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Chapter 6

Culturing Adult Stem Cells for Cell-Based Therapeutics: Neuroimmune Applications

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Additional information is available at the end of the chapter

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Abstract

Pluripotent stem cells can be successfully isolated from a variety of tissues from adult organisms. This fact opens the exciting possibility of cell-based therapies for a large number of clinical treatments. However, the development of optimized protocols to obtain, grow, and cryopreserve cells, as well as that of effective clinical treatment procedures, is no easy task. The therapeutic potential of cells expanded *in vitro* depends on a multitude of factors including isolation procedures, donor and tissue types, expansion and preservation methods, etc. Researchers are investing great efforts to determine which of these many variables significantly impact downstream performance of *in vitro* expanded stem cells by studying associated changes in molecular profiles and their effect on the host immune system. This chapter reviews the current status of stem cell production and its derivatives, which are paving the way to different treatments in the clinic. Due to the research interests of our labs, particular emphasis is placed on the potential benefits of stem cell-based therapeutics for the treatment of spinal cord injuries and the neuroimmune disease myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) not only derived from differentiation and cell engraftment mechanisms but also due to the anti-inflammatory and immunoregulatory capacities of these cells.

Keywords: mesenchymal stem cell (MSC), induced pluripotent stem cell (iPSC), spinal cord injury (SCI), myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), extracellular vesicle (EV)

1. Introduction

Stem cells present particular characteristics that make them different from other cell types. Firstly, they are unspecialized self-renewing tissue resident cells, and secondly they can be

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induced to differentiate into a milieu of specialized cell types, thus holding promise for regenerative medicine. When these cells are isolated from adult fully differentiated tissues, they receive the attribute of adult stem cells, even though they are also present in infants and fetus. Therefore, it would be more appropriate to refer to them as tissue stem cells or mesenchymal stem cells to differentiate them from resident progenitors with limited differentiation capacity. MSCs can be isolated from a large number of tissues, such as bone marrow, adipose tissue, dental pulp, hair follicles, amniotic fluid, Wharton's jelly in the umbilical cord, and even from nervous or cardiac tissue. MSCs are multipotent and can be differentiated into chondrocytes, adipocytes, and osteoblasts under proper conditions [1, 2]. MSCs can be cloned and expanded *in vitro* more than a million fold without losing their differentiation potential [3] constituting, theoretically, a rich resource for tissue repair. However, their sensitivity to environmental cues and genetic factors together with a lack of standardized good manufacturing procedures (GMPs) using defined components has hampered their true therapeutic potential. Since the finding by Bartholomew et al. that MSCs inhibit mixed lymphocyte reactions and prevent the rejection of allogeneic skin grafts [4], a large number of reports have evidenced that MSCs are immunosuppressive and immunoregulatory, properties that can be harnessed therapeutically. However, challenges to fully understand and control MSC regenerative potential remain.

In addition to MSC, the reprograming of terminally differentiated cells or induction of de-differentiation by the introduction of particular sets of transcription factors [5–7] opened an additional avenue of opportunities in the field of regenerative medicine. iPScs or induced pluripotent stem cells facilitate the production of patient-specific cells overcoming immune rejection and also ethical concerns. Although they have shown their value in the generation of *in vitro* models of human disease [8, 9], the low efficiency of reprogramming events and the safety concerns associated with the process of reprogramming has prevented their use in the clinic [6, 10].

Based on the research interests of our labs, this chapter, while reviewing the advances to generate clinical-grade stem cells or their by-products, highlights the potential benefits of stem cell-based therapeutics for the treatment of spinal cord injuries (SCI) and the neuroimmune disease myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS).

2. Stem cell therapy for spinal cord repair

2.1. Spinal cord injury pathological events and timing sequence

Spinal cord injury (SCI) is often mentioned among the first conditions for which stem cells may provide a new therapy. While recent decades have brought significant improvements in rescuing neuronal activity after SCI at preclinical phases testing several individual approaches, translation to the clinic still remains inefficiently explored. Management for SCI efficient treatment is a difficult task by the intrinsic nature of the pathological cascade of events that makes the SCI a dynamic and progressive disorder. The sentence "Time is spine" defines the crucial importance of timing to rapidly diagnose patients and implement neuroprotective interventions during the acute injury phase (≤2 h) in order to diminish the devastating effects of the secondary phase of the injury (≥2–48 h) which are known to be key determinants of the final extent of neurological deficits. The secondary injury leads to necrosis and/or apoptosis of neurons and glial cells, such as oligodendrocytes, which can lead to demyelination and

the loss of neural circuits. Later, in a subacute phase (2–4 days after injury), further ischemia occurs owing to ongoing edema, vessel thrombosis, and vasospasm. Persistent inflammatory cell infiltration causes further cell death and formation of very toxic cystic microcavities over time. Astrocytes, fibroblast, and pericytes proliferate and deposit extracellular matrix molecules into the perilesional area in the already intermediate and chronic phases, few weeks after SCI, when axons continue degenerating (**Figure 1a**) [11].

Spontaneous regeneration during and after having reached the chronic stage occurs due to the neuroplasticity capacity of the central nervous system (CNS); however, very limited gain of function is obtained decreasing advancing age, attributed to both extrinsic and intrinsic factors that modulate further onset, severity, and progression of the injury [12]. The cumulative myelin-associated protein anchorage to myelin sheet debris, in and around the epicenter of the injury, has a strong inhibitory nature. Nogo-A (reticulon-4 isoform A) and myelin-associated glycoprotein (MAG), among other myelin-associated proteins, bind to NOGO receptors to activate the GTPase Rho A, which activates Rho-associated protein kinase (ROCK), a regulator of further downstream effectors, leading to apoptosis and growth-cone collapse of regenerating axons involving neurite retraction [13–17]. Additional external barriers are potently adding to the inhibition of regeneration like the hypertrophic astrocytes and the reactive chemical scar with a number of axonal growth inhibitory chondroitin sulfate proteoglycans (CSPGs) [18].

The chronic SCI repair demands an intensive effort to overcome the impediments and enhance the intrinsic axon regeneration involving an efficient anatomical reorganization [19, 20]. Fortunately, although long distances for axonal reconnection or spared degenerated tracts are normally required, involving a long-term process (a rate of 1 mm/month for axon growth is estimated), it has been shown that as little as 10% of particular tracts can subserve substantial function [19, 20]. This in fact allows hypothesizing for a real recovery, mediated by bridging and partially reconnecting the spared axons allowing subsequent plasticity. Additionally, both, humans and rats, can regain a degree of function after incomplete injury, thought to be mostly due to local structural rearrangements, such as collateral sprouting from remaining axons in the gray matter, rather than by long-distance regeneration of axons in the white matter (**Figure 1b**) [21].

2.2. Stem cell therapy for SCI repair

Cell transplantation methods constitute a very promising strategy for SCI repair. Numerous studies with a diversity of cell types have clearly showed benefits to different extents, for

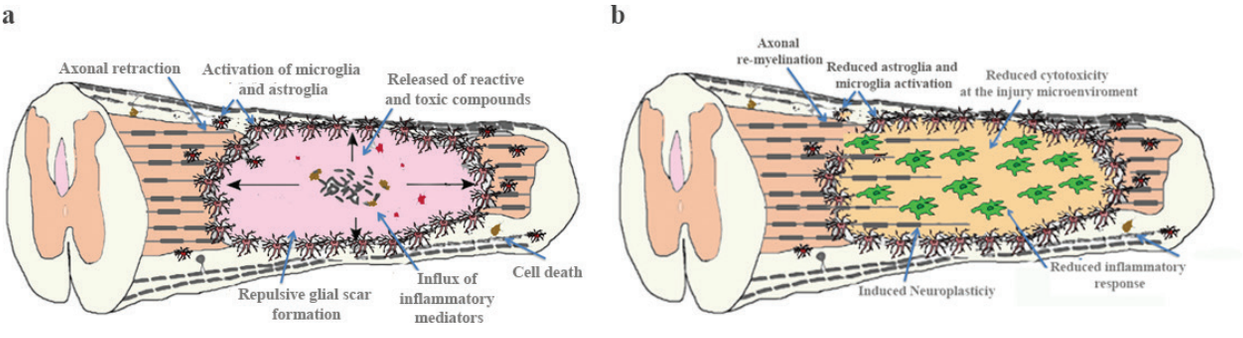


Figure 1. Summary of physiopathological events after SCI (a) and stem cell transplantation (b).

instance, therapeutic effects in sensorimotor function recovery in preclinical models [22, 23] and in several ongoing clinical trials [24–26].

Among these cell types used, mesenchymal stem cells (MSCs) from the adipose tissue, bone marrow [27], umbilical cord, [28] or dental pulp [29], in addition to olfactory ensheathing glial (OEG) cells [30], Schwann cells [31], or neural precursor cells [32], have been used.

Preclinical studies have shown that MSCs have potent anti-inflammatory, anti-apoptotic, immunomodulatory, and angiogenic effects post-SCI [33]. MSC transplantation, overall, results in substantially improved locomotor recovery among animal models of SCI [34]. There have also been several clinical trials using autologous bone marrow-derived MSCs. These early studies confirm the safety of different administration protocols using MSCs post-SCI. It seems that sufficient quantities of transplanted allogeneic MSCs combined with immunosuppression prolong the survival of engrafted cells and improve functional and morphological outcomes after SCI [35].

Transplantation of neural stem/progenitor cells (NSPCs) has shown promising results in the repair and regeneration of lost neural tissues and the associated restoration of neurological deficits [36] with particular benefits among other cell types. The engrafted transplanted NSPCs generate a favorable non-inhibitory environment for functional recovery creating additional paracrine activity modulating the post-SCI inflammatory response, feeding the injured area with growth factors, and rendering additional neurotrophic support by releasing, among others, GDNF. NSPCs include multipotent stem cells present in the ependymal region lining the central canal of the spinal cord (epSPC) [37]. epSPC represents an ideal candidate for stem cell therapy based on noted functional improvements after transplantation and the absence of malignant transformation, offering a safe and relevant cell type for clinical applications. The rationale for the therapeutic application of epSPC for SCI includes the replacement of damaged neurons and glial cells, secretion of trophic factors, regulation of gliosis and scar formation, prevention of cyst formation, and enhancement of axon elongation. After SCI, epSPCs proliferate and migrate to the injured area and produce new oligodendrocyte precursor cells (OPCs) [37]. Acute [38] and chronic [39] transplantation of undifferentiated epSPCs from SCI donors or *in vitro* differentiated OPCs into a rat model of severe spinal cord contusion produced significant locomotion recovery 1 week after injury. Transplantation of epSPCs provides trophic support and positively modulates the local immune response. It reduces purinergic receptor expression associated with neurodegenerative and neuropathic pain, thereby inducing signals promoting neuronal protection and survival with axonal outgrowth [40]. Interestingly, an immortalized human fetal epSPC line (HuCNS-SC) has been the main focus of cell therapies developed by the company Neuralstem, Inc. (USA), and therapies based on this product have been applied to human subjects in a phase II clinical trial. Phase I/II trial has declared no adverse effects of treatment, with modest functional improvements [41].

Stem cell therapy can contribute to SCI repair not only by restoring the damaged tissue through differentiation and engraftment but also by potentiating endogenous tissue regenerative potential. Both processes are influenced by the action of the immune system controlling local inflammation, and thus stem cell paracrine factor role in this context should be carefully evaluated.

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3. Stem cell therapy for other neuroimmune-related health problems: potential benefits for the treatment of myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS)

Mesenchymal stromal cells (MSCs) have been used in clinical trials (CTs) for a broad range of immune-related health problems such as acute and chronic inflammatory disorders, autoimmune diseases, and transplant rejection by their potent immunosuppressive and anti-inflammatory properties [42–45]. As reviewed by Wang et al., as of April 2016, over 500 MSC-related clinical trials were registered on the NIH clinical trial database (https://clinicaltrials.gov/). Although the immunomodulatory properties of MSCs have more recently been identified, almost half of the registered CTs (230 or 42% of them) have or are being conducted for immune- or inflammation-mediated diseases (see **Figure 2**) [45].

Multiple sclerosis (MS) and its animal model (experimental autoimmune encephalomyelitis or EAE) associate with CNS inflammation, gliosis, demyelination, and axonal loss. MSCs' pleiotropic properties, including immunomodulation, immunosuppression, neurotrophy, and repair-promotion, make them attractive candidates for the treatment of neurodegenerative diseases, including MS [42–46]. The remyelination benefits reported in MS are largely attributed to paracrine signals and secreted soluble molecules such as tumor growth factor (TGF-β1), interferon (INF)-γ, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2) [46–48]. On another side, neural precursors obtained from induced pluripotent stem cells (iPSCs) promote the viability of endogenous OPCs facilitating remyelination through the secretion of leukemia inhibitory factor (LIF) in EAE [46, 49–51]. LIF, a member of the IL-6 cytokine family implicated in the pathophysiology of MS, has shown to offer neuroprotection and axonal regeneration as well as prevention of demyelination [49–51].

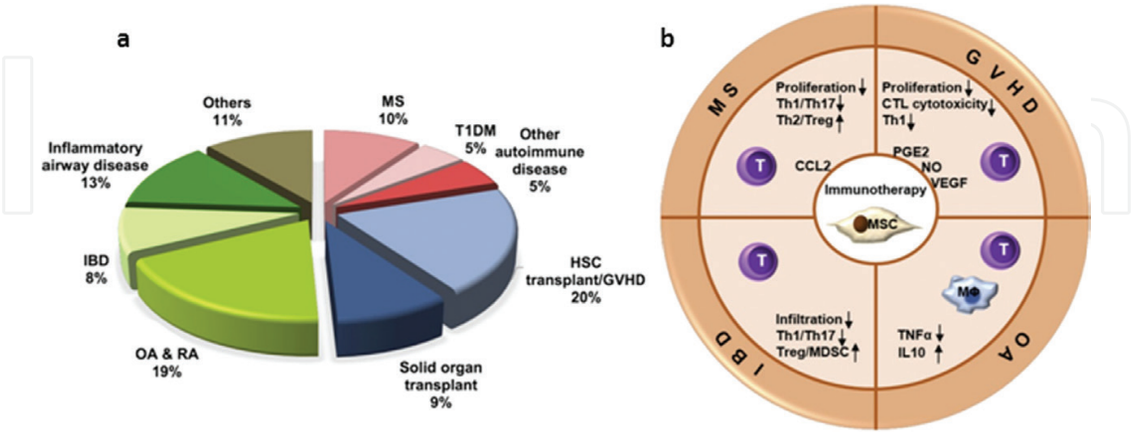


Figure 2. Summary of the number of clinical trials using MSC therapy in immune- or inflammation-mediated diseases, as registered on the website https://clinicaltrials.gov (accessed April 2016). MS, multiple sclerosis; T1DM, type 1 diabetes mellitus; GVHD, graft-versus-host disease; OA, osteoarthritis; IBD, inflammatory bowel disease (a). MSC-derived paracrine factors mediating immunomodulatory functions, particularly toward T lymphocytes, in preclinical animal studies of various immune- and inflammation-mediated diseases (b). Source: Wang et al. [45].

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex, multiorgan system disease, often devastating, for which no single diagnostic test yet exists. The diagnosis of ME/CFS is based on exclusion, meaning other medical conditions, including psychiatric disorders, must be first ruled out. The disease is characterized by profound fatigue and disability lasting for at least 6 months, episodes of cognitive dysfunction, sleep disturbance, autonomic abnormalities, chronic or intermittent pain syndromes, microbiome abnormalities [52], cerebral cytokine dysregulation [53, 54], natural killer cell dysfunction [55], and other symptoms that are made worse by exertion of any kind [56, 57]. The Institute of Medicine (IOM) recently published an update of the diagnostic criteria recommended for CFS [56, 57]. The estimated worldwide prevalence of ME/CFS is 0.4–1%. The disease predominantly affects young adults, with a peak age of onset of between 20 and 40 years, and women, with a female-to-male ratio of 6:1 [58]. Although the etiological agent of ME/CFS remains unknown, the many hypotheses raised based on patient testimonies and clinical observations seem to lead to pathological immune system malfunctioning as one major factor. Autoimmune features on one side [59] and latent infection of unknown microorganisms, with a chronically activated immune system leading to inflammatory type situations, on another [60] have led our group to propose that stem cell-based therapeutics, as evidenced for MS, might be of benefit to these patients as well. The World Health Organization (WHO) has classified ME/CFS as a neurological disorder (International Classification of Diseases, Tenth Revision, Clinical Modification or ICD-10-CM R53.82; G93.3 if post-viral) based on the cognitive and other neurologic associated symptoms these patients suffer from. The neurological symptoms, however, could be explained by microglial activation and the lower-than-normal production of cortisol and adrenocorticotropic hormone (ACTH) these patients show, causing serotonin and corticotropin (CRH) deregulation [61]. A decrease in cortisol production by adrenal glands in turn can influence immune system activity [62]. MSC therapeutics could, at least partially, restore normal immune and, perhaps, neural functioning. Preclinical safety studies, however, should precede CT in ME/CFS.

4. Current protocols for stem cell-based therapeutics

4.1. Mesenchymal stem cells (MSCs)

MSCs do not express major histocompatibility complex class I or II, permitting adoptive transfer between hosts without triggering acute rejection. In 2006, the International Society for Cellular Therapy (ISCT) established minimal criteria to define human MSCs as follows: MSC must be plastic-adherent when maintained in standard culture conditions; MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules; and MSC must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [63, 64]. The latter position paper adds to the original characterization criteria viability and proliferation features [64]. Cells that fulfill these criteria can be isolated from different sources (such as fat, bone marrow, umbilical cord blood, dental pulp, etc.), but tissue source, donor's age, extent, and conditions of *in vitro* expansion, among others, are known to influence the regenerative potential based on engraftment, paracrine effects, and differentiation capacity of these cells [65]. Freeze-thawing effects on the whole genome expression profile of MSC have been observed, although they did not exceed inter-donor differences [66]. This high inherent heterogeneity of MSCs remains a challenge for data

harmonization, particularly across lab comparisons. Despite this limitation, consensus good manufacturing procedures (cGMP) for large-scale clinical-grade MSC have been developed [67–69], based on original low-scale lab preparation methods consisting of tissue trimming, enzyme-based (collagenase) dissociation, cell filtration, and cell-type selection through adherence to plastic and extended survival *in vitro* [70, 71].

Although the numbers of CT with MSCs are already considerable and increasing, only 13 human MSC-based products count with marketing authorization. As shown in **Table 1**, nine are developed for allogeneic therapies and only four for autologous. The main source for MSC manufacturing is the bone marrow, followed by adipose tissue, although others such as umbilical cord, cord blood, placental tissue, and Wharton's jelly are being explored. However, as ASCs (adipose stromal stem cells) possess similar therapeutic potential other than bone marrow MSCs as described by the ISCT and the International Federation of Adipose Therapeutics and Science (IFATS), and since they are obtained by minimally invasive procedures from a generally undesired tissue, the fat, they may shortly become the main choice of adult stem cells for clinical applications. In fact, as reported by Nordberg and Loboa, clinical trials using ASC raised from 18 to 152 in less than 5 years (2010 to the first quarter of 2015) [72]. Standard procedures based on single-use bioreactors yield superior quantities and quality of cells when compared to traditional planar multilayer cultivation systems, such as CELLstack, HYPERStack, and CellFactories (Corning, Nalge) [67].

Efficient manufacture of MSC-based products also takes costs into account. Either allogeneic or autologous therapies involve cGMP upstream processing (USP) through master and working cell banks (MCB and WCB, respectively) and downstream processing (DSP) events, a summary of which are shown in **Figure 3**.

These manufacturing processes are tightly regulated by the Advanced Therapeutic Medicinal Product (ATMP) path [73], the European Medicines Agency (EMA) in Europe, the Center for Biologics Evaluation and Research/Food and Drug Administration (FDA) in the USA, and the Central Drugs Standard Control Organization in Asia (readers are directed to selected reviews for further legal regulatory details) [67, 73, 74].

A potential formulation to standardize cell source has been proposed by Yi et al. who using GMPs could expand clonal MSCs from a single colony-forming unit (CFU)-derived colonies derived from a small amount of bone marrow to treat a number of patients [75].

Typically, the conventional media used for clinical production of MSCs are the common, defined Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium (MEM) basal media supplemented with 10–20% fetal bovine serum (FBS), due to limitations of human alternatives and to cost reasons, although FBS is not cGMP compliant. FBS is prone to batch-to-batch variation and to contamination with prions, viral and zoonotic agents [76]. Thus, most clinical trials (phases I to III) used ASCs or other MSCs produced in the presence of FBS, some of them reporting immunogenic effects in patients, elicited by components of FBS (antibodies against components of FBS, Arthus, and anaphylactic reactions) [77–79]. In addition, the immune responses elicited by FBS could turn into rejection of the transplanted cells in cell-based therapies restricting their therapeutic efficacy. FBS-free alternatives can be basically grouped into serum-free (SF) medium containing animal-derived or human serum albumin and growth factors (GFs). Among human alternatives, the use of autologous products obviates the need for infectious or other pathological agent testing but limits the

Medicinal product	Company	hMSC type	Indication	Marketing authorization
Allostem	AlloSource	Allogeneic ASC	Bone regeneration	US medical device
Cartistem	Medipost	Allogeneic UCB-MSC	Osteoarthritis	Korea
Grafix	Osiris Therapeutics	Allogeneic BM-MSC	Soft tissue defects	US medical device
Prochymal	Mesoblast	Allogeneic BM-MSC	Graft-versus-host disease	Canada and New Zealand
OsteoCel	NuVasive	Allogeneic BM-MSC	Spinal bone regeneration	US medical device
OvationOS	Osiris Therapeutics	Allogeneic BM-MSC	Bone regeneration	US medical device
TEMCELL HS	JCR Pharmaceuticals	Allogeneic BM-MSC	Graft-versus-host disease	Japan
Trinity Evolution	Orthofix	Allogeneic BM-MSC	Bone regeneration	US medical device
Trinity Elite	Orthofix	Allogeneic BM-MSC	Bone regeneration	US medical device
Hearticellgram-AMI	Pharmicell	Autologous BM-MSC	Acute myocardial infarction	Korea
Cupistem	Anterogen	Autologous ASC	Crohn's fistula	Korea
QueenCell	Anterogen	Autologous ASC	Regeneration of subcutaneous adipose tissue	Korea
Ossron	RMS	Autologous BM-MSC	Bone regeneration	Korea

Source: Adapted from Jossen et al. [67]. ASC human adipose tissue-derived stromal/stem cells, BM-MSC human bone marrow-derived mesenchymal stem cells, and UCB-MSC umbilical cord-derived mesenchymal stem cells.

Table 1. MSC-based products with marketing authorization for allogeneic and autologous therapies

production to few doses. The allogeneic alternative permits larger cell production by pooling samples from different donors but requires pathological agent screenings. Human derivatives show improved proliferation when compared to FBS-supplemented media reducing the time for cell expansion and lowering threats of senescence and transformation; however, human serum seems to limit osteogenic differentiation [63, 64, 80], and human platelet-poor plasma limits chondrogenesis [81, 82], while human platelet-rich plasma or platelet lysate preserves trilineage differentiation [83–85]. In addition, human platelet lysate, obtained by temperature-shock protocols (freezing platelets from −30 to −80°C during 24 h followed by a thawing a centrifugation step), can be prepared from banked blood with 4 or 5 days passed expiration date [86], making platelet lysate a preferable choice. To avoid MSC senescence, forced expression of telomerase reverse transcriptase (TERT) has been tried [86, 87]; however, nongenetic manipulations will be more suitable for clinical translation. On another side, the serine/threonine kinase AKT activation by plasma rich in growth factors leads to enhanced

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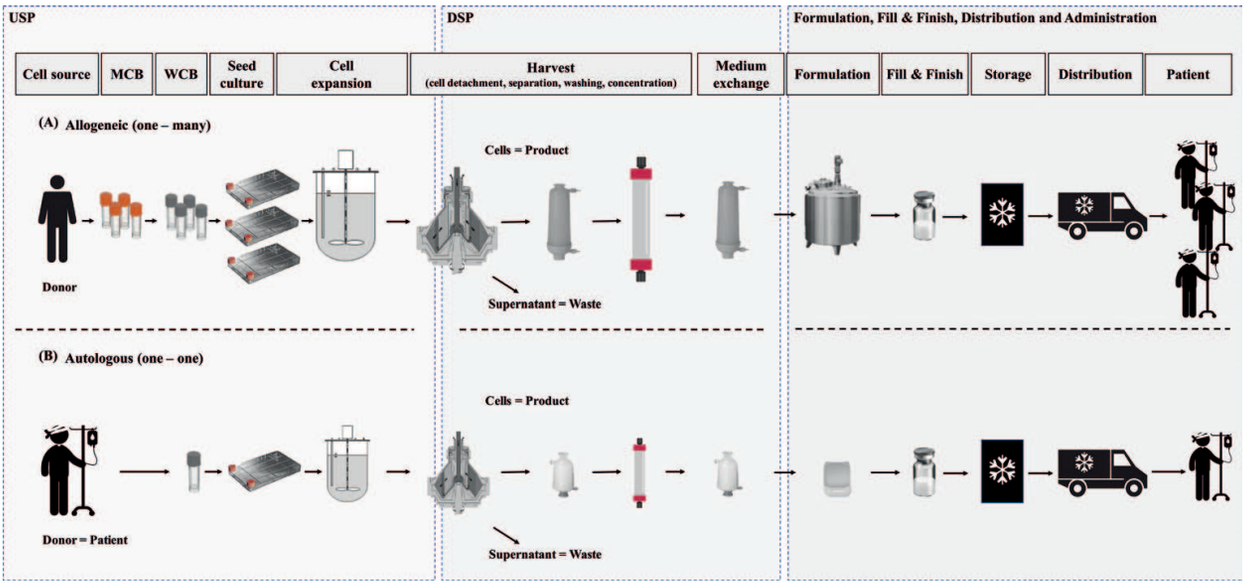


Figure 3. Main operations required to manufacture clinical-grade human MSCs for allogeneic (A) or autologous (B) therapies. USP operations typically include manufacturing of the MCB and WCB, seed cell production, and expansion at a large scale. DSP steps include cell harvest, cell detachment and separation, washing, concentration procedures, and medium exchange. Formulation, fill and finish, and storage and distribution will complete processes prior to clinical administration. Source: Jossen et al. [67].

survival and regenerative potential of MSCs and also confers resistance to hostile environments associated with inflammation which induces cell death by oxidative stress [88–90], evidencing the presence of GFs as a conditioning advantage for stem cell production.

Chemically defined, xeno-free medium does not involve donor or batch-to-batch variation; neither requires pathogenic agent screenings and presents minimal immunogenicity [91]. Drawbacks are the high cost of commercial versions and the fact that cells grown under these conditions lose their ability to adhere to plastic, requiring additional coating agent steps [92, 93]. Cells grown in xeno-free protocols also show improved proliferation potential when compared with FBS-based growing media with the previously mentioned consequent advantages [76, 92–94]. In addition to the choice of source of proteins and growth factors, basal media, seeding density, oxygen tension, confluency, and dissociation protocols may also influence outcomes. Further studies that carefully control growing conditions and at the time explore aspects such as senescence, genetic stability, immunogenicity and cytokine production, and transcriptome and proteome are needed.

In vivo ASCs reside under low oxygen tension (physioxia); Chen et al. have recently shown that the simulation of physioxic conditions (2% O_2) instead of the typical atmospheric oxygen concentration used in cell culture (20–21%) leads to increased proliferation, migration, and angiogenesis plus decreased senescence and apoptosis suggesting that the maintenance of native bioactivities may translate into production of superior cell products [95].

It is clear that tissue source and optimal growing conditions for MSC manufacturing will depend on the needs of downstream applications. In this sense it is important to mention that Okolicsanyi et al. have shown that MSCs isolated from the bone marrow of normal donors

(from Lonza, Australia), expanded as monolayer cultures, retain multilineage differentiation capacity, including neural marker expression, after 43 days of *in vitro* expansion in the commercial synthetically defined human mesenchymal stem cell-growth media MSCGM-CD™ (Lonza, Australia) [96]. Therefore, these cells could in principle be a source for cell-based therapies of the nervous system.

The regenerative capacity of MSCs has been attributed to their anti-inflammatory immunoregulatory properties. Depending on the milieu composition, MSCs, in fact, exhibit anti- or pro-inflammatory properties (see **Figure 4**) [42, 97–100].

In an early stage of trauma or microbial invasion, when concentration of pro-inflammatory cytokines is low, MSCs present with antimicrobial pro-inflammatory properties of neutrophils [97–102]. As inflammation proceeds and pro-inflammatory cytokines build up, MSCs switch to an anti-inflammatory phenotype. Some of these anti-inflammatory actions include inhibition of anti-inflammatory activities of T cells, natural killer cells, and B cells; skewing macrophages to an M2 immunosuppressive state and monocyte-derived dendritic cells to a regulatory phenotype; and increasing their phagocytic capacity and inhibit mast cell degranulation [103, 104]. Increased immunomodulatory capacity of MSCs correlates with high levels of activated complement C3 [105, 106]. As MSCs express the complement factor H and the complement regulatory protein CD59, MSCs are protected from lysis. All this endows MSCs with the potential to suppress uncontrolled immune responses making them a suitable candidate for inflammation and immune dysfunction therapeutics by themselves or in combination with other cell types.

4.2. Induced pluripotent stem cells (iPSCs)

NSPCs may differentiate into neural cells after transplantation into an injured spinal cord, replacing lost or damaged cells, providing trophic support, restoring connectivity, and facilitating regeneration as a large number of studies have reported [107]. NSPC has produced some degree of functional recovery. The fetal, adult brain and adult spinal cord are the main sources for NSPCs resulting in advantageous cells for transplantation because they can be expanded and self-renewed in culture. Fetal NSPCs can be expanded for long periods by *in vitro* conditions, while adult NSPCs have more limited capabilities.

Despite a large number of studies using NSPCs, reviewed by Mothe et al. [108], some important issues such as isolation from their natural niche and their purification and expansion have to be taken in consideration [109]. Also, NSPCs have been reported to promote neuropathic pain, a concerning adverse effect. Most experimental SCI studies with NSPC transplants have involved rodent NSPCs because human NSPCs were either not available or difficult to grow. Human NSPCs have been isolated from the fetal brain and spinal cord of aborted fetuses [110] and postmortem tissue, but actually NSPCs can also be derived from human iPSCs [111].

Human iPSC-derived NSPCs have been transplanted into SCI models [112–114]. In these studies, nonobese diabetic (NOD)-severe combined immunodeficient (SCID) mice were used for SCI. The studies revealed an improved functional recovery with expression of neurotrophic factors from the grafted cells, axonal growth and stimulation of angiogenesis, increased myelination, and new forming synaptic connections between grafted cells and host neurons. In addition these studies showed the safety of human iPSC-derived NSPCs. All studies were performed in the subacute stage with just epicenter transplants.

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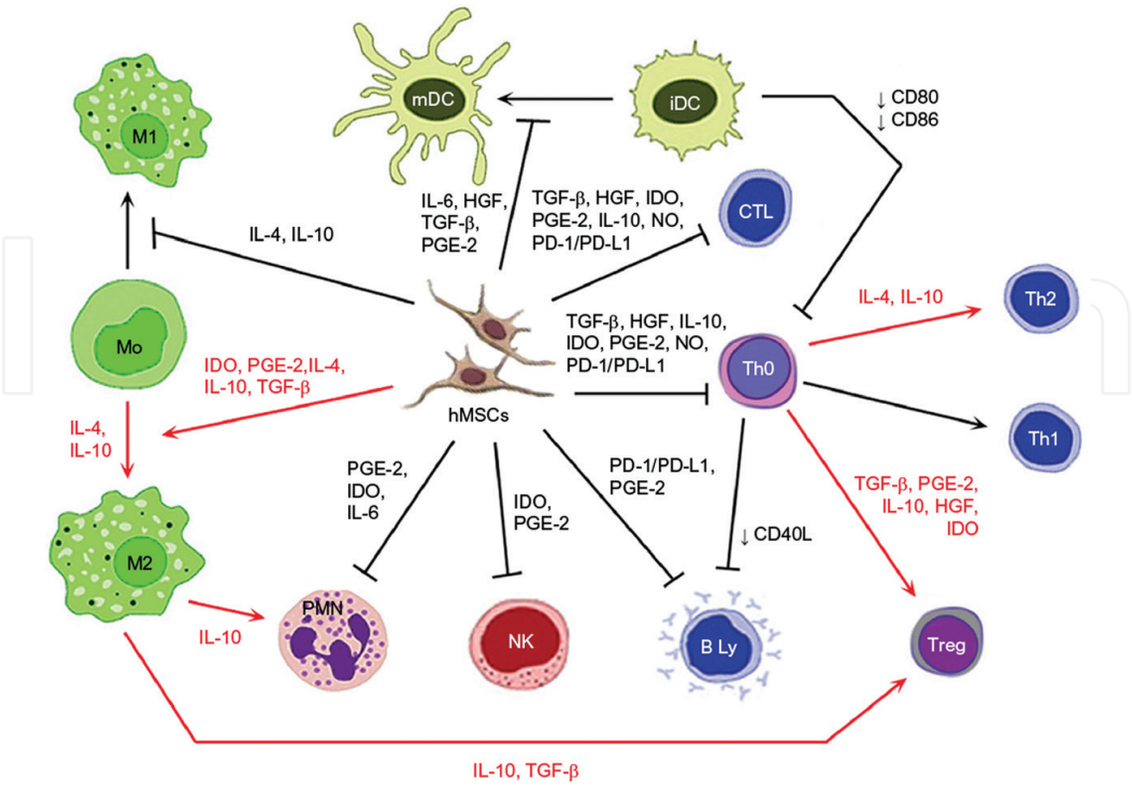


Figure 4. Immunomodulatory action of activated MSCs. Notes: Red arrow, stimulation; black arrow, suppression; blunt-ended arrow, direct inhibition. Abbreviations: iDC, immature dendritic cell; IL, interleukin; HGF, hepatocyte growth factor; TGF-β, transforming growth factor-β; PGE-2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase; NO, nitric oxide; PD-L1, programmed death-ligand 1; hMSC, human mesenchymal stem cell; Treg, T regulatory; Th, T helper; CTL, cytotoxic T cell; mDC, mature dendritic cell; PD-1, programmed cell death protein 1; PMN, polymorphonuclear leukocyte; NK, NK cell. Source: Zachar et al. [97].

All the previous reports support the potential use of iPSC-derived NSPCs in SCI. They have significant advantages, such as the lack of ethical controversy regarding their source and the potential for providing autologous transplants, thus avoiding the risk of rejection or side effects associated with immunosuppression. Recent data demonstrated the effect of the microenvironment of the injured spinal cord in the grafted iPSC-derived NSPCs. This pro-inflammatory environment induced proliferation of grafted cells [115]. Therefore, new approaches are needed to promote and guide cell differentiation, as well as to reduce tumorigenicity. Protocols for NSPC reprogrammed cells are actually improved to avoid rejection [116].

The most current iPSC protocols for neural differentiation require GFs or embryoid body formation, decreasing yields and limiting medical applications. Our lab recently developed a simple animal-free medium formula based on the inclusion of insulin and human extracellular matrix components leading to direct conversion of >98% of iPSCs into expandable and functional neural progenitors with neural rosette characteristics [111]. Further differentiation into dopaminergic and spinal motoneurons as well as oligodendrocytes and astrocytes supports the proposal that these neural progenitors retain responsiveness to environmental cues supporting applicability of the protocol for the treatment of neurodegenerative diseases. The fact that this protocol avoids embryoid body formation makes it suitable for the clinic [111].

Formerly, a feeder-free, single-step, and quick (less than 40 days) generation of mature neurons from iPSC strategy using the chemically defined medium mTeSR from STEMCELL

Technologies, overcoming the need for embryoid body formation and neuronal rosette isolation, was developed by Badja et al. Authors show that after induction the cells express voltage-gated and ionotrophic receptors for GABA, glycine, and acetylcholine (ACh) receptors and recommend the method to model human pathologies [117].

Apart from efficient differentiation methods, iPSC generation presents with the limitations of low reprogramming efficiencies (below 0.02%) and genetic modification requirements, as described by Yamanaka et al. [5, 6] and concurrently James Thompson's group [7]; thus, chromosomal instability and tumorigenic potential derived from oncogene overexpression concerns arise for their use in the clinic. An advance for safety is provided by the use of polycistronic plasmids to lead ectopic expression of the transcription factors OCT4, SOX2, KLF4, and C-MYC [118]. Other improvements based on the choice of somatic cell source, choice of reprogramming factors, culture procedures, and delivery methods have been described. For example, reprogramming kinetics and efficiencies vary between somatic cell types, in particular, keratinocytes reprogramed 2 times faster and 100 times more efficient than skin fibroblasts [10], and in general, immature cells are more readily reprogrammed than terminally differentiated cells [119]. The requirement of reprogramming factors also varies according to the cell type, so neural stem cells need only the introduction of OCT4 to be reprogrammed [120]. Reprogramming efficiency can be increased by different methods including adjustment of expression levels of noncoding RNAs, such as microRNAs or lincRNAs [111, 121]. ncRNAs can reduce the amount of reprogramming factors as they specifically target multiple pathways. Traditionally, lentivirus has been the reprogramming vector of choice; other viral vectors such as Sendai and adenovirus to lower transformation risks have been used with lower efficacy [122–124]. Excellent reviews describing more details for reprogramming protocol improvements are available [125–127].

In addition to iPSC-derived NPCs, other neural types such as neurons or astrocytes have shown some potential for SCI recovery either to improve synaptic connections or reduce neuropathic pain for the first or to protect the lesion epicenter from infiltrating peripheral inflammatory cells for the second [122, 128]. Peripheral inflammatory cell infiltration can be reduced by the immunoregulatory functions of central nervous system perivascular stromal cells (PSCs). Their low abundance, inaccessibility, and limited proliferation capacity hampers its clinical use. However, PSCs can be successfully generated from iPSCs [129] and expanded *in vitro* without senescing. Thus, SCI stem cell-based therapeutics may benefit including PSCs as part of combinatorial treatments.

4.3. Preconditioning of stem cells

It may result advantageously to precondition MSCs or iPSCs before transplantation according to the particular application or therapy, so that the cells are committed to a particular desired phenotype. Okolicsanyi et al. have more recently shown that heparan sulfate proteoglycans (HSPGs) act as drivers of neural progenitors in expanded bone marrow-derived MSCs [96]. Treatment of MSCs with heparin increased proliferation in addition to the expression of neural markers although these changes were not uniform across growth phases indicating that the direct lineage specifications and functionality of expanded cells might need fine-tuning. Nevertheless, the data sustains that MSCs may provide an abundant source that can be manipulated for purposes of neural repair and regeneration. Biomimetic approaches

to exploit the role of HSPGs in neurogenesis, such as synthetic glycopolymers or heparin conjugates, are being developed so that neural differentiation into specific lineages can be controlled and tailored [96, 130].

Stem cell transplantation has shown an important limitation due to its poor survival and engraftment at the injured spinal cord, where cells are exposed to hypoxic conditions, nutritional deficiency, or oxidative stress among others. We recently showed that FM19G11, a small chemical, first described as a HIFα protein inhibitor, is able to allow progenitor cells to differentiate into more mature oligodendrocytes under hypoxia without cytotoxic effects at nanomolar doses [IC50 (80 nM)]. Moreover, FM19G11 induces self-renewal by inducing insulin-like signaling pathway and inducing ATP accumulation, activated glucose metabolism with glucose uptake by upregulation of the GLUT4 transporter. The over-induction of AKT/mTOR signaling was directly correlated to the FM19G11-dependent induction of the self-renewal-related markers Sox2, Oct4, Nanog, and Notch1 [130]. Interestingly, the use of a combination of FM19G11 treatment and epSPC transplantation for SCI therapy reduced the glial scar extension and increased the number of neuronal fibers at the epicenter of the lesion. It also increased expression markers for neuronal plasticity and induced oligodendrocyte turnover for potential remyelination [131, 132].

In addition to preconditioning toward enhancing cell survival and proliferation or inducing differentiation into particular cell types, various treatments have shown to impact MSC secretome which could be advantageous for particular therapies. For example, the treatment of MSCs with IL-1β increases the expression levels of a number of cytokines and chemokines as well as induces the expression of cell adhesion molecules improving the migration ability of preconditioned cells to the site of inflammation *in vivo* [133]. Preconditioning protocols typically include physical treatments such as different degrees of hypoxia, mechanical stretching, application of electromagnetic fields or mimicking of three-dimensional environments on one side, and chemical or pharmacological treatments, including herbal medicines or natural extracts on another. For a recent quite complete review of preconditioning treatments of MSCs and their effects, readers are directed to the review by Hu and Li [134]. It is interesting to note that preconditioning of MSCs with low-dose lipopolysaccharide (LPS), a major component of Gram-negative bacteria, preserves mitochondrial membrane potential inhibiting cytochrome c release in hypoxia serum-deprived cultured cells [135], suggesting that a mild local infection could in fact potentiate stem cell treatment. Therefore, it should be taken into account that patients undergoing stem cell therapies are often subjected to additional pharmacological treatments and exposed to particular environmental factors which may impact the performance of the introduced stem cells at the post-implant level. To circumvent the uncertainty associated with these hard-to-control variables, genetic modification of MSCs toward the production of defined immunoregulatory effects or homing molecules is starting to be explored. For example, EAE was shown to be consistently attenuated by using engineered MSCs with CNS-homing ligand genes along with overexpression of IL-10 [136].

4.4. Extracellular vesicle-based therapeutics

Although autologous MSCs constitute a safer choice in terms of avoiding unwanted immune responses, donor comorbidities may hamper the use of their own stem cells. Expanded allogeneic MSCs were initially believed to be immune privileged due to their low expression of

major histocompatibility complex (MHC) and costimulatory molecules and the fact that they can suppress the activity of numerous immune cell populations [42–45]. Despite the overall safety reported by a large number of CTs [25, 41, 45], substantial evidence now supports both cell-mediated and humoral immune responses against donor antigens following administration of these cells highlighting that MSCs can be recognized by the host immune system (reviewed by Berglund et al. and Lohan et al.) [137, 138]. On another end, iPSCs are envisioned as a source to eliminate immune rejection; however, this remains theoretical, as therapeutic human trials have yet to be conducted. It will be important to monitor DNA methylation status and gene expression changes that could evoke immune responses in transplanted hosts even if iPSCs are autologously derived. Therefore, the possibility of a therapeutic cell-free product could be highly relevant on safety terms.

GFs and cytokines packed and secreted by MSCs (secretome) are thought to play a significant role in SCI repair, mainly by lowering pro-inflammatory cytokines (i.e., IL-2 or IL-6 and TNF-α) [139]. In fact, Cizkova et al. attributed motor function recovery, attenuated inflammatory response, and spared spinal cord tissue to a molecular cocktail found in the MSCs after transplantation [140]. MSC paracrine secretion or secretome was first described by Haynesworth et al. in 1996 [141]; since then multiple actions are endowed to MSC secretome rather than to their engraftment. Such actions include increased angiogenesis, decreased apoptosis and fibrosis, enhanced neuronal survival and differentiation, restriction of local inflammation, and adjustment of immune responses, effects that translate into induction of regeneration of damaged tissues [142]. Therefore, the therapeutic value of stem cells may mainly derive from the released factors or secretome including soluble and vesicle-packed factors. This latter fraction, termed extracellular vesicles (EVs), is a heterogeneous mix of vesicles including exosomes, a subset of double-membrane vesicles characterized by the expression of a set of markers, including tetraspanins CD9, CD63, and CD81 with attributed intercellular communication role including the transfer of their cargo (DNA, RNA, and proteins) [143].

The first documented clinical administration of EVs was performed in 2011, by administration of EVs intravenously infused at intervals of 2 or 3 days during a period of 2 weeks to a steroid-refractory GvHD patient who showed declined symptoms and stability for over 4 months [144]. Many preclinical models have shown the benefit of EV-based therapy including long-term neuroprotection. Treatment with MSC-derived EVs promoted long-lasting recovery of cognitive functions in inflammation-induced preterm brain injury [145]. EV-based therapy of SCI in rats showed a reduction of inflammatory response with apparent astrocyte and microglia disorganization in cord tissue up to 10 mm caudal to the injury site as well as locomotor recovery [146]. This illustrates the multiple potential benefits of EV-based therapies to treat neuroimmune defects. EV superiority with respect to cell-based therapeutics resides in its ready availability, ease of storage and distribution, reduced immunoantigenicity, scalability, and possibility of multiple routes of administration. EVs can also be used as delivery particles by directionally packaging molecules of interest from genetically modified cells while avoiding the risk of transfer of transformed live cells and could be obtained from iPSCs as well. Guidelines and recommendations for production, quality assurance, and application of EV-based therapeutics have been provided in an International Society for Extracellular Vesicles (ISEV) and European Network on Microvesicles and Exosomes in Health and

Disease (ME-HaD) position paper [147]. Also, the International Council for Harmonization of Technical Requirements for Pharmaceuticals of Human Use (ICH) mission guidelines to ensure the production of safe and effective high-quality medicines can be accessed on the following link: http://www.ich.org/products/guidelines.

Although clinical trials using EVs are still seldom, several companies have already engaged in EV or secretome production. A list of these companies with the corresponding links to their web pages can be found in the recent review by Gimona et al. [148]. This review includes a further in-depth review of clinical-grade EV production current status and remaining challenges. Involvement of biobank networks with pharmaceuticals may be relevant for granting standardized GMP production consistency of EVs [149].

Lastly, it should be mentioned that EVs can also be used as a sensor of stem cell plasticity or other cell features as they reflect characteristics of the cell of origin [150] constituting a helpful tool to develop optimized differentiation and preconditioning protocols.

5. Conclusions

Although CTs have in general evidenced MSC safety, the removal of FBS from clinical-grade stem cell protocols results imperative. The pooling of a large number of donors of cells and human blood fraction-based media through the use of stem cell banks or the use of xeno-free synthetic defined media should translate into allogeneic MSC preparations leading to more homogeneous clinical results. Thus, allowing minimal immune-related safety concerns derived from FBS and unveiling the real therapeutic value of *in vitro* expanded off-the-shelf MSCs.

The iPSC manufacturing technology offers the possibility of developing patient-tailored cell therapies with the consequent safety and immune-related advantages, as genetically identical cells should prevent immune rejection. iPSCs can differentiate into all three germ layers and, by their nature, do not raise bioethical debate. However, safety concerns related to *in vivo* properties of immortal cell types and the use of genetically manipulated cells raise regulation hurdles for their use in the clinic.

Preconditioning of *in vitro* expanded MSCs to ensure cell lineage commitment might result advantageously for improved treatment of particular diseases. Optimizations for the treatment of SCI and other neuroimmune health problems such as ME/CFS remain. Also, EVs and in particular exosome-enriched MSC-derived fractions may eventually become the treatment of choice for cell-based-free therapeutics by themselves or in combination with other clinical treatments once GMP production is optimized.

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Conflict of interest

Authors declare no conflict of interest.

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