PERSPECTIVE

The mutual interaction between the host spinal cord and grafted undifferentiated stem cells fosters the production of a lesion-induced secretome

Pathophysiology of spinal cord injury: Injury to the spinal cord results in loss of gray and white matter i.e., it produces a segmental spinal cord lesion and, as a consequence leads to a fatal loss of motor, sensory and autonomic functions. Spinal cord injuries in humans and other mammals are not followed by the replacement of lost neurons and oligodendrocytes and regrowth of injured axons, instead they lead to permanent, fatal and incurable functional deficits. The primary physical injury is followed by a cascade of tissue-decaying events, called secondary injury, which leads to ischemia, vascular disruption, neuroinflammation, excitotoxicity, demyelination and death of neurons and glial cells (Silva et al., 2014; **Figure 1A**). The direct mechanical disruption of the vasculature results in the increased permeability of the blood-spinal cord barrier. Due to the leakiness of blood-spinal cord barrier and production of cytokines by activated microglia, a number of various immune cells (T lymphocytes, neutrophils and monocytes) invade the injury site. The macrophages generate a set of inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1α, IL-1β, and IL-6, which may further augment the secondary events (Ahuja et al., 2017). In addition, a number of other disruptive processes contribute to the heterogeneous and time-sensitive pathophysiology of spinal cord injury.

Limited regenerative capacity following spinal cord injury: The regenerative ability of axons is completely inhibited in the mammalian spinal cord while it is supported by the peripheral nervous system environment. The lack of regeneration in the injured spinal cord is due to limited neuroplasticity and the growth-prohibiting nature of the central nervous system (CNS) microenvironment. The inhibitory factors present in the CNS include numerous proteins such as myelin-associated glycoproteins, oligodendrocyte-myelin glycoprotein and ephrins and this widespread inhibition of axon growth leads to apoptosis, growth cone collapse and neurite retraction (Beattie et al., 2000). In addition, external barriers set limitations to regeneration. Hypertrophied astrocytes and their products form a physical/chemical barrier that collectively contributes to the limited recovery following injury. These barriers include a tightly crossed network of astrocytic processes and a dense deposit of growth-inhibitory chondroitin sulfate proteoglycans that further inhibit the neurite outgrowth. As an end effect, inflammatory processes, reactive gliosis, and an expanded glial scar create an inhibitory milieu within the injured spinal cord that directly prevents axon regrowth and consequent functional recovery.

Secretome or lesion induced-secretome: Stem cell therapy is the most promising treatment in regenerative medicine. An increasing number of studies have demonstrated that a wide range of stem cells, such as mesenchymal, embryonic, neural or recently induced pluripotent stem cells (iPSCs) exert significant therapeutic potential after spinal cord injury. Their positive effect on functional recovery has already been demonstrated (Salewsky et al., 2015), but the mechanism of action remains to be understood. Grafted stem cells or their derivatives can differentiate into various cell types including neurons and glial cells with a limited potential to replace the missing or damaged host cells, support the survival or regeneration of the injured cord via secretion of biomolecules (referred to as "secretome"). Mesenchymal stem cells are multipotent cells derived from various origins including bone marrow, adipose tissue or umbilical cord. In experimental and (pre)clinical studies they play a very important role due to their easy availability, various biological effects, lack of ethical concern, and early immune reactions. Mesenchymal stem cells can release a wide range of bioactive substances including growth factors [vascular endothelial growth factor, brain-derived neurotrophic factor, glial cell derived neurotrophic factor (GDNF), neurotrophin-3/4 (NT-3/4)] or cytokines (granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein 1) resulting in the protection/regeneration of injured host tissue or the modulation of immune reactions (Kolar et al., 2014). Cell-cell contact enables mesenchymal stem cells to modulate their immunomodulatory effects and promote cell viability. Embryonic, neural or induced pluripotent stem cells can also produce growth factors (vascular endothelial growth factor, fibroblast growth factors, platelet-derived growth factor, brain-derived neurotrophic factor, GDNF, NT-4/5 and nerve growth factor) which all have an important role in immunomodulatory reactions and cell survival (Hawryluk et al., 2014). Nevertheless, they are able to modify the microenvironment of the lesion area providing trophic support and axonal sparing/regeneration. This line of evidence suggests that grafted stem cells have the ability to induce prosperous responses via paracrine mechanism at the level of the injured spinal segment. However, the experimental studies often do not provide unequivocal evidence whether the secretome produced by grafted cells is a response to the lesion rather than a simple production of otherwise already secreted factors. It would be also important to address whether identical cells or cell lines produce the same therapeutic profile in different spinal cord or other CNS injury models. It appears likely that most grafted stem cell lines do not enter a communication with the injured CNS, but only continue the secretion of intrinsically determined set of molecules after grafting. More detailed studies would be needed to elucidate the mechanisms of bioactive factor secretion both in native status of the cells and after transplantation.

To answer this question, we used neuroectodermal stem cells (NE-GFP-4C cell line, ATCC No: CRL-2926) to induce significant functional recovery following ventral root avulsion or thoracic contusion injury supported by neuroprotection and extensive axonal regeneration (Pajer et al., 2014, 2019). The grafted stem cells have minimally contributed to exogenous cellular replacement, but produced an array of proteins such as growth factors and cytokines [IL-1 alpha, IL-6, IL-10, tumor necrosis factor-alpha and macrophage inflammatory protein 1 alpha (MIP-1α) in avulsion injury; GDNF, IL-6, IL-10, and MIP-1α in contusion injury]. As reported by our laboratory earlier, native NE-GFP-4C cells do not produce any of these molecules in culture (Pajer et al., 2014). The grafted cells had the capacity to adapt their fate and functions to the specific lesion microenvironment such as segmental motoneuron damages and spinal cord contusion injury. These results have clearly demonstrated the presence of a strong communicative interaction between the surrounding injured host microenvironment and the grafted cells that leads to the secretion of the lesion-induced secretome by transplanted and host cells. It is well established that the cells of the lesion environment express high number of cytokines and these molecules have a strong effect on the grafted cells. Presumably this process gives rise to the factor secretion of the transplanted cells and these bioactive molecules are able to interact with the damaged environment by inducing significant neuroprotection and regeneration. Based on the result above, it can be stated that transplantation of an identical neuroectodermal stem cell line results in different, but effective therapeutic profile in various injury models. Similarly, grafted undifferentiated mouse iPSCs produced MIP-1α, IL-10, GDNF and NT-4 resulting in decreased microglial and astrocytic reactions accompanied by significant neuroprotection and functional recovery (Pajer et al., 2015). Functional blocking experiments proved that these factors played a pivotal role in the significant functional recovery following injury (Pajer et al., 2014, 2019), as blocking the secretome with neutralizing antibodies the morphological and functional improvement was completely abolished. In our unpublished study we have shown that grafted undifferentiated human iPSCs (SB5, Davis et al., 2013) were also able to produce bioactive molecules that may have contributed to functional recovery. Interestingly the factor production of transplanted cells was characteristic only in a narrow time window before expression was ceased. It should be noted that the lesion-induced secretome was produced only as long as the undifferentiated state of the grafted stem cells was maintained, i.e. the stem cells expressed their embryonic stem cell markers SSEA-1 or -4 (Pajer et al., 2014, 2015). The lesion-induced secretome has been shown to be highly potent in tissue sparing without any adverse effects (**Figure 1B**). It should be highlighted that we used undifferentiated cells with embryonic stem cell properties which have in common that two factors (IL-10 and MIP-1α) appeared in their secretome. The positive

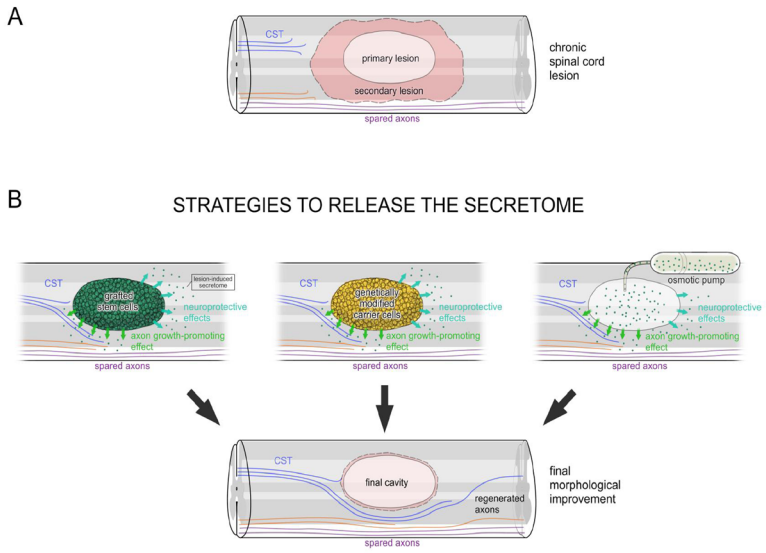

Figure 1 Schematic drawing illustrating the events after spinal cord contusion injury in an untreated cord and after secretome-based therapy.
(A) The major features after spinal cord contusion injury. The injury induces a physical primary lesion which is enlarged by a series of marked inflammatory processes leading to a large secondary damage. The lesion will interrupt a large number of ascending and descending axons, only a limited number of spared axons maintain the connection between the spinal cord segments rostrally and caudally from the injury. (B) The various ways how the secretome can be released in the lesion cavity in our experiments (stem cell-based cellular secretion, by transfected carrier cells, e.g., embryonic fibroblasts and via an osmotic pump). Undifferentiated stem cells interact with the injured host cord and begin to secrete a set of molecules collectively called lesion-induced secretome, specific to the type of injury. This effect can be mimicked by delivering the known secretome directly to the lesion site by cellular or other means. The secretome is able to induce axonal regeneration and neuroprotection, thus preventing the formation of the secondary lesion. The final cavity and regenerated fibers along with the spared axons are shown. The small size of the cavity and the considerable number of spared and regenerated axons result in significant functional improvement. The administration of a set of biomolecules (based upon the lesion-induced secretome of grafted stem cells) via genetically modified carrier cells or osmotic pump induces the same extent of functional and morphological improvement as transplantation of the stem cells.

effect of IL-10 is well known, but the role of MIP-1α is not fully understood. In our most recent study, the administration of a set of biomolecules (via osmotic pump or genetically modified fibroblasts) based upon the contusion-induced secretome of grafted NE-GFP-4C stem cells induces the same extent of functional and morphological improvement in rats with spinal cord contusion injury as transplantation of the stem cells (**Figure 1B**).

Future perspective of secretome based therapy: These enthusiastic results led to the idea of mimicking the beneficial effects of undifferentiated stem cells in an injured cord without using stem cells as cellular therapy. However, it cannot be concluded based upon these results, that any undifferentiated stem cell of any origin with respond appropriately to the injury environment and enhance the neuroprotective mechanism by secretome production. On the other hand, the exploration of stem cell-induced neuroprotective mechanisms may open new horizons of new therapeutic approaches that open the way to the development of new cell-free, but stem cell-based applications. These mechanisms may overcome the limitations of stem cell-based cellular therapy [ethical issues, immune response, teratoma formation (Nori et al., 2015)] and are more controllable and reproducible. Thus, an opportunity to transfer preclinical results into clinical practice will open.

Secretome-based therapy may raise several challenging questions that need to be addressed before the clinical translation of the exciting results based on basic research. Administration of well identified and characterized factors provides more controlled effects, which may overcome the limitations of the stem cell-based applications. After precise characterization of a secretome, the next aim is to provide delivery strategies for this combination therapy where the combined action of the identified biomolecules points towards definite morphological and functional improvement without the adverse effects of any of these factors normally experienced in the rest of the body.

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