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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/102688 since 2017-01-11T18:55:50Z

Published version:

DOI:10.1007/s10143-012-0385-2

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12 October 2024

This is the author's final version of the contribution published as:

Garbossa D; Boido M; Fontanella M; Fronda C; Ducati A; Vercelli A. Recent therapeutic strategies for spinal cord injury treatment: possible role of stem cells.. NEUROSURGICAL REVIEW. 35 pp: 293-311.
DOI: 10.1007/s10143-012-0385-2

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http://www.springerlink.com/index/pdf/10.1007/s10143-012-0385-2

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Recent therapeutic strategies for spinal cord injury treatment: possible role of stem cells.

D. Garbossa[#*], Boido M[°*], M. Fontanella[#], C. Fronda[#], A. Ducati[#], A. Vercelli[°]

#Department of Neurosurgery, S. Giovanni Battista Hospital, University of Torino, [°]Department of Anatomy, Pharmacology and Forensic Medicine, Italy; [§]

[*]These two authors equally contribute to this article

Abstract

Spinal cord injury (SCI) often results in significant dysfunction and disability. A series of treatments have been proposed to prevent and overcome the formation of the glial scar and inhibitory factors to axon regrowth. In the last decade, cell therapy has emerged as a new tool for several diseases of the nervous system. Stem cells act as minipumps providing trophic and immunomodulatory factors to enhance axonal growth, to modulate the environment and to reduce neuroinflammation. This capability can be boosted by genetical manipulation to deliver trophic molecules. Different types of stem cells have been tested, according to their properties and the therapeutic aims. They differ from each other for origin, developmental stage, stage of differentiation and fate lineage. Related to this, stem cells differentiating into neurons could be used for cell replacement, even though the feasibility that stem cells after transplantation in the adult lesioned spinal cord can differentiate into neurons, integrate within neural circuits and emit axons reaching the muscle is quite remote. The timing of cell therapy has been variable, and may be summarized in the acute and chronic phases of disease, when stem cells interact with a completely different environment. Even though further experimental studies are needed to elucidate the mechanisms of action, the therapeutic and the side effects of cell therapy, several clinical protocols have been tested or are under trial. Here we report the state-of-the-art of cell therapy in SCI, in terms of feasibility, outcome and side effects.

Corresponding author:

Diego Garbossa MD

Department of Neurosurgery, S. Giovanni Battista Hospital, University of Torino

Via Cherasco 15

10126 Torino, Italy

Phone: +39-0116335427

Fax: +39-011677078

E-mail: garbossa@libero.it

Keywords: spinal cord injury, stem cells, transplantation, regenerative approach, cell replacement, axonal reorganization

Background

Spinal cord injury (SCI) often results in significant dysfunction and disability. It physically and psychologically affects not only the individual, but also his/her family and the whole society. Worldwide SCI affects approximately 6 million people, most often of young age; the chances of recovery are very low and the disability is permanent, with long-lasting deficits, such as partial or complete paralysis and loss of sensation below the level of the injury. Early rehabilitation in an organized multidisciplinary SCI care system lowers mortality, decreases pressure sores, slightly increases chance of neurologic recovery, and shortens lengths of stay, thus reducing hospital charges. Nevertheless, continued functional dependency, healthcare needs and costs, as well as caregiver burden and stress often remain tremendous.

Functional deficits following SCI result from damage to axon fibers, loss of neurons, activation of astrocytes and microglia, and degeneration of oligodendrocytes [32] and demyelination. The outcome is determined by the mechanical insult, i.e. the primary damage, followed by several secondary processes as ischemia, anoxia, free-radical formation and excitotoxicity [56,96].

The first mechanism of injury consists of traction and compression forces. Compression by bone fragments or soft tissue injures both central and peripheral nervous structures. Within minutes, the spinal cord swells and exceeds venous pressure and results in secondary ischemia. The spinal neurogenic shock leads to systemic hypotension that exacerbates the ischemia. Finally, the release of toxic molecules leads to secondary damage [130]. The wave of secondary cell death, which mainly affects neurons and oligodendrocytes, spreads rostrally and caudally from the site of impact, leading to structural and functional damage. Key secondary injury mechanisms include damage of spinal cord vasculature and ischemia, glutamatergic excitotoxicity, oxidative cell stress, lipid peroxidation and inflammation, all of which alone or in concert can stimulate apoptosis.

Toxic agents such as intracellular Ca^{++}, free radicals and excitotoxic aminoacids are responsible for triggering molecular pathways leading to cell death, such as caspases and MAP kinases, and inflammation. In addition, neutrophils and microglial cells, stimulated by chemokynes released at the lesion area, migrate into the site of injury, and cause enlargement of the lesion area. The traumatic lesion is followed by the degenerative changes of astroglia, oligodendroglia and neurons in and around the lesion site [105,141,224]. The outcome of this sequence of events is the formation of the glial scar, a cavity surrounded by reactive glia which represents a physical obstacle to axonal regrowth [54,148,186,224].

Inhibition of axonal growth

Highly vulnerable to insults, neurons and oligodendroglia are spontaneously replaced after SCI to a very limited extent due to restricted regenerative potential of endogenous neural stem/progenitor cells. Furthermore, axonal regeneration and remyelination, especially from mature neurons, are also extremely limited if present at all. These disappointing limitations can be ascribed to the presence of the glial scar, neurotrophic factor deprivation, decreasing cyclic adenosine monophosphate levels, inhibitory sulfated proteoglycans, and inhibitory myelin-associated molecules. Formation of the glial scar represents an attempt by glial cells to limit the extent of the injury site and promote healing. Scar formation involves oligodendrocyte precursor cells, microglia, macrophages and extracellular matrix.

Besides the glial scar, there are other inhibitory obstacles to axonal regeneration, such as myelin inhibitory molecules including the myelin-associated neurite outgrowth inhibitor Nogo-A, the myelin-associated glycoprotein, the proteoglycans brevican and versican V2, and several potentially repulsive/inhibitory axonal guidance molecules [129,177]. After injury, upregulation of chondroitin sulphate proteoglycans (CSPGs), which are associated with astrocyte and oligodendrocyte precursors, is a major contributor to the inhibitory properties of the adult central nervous system (CNS).

Therefore, one major therapeutic aim is to modify the extracellular matrix. In vitro experiments have demonstrated that proteoglycans associated with reactive astrocytes can be modified to increase axonal growth by removing or preventing the production of sugar epitopes on the proteoglycan molecules [21,64,132,133,191]. An in vivo transgenic model whose astrocytes express a CSPG degrading enzyme showed enhanced axonal growth after injury [26]. Neutralizing a heparan/chondroitin sulphate proteoglycan expressed after brain injury with a blocking antibody allows neurite outgrowth and prevents growth cone collapse in vitro [20]. Injured corticospinal fibers and uninjured serotonergic fibers increase sprouting after enzymatic treatment to remove sugar epitopes [10] and in vivo studies have revealed remarkable long-distance regeneration of adult axons though CNS white matter tracts after enzymatic treatment to digest these proteoglycan side chains [191].

Timing of transplantation

The success of any treatment seems strictly dependent on the timing of SCI therapy. In fact, the therapeutic time window in which the spinal microenvironment is not compromised is very narrow [55,72].

After the initial injury, the damage site expands from the injury epicenter, i.e. many centimeters in a human. Analysis of chronic SCI shows that, typically, portions of the outer white matter are spared

while there is extensive damage of the inner grey matter. Within white matter, both ascending and descending axons degenerate, and demyelination occurs due to loss of oligodendrocytes. Chronic, progressive demyelination is a persistent feature of SCI [203]. The astrocyte response begins immediately after injury (proliferation, hypertrophy, etc.) and evolves over time. Reactive astrocytes produce extracellular matrix components such as chondroitin and keratan sulfate proteoglycans. Ultimately, a scar-encapsulated cavity many times the size of the initial injury forms [54,186].

The occurring cellular events are characterized by the time-dependent expression of specific molecules, such as the early activation of inflammatory cytokines (interleukin-1 alpha and interleukin-1 beta, tumor necrosis factor- alfa and interleukin-6), and the glial cyst and scar formation in the following days [147]. The weeks before the complete formation of the glial scar may offer a potential temporal opportunity to facilitate and maximize benefit from endogenous or transplanted stem cells. This is thought to be the interval whereby stem cells can achieve maximum repair in the injured CNS.
Therefore, SCI involves different phenomena, which have a specific chronology summarized in an acute and a chronic phases. Consequently, stem cell transplantation occurring at different time intervals from the injury may interact with different mechanisms of damage or repair. Transplantation in the acute phase occurs before the formation of the glial scar and cyst, and also modulates the early phases of microglia activation and astrogliosis. On the other hand, since it is quite often difficult to foresee the outcome of an injury in the acute phase, the lesion must be stabilized before invasive treatment. Transplantation in the chronic phase could give new hopes to people who are functionally impaired since months or years: in fact, several authors have demonstrated the stem cell ability to fill the lesion cavity and eventually to bridge the gap on the lesion area (Figure 1A) [141].

Experimental models of SCI

Treatment for SCI in patients still remains limited to the reduction of the lesion and to the control of inflammation and of glial activation [4,49,53]. Even though this approach has been questioned, cortisone remains the elective treatment in many countries, such as Italy. Most of the studies involving new therapeutic strategies, and in particular those involving stem cell therapy, undergo a preclinical phase. To this aim, the major challenge consists in creating a reproducible experimental model which can mimic the human pathology. Actually, two models in mice and rats are commonly used in studies on SCI treatment: the compression and the transection models. The first one is quite adherent to the SCI due to trauma, and is the most relevant for human SCI. However, the spared axons and regenerating axons in the injured spinal cord are not easy to distinguish [197]. Specific devices have

been used to create reproducible SCI compression lesions, such as the IH device (Precision Systems and Instrumentation LLC) [172] or the a 23 g clip (Walsh) [90]. On the other hand, unilateral [45] or bilateral [164] transection of the dorsolateral funiculus in rodents allows to study axon regeneration and sprouting while reducing the role of the glial cyst. In particular, unilateral transection allows to study axonal sprouting from the contralateral side and the effects of stem cell transplantation [19]. In any case, it must be considered that there are consistent differences in the anatomy of the descending pathways controlling movement between humans and rodents [176].

In order to assess the outcome of treatment in preclinical studies, a general consensus must be achieved on the parameters to consider. First of all, a battery of behavioral tests is currently used for testing the improvement of motor performance following treatment in terms of muscular strength, resistance and coordination [180]. Nevertheless, it must be considered that in rodents the relevance of intrinsic spinal circuits is much higher than in primates [169]. Second, the morphological outcome includes a series of parameters relative to i) fiber sprouting [number of myelinated fibers in the white matter, of Growth Associated Protein 43 or 5HT (serotonin)-positive fibers], ii) glial activation (microglial activation and astrogliosis), iii) glial scar and cyst size. [4,18,19,88,104].

Treatments that promote recovery following SCI

The limited spontaneous regeneration and regrowth of the proximal segment of the injured spinal cord represents an important target for research. Treatments should act on both on the intrinsic neuronal mechanisms and on extracellular matrix (ECM), and on non neuronal cells located beyond the lesion, especially neutrophils and microglia. On the extrinsic side, the ECM is responsible for the formation and stabilization of perineuronal nets and matrix networks containing potent growth inhibitory molecules, such as CSPGs. Interventions that have been combined with transplants to promote repair and/or recovery include the application of neurotrophic or growth factors, pharmacological agents that mimic the action of neurotransmitters, antiapoptotic agents, agents that interfere with axonal growth inhibitors and physical rehabilitation and training [56,105,178]. Most treatments are devoted to cure acute injury, whereas chronic injury is a more challenging condition. Recent advances in stem cell biology have provided new tools in therapeutic strategies in neurodegenerative diseases and injury, aiming both to cell replacement and trophic support.

Potential approaches aim to optimize functional recovery after SCI. They include minimizing the progression of secondary injury, manipulating the neuroinhibitory environment of the spinal cord, replacing lost tissue with transplanted cells or peripheral nerve grafts, remyelinating denuded axons,

and maximizing the intrinsic regenerative potential of endogenous progenitor cells. Their primary goal has been to replace lost cells [148]. While early studies transplanted differentiated neural cells and glia, more recent studies have proposed transplantation of stem cells or unrestricted progenitors, committed to the neural lineage (Figure 1B). These cells have a remarkable ability to differentiate into appropriate cells by taking cues from their close environment. Moreover, transplanted cells can provide molecules, such as neurotrophic factors, supporting nervous system regeneration.

The issue of stem cell research is politically and ethically charged. As a result, stem cell technology is imbued in an ethical conflict between human embryo research raising moral concerns on the one hand, and the magnitude of the potential benefits to patients, on the other. Stem cells may be derived from a variety of sources, including early embryos, fetal tissue and some adult tissues (e.g., bone marrow and blood).

This review reports the state-of-the-art of cell therapy in SCI. First, the limit to regeneration present in the injury site as well as the issues involved in cell sourcing are detailed. Next, the different types of cell therapy are defined with the benefits and drawbacks of each type given. Methods for delivering cells are also discussed. Finally, suggestions are made for future work in developing therapies for SCI. Recent advances in neural injury and repair, and the progress towards development of neuroprotective and regenerative interventions are discussed.

Regenerative approach vs cell replacement strategies (Figure 2)

Spinal cord repair consists in axonal regeneration, in the restoration of former neural circuits and eventually in the formation of new ones. Cell-based approaches for spinal cord functional repair center on two fundamental directions that are not mutually exclusive: restitution of white matter long-tracts ("regenerative" approaches) and cell (i.e., neuronal or oligodendrocyte) replacement [54,87,186]. The challenges to obtain functional recovery are the following: i) cell survival or replacement; ii) axon regeneration or growth; iii) correct targeting by growing axons, and iv) establishment of correct and functional synaptic connections. The solutions proposed include intraspinal transplants with fetal cells or progenitor cells to restore the intraspinal circuitry or to function as relays for damaged axons. The physiologically disrupted but anatomically preserved axons can be remyelinated by Schwann cells, oligodendrocytes, and olfactory ensheathing cells (OECs) transplantation.

When cell replacement is considered, stem cells can differentiate into neurons and form new circuits eventually bridging the gap on the lesion area. Nevertheless, the loss of neurons contribute only minimally to the functional deficits in SCI, as only neurons at the injured segmental level are lost. Cell-

based therapies can substitute lost glial cells to some extent and provide growth promoting factors; however, structural and functional recovery is moderate at best [166]. Ideally, cell candidates for transplantation should be able to replace the function of astrocytes, which build the cellular scaffold of the spinal cord and provide guidance cues for regenerating axons and oligodendrocytes, which myelinate axons, thus allowing proper nerve conduction [33,178]. We have recently obtained a good integration of neural precursors collected from the spinal cord of embryonic day 12 (E12) mice into the lesioned spinal cord of the adult mouse, with the elongation of axons through several neuromers, emitting segmental collaterals [18] (Figure 1C).

Regarding the bystander role, it has been shown that different types of stem cells can produce and release in the environment trophic factors and immunomodulatory molecules [209]. Various cell types such as fibroblasts, Schwann cells and OECs have been analysed for their regenerative potential after transplantation into the injured spinal cord [16,53,200,208]. Successful axon regeneration requires that injured neurons activate a specific gene program, which includes activation of regulatory genes and growth-associated proteins needed to sustain the elongation of the axon stem. The expression of these genes is modulated by extrinsic signals issued by different sources. On the one hand, intrinsic neuronal properties are influenced by molecular cues present in the axonal microenvironment. On the other, there is increasing evidence that interaction with the external world through sensory stimulation or physical exercise also exerts a strong modulatory effect on neuritic growth and plasticity. Therefore, the growth potential of injured neurons (but also of their uninjured counterparts that may contribute to repair through circuit reorganization) depends on the interaction between intrinsic neuronal properties, environmental regulatory molecules and experience-related mechanisms.

Cell replacement [113] can be achieved by transplanting stem cells either differentiated in vitro [94], or naïve, expected to differentiate in vivo [15]. Undifferentiated cells, however, may aim to replace different cell types such as glia as well as neurons. In addition to undifferentiated or progenitor neural stem cells, many different cell types such as genetically modified fibroblasts, OECs and Schwann cells have been used to promote axonal regeneration [109,115,200,205,224]. Functional recovery has been obtained in experimental models of SCI following transplantation of embryonic stem cells (ESCs) [129], mesenchymal stem cells (MSCs), neural stem cells (NSCs) and glia restricted precursor cells [18,29,44,76,83,89,92,129,146,200].

EMBRYONIC STEM CELL TRANSPLANTATION

ESCs have the broadest potential of any true stem cell. ESCs are isolated from the inner cell mass of the blastocyst [62,131,220], derived through in vitro fertilization [52,126,183]. They a) can replicate indefinitely without aging, b) are pluripotent, i.e. can give rise to all the different types of cells in the body, c) give rise to genetically normal cells and d) can be easily manipulated genetically [110]. ESCs can be differentiated in vitro before transplantation: culture in media containing Vitronectin and retinoic acid, Sonic hedgehog, Noggin, or SB431542 can promote oligodendrocyte differentiation [57]. Therefore, ESCs can differentiate into functional neurons [13,60,95,114,118,229], which integrate in vivo into host circuits as shown electrophysiologically after transplantation [24,71,215]. In addition, they are able to differentiate into glia [23,174], such as oligodendrocytes, capable of rapid differentiation and myelination in mixed neuron/glial cell culture [98,101] and which remyelinate and promote regeneration in the injured axons in SCI [131] and functional recovery [61,106,144,166]. On the other hand, ESCs have an intrinsic potential to give origin to teratomas after transplantation [15,31,173], even after fluorescent-activated cell sorting [35,199,214] during or after differentiation to purify the neural cells. Moreover, they can give rise to normal cells in the wrong place. Therefore, before transplanting ESC-derived cells, it is critical to get rid of undifferentiated pluripotent cells, to gain control of ESCs and guide their differentiation toward the neural lineage. Elimination of pluripotent cells has been convincingly obtained by spontaneous differentiation of ESCs cultured at low density, followed by propagation as a monolayer in Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) [38]. ESCs in the presence of appropriate signaling molecules can be maintained as a relatively homogeneous population of stem cells. Prolonged differentiation protocols [22], or inhibition of proliferation signaling pathways by genetic manipulation [111] decreases the incidence of tumor formation. ESC-derived tumor formation can be also prevented by co-transplantation with BMSCs [127].

STEM CELLS OF NEURAL ORIGIN

NSCs can be isolated from many regions of the CNS of embryonic as well as adult mammals [79], and propagated in culture in the presence of EGF and/or bFGF as neurospheres. Neurospheres, differently to ESCs, represent heterogeneous clusters of proliferating cells including stem cells, committed progenitors and differentiated cells [1]. NSCs also seem to be restricted in the variety of neurons they can generate. In fact, NSCs did not give rise to motoneurons, whereas they could be successfully differentiated into cortical projection neurons [50], interneurons [175], and hippocampal pyramidal neurons [39]. Differentiated NSCs fully integrated into the host circuits, since they could be

retrogradely labeled from the axon terminals, formed synaptic contacts, and generated action potentials in vivo [50]. In addition to be able to differentiate into neurons [93,189], they can differentiate into glial cells, such as astrocytes [51,75,219] and oligodendrocytes [155,226], and endothelial cells [223]. NSCs can protect against glutamate-induced excitotoxicity and secrete GDNF and NGF (nerve growth factor), thus promoting survival of injured motoneurons [117]. Embryonic neural precursors can integrate into host damaged tissue, differentiate into neurons, astrocytes and oligodendrocytes, and promote regeneration and repair [69,107], enhancing sprouting of serotonergic and noradrenergic fibers [18,137].

Organotypic cell replacement can be achieved with neural precursors cells (NPCs). NPCs from embryonic as well as adult CNS tissue have the capacity for self-renewal and multipotency [162,193]. After delayed transplantation of embryonic derived NPCs into the injured rat spinal cord, differentiation into glial and neuronal lineages as well as modest functional improvement have been reported [68,129,200]. Even though NSCs display a lower tumorigenic risk than ESCs, it has been reported at least in one case that they can give rise to brain tumor [6].

Transplantation of neural stem cells and progenitors entails ethical concerns, limited availability and need for immunosuppression in the host, which can be avoided by the isolation and expansion of autologous adult NPCs. Autologous adult NPCs transplanted into the intact spinal cord of adult rats survive, migrate over considerable distances and differentiate into astroglial and oligodendroglial cells [184]. Subventricular zone-derived adult NPCs survive after delayed transplantation - at least seven days post injury- into the spinal cord parenchyma surrounding the injury site [28].

Acutely injured spine area is an inhospitable CNS environment for any cell graft, due to the release of inflammatory molecules and the upregulation of mediators of cell death/degeneration and secondary ischemic events. It has been proposed that neurospheres can survive in the host better than single cell suspensions, since cell–cell contacts remain intact and detrimental effects by dissociation methods are avoided [194]. Adult NPCs transplanted into the fluid-filled lesion cavity (cyst) fail to produce extracellular matrix needed to survive [129]. On the other hand, adult NPCs migrate and align along injured axon pathways caudal and rostral to the lesion site, suggesting that they are not sealed off by the surrounding host spinal cord, in contrast to other cell types such as fibroblasts or Schwann cells [28,34,49,63,67]. Remyelination of host axons by neural stem cells may be one mechanism generating functional recovery [116], moreover, stem cells can also promote regeneration, enhancing sprouting of descending fibers [18]. Peripheral nervous system (PNS) myelinating Schwann cells derived from neural crest-like skin-derived precursors, used for autologous graft, transplantated into the injured rat

spinal cord, reduced contusion cavity size, myelinated endogenous host axons, recruited endogenous stem cells, provided a bridge across the lesion site, increased the size of spared tissue rim, reduced reactive gliosis, and generated an environment highly permissive to axonal growth [150,151]. Transplantation of Schwann cells results in a strong integration in the host tissue with anatomical and functional improvement, excepted when transplanted directly into lesion cavity, where they show poor survival rate [49].

OECs surround olfactory axons and facilitate their lifelong regeneration. They are attractive for their plasticity and allow axons to cross glial scars as well as the PNS-CNS boundary. OECs can stimulate tissue repair and neuroprotection, enhance axonal regeneration and remyelination, activate angiogenesis, influence the endogenous glia after lesion [163], even though there are contrasting reports probably due to the changes in their biological properties with increasing age and/or passage number. A recent study on 7 patients receiving autologous OECs into the lesion site after chronic SCI demonstrated a variable extent of functional recovery, as far as restoration of bladder sensation, voluntary anal sphincter contraction and improved motor and sensory function to variable extent [157].

Bone marrow-derived cells

Adult bone marrow is easily accessible, containing both hematopoietic stem cells (HSC) and bone marrow MSCs, and their usage does not imply the ethical concerns associated with ES cells. In fact, they can be collected from the patient himself or from donors following informed consent.

Rodent HSCs transplanted into mice with compression SCI generate oligodendrocytes, resulting in significant functional recovery of hindlimb function and general locomotion [3]. As neural differentiation of HSCs is controversial, they are thought to impart beneficial neuroprotective and/or immunomodulatory effects by releasing growth or antiinflammatory factors [40,145,190].

Azizi et al. [7] infused rat brains with human marrow stromal cells/MSCs, capable of expansion, self-renewal, and differentiation into several different cell lineages. Their migration into the brain resembles that of paraventricular astrocytes, but whether the cells adopt neural cell fates remains uncertain. Several studies have suggested that MSCs, like bone marrow mononuclear cells and umbilical cord cells, may generate neurons both in vitro and in vivo. Nevertheless, these conclusions have been drawn mostly on morphological criteria in vitro [46,221] and in vivo on studies employing transferable labels [100,140,185], without electrophysiological evidence [122]. Therefore, these studies have been challenged [25,41,121,195]. More recently, the appearance of neuronal-like MSCs has been ascribed to a process of cell fusion rather than that of transdifferentiation [222]. Nevertheless, cell

fusion is probably very rare, and the acquisition of neural antigens by MSCs might simply reflect their extreme immaturity and their undetermined fate [47,70,122].

On the other hand, MSCs, which are currently used in association with HSCs in order to decrease immunoreaction in the host, have an anti-inflammatory potential, i.e. decrease microglia and astroglia activation, and their usage in diseases of the nervous system has a positive functional outcome [70,143]. Recently, MSCs have been transplanted into the injured spinal cord, either embedded in a polymer, or by lumbar puncture, associated with neurotrophic factor such as IL-6, BDNF (brain derived growth factor), NGF, VEGF (vascular endothelial growth factor), NT-3 (neurotrophin-3) [42,81,103,108,122,135,142,167,207], with promising results in terms of functional outcome [8,99,210,217]. MSCs probably display their action by modulating inflammatory response to the insult and providing trophic factors for neurons and regenerating fibers. Transplantation and intravenous injection of bone marrow cells, and especially MSCs, has been repeatedly shown to significantly improve locomotion and hindlimb sensitivity after contusion, hemisection, and compression SCI in mice and rats compared with controls, with an increase in spared white matter in treated animals. The mechanisms of action underlying these benefits are only beginning to be understood [218].

In our experience, MSCs were able to survive for a long time into the injured spinal cord, promoting sprouting of raphespinal axons and functional recovery of motor behaviour, probably acting as biologic minipumps able to deliver trophic factors and immunomodulatory molecules [18]. Furthermore such ability could be exploited by transfecting MSCs to produce and release constantly neurotrophic factors, thus providing a continuous and *in loco* source with neuroprotective effects [9,70,153].

MSCs migrate toward injury-associated signals in vitro, attracted by cytokines and chemokines [136,156,188]. Notwithstanding the rather poor survival of MSCs following transplantation into the brain [41], endovenous administration of MSCs lead to significant functional improvement in a cerebral ischemia model [78], making unclear their mechanism of action. Nevertheless, the therapeutic potential of MSCs is currently being investigated in several clinical trials for neural diseases [58]. To this aim, accurate pre-transplantation analyses should be carried such as demonstration of a normal karyotype. In fact, even though MSCs are thought to be potentially free from side effects, malignant transformation has been found after extended culture in vitro both for mouse [138,202] and human [170,181]. Their uncontrolled proliferation could be further supported in vivo by their immunosuppressive properties [48,171,233].

Another bone marrow-derived stem cell population, called multipotent adult progenitor cells (MAPCs), has been described displaying greater potential than MSCs [84,161]. In vitro MAPCs can give rise to functional cell types from the three embryonic germ layers, including functional neuronal cells [168,179,181,228]. Zhao and coll. [232] have employed MAPCs in a rodent model of stroke, showing a behavioral function improvement, even if there is no evidence for their neural differentiation in vivo [25].

In conclusion bone marrow-derived cells have been the focus of a significant debate in stem cell biology pertaining to whether stem cells can transdifferentiate. Some studies have shown MSC differentiation to oligodendrocytes after implantation, whereas others demonstrated the cells only to localize to Schwann and oligodendrocyte cells post-transplantation. Other studies detected no transdifferentiation, even though functional improvement was noted. In vivo differentiation to highly pure neural populations has not been clearly documented; however, some ex vivo bone marrow-derived cells have been shown to express neuronal and oligodendroglial markers [9,218].

EX-VIVO TRANSDIFFERENTIATED CELLS

A recent innovative approach in regenerative medicine is represented by ex-vivo transdifferentiared cells, consisting in reprogramming somatic cells (e.g. fibroblasts) by inserting some transcription factors (OCT4, SOX2, KLF4 and MYC), thus obtaining the so-called induced pluripotent stem cells (iPSCs). These factors play a specific role in maintaining cellular pluripotency, assuring the typical morphology, growth properties and genetic features of ESCs, and allowing the differentiation into endoderm, mesoderm and ectoderm germ layers [196].

Moreover, there are both advantages and disadvantages relative to iPSCs: in fact, their use circumvents many ethical issues generally related to ESCs, but similarly to ESCs they can cause teratomas, specific embryonic tumors composed by different cell types [111]. Indeed this problem is related to their pluripotency, therefore the aim is now to induce a partial cell commitment: new techniques allow to reduce the number of employed transcriptional factors and chemicals, generating partially reprogrammed iPSCs able to self-renew and differentiate into specific cell lineages, or aberrantly reprogrammed cells that can only self-renew [225].

A valid alternative consists in differentiating in vitro iPSCs into neural precursors, successfully obtaining neurons and glial cells: when transplanted in the murine brain iPSCs gave rise to glutamatergic, GABAergic and catecholaminergic neurons [216]: in addition, to limit the teratoma

formation, authors have separated the undifferentiated cells, representing a limited contaminating population, from committed neural cells.

Hence, before using iPSCs in clinical trials, it is fundamental to evaluate their safety and their efficacy. Tsuji and coll [204] have already transplanted neurospheres obtained from iPSCs into injured murine spinal cords, showing a consistent functional recovery due to remyelination and serotoninergic fiber regrowth.

Finally a consistent advantage from iPSC use consists in that cells can be obtained directly from patients and expanded, eliminating graft rejection problems.

CO-TRANSPLANTATION OF DIFFERENT STEM CELL TYPES

As mentioned, every stem cell type entails both advantages and disadvantages: ESCs, even though totipotent, imply ethical problems and can give rise to tumors; on the other hand, adult stem cells are easily obtained from many tissues and are safe (e.g., MSCs are already employed in several clinical trial for hematological pathologies, heart/vascular diseases, osteogenesis imperfecta, amyotrophic lateral sclerosis), even though they are more restricted in their differentiation potential than ESCs [59,65].

A promising approach can consist in co-transplanting different stem cell types: in fact some authors have already demonstrated the synergistic effects obtained by combined graft of stem cells. For example, Zhang and coll. [231] co-transplanted in a rat transected spinal cord NSCs and Schwann cells transfected with adenoviral vectors carrying human NT-3, observing functional recovery due to axonal regeneration, remyelination and neuron survival. Similarly, Wang and coworkers [213] have employed a stem cell cocktail (NSCs and OECs) in a rat injured spinal cord, obtaining locomotor recovery, and NSC differentiation into neural cells and the regeneration of nerve fibers crossing the lesion site from OECs. Therefore, co-transplanted stem cells have a synergistic effect, mutually acting on neural regeneration and on behavioral recovery: indeed co-transplantation seems more effective than a single cell type graft. It is also possible to exploit the MSC characteristics, in terms of homing, delivery of trophic factors, immunosuppressive properties, to enhance the integration of other stem cells, such as neural/embryonic stem cells into the injured CNS.

Recently, MSCs have been injected with embryonic-derived oligodendrocyte progenitor cells (OPCs) in dysmyelinated mice, obtaining the enhancement of OPC survival and their oligodendroglial maturation, in addition to modulating neuroinflammation [43].

Proteomics of stem cells

Due to their enormous potential, stem cells are investigated in many molecular profiling studies in order to find new markers and regulatory pathways governing their self-renewal and differentiation. Neuroproteomics represents the most recent approach to study the nervous system proteome: in fact, techniques like microarrays, two-dimensional gel electrophoresis, mass spectrometry and protein arrays for proteomic analysis allow to monitor the changes in gene or protein expression of a variety of different molecules [11].

First of all, gene expression profiling stem cells at different stages of differentiation is very important to define and characterize the cells which are harvested, cultured, expanded and finally transplanted. Some "stemness" genes specifically expressed in ES cells and involved in maintaining pluripotency and self renewal, such as Oct-3/4, Sox-2 and UTF-1, can be identified [14]. On the other hand, Wang and Gao [211] identified 23 proteins with changing expression levels or phosphorylation states after neural differentiation of murine ESCs: in particular, translationally controlled tumor protein (TCTP) is downregulated and alpha-tubulin upregulated, suggesting a role of TCTP in modulating neural differentiation through binding to Ca(2+), tubulin and Na,K-ATPase. Differentiation of fetal NSCs into neural cells is accompanied by changes in the expression of proteins involved in DNA and RNA binding (hnRNPs A1, A2/B, and H), iron storage (Ferritin L subunit), redox regulation (Protein DJ-1), mRNA processing and transport (hnRNPH and hnRNP A1) [187]. Seven proteins increase their expression and one (HS90B, a tumor-specific transplantation antigen) decreases in the transition from ES cells to NS cells, whereas eight decrease their expression from NS cells to neurons [2]: therefore, protein synthesis and folding, oxidoreduction, signal transduction, and changes in the cytoskeleton are up-regulated in the transition from ES cells to NS cells, and protein synthesis and folding, oxidoreduction, and signal transduction are down-regulated in that from NS cells to neurons.

Proteomics can represent a resource for the treatment of neurodegenerative diseases. Transplanted stem cells and the injured environment following ischemia or nerve injury can be analysed by i) studying the expression of growth and neurotrophic factors secreted at injury site, such as insulin growth factor-1, VEGF-A, transforming growth factor-beta1, brain derived neurotrophic factor, stromal derived factor-1alpha and NGF, and ii) evaluating the possible interaction between stem cells and the environment [30,42].

Stem cells, either constitutionally or upon stimulation by the injured environment, can change their pattern of expression and secrete neurotrophic and/or immunomodulatory molecules. MSCs

display immunological, reparative and anti-inflammatory properties, making them a promising tool in approaches of regenerative cell therapy. MSCs are able to secrete several bioactive molecules with trophic (stem cell factor SCF, leukemia inhibitory factor LIF, macrophage colony-stimulating factor M-CSF, NGF, NT-3), immunomodulatory (prostaglandin E2 PGE-2, trasforming growth factor beta1 TGF-β1, LIF), anti-apoptotic and angiogenetic (VEGF, bFGF, interleukin 6 IL-6, insulin-like growth factor-1 IGF-1) properties, fundamental in leading to tissue repair at sites of injury [122,125,134,142]. In a microarray study, the global transcriptional profile of mouse MSCs put in evidence secreted proteins that play a role within the HSC niche such as fibronectin-1 (Fn1), osteopontin (Spp1), chemokine C-X-C motif ligand- 12 (Cxcl12), thrombospondin-1 (Thbs1), thrombospondin- 2 (Thbs2), transforming growth factor-β2 (Tgfb2), angiopoietin-1 (Angpt1), insulin-like growth factor binding protein-4 (Igfbp4), fibroblast growth factor-7 (Fgf7), secreted frizzled-related protein-1 (Sfrp1), secreted frizzled-related protein-2 (Sfrp2), dickkopf-3 (Dkk3), vascular cell adhesion molecule-1 (Vcam1), and bone morphogenetic protein receptor type 1a (Bmpr1a) [154]. Therefore, MSCs express a variety of genes, some of which they share with other cell types, and some are specific. The result is the expression of a cocktail of proteins that can modulate immune system, modulate microglia activation and astrogliosis, and provide trophic support for neuron survival and axonal growth.

Neuroprotection can be provided also by neural stem/progenitor cells: they can interfere with production of free radicals and increase the expression of neuroprotective factors by secretion of ciliary neurotrophic factor and VEGF [123,128]. Neural stem cells show also immunomodulatory potential, determining a bystander inhibitory effect on T cell activation and proliferation in lymph nodes [12].

Finally embryonic stem cells are able to delivery several factors (hepatocyte growth factor, TGF beta, BDNF) that can act both locally and systemically, promoting biological repair and regeneration; moreover, when transplanted, they can establish synergistic interactions with endogenous adult stem cells, enhancing in this way tissue regeneration [74,117,119,230].

Indeed, the study of the reciprocal influences of the lesioned environment and the transplanted stem cells at a proteomic level is drawing an increasing interest: the functional roles of neuroproteoma can represent a powerful tool in regenerative medicine.

Usage of genetically-modified stem cells

Genetically-modified cells include fibroblasts, Schwann cells, macrophages, ES cells, OECs, MSCs and NSCs: i.e. all cell types tested for cell therapy have been genetically modified to enhance their therapeutic potential. For genetic modification, retroviral vectors provide a stable and safe means to

modify cells to express high levels of neurotrophic factors without the expression of wild-type viral genes [17].

Gene therapy can provide injured axons and neurons with a local source of trophic molecules, to stimulate neuronal survival and possibly axonal growth [17]. The expression of receptors for neurotrophic factors and their pleiotropic effects on different neuronal and glial cell types makes it necessary to target trophic molecules to either a specific subpopulation of neurons or to the injury site itself and its immediate proximity.

NT-3-genetically modified-cells grafted into the lesioned spinal cord provide not only trophic molecules at the injury site, but also support axonal growth: moreover, they can provide "bridges" for growing axons to potentially connect injured spinal cord "stumps" [63].

Genetically modified cells producing BDNF and/or neurotrophin (NT)-4/5 have been investigated in several studies. After midthoracic dorsal hemisection lesions, increased growth of primary sensory, noradrenergic coerulospinal and motor axons into BDNF- and NT-4/5-secreting fibroblast grafts was observed. BDNF-mediated growth responses of sensory and cholinergic axons were also found after midthoracic contusion injuries [135].

Growth of raphespinal and coerulospinal axons following BDNF delivery have also been reported but its extent varies from study to study, perhaps depending on differences in the type or site of spinal cord injury, in the quality and extent of BDNF delivery, and in the cells which vehicle BDNF gene delivery [135]. Following complete transection at the midthoracic spinal cord, placement of Schwann cell grafts and infusions of BDNF and NT-3 induced some growth of coerulospinal and raphespinal axons [224]. Similar results were obtained using grafts of Schwann cells genetically modified to secrete BDNF [135]. Growth factors can also serve to coax sprouting axons to cross the gap across the lesion. Once regeneration is stimulated, molecules that provide directional cues are critical for routing axons to their correct targets [178].

When different growth factors were used in combination (for example BDNF and GDNF), they resulted in significant reduction of motor dysfunction and spinal cord pathology. However, transplantation of fibroblasts genetically modified to produce neurotrophic factors BDNF or NT-3 onto rat spinal lesion did not show advantage in sensorimotor recovery to either grafting with gelfoam or gelfoam plus fibroblasts, in addition to increase termal hyperalgesia [77,102,167].

Genetically engineered fibroblasts secreting NGF can stimulate axons to regenerate into the center of an injury when implanted in this region [87,91,206], although this sprouting remains within the area of trophic support and does not lead to long distance functional regeneration. Intrathecally

delivered NT-3, NGF, and GDNF can promote axonal regeneration across the dorsal root entry zone [158,159], and glial overexpression of NGF can stimulate regeneration of nociceptive axons from the dorsal roots which in some circumstances may lead to thermal hyperalgesia [165,198].

More recently, in order to achieve axonal growth, Hamada's group (2006) has transfected ES cells with the MASH1 gene, yielding purified spinal motoneuron precursors deficient in the expression of Nogo receptor: after graft on spinal cord injured mice, animals showed excellent improvement of the motor functions, confirmed by electrophysiological studies.

Moreover, combination therapy such as treatment with NT-3 and cAMP together to stimulate neuronal cell bodies has been shown to allow regeneration beyond the lesion site of spinal injuries [120], suggesting that the use of multiple simultaneous approaches to this complex problem may prove important when constructing future strategies for improving rehabilitation and functional recovery.

Implantable scaffolds

As discussed above, the glial scar represents the main barrier to axon regeneration at the injury site [186]. Even though stem cells can penetrate the lesion cavity and establish a permissive environment to axonal growth, this potential is limited by many other inhibitory factors [129,177]. An innovative means for delivering stem cells to the injury site and promoting axonal growth consists in seeding stem cells into scaffolds which support regenerating axons.

A variety of materials have been evaluated to this aim [124]. One consists in fibrin, due to its biocompatibility, biodegradability, flexibility and plasticity [201]. Alone, fibrin promotes regeneration and delays accumulation of reactive astrocytes at the lesion site [86]. Alternatively, fibrin, enriched with stem cells - usually bone marrow stem cells (BMSCs) or NP - and/or growth factors – such as NT-3, NGF, platelet-derived growth factor-, improves survival, differentiation and migration of grafted cells, as well as it determines an increase in neural fiber density [82,85,234]. Others natural polymers find extensive use in SCI treatment, such as collagen and hyaluronic acid, due to their elasticity, support to cell adhesion and migration, and time of degradation: in fact, the implantation of these scaffolds with or without stem cells creates a favourable environment for nerve regeneration, significantly improving the recovery of locomotor and sensory functions [152,212]. NVR-N-Gel (biodegradable co-polymer neurotube containing viscous gel) is a crosslinked hyaluronic acid with the adhesive molecule laminin, and growth factors [164]. Time of degradation is important in order to be progressively replaced by the matrix produced from transplanted and host cells. When drug or neurotrophic factors were loaded in a polymer nanocarrier: this system can reduce neuroinflammation

in acute stage [36] and support the axonal outgrowth and the synaptic reconnection in the delayed phase [192].

In alternative to natural scaffolds, synthetic polymers allow a wider control of chemical and physical characteristics of the material, even succeeding to mimic the structure of white and grey matter found in the uninjured spinal cord. They consist of biodegradable hydrogels (polyester of lactic acid - PLA, polyester of glycolic acid - PGA and polyethylene glycol - PEG) or non-biodegradable hydrogels, methacrylate-based. The delivery of NSCs and Schwann cells via PGA scaffold enhances their growth promoting properties on axons across the transected spinal cord [149]. These results have been confirmed both by histological analysis in terms of reduction of lost tissue and diminished glial scarring, and behavioral assessment in terms of coordinated, weight-bearing and hindlimb stepping [200]. Moreover, other promising scaffolds such as poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) and HPMA-RGD hydrogels [N-(2-hydroxypropyl)-methacrylamide with attached amino acid sequences-Arg-Gly-Asp], seeded with MSCs, have obtained positive results in SCI [37,73].

Finally, as reviewed by Madigan [124], the scaffolds seem to support stem cell differentiation, especially fibrin scaffolds. Therefore, combining scaffolds with cell transplantation represents a promising alternative to injection methods, since such scaffolds not only support regeneration but also enhance cell survival after transplantation and promote differentiation into desired phenotypes. On the other hand, non-biodegradable biomaterial-based treatment can cause a chronic compression of regenerating axons, and the transplant site can act as a sink, precluding sprouting to escape and reconnect with the host spinal cord.

Clinical Trials

Most in vivo studies relative to therapy of SCI are performed in rodents. There are, however, many species-specificities when compared to humans, for example in the anatomy of motor pathways and behavior. Therefore, although preliminary studies in animals are necessary and fundamental, research on SCI should aim to trials in humans.

In a clinical trial in 2004, intravenous injection of autologous BMSCs resulted in a significant improvement, from American Spinal Injury Association score B to D, in only one over 9 SCI patients [160]; however, BMSCs were well tolerated during the observation period. A phase I study established

safety in acute complete SCI: of 8 enrolled ASIA A patients, 2 improved to ASIA C after 6 months and 3 to ASIA C after 1 year, with some bladder recovery [97]. In the first cell-based therapy for acute complete SCI multicenter randomized controlled phase II study (ProCord), enrolling only patients with complete SCI, treated macrophages promoted recovery from SCI [97]. Phase II was stopped when Proneuron Biotechnologies, Inc. (Los Angeles, CA), the parent company, found that most patients had undergone surgery twice, a first time for biomechanical stabilization, and a second, approximately 2 weeks postinjury, concomitantly with macrophage injection. In addition, surgeons had some difficulties to accurately identify the border of the lesion where activated macrophages were to be injected.

It has also been suggested that delivery of autologous BM precursors via lumbar puncture or activated macrophages into the CNS might be an acceptable alternative to the intravenous administration [5,27,97]. A phase I/II nonrandomized study, using autologous BMCs injection into the perilesional area at different time points post injury (within 14 d, between 15 d and 8 wk, and after 8 wk) in 35 patients with complete SCI, combined with administration of GMCS-F (granulocyte macrophage-colony stimulating factor), obtained an improvement in the American Spinal Injury Association Impairment Scale (AIS) impairment scale grade in 30% of patients (from A to B or C) compared to controls [227]. Whereas no side effects at a 10 month follow-up are reported, neuropathic pain during the treatment and tumor formation at the site of transplantation still remain to be investigated in the long term [227].

Other studies tested the inflammatory microenvironment before stem cell administration. Patients first underwent intravenous injection of autoimmune T cells. NSCs, trandifferentiated from BMSC in culture with autologous autoimmune T cells, were injected into the lesion site of two patients with chronic SCI, demonstrating some level of motor and sensory recovery [139]. In another preliminary safety trial in 2006, safety, feasibility, and good tolerance of HSC transplantation via lumbar puncture were demonstrated in a group of 10 patients [139].

Finally, olfactory mucosa autografts into the SCI lesion succeeded to fill the site and trigger some functional recovery [112]. In another study [80], olfactory bulb derived from aborted fetuses were transplanted, but the short follow up precluded any conclusion.

Clinical trials basically showed the safety of the transplantation procedures. The benefit of cell replacement therapy however can only be established by further clinical phase II/III trials. Actually, the variable and sometimes inconclusive findings from human trials would argue that a better understanding of the biological mechanisms and potential of cell replacement therapies in animal injury models must be obtained before clinical translation. Even a stem cell-based therapeutic strategy

eventually successful in clinical trials still faces practical issues to be translated to the clinical practice. It must be made easily accessible for hospitals, and this ideally includes the availability of cell banks and the possibility to distribute stem cells. This is an exciting but not immediately actual scenario. Regulatory organizations such as Food and Drug Administration will guide the translation of stem cells therapy for SCI in animals to human trials. Companies, researchers, patients should be aware of the regulatory issues that must be addressed before starting human experimental trials, because of some special concerns: a) the spinal cord is a site sensitive and dangerous, b) stem cell therapy is a new treatment and entails a number of complex issues; such as toxicities, risk for tumorigenicity, interactions of cells with drugs, c) clinical deficit could be worsened by inappropriate stem cell differentiation. Operating procedures must be compiled to standardize: a) the inclusion criteria and potential benefits of participating in the trials with stem cells, since patients have done all therapeutic alternatives; b) informed consent; c) the methods in preparing and delivering cells; d) equipment and surgical approach; e) evaluation in the follow-up.

CONCLUSION

Although we may never be able to "regrow" large areas of spinal cord injured, stem cell technology is a rapidly evolving field that will impact the future treatment of SCI, exploiting both limited CNS neurogenesis and exogenous stem cells complementary advantages. Transplantation of stem cells represents an important new approach to managing SCI. Improvements in molecular and microscopic techniques along with the availability of modified stem cell lines have accelerated research into stem cell transplantation. The fact that functional gains have been demonstrated in animal models after delivery of cells of both neural and non-neural origin in preclinical models of CNS injury is encouraging. Nevertheless, caution is necessary to ensure the highest standards of safety and scientific method as this exciting field moves forward. Cell-based therapy in SCI had the goal to: 1) replace new neurons that die within the first minutes to days after injury; 2) provide a source of cells to promote remyelination and axonal sprouting; and 3) delivery trophic molecules that can promote cellular protection and plasticity.

In order to apply these techniques, however, it is critical that we continuously consider that patients might benefit from additional treatment. Work from multiple labs is suggesting that the cells microenvironment is important in establishing the sequence of what cells will and will not do,

particularly in vitro systems. With this combination of expertise, and with important links to national resources, the hope is to quickly translate basic science studies into clinical trials, avoiding the usual roadblock between the two areas of endeavor.

LEGENDS TO THE FIGURES

FIGURE 1

(a) After compression injury and transplantation, neural precursors (in green) are able to fill the lesion cavity, here well highlighted by glial fibrillary acidic protein (GFAP) immunostaining (in red). **(b)** In vitro neural precursor display heterogeneous morphology, frequently showing more or less elongated processes. **(c)** Also in vivo, when transplanted 2 weeks after hemisection injury, NPs are distributed in clusters, display variable aspect and emit processes of different lengths directed caudally and laterally with synaptic boutons in contact with local neurons (labeled in red labeled with anti-5HT antibody). Scale bar = 200 μm in **a,** 50 μm in **b,** 100 μm in **c**.

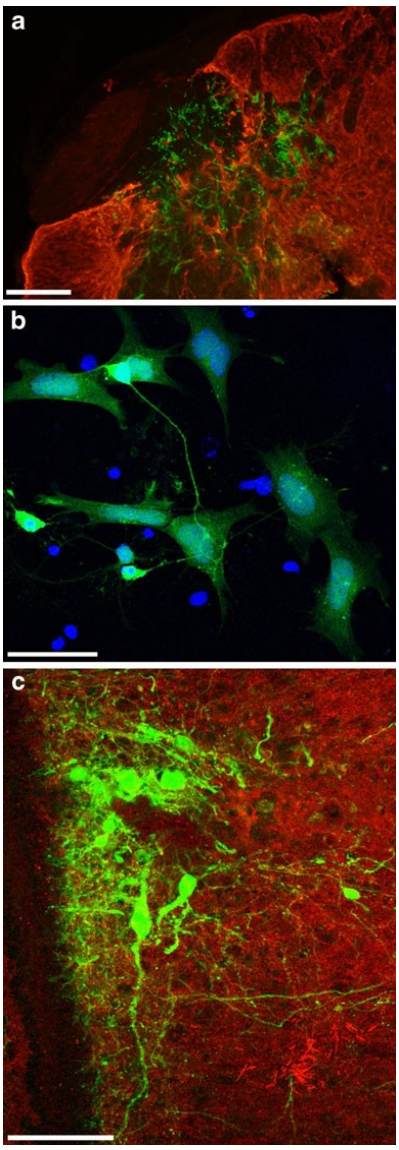
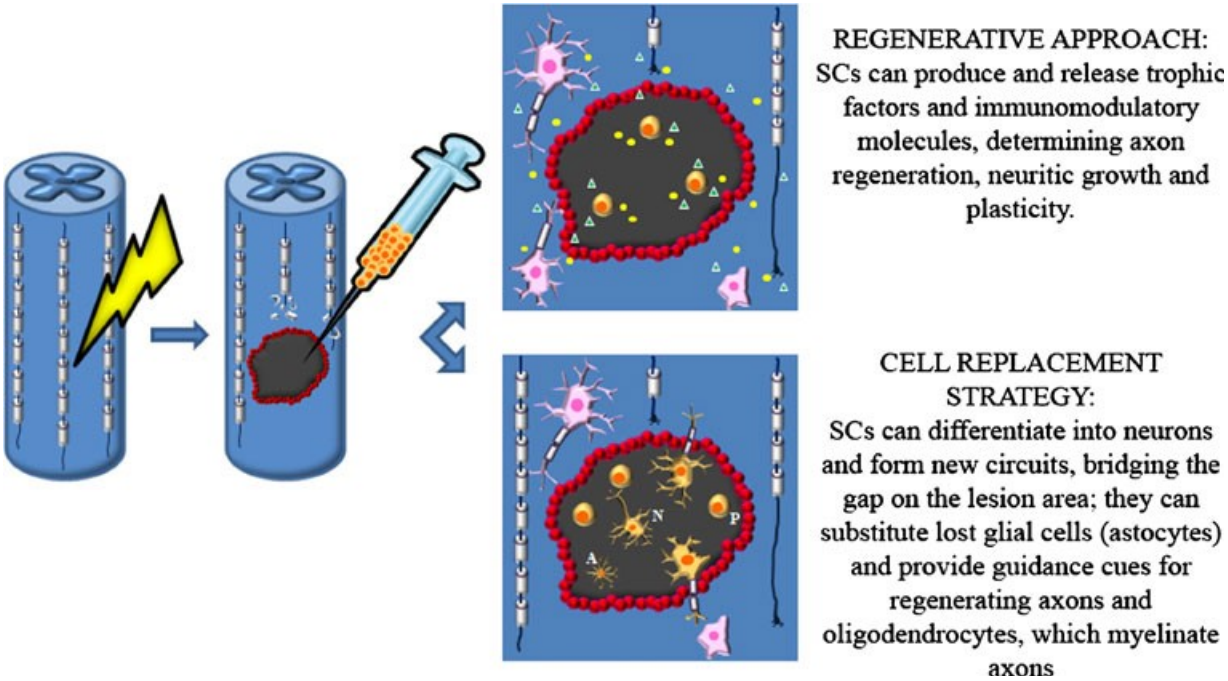


FIGURE 2

Regenerative approach vs cell replacement strategy.

Following an injury, spinal cord regeneration can be supported by stem cell transplantation. In particular, stem cells can stimulate a regenerative process, by delivering trophic factors (yellow dots) and immunomodulatory molecules (green triangles), which can enhance axon sprouting [see host surviving motoneurons (pink) with axonal growth cones]. Alternatively, stem cells can differentiate into neurons (N) and integrate into the host circuits, emitting axons able to bridge the lesion gap; moreover they can integrate the remaining functional glia, for example differentiating into astrocytes (A), or they may remain as undifferentiated precursors (P).



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