SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: borsa di ricerca all'estero

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): finale

TITOLO DELLA RELAZIONE: An innovative approach for spinal cord injury: induced-neural stem cells (iNSCs) and pRNA nanotechnology

RELAZIONE:

Spinal cord injury (SCI) is a complex and debilitating pathology that involves thousands of individuals each year. Despite improvements in modern medicine leading to a normal life expectancy, there are limited treatment options, such as surgical decompression and stabilization of the spinal cord, corticosteroid administration and physical therapy aiming to minimize the damage to the spinal cord and optimize the functionality of spared connections [1] and there are still no fully restorative therapies for SCI [2]. Although it has been observed that neural tissue repair may spontaneously occur in patients affected by acute and chronic inflammatory and degenerative disorders of the nervous system, this process is not robust enough to promote a functional and stable recovery of the nervous system architecture [3]. From this perspective, promotion of tissue repair and regeneration represents one of the most intriguing although challenging therapeutic approaches, and different experimental regenerative therapies have been developed in the last decades aiming to diminish or modulate the devastating consequences of SCIs [4]. Several experimental therapies have been employed to ameliorate the hostile injured environment, amongst which stem cell transplantation is a standout. Neural stem cell (NSC) transplantation has shown promising results in promoting functional recovery in SCI models. However, clinical translation of stem cell transplantation is

limited by major issues of accessibility, immunocompatibility of cell sources, and ethical concerns; moreover, the lesion site in SCI pathology is an hostile and inhospitable environment that limits the survival, differentiation and engraftment of transplanted cells. To address the first translational issues, the use of induced neural stem cells (iNSCs) [5] can overcome allogeneic limits arising from transplantation of foetal NPCs used in clinical trials, leading to the possibility of fully immunocompatible transplants.

Nevertheless, the inhibitory nature of the injured site still represents the major impediment for a successful engraftment of transplanted cells[6]. The major contributors to this hostile environment and inhibitory mechanisms are represented by reactive astrocytes, thus combinations of strategies, such as stem cell transplantation augmented by modulation of secondary mechanisms, may lead to cumulative improvements in outcome after SCI [7]. Astrogliosis`, as is called this phenomenon of reactivity observed in astrocytes`, involves a broad range of mechanisms and its experimental modulation has yielded controversial outcomes. Indeed`, it is now clear that complete ablation of reactive astrocytes leads to detrimental effects in SCI pathology[8] [9], but on the other hand chronic astrogliosis can limit and inhibit axonal regeneration. Most of the approaches aimed at modulating two of the hallmarks of reactive astrocytes, i.e. upregulation of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, yielded controversial outcomes. The targeted ablation of reactive astrocytes after CNS injury, by using the mouse GFAP promoter in transgenic mice, showed an exacerbation of tissue degeneration [8, 10] suggesting that loss of astrocytes in the acute phase after CNS injury may lead to secondary degeneration of other cell types, on the other hand some studies showed increased axon regeneration by deletion of GFAP and vimentin genes [11, 12]. More intriguingly, the application of siRNA targeting GFAP and vimentin in *in vivo* SCI models showed functional recovery and improvements in urinary dysfunctions, by injecting siRNA soon after the injury [13, 14]. Contrary to the knockout technique, the RNAi systems achieves downregulation of dysregulated mRNA/proteins without the loss of genomic information of the targeted gene; moreover, they allow for the possibility of modulating the extent and timing of gene regulation [15]. Thus, based on the dual role of astrogliosis in SCI, but more generally in CNS diseases, we strongly believe that a time-specific modulation of this phenomenon may lead to remarkable beneficial outcomes: while the presence of reactive astrocytes in acute stage of the disease would restrict the spread of secondary damages, their modulation in sub-acute stage would avoid the consolidation of such phenomena that have been shown to become detrimental, especially in the chronic phase.

However, based on the fact that reactive astrocytes exert contradictory effects after CNS insults, beneficial or harmful depending o context and timing, it may be efficacious to look for new therapeutic targets that modulate these different activities in a controllable fashion and test these mechanisms. Amongst potential candidates for modulation, Lipocalin 2 (Lcn2), a siderophore-binding protein implicated in the modulation of the inflammatory response in several CNS diseases, may represent a more promising target [16]. Indeed, silencing of Lcn2 in sub-acute phase may lead to a modulation of the inflammatory response and secondary damages upon SCI, creating a more favourable environment for iNSCs transplantation and engraftment.

Small interfering RNA (siRNA) represents a great promise for targeted gene silencing in therapeutic applications, and advances in knowledge about the molecular mechanisms of endogenous RNA interference (RNAi) has been used to develop innovative nucleic-acid medicines as treatment of diseases, most

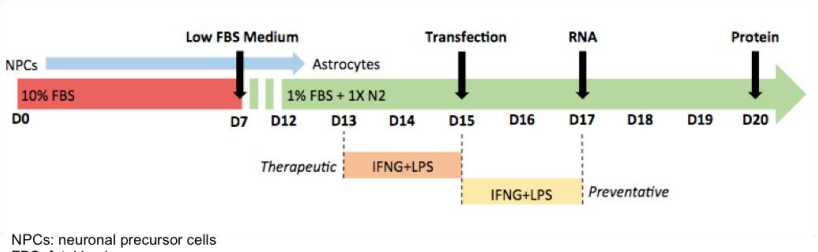
commonly cancers [17]. However, clinical translation of RNA-based therapeutics is impeded by difficulties in delivery; nevertheless, over the last couple of decades nanotechnology has advanced the development of drug-delivery platforms, including siRNA–based drugs [18]. Specific to the purposes of this study, packaging RNA (pRNA) nanostructures, bio-inspired constructs, are a promising example of this technology. Its advantageous size, thermodynamic stability,resistance to chemical denaturation, and modular nature make it a suitable candidate for translational approaches.

Therefore, the overall goal of this project is to conduct preliminary investigations into a combinatorial approach for SCI therapy, aiming to ameliorate the deleterious injury environment through the downregulation of Lcn2 expression by delivering pRNA nanostructures, to ultimately create a more amenable niche for the engraftment and differentiation of therapeutically-potent transplanted iNSCs.

Thus, the aims of this project have been:

1) establishment of a reproducible and reliable protocol for *in vitro* reactive astrocyte culture that would allow for screening of the safety and efficacy of pRNA nanostructures:

In order to assess the safety and efficacy of nanotherapeutics we developed a reproducible and reliable protocol (Fig.1) for differentiating neural stem cells (NSCs) obtained from the subventricular zone of adult mice into mature resting astrocytes, as platform for testing pRNA nanoparticles.



NPCs: neuronal precursor cells
FBS: fetal bovine serum
N2: n2 supplement
IFNG: interferon γ
LPS: lipopolysaccharide
Day 15: transfenction with nanostructures
Day17: RNA collection
Day 20: protein collection

Fig.1

NSCs differentiated via ourprotocol showed a progressive maturation of the astrocytic phenotype and morphology over the course of the protocol, from day 1 to day 15, assessed by immunofluorescence techniques.

Then, in order to mimic *in vivo* astrocyte reactivity after SCI, we activated resting astrocytes by using a cocktail of lipopolysaccharide (LPS) and interferon γ (IFNG) [19] at two different stages of the differentiation protocol (Fig.1). Both therapeutic and preventative activation resulted in an upregulated gene expression of the most common reactivity markers: glial fibrillary acidic protein (GFAP), vimentin, and Lcn2 (as assessed by qPCR techniques and western blot techniques), allowing for the assessment of the efficacy of siRNA-functionalised 3WJ nanostructures to modulate this overexpression. Moreover,

activated astrocytes showed a classical pro-inflammatory polarisation (upregulation of nitric oxide synthase 2, interleukin-6 and tumor necrosis factor, as assessed by qPCR techniques), thus making them a convenient model system for proof-of-concept studies of pRNA nanotherapeutics.

2) evaluation of the safety and efficacy of pRNA nanostructures in silencing *Gfap* and *Vim* genes as *a proof of concept*, and the evaluation of *Lcn2* silencing in the perspective of a translational approach:

These proof-of-concept studies confirmed the powerful potential of siRNA-3WJ constructs. 3WJ nanotherapeutics did not show any cytotoxic or immunogenic effects and in particular their employment showed an efficacious knockdown, up to 80%, of astrogliosis-associated genes (*Gfap*, *Vim* and *Lcn2*), confirmed by a significant reductions in mRNA and protein expression using relatively small doses of siRNA-3WJ, by using qPCR techniques and western blot techniques. Moreover, no off-target effects were observed in our limited study, by employing as negative control a non-targeting nanoparticle, containing a sequence that does not target any known mammalian gene product. Furthermore, by using astrocyte-condition media experiments we confirmed the key role of Lcn2, as protein secreted by reactive astrocytes, as a potentially significant contributor to the propagation of inflammation and astrogliosis, and consequently the potential of therapeutic intervention employing anti-*Lcn2* 3WJs to ameliorate astrocyte reactivity.

- The manuscript showing these results "Smith J.A., **Braga A.**, Verheyen J., Basilico S., Alfaro-Cervello C., Haque F., Guo P. and Pluchino S., *RNA nanotherapeutics for the amelioration of astrocyte reactivity."* is under revision in Molecular therapy-Nucleic Acids.

3) evaluation of the potential for clinical application of iNSCs compared to the most commonly used and characterized NSCs, by analysing the effects of subacute iNSC transplantation-induced functional recovery in a mouse model of moderate contusive spinal cord injury (SCI).

The therapeutic potential of sub-acute transplantation of iNSCs was assessed in a moderate contusive model of SCI, 70 kdyne, by using Infinite Horizon impactor. Locomotor activity of SCI mice was assessed at day 1, 3 and 5 by using the Basso Mouse Scale (BMS) score in the open field arena and at day 7 after SCI (subacute phase), mice were randomized into a total of three different treatment groups (vehicle-only control group, GFP-iNSCs transplanted group and GFP-NSCs transplanted group) based on individual BMS scores assessed on the same day.

Preliminary data showed that transplantation of iNSCs retains the potential to ameliorate the hostile injured environment upon SCI.

To investigate the survival of transplanted cells, mice from GFP-iNSCs transplanted group were sacrificed 4 and 7 weeks after transplantation, and 5.23 ± 1.34 % and 1.94 ± 0.43 % of corresponding percentage of surviving cells on the total number of transplanted cells were found, respectively. Moreover, 7 weeks after transplantation we observed an improvement in fine locomotor capabilities that may suggest a modulation of the secondary mechanism involved in the pathology that further investigations are aimed at elucidating, such as a reduction in lesion volume, promotion of remyelination or modulation of inflammatory response. Ultimately, iNSCs have a greater latent translational value compared to the most commonly used NPCs,

since their employment may overcome the ethical and practical barrier to translation faced in using fetal NSCs or the teratogenic potential of autologous induced pluripotent stem cells (iPSCs).

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