COMBINATORY TREATMENT OF NEURAL PRECURSOR CELLS AND A NEW NANOCONJUGATE OF FASUDIL FOR THE CLINICAL APPLICATION IN ACUTE SPINAL CORD INJURY

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1. Summary

We proposed here to translate the combination of neuronal precursor cells, derived from the spinal cord tissue (NPC) and the local administration of a new fasudil nanoconjugate (PGA-SS-FAS) for the treatment of acute spinal cord injuries (SCI) from preclinical experimentation into a closer clinical practice. To do so, we have generated a cell bank of human NPCs from fetal tissue (hfNPCs) from spinal cords from legal gestational interruptions during second semester (19.21 weeks), and manufactured clinical grade PGA-SS-FAS at a large scale. In vitro expanded hfNPCs retained neural features and multipotency keeping self-renew capacity allowing the generation of cell banking for allogenic application. Second, we established a simple procedure to prime the hfNPCs by overnight treatment with 50 μM of PGA-SS-FAS to induce improved neuronal differentiation and to overcome neurite-like retraction by Rho/ROCK activation, mimic in vitro by the presence of lysophosphatidic acid. Primed hfNPCs transplanted in an immune-deficient mouse (NU(*NCr)*-Foxn1nu) immediately after SCI by thoracic compression, in comparison with non-primed cells: 1) enhanced grafted cells migration throughout the spinal cord injured tissue; 2) increased preservation of GABAergic inhibitory Lbx1 and glutamatergic excitatory Tlx3 somatosensory interneurons; 3) increases the number of preserved and activated neurons, positive for cfos, surrounding the injury epicenter. Overall, the new generate hfNPC lines, primed by an in vitro treatment with a new polymer-conjugate of fasudil, would provide an improved translational approach for the treatment of acute SCIs.

2. Results

Pseudo-GMP manufacture of the PGA-SS-FAS conjugate, a nanoconjugate with ROCK-2 inhibitor properties

A GMP-like manufacture process is in place for the production of PGA-S-S-fasudil. The manufacturing protocol involves 3 steps: 1) The polymerization and deprotection of poly-L-glutamic acid, 2) the synthesis and purification of the disulphide-Fas linker and 3) the covalent conjugation of the Fas-linker moiety to the poly-L-glutamic acid carrier. A 5g testing sample with the optimized synthesis was obtained following this process as a validation. This was together with a batch of 20g of the non-GMP fasudil linker that had been stored already without degradation. A risk pre-assessment in non-GMP

environment, non-GMP batch production for preclinical studies, in-process controls, and batch Release were included. After the risk-analysis (RA) study under Quality-Risk Management ICH Q9 guidelines following a FMECA methodology executed with the support of PTS based on the optimized PGA-SS-fasudil synthesis, the self-immolative fasudil linker is the critical step due to its instability.

Also, all methods of analysis (MoAs), including qualified MoAs, reference standards set, in-process controls, and intermediate and final products MoAs have been developed and adjusted to be used in future clinical trials with nanoconjugate involvement. Finally, a technical package for process transference to GMP manufacture has also been elaborated to accelerate the translation process.

hfNPCs populations proliferate, expand and express canonical neural markers and neural progenitor cells features *in vitro:* We tested whether whole human fetal spinal cord homogenates could be used to isolate and expand hfNPCs with minimal intervention since neurosphere-like forming cultures make it possible to isolate self-renewing and clonally-like growing neural precursors (Reynolds & Rietze, 2005). Dissociation and culture of human fetal spinal cord efficiently generated neurospheres in free-floating conditions after 2 DIV in the presence of the mitotic factors bFGF, EGF and LIF, although as shown in Figure 1A (left panel), cultures also presented cell aggregates and debris. As previously described for adult human NPCs (Mothe, Zahir, Santaguida, Cook, & Tator, 2011), we found that propagation of hfNPCs was more efficient when hfNPCs were cultured as an adherent monolayer than in suspension (Figure 1B). Following subculture on reaching 80% confluence, the cell doubling time calculation (PDL-t) for the NPCs population was calculated until passage four (Figure 1C). In order to study if our hfNPCs also form neurogenic niche-like structures, we performed immunostainings against γ-tubulin, that recognises in microtubule-organizing centres, centrosomes and basal bodies and β-catenin to delineate cell borders as previously described (Rodriguez-Jimenez, Clemente, Moreno-Manzano, & Erceg, 2019). We thus found for the first time that *in vitro* cultures of hfNPCs neurospheres derived from spinal cord adopt pinwheel structures and show neural features during fetal developmental stages (Figure 1D). hfNPCs (at passage 4) showed proliferative ability, 21.4 ± 3.3% cells were at mitosis, since they incorporated BrdU and around 37.5 ± 4.7% were positive for Ki67 (Figure 1E). To ascertain if hfNPCs expressed canonical neural markers (Figure 1F), we examined the expression of

neurogenic locus notch homolog protein 1 (Notch1), paired-box protein 6 (Pax6), nestin, Sox2, Forkhead Box J1 (FoxJ1), doublecortin (DCX) and neurogenin. hfNPCs highly expressed Notch1 and Pax6 (90.5 ± 13.8% and 91.7 ± 4.4% positive cells respectively). Pax6 regulates proliferation and self-renewal of NPCs (Sansom et al., 2009) and Notch translocates on activation to the nucleus and leads the maintenance of stemness (Imayoshi, Sakamoto, Yamaguchi, Mori, & Kageyama, 2010). hfNPCs were also positive for nestin (83.4 ± 2% positive cells) and Sox2 (79.7 ± 2.2% positive cells) which are conserved in NPCs (Collignon et al., 1996; Lendahl, Zimmerman, & McKay, 1990). We also determined the expression of FoxJ1, which is specifically involved in the maintenance of neural stem cells in the spinal cord (X. Li et al., 2018), accounting for 65 ± 6.4% of positive cells.

In addition, half of the population of hfNPCs was positive for DCX accounting for 56.4 ± 7.4% of positive cells and neurogenin accounting for 45.8 ± 9.5% of positive cells, both neurogenin and DCX being involved in promoting neurogenesis (Brown et al., 2003; Sun et al., 2001).

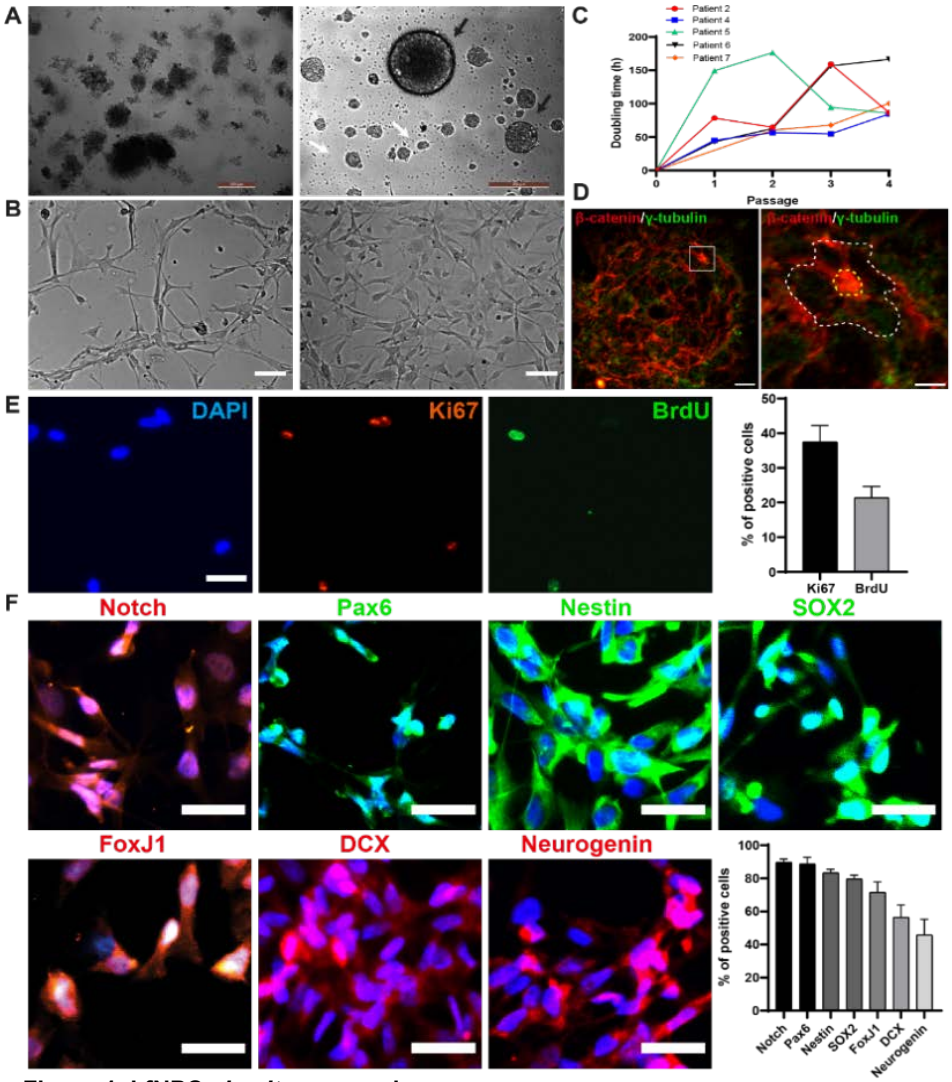

Figure 1. hfNPCs *in vitro* expansion.

PGA-SS-FAS enhances ventral engraftment and promotes hfNPCs neural differentiation: To determine safety of the therapy, we studied the tumorigenic and invasive capacity of the transplants 4 weeks after transplantation in healthy spinal cord tissue. We did not find any overgrowth or phenotypic transformation of the grafted cells in the spinal cord (Fig. 2A) and we do not detect any invasion of eGFP positive expressing cells in the brain, heart or liver (Fig. 2B).

Immunostaining analysis demonstrated that hfNPCs survived at the lesion site, migrated and integrated within the host spinal cord 4 weeks after transplantation in non-injured (Figure 2B) and injured animals (Figure 2F-G). Interestingly, we show similar survival levels on non-injured and injured animals, indicating that the lesion environment was not affecting the survival. Although PGA-SS-FAS did not show any effect on cell survival on injured animals (Figure 2H), PGA-SS-FAS treated hfNPCs

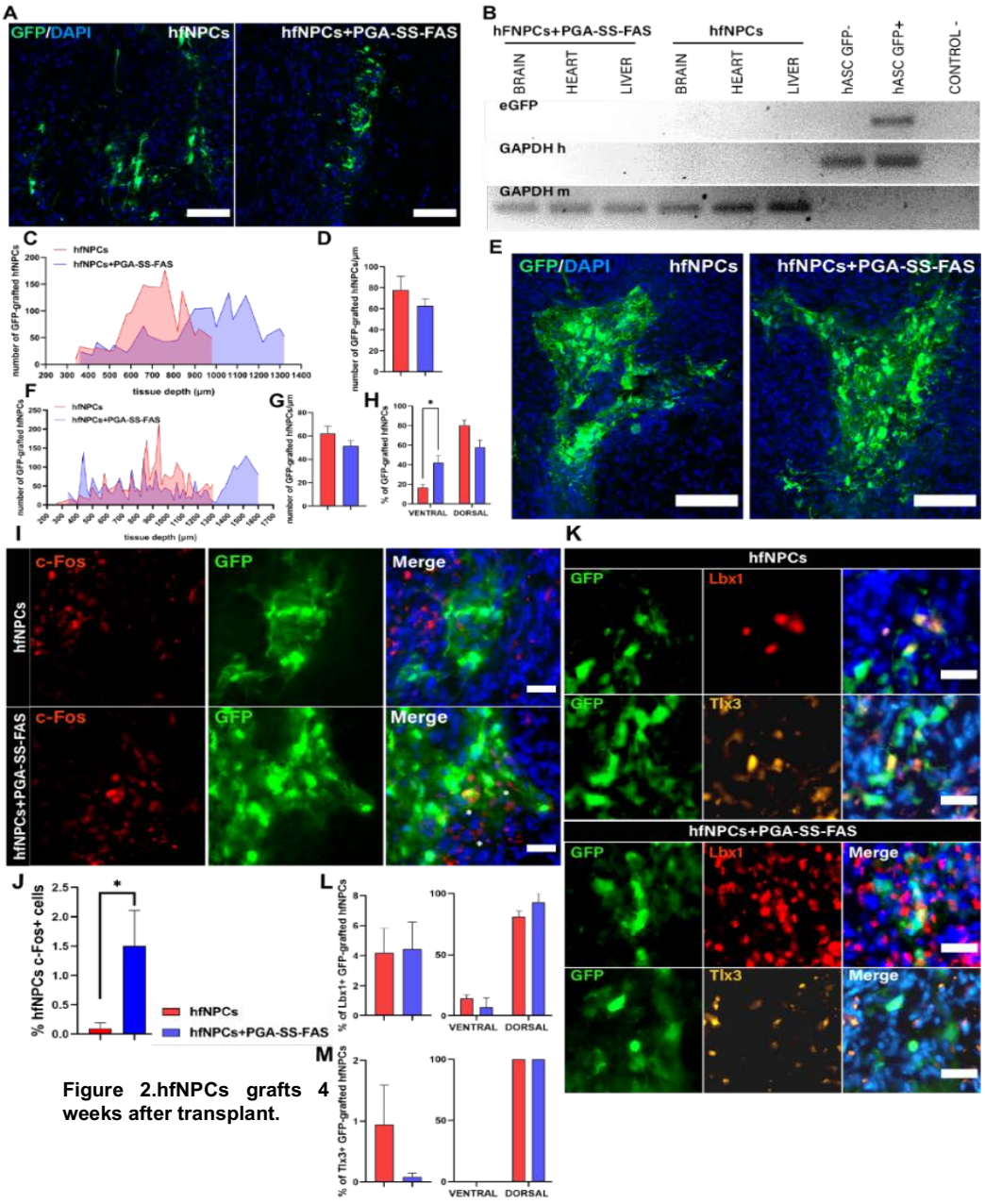

Figure 2.hfNPCs grafts 4 weeks after transplant.

showed increased co-localization with c-Fos ($GFP^+/c\text{-}Fos^+$ cells) (Figure 2I), c-Fos being a marker associated with neuronal activity (Hudson, 2018), which suggests functional integration into the host spinal cord circuitry. Furthermore, PGA-SS-FAS treatment enhanced hfNPC migration in the spinal cord parenchyma (Figure 2F) and we also found a significantly higher percentage of the grafted hfNPCs (PGA-SS-FAS hfNPCs) in the ventral areas of the spinal cord (Figure 2G) compared to untreated group (hfNPCs). In addition, analysis performed on non-injured animals (data not shown) revealed that the lesion environment was not influencing hfNPC survival or migration patterns, and in

this case PGA-SS-FAS-treated hfNPCs were also distributed more evenly throughout the spinal cord tissue.

Furthermore, four weeks after grafting, some of the grafted cells expressed the neuronal markers of specific GABAergic inhibitory Ladybird homeobox 1 (Lbx1) and glutamatergic excitatory T cell leukemia homeobox 3 (Tlx3) somatosensory interneurons (Figure 2J-L). These markers are involved in neuronal fate determination of interneuron populations located in the dorsal horns of the spinal cord and Lbx1 and Tlx3 interneurons, modulate and integrate peripheral somatosensory inputs (Monteiro et al., 2021). We found that both $Lbx1^{+}$ and $Tlx3^{+}$hfNPCs were located in dorsal areas of the spinal cord as the endogenous populations of these neurons which are located in the dorsal horns. However, we did not see any effect of PGA-SS-FAS treatment on the differentiation process.

3. Relevance and possible clinical applicability of the final results:

The complex pathological nature of traumatic spinal cord injuries requires the implementation of a multifaceted and versatile therapeutic perspective regarding the development of treatments. The single application of unique strategies based on cell transplantation including the use of neural precursor cells (NPC) has already shown beneficial effects on clinical applications. We have accomplished a new strategy for improving individual cell therapy by a combination of the NPC with a new compound PGA-SS-F which has shown to be a good candidate to reinforce the regenerative capabilities of the NPCs. We have implemented here a methodology to prime the cells before transplantation in order to achieve a unique treatment application with improved characteristics, such as increased migration into the grafted and injured area and higher cellular activation, in a model of SCI by severe compression. This strategy will contribute to provide a versatile and clinically relevant approach to be implemented in the near future in the treatment of acute SCI.

4. Publications, communications and training of personnel derived from this research

Primed Human Neural Precursor Cells with a Polymer-conjugate of Fasudil, a Rho/Rock inhibitor, for the treatment of acute spinal cord injuries. Esther Giraldo, Pablo Bonilla, Mara Mellado, Pablo Garcia-Manau, Carlota Rodó-Rodriguez, Ana Alastrue, Eric Lopez, Elena Carreras Moratonas, Ferran Pellisé, Đorđević S, M Jesus Vicent, Victoria Moreno (manuscript under preparation)

Ependymal cells in the spinal cord as neuronal progenitors. Moreno-Manzano V. CurrOpinPharmacol. 2019 Dec31;50:82-87. doi: 10.1016/j.coph.2019.11.008. Review.

Combinatory treatment of Neural precursor cells and a new nanoconjugate of Fasudil for the clinical application in Acute Spinal Cord Injury Raquel Rojas-Márquez, Esther Giraldo-Reboloso, Maravillas Mellado-López, Sergi Querol, Victoria Moreno-Manzano, Joaquim Vives. (Poster) Annual Meeting TerCel, Red de Terapia Celular, isciii. Santiago de Compostela, 28-29 November 2019. Poster.

Fasudil polyglutamate conjugate enhanced neural stem cells survival and neurite elongation for cell therapy in spinal cord injury. V. Moreno-Manzano[1], E. Giraldo[1], V. Nebot[2, 3], A. Alastrue-Agudo[1], O. Zagorodko[2], Ana Armiñan, M. Mellado[1], E. López[1], S Dordevic[2], MJ Vicent[2]. SENC conference, Sept 2019

Fasudil polyglutamate conjugate enhanced neural stem cells survival and neurite elongation for cell therapy in spinal cord injury. E. Giraldo[1], V. Nebot[2, 3], A. Alastrue-Agudo[1], O. Zagorodko[2], Ana Armiñan[2], M. Mellado[1], E. López[1], S Dordevic[2], MJ Vicent[2], V. Moreno-Manzano[1]. Gordon Conference, June 2019

A New Nanoconjugate Of Fasudil, For Spinal Cord Injury Treatment In Combination With Neural Precursor Cells. E. Giraldo, V. Nebot, O. Zagorodko, A. Alastrue-Agudo, B. Martinez, S Dordevic, MJ Vicent* and V. Moreno-Manzano* Oral and Poster, *ISCORE19,* Barcelona 13-14 Dec 2019

M. J. Vicent. Invited Speaker. Rationally Design Polypeptide-based Therapeutics. Gordon Research Conference on Cancer Nanotechnology. June 2019

M. J. Vicent. Invited Speaker. PAPER ID: 3196043. Polypeptide-based conjugates as versatile therapeutics as single agents or in combination therapy (final paper number:

PMSE 199). American Chemical Society (ACS) Annual Meeting & Exposition in San Diego, CA, August 25 - 29, 2019.

European Patent application: nº EP19382225.1, and PCT Application: PCT/EP2020/058940. WO2020193802A1. Co-inventors: MJ Vicent, V Moreno, E Giraldo, VJ Nebot, R Requejo, A Armiñan, A Alustre, O Zagorodko, JJ Arroyo-Crespo por "CONJUGADOS POLIMÉRICOS Y USOS DE LOS MISMOS", in the name of FUNDACIÓN DE LA COMUNIDAD VALENCIANA CENTRO DE INVESTIGACIÓN PRÍNCIPE FELIPE y FUNDACIÓN STEP BY STEP

A rationally designed self-immolative linker enhances the synergism between a polymer-ROCK inhibitor conjugate and neural progenitor cells in the treatment of spinal cord injury. Giraldo E, Nebot VJ, Đorđević S, Requejo-Aguilar R, Alastrue-Agudo A, Zagorodko O, Armiñan A, Martinez-Rojas B, Vicent MJ, Moreno-Manzano V. Biomaterials. 2021;276:121052. doi: 10.1016/j.biomaterials.2021.121052. Epub 2021 Jul 2

DOCTORAL THESIS, TITLE: Human-Induced Neural and Mesenchymal Stem Cell Therapy Combined with a Curcumin Nanoconjugate as a Spinal Cord Injury Treatment.

DOCTORAL STUDENT: PABLO BONILLA (under preparation, expected to be defended in November 2021)