PERSPECTIVE

Combinatorial therapies for spinal cord injury: strategies to induce regeneration

Spinal cord injury (SCI) is a condition without treatment, mainly characterized by the loss of motor and sensory function below the level of injury. This is accompanied by several complications such as cardiac and respiratory compromise, and often patients present psychological ailments associated with the drastic alteration of their normal lifestyle. SCI pathophysiology derives from a massive damage to the spinal cord tissue, which is propagated by secondary events such as inflammation, excitotoxicity among others, that increase neuronal loss. In a later stage, a glial scar composed by reactive astrocytes and a cystic cavity are formed, creating a physical and chemical barrier for axonal regrowth. Strategies capable of inducing neuronal regeneration are therefore a fundamental part for a successful recovery of function for these patients.

In the last two decades, the tissue engineering (TE) field has shown promising advances, mainly at the pre-clinical level, aiming at SCI repair. Based on TE principles, the first studies were focused on developing a matrix (or scaffold) with physical properties similar to the spinal cord tissue. At the same time, these matrices are required to be biocompatible and biodegradable, while avoiding any enhanced inflammatory response. One of the first studies, mentioning the application of a matrix after a SCI, was done by de la Torre and colleagues, in 1984 (de la Torre et al., 1984). Using a collagen matrix (CM) implanted *in situ* at a T10 complete transection injury in rats, the authors report that the CM supported the growth of central neurites, fibroblasts and a network of blood vessels, which did not happen in control animals. Even though no improvements were seen in motor behavior, early waveforms of somatosensory evoked potentials were observed in CM-treated rats (de la Torre et al., 1984).

The concept of creating a bridge over the lesion is even older, with peripheral nerve grafts being performed after an injury, resulting in impressing levels of regeneration (David and Aguayo, 1981). However, unlike peripheral nerve, CNS tissue has a very limited capacity of self-regeneration, which has been hindering the development of successful therapies. Growth factors for instance, have been commonly associated with scaffolds to increase axonal regrowth. An elegant work from Anderson et al. (2016) demonstrated that a synthetic diblock co-polypeptide hydrogel, composed of lysin and leucine residues, served as a depot for neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) delivery. This approach, when conjugated to a peripheral conditioning lesion, led to robust ascending sensory tract axons regrowth into the lesion core, after a compression injury at T10 level, in a mice SCI model. Interestingly, the axons even grew through and beyond dense astrocytic scars (Anderson et al., 2016).

Cellular therapies have also been on the forefront of TE regeneration strategies. Either with the objective of replacing the neural tissue, or simply providing trophic support to increment endogenous regeneration, transplanted cells may play a key role in SCI treatment. However, cell survival in transplants to the injured spinal cord is very low, a fact that partially hinders their success as a therapeutic strategy. In this sense, the conjugation of cellular therapies with biomaterials is of great interest, since these scaffolds could serve not only as bridges for axonal regeneration, but also as vehicles for cellular transplantation.

In vitro testing of different biomaterials' suitability to induce cellular growth and neurite extension is therefore crucial. Our group directly compared three different extracellular matrix (ECM)-derived hydrogels in their capability to serve as a matrix for adipose stem cells (ASCs) growth and neurite formation and extension, using dorsal root ganglia (DRG) explants (Oliveira et al., 2017). One hydrogel was made of gellan gum (GG), a polysaccharide produced by bacteria; other was made of collagen and another one made of hyaluronic acid and rich in laminin epitopes. Interestingly, ASCs grew differently in all the three matrices, presenting altered levels of metabolic activity and gene expression, and even DRG neurite outgrowth mediated by ASCs was different according to each type of hydrogel (Oliveira et al., 2017). In this particular study, even though all three hydrogels were derived from natural polymers, the different source might have been a key aspect for the results obtained. Moreover, the physical properties of each hydrogel were most certainly very important. Differences in porosity and stiffness are known to affect the behavior of cells, particularly stem cells (Engler et al., 2006). For instance, Engler et al. (2006) demonstrated that matrices that mimic striated muscle elasticity (8–17 kPa) induce mesenchymal stem cells (MSCs) differentiation to myoblast-like cells, while stiffer matrices mimicking collagenous bone (25–40 kPa) can be osteogenic. Curiously, it was demonstrated that although reprogramming MSCs differentiation is possible with soluble factors, after several weeks in culture, cells eventually commit to the lineage specified by matrix elasticity, highlighting the importance of the matrix physical properties in cellular behavior.

In addition to physical characteristics, the presence of chemical cues is fundamental for cellular growth within any type of scaffold. In this matter, our group has done an extensive characterization on the role of RGD motifs and their integration into GG-based hydrogels, evaluating the adhesion of different cell types, growth and paracrine secretion. By using Diels-Alder click chemistry to modify GG with a fibronectin-derived peptide (GRGDS), we improved the morphology and proliferation of neural stem/progenitor cells (NSPCs) (Silva et al., 2012), a cell population commonly used in SCI-based pre-clinical studies. The same GG-GRGDS hydrogel formulation induced higher proliferation and metabolic activity of bone marrow MSCs (BM-MSCs), with cells presenting a normal morphology only in modified GG hydrogels (Silva et al., 2013). This chemical alteration also led to changes in the molecules secreted by BM-MSCs (the secretome), since they induced higher metabolic viabilities and neuronal cell densities, when compared to the secretome of cells

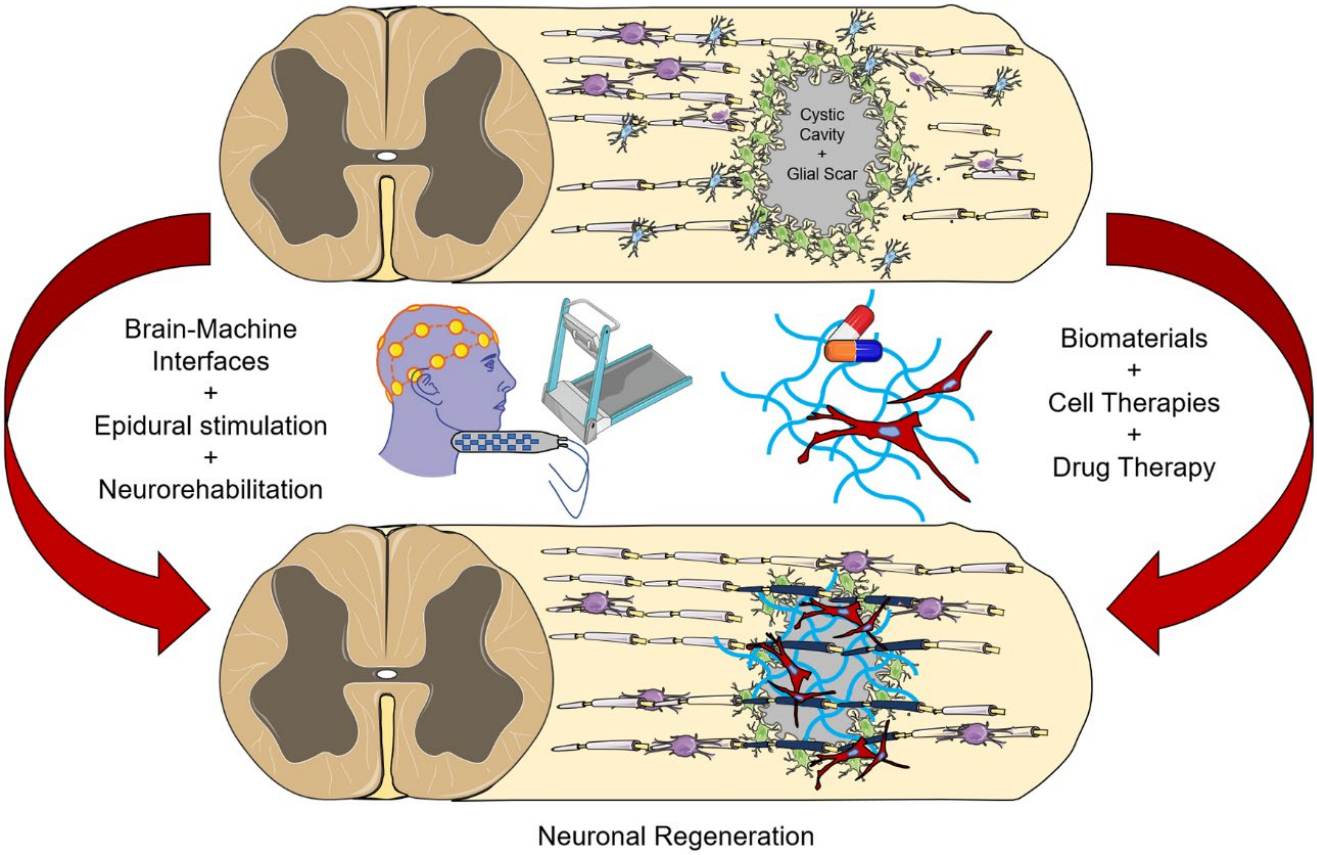

Figure 1 Combinatorial therapies for spinal cord injury (SCI) regeneration.
The use of brain-machine interfaces (BMIs), Epidural Stimulation and Neurorehabilitation strategies could be combined in the future with Tissue Engineering approaches (biomaterials, cell therapy and drug administration) to maximize the therapeutic effect and induce neuronal regeneration.

cultured in unmodified GG.

Nevertheless, although *in vitro* testing is important as a first assessment measure, *in vivo* experiments are crucial, because it is still not possible to fully recreate the lesion microenvironment. Taking this into account, our group showed a promising role for GG-GRGDS hydrogels *in vivo* (Gomes et al., 2016). When transplanted into a lumbar hemisection model of SCI, GG-GRGDS combined with ASCs and olfactory ensheathing cells (OECs) induced a significant recovery of motor scores of SCI rats, together with reduced levels of astrocytes and inflammatory cells, at the site of injury. Importantly, higher number of neurofilament positive cells indicate a possible increase in neuronal preservation after injury and/or increased regeneration (Gomes et al., 2016). Even though these results are encouraging, especially considering the aggressive injury induced (lumbar lesion model), there was still a restricted number of neurons/axons invading the lesion core, and the recovery of motor function was only partial.

Although these results are positive, there is a lack of studies combining a strategy that tackles both the secondary events responsible for neuronal death and inhibition of axonal growth, enabling at the same time, the tissue to regenerate. On one side, the ideal therapeutic approach would conjugate a biomaterial that could serve both as a vehicle for different treatment options (cells, drugs, neurotrophic factors) and simultaneously could be a suitable matrix, allowing the tissue to regenerate through it. This kind of scaffold should not induce any type of additional inflammatory response, hence the frequent use of natural and ECM-derived materials. Then, it should degrade with time, not too quickly so the cells or drugs would be rapidly lost and not integrated into the host tissue, but not too slowly, so it can be gradually replaced by newly formed neuronal tissue. On the other hand, this kind of strategy should be complemented with treatments focused on the abovementioned secondary events: drugs that could 1) target inflammation, so that it remains as a normal response to injury and does not develop into a chronic inflammatory state; 2) deal with the neurotoxicity derived from the excessive release of glutamate; 3) find ways to diminish the inhibitory signals of axonal regrowth, for instance degrading the glial scar.

The source of cells to be used in TE applications is another critical issue. The cells should derive from a source as much available as possible, and autologous sources are preferable, so immune rejection can be avoided.

Another great challenge in this field is the regeneration specificity of the damaged spinal cord. Usually, the regrowth of damaged axons is made in a disorganized fashion, losing completely the distinct organization of the spinal cord axonal tracts. In order to minimize this effect, Stokols and Tuszynski developed freeze-dried agarose scaffolds, with uniaxial individual channels extending through the entire length of the scaffold (Stokols and

Tuszynski, 2006). One month after an injury in rats, the scaffolds integrated the host tissue, being penetrated by different cells and amazingly axons grew in a linear fashion. The incorporation of BDNF into the walls and lumen of the scaffold augmented axonal regeneration.

Some of the most promising works for the future of SCI field include epidural stimulation and the use of brain-machine interfaces (BMIs). These strategies represent big hopes for SCI patients and have different principles underlying their application. Gregoire Courtine's group for instance, has been working on different models of electrochemical neuromodulation, being capable of inducing circuit reorganization after a SCI in rats (Asboth et al., 2018). On the other hand, Miguel Nicolelis' group has paved the way of BMI applications, that could be used for the creation of neuroprostheses and applied to a SCI context (Lebedev and Nicolelis, 2017). The use of both kinds of approach is exciting because they are associated to neuronal plasticity events. Nevertheless, inherent differences between species have to be taken into account, since the functional state of the spinal cord pre- and post-lesion varies from lower vertebrates, to small mammals or humans. In the same line of thought, the response to drug therapy might differ among species, making difficult to predict the success of a given treatment. Therefore, there is a need for combined therapies that could promote and guide the often disorganized neuronal plasticty, in order to induce some recovery of function, which is of the utmost importance for patients. Treadmill training and other rehabilitation regimens have been commonly associated with epidural stimulation (Angeli et al., 2014). This kind of repetitive and active training could be essential for stimulating neuronal circuitry reorganization and for the initiation of voluntary movements. In this sense, in the future, BMIs, epidural stimulation and neurorehabilitation should be combined with TE guidance strategies, increasing the chances of recovery after SCI (**Figure 1**). By using an integrated and combinatorial therapeutic approach, based on biology, as well as tissue and computer engineering concepts, we may achieve a successful therapy for SCI.

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References

Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, Coppola G, Khakh BS, Deming TJ, Sofroniew MV (2016) Astrocyte scar formation aids central nervous system axon regeneration. Nature 532:195-200.

Angeli CA, Edgerton VR, Gerasimenko YP, Harkema SJ (2014) Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. Brain 137:1394-1409.

Asboth L, Friedli L, Beauparlant J, Martinez-Gonzalez C, Anil S, Rey E, Baud L, Pidpruzhnykova G, Anderson MA, Shkorbatova P, Batti L, Pages S, Kreider J, Schneider BL, Barraud Q, Courtine G (2018) Cortico-reticulo-spinal circuit reorganization enables functional recovery after severe spinal cord contusion. Nat Neurosci 21:576-588.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214:931-933.

de la Torre JC, Hill PK, Gonzalez-Carvajal M, Parker JC Jr (1984) Evaluation of transected spinal cord regeneration in the rat. Exp Neurol 84:188-206.

Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677-689.

Gomes ED, Mendes SS, Leite-Almeida H, Gimble JM, Tam RY, Shoichet MS, Sousa N, Silva NA, Salgado AJ (2016) Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model. Biomaterials 105:38-51.

Lebedev MA, Nicolelis MA (2017) Brain-machine interfaces: from basic science to neuroprostheses and neurorehabilitation. Physiol Rev 97:767-837.

Oliveira E, Assunção-Silva RC, Ziv-Polat O, Gomes ED, Teixeira FG, Silva NA, Shahar A, Salgado AJ (2017) Influence of different ECM-like hydrogels on neurite outgrowth induced by adipose tissue-derived stem cells. Stem Cells Int 2017:6319129.

Silva NA, Cooke MJ, Tam RY, Sousa N, Salgado AJ, Reis RL, Shoichet MS (2012) The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate. Biomaterials 33:6345-6354.

Silva NA, Moreira J, Ribeiro-Samy S, Gomes ED, Tam RY, Shoichet MS, Reis RL, Sousa N, Salgado AJ (2013) Modulation of bone marrow mesenchymal stem cell secretome by ECM-like hydrogels. Biochimie 95:2314-2319.

Stokols S, Tuszynski MH (2006) Freeze-dried agarose scaffolds with uniaxial channels stimulate and guide linear axonal growth following spinal cord injury. Biomaterials 27:443-451.