechanical properties were previously reported for homologous structures (Vilariño-Feltrer et al., 2016).

In order to test a potential cytotoxic effect of the HA- PLLA conduits based-materials, epSPCs (Figure S2) and mouse fibroblasts (data not shown) were exposed to conditioned medium obtained by 24h incubation of HA or HA-PLLA into culture medium or to non-conditioned medium (control group) for 24, 48 or 72 hours. MTS assay showed that neither HA or HA-PLLA extracts affected cell viability (Figure S2).

The neural precursor cells colonized the HA and the HA-PLLA conduits

Due to the highly hydrophilic nature of high molecular weight (1.5-1.8 MDa) HA the cells seeded in contact with the HA scaffold showed a low or null cell adhesion (Vilariño-Feltrer et al., 2016). When epSPC were seeded into the lumen of the scaffolds, the low cell adherence of the HA potentiates preferentially cell-cell interactions, which lead to the generation of a continuous monolayer of the seeded cells not attached to the biomaterial's channel inner surface (Figure 2), similarly to what previously found with Schwann cells (Vilariño-Feltrer et al., 2016). A cross section of the hollow HA conduit shows that no cells protrude across the scaffold's walls (Figure 2A, *), while at the internal surface of the lumen a continuous layer of epSPC is formed (Figure 2A-B, **). The scaffold tube thus acts as an efficient cell containment biohybrid structure. The epSPC seem to arrange within the HA conduit mimicking the ependymal aligned organization of the spinal cord central canal (Alfaro-Cervello et al., 2012). DAPI nuclear staining showed a compact cell distribution at the channel inner surface (Figure 2C). When the epSPC were seeded into HA-PLLA conduits the cells also attached to the PLLA fibers (Figure 2D-F). Morphological differences were evident between the epSPC covering the lumen and those attached to the PLLA fibers. The epSPC on the PLLA fibers showed prominent projections from a voluminous cell body (Figure 2E, #) in comparison with the flat, compact and homogeneous organization pattern found close to the HA internal surface of the lumen (Figure 2B).

PLLA fibers induce neuronal differentiation of epSPC in HA-PLLA biohybrids

The multipotency of epSPC in vitro and in vivo has already been demonstrated, showing the directly induced or spontaneous differentiation capability of epSPC to generate neurons, astrocytes and oligodendrocytes (Rodriguez-Jimenez et al., 2016). To test whether the HA-PLLA conduit maintains this multipotency capacity in spontaneous differentiation, immunostaining of NeuN, GFAP and Olig2 were used to identify neurons, astrocytes and

oligodendrocytes respectively (Figure 3A). On the other hand, the proliferative or self-renewal capacity of the epSPC in the conduits was evaluated by the detection and quantification of Ki67 expression (cell proliferation marker; green) (Figure 3B). Confocal images showed that epSPC growing on the lumen of the HA conduits showed a higher proliferative activity than the epSPC attached to the PLLA fibers in the HA-PLLA (Figure 3B) indicating a potential differentiation stimulus. To evaluate a preferential differentiation into neuronal or glial cell fate of the epSPC, the astrocyte cell marker GFAP (red) and β-tubulin III (TUJ-1, green), for neuronal cells were used (Figure 3C). The epSPC monolayer coating the inner surface of the HA conduit had a glial preferential differentiation pattern, showing significant higher amount of GFAP positive cells and lower number of TUJ-1 positive cells (Figure 3C, E). Contrarily, the quantification of positive cells for the neuronal marker showed a significant increased number of TUJ-1 positive epSPC seeded on the HA-PLLA conduits when compared with epSPC seeded either on the HA conduit or in a 2D monolayer using Matrigel® as a substrate (Figure 3C, D, E). Indeed, the 3D cultures of epSPC on both, HA and HA-PLLA structures, significantly favor neuronal differentiation in comparison with Matrigel® 2D culture (Figure 3D, E).

Importantly, on the HA conduits the GFAP-positive astroglial cells (red) showed long but non-orientated projections and the TUJ-1 positive cells showed poor and non-organized cell expansions with rare interactions between each other (Figure 3C, *). In contrast, the TUJ-1 positive cells (green) in the PLLA fibers showed longer projections, even reaching contiguous fibers (Figure 3C, #) and often guided by the astrocyte projections (Figure 3C, arrow). Remarkably, the HA-PLLA biohybrids thus generated a niche mimicking the *in vivo* neuronal cell network with neuronal projections accompanied by the glial extension in a guided architectural organization.

HA-PLLA scaffold allows cell transplantation with improved neuronal tissue preservation in a model of complete SCI

The HA-PLLA conduits or the biohybrids, HA-PLLA + epSPC, were longitudinally open with a scalpel and placed covering both stumps immediately after sectioning the spinal cord (Figure 4A, right). A transversal section view of the biohybrid organization is illustrated in Figure 4A, left. Animals with spinal cord complete sections without the implant were included as the control group. All animals were individually videotaped and locomotor functional recovery was studied by using the BBB scale (Basso et al., 1995) during three weeks after injury, however, the analysis did not show any significant difference between groups. All rats showed scores between 0-1 out of a 21 scale due to the severity of the complete lesion (Figure S3). The rats were sacrificed and perfused three weeks after injury and the spinal cords were subjected to histological analysis. The E&H anatomical staining of the spinal cord longitudinal sections as is shown in a representative spinal cord tissue form one animal per each group (Figure 4B; sections from dorsal to ventral side) allows a comparison between groups of the cavities or cyst formation (Figure 4C) and tissue degeneration at the injured area (Figure 4D). The control group showed the higher number of cavities and percentage of tissue degenerated area in comparison with the HA-PLLA or HA-PLLA + epSPC implanted groups (Figure 4C and D). epSPC-GFP (green) were efficiently transferred to the spinal cord, as soon as 3 days after injury and biohybrid implantation, as is shown in Figure 4E. However, few alive GFP positive cells were detected at the injured area three weeks after injury and implantation (Figure 4E). Infiltrated macrophages and endogenous microglia contribute to the inflammatory response and secondary damage contributing to tissue degeneration and remodeling (Donnelly & Popovich, 2008). Three weeks after injury we did not find significant differences among groups on macrophages and activated microglia positive for ED1/CD68 at the injury site (Representative images are shown in Figure S4). It is also well established that after SCI, surviving oligodendrocyte

progenitor cells (OPC) are the major source for oligodendrocyte replacement and remyelination (Hesp et al., 2015) after massive oligodendrocyte cell death. We then explored the influence of the conduits on induction OPC at the injury, by the detection of Olig2 positive cells; however, no significant differences were found in comparison with the control groups (Representative images are shown in Figure S4).

HA-PLLA conduits reduce astrogliosis and the biohybrid increase the number of re-growing neuronal fibers at the epicenter of the injury

Three weeks after complete section and treatments, the analysis of the astrogliosis by the quantification of the astrocytes surrounding the scar, positive for GFAP (green), showed a significant decrease when the lesion was covered by the HA-PLLA conduits, with or without epSPC (Figure 5A, B). However, only the HA-PLLA + epSPC biohybrid significantly increased the number of preserved neuronal fibers, TUJ-1 positive fibers at the injury site (red; Figure 5A, C). Importantly, a significant preservation of the longitudinal axis orientation of the surviving neuronal fibers was found in all animals with the HA-PLLA conduits (Figure 6A, B) with higher number of fibers showing an inclination degree close to 0 (horizontal plane) in comparison with the control condition. However, only the HA-PLLA + epSPC conduit significantly enhanced the percentage of neuronal fibers expressing GAP43, a growth cone marker (Morita & Miyata, 2013) (green; Figure 6C, D), indicating an activated process for axonal re-growth.

Discussion

SCI is a multi-factorial disorder determined by a combination of both extrinsic and intrinsic processes that inhibit neural regeneration and repair (Kumar et al., 2015). Any effective SCI therapy should then attempt a highly minacious intervention including multidisciplinary and combinatorial approaches to overcome the limited regeneration capacities of the spinal

neurons. Immediately after SCI, a rapid and massive necrotic cell death, local ischemia, vascular network loss with edema and disruption of the blood-spinal cord barrier (Wilson & Fehlings, 2011) make mandatory a local and fast direct intervention. The design and selection of new biomaterials for SCI applications should be based on this necessity to maximize the chances to preserve the neuronal function with minimal and efficient intervention.

HA has been used in tissue engineering of the central nervous system showing important benefits based on its biodegradability, biocompatibility and good integration with the host tissue (Collins et al., 2012). Here we employed a tubular scaffold made of cross-linked HA offering a stable moldable structure. The conduit can host seeded cells, allowing *in vitro* self-renewal and cell differentiation. The porosity of the conduit´s matrix permits exchange of oxygen and nutrients as well as the disposal of waste products, but impedes cell migration across the conduit's walls. Thus, the scaffold acts as a protecting barrier against environmental inflammatory cell infiltration, as well as against the invasion of endogenous astrocytes, pericytes or microglia. Guidance concepts have been implemented in a variety of matrices (Straley et al., 2010 Simitzi et al., 2017). Longitudinally oriented channels and fibers are thought to provide appropriate guiding platforms to bridge the scar and repulsive areas (Lim et al., 2010; Stokols & Tuszynski, 2006). Besides, it is believed that channels and fibers promote regeneration by providing increased surface area to which cells and regenerating axons can attach (Simitzi et al., 2017; Xie et al., 2014), PLLA fibers have been described as a positive environmental stimulus for nerve fiber regrowth (Tian et al., 2015). Here, a set of PLLA fibers placed in the HA conduit lumen also serve to enhanced the adhesion and differentiation of *in vitro* seeded epSPC. On PLLA epSPCs showed a preferential neuronal cell fate and a reduced percentage of astroglial features, by spontaneous differentiation in comparison with the HA conduits or Matrigel® cultures. This phenomenon occurred without modifying the regular growth medium conditions, thus representing a very attractive alternative to the complex direct differentiation protocols, reducing the need of extra growth

factors or morphogen stimuli, to induce the neuronal preferential differentiation. Moreover, the reduction of the astroglial population prior to transplantation would reduce the contribution to the fibrotic astrocytic dependent processes, while maintaining a percentage of glial cells needed for further nutritional support and blood spinal cord barrier neuronal network reconstruction (Lu et al., 2012).

HA-PLLA biohybrids could provide structural support for guided regrowth of axons *in vivo*. They may promote the axon extension in a fiber-guided manner. This has been shown here by the improved directionality orientation, better maintained in the implanted animals, of the more preserve neuronal fibers expressing the axon growth marker, GAP43. Our ultimate goal is to establish a biocompatible conduit composed of the HA scaffold and PLLA fibers allocating epSPC providing a permissive environment for axon regrowth for a reliable translational application. The manufactured conduits showed adaptive and moldable characteristics were able to adapt to the shape of the damaged spinal cord keeping the needed elasticity not to harm or stress the tissue when the vertebrae move. Here we first demonstrated that the HA-PLLA biohybrids allow the transfer of the epSPC into the spinal cord from 3 days after implantation, showing a significant viability of the transplanted cells during the acute stages, immediately after injury, however, the survival of grafted cells remains very limited 3 weeks after implantation as we previously described in epSPC transplantation by intramedullary injections (Alastrue-Agudo et al, 2018). Although expression of neuronal positive marker was found *in vitro* on the PLLA fibers in the HA biohybrids, there was no detectable co-localization neither of TUJ-1 nor of GFAP in the GFP-epSPC migrating cells into the spinal cord tissue three weeks after SCI. This might be explained by an early cell death of the more differentiated cells or by a preferential undifferentiated stage of the neural precursor cells *in vivo.* The locomotor analysis, by the BBB test did not showed significant differences, with scores between 0-2, due to the severity of the lesion, and the short time for

evaluation after injury and sacrifice (3 weeks). A long-term study is required for significant locomotor functional improvements in such as several injuries. However, the histological analysis reveals significant improvements on neuronal tissue preservation, higher number of neuronal fibers at the epicenter of the injury, showing higher expression of GAP43 with a better-guided pattern, indicating a better condition for neuronal regeneration. Therefore, in acute stage, the conduits could be implanted during first surgical intervention of decompressed approaches. The intrathecal or subarachnoid space, the fluid-filled area, between the dura mater and arachnoid pia mater, constitutes an efficient local administration access to supply direct therapeutic support. The denticulate ligaments maintain anchorage the spinal cord to the meninges, keeping the sub-arachnoid space. However, after SCI this network is destroyed at the injured area, allowing the fibrotic newly formed tissue to create new focal adherences between the cord and the dura mater interrupting the dynamic of cerebral spinal fluid (CSF) flow and increasing the incidence of syringomyelia. Due to the high water content of the HA tube, cells on its outer surface are prevented to attach, and thus the HA-PLLA conduits did not show adherences to the neighboring tissues three weeks after *in vivo* implantation, no visible fibrotic scar nor additional endogenous cell invasion and no related CSF cystic accumulation.

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Disclosure:

There is no conflict of interest within any of the authors, including any financial relationship conflict

Author Contribution:

MMP and VMM conceived the idea. MMP provided conceptual advice and contributed materials. CMR, VMM, LRD, AAA and MMP designed research. CMR, LRD, AAA, EGR, PIS, VMM, MSP and ELM performed research. VMM, CMR, LRD, AAA and MMP analyzed the data. VMM, MMP and CMR wrote and revised the manuscript.

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Figure legends:

Figure 1. Macro- and micro-views of HA and HA-PLLA conduits. Macroscopical view of hydrated HA (A) and HA-PLLA (D) conduits in cell culture wells. PLLA fibers are visible in the lumen of the translucent HA conduits (D). Scale bar: 1mm. Right panels show SEm images of the longitudinal section of both HA (B) and HA-PLLA conduits (E). Also it is shown the detail of transversal section of HA conduits (C) and the bundle of PLLA fibers coming out the lumen of the conduit after longitudinal sectioning of HA-PLLA conduits (F). Scale bar:1mm (A, D and E), 400 μm (B, C) and 300 μm (F).

Figure 2. epSPC distribution in the conduits. SEm images of longitudinal sections of HA (A, B) and HA-PLLA conduits (D, E) are shown 14 days after 3D cell culture. * indicates the acellular porously wall of the conduits; **, indicates the internal lumen space populated by the epSPC and # when epSPC are seeded and attach to the PLLA fibers. Confocal fluorescence images show the internal epSPC distribution after nuclear staining with DAPI in the lumen of the HA (C) and HA-PLLA (F) conduits 14 days after 3D cell culture. Scale bar: 100 μm (A, C, F), 300 μm (D), 20 μm (B), 50 μm (E).

Figure 3. Immunofluorescence analysis of glial and neuronal spontaneous epSPC differentiation seeded into the HA or HA-PLLA conduits (A) Confocal representative images show that HA-PLLA conduits promote epSPC differentiation to all neural linages and provide structural support for mature neurons (NeuN positive marker), astrocytes (GFAP) and oligodendrocytes (Olig2). Bridge field (BF) and fluorescence immunostaining merge images show the differentiated cells attached to the PLLA fibers. (B) right: Confocal representative images show the expression of ki67 (cell proliferation marker) of the epSPCs seeded in the lumen of HA or HA-PLLA conduits; left: Quantification of ki67 positive cells show increase proliferation of epSPCs in HA. The epSPC population attached to the PLLA fibers show significant lower Ki67 expression due to potential induction of cell differentiation; (C)

Confocal representative images show the expression of TUJ-1 (green; a neuronal marker) and GFAP (red; an astrocytic marker) of the epSPC seeded in the lumen of the HA indicated by * or HA-PLLA conduits for 14 days, indicated by # ; (D) epSPC seeded on Matrigel coated coverslips (2D culture) were subjected to TUJ1 (red) and GFAP (green) immunostaining. DAPI nuclear staining is shown in blue; (E) Graphical quantification of TUJ-1 (+) and GFAP (+) cells is shown, expressed in percentage, of epSPC in HA or HA-PLLA conduits in comparison with epSPC seeded on Matrigel® coated plates.

Three independent experiments were performed and a minimum of 100 cells per condition were quantified. Data is expressed as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001). Scale bar: 40 μm (white), 20 μm (red).

Figure 4. HA-PLLA conduits implantation after complete rat SCI. (A) *Left:* Sketch of a frontal view representing the HA-PLLA conduit covering the spinal cord; *Right*: Representative images showing the complete sectioned spinal cord and the conduit covering the injury (delimited by discontinue lines); (B) Representative images of E&H staining from one individual of longitudinal spinal cord sections for all three tested groups three weeks after injury. (C) Quantification of the cavities and cysts and (D) the neuronal degenerated tissue from E&H staining at the injured site expressed as mean ± S.E. M.. *p<0.05, ***p<0.001 (Student's t test of pair comparisons with Control condition); (E) *left*: Representative images of TUJ-1 (orange; alexa 555) and the GFP-epSPC (green) in longitudinal sections at 3 days (upper images) and 3 weeks (bottom) after biohybrid implantation (HA-PLLA + epSPC); Scale bar: 500 μm; *right:* higher magnification images at the indicated area (by discontinued line) in the panoramic image. DAPI is used for nuclear staining (blue). Scale bar: 50 μm. n=6

Figure 5. Astrogliosis and Neuronal fibers analysis at the injury site. (A) Immunohistochemically detection of TUJ-1 (Alexa-555; shown in red as a pseudo-color; neuronal marker) and GFAP (Alexa-647; shown in green as a pseudo-color; astroglial marker) in longitudinal sections, from dorsal (top) to ventral (bottom) side, rostral left side, including the epicenter of the lesion, of the spinal cords for each experimental group three weeks after complete section and conduits implantation. (B) Graphical representation of GFAP and (C) TUJ-1 immunostaining quantification expressed as mean ± S.E.M. *p<0.05, ***p<0.001 (Student's t test of pair comparisons with Control condition).n=6

Figure 6. HA-PLLA biohybrid significantly guide and induces axonal growth of the neuronal fibers. (A) Representative images of TUJ1 staining for each group employed for (B) directionality analysis of the neuronal fibers at the epicenter of the injury. The graphical quantification shows the number of segment of fibers positive for TUJ1 showing all varieties of inclinations from a horizontal (0) to a perpendicular plane (90ºC or -90º (180ºC)); (C) Representative images of TUJ1 and GAP43 merge staining's for each experimental group, *right*: higher magnification images from the indicated area at the panoramic images (indicated with discontinued lines);(D) quantification of GAP43 and TUJ1 co-expressing neuronal fibers. ****p<0.0001, *p<0.05 (Student's t test of pair comparisons with Control condition); Scale bar 500 μm. n=6

Supporting Information

Figure S1: epSPC in vitro cultures characterization: (A) morphological view in phase contrast microscopy of epSPC growing as neurosphere-like cultures (left) or seeded onto matrigel coating plates (right); (B) Semi-quantitative PCR mRNA expression analysis of the indicated transcripts from total RNA extracts of epSPCs growing as neurospheres-like

cultures; (C) Immunostainings for Sox2, Nestin, DCX and Glast detection in epSPCs 24 hours after seeded onto matrigel coated plates in proliferative medium.

Figure S2: Cell viability assay. epSPCs as neurospheres-like , in ultra-low attach 96 well plates, were exposed to HA-PLLA conditioned medium or control growth medium (control group) for 24, 48 and 72 hours in standard cell culture conditions (5% CO2, 37 C) and then subjected to MTS cell viability assay determination. Three independent experiments were performed in triplicates. Data are expressed as mean± S.E.M. No statistical differences were found among the groups.

Figure S3: Functional locomotion test after SCI: Functional locomotor analysis was evaluated using the BBB score over the three weeks post-injury and implantation. No significant differences were found among the groups.

Figure S4: Immunohistochemically detection of Olig2 (red; oligodendrocyte marker) and ED1 (green; macrophage marker) in a representative longitudinal section of the spinal cords of the control, HA-PLLA and HA-PLLA + epSPC experimental conditions three weeks after injury and implantation. Scale bar, 500 μm