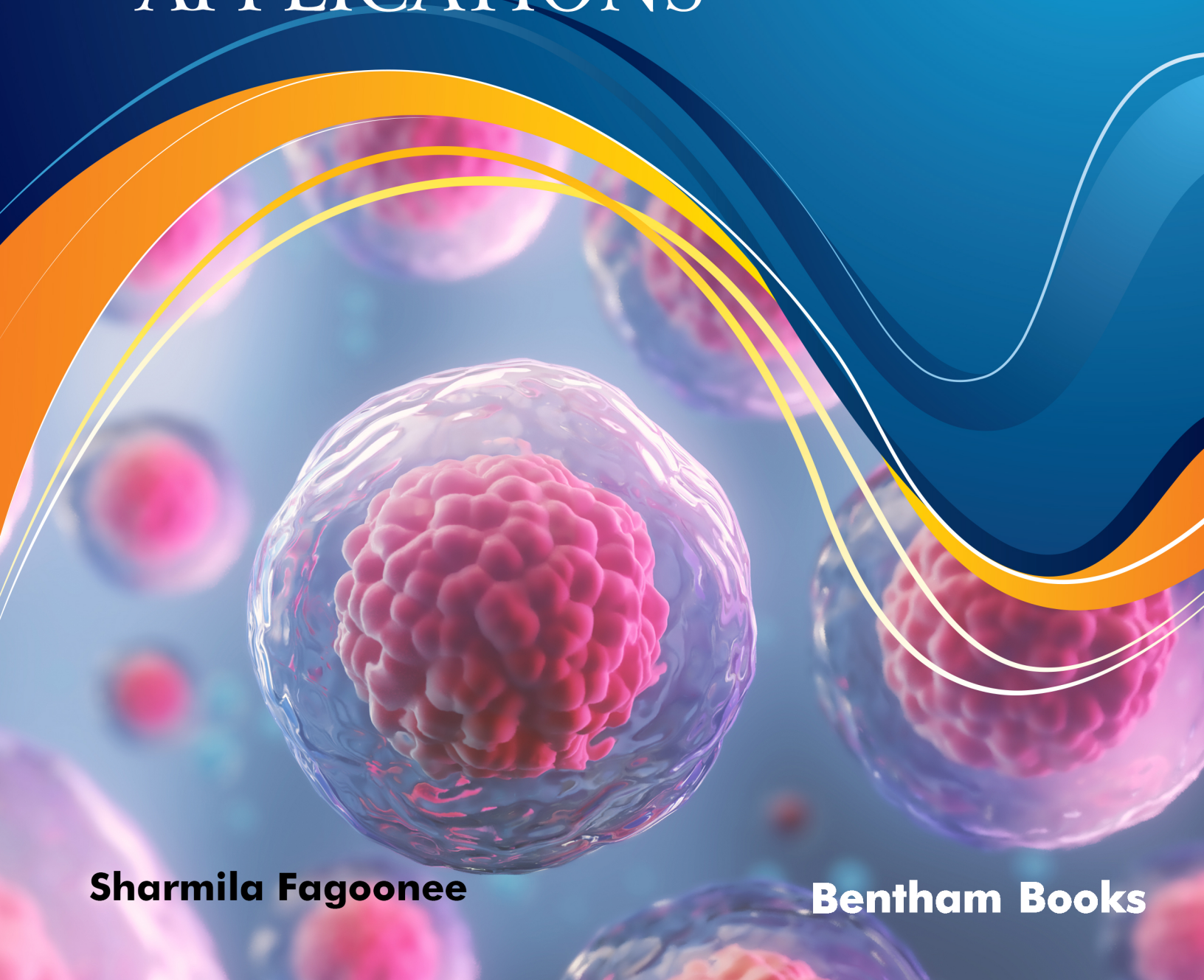
STEM CELL DELIVERY ROUTES

FROM PRECLINICAL MODELS TO CLINICAL APPLICATIONS



Sharmila Fagoonee

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Stem Cell Delivery Routes: From Preclinical Models to Clinical Applications

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Stem Cell Delivery Routes: From Preclinical Models to Clinical Applications

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FOREWORD

It is a great pleasure to present *"Stem Cell Delivery Routes: From Preclinical Models to Clinical Applications"*, a book that focuses on different strategies for stem cell delivery in pre-clinical models and clinical trials. This book provides a very focused and complete overview of the topic, and will certainly be of great utility for researchers and clinicians involved in stem cell therapeutic application. The book is written by Dr. Sharmila Fagoonee, a brilliant scientist with a long-standing experience in Regenerative Medicine. In particular, the researcher's experience on stem cell administration in pre-clinical murine models of liver, kidney and ocular pathologies is reflected by the specific deepening of the stem cell delivery in those pathologies.

The book reflects the state-of-the-art knowledge on stem cell administration that involves not only stem cell therapies, but also the more recent involvement of stem cell bioproducts, such as extracellular vesicles. Indeed, stem cell-derived extracellular vesicles, and in particular mesenchymal stromal cell (MSC)-derived vesicles, are considered to mediate several of the beneficial effects of stem cell therapies. The book also takes into account labelling, biodistribution and tracking of stem cells, and the hurdles involved. The main possible routes of administration within the circulation or intra-tissue are depicted for liver and kidney, as a paradigm of parenchymal organs. Moreover, the book takes into consideration the administration routes inside the different ocular compartments, as eyes appear for their accessibility of particular relevance in stem cell research.

Finally, the clinical aspects of stem cell delivery for clinical trials are presented. Among several possible applications, a whole sub-chapter is dedicated to MSC-based therapeutics for COVID-19, considering how this emergency expanded the use of stem cells in the clinic. Indeed, proper stem cell/extracellular vesicle delivery is of utmost importance for their activity, and the main setbacks and solutions for improving MSC clinical application are also outlined.

I am convinced that this book will greatly help researchers and clinicians involved in cell therapies, who will benefit from the knowledge mentioned and illustrated in the book.

Benedetta Bussolati
Prof. of Laboratory Medicine
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PREFACE

In an era of organ-shortage crisis, cell-based products are receiving more and more attention as lifesaving therapeutics. For several decades now, stem cells have been the object of keen interest in the field of regenerative medicine due to their dynamicity, flexibility and interactiveness. Stem cells are present in all adult tissues and participate in regenerative processes. In particular, mesenchymal stromal/stem cells (MSCs) and their bio-products benefit from extensive research and literature due to their isolation from easily sampled tissues. Huge progress has been made in the stem cell transplantation area largely due to the use of animal models of human diseases. Nevertheless, MSCs for clinical applications are "equal, but some are more equal than others" (from G. Orwell's Animal Farm). In fact, source tissue-associated differences exist, and can affect MSC functionality in a disease context-wise manner. Some issues regarding the delivery route, homing and engraftment still need to be dealt with in order to safely reach the desired clinical application. This book deals mainly with the various MSC delivery routes and cell carrier materials employed. The cell tracking methods in preclinical and clinical studies will be discussed, with specific emphasis on the liver, ocular surface and kidney whilst discussing factors that affect the residence time, viability, and homing of MSCs. The discussions are accompanied by key descriptions of MSC-based therapeutic applications in rodent models and human clinical studies.

The advantages and bottlenecks in MSC application in the clinics and ways to improve the therapeutic efficacy of transplanted cells are also tackled. This field requires serious standardisation in order to obtain reproducible, comparable and interpretable inter-studies results. This is an area where not all negative results are negative, and publication of results should be encouraged. Data and experience sharing will accelerate the pace towards the common goal of cell-based organ repair and regeneration.

Where are we, and where are we heading with MSC-based therapy? From single stem cells to xenorobots, this amazing field never stops surprising us. What's in a cell and what's around a cell all matter in the regenerative medicine field. And as we worry about the ingredients in our food, what stem cell-based bio-products we allow to inject into our body are also important. Thus, unregulated stem cell tourism should be strongly discouraged.

To the best of my knowledge, this is the first book on stem cells and derivatives delivery routes in preclinical models and clinical applications. The contents are adapted to suit undergraduates to lecturers, clinical researchers to biomedical engineers, as well as those just curious to understand more about this important and revolutionary clinical opportunity that we constantly hear about and that seems to fit well in the medical puzzle.

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CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

DEDICATION

This book is dedicated to…

My Mum, Mala, a very strong and kind person, a loving mother, who courageously fought a carcinoma and passed away on 3rd July 2020, a time when the world came to a stop, and travelling for the last salute was impossible,

My Father and Sister, who, even far away, never make me feel the gap,

My Husband and Kids, especially, who lovingly support me every day

All those who give without expecting.

CHAPTER 1

Stem Cells for Clinical Application

Abstract: Basic experimental research on stem cells has paved the way towards an array of possible clinical applications. Mesenchymal stromal/stem cells (MSCs), due to their multipotent properties and easily accessible sources, are the most studied stem cell types in a spectrum of diseases and injuries. Cell viability and dosage, delivery routes, homing and engraftment are some of the crucial factors that ensure the therapeutic efficacy of transplanted stem cell therapy in preclinical as well as clinical studies. In this chapter, we will introduce the types of stem cells and their derivatives that can be used for tissue repair and regeneration. In particular, the reasons behind the choice of certain cell types for transplantation and associated strategies are discussed based on knowledge gained on MSC research and its application for the treatment of human diseases. The administration route and cell carrier materials are among the factors that can influence the residence time, viability, and homing of stem cells.

Keywords: Adult stem cells, Allogeneic cell transplantation, Cell dosage, Cell homing, Cell transplantation, Clinical applications, Embryonic stem cells, Hematopoietic stem cells, Human diseases, Immunomodulation, Induced pluripotent stem cells, Local cell delivery, Mesenchymal stromal/stem cells, MSC engraftment, Preclinical studies, Secretome, Sources of MSCs, Spermatogonial stem cells, Systemic delivery, Transdifferentiation.

1. TYPES OF STEM CELLS FOR CLINICAL USE

The pioneering work done by E. Donnall Thomas in 1957, who first performed allogeneic bone marrow (or hematopoietic stem cell) transfusion in patients, earned him a Nobel Prize in 1990 for his discoveries regarding cell transplantation for human diseases treatment [1, 2]. In this study, six patients were treated with radiation and chemotherapy followed by intravenous infusion of marrow-derived from a normal donor. Only two patients had cell engraftment, but none survived up to 100 days post-transplantation. Thereafter, with the discovery of the human leukocyte antigen (HLA) antigens and typing methods, E. Donnall Thomas began a clinical trial program, in which 100 allogeneic transplantations were performed in 54 patients with acute myeloid leukemia and 46 patients with acute lymphoblastic leukemia undergoing combination chemotherapy. Only 13 patients had disease-free survival for 1 to 4.5 years following marrow graft [3]. It was

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concluded that bone marrow transplantation should be performed early in the management of patients with acute leukemia using HLA-matched marrow. This work revolutionised medicine and paved the way for human cell-based therapy, through at least three crucial hints: 1) allogeneic cell transplantation was possible, 2) timing of therapy was important, and 3) HLA-matching was necessary. Since then, cells (and thereafter, stem cells) have gained tremendous attention from the scientific and medical communities. Advances in stem cell research have led to the identification of multipotent cells in adult tissues, not only in the bone marrow, but also in the easily accessible adipose tissue, umbilical cord, amniotic fluid, placenta, breast milk as well as donated organs like the liver [4, 5]. The adult stem cells are present in limited amounts in all organs and are essential for tissue homeostasis and repair.

Stem cells, due to their capacity for self-renewal and differentiation into various cell types, are indeed very promising for the treatment of human diseases (Fig. **1**). Preclinical studies in appropriate animal models are necessary to obtain important insights into how transplanted stem cells will behave in human subjects. Animal models provide information about stem cell behaviour when surrounded by an immune system or vasculature as well as upon complex interaction with different cell types in the receiving microenvironment [6, 7]. Thus, preclinical studies are requisite to test for the safety and efficacy of stem cell administration as well as to undertake stem cell-based clinical studies on humans. Several successful cases of stem cell transplantation in the rodent models of hepatic, corneal and renal diseases, for example, have been reported [8 - 11]. However, despite their potential therapeutic applications, stem cells use in the clinic has been limited by setbacks encountered. In the case of allogeneic stem cell transplantation, there have been several reports of immune rejection or graft *versus* host disease and the need for life-long maintenance on immunosuppressive therapy to avoid immune clearance of injected cells [12]. Moreover, the most studied and promising stem cells, that is, the embryonic stem cells (ESCs), are hurdled by ethical concerns regarding embryo use [13]. Especially their intrinsic capacity for self-renewal and highly plastic nature (with the possibility of teratoma generation *in vivo*), shared by stem cells and cancer cells, are among the most worrisome features. Since the discovery by the group of Shinya Yamanaka, awardee of the 2012 Nobel Prize in Physiology or Medicine, of a way to make somatic cells adopt a pluripotent state, the induced pluripotent stem cells or iPSCs have revolutionised medicine and expanded the horizon of possibilities of cell sources for patient-tailored therapy. In 2017, a Japanese patient with neovascular age-related macular degeneration was the first to receive transplantation of a sheet of allogeneic retinal pigment epithelial cells differentiated from skin fibroblasts-derived iPSCs into the subretinal space [14]. Twelve months post-transplantation, no reject or complications occurred, showing that iPSC-based therapy was safe and feasible.

However, there was no significant improvement in clinical outcome, suggesting that further work is needed to improve the efficacy of iPSCs *in vivo*. Few clinical studies have been registered in the US Food and Drug Administration (FDA) clinical trials database with the primary aim being transplantation in patients despite the global trend showing a rise in the use of human iPSCs in clinical studies (Table **1**) [15]. One particular concern in using the iPSCs is that, despite their induced differentiation *in vitro* before transplantation, some residual undifferentiated cells may remain, which with time, may lead to tumour formation *in vivo*. Before ESCs or iPSCs can be routinely employed in the clinics, further studies, assisted by surface marker discovery to sort only cells differentiating in specific lineages, are needed to circumvent current limitations. Other types of pluripotent stem cells, such as those deriving from spermatogonial stem cells, or trans-differentiated cells are also promising but not ready to be used in the clinics [16, 17]. This brings us to the most widely studied mesenchymal stromal/stem cells (MSCs), which can be included in treatment regimens for certain pathologies. In fact, MSCs are currently being studied in the clinical setting, for example, in orthopaedic surgery for joint degenerative and inflammatory diseases [18]. The autologous MSCs can be harvested from adipose tissue or bone marrow, and prepared using available commercial systems for one-step infusion in the patients or expanded *in vitro* for two-step interventions [18].

Table 1. Clinical studies using iPSCs for transplantation in patients. The search terms were "induced pluripotent stem cells", "transplantation", (www.clinicaltrials.gov, downloaded on 02/06/21); LVEF: left ventricular ejection fraction; iPSC: induced pluripotent stem cells; RPE: retinal pigment epithelium; PLGA: poly lactic-co-glycolic acid.

NCT Number	Title	Status	Conditions	Interventions	Outcome Measures	Phases	Enrollment	Study Type	Study Period	Locations
NCT04339764	Autologous Transplantation of Induced Pluripotent Stem Cell-Derived Retinal Pigment Epithelium for Geographic Atrophy Associated With Age-Related Macular Degeneration	Recruiting	Age-Related Macular Degeneration	Combination Product: iPSC-derived RPE/PLGA transplantation	Visual acuity change, adverse events	Phase 1|Phase 2	20	Interventional	2020-2029	United States
NCT04696328	Clinical Trial of Human (Allogeneic) iPS Cell-derived Cardiomyocytes Sheet for Ischemic Cardiomyopathy	Recruiting	Myocardial Ischemia	Biological: Human (allogeneic) iPS cell derived-cardiomyocyte sheet	Improvement in LVEF, Incidence of adverse events and defects	Phase 1	10	Interventional	2019-2023	Japan

CHAPTER 2

Stem Cells and Derivatives Delivery Modes in the Liver

Abstract: The liver is at the crossroad of several vital processes, including metabolism, detoxification and immune surveillance. Chronic insults caused by a multitude of factors reduce liver functionality and, if left unchecked, can lead to lethality. Definitive cure of the damaged liver occurs through orthotopic organ transplantation, but the shortage of suitable organs, high costs and its invasiveness limit such an approach. Thus, new strategies to attenuate liver disease progression and restore function are being searched for. Cell therapy is resolute in some cases, and act as bridging therapy in others. Several cell types have been investigated both preclinically and clinically for their therapeutic efficiency. Stem cells are optimal candidates for reversing liver damage, due to their plasticity and capacity to secrete reparative factors. Among stem cells, MSCs are the most studied for their manipulability *in vitro*, and efficacy *in vivo*. MSCs play a therapeutic role in liver disease by homing to and engrafting in the injured liver, and by its ability to adopt a hepatogenic fate in some cases. In other instances, the secretome of injected MSCs favour liver regeneration and injury repair. When delivered through different routes including intravenous, intraportal, intrahepatic, intraperitoneal and through the hepatic artery, MSCs may confer different therapeutic efficacy. Cell survival *in vivo*, cell dosage, the extent of liver damage and microenvironment are other factors that determine the success of MSC-based therapy. In this chapter, the delivery routes used to target MSCs to the liver will be addressed.

Keywords: Bone marrow-derived mesenchymal stem cells, Cirrhosis, Clinical studies, End-stage liver disease, Fibrosis, Induced pluripotent stem cells, Intrahepatic injection, Intraportal delivery, Intrasplenic route, Intravenous, Liver, Liver-derived mesenchymal stem cells, Liver function, Mesenchymal stem cells, Mouse models, Orthotopic liver transplantation, Preclinical, Stem cells engraftment, Stem cell homing and engraftment, Transdifferentiation.

1. THE LIVER AND HEPATIC DISORDERS

The liver is the largest internal organ of the body and performs several vital functions, such as toxins scavenging, regulation of metabolism and control of homeostasis, as well as protein synthesis and glycogen storage. Several cell types constitute the liver, including hepatocytes, cholangiocytes, the resident macro-

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phages or Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), fibroblasts, lymphatic vessel cells, oval cells, lymphocytes and other immune cells [1]. Blood supply to the liver moves through the hepatic artery and portal vein, which are patrolled by the liver-resident lymphocytes and the Natural Killer T cells. The latter screen for both systemic and gut-derived pathogens and toxins. The liver thus holds a key immunoregulatory position in the defence against blood-borne infections [2]. Activation of sentinel immune cells induces the rapid recruitment of a large number of peripheral leukocytes to mount the immune responses in the liver, further contributing to the elimination of pathogens and antigen presentation to lymphocytes. Tightly controlled processes, taking place through the coordinated action of all these cell types, ensure maintenance of the delicate immunological balance and the correct functioning of the liver.

The liver is thus exposed to pathogens that may cause acute or chronic injury. Other repeated and prolonged insults, such as those caused by viral infection (Hepatitis B virus, Hepatitis C virus, for instance), alcohol abuse, drugs (for example, an overdose of aminoacetophen) and autoimmune attack, can also trigger liver injury and inflammation. Added to this scenario, genetic defects (such as mutations in genes responsible for bile metabolism) and metabolic diseases (caused by fat deposition, such as non-alcoholic fatty liver disease) may add another layer of stress onto the liver. Necrosis of hepatocytes and/or cholangiocytes following injury can result in fibrogenesis, a highly dynamic process and mechanism of wound healing, leading to fibrous tissue accumulation in the liver. The process can be reparative or reactive, and involves a plethora of different hepatic cell types [3]. KCs and LSECs are two of the non-parenchymal cell populations involved in the early response to injury [4]. KCs release chemokines, including CCL2, CXC ligand (CXCL)-1 and -2, which attract other immune cells, such as monocytes and neutrophils, as well as cytokines like Tumor Necrosis Factor (TNF)-α, interleukin (IL)-1, and IL-6, into liver tissue to start the process of liver repair [5, 6]. Dendritic cells, natural killer cells and natural killer T cells are the other immune cells that respond to liver injury by generating cytokines to initiate anti-inflammatory responses [7]. On the other hand, the LSECs, which constitute ~50% of the non-parenchymal cells of the liver and are found at the lining of the hepatic sinusoids, perform important filtration and scavenger functions, and act as important immune sentinels [2, 4, 8]. The HSCs, located in perisinusoidal space between hepatocytes and LSECs, play a crucial role in the initiation and progression of fibrosis and are the main producers of extracellular matrix (ECM) in the liver. Quiescent HSCs undergo activation and myofibroblastic transformation following stimulation by pro-inflammatory cytokines, such as Transforming Growth Factor (TGF)-β, Platelet Derived Growth Factor (PDGF), and abundantly secrete ECM proteins, tissue inhibitors of

metalloproteinases (TIMPs), and matrix metalloproteinases (MMPs) that remodel the architecture of the injured area [9]. HSCs also secrete fibrogenic factors that further stimulate the production of ECM by portal fibrocytes, bone marrow-derived myofibroblasts, fibroblasts, thereby enhancing fibrogenesis [10]. Thus, the normal liver architecture is gradually replaced by a nodular structure of fibrous septa, and functionality is impaired.

In cases of acute injury, the removal of a causal agent can reverse liver injury by activation of endogenous repair mechanisms [11]. The liver has a remarkable capacity to regenerate. In 1931, Higgins and Anderson demonstrated that the liver is a unique organ which is capable of regenerating completely after two-thirds partial hepatectomy in rats [12]. Thereafter, several studies have demonstrated that the healthy liver of several species, including humans, can regenerate when partial hepatectomy (as much as 70%) is performed. However, when the insults are chronic, the liver's regenerative capacity is affected, and chronic deposition of ECM can lead to severe and life-threatening cirrhosis and associated hepatocellular carcinoma. With high morbidity and mortality, severe liver diseases present a major threat to human health and have become an important burden on healthcare systems worldwide [13].

Orthotopic liver transplantation is the mainstay therapy for end-stage liver diseases and some liver-based metabolic diseases [14]. However, in countries where liver transplantation is possible, organ shortages and elevated costs associated with such an invasive procedure render this option not accessible to all patients. Moreover, this issue is further complicated by ethical concerns caused by the use of deceased or living donor organs, and transplantation of virus-infected donor livers, such as by Hepatitis C virus or by SARS-CoV-2, into the liver recipients [15 - 17]. There is thus an urgent need to search for resolute and lasting alternative treatment approaches for patients with liver cirrhosis and liver failure. The quest for novel therapeutic options has resulted in the emergence of growth factor-, gene-, probiotic-, and cell-based therapies [18]. So far, cell therapy with primary hepatocytes, hematopoietic cells, immune cells, endothelial progenitor cells have offered promising options for the treatment of liver diseases [19]. However, cells like hepatocytes lose their viability and functionality when expanded *in vitro*. Thus, transplantation of stem cells from various sources, such as MSCs, hematopoietic stem cells, iPSCs, and human liver stem cells for liver repair has been investigated (Fig. **1**) [11, 20, 21]. IPSCs are very promising in the field of liver regeneration. These cells, derived from the reprogramming of somatic cells, share characteristics with ESCs and have a great capacity for differentiation but are not subject to ethical concerns which currently limit the use of ESCs. Hepatocyte-like cells have been generated from iPSCs using different approaches and have shown hepatocyte functionality *in vitro* and preclinical

CHAPTER 3

Stem Cells and Derivatives Delivery Modes to the Ocular Surface

Abstract: The ocular surface is constantly exposed to the environment and is prone to severe injury or disease which may be responsible for vision loss. Serious corneal injuries may result in permanent vision loss and their treatment remains a clinical challenge. MSCs and their secreted factors (secretome) have extensively been studied for their regenerative properties in preclinical models. The plethora of cytokines and growth factors, as well as EVs released by MSCs, act in concert against scarring, neovascularisation and inflammation, and assist in the re-epithelialisation process of the ocular surface after injuries. Different routes of MSC and EV administration have been studied in preclinical models, and thereafter employed in the clinical setting in order to maximise the efficacy of MSC-based treatment for corneal disturbances. This chapter describes the possible routes of administration, including systemic, local and topical delivery of stem cells and their bio-products, and the associated efficiency of repair.

Keywords: Alkali burn, Clinical studies, Corneal regeneration, Extracellular vesicles, Inflammation, Injured ocular surface repair, Intrastromal injection, Limbal stem cell deficiency, Mesenchymal stromal/stem cells, Mouse model, Neovascularisation, Periorbital delivery, Preclinical studies, Secretome, Stem cells, Subconjunctival injection, Systemic delivery, Topical application, Transplantation routes, Wound healing.

1. THE OCULAR SURFACE AND INJURY

The cornea is a highly organised tissue at the ocular surface and plays a crucial role in maintaining proper vision [1]. The cornea consists of 5 layers: an epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (Fig. **1**). The corneal transparency and curvature, which provide the major refractive power necessary to focus an image on the retina, are maintained by the underlying stroma composed of uniformly arranged collagen fibrils and heterogeneously distributed keratocytes [1, 2]. The cornea is avascular, and tear film, mainly constituted of mucin and lipid, protects the outer mucosal surface from epithelial debris, mechanical and microbial insults, as well as ensures the

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correct functioning of limbal epithelial cells [3, 4]. Moreover, tight immunoregulatory mechanisms, involving both the innate and adaptive immune systems, maintain the cornea healthy [5].

Causes of corneal pathologies and vision loss are numerous, and include chemical burns, complications related to the use of contact lenses, dry eye disease, allergic eye diseases and trauma [3, 6]. Homeostatic balance at the ocular surface is perturbed and inflammation ensues [7]. The high turnover of the corneal epithelial cells ensures the continuous replacement of the damaged cells at the ocular surface [8]. The limbus, where the adult (epithelial and mesenchymal) stem cells reside, is the main source of corneal epithelial cells. The limbal stem cells generate new corneal epithelial cells to replace the old cells or damaged ones for corneal epithelium maintenance [9]. However, upon severe injury, when the ocular surface can no longer be repaired by the action of endogenous systems due to limbal damage or endothelium loss, alternatives are needed. Corneal transplantation is the last resort to treat most corneal debilitating diseases, but several limitations hinder its suitability in the clinics, such as high cost, shortage of corneal tissue donors, and need for sophisticated instruments and trained personnel [10]. Thus, other strategies have been evaluated to address the problems related to corneal injuries. Medical management to minimise ocular injury comprises removal of the offending agent, copious irrigation of the ocular surface, use of agents that can promote epithelialization, such as artificial tears, fibronectin, epidermal growth factor (EGF) and retinoic acid, minimising ulceration through the use of ascorbate, tetracyclines, and collagenase inhibitors, and regulation of inflammation with drugs such as corticosteroids, progestational steroids, nonsteroidal anti-inflammatory drugs, and citrate [11]. Topical biological fluids, including autologous serum, umbilical cord serum, amniotic membrane suspension, and autologous platelet-rich plasma, are increasingly being used in acute ocular burns, for instance, as a source of growth factors in order to promote corneal wound healing and repair [11]. Debridement of necrotic tissue, application of tissue adhesives are among the indicated procedures for the surgical treatment of acute ocular burns, for instance [12]. Some of these procedures are associated with a high rate of side effects. Use of corticosteroids, for example, has been shown to increase the risk of keratitis and inhibit corneal wound healing [3].

Bioengineered corneas can overcome the limitations of the aforementioned procedures. Cell sheets with adhesive ECM proteins do not require any biomaterial or suturing process to stay on the ocular surface [13]. Okano *et al.*, for instance, employed a thermo-sensitive polymer, poly (N-isopropyl acrylamide), which is non-adhesive at below 32°C but becomes adhesive at 37°C, to construct epithelial and endothelial cell sheets [14, 15]. The authors reported successful attachment of the sheets in rabbits as well as in a patient suffering from Saltzman

syndrome [16, 17]. Thus, cell sheets offer a promising platform to deliver stem cells, such as fetal cartilage-derived stem cells, to the injured cornea [18].

The possibility of using stem cells, which are carriers of therapeutic factors, has opened up a new horizon in the field of corneal regeneration. Stem cells such as MSCs of different origins have been the most widely employed in the regeneration of the damaged cornea, mainly due to their immunomodulatory properties [10]. However, recently, adult skin cells were reprogrammed into iPSCs for human corneal repair in a Japanese clinical study, indicating that other cell types are also promising in ocular surface regeneration [19, 20]. In this chapter, the potentiality of MSCs in ocular surface repair will be discussed in depth.

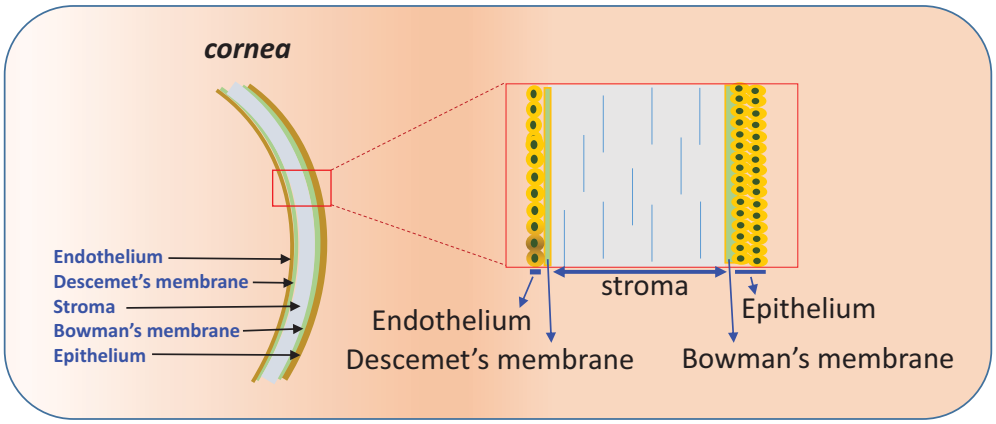

Fig. (1). The structure of the cornea. The cornea consists of 5 layers: (outer part) an epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (inner part).

2. MSCS IN THE THERAPY OF OCULAR SURFACE INJURIES

MSCs from various sources have been investigated for their possible therapeutic effects on ocular surface repair and regeneration. Bone marrow-derived MSCs have been commonly employed for this purpose. These MSCs have shown immunomodulatory effects and improved functionality *in vivo* in several models of ocular injuries with accompanying inflammation, such as chemical burns and inflammation-induced dry eye [21, 22]. However, bone marrow isolation is invasive and painful, and the MSCs represent only 0.001% to 0.01% of the total cells [23]. Thus, adipose tissue-derived MSCs are more accessible for use in cell therapy of ocular surface injuries. The yield of MSCs is higher than that of the bone marrow amounting to 5000 cells per gram of adipose tissue [24]. However, studies using adipose tissue-derived MSCs for corneal regeneration have given scarce and conflicting results. For instance, adipose tissue-derived MSCs were

CHAPTER 4

Stem Cells Delivery Modes in the Kidney

Abstract: Stem cell-based therapies are promising for the treatment of various kidney diseases. MSCs have conferred protective and regenerative effects on renal cells. However, the major hurdle encountered is the delivery of a sufficient number of MSCs to the kidney to achieve therapeutic benefits. Several injection routes have been utilised to deliver cells to the kidney parenchyma. Only a small proportion of MSCs journey to the kidney when the systemic route is employed. Direct delivery routes, like renal artery injection, are promising but require surgery. Other cell delivery methods include kidney capsule injection, intraperitoneal delivery and intraparenchymal administration. Recently, a minimally invasive renal artery injection was also implemented to promote the delivery of a significant number of transplanted cells to the kidney. Several clinical trials have been performed using MSCs from different sources for the treatment of kidney diseases. The limited results available from clinical studies show that MSCs administration for the management of kidney diseases is safe and feasible.

Keywords: Acute kidney injury, Cell delivery, Cell therapy, Chronic kidney disease, Clinical trials, Decellularised kidney, Diabetic nephropathy, Extracellular vesicles, Induced pluripotent stem cells, Intraparenchymal administration, Intraperitoneal injection, Intravenous delivery, Ischemia/reperfusion injury, Kidney, Kidney capsule, Kidney transplantation, Mesenchymal stromal/stem cells, Preclinical studies, Renal function, Repair and regeneration.

1. STEM CELLS FOR CELL THERAPY OF KIDNEY DISEASES

The kidney is a complex organ that performs highly specialized tasks, such as removal of metabolic wastes, maintenance of electrolyte balance, and regulation of blood pressure, crucial for body homeostasis. This organ, which is constantly exposed to injurious stimuli like toxins and ischemia, possesses an inherent ability to regenerate in order to restore functionality [1]. Tissue repair can occur with the coordinated action of endogenous factors that stimulate surviving tubular cells to dedifferentiate and migrate to areas with tubular injury, followed by proliferation and differentiation into functional cells [2 - 5]. When the renal structure cannot be replenished by regenerating mechanisms, kidney functionality is affected and diseases emerge. Kidney diseases, including acute kidney injury (AKI), chronic kidney disease (CDK), lupus nephritis, diabetic nephropathy, have become a

major health issue worldwide due to their rapidly growing incidence and fatality [6]. AKI is a multifactorial disease involving ischemia, infection, toxins such as radiological contrast agents or autoimmune reactions that lead to reduced blood flow to the kidneys and induce rapid apoptosis and necrosis of renal cells. If left untreated, AKI may progress to CKD and lead to kidney failure [7]. CKD arises as a consequence of continuous insidious renal damage and scarring in the presence of high blood pressure, diabetes (diabetic nephropathy), or autoimmune disease (lupus nephritis) [8]. Loss of renal function ensues, ultimately leading to end-stage renal disease (ESRD).

Treatment for kidney diseases includes multidrug therapy, which however, cannot prevent the progress to ESRD in most patients. ESRD patients require renal replacement therapy, that is, maintenance dialysis or kidney transplantation [9]. These procedures, whilst necessary, suffer from severe limitations. Dialysis impacts the social life of patients and has high medical costs [10]. On the other hand, kidney transplantation restores renal function, but compatible donor organs are scarce. Thus, novel and better therapeutic options are required to alleviate, resolve, or prevent kidney diseases, as well as to improve the survival and quality of life of patients. Several artificial devices have been prepared to substitute the non-functional kidneys, such as those making use of artificial intelligence and machine learning to improve dialysis options [11]. Different types of stem cells have also been infused into the decellularised kidney. For instance, Ross, *et al.*, seeded undifferentiated mouse ESCs in decellularised whole rat kidneys, which preserve the appropriate ECM-based differentiation signals [12]. Interestingly, in this xenograft study, mouse ESCs, delivered through the renal artery of the decellularised rat kidneys, were distributed into vascular structures and their associated glomeruli. On the other hand, cell delivery through the ureter provided access to the renal collecting system ECM, rendering complete organ repopulation possible. In this case, ESC distribution into the collecting system was observed [12].

Stem cells from exogenous sources, such as the ESCs, MSCs, iPSCs, human liver-derived MSCs have been reported to participate in kidney regeneration and repair processes in preclinical animal models [10]. Human ESCs were induced to differentiate into functional renal proximal tubular cells *in vitro* and could generate tubular structures when injected into the cortex of kidneys excised from newborn mice [13]. Moreover, directed differentiation of human iPSCs into two embryonic kidney progenitors, nephron progenitor cells and ureteric bud, has been described [14]. Cell therapy using nephron progenitor cells derived from human iPSCs improved AKI induced by ischemia reperfusion injury (IRI) in mice [15]. Transplantation of hiPSC-derived kidney organoids into the renal subcapsular space of immunodeficient mice also revealed that these structures

integrated into the blood vessels of the host mice [16]. However, due to the well-known limitations of ESCs and iPSCs, alternative stem cell types have also been studied for their potentiality in kidney repair and regeneration in preclinical models with the ultimate aim of clinical translation. Among the stem cells tested for their reparative efficiency in the context of kidney regeneration, MSCs emanated as a highly promising therapeutic approach with higher accessibility to patients.

2. MSCS IN THE THERAPY OF KIDNEY DISEASES

A rapidly increasing number of reports have addressed the use of MSCs for the cure of kidney diseases. MSCs derived from the bone marrow, adipose tissue, placenta or umbilical cord are the most studied cells in preclinical models of AKI and CKD. AKI was induced in several ways, including treatment with toxic agents such as glycerol or cisplatin, or by surgery like IRI [17]. MSCs conferred protective and regenerative effects on renal cells. For instance, injection of MSCs derived from male bone marrow accelerated tubular proliferation, thus restoring renal tubule structure and ameliorated renal function in cisplatin-treated syngeneic female mice [18]. Some MSCs were found to proliferate in the tubuli; however, the functional benefit could also be attributed to the ability of MSCs to secrete growth and trophic factors or EVs. MSC treatment also showed evidence of reducing the progression of CKD in animal models [19]. After MSC therapy, the marked reduction in plasma urea was observed correlated with the decrease in both glomerulosclerosis and interstitial fibrosis. In addition to amelioration in renal function, MSC injection enhanced anti-inflammatory effects in the damaged tissue [20]. MSCs can also modulate renal blood flow, vascular permeability and immunological responses [21]. Moreover, it was also shown that xenotransplantation of human adipose tissue-derived stromal vascular fraction or MSCs, directly administered in the kidney parenchyma following IRI, conferred renoprotective effects [22]. MSCs can also migrate to the sites of injury in preclinical models of AKI; transdifferentiation events, however, were rare and could not account for the beneficial effects observed within 2 days of MSC injection [23]. Thus, a paracrine action of MSCs was more plausible through the release of soluble proteins or membrane-enclosed entities bearing bioactive molecules, the EVs.

3. MSC TRANSPLANTATION ROUTES

Either by limited differentiation or, especially, by secreting paracrine factors with beneficial effects on the renal cells, MSCs can rescue kidney function and are indeed promising for translational research. However, therapeutic success is

CHAPTER 5

The Secretome of Stem Cells

Abstract: Stem cell transplantation is promising for the treatment of injuries and diseases. Some concerns raised about certain aspects of cell therapy and associated risks have solicited utilising the "secretome" or proteinaceous secretions of the stem cells as an alternative therapy. The secretome of stem cells has been shown to be loaded with therapeutic biomolecules, such as growth factors, cytokines and EVs. Due to technological advances, knowledge and extensive molecular data on the secretome of stem cells, especially MSCs, are getting constantly updated. Soluble proteins or EVs are the main paracrine effectors of MSCs in tissue repair and regenerative activity at sites of injury. Extracellular vesicles, in particular, are currently under intensive investigation and can develop into a practical option for patient treatment in clinics. This chapter will deal with the promises of MSC secretome, taking as examples the data available from studies on the liver, cornea and kidney.

Keywords: Acute kidney injury, Bioengineering, Cell-free therapy, Chronic kidney diseases, Cornea, Cytokines, DNA, Drug delivery, Exosomes, Extracellular vesicles, Growth factors, Kidney, Liver, Liver fibrosis, Mesenchymal stem cells, Microvesicles, MiRNA, MRNA, Secretome, Tissue repair.

1. WHAT'S IN A SECRETOME?

Stem cells have an essential role in preserving cellular homeostasis and tissue restoration. In several studies, it has been shown that stem cells can impart their therapeutic effects by differentiating into target cells at the sites of damage [1, 2]. MSCs, due to their availability and potentiality, have been regarded as highly promising therapeutic agents in the treatment of inflammatory and degenerative diseases [3]. Their mechanism of action is multifaceted and has been described in the previous chapters. Transdifferentiation, however, is not considered a principal mechanism of action of MSCs *in vivo*, albeit the beneficial effects conferred upon the target organ. The immunosuppressive and angiomodulatory action of MSCs has been mainly attributed to the secretome of these cells. In fact, injection of MSC-derived secretome was found to be safe in both animal models and patients in several studies.

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MSC-sourced secretome is enriched in therapeutic bio-products, released in free forms as soluble factors (growth factors, cytokines), or enclosed within membranes (EVs). However, MSCs isolated from different tissues and cultured *in vitro* may differ in some fine details. Albeit no gross difference in cytokine profile among MSCs derived from diverse sources has been reported, the secretion of the anti-inflammatory cytokine, IL-10, for instance, is highly contradictory, and could be due to the source of these cells [4]. Moreover, EV contents are known to vary according to cell source and physiological conditions. The therapeutic utility of MSC-derived secretome in tissue repair and regeneration with regard to the liver, ocular surface and kidney is discussed herein.

2. THE SECRETOME: SOLUBLE MEDIATORS

MSCs are known to exert their immunomodulatory functions *in vivo* through cell-to-cell contacts. Moreover, the reparative activity of MSCs occurs through a repertoire of secreted trophic factors, growth factors, chemokines and cytokines. Several cytokines, including IL-10, IL-6, TGF-β, chemokines comprising CCL-2/ Monocyte chemotactic protein (MCP)-1, CCL-5/RANTES, and growth factors such as VEGF are among the most documented soluble bioactive molecules released by MSCs [4]. Trophic factors secreted by MSCs regulate both intra- and extra-cellular signalling pathways which assist in promoting liver regeneration and angiogenesis, whilst reducing inflammation, apoptosis and fibrosis [5]. The systemic infusion of bone marrow-derived MSCs expressing flk1, a receptor for VEGF, could engraft in the liver of CCl_4-treated mice, and differentiate into albumin-producing cells (at low frequency). As a consequence, there was a significant reduction in fibrosis and hepatic injury [6]. MSCs can also secrete anti-apoptotic factors, including IL-10 and TNF-α that inhibit HSC proliferation and reduce collagen synthesis, and HGF, for example, which promotes the apoptosis of HSCs [7]. Hepatoprotective effects are also conferred by the transplanted MSCs. For instance, stromal cell-derived factor 1, or HGF, insulin-like growth factor 1 (IGF-1), and VEGF, mitogenic EGF, HGF, nerve growth factor, and TGF-α, as well as angiogenic (VEGF) factors are released by MSCs and exert an anti-apoptotic effect on hepatocytes at sites of injury [5]. MSCs also decrease the expression of pro-inflammatory factors (such as TNF-α, IFN-γ and IL-1β) as well as the expression of chemokines (such as CXCL1 and CXCL2) following transplantation to dampen liver inflammation [8]. Further details of paracrine action of trophic factors released by MSCs for liver repair and regeneration have been extensively reviewed elsewhere [5, 8, 9].

Conditioned media from adipose tissue-derived MSCs showed potentiality as ophthalmic eye drop on the basis of their growth factor-rich content [10]. The

cell-free conditioned media contain numerous mediators capable of enhancing tissue repair in the damaged cornea of a chemical burn model. Bone marrow-derived MSCs were found enriched in growth factors including keratinocyte growth factor, VEGF, FGF, EGF, and HGF [10]. Interestingly, the application of MSCs embedded in fibrin gel on the cornea of a murine model of corneal debridement prevented neovascularisation [11]. The effect of MSCs on wound closure was also investigated by applying MSC secretome in hyaluronic acid/ chondroitin sulphate gel carrier topically on corneal wounds once daily *in vivo*. Mice treated with MSC secretome had accelerated wound closure and absence of sub-epithelial scarring and fibrosis with respect to saline control groups [12]. Regarding renal regeneration, MSCs could contribute to anti-apoptotic, anti-inflammatory and matrix remodelling activities through the production of growth factors such as IL-6, VEGF, and IGF-1 to dampen cisplatin-induced renal injury or bone morphogenetic protein (BMP) 7 to improve diabetic glomerular fibrosis [13 - 15]. Growth factors and cytokines responsible for the protective effects on the kidney following AKI, CKD and kidney transplantation have been recently reviewed elsewhere [16]. MSC secretome thus shows great potential for the use of the MSC as an acellular regenerative therapy in various pathological processes [17].

3. THE SECRETOME: MEMBRANE-ENCLOSED MEDIATORS

3.1. Extracellular Vesicles

EVs are a heterogeneous population of membrane-enclosed nano-sized particles released by all cell types. Release of EVs, occurring in physiological fluids such as urine, saliva, blood, amniotic fluid, synovial fluid, breast milk, becomes particularly copious after induction by various stimuli such as stress and injury [18]. EVs participate in intercellular and inter-organ communication through the exchange of bioactive molecules. Cells can internalise EVs *via* direct membrane fusion, endocytic uptake or lipid-ligand receptor-mediated interaction [19]. They have physiological roles in key processes such as immune surveillance and tissue homeostasis, but can also be important determinants of inflammation, angiogenesis and cancer progression [20 - 22]. EVs can be classified into different subclasses according to their biogenesis and size (Fig. **1**). Despite the fact that the main classes of EVs are exosomes and microvesicles, several studies describe these entities generally as EVs, due to the difficulty imposed by overlapping sizes in separating pure populations. Thus, the term EVs will be used herein. The isolation and purification of EVs from various sources have been extensively described and compared elsewhere [23]. The therapeutic effects of EVs from

CHAPTER 6

Delivery Strategies for Cell-based Therapeutics

Abstract: Improvement in MSCs culture and expansion, delivery, homing and engraftment are needed in order to achieve optimal therapeutic outcomes in the clinic. Strategies to enhance safe and efficient stem cells as well as associated bioproducts delivery to target organs *in vivo* are currently being implemented. Stem cell delivery medium including natural and synthetic biomaterials, cell encapsulation devices, biologic and artificial scaffolds have received much attention lately. An ideal cell delivery vehicle must fulfill certain criteria, such as maintaining the vitality and function of embedded cells, being biologically compatible and biodegradable, and allowing controlled release of biomolecules from the cells towards the target tissue. In this chapter, the strategies adopted to deliver MSCs to or enhance their therapeutic activity at sites of injury in the liver, ocular surface and kidney are described. New and remodelled delivery systems are required to ensure the successful translation of cell therapies to the clinics.

Keywords: Bioartificial devices, Biocompatible delivery medium, Biodegradable materials, Biomaterials, Biopolymers, Cell delivery medium, Cell engraftment, Cell transplantation, Clinical translation, Cornea, Decellularised scaffolds, Encapsulation, Extracellular vesicles, Homing and engraftment, Host immune system, Kidney, Liver, Mesenchymal stromal/stem cells, Synthetic polymers, Tissue engineering.

1. IMPORTANCE OF CELL-BASED THERAPEUTICS

Small-molecule drugs, biological agents and cell-based therapeutics are the most important pillars of medicine [1]. Cells such as the MSCs, offer several advantages over small molecules and biological agents, in that the former can adapt and react to the surrounding environment and selectively synthesise therapeutic molecules to induce reparative processes and restore functionality at sites of injury. Moreover, cells can replace dead ones and offer architectural support to the injured organ. Stem cells are dynamic and flexible living entities that are capable of interacting with other cells, such as immune cells, of regulating their activities, hence justifying their increasing interest in the field of regenerative medicine [2]. The most classical ways of introducing cells in the body are *via* injection of cells resuspended in an appropriate medium, either syste-

mically or locally into the target organ. However, large-scale cell death, poor homing and engraftment, and difficulties in cell tracking and monitoring cell fate *in vivo* have stimulated the search for tissue engineering-based approaches for improving the outcome of cell transplantation. Cell delivery medium aiming at increasing the success of organ repopulation as well as encapsulation systems to create a shield against the host's immune system, and scaffolds for therapy in advanced disease phases are under currently under investigation.

2. STRATEGIES TO OPTIMIZE CELL DELIVERY

Tissue engineering techniques have been used to study the organs of interest in order to devise target-specific *in vitro* conditions for growth of stem cells in the presence of signals that prime these cells to adopt the right fate *in vivo*. Coupled with microfluidics, the bioengineered systems regulate the circulation of nutrients and oxygen for better growth and differentiation of stem cells prior to transplantation *in vivo*. It is important that cell delivery materials or scaffolds provide the appropriate signals to cells, that the host does not mount an immune reaction in response to the carrier, and that the biomaterials injected undergo degradation following cell delivery to the target organ. Thus, the stem cells injection medium and biomaterials used are of utmost importance in ensuring optimal organ repopulation *in vivo*. Wherever the microenvironment is destroyed due to an advanced disease state, there is also the possibility to use scaffolds seeded with cells, such as MSCs, for supporting organ function.

3. STEM CELLS AND DERIVATIVES INJECTION MEDIUM

A wide variety of MSC infusion mediums has been used both in preclinical animal studies and in human clinical applications. The most employed cell delivery medium is phosphate-buffered saline (PBS). MSCs were delivered in PBS in a common bile duct ligated rat model, and this resulted in reduced fibrosis and inflammation compared to PBS-injected rats [3]. Glomeruli derived-MSCs (GI-MSCs) were also employed in a model of ischemia/reperfusion injury in mice. Intravenous injection of 1×10^5 GI-MSCs in PBS contributed towards dampening kidney ischemic injury in these mice [4]. Type 1 diabetic mice were injected subconjunctivally with 5×10^4 MSCs and there was an enhanced wound healing with respect to PBS-injected control [5]. MSC-derived EVs were also delivered in PBS in the *Mdr*2-/- mouse model of primary sclerosing cholangitis. Injection of 100 µl of EVs ($\pm$ 9.1×10^9 particles/mL) once a week, for three consecutive weeks caused a reduction in cholestasis and fibrosis biomarkers and reduced collagen deposits histologically [6]. In a mouse model of aristocholic acid-induced neuropathy, EVs were resuspended in PBS and injected

intravenously at 1×10^{10} EVs/ml/mouse [7]. MSC-EV administration restored kidney functionality as seen by the reduction in blood urea nitrogen and creatinine levels, and decreased fibrogenesis compared to PBS-injected controls. MSC-derived EVs (100 μg EVs) applied in PBS to the cornea of an alkali burn injury mouse model once per day for two weeks enhanced proliferation, suppressed inflammation and apoptosis of corneal epithelial cells, hence promoting wound healing [8]. Thus, injection of cells in a physiological solution devoid of growth factors or impurities provides beneficial effects *in vivo*.

4. BIOMATERIALS FOR MSC ENCAPSULATION

Adhesion of cells to biological carriers is important for their survival and journey to the target organ *in vivo*. A number of natural ECM molecules can act as biological carriers and can participate in the remodelling of different tissues in preclinical studies as well as in clinics [9]. Animal-derived biomaterials such as ECM-derived collagen gels and basement membrane-derived Matrigel have been used as a matrix for cell delivery due to their capacity to support cell growth and differentiation. These animal-derived biomaterials are of undefined constituents and may contain residual growth factors or impurities, rendering their translation into human studies difficult. Despite the drawbacks, these biological carriers are still employed in preclinical studies. For instance, bone marrow-derived MSCs were co-transplanted in Matrigel plugs, with the aim of promoting engraftment of the latter *in vivo* [10]. Interestingly, subcutaneous injection of these co-delivered cells in mice showed that, at day 7, cell engraftment and vessel forming capacity of endothelial colony forming cells in the Matrigel implants had improved with respect to injecting these cells alone, without MSCs. Clinically acceptable alternatives to Matrigel are also being devised [11].

A number of xeno-free, chemically-characterised, and highly tunable synthetic alternatives to Matrigel are being devised and tested to promote the injection of encapsulated exogenous MSCs prior to transplantation to protect the cells from the host's immune attack [10]. Cells can be incorporated in polymerised, biocompatible and semi-permeable structures, called microspheres or microcapsules. Several biopolymers have been employed in the development of an artificial matrix for cell delivery. These include sustainable and biodegradable Poly(lactic acid) (PLLA), poly lactic-*co*-glycolic acid (PLGA), PLLA-PLGA copolymers, as well as biomaterials such as agarose, hyaluronic acid, alginate and collagen gels, used to support 3D growth of cells [12, 13]. These biologically active materials are prepared with adjustable permeability to allow the controlled and bidirectional exchange of oxygen and metabolic products between the host and the transplanted cells. This ensures that the correct differentiation signals

CHAPTER 7

Stem Cells and Derivatives Homing and Tracking *In Vivo*

Abstract: Stem cell-based therapeutic possibilities have revolutionised medicine. In order to maximise clinical outcome, it is essential to use the optimal cell type and dosage, and cell infusion routes, as well as determine the post-transplantation homing and engraftment efficiency of infused cells. Tracking the fate of transplanted cells is pivotal to monitoring their viability and distribution to the target organ. Several labelling techniques are employed to trace transplanted cells *in vivo*. In rodents, magnetic-, fluorescence- or luminescence-based imaging methods have been developed and tested for their capacity to evaluate the engraftment of transplanted cells. The majority of these modes of *in vivo* cell tracking are still in the preclinical phase of investigation. Acquisition of reliable images depends on the specificity of the signal of the labels used at a certain tissue depth. While longitudinal analysis is feasible in preclinical models, and usually relies on histological or molecular analyses for its confirmation, this is still undoable in the clinical setting. Further research in molecular imaging approaches and in ways to follow the *in vivo* fate of injected cells in humans are required.

Keywords: Biodistribution, Bioluminescence, Biomarkers, Cell delivery route, Cell labelling, Cell tracking, Cornea, Engraftment, Extracellular vesicles, Fluorescence, Homing, Kidney, Liver, Magnetic resonance imaging, Mesenchymal stromal/stem cells, Molecular imaging, Nanoparticles-based tracking, Non-systemic cell delivery, Preclinical research, Systemic cell infusion.

1. SYSTEMIC AND NON-SYSTEMIC HOMING AND ENGRAFTMENT OF STEM CELLS.

Successful organ repair relies on the homing and engraftment of administered cells in the target organ. The routes of administration, systemic or non-systemic, largely influence the migration and homing of cells *in vivo*. In systemic homing, exogenous MSCs, administered into the bloodstream, must undergo a multistep process to exit the circulation and be recruited to the injury site. The process of systemic homing, most probably involving the leukocyte-like properties of MSCs, comprises five steps [1]: tethering and rolling [2], activation [3], arrest [4], trans-

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migration or diapedesis, and [5] migration (Fig. **1**), extensively reviewed in [1]. Briefly, *tethering* is started by CD44 molecules expressed on MSCs , which bind to selectins expressed by endothelial cells. Flowing MSCs are captured and start rolling along the endothelium [2]. Activation then ensures thanks to the expression of stromal cell-derived factor (SDF)-1 on endothelial cells. SDF-1 binds to the chemokine CXC receptor (CXCR)-4 or -7, which are among the receptors expressed by MSCs, and facilitates homing to target tissues [1, 3]. This activation step proceeds to the arrest phase, which involves integrins. For example, adhesion of MSCs partially depends on CXCR4-SDF-1 and α4β1 (very late activation antigen (VLA)-4)/ *vascular* cell adhesion molecule (VCAM)-1 interaction which allows firm attachment of these cells to the endothelium [4]. The fourth step regards *transmigration* or *diapedesis*, in which MMPs actively participate in the breakdown of the endothelial basement membrane to promote the migration of MSCs through the endothelial cell layer [5]. The MSCs then need to *migrate* through the interstitium to the target organ and to the site of injury. A gradient of chemotactic signals guides the MSCs to the site of tissue damage [6]. MSCs migrate in response to many chemotactic factors released by injured or inflamed tissues, including growth factors such as IGF-1, PDGF-AB, chemokines such as RANTES, macrophage-derived chemokines (CXCL2-4) and SDF-1 [7, 8]. MSCs bear receptors for these factors on their surface, as for instance, the receptors for IGF-1, PDGF and the macrophage-derived chemokine receptors CCR2-4 and the SDF-1 receptor, CXCR4. A study using a mouse model of glycerol-induced acute renal failure (ARF), for instance, demonstrated that the migration of MSCs to the injured kidney was dependent on CD44 expression on MSCs [9]. These murine bone marrow-derived MSCs intravenously injected into mice with ARF migrated to the injured kidney that expressed abundant hyaluronic acid in the renal cortex compared to the healthy tissue. The expression of the chemokine receptor CXCR4 on MSCs also appears to play a role, as overexpression of CXCR4 increased the homing of MSCs to the injured kidneys [9].

In non-systemic homing, MSCs are delivered locally at or near the target organ, and cell recruitment takes place through the release of trophic factors by the injured tissue. Most of the processes described above are not necessary in this mode of homing. As described in the previous chapters, the majority of MSCs delivered intravenously remain entrapped in the lungs due to “first-pass” effect [10]. Thereafter, MSCs can be found in other organs, such as the liver and spleen as well as at sites of injury. It has also been reported that freshly isolated MSCs show superior homing ability compared to *in vitro* expanded MSCs and that different MSC subtypes, like the “classical” MSCs and multipotent adult progenitor cells (MAPCs) have different transmigration potentials [10, 11]. Adherent MSCs home to filter organs after intravenous injection, whereas

MAPCs may require local delivery for best functioning. Thus, non-systemic delivery is an important solution to resolve these homing and migration issues, especially because it is not clear how *in vitro* expansion and Good Laboratory Practice (GLP)-related processes, requested for preclinical studies, may influence the homing properties of MSCs *in vivo*. Cell culture duration and the number of passages clearly alter MSC morphology, phenotype, differentiation, viability, and migratory properties by inducing molecular changes in the cells [8, 12]. Thus, different MSC preparations may show variation in homing receptor expression, and consequently affect the therapeutic outcome of MSC administration [8]. Thus, there is an urgent need to monitor the fate and biodistribution of injected stem cells and their derivatives *in vivo*. Imaging approaches and strategies to verify target organ functionality restoration are very important in achieving this goal.

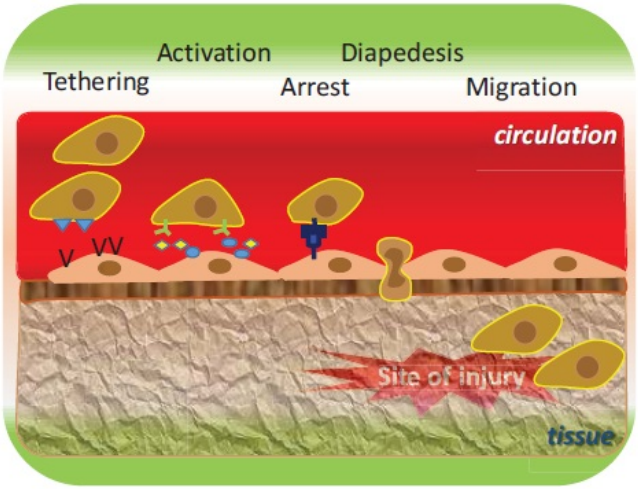

Fig. (1). MSC homing mechanisms. MSCs due to their leukocyte-like properties undergo a 5-step process to exit the circulation and be recruited to the site of injury.

2. LABELLING STRATEGIES AND ASSOCIATED TECHNOLOGIES

An essential step in the demonstration of the therapeutic activity of MSCs *in vivo* regards the labelling and tracking of these cells. The appropriate cell delivery route, choice of MSC source, cell dosage and the time of intervention are still unanswered questions that can be partially tackled by following the fate of the injected cells. Cell labelling strategy is a critical determinant in the success of cell tracking *in vivo*. Labelling helps in distinguishing transplanted cells from host cells, in monitoring biodistribution and migration after transplantation, and in assessing the efficacy of the transplanted cells [13]. Continuous, long-term monitoring of transplanted stem cells with safer, non-invasive, and repeatable

CHAPTER 8

Current Hurdles in Stem Cells Tracking *In Vivo*

Abstract: The promises linked to stem cell-based therapy have encountered several hurdles on their way to clinical trials. Albeit the results obtained in preclinical studies have been encouraging, especially regarding therapeutic outcomes in several models of human diseases, this has not been the case in clinical trials using stem cells. MSCs have been mostly used in clinical studies, which limits us mainly to this cell type for the time being for clinical applications. A point that should be urgently evaluated before proceeding with cell therapy is whether this approach is applicable to all sorts of diseases, especially in cases where the microenvironment is no longer cell receptive. From a technical point of view, improvement is required at several steps of cell therapy: standardisation of MSC source and production, choice of cell injection route, cell dosage, frequency and timing of cell administration, cell tracking, cell homing and engraftment. Data regarding long-term MSC survival *in vivo* are scarce. In this chapter, the bottlenecks that refrain from the widespread use of MSCs in clinical applications will also be considered. Currently available and innovative solutions to tackle all these issues are discussed.

Keywords: Adverse effects, Biodistribution, Biomarkers, Clinical applications, Cell dosage, Cell entrapment, Cell labelling, Engraftment, Extracellular vesicles, Frequency of injections, Genetic engineering, Homing, Hurdles, *In vivo* tracking, Long-term survival, Mesenchymal stromal/stem cells, Preclinical studies, Preconditioning, Tissue engineering, Standardisation.

1. CHALLENGES IN STEM CELLS HOMING AND ENGRAFTMENT RESEARCH

MSCs are the most considered cell type in the field of regenerative medicine. Stem cells originating from other adult tissues have also been employed but to a limited extent. Thus, most information available on the beneficial effects of stem cells in preclinical models and clinical settings is mainly on MSCs and their therapeutic utility in organ regeneration and repair. Data obtained so far in preclinical studies are very promising. From basic research, we have defined MSC isolation and characterisation procedures, culture, expansion and conservation conditions, somewhat established cell dosage, frequency and route of injection in animal models, and devised new ways of tracking cells *in vivo* in order to assess

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homing and engraftment, and correlate the results to therapeutic outcome. All these are feasible in animal models that have been humanised in some cases or immunodepressed in others but can be hardly performed in patients. Considering the similarity in anatomical, physiological and genetic features, human and rodents are different when finer details are considered, such as the signalling pathways or gene usage, and somewhat in macroscopical organisation such as the pancreas or the eye (Fig. **1**) [1 - 3]. Thus, preclinical research provides us with an approximate protocol that can be used in the clinics.

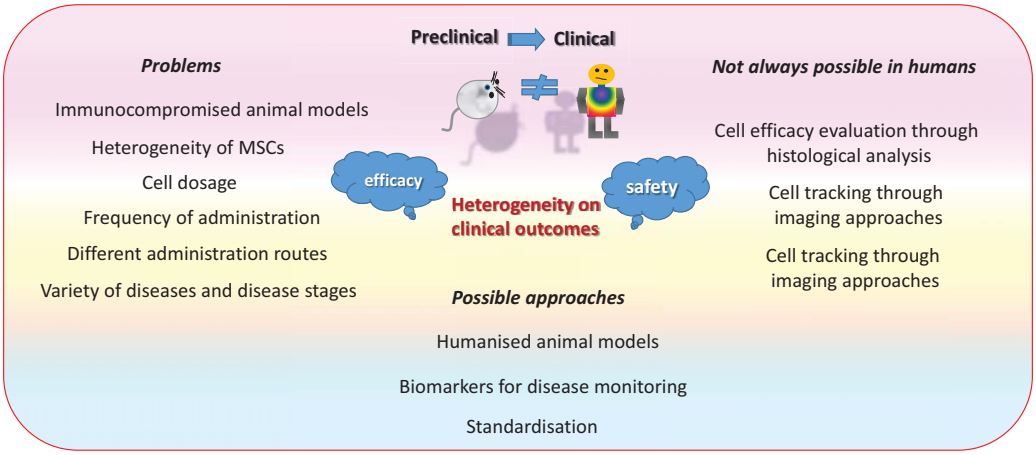

Fig. (1). Hurdles associated with the translatability of preclinical data to MSCs application in clinics. The translation of preclinical outcomes of MSC therapy has encountered several problems and some technical problems that have generated heterogeneous results in clinical studies. Some strategies have been proposed to improve the efficacy and safety assessment in patients.

The medical usage of stem cell-based products in humans is regulated by guidelines and directives, as for drugs. However, stem cells as living, dynamic, adaptive and autonomous entities may react and behave differently from drugs when injected *in vivo* [4]. Thus, the standards used to defined drugs (chemical compounds or purified recombinant proteins) may not be strictly applied to cells in cell-based therapies. Heterogeneity of MSC populations, arising from several factors including the tissue of origin, MSC injection routes as well as inter-individual variability among patients, give different outcomes in the clinics. On the other hand, the effects induced *in vivo* by MSC-EVs in a certain disease context could be more predictable than their cells of origin. EVs, once released from cells, cannot autonomously change their biomolecular contents.

Several critical issues in clinical protocols require further improvement: which source of MSCs is more therapeutically suitable for the target organ, what is the optimal timing for cell administration, what is the most effective dosage of MSCs,

which is the best route of administration and what are the primary end-points. There are also concerns regarding disease context and how it can induce MSCs to promote tumorigenesis or fibrogenesis. For instance, one study reported that autologous adipose tissue-derived MSCs, administered intravenously into the systemic circulation in a patient with chronic kidney disease, resulted in deterioration of renal function and worsening of fibrosis in the interstitial tissue [5]. Inflammatory cell infiltration and atrophy of the tubules were also observed at 5 months following cell injection, suggesting that the infused MSCs could have induced nephrotoxicity. Therefore, it is essential to perform large-sized randomised controlled clinical trial to confirm the long-term clinical benefits and safety of MSC-based therapies obtained in clinical studies. Any histological alterations caused by transplanted MSCs in the target organs should be analysed to assess possible fibrotic changes. However, obtaining a biopsy of the target organ is not always possible. Further studies will provide insights into the effects of infused stem cells on the injured microenvironments and vice [6].

Another critical issue regards the fate of transplanted cells in MSC recipients in preclinical studies. Most techniques employed to track cells *in vivo* rely on the detection of dyes or nanoparticles that do not necessarily imply corresponding living cells. It cannot be excluded that the label was associated with apoptotic MSCs or was present in macrophages that may have engulfed the infused cells or to bystander endogenous cells [3]. Label detection may not correspond to the localisation and persistence of live MSCs. Moreover, some stem cells function can be affected by labelling. For instance, bone marrow-derived MSCs showed substantial changes in metabolic activity and morphology after eGFP (enhanced green fluorescent protein) and Cell Tracker™ Green CMFDA (5-chloromethylfluorescein diacetate) staining [7].

The translation of results obtained from safety and toxicity studies on animal models to humans is not always feasible. The immunosuppressive properties of human MSCs, for example, have been investigated in the immuno-compromised recipient animals prior to proceeding with clinical studies. The human MSCs, however, differ in their immunomodulatory mechanisms compared with murine cells [8]. Moreover, humans and mice differ in their expression of the major histocompatibility complex (MHC) and costimulatory molecules [8]. Thus, the immunocompromised animals cannot fully reflect the complexity of a human immune response, thus limiting insights into pathological inflammatory responses to the cells [9]. Human MSC recipients have a functional immune system which may affect the survival of injected MSCs. The idea that MSCs may not survive long after administration stems from work performed *in vivo* in immuno-compromised mice. It was shown that systemically infused human MSCs acquire

CHAPTER 9

MSCs in the Clinics: Applications and Outcomes

Abstract: MSCs are promising for cell therapy of a variety of pathological conditions. MSCs can interact with the surrounding cells and environment and can be harnessed to confer therapeutic effects in several ways, as witnessed by progress in preclinical studies performed to date. However, translation into routine clinical practice is still trailing behind, as the beneficial effects seen in preclinical models could not be fully reproduced in clinical trial settings. The heterogeneity in bioprocesses that surround MSCs from their isolation to their transplantation is mostly responsible for the uncertain clinical outcomes. Yet, MSCs continue to be studied in a broad spectrum of clinical trials due to the MSC attributes that suggest that these cells will tip the balance towards finding an effective therapy for diseases hitherto incurable by other strategies. MSCs production should be standardised in order to optimize their output in the clinical settings. Very few of the registered clinical trials, performed with MSCs from diverse sources to date have published data. This is an area where not all negative results are negative, and publication of results should be encouraged. Negative results can help in devising better strategies in order to overcome difficulties and take us a step forward towards real therapy.

Keywords: Administration route, Automation and robotics, Advanced therapy medicinal products, Clinical outcome, Clinical trials, Cryopreservation, Current GMP, Exosomes, Extracellular vesicles, High-volume cell expansion, Kidney, Liver, Mesenchymal stromal/stem cells, Ocular surface, Preclinical model, Regulatory framework and guidelines, Standardisation, Stem cells bio-products, Therapeutic efficacy, Xenogeneic-free cultures.

1. MSCS IN THE CLINICS

MSCs are a heterogeneous population of cells derived from various sources such as the bone marrow, umbilical cord, adipose tissue as well as vascularised organs, including the liver and pancreas. MSCs are multipotent cells, but despite their shared MSC characteristics, not all MSCs are equal, as previously described. Several factors control MSC functional activity. Age of tissue donors, culture conditions, expansion *in vitro* and administration routes all decide the therapeutic outcome of the MSC injection. Preclinical models have somewhat revealed their differences. The secretome of MSCs also depends on the tissue of origin [1]. It is

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thus difficult to predict how MSCs, even derived from the same type of tissue, will behave *in vivo* in humans. The journey of MSCs to the clinic has not been straightforward and is still full of controversies. The European Medicines Agency (EMA) approved the first marketing authorization for an MSC product (Cx601, derived from adipose tissue and tested in phase 3 randomised, double-blind controlled trial, showing the efficacy of MSCs) for the treatment of complex perianal fistulas in Crohn's disease patients [2]. MSCs are considered advanced therapy medicinal products in Europe, and specific regulatory frameworks and guidelines of medical devices govern their use. Moreover, adherence to current GMP for clinical-grade MSC production is one of the requisites imposed by European guidelines [3]. The MSC therapy roadmap puts patient safety and well-being in a prime position. Recently, to optimise MSC therapeutic efficacy in patients, major current GMP considerations and challenges have been addressed in order to rewire towards standardised procedures (Fig. **1**). These considerations have been reviewed by Sanz-Nogués *et al.* and include 1) full screening of donor health status and preferred use of autologous cells for rapid intervention in the clinics; 2) most commonly used sources of MSCs are bone marrow, adipose tissue and umbilical cord; decision also dependent on proprietary issues; 3) cell karyotypic analysis required for batch release as several factors from isolation, expansion to freezing and thawing affect MSC growth kinetics and therapeutic efficacy; 4) use of cGMP compliant, defined, and xenogeneic-free culture supplements instead of fetal bovine serum or human platelet lysate; 5) better use freshly cultured cells rather than cryopreserved ones; 6) use of cGMP-grade reagents and cell sorting technologies to enrich for subgroups of MSCs, based on the presence of specific surface markers; 7) high volume cell expansion systems preferable with respect to the plastic culture dishes; 8) use of automation and robotics for large scale MSC production; 9) quantitative measurement of target organ functionality; 10) use of MSCs combined with tissue engineering approaches and medical devices [3]. Standardisation of MSC preparation procedures is the key to therapeutic success in context-dependent clinical applications. Some examples of clinical use of MSCs in the fields of hepatology, nephrology and ophthalmology are described below.

2. CLINICAL TRIALS EMPLOYING MSCS FOR TREATING DISEASES

2.1. Liver

The results obtained on the therapeutic efficacy of MSCs in preclinical models are gradually being translated onto clinical studies. MSCs have been employed in the clinics for over two decades, and most studies have evaluated the safety and feasibility of MSC administration, with a small preview on therapeutic outcomes.

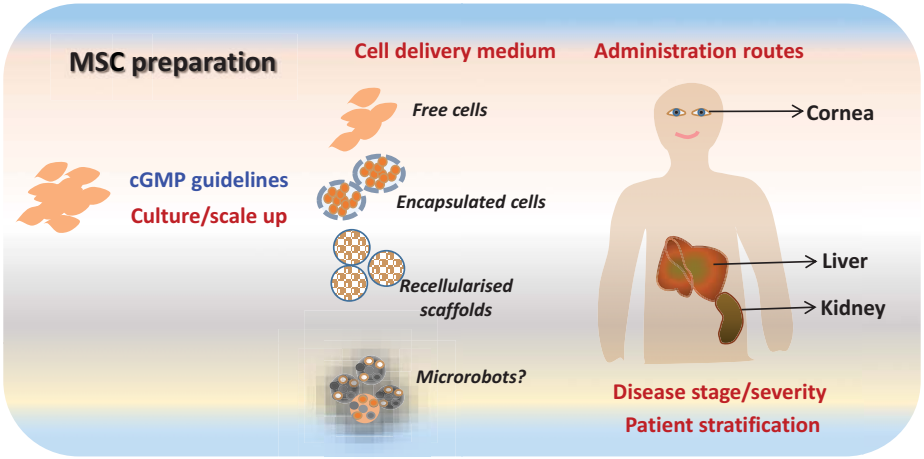

Fig. (1). MSCs delivery in the clinics. Improvements are required at several steps, from MSC preparation to cell administration route. Standardisation of MSC production according to current GMP guidelines is the first step towards getting comparable inter-studies results, followed by the choice of delivery medium. Disease staging and severity are the other factors that affect stem cell therapy and should be appropriately integrated into the workflow.

Several clinical trials have been carried out using MSCs in patients suffering from liver diseases with diverse aetiologies. For acute liver failure, the use of MSCs is mostly limited to preclinical models. One open-label, non-blinded randomised controlled study showed that allogeneic bone marrow-derived MSCs administered through the peripheral vein in hepatitis B virus (HBV)-related acute-on-chronic liver failure HBV-related cirrhosis caused an improvement in survival and clinical parameters such as serum total bilirubin and MELD Disease scores (which evaluate survival in patients with end-stage liver diseases) [4]. Another study in HBV-induced liver failure patients showed that autologous marrow-derived MSCs delivered through the hepatic artery were safe and improved the patients' conditions in the short-term, but not in the long-term [5]. In the case of liver cirrhosis, there are more reports on the use of MSCs in clinics. For instance, autologous bone marrow-derived MSC infusion, through the peripheral vein, in patients with liver cirrhosis improved liver function as seen by an increase in serum albumin and total protein levels, and a reduction in Child-Pugh score (a system for assessing the prognosis of chronic liver diseases) [6]. Reduction in hepatic fibrosis was also observed in cirrhotic patients following autologous bone marrow-derived MSCs injected through the hepatic artery [7]. In patients with alcoholic cirrhosis, MSC injection through the hepatic artery reduced collagen deposition and improved liver function and MELD score [8].

CHAPTER 10

Perspectives

Abstract: The preclinical successes of MSC-based therapy are not equalled in the clinical setting. Moreover, the translational advances of cell-based therapy are hindered by a plethora of factors that result in the heterogeneity of the clinical outcomes. Decades of research and development of MSC-based therapy have shown that transferring MSCs from bench to bedside is possible, but few clinical studies have reported favourable results. Rigorous control over MSC manufacturing steps, clarifying the mechanisms of action in each organ and disease, and the control of cell quality, as well as in-patient fate, are areas where much improvement is needed. Due to these critical points, stem cell medical tourism is not recommended. Especially, lack of patient protection, the use of MSC preparations with insufficient evidence of safety and efficacy are among factors that may lead to deterioration of health conditions. MSC-based interventions backed by preclinical studies and clinical trials showing feasibility and safety are clearly important before routine treatment with stem cells can be envisaged. Helped by artificial intelligence, data generated by high throughput technologies can be gathered and interpreted in order to increase patient-tailored life-saving therapeutic efficacy of MSCs and MSC-based product such as extracellular vesicles.

Keywords: Artificial intelligence, Bioengineering, Clinical outcome, COVID-19, Drug development, EV engineering, Extracellular vesicles, High throughput technology, Mesenchymal stromal/stem cells, MSC administration, MSC-based therapy, MSC delivery routes, Patient-tailored treatment, Secretome, Stem cell clinics.

1. CURRENT SETBACKS OF MSC-BASED THERAPY IN THE CLINICS

MSC-based therapy represents an exciting but challenging option for the treatment of patients with acute and chronic diseases. The variables described in the previous chapters regarding different aspects of MSC preparation to injection have made inter-studies comparisons difficult. Thus, it is important, at least until a standard MSC regimen is chosen by regulatory authorities, that stem cell therapy is tailored to each patient rather than generalised. At present, despite the tremendous progress in the field of stem cell therapy, successful MSC-based interventions in the clinic are still too few.

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Preclinical studies assist in deciding which route of stem cell delivery is best and safest whilst ensuring homing and long-term engraftment of an adequate number of cells to improve organ function. There are several crucial decisions that a clinical investigator has to take before undertaking a clinical study. Which is the best MSC injection route for the patient under consideration? Which cell dosage is optimal? What is the correct timing for treatment? Even if cell tracking in the patient is not possible, which readouts (biomarkers) will help in finding out if the MSC therapy is effective? Is a single administration enough? These are only some questions that need to be addressed before proceeding to cell therapy in patients.

Another problem is the unregulated use of MSCs in certain stem cell clinics and the widespread diffusion of medical tourism. Often uncharacterised MSC products are used in patients with the promise of successful outcomes, and there is no control on the *in vivo* fate of the injected cells [1]. As highlighted by Sipp *et al.* and Galipeau *et al.*, illegal and unethical selling of stem cell products, especially MSCs, has generated a stem cell-mess, and efforts are required from both international research communities and medical practitioners to better inform patients about the importance of controlled MSC production and appropriate use in the clinics for their own safety [2, 3].

2. POSSIBLE SOLUTIONS FOR IMPROVING MSC CLINICAL APPLICATION

2.1. Artificial Intelligence

Artificial intelligence is getting more and more integrated into medical decisions. The use of machines and software to analyse and interpret large amounts of data generated by high throughput screening has become an inseparable companion for scientists. Computer-assisted biologically active molecule design is, for instance, an excellent opportunity for drug discovery [4]. There is now great interest in using artificial intelligence in understanding and predicting the outcomes of MSC therapies. For instance, artificial intelligence can improve the accuracy of scaffold fabrication in regenerative medicine for the rapid and accurate generation of bioengineered tissue [5]. Slowly but gradually, artificial intelligence is becoming part of the workflow of stem cell-based intervention in the clinic.

2.2. Engineered MSC-EVs

EVs, as previously described, transport biomolecules from MSCs to target cells. They are the major paracrine effectors present in the secretome. The molecular composition of EVs is dependent on the cell of origin. Therapeutically safe MSCs

will produce therapeutically valid EVs, while MSCs from diseased tissues will emanate EVs capable of negatively modifying the target tissue microenvironment and participate in tumorigenesis. Thus, controlled production of EVs, standardisation in purification methods and characterisation are also requisite for medical therapy. Moreover, the costs related to producing high quality and sufficient EVs for clinical applications are still too high. Still, EVs are regarded as the entities with the propensity to solve most problems associated with cell therapy.

Thanks to bioengineering approaches, it is now possible to modify EV contents, as well as their surface properties in order to amplify their therapeutic potential *in vivo*. Several approaches have been studied to enrich EVs with therapeutic molecules, including co-incubation, electroporation, transfection, sonication and permeabilisation [6, 7]. Engineered EVs can also be used for drug delivery to sites where MSCs, due to physical hindrance, cannot reach. EVs have thus become promising tools for the treatment of human diseases. Several clinical trials regarding the use of EVs have been registered in www.clinicaltrial.gov and are listed in Table **1**, and the results of which are much awaited.

Table 1. Registered clinical trials of treatment using EVs or exosomes derived from MSCs (https://www.clinicaltrials.gov).

NCT no.	Title	Status	Conditions	Interventions	Locations
NCT04602104	A Clinical Study of Mesenchymal Stem Cell Exosomes Nebulizer for the Treatment of ARDS	Not yet recruiting	Acute Respiratory Distress Syndrome	Biological: low, medium or high dose hMSC-Exos; Dosage 1of hMSC-Exos; Dosage 2 of hMSC-Exos; No hMSC-derived exosomes	China
NCT0427698	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes Treating Severe Novel Coronavirus Pneumonia	Completed	Coronavirus	Biological: MSCs-derived exosomes	China
NCT04313647	A Tolerance Clinical Study on Aerosol Inhalation of Mesenchymal Stem Cells Exosomes In Healthy Volunteers	Recruiting	Healthy	Biological: 1X, 2X, 4X, 6X, 8X or 10X levels of MSCs-Exo	China

SUBJECT INDEX

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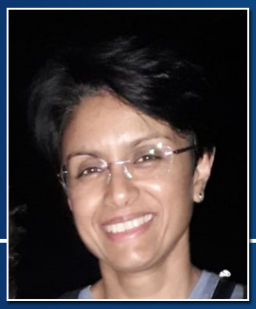

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Dr. Sharmila Fagoonee (Ph.D.) is a researcher at the Institute of Biostructure and Bioimaging of the Italian National Research Council (CNR) and Molecular Biotechnology Center in Turin, Italy. After her B.Sc. (Hons.) in Biology in Mauritius and M.Sc. in Cellular Biology in Bordeaux, France, she got her Ph.D. in Human Cell and Molecular Biology at the University of Turin. Her Ph.D. thesis was centered on analyzing heme and hemoglobin metabolism in mice models. During her post-doc studies, she started working in the field of regenerative medicine, especially on embryonic stem cells and spermatogonial stem cells and their differentiative capacity, after attending a training at BIDMC, Harvard, Boston, USA. Using in vitro and in vivo multidisciplinary approaches, her two current research areas are: 1) Regenerative Medicine: the investigation of the potential of adult stem cell-based therapy for human pathologies in preclinical models and the search for extracellular vesicles-based disease biomarkers and 2) Post-transcriptional regulation of gene expression: finding the molecular mechanisms underlying the role of the RNA binding proteins and splicing factors, ESRPs, in human diseases. She is a coordinator of internships, a scientific committee member, and a lecturer of the Master's course in Stem Cells, Regenerative Medicine, and Cell Factory Management at the University of Turin. Dr. Fagoonee is the chief editor of Minerva Biotecnologica (Minerva Biotechnology and Biomolecular Research) journal and is active as an Editorial Board member as well as a reviewer of several scientific journals.