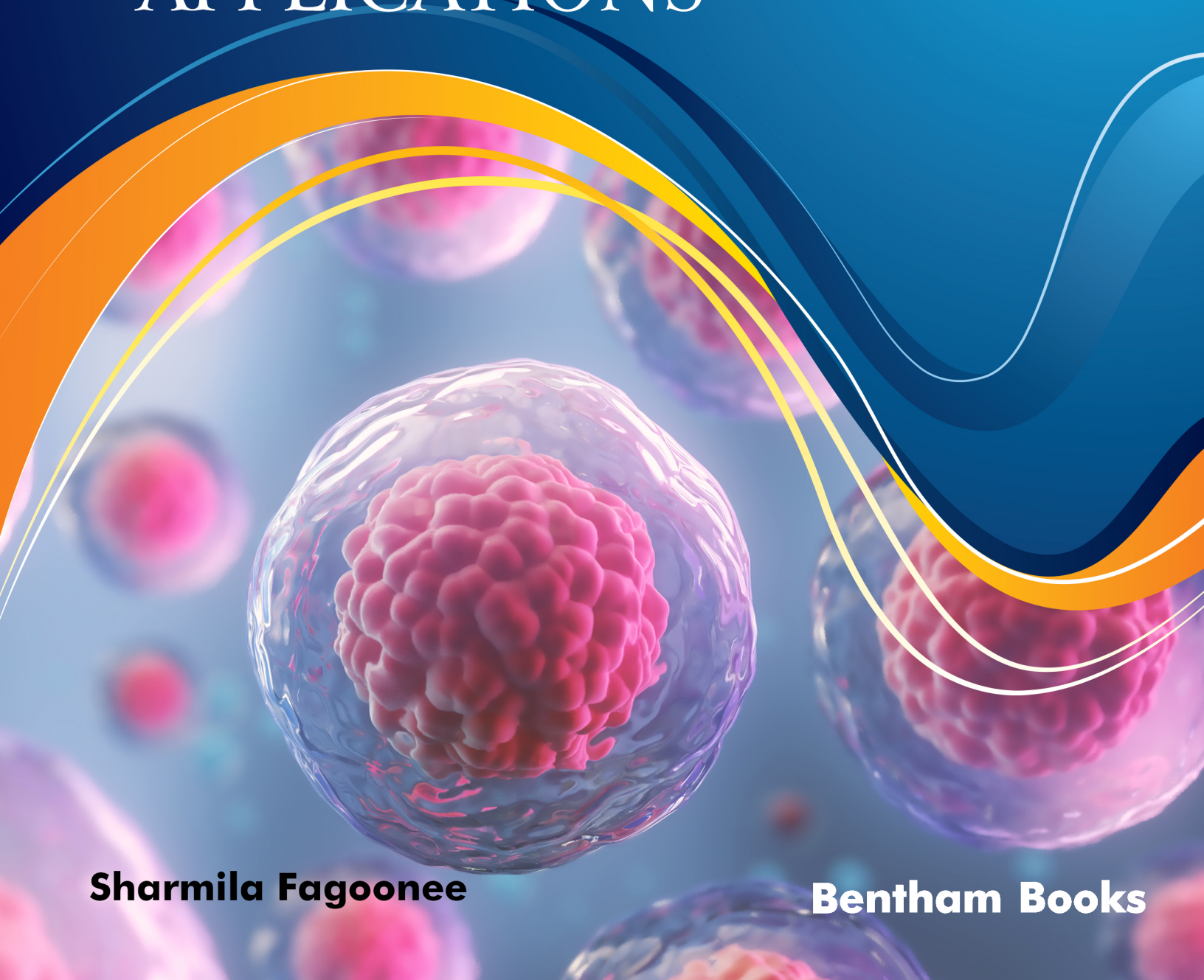
STEM CELL DELIVERY ROUTES

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.

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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

(Table 1) cont.....

NCT Number	Title	Status	Conditions	Interventions	Outcome Measures	Phases	Enrollment	Study Type	Study Period	Locations
NCT04396899	Safety and Efficacy of Induced Pluripotent Stem Cell-derived Engineered Human Myocardium as Biological Ventricular Assist Tissue in Terminal Heart Failure	Recruiting	Heart Failure	Biological: EHM implantation	Target heart wall thickness, Heart wall thickening fraction	Phase 1|Phase 2	53	Interventional	2020-2024	Germany

2. MESENCHYMAL STROMAL/STEM CELLS

After over five decades of research since its first description, MSCs have become one of the most promising and widely investigated stem cells for treating a vast gamma of diseases comprising neurodegenerative, cardiac, hepatic and renal disorders, graft-*versus*-host disease and cancer as well as lately for treatment of Coronavirus Disease 19 (COVID-19) patients [19, 20]. As mentioned above, MSCs are isolated from easily donated tissues, such as bone marrow, umbilical cord or adipose tissue. A minimal criterion has been established for defining MSCs [21, 22]. MSCs are adult stromal/stem cells with plastic-adherent properties, capacity for self-renewal and multipotency (Fig. **1**). In fact, these stem cells can give rise to mesodermal lineages including adipocytes, osteocytes and chondrocytes, as shown by several studies *in vitro* [20]. MSCs are characterised by the presence of certain surface markers, such as CD73, CD90, CD105, CD44 and CD29, and absence of CD34, CD45, CD14, CD11b, CD79a, CD19, and HLA class II [23]. Moreover, MSCs are endowed with immunomodulatory properties and can modulate immune responses. For example, Melief *et al.* showed that bone marrow-derived MSCs enhanced the formation of Regulatory T cells *in vitro*, and skewed the polarisation of monocytes towards an anti-inflammatory (type 2) phenotype [24].

Fig. (1). Properties of MSCs. MSCs can be characterised by their capacity to adhere to plastic, their self-renewal and differentiation potential as well as by the presence of some surface markers.

3. SOME CONSIDERATIONS BEFORE MSC TRANSPLANTATION *IN VIVO*

Before envisaging their clinical applications, it is important to be aware that not all MSCs are equal. Albeit their similar behaviour in culture, MSCs from different sources have varying proliferative and immunomodulatory properties. For instance, higher proliferative capacity and faster osteogenic differentiation were observed with MSCs derived from umbilical cord blood, with respect to those from bone marrow [25]. Moreover, immunosuppressive capabilities may also vary among MSCs derived from adipose tissue, bone marrow and umbilical cord blood [26]. Overall, stem cell types or cell sources are not the only ingredients for a successful clinical stem cell therapy recipe. Other parameters that need to be carefully evaluated in the clinics are the route of stem cell delivery, cell dosage, frequency of cell administration and timing of intervention. Every time MSCs are isolated and prepared, these cells should be accurately characterised in order to determine their potency prior to considering preclinical and clinical studies. MSCs are very sensitive to their surroundings, and their properties may vary from lab to lab because of changes in simple conditions used for their culturing, such as media components, hypoxia, pH, CO_2 and the number of passages.

MSCs show pleiotropic effects and exert their action by either being present at the site of injury/inflammation or distally. For instance, hepatic differentiation of MSCs has been reported *in vitro*. These differentiation events have also been found to occur *in vivo*, albeit more rarely, and can participate in regenerative processes. On the other hand, these cells may not necessarily localise to the specific site of injury to confer therapeutic effects. MSCs can, in fact, release paracrine factors (cytokines, chemokines and extracellular vesicles) which can favour tissue regeneration and repair (Fig. **2**) [27]. The fact that the MSC secretome has quasi-inexistent immunogenicity and therapeutic properties may resolve problems related to whole-cell therapy and has opened up numerous possibilities in the field of regenerative medicine. It may be possible to consider generating current Good Manufacturing Practice (GMP)-compliant reserves of accurately characterised MSC-derived extracellular vesicles (EVs) that can be conserved as ready-to-use biologics for the treatment of patients. Studies in this direction are ongoing.

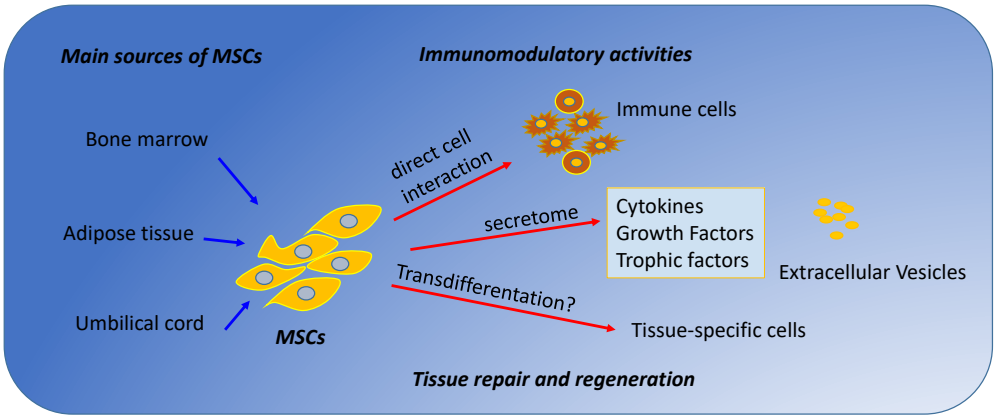

Fig. (2). Mode of action of MSCs. The main sources of MSCs are the bone marrow, adipose tissue and umbilical cord. MSCs play an important role in immunomodulation through the regulation of immune cells' activity, in tissue repair and regeneration through the secretome which is rich in growth factors, cytokines, trophic factors, as well as extracellular vesicles.

4. ROUTES OF STEM CELL ADMINISTRATION

The ideal administration route is the one that ensures that most injected cells safely target the organ under study and confers the highest therapeutic efficacy [28]. Thus, of all the administration routes available to reach the target tissue, the one that gives the least adverse effects whilst considering the disease context and patient conditions, and provides maximal benefit should be chosen. The two main ways for introducing cells into the body include local intervention and systemic infusion. Local delivery involves direct injection in or near the organ of interest, while systemic delivery includes venous or arterial routes. Direct delivery of MSCs to a target tissue could result in higher retention [29]. The stem cell delivery route chosen influences the dynamics of their biodistribution in the body whilst having a profound effect on the mechanism of action, and possibly, on the clinical outcome. Usually, preclinical animal models are employed to explore the best route of delivery, dosage and timing of intervention, as well as assessment of side effects, of stem cells as done for drugs. MSCs are known to confer both local and systemic actions on injured tissues [30]. Both local and systemic routes of injection of MSCs have been tried and successfully adapted to the organ under study in animal models. In clinical practice, local delivery of cells can prove more invasive, and require special medical devices and preparations, with respect to injection in the peripheral vein.

Regarding patients affected by life-threatening diseases, several factors are weighed carefully before the administration of stem cells is performed. It is of utmost importance to perform in-depth clinical evaluations of the MSC recipient for the presence of comorbidities. A factor that may nuance the benefits of cell-

based interventions is pulmonary dysfunction. The latter should be ruled out if stem cell infusion through the peripheral vein is considered as complications related to cell entrapment in the lungs, as the first-pass organ, may ensue. The circulatory system should also be fully functional. Any change in vascular permeability may affect cell extravasation from the circulation and intravasation into the damaged tissue.

Successful engraftment of injected stem cells depends on several factors. Cell vitality is of utmost importance as transplanted cells must adapt to the new environment and survive for enough time to replenish missing cells and restore function *in vivo*. The degree of cell engraftment is also dependent on the receiving organ's microenvironment. In end-stage diseases, when the organ is hardly functional and the microenvironment is mostly composed of scar tissue as well as inflammatory cells, stem cells may not engraft and get quickly cleared from the system. Thus, optimal MSC delivery methods depend on the type of lesions that need to be treated.

CONCLUSION

Several issues still need to be addressed before MSCs can be routinely applied in patient organ regeneration and repair. It is still not clear, for instance, 1) which types and stages of human diseases cannot be treated with MSCs, 2) what are the best sources of MSCs for clinical use or if the source should be chosen target organ-wise, 3) what is the optimal dose of cells and frequency of administration to confer the greatest therapeutic efficacy, 4) what is the best route and timing of cell delivery to ensure maximal cell engraftment in patients, 5) the complete molecular mechanisms by which MSCs exert their therapeutic effects in humans. Insights regarding these aspects will definitely come from future experimental research as well as further well-designed clinical studies.

LIST OF ABBREVIATIONS

COVID-19	Coronavirus Disease-19
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
HLA	Human Leukocyte Antigen
iPSCs	Induced pluripotent stem cells
LVEH	Left ventricular Ejection Fraction
MSCs	Mesenchymal stem/stromal cells

PLGA Poly lactic-co-glycolic acid

RPE Retinal pigment epithelium

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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

models. The iPSC-derived hepatocyte-like cells offer a platform for drug testing as well as for liver disease modelling *in vitro* [22, 23]. However, *in vivo* demonstration of the therapeutic utility of these cells is mainly limited to animal studies. Further work is needed for the clinical translatability of this iPSC-based approach, especially due to reports of possible tumorigenic alterations or non-specific lineage differentiation that may occur *in vivo* by residual undifferentiated iPSCs [24].

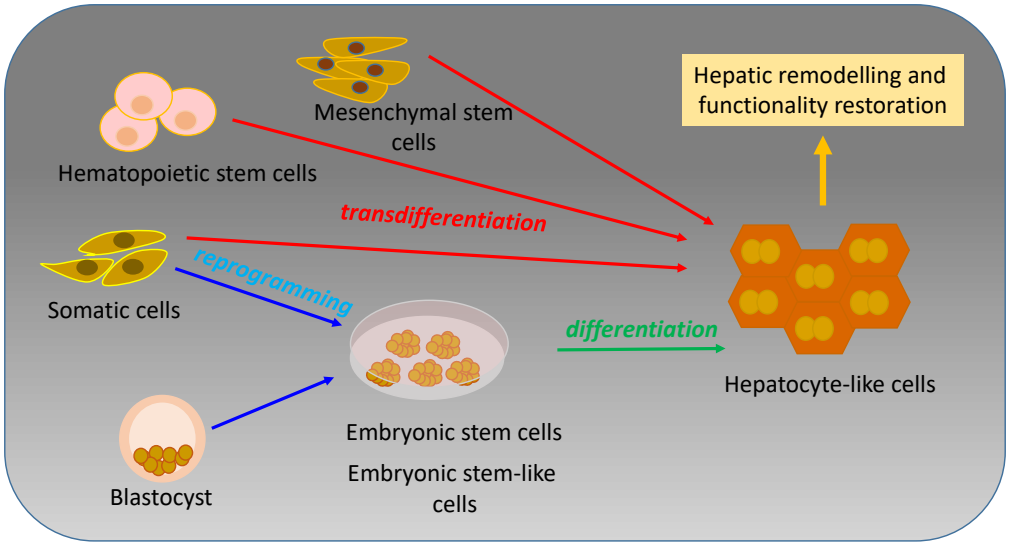

Fig. (1). Main sources of hepatic-like cells for cell therapy of liver disease. Hepatocyte-like cells can be differentiated from pluripotent stem cells (embryonic origin or induced from somatic cells) or from transdifferentiation of adult somatic cells such as fibroblasts or hematopoietic stem cells or mesenchymal stem cells. Hepatocyte-like cells obtained from these sources can participate in hepatic tissue remodelling and repair.

So far, MSCs, due to their availability and low immunogenicity, have been the most employed stem cell type at the preclinical and clinical levels in the context of liver diseases. This chapter will thus describe the main advances regarding MSC-based delivery routes for liver therapy.

2. MSCS IN THE THERAPY OF LIVER DISEASES

MSC-based therapy represents a promising strategy for liver regeneration, and has yielded quite satisfactory results in attenuating liver injury *in vivo*. These cells are attractive as candidate cells for restoring hepatic functionality due to their abundant sources, high proliferative ability ensuring their expandability *in vitro*, multilineage differentiation potential, and lack of ethical restraints compared to

the pluripotent stem cells. MSCs' potentiality to repair the liver and restore function justifies their choice for the therapy of hepatic diseases [25]. Three main sources of MSCs have been investigated for their capacity to regenerate the diseased liver, namely the bone marrow, umbilical cord, and adipose tissue. Usually, MSCs derived from these sources show no significant differences concerning the morphology and immune phenotype [26]. *In vitro*, culture in hepatogenic culture media, containing specific growth factors, such as hepatocyte growth factor (HGF), oncostatin M, dexamethasone, can induce MSCs to adopt a hepatocyte fate [27]. Cells with typical hepatocyte morphology, that is, binuclear cells with prominent glycogen-loaded cytoplasm, and functionality, including abilities to uptake low-density lipoprotein and indocyanine green, secrete albumin, produce urea when challenged with ammonium salts, store glycogen and bear cytochrome P450 activity, have been obtained from MSCs [28]. *In vivo*, a small number of MSCs can migrate towards the injured liver, and promote hepatocyte proliferation and differentiation as well as neovascularisation in the liver, and regulate repair processes in a paracrine way mainly through their anti-inflammatory and immunomodulatory actions, as seen in animal models [29, 30].

However, MSCs from different sources may have unique properties that explain the success of some sources of MSCs in liver diseases in a context-wise manner. For instance, in a comparative study, Sayyed *et al.* showed that umbilical cord-derived MSCs, sorted on the basis of CD34 positivity, was more efficient compared to the bone marrow-derived MSCs in elevating albumin levels as well as reducing liver injury markers (Alanine aminotransferase) and profibrotic genes (Collagen 1a1, TGF-β1, and α-smooth muscle actin (SMA)) in an experimental model of fibrosis in rats [31]. On the other hand, using the carbon tetrachloride (CCl_4)-induced liver fibrosis mouse model with a Metavir fibrosis score of 3, Baligar *et al.* showed that $CD45^+$ bone marrow-derived cells conferred enhanced anti-fibrotic effects and liver repair capacity, compared to adipose tissue-derived MSCs [32]. This was promoted through the induction of elevated expression of MMPs such as MMP-9 and MMP-13, as well as inhibition of HSC activity through the secreted FasL-induced apoptosis of activated HSCs by the $CD45^+$ cells. In contrast, adipose tissue-derived MSCs favoured the secretion of TGF-β1 and insulin growth factor-1 which promoted myofibroblastic differentiation of HSCs and promoted their proliferation [32]. Thus, the source of cells for liver repair and regeneration should be appropriately chosen to achieve optimum therapeutic effects *in vivo*.

The degree of liver damage and its persistence may also play an important role in the recruitment of cells. Interestingly, bone marrow-derived MSCs administered through the peripheral vein of NOD/SCID mice were shown to home to the liver parenchyma in the context of chronic injury generated by multiple intraperitoneal

injections of CCl_4 *in vivo* [33]. In contrast, limited MSC engraftment was observed in an acute injury environment caused by a single dose of CCl_4 injection. The authors showed that hepatocellular differentiation was rarely observed (ranging from less than 0.1% to 0.23%) as judged by barely detectable levels of human alpha-fetoprotein, cytokeratin-18, -19 and albumin in the injured livers subjected to MSC therapy. Thus, the success of MSC-based liver therapy is dependent on multiple factors.

Resident cells with MSC properties have also been isolated and cultured from the human liver. These cells show characteristics of mesenchymal cells, as well as pluripotent stem cells [34 - 36]. There is some evidence of the ability of these cells to differentiate into hepatocyte-like cells *in vivo* after transplantation in several animal models of severe liver diseases as shown by Herrera *et al*. [37]. Najimi *et al.* demonstrated that 10 weeks following intrasplenic transplantation of MSC-like cells isolated from adult human livers (human liver stem cells or HLSCs) into 14-day-old uPA+/+ severe combined immunodeficiency (SCID) mice, positivity for human albumin, prealbumin, and alpha-fetoprotein could be seen in the recipient's livers 8–10 weeks post-injection [38]. The same results were obtained after injection of HLSCs in 6–8-week-old SCID mice following two-thirds partial hepatectomy, where human hepatocyte-like cells were found mainly near the vascular structures 56 days after transplantation. Thus, hepatogenic differentiation of HLSCs occurred upon homing to the mouse liver. These liver-derived MSC-like cells were also shown to engraft, differentiate into hepatocyte-like cells expressing both albumin and ornithine transcarbamylase, and remain for as long as 60 days post-transplantation in the periportal area of the liver of SCID mice subjected to 20% hepatectomy [39]. Our group demonstrated that HLSCs successfully engrafted in the liver and partially restored UGT1A1 enzyme activity in an immunocompromised mouse model of Crigler-Najjar Syndrome type I. Cell therapy promoted survival and improved UGT1A1 enzyme activity in these mice [20].

3. ROUTES OF TRANSPLANTATION

The relatively superficial position of the liver provides access to this organ through different modalities. The MSC delivery routes considered are the direct cell injection routes (intraparenchymal or through the portal vein and hepatic artery) and the indirect routes (intravenous, intrasplenic and intraperitoneal) (Fig. **2**). The systemic route is most commonly used, followed by the hepatic artery and portal vein infusion, intrasplenic delivery, intrahepatic injection and intraportal route [40]. Although MSCs are very promising for the therapy of liver diseases, it was observed that different cell transplantation routes resulted in different therapeutic efficiency. Moreover, there are controversies regarding the

localisation and persistence of MSCs in the body after administration. For instance, MSCs administered *via* the portal vein localise to the liver, whereas when these cells are administered in tissues like muscle and fat pads, detainment and permanence for several weeks in these sites may occur [41]. The route of administration is thus an important factor determining the correct delivery of MSCs to target organs *in vivo*.

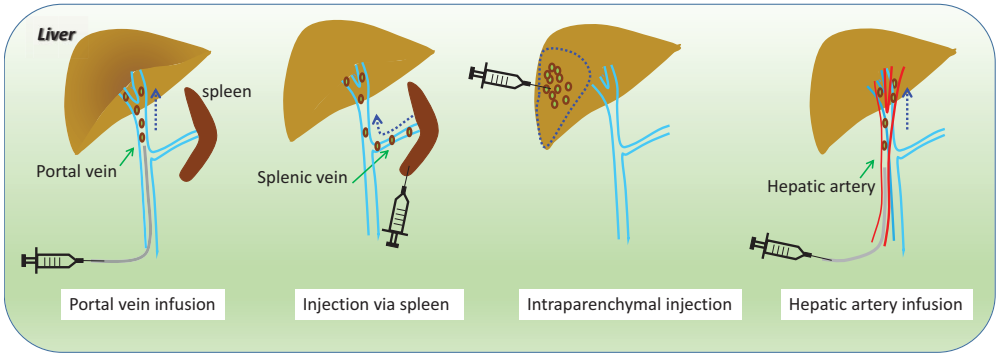

Fig. (2). Routes of cell delivery to the liver. The main cell local delivery routes are shown. Distribution of injected cells occurs over the whole liver, except in the case of intraparenchymal injection.

3.1. Systemic Administration

The intravenous route is the easiest, less invasive and most popular route of infusion of MSCs, and has been employed in numerous preclinical studies as well as in clinical trials. Intravenous injection favours repeated delivery of cells, which remain close to the oxygen- and nutrient-rich vasculature after extravasation into the liver [42]. Regarding the latter, stem cells can be infused into the peripheral vein of the arm of the patient like any simple pharmacological substance. In rodents, cells are infused through the tail vein. Numerous preclinical studies have demonstrated that treatment with MSCs promotes functional recovery in rodent models of liver injury. In almost all cases, the transpulmonary "first-pass" attenuation effect was observed with systemic stem cell injection [43]. MSCs get entrapped in the first micro-capillary network they encounter, from where they can participate in immunomodulation by paracrine mechanisms [44]. Lung entrapment of MSCs is induced by space restriction because cultured MSCs are larger than the diameter of the lung micro-capillaries [41]. A small percentage of cells delivered through the peripheral vein may thereafter migrate to the diseased organ due to their leukocyte-like properties. Factors such as stromal-derived factor (SDF)-1, basic fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), released by the injured tissue guide MSCs to the target area. MSCs injected through the intravenous route, however, do not survive in mice lungs beyond 24 hours after cell injection, and disappear without migrating

to the injured organs, probably due to clearance by immune cells. Eggenhofer *et al.* injected DsRed-labelled bone marrow-derived MSCs in mice undergoing liver ischemia-reperfusion injury. Analysis of tissues after 24 hours revealed that living MSCs localised mainly in the lungs, but not in the injured livers [41]. Moreover, the infused MSCs did not survive beyond 24 hours *in vivo.*

MSCs entrapped in the lungs may also have beneficial effects on the damaged organ, even distantly. This can be attributed to factors (cytokines, chemokines) or extracellular vesicles (EVs) containing bioactive molecules in reprogramming the non-injured liver cells towards a regenerative fate through immunomodulatory activities, amelioration of the injury and anti-fibrotic effects [45]. Bone marrow-derived MSCs conditioned media were found to favour an immunosuppressive environment and to suppress fibrogenesis and necroinflammation in the CCl_4-induced mice model of chronic liver injury by acting on hepatocytes, macrophages, $CD4^+$ T lymphocytes, and HSCs [46]. Several studies have highlighted the contribution of MSC-derived EVs in contributing to the amelioration of liver injury. For instance, in the lethal murine model of hepatic failure induced by D-galactosamine/TNF-α, systemic administration of MSC-EVs decreased hepatic necrosis and increased the survival of mice [47]. Anti-fibrotic effects were also observed with MSC-EV injection in preclinical models [48].

The extent of liver damage is probably another determinant in the homing and engraftment of MSCs *in vivo*. It has been shown that MSCs administered through the peripheral vein show enhanced migration into the liver parenchyma in chronic injury *in vivo*, compared to the limited engraftment observed in an acute injury environment [26]. Importantly, there is need to further understand the interaction between transplanted MSCs and the injection route. Some studies have shown that MSCs have pro-coagulant activity and express tissue factor that plays a key role in the activation of platelets [42]. For instance, Oeller *et al.* infused bone marrow-derived MSCs (6×10^6 cells/kg) with low tissue factor expression or umbilical cord-derived MSCs with elevated tissue factor expression through the tail vein of rats [49]. One hour post-infusion, massive intravascular thromboembolism was seen in the lung, liver and spleen of umbilical cord-derived MSCs-injected rats, with respect to bone marrow-derived MSCs-injected ones [49]. Thus, thrombogenic events may ensue following the systemic delivery of MSCs. Other studies, however, failed to show such activity. Netsch *et al.* showed that MSCs had no effect on platelet activation and thrombus formation, but these cells could actively inhibit platelet activation by CD73 activity responsible for antithrombotic adenosine formation [50]. Thus, in order to improve the therapeutic effects of MSCs, any thrombogenic activity should be accurately detected and monitored.

3.2. Direct Delivery Through The Portal Vein or Hepatic Artery

The liver has two blood supplies through the portal vein which brings in venous blood, and the hepatic artery distributes arterial blood in this organ [51]. Portal vein is an often-used route for stem cell transplantation due to multiple vascular accesses. Studies in rats have shown that cells delivered into the hepatic sinusoids through the portal vein most effectively integrate the liver parenchyma. This involves cell accumulation in the hepatic sinusoids, the passage of cells into the space of Disse which requires retraction of the sinusoidal endothelial cells, followed by transient disruption of the gap junction and tight junction between adjacent hepatocytes in the proximity of the transplanted cells, to allow the latter to insert themselves between host hepatocytes [52]. In patients, the portal vein is reached either by trans-jugular or trans-hepatic percutaneous approach. MSCs injected through the portal vein bypass the transpulmonary route and its related complications to engraft in the liver. MSCs translocate to the liver and get trapped in the hepatic sinusoids, probably due to sinusoid occlusion or receptor-mediated interactions between transplanted cells and LSECs and matrix components [53]. Retraction of the LSECs allows the passage of transplanted cells into the space of Disse after which there is a transient disruption of cell-cell junctions between neighbouring hepatocytes, hence allowing the insertion of the transplanted cells among host hepatocytes. The bile canaliculi and gap junctions reform after 72 hours *in vivo* [54]. Interestingly, adipose tissue-derived MSCs injected through the portal vein improved microcirculation in the fibrotic liver as well as function with respect to tail vein injection. Liver fibrosis caused by CCl_4 injection in rats was reduced through MSC-induced downregulation of VEGF expression [55]. Another study examining the engraftment of MSC in the liver concluded that MSCs administered *via* the portal vein showed higher rates of engraftment compared to the vena cava delivery [56]. However, portal vein administration becomes risky in the presence of ascites and can cause bleeding, diathesis and coagulopathy [57].

In humans, it is also possible to infuse stem cells through the hepatic artery into the liver *via* trans-femoral, trans-radial and trans-brachial approaches. The hepatic artery enters sinusoids adjacent to portal tracts, and forms a capillary plexus around the bile ducts [51]. Interestingly, increased engraftment efficiency was found upon intra-arterial delivery compared to intravenous delivery [58].

3.3. Intrasplenic Injection

Blood travelling through the splenic vein may go to the left lobe or the right lobe of the liver [51]. The ease of cell transplantation and high natural blood flow makes the spleen an ideal ectopic *route* for delivering stem cells to the *liver*. In

rodents, the splenic injection of stem cells is easy. The spleen is exteriorised and cells are injected in the lower pole, after which the latter is ligated in order to prevent cell leakage or bleeding [59]. The spleen then serves as a conduit for homogeneously directing MSCs into the liver parenchyma of all liver lobes through accumulation in the hepatic sinusoids, as happens in the case of intraportal injection, described above [60].

Bone marrow-derived MSCs injected intrasplenically, under ultrasound guidance, in the CCl_4-induced liver fibrosis rat model were shown to ameliorate liver function [61]. This study also demonstrated that cells delivered intravenously inhibited the inflammatory response more effectively than the intrasplenic injection, but both intravenous and intrasplenic BM-MSC injections conferred an anti-fibrotic effect, as shown by the reduction of pro-fibrogenic factor expression (TGF-β1 and α-SMA) and the increase in anti-fibrogenic factor expression (cytokeratin-18 and HGF), complemented by histopathological evaluation (Masson's trichrome staining) [61]. In humans, the spleen can be accessed by direct injection into the splenic artery through a catheter inserted into the femoral artery [62].

3.4. Intraparenchymal Route

Liver parenchyma is composed mainly of hepatocytes arranged in plates. The parenchymal cells undergo slow turnover, but are capable of entering a proliferative state upon liver injury and restore tissue loss [63]. In animal models, we have shown that injection directly in the liver parenchyma successfully delivers stem cells to the liver. The engraftment, in this case, was regional and heterogeneous, and localised exclusively to the injected liver lobes [20, 64]. The cells could be found up to 1 month after injection. Baertschiger *et al.* injected bone marrow-derived MSCs in a mouse model of acute liver injury induced by two-thirds hepatectomy [65]. The efficiency of intrasplenic and intrahepatic delivery of stem cells was compared. Interestingly, stable engraftment in the liver was achieved after intrahepatic injection but not after intrasplenic injection of MSCs (0.5 to 1×10^6 cells). The MSCs that remained up to 8 weeks in the liver following intrahepatic delivery maintained a mesenchymal phenotype and expressed vimentin and α-SMA, but showed no expression of hepatic markers. Successful engraftment of injected MSCs by intrahepatic route was also observed in larger animals. Chamberlain *et al.* injected human MSCs through an intraperitoneal or intrahepatic route into preimmune fetal sheep without liver injury [66]. The intrahepatic injection more efficiently generated hepatocytes following 70 days of xenotransplantation (13% of the hepatocytes) with respect to the intraperitoneal injection [66]. With intrahepatic delivery, hepatocytes were

distributed throughout the liver parenchyma of the injected lobe, while the intraperitoneal injection caused a preferential periportal localisation of human hepatocytes.

The fact that stem cells surviving upon intraparenchymal injection do not migrate to the other lobes of the liver but remain localized to the injection site offers the advantage of not requiring complicated procedures for cell tracking. Knowing in which lobe the cells were injected, it is easy to analyse the fate of these cells. On the other hand, however, if the aim is to restore liver function over the whole liver, then this is not possible through a single-lobe injection. Cells need to be injected into multiple liver lobes in order to optimise their homing and increase their therapeutic effects. Direct cell injection into the liver parenchyma is, however, not exempt from risks. There may be an inadvertent entry into the hepatic vein that carries the cells off to the pulmonary capillaries, hence inducing emboli, and organ infarcts [67].

In a study conducted by Zhao *et al.* intravenous, intrahepatic and intraperitoneal injection routes were compared in the treatment of liver fibrosis, using MSCs. It was found that the intravenous route was the most efficient in increasing the serum levels of the anti-inflammatory cytokine, IL-10, and decreasing those of the pro-inflammatory cytokines, IL-1β IL-6, TNF-α, TNF-β, which induced a reversal in liver fibrosis and improved function, compared to the other two routes [68]. Bone marrow-derived MSCs endured in liver tissues when injected through the intrahepatic artery, indicating that these MSCs engrafted but did not differentiate into hepatocytes. Moreover, the intraportal infusion was found to be more efficient than the peripheral route in clinical trials.

3.5. Intraperitoneal Administration

Some studies have shown that MSCs injected intraperitoneally can home to sites of injury. The intraperitoneal route exposes the cells to a nutrient-rich and hemodynamically stable environment [69]. Recently, Putra *et al.* compared the intravenous and intraperitoneal MSC delivery routes in rats subjected to CCl_4 treatment to induce acute liver failure [40]. One million MSCs were introduced through the tail vein or intraperitoneally, and liver functionality was assessed after up to 5 days post-cell injection. There was a marked decrease in the liver damage indicators, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, and bilirubin levels, and an increase in VEGF levels which correlated with the extent of migration of MSCs. More specifically, the levels of liver injury enzymes decreased, while VEGF was significantly higher in the intravenous group with respect to the intraperitoneal group. Analysis over a 5-day period revealed that the intravenous route gave better results than the

intraperitoneal injection. In particular, MSCs could migrate faster to sites of injury when delivered by intravenous route compared to intraperitoneal delivery, and this could be attributed to the leukocyte-like features of these cells. However, in order to confer therapeutic effects, MSCs delivered intraperitoneally do not necessarily have to migrate to the site of injury. It was shown that MSCs co-embedded in biomaterials (poly(lactic acid-glycolic acid) (PLGA) scaffolds) with hepatocytes, followed by transplantation into the abdominal cavity, could provide metabolic support and paracrine factors to enhance liver regeneration and survival in an acute liver failure mouse model [70]. Recently, human umbilical cord-derived MSCs administered systemically or intraperitoneally (encapsulated in alginate capsules) in a CCl_4-induced model of liver cirrhosis in rats reduced collagen deposition and fibrosis, and enhanced liver recovery [71]. The authors showed that with encapsulated MSCs, the recovery rate (in dynamics of changes in liver epidermal growth factor (EGF) expression and fibrosis levels) was faster especially at 3 weeks after cell administration compared to the systemic cell injection mode. Thus, in animal models, intraperitoneal injection of MSCs is a useful resource, which has been hitherto scarcely explored in humans, except for some clinical studies on ovarian cancer (*clinicaltrials.gov* NCT02530047).

The rationale for MSC transplantation in patients in clinical studies stemmed from data obtained from animal studies showing that MSCs transplanted into intrahepatic or extrahepatic sites could engraft and proliferate in the liver or other sites. Moreover, these cells could restore enzymatic activity, secrete hepatic proteins such as albumin, improve lipid metabolism, as well as improve survival of animals with induced acute liver failure [20, 67, 72]. For instance, injection of liver MSCs or conditioned media in a mouse model of fulminant hepatic failure attenuated mortality and enhanced liver regeneration [37]. It is possible to perform repeated MSC injections over time without deleterious consequences on the hepatic vasculature or liver sinusoids, only by the intravenous route. The other injection routes involve invasive procedures that can be performed only once. Transient portal hypertension may ensue cell transplantation, which is dependent on injected cell size-induced occlusions in the periportal vasculature. This is usually temporary and is resolved by the opening of alternative vascular channels or by the integration of cells into the liver parenchyma a few hours post-injection [67]. This aspect should not be neglected when considering cell transplantation in patients with portal hypertension or severe liver diseases, such as cirrhosis. The blood flow velocity is important in ensuring the successful delivery, persistence and engraftment of MSCs. If the blood flow is high, cells may be destroyed by shear forces, while if the flow is reduced, cells have the time to attach to ECM components and extravasate through the endothelium to settle into the target organ.

Regardless of the route of administration, human liver-derived MSCs, for example, can home to and engraft in the damaged mouse liver. In general, the injected cells persist in the recipient mice's liver for 30–56 days [34]. In humans, it is difficult to assess the persistence of injected cells over time, apart from the dosage of liver functionality biomarkers. Importantly, by altering the site of injection, we can generate new hepatocytes in different hepatic zones with the ability to synthesize different proteins/enzymes [66].

4. MSC DELIVERY ROUTES IN CLINICAL TRIALS

Based on the promising results generated from animal models, MSCs were delivered *via* different routes in clinical trials. The intravenous route was most commonly employed with respect to portal vein or hepatic artery delivery and intrasplenic injection. A systematic review performed by Yang *et al.* pointed out the fact that the latter was the least used mode of MSC infusion [19]. In this study, 22 clinical trials were analysed to determine which liver diseases MSC treatment was most recommended. Of these clinical trials, 14 chose the intravenous cell delivery route, while in only 2 intrasplenic route was employed. MSCs were delivered through the hepatic artery in 5 clinical trials. Amer *et al.* injected bone marrow-derived MSCs in end-stage liver cell failure due to chronic hepatitis C *via* the portal vein or the spleen and compared the outcomes according to the Model for End-Stage Liver Disease (MELD) score, fatigue scale and performance status [73]. Interestingly, there was a significant improvement, in the MSC-injected patients, regarding ascites, lower limb edema, and serum albumin with respect to patients who received traditional supportive treatment. There was no difference between intrahepatic and intrasplenic groups. The hepatic artery route was also explored in several clinical studies. For instance, the short-term efficacy and long-term prognosis of liver failure patients caused by hepatitis B were assessed following single transplantation with autologous bone marrow-derived MSCs transfused into the liver through the proper hepatic artery [74]. No serious side effects or complications were observed, and there was a marked improvement in liver functionality and MELD score of patients in the transplantation group 2-3 weeks after MSC transplantation *versus* the control group. The direct delivery routes are preferred as off-target cell accumulation can be bypassed. However, in clinical studies, it is difficult to compare the efficacy of cell delivery modes. Patient-specific decisions are taken in the clinical setting, in order to avoid risks surging from the potential presence of thrombosis in the portal vein, for instance.

The safety of liver-derived MSCs injection *via* a percutaneous transhepatic portal catheter was also evaluated in a Phase I/II clinical trial involving pediatric patients with urea cycle disorder and Crigler-Najjar syndrome [75]. In the patients with

urea cycle disorder, cell transplantation resulted in a significant increase in urea production at 6 months post-treatment with respect to baseline. In 2 patients with Crigler-Najjar syndrome, cell infusion with daily phototherapy reduced total serum bilirubin levels by approximately 20%. In another recent study, the safety and feasibility of percutaneous administration of HLSCs into the liver parenchyma of infants with inherited neonatal-onset hyperammonemia were evaluated [76]. Ammonia concentration remained stable in all treated patients despite the increased dietary protein intake. These studies show that the transplanted human liver-derived MSCs somewhat restored functionality in cases of inborn errors of metabolism.

5. MODE OF ACTION OF DELIVERED MSCS IN LIVER DISEASE

Preclinical studies have demonstrated how MSCs can exert a plethora of therapeutic effects, through multiple mechanisms of action, in the setting of liver diseases. MSCs act by coordinating dynamic and integrated hepatic reparative processes through hepatogenic differentiation or indirectly by suppressing immune reactions, fibrogenesis, and hepatocellular apoptosis/necrosis, whilst stimulating liver regeneration [18]. For instance, MSCs transplanted *in*trasplenically into CCl_4-treated mice engrafted in the liver and underwent differentiation into cells with typical hepatocyte morphology expressing human albumin and alpha-1-anti-trypsin [77]. Banas *et al.* further showed that engrafted adipose tissue-derived MSCs that had undergone hepatogenic differentiation attenuated hepatocyte necrosis and promoted liver regeneration in mice with acute liver failure. MSC injection restored liver functions as ammonia and purine metabolism, decreased liver injury markers and improved survival of mice [78]. On the other hand, contrasting results were obtained by Di Bonzo *et al.* who demonstrated that the MSCs that homed to the liver of sublethally irradiated NOD/SCID mice, subjected to acute or chronic liver injury by a single or chronic intraperitoneal injection of CCl_4, respectively, rarely underwent hepatogenic differentiation [33].

The paracrine mechanism of action of MSCs has gained much interest lately in the field of liver repair and regeneration. MSCs can release various bioactive molecules in free forms (cytokines, growth factors) or enclosed within EVs which can induce disease regression. It was shown that MSCs can secrete molecules that reduce liver inflammation and fibrosis, and replenish the functional hepatocytes, hence counteracting alterations in the hepatic architecture [79]. Several soluble factors, such as HGF, TNF-α and IL-10, produced by MSCs can induce apoptosis of activated HSCs and cause a decrease in collagen synthesis [80].

Interestingly, MSCs can also modulate tissue immune responses through direct cell-to-cell interaction or paracrine action. MSCs, through the regulation of innate and adaptive immune responses, and by modulating the proliferation and function of T and B lymphocytes and of Natural Killer cells as well as the maturation of dendritic cells can generate a tolerogenic environment for cell engraftment *in vivo* [81]. For instance, Shi *et al.* conducted a pilot study to assess the safety and clinical feasibility of umbilical cord-derived MSC treatment in liver transplant patients as a therapeutic intervention for liver allograft rejection [82]. Intravenous infusion of MSC suppressed acute allograft rejection by inducing the formation of Regulatory T cells at the expense of Th17 cells, hence dampening alloreactive responses in the liver transplant recipients. However, MSCs barely engraft in cases of severe liver diseases, where inflammatory and toxic conditions cause irreversible damage to the niche.

MSCs can be induced to adopt hepatocyte-like features *in vitro* by culturing with HGF, fibroblast growth factor-2/4, EGF, oncostatin M, dexamethasone, insulin-transferrin-selenium, or nicotinamide, for example, before transplantation [83]. These hepatogenic MSCs were also investigated for their therapeutic effects on the liver. Hepatogenic MSCs homed at higher rates to the liver and reduced CCl_4-induced liver fibrosis to a greater extent compared to undifferentiated MSCs [84]. However, transplantation of undifferentiated MSCs gave better results in terms of liver function maintenance compared to hepatogenic MSCs [85].

Interestingly, some studies have shown MSC fusion with hepatocytes *in vivo* as another mechanism to promote liver regeneration [86]. For instance, the fusion between preconditioned bone marrow-derived MSCs and hepatocytes occurred in the periportal region of the liver lobule after partial hepatectomy and the intrasplenic delivery of cells in irradiated immunodeficient mice [87]. In case of severe diseases, cell fusion events can occur only in the presence of a sufficient number of viable cells. Overall, the multifaceted action of MSCs puts these cells under the limelight for liver regeneration and repair.

CONCLUSION

Stem cell therapy is a novel approach for the treatment of liver diseases. Preclinical studies have largely served as a guide for dissecting the mechanism of action and understanding the role of MSC-based therapies for liver diseases in the clinic, due to the difficulties in analysing MSC fate in patients [88]. However, low migration, poor cell survival and engraftment in the injured liver, and the risk of carcinogenesis and viral transmission are problems that are still encountered in preclinical studies. Thus, strategies to enhance the therapeutic efficacy of injected MSCs or their derivatives are much awaited and are currently being investigated.

In conclusion, in order to broaden the applicability of MSC-based cell therapy for chronic liver disease, improvements in several steps are required starting from the cell source to the route of cell delivery. Standardisation of the cell source, culture conditions, cell dosage, timing of injection, administration route, are among the starting points that require immediate attention in order to enhance the therapeutic outcomes of MSC treatment in the clinics.

LIST OF ABBREVIATIONS

CCl_4	Carbon tetrachloride
CXCL	CXC ligand
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cell
EV	Extracellular vesicle
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HLSCs	Human liver stem cell
HSC	Hepatic stellate cells
IL	Interleukin
iPSC	Induced pluripotent stem cell
KC	Kupffer cell
LSEC	Liver sinusoidal epithelial cell
MELD	Model for end stage liver disease
MMP	Matrix metalloprotease
MSC	Mesenchymal stromal/stem cell
PDGF	Platelet-derived growth factor
PLGA	Poly (lactic acid-glycolic acid)
SDF	Stromal-derived factor
SMA	Smooth muscle actin
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloprotease
TNF	Tissue necrosis factor
VEGF	Vascular endothelial growth factor

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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.

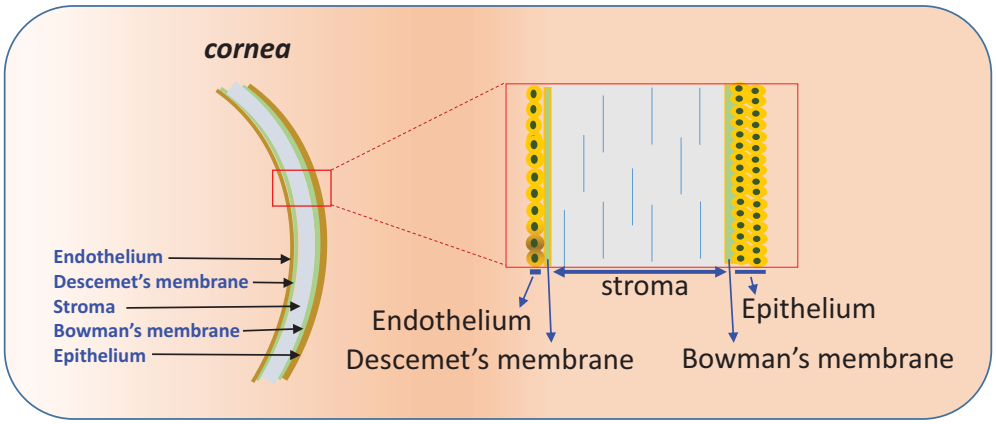

Fig. (1). The structure of the cornea. The cornea consists of 5 layers: (outer part) an epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (inner part).

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

employed in an attempt to prevent transplant rejection and increase the duration of graft survival in a rabbit model of corneal allograft rejection. It was found that injection of MSCs into the graft junction during surgery increased inflammation (corneal oedema with increased thickness, higher level of leukocyte infiltration) [25]. Graft survival was also reduced compared to sham controls. MSCs from other sources have also been investigated in the setting of ocular surface injuries. For instance, corneal stromal MSC-like cells express specific markers (such as the cytokeratins, CK3, CK12, and CK19) of the cornea compared to other sources of MSC, can suppress inflammatory response and reduce corneal scarring after injury [26]. Although not yet extensively employed for corneal regeneration, an *ex vivo* study has demonstrated the capacity of dental pulp-derived MSCs to enhance the ocular surface reconstruction in a rabbit model of total limbal stem cell deficiency by decreasing corneal opacity and neovascularisation [27]. These data encourage further studies on the use of MSCs derived from various sources for ocular surface repair.

The appropriate MSC source should thus be chosen for cell therapy in order to maximise their therapeutic effects in the target organ. MSCs are cells with low immunogenicity due to the fact that under the resting state, these cells express minimal levels of the Major Histocompatibility Class (MHC) II proteins, but no CD40, CD40 ligand, CD80 and CD86 co-stimulatory molecules [28]. Importantly, post-operative bone marrow-derived MSC injection in a rat model of corneal allograft rejection resulted in prolonged corneal allograft survival compared to vehicle-treated rats subjected to transplant surgery. Similarly, graft-*versus*-host disease (GVHD) is a contraindication following ocular allogeneic stem cell transplantation in a considerable number of patients (40-60%) [29]. In these cases, the immunomodulatory properties of MSCs may come to the rescue and protect from GVHD. In fact, there are several reports of clinical trials using MSCs to both prevent and treat GVHD [30]. For example, treatment of ocular GVHD with human MSCs by subconjunctival injection efficiently reduced corneal inflammation and squamous metaplasia [29]. Although allogeneic MSC therapy is beneficial, any small sign of adverse effects should not be neglected. Unfortunately, uncontrolled treatments offered to patients in stem-cell clinics can lead to devastating outcomes, like those observed in the recent case of MSC injection for the treatment of retinal disorders. Autologous adipose tissue-derived MSCs, injected intravitreally in age-related macular degeneration patients were found to cause severe bilateral visual loss due to retinal detachment and increase in intraocular pressure [31]. For successful translation to the clinic, it is important that multiple sources of MSCs, backed by preclinical studies, are explored in order to develop the most efficient, standardised and cost-effective treatment for ocular surface diseases.

3. MSC DELIVERY ROUTES

The therapeutic efficacy of MSCs is largely influenced by the injection routes. There is, however, a lack of consensus regarding the optimal route of MSC administration to achieve the maximum therapeutic effect. The main stem cell delivery routes for corneal repair are intravenous, subconjunctival, intrastromal, and periorbital, as well as a topical application (Fig. **2**). The intraperitoneal route can also be used but is not discussed herein.

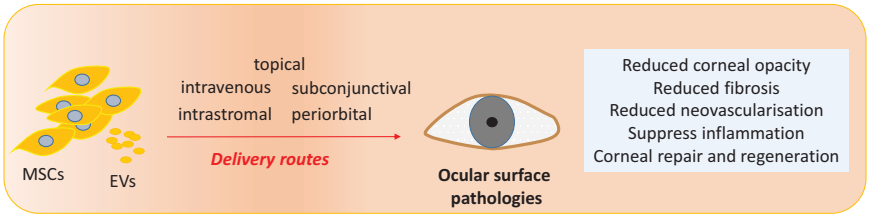

Fig. (2). MSC delivery routes to the cornea. Mesenchymal stromal/stem cells (MSCs) and their derivatives such as extracellular vesicles (EVs) can be administered to the ocular surface in different ways: topical, intravenous, subconjunctival, intrastromal and periorbital. This leads to improvements in corneal function.

3.1. Intravenous Route

The intravenous route is a practical way of injecting stem cells targeted to injured organs in animal models or patients. Systemically administered MSCs have been demonstrated to home to injured tissues following intravenous injection in several models of internal organ damage, including brain, lung and liver. Interestingly, even for external tissues like the ocular surface, systemically administered MSCs were shown to home to thermal cauterization-injured corneas but not to the healthy ones in mice, and also to accelerate wound closure in another mouse model of corneal injury created using an Algerbrush [32, 33]. MSCs injection reduced corneal opacity, tissue fibrosis and inflammation compared to controls. Importantly, intravenous administration promotes the survival of MSCs journeying in the nutrient and oxygen-rich vascular environment, and when MSCs extravasate, they are usually found in the vicinity of the vasculature, hence facilitating their tracking [32]. However, barely 1% of systemically infused MSCs reach the target tissue due to entrapment in the lungs, as previously described [34]. Thus, to obtain the desired therapeutic outcome, injection of huge quantities of cells is required, making this cell delivery method less promising for ocular surface diseases.

3.2. Subconjunctival Injection

The subconjunctival injection is a local administration route that permits targeted delivery of MSCs and circumvents the pulmonary first-pass effects, while dampening potential off-target homing as well as immunomodulatory activities. MSCs have been administered subconjunctivally to analyse the effects on corneal wound healing in the acute stage of sodium hydroxide-induced alkali-burn injury in rat [35]. MSC injection accelerated corneal wound healing and reduced the neovascularisation area compared to the control group. Moreover, recruitment of $CD68^+$ cells at the sites of injury was reduced and the expression of macrophage inflammatory protein-1 alpha (MIP-1α), TNF-α and VEGF were found at lower levels in the MSC-treated corneas with respect to controls. Thus, the subconjunctival injection of MSCs, whilst being minimally invasive, efficiently promotes the recovery of the corneal epithelium. Importantly, the subconjunctival injection can be easily done by ophthalmologists in clinical practice.

3.3. Intrastromal Injection

Intrastromal cell injection is a minimally invasive, painless procedure and therapeutically practical approach that can be easily performed by ophthalmologists. In preclinical models, it was shown that bone marrow-derived MSCs transplanted intrastromal into the keratocan-null mice persisted in the cornea without inducing any immune or inflammatory responses and restored the expression of the missing protein [36]. The transplanted MSCs initially localised to the site of injection and then successively migrated to the corneal periphery to spread homogeneously in the entire cornea after 4 to 6 weeks of injection. In another study, human corneal stromal keratocytes were injected intrastromally in rat corneas with early opacity induced by irregular phototherapeutic keratectomy [37]. The clarity and thickness of the cornea were restored in the cell-injected corneas with respect to the vehicle-injected injured corneas. The intrastromal injection route is safe and efficient as shown by these results.

3.4. Periorbital Delivery Of Mscs

The therapeutic potential of MSCs was investigated in a murine model of inflammation-mediated dry eye caused by the intraorbital injection of concanavalin A (21). Periorbital administration of human or mouse bone marrow-derived MSCs decreased $CD4^+$ T cells infiltration as well as the levels of IL-2 and IFN-γ inflammatory cytokines in the intraorbital gland and ocular surface. Importantly, aqueous tear production, as measured by phenol red thread test, and the number of conjunctival goblet cells, revealed by Periodic Acid Schiff staining

of the conjunctiva, increased. Corneal epithelial defects were reduced upon MSC injection compared to controls. Thus, periorbital injection of MSCs protected the ocular surface by dampening inflammation in dry eye syndrome in the mouse. In this case, MSCs conferred therapeutic effects without long-term engraftment.

3.5. Topical Administration

The position of the cornea facilitates the topical application of stem cells. Several studies have reported the topical application of MSCs, resuspended in a different medium, to the injured cornea. MSCs applied directly to the ocular surface provided anti-inflammatory and anti-angiogenic activities in chemically burned corneas of rats [38]. MSCs in fibrin gel or seeded onto the amniotic membrane were also grafted onto the injured corneas [39, 40]. Umbilical cord-derived MSCs were transplanted *via* intrastromal injection or applied in fibrin plug in a mouse model of congenital and acquired corneal opacity associated with the loss of collagen V [1]. In this study, the intrastromal injection was employed in the congenital model, and fibrin application was used in the acquired model [1]. Interestingly, intrastromal injection of umbilical cord-derived MSCs increased corneal thickness and reduced corneal opacity in the congenital mouse model, but no effect was seen with MSC application in the acquired scarification model. Thus, the cell delivery mode is of utmost importance in ensuring the proper action of injected cells at the target site.

The non-invasive, topical application of MSCs may offer several advantages such as direct delivery of a concentrated population of MSCs and associated paracrine signals to the target area, fast wound healing process and avoidance of allograft rejection [32]. However, for topical application of MSCs to confer optimal therapeutic effects, a carrier that does not alter the properties of MSCs is required to ensure the persistence of cells in the injury-altered microenvironment.

4. MSC DELIVERY ROUTES IN CLINICAL STUDIES

Few clinical studies are ongoing or have been completed on the use of MSCs for corneal regeneration as shown in the registry at www.clinicaltrials.gov. For instance, Calonge *et al.* conducted a 6 to 12 months proof-of-concept, randomised, and double-masked pilot trial with allogeneic bone marrow-derived MSCs for the treatment of limbal stem cell deficiency. MSCs were grown on the human amniotic membrane, and during transplantation, the membrane was applied with the cells in contact with the wound, followed by suturing of the transplant to the bare sclera. This procedure showed that MSCs transplantation

was safe and improved corneal epithelial damage caused by limbal stem cell deficiency [41]. Importantly, MSC source, mode of stem cell administration, dosage, and timing of injection need to be standardised to compare the clinical results.

5. MODE OF ACTION OF DELIVERED MSCS IN OCULAR SURFACE INJURIES

Some studies have shown that MSCs can interface with the injured tissue directly *via* cell-cell contact [3]. Intercellular mitochondrial exchange can take place between MSCs and the target cells to mediate repair and enhance the survival of the injured cells [42]. This biological event is seen both under physiological and pathological conditions. For instance, mitochondrial transfer was seen in renal tubular cells to bone marrow-derived MSCs by tunneling nanotubes, suggesting that this passage of organelles could mediate renal differentiation of the MSCs in the co-cultures [43]. The mitochondrial transfer was also demonstrated between MSCs and corneal epithelial cells [44]. The mitochondrial transfer protected the corneal epithelial cells against oxidative stress-induced mitochondrial dysfunction. This mechanism could also be responsible for the protection of cells at the ocular surface following MSC injection, and requires further investigation.

The paracrine action of MSCs has received much attention in recent years, as MSCs can confer therapeutic effects albeit their failure to reach the target tissue. Thus, the interest surrounding MSCs has shifted to their paracrine function. A positive therapeutic response can be achieved irrespective of whether the cells reach the target organ or not, especially when MSCs are delivered systemically and undergo lung entrapment. The MSC secretome is rich in anti-inflammatory factors, cell-mobilisation factors, and growth factors that can travel in the vascular system to reach the target organ to exert therapeutic effects [45, 46].

MSC's immunomodulatory properties can also participate in dampening inflammation and injury. Several studies on the corneal and ocular surface showed that MSC treatment reduced the levels of inflammatory mediators. For instance, intravenously delivered bone marrow-derived MSCs could home to the inflamed ocular surface and enhance corneal allograft survival by suppressing alloimmunity mediated by antigen-presenting cells and alloreactive T cells [47].

An array of possible mechanisms of action of MSCs can be evaluated in cellular systems and animal models. Dissecting the mechanism of action and pathways involved in each disease or injury context will help improve the safety profile and therapeutic value of MSCs in ocular surface regeneration and repair.

CONCLUSION

There is extensive evidence from *in vitro* and *in vivo* studies indicating that MSC-based strategies are indeed promising for corneal repair and regeneration. Dosage of the bio-products employed and frequency of administration depends largely on the delivery routes. It is now time to incorporate all information available so far to further the therapeutic efficacy of MSCs in the resolution of ocular surface disturbances in the clinics.

LIST OF ABBREVIATIONS

ECM Extracellular matrix

EV Extracellular vesicle

GVHD Graft-*versus*-host disease

IL Interleukin

iPSC Induced pluripotent stem cell

MHC Major histocompatibility complex

MIP1α Macrophage inflammatory protein-1α

MSC Mesenchymal stromal/stem cell

TNF Tissue necrosis factor

VEGF Vascular endothelial growth factor

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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

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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

largely dependent on the route of MSC delivery. Engraftment of MSCs in the injured kidney was observed following different administration routes, including intravenous, intra-arterial, intraperitoneal, intraparenchymal, subcutaneous and intramuscular, discussed herein (Fig. **1**). In a few studies, the efficiency of MSC homing among delivery routes was compared.

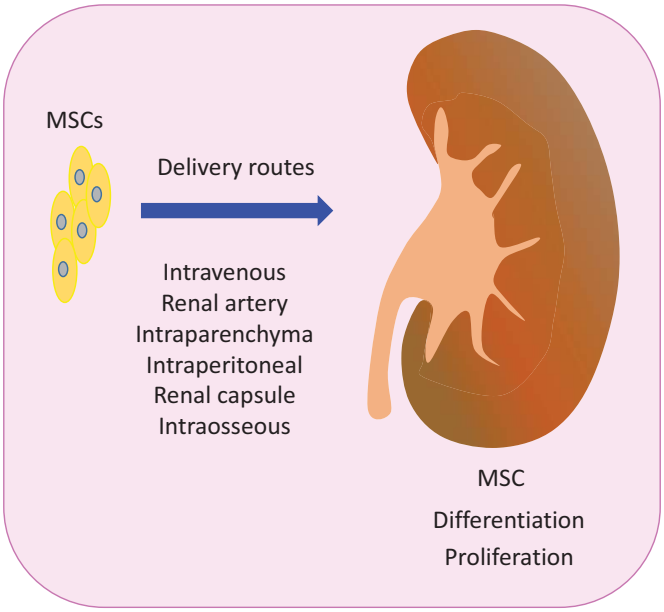

Fig. (1). MSC delivery routes to the kidney. The most frequently employed routes of administration to direct stem cells to the kidney are shown.

3.1. Intravenous Infusion

Intravenous cell administration, whilst being easy and non-invasive, results in pulmonary entrapment of cells. Thus, only a small proportion of cells is redistributed to the liver and spleen, while an even smaller number of cells may reach the kidney. Despite this, intravenous infusion of MSC (derived from iPSCs) conferred renoprotection in the model of Adriamycin-induced AKI [24]. Briefly, administration of 2×10^5 MSC mitigated proteinuria and renal failure in the AKI mice compared to controls. MSCs attenuated apoptosis through reduced Bax expression and Bax/Bcl2 ratio, and restoration of survivin loss in the renal cortex, as well as tubulointerstitial fibrosis through decreased cortical deposition of total collagen, hence protecting the mice against renal function loss. Again, most of the injected MSCs were found in the lungs.

In the experimental model of type 1 diabetes induced by streptozotocin injections, allogeneic bone marrow-derived MSCs conferred nephroprotection following

intravenous infusion [25]. No MSC-to-renal cell trans-differentiation events were observed in the kidney of these mice. Renal function improved principally through the paracrine action of MSCs. Levels of proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) and fibrogenic growth factors (TGF-β) decreased, while those of anti-apoptotic (Bcl2 and VEGF) and anti-inflammatory proteins (IL-10, FGF-1) increased. Albeit the successful engraftment of some cells at the sites of injury, intravenous delivery, although doable and less invasive, is not the optimal mode for directing a sufficient number of cells to achieve the best therapeutic activity to the damaged kidneys.

3.2. Intra-arterial Injection

Delivery of MSCs directly into the renal artery of an injured kidney (thus, bypassing the lungs) can result in major cell engraftment in this organ. In an IRI rat model, performed by occluding the left kidney for 45 minutes and nephrectomising the right kidney, Cai *et al.* showed that MSC retention in the kidney was higher when cells were delivered through the renal artery rather than through the tail vein or carotid artery [26]. There was a homogeneous distribution of MSCs in the injured kidney and a significant improvement in renal function and morphology. In a recent study, human bone marrow-derived MSCs delivered through the renal artery persisted in the injured kidney up to 21 days after injection and efficiently reduce renal fibrosis following IRI in rats compared to the intravenous route [27].

The effects of bone marrow-derived MSCs against diabetic podocyte injury were investigated in a rat model of streptozotocin-induced diabetes [28]. MSCs (2×10^6) were delivered through the left renal artery and protected the kidney from albuminuria development and podocyte loss, most probably through the paracrine action of MSCs. In rat mesangioproliferative anti-Thy1.1 glomerulonephritis, injection of MSCs into the left renal artery led to the accumulation of cells in the glomeruli and intrarenal vessel mostly, protecting from acute renal failure and leading to glomerular recovery and restored function. MSC ameliorated mesangiolytic damage, caused an increase in glomerular cell proliferation and significantly reduced proteinuria compared to tail vein injection during the 6-day follow-up period.

In a systemic review and meta-analysis, Papazova *et al.* investigated the efficacy of cell-based therapy in preclinical models of CKD [19]. Direct intrarenal delivery was applied in 17 studies, subcapsular or parenchymal administration in 5 articles and renal artery delivery in 12 studies. The results of the meta-analysis showed that bone-marrow-derived progenitors and MSCs, through intravenous or renal artery injection, were the most effective in reducing the development of CKD.

Some problems are associated with cell delivery through the renal artery. This method is invasive and mostly performed at the end of the open abdominal surgery following renal ischemia induction in rodents [29]. Recently, a non-surgical ultrasound-guided renal artery injection method was developed to improve stem cell delivery to the kidney without the need for open abdominal surgery [29]. Cells were delivered through the paravertebral muscle into the renal artery in order to avoid damage to surrounding organs, and hyperechogenic contrast from an alginate solution around the renal cortex helped in controlling successful injection. This study shows that there is much room for technical improvement in order to achieve the most efficient MSC delivery with the least adverse events.

As with all exogenously administered therapy, caution is necessary for intra-arterial cell administration, too. There is evidence of vascular occlusion occurring after intra-arterial MSCs administration due to their relatively large cell size, hence raising safety concerns [30]. There is also the possibility of long-term mal-differentiation of injected MSCs and resulting fibrosis. It was found that intraglomerular MSCs could partially differentiate into adipocytes in the injured kidneys 2 months post cell injection, and therefore, offset the initial beneficial effects observed in the short-term [31]. Moreover, renal artery puncture and injection are highly invasive in rodents and can result in massive bleeding, which can lead to further damage with respect to renal capsule injection, for instance. A solution can be to use the sub-adventitial renal artery puncture as developed by Cai *et al.* in order to avoid bleeding from the puncture site [26]. The right choice of MSC injection route and cell dosage coupled with technological improvements will surely lead to better cell retention and therapeutic effects in the kidney with minimal adverse events.

3.3. Intraparenchymal Administration

Few studies addressed the possibility of delivering MSCs directly in the renal parenchyma to circumvent the problem of poor homing of cells after systemic delivery. Caldas *et al.* employed a 5/6 renal mass reduction rat model and showed that the infusion of bone marrow-derived mononuclear cells could reduce or stabilise the rate of progression of chronic renal failure [32]. Cells were delivered in the renal parenchyma of the remnant kidney. A similar trend was observed with the intraparenchymal injection of MSCs. In another study performed by Alfarano *et al.*, bone marrow-derived MSCs were directly injected into the renal parenchyma in rats subjected to IRI and unilateral nephrectomy [33]. Cells were delivered on day 7 after IRI. Kidney analysis at day 21 post-surgery showed that there was a significant reduction in chronic tubular injury and interstitial fibrosis,

and an improvement in renal function. Thus, MSC direct delivery into the renal parenchyma showed some therapeutic efficacy. Intraparenchymal cell delivery is also possible without laparotomy. Following the location of the kidney by palpation, the organ was held firmly to inject cells through the skin of mice [34]. Interestingly, labelled cells were found in the renal tissue. Despite the efficient local delivery of MSCs through this route, the cells tend to localise only at the injection site without being distributed throughout the kidney. This route is also difficult to implement in the clinics, and the risk of causing additional injury to an already damaged kidney and compromise recovery of renal function has not much encouraged research in this direction [26].

3.4. Renal Capsule Injection

As described above, the targeted delivery of cells into the kidney is strenuous. As an alternative strategy, cell delivery under the renal capsule can be considered. The renal capsule is the fibrous membrane enveloping the outer surface of the renal parenchyma is regarded as a niche for stem cells [35, 36]. For instance, organoids generated from human pluripotent stem cells connected efficiently with existing vascular networks upon subcapsular kidney implantation in mice and differentiated into kidney-like structures, with glomerular filtration barrier and formation of slit diaphragm formation, and showed tubular epithelial maturation 4 weeks after implantation [37]. Migration of cells injected under the kidney capsule to sites of injury has also been observed. Cavaglieri *et al.* injected rat MSCs under the renal capsule of the remnant kidney of rats following 5/6 nephrectomy [38]. Improvement in renal function and reduction in glomerulosclerosis were observed 30 days after cell delivery. Interestingly, MSCs were found in the renal parenchyma following subcapsular injection. The biodistribution of MSCs was also analysed in a cisplatinum-induced AKI mouse model, after injection of MSCs *via* the intraperitoneal and renal subcapsular routes [39]. Enhanced cell survival *in vivo* was observed and there was an improvement in renal morphology and function irrespective of the intraperitoneal or subcapsular MSC administration, suggesting that the paracrine action of MSCs conferred the renoprotective effects seen.

3.5. Intraperitoneal Administration

Intraperitoneal delivery of MSCs is useful in cases where the secretome of the cells can improve the disease outcomes. Adipose tissue-derived MSCs were intraperitoneally injected 4 hours post-IRI in mice in order to assess the efficacy of these cells in preventing the development of renal fibrosis over a period of 6 weeks post-surgery [40]. MSC injection resulted in reduced renal type I collagen

deposition and fibrosis. Systemic and tissue inflammation was also regulated by MSCs as shown by decreased Th1 cytokines (IL-1β, IL-6, and TNF-α) and increased Th2 cytokines (IL-4 and IL-10) levels. Thus, MSCs show renoprotective activity upon intraperitoneal delivery.

In another setting, it was demonstrated that intraperitoneally delivered human MSCs could be recovered as aggregates of varying size that contained mouse immune cells attached to the peritoneal cavity, including the omentum and mesentery, but not in the peritoneal lavage fluid, thus limiting the passage of the cells into the systemic circulation [41]. This could be the reason behind the paracrine action of intraperitoneally administered MSCs on organ regeneration. MSCs, especially xenografts, may also be encapsulated in biomaterials before intraperitoneal injection to enhance their survival *in vivo* [42].

4. MSC DELIVERY ROUTES IN CLINICAL TRIALS

Several routes of MSC administration have been employed in clinical studies. A search for completed clinical trials regarding "kidney diseases" and "mesenchymal stem cells" and registered on www.clinicaltrials.gov website yielded 6 studies (Table **1**). In most studies, the intravenous route of MSC delivery was employed. No results have yet been published for these completed clinical trials. In the published clinical studies, there are controversial results regarding renal artery delivery of MSCs. For instance, autologous adipose-derived MSC were administered through the renal artery and resulted in increased blood flow in stenotic and contralateral kidney, 3 months post-cell injection compared to controls [43]. On the other hand, intra-aortic infusion of allogeneic bone marrow-derived MSCs did not improve kidney function, but seemed to worsen prognosis probably due to the inflammatory environment in patients with AKI resulting from cardiac surgery [44].

Interestingly, the first clinical studies on the use of MSCs in living-donor kidney transplant recipients showed that intravenous administration of autologous bone marrow MSC one week after or 24 hours prior kidney graft transplantation slowed mean renal function yearly decline rate by ~70% compared to control transplanted subjects over 5-7 years follow-up [45]. This phase I study showed that intravenous MSC injection in renal transplant recipients was safe and feasible. In an open-label phase I-II trial, third-party MSCs were infused intravenously in patients after kidney transplantation [46]. The injection was safe and no adverse events were observed. Moreover, there was an improvement in allograft function

1 year after transplantation. These results warrant further long-term investigation in the clinical setting.

MSC delivery through the intraparenchymal route, though feasible in the preclinical models, is not practical in clinical studies. No intraperitoneal injection of MSCs in patients with kidney diseases seems to have been undertaken in clinical trials.

Table 1. Clinical trials with MSCs for kidney diseases. Data were downloaded from http://www.clinicaltrials.gov on 31/05/21 and show the MSC delivery routes employed in the clinical studies.

NCT Number	Title	Study Results	Conditions	Interventions	Outcome Measures	Phases	Enrollment	Locations
NCT02166489	Mesenchymal Stem Cells Transplantation in Patients With Chronic Renal Failure Due to Polycystic Kidney Disease	No Results Available	Chronic Renal Failure|Polycystic Kidney Disease	Biological: **Intravenous** injection of autologous mesenchymal stem cells	Mass formation| Glomerular Filtration Rate (GFR)	Phase 1	6	Iran
NCT02195323	Autologous Bone Marrow Derived Mesenchymal Stromal Cells (BM-MSCs) in Patients With Chronic Kidney Disease (CKD)	No Results Available	Chronic Kidney Disease	Biological: **Intravenous** injection	Mass formation| Creatinine| GFR	Phase 1	7	Iran
NCT02266394	Hypoxia and Inflammatory Injury in Human Renovascular Hypertension	No Results Available	Renal Artery Stenosis| Ischemic Nephropathy| Renovascular Disease| Chronic Kidney Disease	Drug: Mesenchymal stem cell| Procedure: Mesenchymal stem cell delivery with **stent placement/ Intra-arterial infusion**	Change in Kidney function| Safety of Mesenchymal stem cell infusion| Decrease in Kidney inflammation	Phase 1	42	United States
NCT01840540	MSC for Occlusive Disease of the Kidney	No Results Available	Atherosclerotic Renal Artery Stenosis| Ischemic Nephropathy| Renovascular Hypertension	Drug: **Arterial infusion** of autologous mesenchymal stem cells	Renal blood flow and function in the treated kidneys.|Level of kidney function.	Phase 1	6	United States
NCT04318600	Allogeneic Amniotic Mesenchymal Stem Cell Therapy for Lupus Nephritis	No Results Available	Lupus Nephritis| Mesenchymal Stem Cells	Drug: human amniotic mesenchymal stem cell/ **Peripheral intravenous infusion**	Incidence of Adverse Events| 24h urine protein quantification pre- and post-treatment| Changes in GFR	Phase 1	16	N/A

(Table 1) cont.....

NCT Number	Title	Study Results	Conditions	Interventions	Outcome Measures	Phases	Enrollment	Locations
NCT03174587	Evaluate the Safety of CS20AT04 Inj. in Subjects With Lupus Nephritis	No Results Available	Lupus Nephritis	Biological: allogeneic bone marrow derived mesenchymal stem cells/ **intravenous-peripheral vein**	Safety assessment (evaluation)	Phase 1	7	Korea

5. MODE OF ACTION OF DELIVERED MSCS IN KIDNEY DISEASE

Multiple modes of action of MSCs in kidney diseases have been described depending on the delivery route. When injected intravenously, most MSCs get entrapped in the lungs, and thus, any beneficial outcome obtained is mainly due to the paracrine action of the infused cells. Several growth factors and cytokines as well as the components of extracellular vesicles, have been correlated to the renoprotective and regenerative effects in this case. When delivered locally, in the parenchyma, for example, MSCs may differentiate into renal cells. However, there is only scarce evidence that this happens *in vivo*. Bone marrow-derived MSCs from syngeneic GFP-positive mice were found to differentiate into glomerular mesangial cells in lethally irradiated mice and persisted in the kidney till week 24 [47]. Moreover, in a mouse model of AKI induced by IRI, human adipose tissue-derived MSCs differentiated into renal tubular epithelial cells at the early stages of injury and participated in maintaining tissue integrity [48]. Most of the data highlight that MSCs promote tissue repair processes by paracrine action. The MSCs secrete a plethora of factors (antioxidant, anti-apoptotic and growth factors) that stimulate remaining healthy cells in the damaged site to dedifferentiate, proliferate and substitute injured cells in the kidney (Fig. **2**) [8]. EVs largely assist in this regenerative process.

Besides their regenerative potential, MSCs also have immunomodulatory properties [49]. Interestingly, it was shown by Bulati *et al.* that the human amniotic membrane-derived MSCs increase their immunomodulatory properties through stimulation by IFN-γ released by activated lymphomonocytes [50]. This activation is largely dependent on cell-to-cell contact involving the programmed death-ligand 1 (PDL-1)/PD-1 axis. The resting MSCs, on the other hand, have very low immunomodulatory effects.

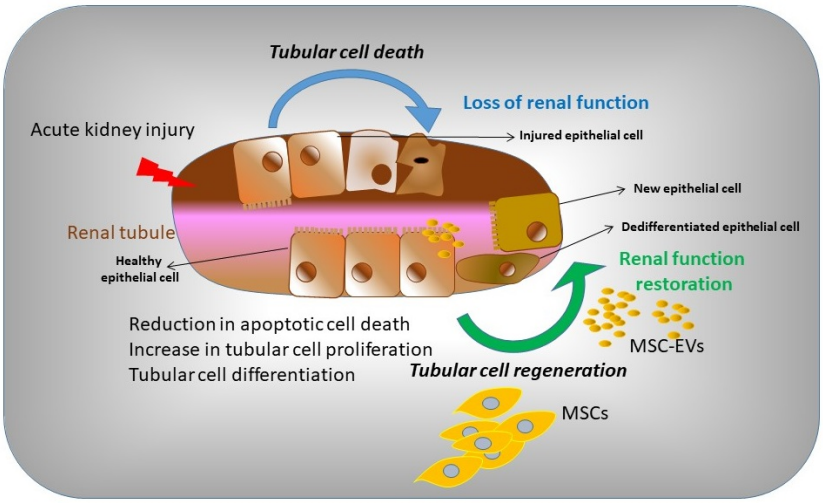

Fig. (2). Mechanism of tubular regeneration by MSCs.

CONCLUSION

Cell therapy strategies for kidney diseases have developed at a very fast pace in recent years, due to preclinical research conducted on several animal models of human diseases. The routine clinical treatment, however, is still hindered by the inadequate cell delivery to injury sites and limited retention of stem cells after transplantation. Therefore, the development of a less invasive and more efficient injection route is urgently required to improve stem cell delivery in clinical studies. Moreover, the efficacy of MSC-released factors in the milieu of an already established renal inflammation or injury appears to be less promising [8]. Thus, further studies are imperative to decide where and when to use MSCs in the clinical setting.

LIST OF ABBREVIATIONS

AKI	Acute kidney injury
CKD	Chronic kidney disease
ECM	Extracellular matrix
ESC	Embryonic stem cell
ESRD	End-stage renal disease
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
IFN	Interferon
IL	Interleukin

iPSC Induced pluripotent stem cell

IRI Ischemia reperfusion injury

MSC Mesenchymal stromal/stem cell

PD-L1 Programmed death ligand 1

TGF Transforming growth factor

TNF Tissue necrosis factor

VEGF Vascular endothelial growth factor

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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

different sources have been interrogated in different animal models of human diseases [24].

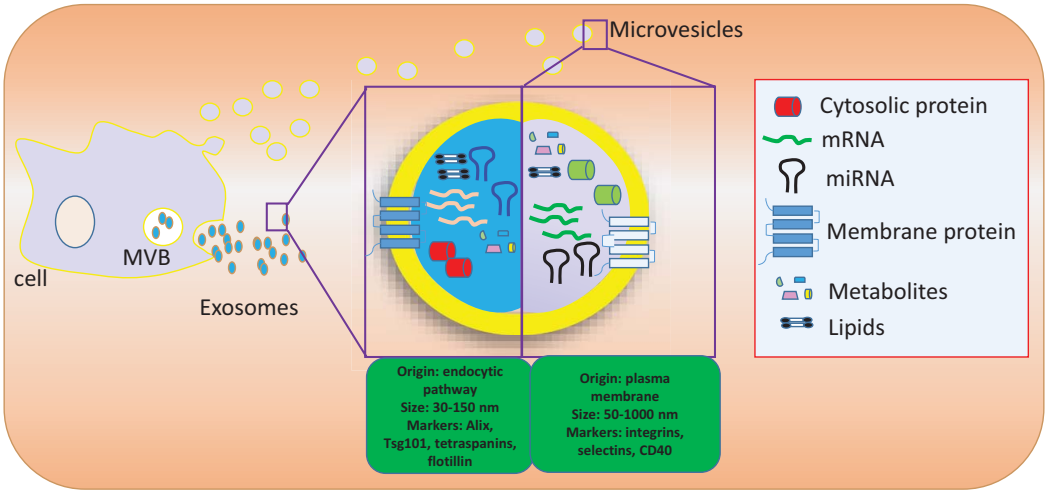

Fig. (1). Comparison between extracellular vesicles. Exosomes and microvesicles are the major types of EVs considered in regenerative medicine. These EVs can be distinguished according to surface markers and biomolecular contents [25].

3.2. EV Therapeutic Contents

In the context of cell-based therapy, EVs, due to their small size, do not get entrapped in the lungs or other filtering organs such as the spleen, liver and kidney as may happen upon infusion of exogenous stem cells [26]. Interestingly, it was shown that entrapped stem cells disintegrate into smaller membrane-entities (EVs including apoptotic bodies) which travel to distant organs upon release in the circulation to exert their therapeutic effects. EV contents are protected from the action of nucleases and proteases by the presence of the bilayered lipid membrane [27].

Several types of bioactive molecules have been identified inside EVs. The contents and concentrations of these molecules vary according to the pathophysiological condition of the source organ. A cargo of genetic materials including different RNA species (mRNA as well as microRNAs (miRNAs) and other non-coding RNAs), proteins (enzymes, cytokines, chemokines, receptors, immunoregulatory proteins and growth factors) and lipids (including cholesterol, sphingolipids) have been found in EVs [28]. mRNAs, for instance, can influence the biological function of the cells in the vicinity by regulating their differentiation, proliferation and transcription. MiRNAs also modulate several key processes such as apoptosis, cell cycle as well as migration [29]. It is possible to

distinguish exosomes from microvesicles based on their protein contents. To a limited extent, DNA molecules have also been documented inside EVs, which could account for maintenance of cellular homeostasis or protection from unwanted, damaged DNA, for DNA transfer for modulation of recipient cells and regulation of immune responses [30]. Exosomes are rich in annexins such as Annexin A1, tetraspanins (CD63, CD9, CD81 and heat shock proteins, such as HSP70), as well as in Alix, Tsg101 and clathrin [31]. Microvesicles, on the other hand, have large amounts of phosphatidylserine-containing proteins associated with lipid rafts and CD40 surface markers. Modern technologies such as RNA-sequencing, have enabled the high throughput characterisation of EV composition and have added a yet new layer to the search of biomarkers (EV-based) for several pathologies [32]. In fact, the molecular content of EVs reflects that of the cell origin at a given time and in a given context.

3.3. EVs in Cell-free Therapy

The ease with which MSCs can be expanded *in vitro* renders these cells excellent sources of EVs for clinical use. MSCs have been the most widely used for experimental cell therapy, and consequently, their EVs have been studied more extensively than those derived from other stem cell sources. EVs retain similar therapeutic advantages to the parental cells, thus encouraging their use in preclinical studies as a substitute of cell transplantation [33]. Cell therapy raises several concerns that still have to be dealt with. For instance, the survival rate of injected stem cells and therapeutic properties are largely dependent on the type of microenvironment these encounter *in vivo*. Intravenously injected stem cells may be trapped in the filtering organs, which reduces the therapy efficiency. On the contrary, EVs derived from MSCs (MSC-EVs) are nano-sized particles that, for instance, have been found at higher concentrations in the injured sites, and are less susceptible to degradation compared to cells, hence supposedly more persistent at sites of injury [26]. EVs cannot replicate like MSCs, thus bypassing the concerns raised about possible uncontrolled cell division that may inadvertently occur *in vivo* during the process of transformation.

4. EVS IN LIVER REPAIR AND REGENERATION

In a structurally complex organ like the liver, in which the interaction among the different cell types ensures maintenance of homeostasis and multiple vital functions, cell-to-cell communication is a crucial modulator of physiological as well as pathological events. This communication system is based on bio-products which are released into the extracellular milieu to mediate intercellular signalling.

MSC-EVs have received much attention in the treatment of liver diseases due to their therapeutic contents. The use of MSC-EVs in the treatment of liver diseases has been reported in several preclinical studies. After intravenous injection of EVs (microvesicles) obtained from human liver MSCs into rats with 70% hepatectomy, morphological and functional recovery of the liver was observed. This effect was associated with an increase in hepatocyte proliferation in the animal livers, and was abolished when EVs were pretreated with RNase, thus confirming the role of specific RNA patterns in the hepatoprotective function of the EVs (microvesicles) (Fig. **2**) [34].

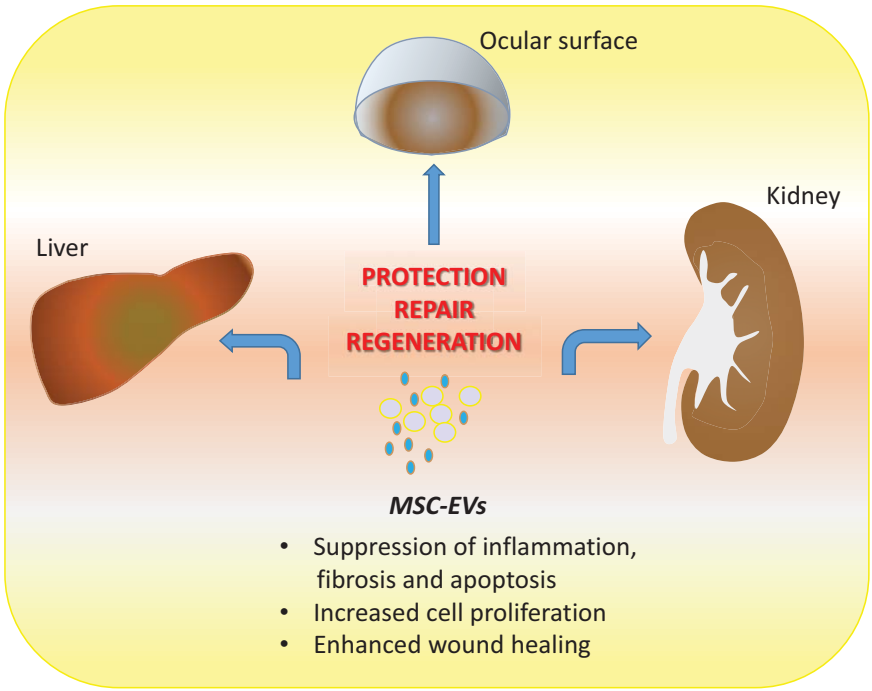

Fig. (2). Effects of MSC-EVs on liver, ocular surface and kidney repair and regeneration. The main beneficial effects are listed.

MSC-EVs administration induced liver protection by acting on survival, growth and migration of hepatic cells and regulating inflammatory events. Li *et al.* showed that umbilical cord-derived exosomes inhibited liver fibrosis by counteracting the epithelial-to-mesenchymal transition of hepatocytes, a process for deriving fibroblasts from these cells, which is essential for the progression of fibrosis, and reducing collagen production in a mouse model of CCl_4-induced liver fibrosis [35, 36]. The TGFβ1/Smad2 signaling pathway, involved in fibrogenesis, was reduced by these MSC-EVs delivered into the left and right

lobes of the liver of mice treated with CCl_4 [35]. The same group further showed that a single dose of EVs administered intravenously could protect mice subjected to liver transplantation from oxidative stress and apoptosis, hence diminishing the possibility of liver failure [37]. Glutathione peroxidase 1, one of the endogenous antioxidant enzymes, contained inside the MSC-EVs could be responsible for these effects. EV-contained biomolecules such as miRNAs are also useful in controlling fibrogenesis. In a CCl_4-induced liver fibrosis mouse model, tail vein delivered adipose tissue-derived MSC-EVs, modified to express high levels of miR-122, caused a decrease in proliferation and activation of hepatic stellate cells (HSCs) and effectively blocked fibrogenesis [38]. Expression of miR122 target genes IGF receptor 1, Cyclin G1 and prolyl-4-hydroxylase α1, implicated in the proliferation and collagen maturation of HSCs, were significantly downregulated in an HSC line, LX-2 [38]. The anti-inflammatory effects of bone marrow MSC-EVs were demonstrated in the concanavalin-A-induced liver injury model, in which intravenous EV administration caused a reduction in serum aminotransferase (alanine aminotransferase) and proinflammatory cytokines (IL-2), while inducing the anti-inflammatory cytokines (TGF-β, HGF) and levels of Regulatory T cells [39].

MSC-EVs also carry metabolically active enzymes such as those involved in glycolysis [40, 41]. EVs derived from umbilical cord-derived MSCs, delivered in poly (ethylene glycol) hydrogels to enhance sustained retention at the site of injury, could attenuate oxidative stress by decreasing the infiltration of neutrophils, thereby protecting the liver against apoptosis [42]. Lately, EVs derived from human bone marrow MSCs were injected intraperitoneally in the Multidrug Resistance 2 (*Mdr2*)-/- mice which partially recapitulate the Primary Sclerosing Cholangitis patients' phenotype [43]. EVs injection once weekly for 3 consecutive weeks markedly reduced inflammation in these mice as seen by the decrease in NFκB-induced VCAM1 expression by inflamed liver endothelium and infiltrating immune cells. Moreover, reduced α-smooth muscle actin immunoreactivity and peribiliary fibrosis were noted in the livers of the EV-treated *Mdr*2-/- mice.

EV-based therapy could develop into a very useful tool for the treatment of liver diseases. Further preclinical studies are required to better understand the mechanism of action and effectiveness of EVs with respect to the type and severity of liver diseases.

5. EVS IN OCULAR SURFACE REPAIR

The use of EVs derived from MSCs for the treatment of corneal injuries and scars is quite recent. It was shown that human placenta-derived MSC-EVs promoted

corneal wound healing by blocking inflammation and apoptosis of epithelial cells, as well as angiogenesis when applied topically in a mouse corneal alkali burn model (Fig. **2**) [44]. EVs obtained from corneal stromal/mesenchymal stem cells (CSSCs)-derived MSCs also provided encouraging results in mice following corneal epithelial debridement [45]. EVs applied in fibrin gel reduced corneal scarring, reduced fibrogenesis and inflammation, thus promoting corneal regeneration. These effects could be attributed to the EV cargos, such as miRNAs, as shown by the reduced scar-reducing potential of CSSCs subjected to knockdown of Alix protein, required for packaging of miRNAs into exosomes. Much work is needed in this field in order to develop the best therapeutic approach using MSC-EVs for corneal regeneration and repair.

The use of EVs for the treatment of corneal burns is mostly in the preclinical stage, but encouragingly, few clinical trials using cell-derived EVs in ocular diseases can be found on www.clinicaltrials.gov website. The route of EV administration and treatment schedule are very important determinants in the duration of these biological entities in the eye. For instance, neuroprotection was achieved by the local, intravitreal injection of a smaller number of MSC-EVs with respect to those applied intravenously (for the latter, five times more EVs were required). One single intravitreal injection revealed that the MSC-EVs lasted in the eye for up to 30 days post-injection. Multiple, weekly intravitreal injections enhanced MSC-EVs therapeutic efficacy and conferred permanent neuroprotection [46]. Thus, the route of EV delivery, among other factors, will determine the success of EV-based therapy in clinical studies.

6. EVS IN KIDNEY REGENERATION

The use of MSC-EVs in the functional recovery of the kidney upon injury has also received increased attention in recent years. Several studies have used MSC-EVs for kidney regeneration in settings of AKI, metabolic kidney disease and CKD. EVs promoted the restoration of injured tubular cells, cell proliferation and differentiation, while protecting from apoptosis and necrosis (Fig. **2**). For instance, in a rat model of AKI induced by ischemia-reperfusion injury, umbilical cord-derived MSC-EVs, infused through *via* the left carotid artery, protected from tubular cell necrosis and improved kidney functionality as seen by the dosage of serum creatinine and urea levels [47]. Tail vein delivery of EVs from bone marrow-derived MSCs also ameliorated renal function and morphology in glycerol-induced AKI in SCID mice by inducing the proliferation of remaining tubular cells [48]. Survival of mice exposed to cisplatin-induced AKI was also improved by multiple injections (a first intravenous infusion at 8 hours post-cisplatin administration followed by tail vein injections at days 2, 6, 10, 14 and 18

post-cisplatin injection) of bone marrow-derived MSC-EVs due to reduction of apoptosis in the epithelial cells, accompanied by significant improvement in tubular histology and renal function [49]. On the other hand, a single EV injection improved only survival in this model without significant improvement of kidney function or morphology. EVs from different sources also exerted anti-inflammatory and immune regulatory effects in the damaged kidney, and through different bioactive molecules [50]. Anti-oxidant effects of a single intravenous injection of human Wharton's Jelly MSC-EVs were observed in a rat model of ischemia-reperfusion injury [51]. Apart from the improvement in tubular morphology and function, markers of oxidative stress, malondialdehyde and 8-hydroxy-2′-deoxyguanosine, induced by tubular injury, were significantly reduced by the injection of EVs. The antioxidant enzyme heme-oxygenase-1 was upregulated in MSC-EV-treated rats. Autologous adipose tissue-derived MSC-EVs delivered intrarenally attenuated renal inflammation and ameliorated medullary oxygenation in a porcine model of metabolic syndrome with unilateral renal aortic stenosis [52]. The renoprotective effects were imparted by IL-10 contained in EVs. Thus, administration of MSC-EVs promoted renal regeneration and improvement in tubular function in preclinical models of kidney injury.

The safety and therapeutic efficacy of MSC-EVs were also assessed in clinical trials. For instance, in a clinical trial involving 40 grade III-IV CKD patients, human umbilical cord-derived MSC-EVs were administered 2 doses 1 week apart in 20 patients [53]. The other 20 patients were placed in the placebo group and were administered saline intravenously. The first dose was injected intravenously through the median cubital vein, while the second dose was given after 1 week *via* the renal artery under computed tomography guidance and directed to the diseased kidney. MSC-EVs alleviated inflammation and improved renal function, and no adverse effects were reported during the 1 year follow-up with respect to the other 20 patients in the control group, indicating the safety and efficacy of MSC-EVs under these conditions.

CONCLUSION

On the whole, EVs have been accepted as a valid alternative to cells for the treatment of human diseases. This field of research is in constant evolution as new technologies are devised and characterisation problems are tackled. More studies are needed to analyse the dosage and frequency of EV administration or what happens upon EV treatment in case of more advanced diseases. To what extent EVs can repair an injured tissue is still under scrutiny. And in the case of severe diseases with complications like portal hypertension, whether EVs can confer still therapeutic activity remains to be investigated [54]. Standardisation of source,

purification and conservation methods, as well as delivery routes are urgently required to be able to compare preclinical results, and will take us a step further towards the clinical application of EVs. In most of the studies performed hitherto, EVs were delivered through the intravenous route. In few cases, EVs were injected directly into the target organ. Thus, there is the necessity to investigate all the plausible routes of EV delivery in order to achieve the maximum therapeutic activity with the least number of administrations. Of late, studies have been undertaken to investigate the safety and therapeutic efficacy of EVs administered through aerosol inhalation in healthy volunteers and patients (*clinicaltrials.gov* NCT04313647, NCT04276987). The possibility of alleviating COVID-19 symptoms will also be analysed by aerosol inhalation of MSC-EVs [29]. If successful, the aerosol inhalation may also be investigated for diseases involving other organs, such as the liver and kidney, considering that EVs can be transmigrated through the vascular wall into the circulation and get transported to sites of injury. To date, few clinical trials have been undertaken to investigate their therapeutic potential in human diseases [55, 56]. Bioengineering and cellular modification approaches will be most useful in promoting the ability of MSC-EVs in achieving cell-specific targeting, and in delivering high quantities of therapeutic biomolecules to the site of interest.

LIST OF ABBREVIATIONS

AKI	Acute kidney injury
BMP	Bone morphogenic protein
COVID-19	Coronavirus Disease-19
CCL	CC chemokine ligand
CCl_4	Carbon tetrachloride
CCR2	C-C motif chemokine receptor 2
CKD	Chronic kidney disease
CSSC	Corneal stromal/mesenchymal stem cell
EGF	Epidermal growth factor
EV	Extracellular vesicle
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HSC	Hepatic stellate cell
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
KGF	Keratinocyte growth factor

MCP-1	Monocyte chemotactic protein 1
MDR2	Multidrug resistance 2
miRNA	MicroRNA
MSC	Mesenchymal stromal/stem cell
RANTES	Regulated on activation, normal T cell expressed and secreted
TGF	Transforming growth factor
TNF	Tissue necrosis factor
VEGF	Vascular endothelial growth factor

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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-

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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

reach the cells to release the appropriate therapeutic factors. Porosity, stiffness and geometry of the biomaterials all contribute to the successful diffusion of soluble factors and extracellular vesicles, while retaining the cells [14 - 16]. Additional work needs to be done to incorporate the vascular networks, for example. Moreover, the use of single biomaterials can hardly support cell transplantation and organ regeneration. For instance, sodium alginate was coupled with polyethylene glycol, which conferred improved stability and mechanical strength to the microspheres [15]. Development of 3D tissue printing approaches with biomaterials to meet the needs for exogenous organs is under way. Important advances in this field have been made, but further work is needed to reconstruct the complex microarchitecture of ECM components as well as the cellular composition in sufficient resolution to get a fully functional organ [17].

Encapsulation, allowing the three-dimensional cell-cell contact, also ensures that MSCs maintain their vitality, therapeutic effects and differentiation capacity in damaged tissues [18]. It is possible to mimic the *in vivo* microenvironment by incorporating other cell types in the microcapsules. Different technologies are employed for the creation of MSC microcapsules and have been extensively reviewed in [19]. Regarding the cornea, the direct application of EVs would imply rapid clearance due to fast fluid (tear) turnover and exposure to the external environment. Thus, biodegradable or highly porous hydrogels have been used to ensure localised and sustained EV delivery to the cornea [20]. The non-invasiveness of topical application of MSC-derived EVs provides the opportunity to test which delivery medium enhances corneal repair with respect to PBS. Human hepatocyte microbeads, generated in polymerized alginate, showed hepatocyte-specific function and lack of immunogenicity *in vitro* [21]. Moreover, transplantation of these microbeads intraperitoneally in rats provided metabolic support and rescued them from acute liver failure.

In order to translate the encapsulation-based cell therapy strategies in the clinical setting, further studies are ongoing. It is imperative to determine what functional cell mass can be transplanted using this approach and for how long the cells embedded into the grafts will survive *in vivo*.

5. DECELLULARISED SCAFFOLDS FOR MSC DELIVERY

The use of decellularised scaffolds for bioengineering of human organs using stem cells is envisioned as a major advance in the field of regenerative medicine. Since the description of the first small intestinal submucosa matrix by Badylak *et al.*, many changes have been brought to the procedures and a variety of organs from animals and humans, including kidney and liver, have been decellularised [22 - 24]. The process of decellularisation regards the accurate removal of cellular

material with mild detergents, such as Triton-X100, from whole organs with minimal disturbance to the ECM microstructure and function, such as cellular adhesion, signalling processes, binding growth factors, as well as maintenance of the microvascular networks [25, 26]. Functional and transplantable constructs are generated by growing viable cells into these supportive scaffold structures, accompanied by specific biochemical cues to direct differentiation [27].

5.1. Liver

The possible therapeutic applications of stem cell-replenished bioscaffolds are numerous. Several studies demonstrated successful decellularisation of small animal livers, such as rodent livers, and replenishment with stem cells from different sources [28, 29]. We showed that human liver MSCs could successfully repopulate mouse liver scaffolds *in vitro*, and differentiate into hepatocyte-like cells expressing metabolic enzymes, such as UGT1A1 [30]. Efficient decellularisation procedures for livers of superior sizes, such as those of pigs and humans, have been described. These large-sized livers have the potential to develop into an important tool in the clinical setting [31]. Human whole-organ liver decellularisation is feasible, albeit the time required to prepare each liver scaffold varies [27]. Some adjustments, especially regarding the flow rates, are still necessary for larger organs in order to properly perfuse the entire organ. Moreover, the characteristics of the liver, such as age of the donor, presence of steatosis or fibrosis, which considerably alter the ECM contents as well as texture and architecture, should also be considered.

Stem cells, capable of differentiating into liver cells, are candidates for repopulation of human liver scaffolds [32]. Interestingly, it was shown that liver scaffolds can induce MSC differentiation into hepatocytes. Mouse liver scaffolds were seeded with mouse bone marrow-derived MSCs and cultured in hepatic differentiation media for 1 month [33]. With respect to the monolayer culture, the liver biomatrix scaffold significantly promoted MSC differentiation into functional hepatocytes [33]. Sectioning of the MSC-repopulated scaffolds into 1 × 1 mm pieces and their transplantation into the liver parenchyma of a mouse model of CCl_4-induced liver fibrosis improved liver function. Another study showed that systemic transplantation of MSCs harvested from liver scaffolds into a mouse model of CCl_4-induced liver fibrosis, enhanced mice survival and restored function [34]. In addition, seeded liver scaffold sections rescued liver function after transplantation into a mouse model of fulminant hepatic failure. Human liver-derived MSCs were also employed in rat liver scaffold repopulation [35]. Interestingly, these MSCs could efficiently differentiate into hepatocyte-like cells, as well as epithelial-like and endothelial-like cells when seeded for 21 days in the

presence of human liver MSC-conditioned media. These results show that depending on the potency of the cell type seeded in the acellular natural liver bioscaffolds and the culture conditions imposed, it may be possible to obtain several lineages in order to not only replenish these structures with hepatocytes, but also with the vascular and biliary systems.

Human livers unsuitable for transplantation have also been successfully decellularised and reseeded with different cell types. Mazza *et al.* showed that human liver left lobes or whole liver could be successfully decellularised and repopulated for up to 21 days with human hepatic stellate cells and with hepatocellular carcinoma and hepatoblastoma cell lines [24]. Omental or subcutaneous implantation of the dissected liver scaffolds ($5 \times 5 \times 5$ mm) into immune competent mice demonstrated their biocompatibility and did not elicit a host immune reaction. The results obtained from preclinical models and from human decellularised livers are indeed promising and suggest that, with some further optimisation, functional organ replacement is just a few steps away.

5.2. Ocular Surface

Human amniotic membranes, one of the thickest basement membranes of the body, have been studied as naturally occurring biomaterials for ocular surface reconstruction [36]. This membrane is loaded with anti-angiogenic, anti-inflammatory, and neurotrophic factors and can inhibit fibroblast proliferation and myofibroblast differentiation [37]. Amniotic membrane transplantation has been used for a variety of ocular surface diseases and in the management of acute ocular chemical burns to promote epithelialisation, reduce inflammation and restore ocular surface integrity [38]. This procedure has the potential to improve vision, especially when these structures are used as a scaffold and are seeded with therapeutic stem cells [39]. Despite the fact that during the last decades, amniotic membrane transplantation has been the gold standard for the treatment of ocular surface deterioration, there are still risks associated with this technique, such as the formation of hematomas and granulomas [40].

Like the liver, the cornea devoid of cells can also be used as a scaffold. Porcine corneas have been studied for the set-up of decellularisation procedures due to their anatomical similarities with the human cornea [41]. The main problem associated with the use of porcine scaffolds for xenogeneic transplantation is that any remaining cells may elicit a strong immune response and graft rejection in humans, thus hindering their use in clinical studies. Thus, the perfect corneal scaffold would be human. Human corneas have been used in decellularisation procedures [42]. Ideally, corneas deemed unsuitable for corneal transplantation due to low endothelial cell count could be used to obtain decellularised scaffolds

[41]. Added to this, tissues discarded after refractive surgeries may be repurposed for scaffold preparation. Repopulation of these matrices can be done with different sources of human cells to generate a viable cornea. Usually primary cells are preferred to cell lines for risk of tumour formation. Stem cells with high regenerative capacity, such as MSCs, are also optimal candidates. As described above, repopulation of the decellularised organ can be achieved through the vasculature network present. However, for avascular and thin tissues like the cornea, alternative strategies are required to repopulate the scaffold with the three main cell types of this tissue: epithelial cells, keratocytes and endothelial cells. Thus, the development of new strategies is most awaited in this field.

5.3. Kidney

There is an urgent need to develop a functional kidney graft especially for use in patients with ESRD. Recreating a human kidney *ex vivo* based on decellularised bioscaffolds is extremely challenging. The main problem is the need to combine seeded cells with circulation and microcirculation, which ensure proper kidney functioning. Several cell types can be considered for organ repopulation [43]. Supported by the decellularized extracellular structure (ECM) which helps in maintaining the morphological shape of the kidney as well as the architecture of the parenchyma at micro and macro levels, the bioengineered kidney can develop into fully implantable sources and treatment option for ESRD [43]. Preclinical studies have shown that decellularised rat kidney scaffolds obtained from cadavers and repopulated with epithelial (rat neonatal kidney cells) through the ureter and endothelial cells (human umbilical venous endothelial cells) instilled through the renal artery could produce urine when transplanted in an orthotopic position following nephrectomy [44].

Histocompatible donors are required for kidney transplantation in clinical practice. Thus, it is important to determine whether tissue typing reagents, such as HLA classes I (A, B and C) and II (DR) antigens are absent from kidney grafts. It was shown that the decellularisation process completely removed HLA-ABC and HLA-DR from human kidneys [45]. This widely expands the applicability of decellularised kidney bioscaffolds, seeded with autologous cells, in restoring functionality in multiple recipients [46]. Recently, re-endothelialisation of a complete human kidney scaffold with human inducible pluripotent stem cell-derived endothelial cells in the presence of growth factors was reported [47]. The differential arteriovenous delivery system used successfully repopulated the vasculature, comprising the glomerular and peritubular capillaries, and the bio-engineered kidney was functional as shown by the perfusion of whole blood with reduced clotting and better distribution compared to non-endothelialised scaffolds.

Translation of this technology still requires some optimization before application in clinics can be considered (Fig. **1**). Importantly, different stem cell sources and combinations of cell types should be tested in order to achieve whole organ repopulation and to optimise functionality.

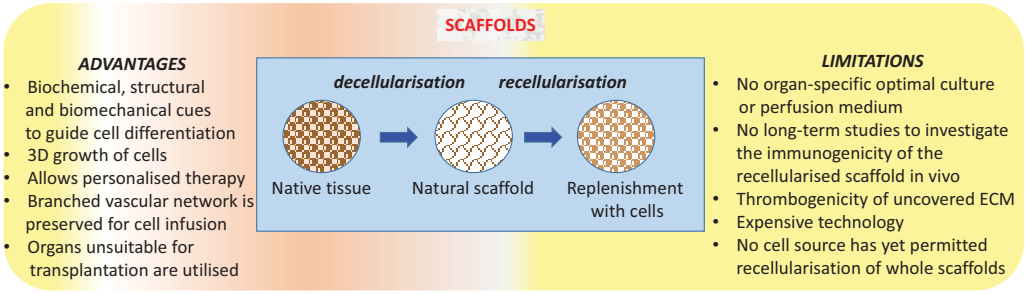

Fig. (1). Decellularised scaffolds for organ regeneration. Some advantages and limitations of this technology are listed [28].

6. MAGNETICALLY-ACTUATED MICROROBOT FOR TARGETED MSC DELIVERY

To improve the targeted delivery of cells to injury sites, most recently cell-loaded microrobots have been implemented. For instance, Li *et al.* devised a magnetically driven microrobot designed to access small regions of the body and to cross the blood barrier to deliver cells like MSCs *in vivo* relying on a magnetic gradient field–driven mechanism [48]. Following *in vitro* verification of the ability to support cell growth and delivery, the MSC-loaded microrobots were taken up with a needle by air sucking and injected into the yolk sac 30-hour post-fertilisation zebrafish embryos, which are transparent and allow easy monitoring and movement of the microrobot. The microrobot successfully moved inside the yolk sac against the magnetic field gradient. In order to verify the release of cells *in vivo*, the authors used GFP-labelled HeLa cells inside the microrobots, and injected these in Matrigel and in PBS (1000 microrobots in 100µl Matrigel and 100µl PBS) subcutaneously in nude mice. Increase in GFP signal was used to monitor tumour growth at the injection site, and it was shown that the microrobots released their cells, which generated tumours *in vivo* compared to microrobots injected without cells. Histological analysis at 4 weeks after microrobot-mediated cell delivery in nude mice revealed that the microrobots localised to the edge of the tumour, confirming that the latter formed from correctly delivered cells. Further studies confirmed that microrobots support cell transport and differentiation *in vivo* [49, 50].

Human adipose tissue-derived MSCs were recently grown in microrobots to test the biocompatibility and biodegradability of these systems, important for use in clinical studies [51]. MSC-loaded microrobots were delivered through open surgery into the knee joint of rabbits with defects in the medial condyle in order to analyse the efficiency of the system in cartilage regeneration. Following magnetic targeting using the electromagnetic actuation system, which is composed of multiple electromagnetic coils that can emanate a magnetic field in a desired direction in the 3D space, all MSC-containing microrobots were moved to the area of injury where these were held in place through a magnet applied outside the femur [51]. Interestingly, during the 3 weeks' observation, the microrobots degraded *in vivo*, cells released in the knee joint increased in number and importantly, no significant inflammatory reaction or damage to the cartilage tissue caused by microrobot degradation were found when compared to PLGA-delivered MSCs. Cartilage regeneration was enhanced in the MSC-loaded microrobot injected knees with respect to non-injected ones, showing the efficacy of this system. Further studies investigating the safety of microrobots in the long-term are required in order to apply these in clinical studies.

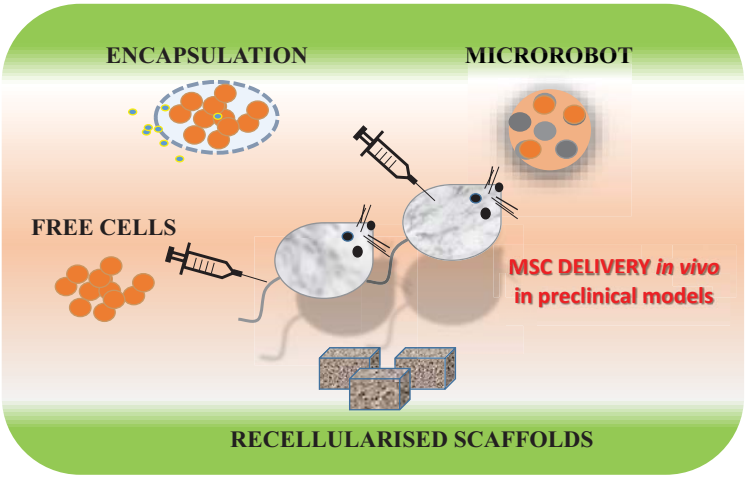

Fig. (2). Strategies to optimise MSC delivery *in vivo*. MSCs can be delivered in various systems, such as in microcapsules or microspheres, or by seeding in microrobots or in decellularised scaffolds. Free cells can be delivered through the tail vein, while intraperitoneal or subcutaneous delivery is preferred for other systems. Studies are hitherto limited to the preclinical setting.

CONCLUSION

Implantable bioengineered cell-based devices and microrobots with the objective of delivering a sufficient number of viable cells or bioactive molecules to injured tissues in order to improve function are the next-generation therapeutic interventions (Fig. **2**). The organ decellularisation and recellularisation procedure

is also very promising but needs automation in order to produce uniform scaffolds for comparing results among clinical studies. From a clinical transplant perspective, the possibility of using discarded human organs as a source of bioscaffold consisting of tissue-specific ECM raises hope in the field of organ bioengineering and regeneration. Thus, finding the optimal cell delivery medium is one of the keys to success in this complex scenario of tissue regeneration with the aid of stem cells.

LIST OF ABBREVIATIONS

PBS	Phosphate-buffered saline
CCl_4	Carbon tetrachloride
ECM	Extracellular matrix
ESRD	End-stage renal disease
EV	Extracellular vesicle
GI-MSC	Glomeruli-derived MSC
HLA	Human leukocyte antigen
MSC	Mesenchymal stromal/stem cell
PEG	Polyethylene glycol
PLGA	Poly lactic-*co*-glycolic acid
PLLA	Poly(lactic acid)

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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.

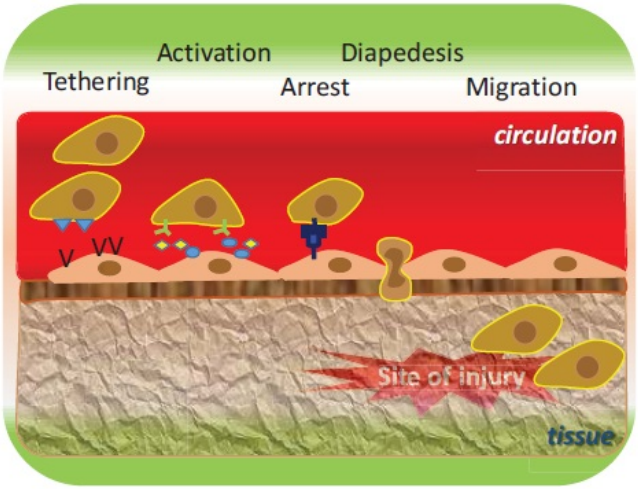

Fig. (1). MSC homing mechanisms. MSCs due to their leukocyte-like properties undergo a 5-step process to exit the circulation and be recruited to the site of injury.

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

imaging modalities allows a careful dissection of the regeneration mechanisms linked to each organ. Assisted by nanobiotechnology, successful labelling and tracking of cells *in vivo* is no longer a vision of the future in preclinical models. Desirable features of these tracking agents include stability to long-term, atoxic to cells, and not affecting cellular function [14].

2.1. Molecular Imaging Methods

In vivo tracking of stem cells using imaging approaches employs different methods such as optical imaging (fluorescence imaging and bioluminescence imaging), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computer tomography (SPECT), computed tomography (CT), photoacoustic imaging (PAI) and ultrasound, which are mainly employed at the preclinical level [15, 16].

2.2. Fluorescence-based Imaging

A series of fluorescent dyes have been developed for cell tracking including chloromethyl-benzamidodialkylcarbocyanine (CM-Dil), carboxy fluorescein diacetate succinimidyl ester (CFSE), Hoechst 33342, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI), and PKH26 (Fig. **2**). Many of these dyes induce significant cellular toxicity and affect the biological and proliferative activity of cells. The lipophilic long-chain carbocyanine dye PKH26 is most widely used as a cell tracer as it stains the stem cell membrane, whilst giving the least cellular toxicity [17]. Use of lipophilic fluorescent stains such as the near-infrared dye DiD **(**1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine) for labelling and tracking transplanted MSCs is also possible. DiD-labelled bone marrow-derived MSCs, for instance, were injected in rats following radiation-induced lung injury, and tracked *in vivo* using fluorescence endomicroscopy imaging [18]. The efficiency of different MSC delivery routes was compared after cell injection, and it was shown that more cells homed to the lungs with endotracheal delivery with respect to intravascular administration. Fluorescent dyes are also used for labelling extracellular vesicles (EVs) to follow their biodistribution *in vivo*. For instance, Grange *et al.* injected DiD-labelled EVs intravenously in a glycerol-induced acute kidney injury mouse model [19]. Optical imaging (using the IVIS 200 small animal imaging system) of the whole body at 15 minutes, 5 hours and 24 hours post-EV injection revealed that EVs accumulated in the injured kidneys. These results were confirmed by analysis of explanted kidneys 5 and 24 hours after DiD-labelled EV treatment, thus showing that optical imaging of labelled cells or their bioproducts can be, with some improvements, feasible.

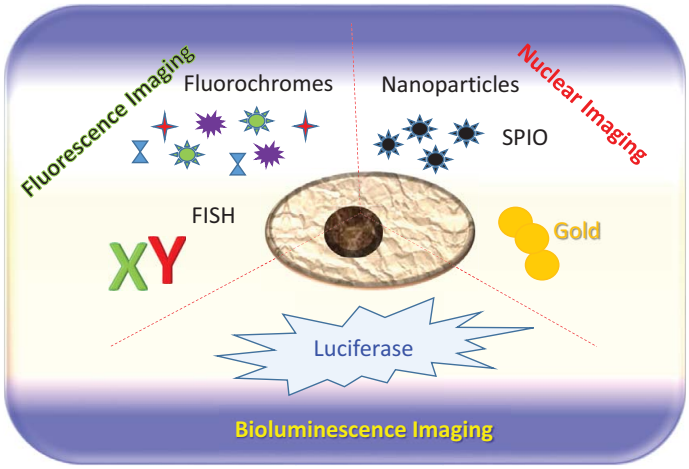

Fig. (2). Main labelling strategies for cell tracking *in vivo*. MSCs labelled with fluorochromes, nanoparticles or express luciferase are revealed by fluorescence microscopy, nuclear imaging techniques such as magnetic resonance or ultrasound/PAI, and bioluminescence imaging, respectively. FISH: fluorescent *in situ* hybridisation; SPIO: super paramagnetic iron oxide.

Limitations: Gradual loss of the dye by leakage from the cell can aspecifically stain neighbouring cells. Reduction during cell division is another limitation of these dyes. There are also recent reports that cell labelling with PKH26 or VybrantDil may result in significantly reduced migration of MSCs *in vitro* [20]. Moreover, light tissue penetration and autofluorescence are serious limitations of optical imaging. Macrophage engulfment of stained MSCs or their derivatives can give aspecific signals. This can be resolved by staining with species-specific antibodies, especially *in situ*ations where xenogenic transplantation is performed. Where it is clear that MSCs transdifferentiate into the cell type of interest *in vivo*, it is also possible to stain tissues for specific markers and analyse the co-localisation of the cell labels with surface markers. Additional studies are, however, required regarding the safety and efficacy of these dyes before their clinical use.

2.3. Bioluminescence-based Imaging

Bioluminescence-based imaging has gained much interest in the monitoring of stem cell-based transplantation studies *in vivo* due to its higher signal-to-noise ratio compared to fluorescence-based methods (Fig. **2**) [21]. Bone marrow-derived MSCs were transduced with a luciferase lentiviral vector prior to intravitreal transplantation into the eyes of rabbits [22]. Bioluminescence signal, followed for 60 days after intraocular D-luciferin injection, decreased gradually from 8 days to 30 days post-MSC injection. At 30 days, the transduced cells

localised exclusively to the vitreous cavity. EVs produced from melanoma cell lines expressing a *Gaussia* luciferase and a truncated lactadherin fusion protein could also be efficiently monitored *in vivo* after intravenous injection [23]. Luciferase activity was measured in blood, and it was shown that EVs disappeared within 2 minutes' of injection from the circulation. Analysis of the tissue distribution of EVs revealed that these entities initially homed to the liver, and then were detected in the lungs and spleen. The results obtained *in vivo* with bioluminescence imaging were further confirmed in *ex vivo* analysis of tissues following PKH26 fluorescently-labelled EVs injection. Thus, bioluminescence offers a superior *in vivo* signal with respect to fluorescence.

Limitations: Bioluminescence imaging is also limited by tissue depth with an imaging depth of 1-2 cm with respect to fluorescence imaging (1 cm) [24]. Moreover, the acquisition time is longer (minutes) following substrate, luciferin, administration with respect to fluorescence (seconds). Moreover, the fur colour of animals may also impact the signal output of bioluminescence imaging. Thus, depilation in preclinical animal studies is highly recommended (Technical note: Pre-clinical in vivo imaging from https://www.perkinelmer.com).

2.4. Fluorescent *in situ* Hybridisation

In preclinical studies, it is possible to detect exogenous cells in organs with fluorescent *in situ* hybridization (FISH) (Fig. **2**). FISH technology shows high sensitivity and specificity in recognising target DNA or RNA sequences, can help in visualising hybridisation signals at the single-cell level, and detects fusion events [25]. Several studies have injected male cells into females or *vice versa* in order to be able to trace the cells of interest [26, 27]. Detection of the Y-chromosome in the target tissue of female recipients was used as an indicator of the efficacy of cell therapy. Others have used FISH to detect the presence of human cells in mice using probes specific for human and mouse centromeres [28, 29]. Fusion of transplanted MSCs with recipient cells may occur spontaneously, albeit rarely, in organs such as the murine heart [30]. In the liver, cell fusion is a physiological process, which is enhanced upon liver injury in mice [31]. The fusion products can perform tissue-specific functions as well as proliferate. Investigations of immunohistochemical expression of cell-specific markers, such as albumin, alpha-fetoprotein, cytokeratin-18 and -19 in liver cells, can help in determining whether transplanted MSCs can adopt a hepatic fate *in vivo*. The FISH technology can be applied to some clinical studies in which biopsy material from the target organ is available. For instance, in a clinical study involving 4 female patients with alcoholic liver injury, the contribution of injected bone marrow-derived MSCs to the liver tissue was determined by FISH. It was shown

that MSCs contributed to the hepatic myofibroblast population but did not generate parenchymal lineages such as hepatocytes or biliary cells [32]. Thus, FISH is important in tracking infused cells inside a structured tissue *in vivo* to answer the questions of "where" and "what" on the whereabouts of the stem cells.

Limitations: Tissue biopsy requirement is the main limitation of this type of cell localisation method. Moreover, the method is costly, requires experienced personnel, and due to limited spectral combinations, not all different RNAs or splice variants can be detected in a single cell [33].

As an advancement of the above-mentioned optical imaging techniques, fluorescent nanodiamond has emerged as an attractive alternative for quantitative tracking of stem cells *in vivo* as this nanomaterial is chemically inert, biocompatible, and does not change labelled cells' properties. The particle has negatively charged nitrogen-vacancy centres that are fluorescence sources with broad excitation and emission spectra in the visible and near infrared range and magnetic field-dependent fluorescence emission [34]. Fluorescent nanodiamonds have been successfully used to label MSCs derived from human placentas for precisely determining the number and position of intravenously transplanted MSCs in miniature pigs [35]. Interestingly, 70% of labelled MSCs were found in the lungs at 24 to 48 hours post-cell injection. This method offers promise for single cell imaging *in vivo* in preclinical studies.

2.5. Nanoparticles-based Tracking

Nuclear imaging, which provides excellent sensitivity and good tissue penetration, has been extensively used in preclinical and clinical studies. MRI has been used to track MSCs in preclinical studies [36]. To visualize MSCs using MRI, the cells were treated with an intracellular contrast agent such as super paramagnetic iron oxide (SPIO) to track them in damaged tissues post-transplantation (Fig. **2**). Non-invasive and repeated *in vivo* monitoring could be achieved in this due to the significant temporal and spatial contrast of SPIO-based MRI [37]. However, ischemic and hypoxic environments can significantly reduce the signals [36]. In another setting, MSCs were labelled with gold nanospheres and were administered through the cornea into the anterior chamber of porcine eyes *ex vivo* [38]. The distribution of labelled MSCs was monitored longitudinally by an ultrasound/PAI platform. EV labelling for nuclear imaging is also possible. Recently, radio-iodine (I^{131})-labelling of thyroid cancer cell-derived EVs was performed, and these EVs were injected intravenously in mice for biodistribution analysis using gamma camera imaging [39]. Nuclear imaging techniques and multimodal approaches are also promising for the detection of small quantities of EVs *in vivo*, but further experimental set-up is required.

3. ASSESSING THERAPEUTIC EFFICACY IN CLINICS.

Tracking cells during regenerative cytotherapy is crucial for monitoring their engraftment, safety, and efficacy. Albeit several cell tracing techniques exist by which the infused stem cell fate can be followed, these are mainly limited to preclinical models. A reliable, clinically applicable cell-tracking agent is needed so that biodistribution and clearance can be defined to better understand potential off-target toxicity and redosing strategies [40]. Hitherto, in none of the clinical trials colonisation or engraftment of transplanted cells has been looked for in the recipient organ.

CONCLUSION

Novel labelling technologies and imaging modalities are urgently required to track transplanted cells *in vivo*, regardless of tissue depth. Localisation and fate mapping of stem cells within the target organ help in understanding the utility of the transplanted cells in the disease model. Several imaging technologies used hitherto rely on target tissue biopsies, which are not always possible to obtain. Thus, *in vivo* monitoring of MSC therapeutic efficacy, albeit challenging, is mostly encouraged. As GMP-regulated bioproducts are essential for clinical studies, it is important for researchers to move towards using GLP/GMP-type cell-based products for preclinical studies, too, in order to use standardised procedures to obtain reproducible, comparable and interpretable inter-studies results.

LIST OF ABBREVIATIONS

CM-DiI	Chloromethyl-benzamidodialkylcarbocyanine
CFSE	Carboxy fluorescein diacetate succinimidyl ester
CT	Computed tomography
CXCR	Chemokine CXC receptor
DAPI	4,6-diamidino-2-phenylindole, dihydrochloride
DiD	1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine
FISH	Fluorescent *in situ* hybridisation
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
IGF-1	Insulin-like growth factor-1
MAPCs	Multipotent adult progenitor cells
MRI	Magnetic resonance imaging

PAI Photoacoustic imaging

PDGF Platelet-derived growth factor

PET Positron emission tomography

SDF-1 Stromal cell-derived factor-1

SPECT Single photon emission computer tomography

SPIO Super paramagnetic iron oxide

VLA Very late activation antigen

VCAM Vascular cell adhesion molecule

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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.

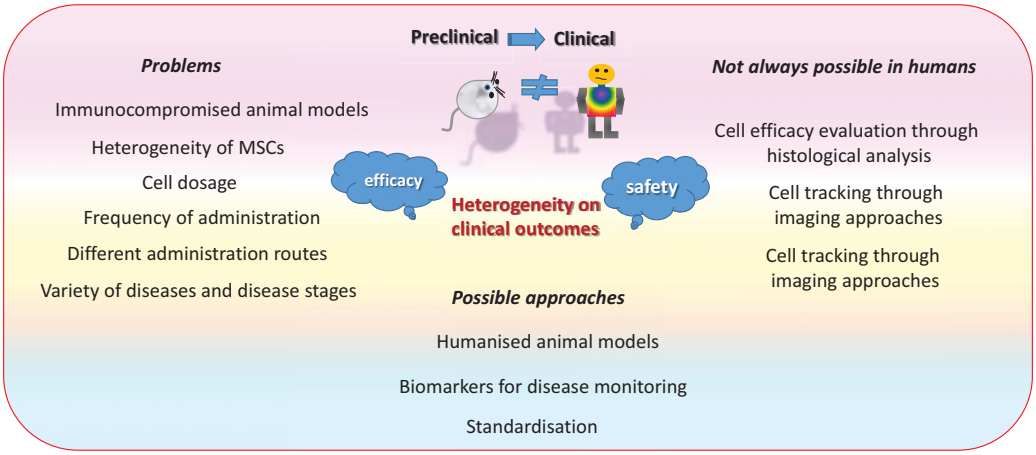

Fig. (1). Hurdles associated with the translatability of preclinical data to MSCs application in clinics. The translation of preclinical outcomes of MSC therapy has encountered several problems and some technical problems that have generated heterogeneous results in clinical studies. Some strategies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

apoptotic and phagocytic cell markers [10]. Most studies, in fact, do not show long-term cell tracing results beyond 2 months post-cell injection.

MSCs are usually cleared rapidly from the body, which raises the question on the mechanism of action behind their therapeutic effect [11]. It can be hypothesised that a small fraction of administered MSCs survives and migrates to sites of injury and inflammation to confer beneficial effects of these cells or that MSCs are able to rapidly mediate tissue repair or immunomodulation by interacting with and imparting their effects onto other cells, such as the immune cells [12]. For instance, it was found that MSCs, following stimulation by factors released by T cells, secrete prostaglandin E2 and IL-10 for example, to attract other T cells in the vicinity in order to inhibit these [12].

Survival after injection is a critical factor in stem cell therapies and is often challenging due to the harsh environmental conditions in cases of severe diseases that MSCs may encounter upon transplantation. MSCs survival rates vary across studies involving various animal and clinical models, from short-term integration to a longer residence. These data are quite heterogeneous and depend on several factors, including the route of MSC delivery as well as pathology under consideration. Cell survival studies in humans are not feasible due to the fact that repeat biopsies are not possible and ethically acceptable [13].

All these unanswered questions and current challenges regarding the use of MSCs in cell therapy approaches need to be addressed before a fully competent MSC can fully resolve any patient's health problem. Thus, ongoing research is focused on finding strategies to improve the therapeutic efficacy of MSCs *in vivo*, for instance, by augmenting survival, homing and residence time of MSCs in the target organs and improving labelling approaches to track infused cells.

2. STRATEGIES TO IMPROVE HOMING AND ENGRAFTMENT OF STEM CELLS AND DERIVATIVES

MSCs may encounter an unfavourable microenvironment *in vivo*, causing most of the transplanted cells subsequently to undergo cell death. Thus, the limited number of MSCs reaching the target organ mitigates their therapeutic action. Various strategies to improve MSC function and survival have been developed and implemented. These include genetic engineering, preconditioning used during the culture phase; tissue engineering used on a 3D matrix and involving signaling molecules; and cell-free therapy achieved through the use of extracellular vesicles.

2.1. Genetic Engineering

MSCs possess various beneficial biological properties. Optimisation of the natural function of MSCs and their derivatives such as EVs in different aspects of tissue regeneration, organ repair and immune-modulation is possible [14]. Genetic engineering approaches have been employed to modify MSCs in order to modulate the production of inflammatory mediators and cytokines, for instance (Fig. **2**). In other cases, crucial therapeutic genes have been introduced in MSCs in order to maximise beneficial effects. MSCs can also be employed as a platform for loading therapeutic cargo into secreted EVs for delivery to target organs. This can enlarge the spectrum of diseases for which MSCs could provide therapeutic benefits.

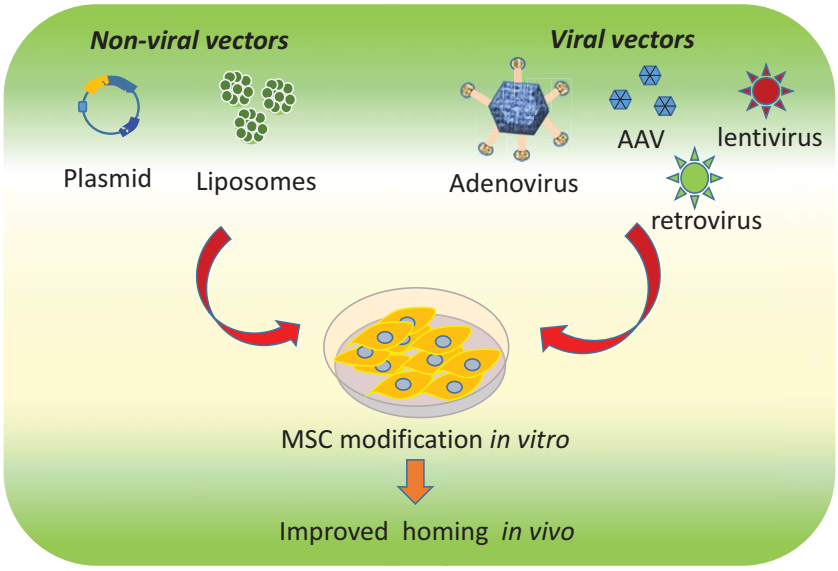

Fig. (2). Genetic engineering strategies to modify MSCs. Non-viral vectors such as plasmids or liposomal formulations can be transfected into MSCs. These cells can be efficiently transduced with different types of viral vectors such as lentivirus, retrovirus, adenovirus, and adeno-associated virus.

Several different tools and molecular engineering techniques are used to modify genomic sequences, such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9. CRISPR/Cas9 has been employed for gene editing of MSCs, for instance, it was shown that site-specific mutagenesis and integration of over 3 kilobases of exogenous DNA (PDGF-B, VEGF-A, and IL-10) in the genome of human MSCs derived from different sources as the bone marrow, umbilical cord and adipose tissue was feasible [14]. This manipulation did not change the characteristics of the MSCs, but assisted in delivering a higher concentration of growth factors to enhance wound healing in the diabetic mouse

model. RNA interference technology using siRNAs or microRNAs that inhibit the expression of messenger RNA of target genes in a sequence specific manner has also been successfully employed in MSCs [15, 16]. Interestingly, miRNA-modified MSCs transmit the beneficial effects to exosomes for tissue repair *in vivo* [17].

Integrating and non-integrating viral vectors, as well as delivery of plasmid-DNA or mRNA, have been used to modify MSCs. Integrating vectors, such as lentiviral vectors, were used to express the chemokine CXCR4, in bone marrow-derived MSCs for the treatment of lung injury in rats [18]. Lentiviral vectors, due to their capacity to infect non-dividing or slowly proliferating cells, are particularly useful in delivering transgenes into MSCs. Despite promoting non-site specific integration of the transgene in the genome, the use of lentiviruses does not significantly change MSCs differentiation potential [19]. Another approach is transgene delivery *via* plasmids. Transient delivery of CXCR4-expressing plasmid DNA in MSCs currently has low efficacy and can affect cell proliferation rate [19, 20]. mRNA transfection by different methods, such as electroporation and cationic lipids, can also help in achieving the transient overexpression of molecules of interest. For example, human MSCs transfected with CXCR4-GFP fusion mRNA showed increased migration potential towards a CXCR4 ligand SDF-1 gradient *in vitro* [21]. Thus, safe genetic modification of MSCs to enhance their therapeutic effects is possible and will help in improving clinical outcomes.

2.2. Tissue Engineering

Tissue engineering approaches have been adopted *in vitro* to promote MSC tissue-specific adaptation *in vitro* before transplantation. These include co-culture systems and the development of 3D scaffold-based (hydrogels, sponges, fibres, for example) or scaffold-free (temperature-responsive polymers for making single layer sheets, for example) structures [22, 23]. For instance, co-culture of bone marrow-derived MSCs with hepatocytes in a ratio of 1:5 in PLGA scaffolds improved liver function *in vivo* in a mouse model of acute liver failure with respect to MSC-PLGA alone [24]. Natural decellularised scaffolds have also been employed to promote MSC differentiation by providing a more physiological environment before transplantation *in vivo* [25]. Cells grown in these systems would be conditioned to home to and engraft in the target organ.

2.3. Preconditioning

Various factors help in preconditioning MSCs in order to preserve their homing and survival *in vivo* against stress and insults in the pathological environment.

Several strategies employing physical, chemical and biological factors have been adopted to precondition stem cells, such as hypoxia, heat shock, pharmacological or chemical agents, trophic factors and cytokines, and physical factors (pulsed electromagnetic fields) and materials (silica particles separated according to size) [26]. Regarding the latter, it was shown that upon silica incubation with culture media, smaller nanoparticles and larger microparticles formed and could be separated on the basis of size. The nanoparticles could promote the proliferation of adipose tissue-derived MSCs by inducing phosphorylation of the survival signalling factors ERK1/2, while the microparticles stimulated apoptosis in the MSCs, showing that particle size matters. Regarding small molecules, An *et al.* showed that treatment of human umbilical cord-derived MSCs with Valproic acid, a histone deacetylase inhibitor, increased the differentiation of MSCs towards a hepatic fate rather than towards an adipogenic fate [27]. This strategy can be adopted before cell transplantation in order to stimulate MSCs to home to or secrete therapeutic factors that can restore hepatic functionality once *in vivo*. Cultivating MSCs under hypoxic conditions is important as the natural microenvironment of these cells is recreated *in vitro*. Different levels of oxygen concentration in culture can affect MSC behaviour. For instance, cultivating bone marrow-derived MSCs at 1% followed by infusion through the portal vein decreased liver injury and promoted liver regeneration after massive (85%) hepatectomy in rats with respect to cells grown in normoxic conditions [28].

2.4. Reducing Cell Entrapment in the Lungs

Stem cell entrapment in the lungs and other organs could be caused by a combination of mechanical and physiological conditions, the small capillary size, the large capillary network as well as the strong adhesive properties of MSCs. Lung entrapment may cause unwanted effects such as embolism in small animals when such cells are studied at preclinical levels [29]. For instance, MSC entrapment in the lungs was significantly dampened upon treatment with the vasodilator sodium nitroprusside prior to MSC transplantation [30]. Pretreatment of mice with sodium nitroprusside in a CCl_4-induced fibrosis model also improved homing and led to a reduction in fibrosis-related gene expression, such as α-SMA; collagen 1a1 [31]. Thus, acting on cell retention in the lungs can improve the therapeutic efficacy of transplanted MSCs. Of note is the fact that sodium nitroprusside is an arterial and venous vasodilator used in clinical practice to lower blood pressure [32]. Its utility as an enhancer of stem cell homing in patients should be investigated.

2.5. Improved Labels for *in vivo* Cell Tracking

Cell labelling and tracking are essential in determining the success of cell therapy and the therapeutic efficacy of inoculated cells *in vivo*. As described in Chapter 7, several approaches and technologies have been devised to track MSCs after transplantation. However, whether the live labelled MSCs themselves emanate the signal or whether tissue macrophages after engulfing labelled MSCs is still not clear. Kang *et al.,* for instance, described a new technology for labelling and tracking cells based on bioorthogonal chemical reporters [33]. Briefly, cells were treated with tetra-acetylated N-azidoacetyl-D-mannosamine (Ac_4ManNAz) to generate unnatural sialic acids with azide groups on the surface of different types of carcinoma cells [33]. The azide-labelled cells were then transplanted into mouse livers, followed by the intravenous infusion of dibenzyl cyclooctyne-conjugated Cy5. The latter chemically bound to the azide groups exposed on the surface of the transplanted cells *in vivo*, hence allowing specific visualization of the target cells. Importantly, near-infrared fluorescence images obtained *ex vivo* and *in vivo* showed that distorted signals generated by macrophages' phagocytosis of target cells were reduced, and as low as 3×10^3 labelled carcinoma cells could be detected in the liver of mice. This strategy can develop into a very useful tool for specifically tracking injected cells *in vivo* effects without interference from macrophages.

3. MSC MODIFICATIONS SPECIFIC FOR TARGET ORGANS

3.1. Genetically Modified MSCs for the Treatment of Acute Liver Injury

The acute liver injury occurs upon insults by a multiplicity of factors and can result in massive hepatocellular necrosis and liver failure if not treated in a timely manner. Cell therapy markedly increases the bridging time for orthotopic liver transplantation in patients with acute liver failure, hence allotting more time to transplant operators to find suitable donor organs [34]. Efficient targeting and homing of MSCs to the injured area is, therefore, one of the key steps towards achieving a better therapeutic outcome in patients with liver injury. Liver macrophage deletion has been shown to enhance MSCs homing to CCl4-treated mice and reduce hepatic fibro-inflammatory reaction [35]. MSCs have also been modified to perform better *in vivo* and genetic engineering has been of utmost relevance [14].

Several studies have shown that the SDF-1/ CXCR4 axis is crucial for homing of MSCs to sites of injury. MSCs express CXCR4, but the level is gradually reduced *in vitro* with culture and expansion [36]. SDF-1 is expressed at higher levels upon injury and acts as a potent chemoattractant to recruit transplanted CXCR4-

expressing [37]. Increasing the levels of CXCR4 on MSCs was a strategy employed to accelerate mobilisation of an immortalised human bone marrow-derived MSC cell line, UE7T-13 [38]. MSCs were transduced with a lentiviral vector expressing the human CXCR4 gene. Intravenous injection of these modified MSCs, following DiR labelling, in a mouse model of CCl_4-induced liver injury showed that the majority of CXCR4-MSCs were localised to the injured liver 5 days post-transplantation with respect to the control MSCs that showed equal homing to both the liver and the spleen. There was a significant improvement in liver regeneration and function, probably due to the increased secretion of HGF and VEGF by the modified MSCs [38]. In another study, bone marrow-derived MSCs were genetically modified to overexpress the HGF receptor c-Met in order to improve the migration and homing ability of these cells [39]. C-Met-MSCs were administered through the tail vein of rats subjected to D-galactosamine/lipopolysaccharide-induced acute liver failure. Fluorescence imaging of DiR-labelled MSCs revealed that the modified cells migrated more efficiently to the liver, improved hepatic function (decreased serum levels of serum aminotransferases and total bilirubin) and increased survival compared to control MSCs.

Other strategies to improve homing and engraftment in the injured liver regard rendering MSCs resistant to apoptosis by overexpressing the pro-survival factor Akt1. Moreover, lowering the expression of HLA-1 on the surface of MSCs may assist in immune evasion *in vivo*, hence allowing allogeneic MSC engraftment with higher efficiency [40].

3.2. Genetically Modified MSCs for Treatment of Corneal Injuries

Genetically modified MSCs have been used in a few studies for corneal repair. Lentivirus-mediated modulation of miR146a expression was performed in bone marrow-derived MSCs [41]. Rats undergoing corneal alkali burn were administered MSCs through the tail vein. It was shown that MSCs expressing high levels of miR146a ($miR146a^{high}$) were protected from apoptosis *in vivo*, induced an improvement in corneal opacity, reduced neovascularisation and inflammation, and improved histology of the corneal tissue, compared to control or $miR146a^{low}$ MSCs. In another study, bone marrow-derived MSCs expressing IL-10 *via* lentiviral transduction were subconjunctivally injected in a rat model of corneal allograft rejection [42]. Injection of the modified MSCs significantly extended the graft survival time from 10 days to 20 days and reduced immune cell (T cells and macrophages) infiltration. These results are indeed promising and warrant further research on the long-term effects of the modified MSCs.

3.3. Genetically Modified MSCs for the Treatment of Acute Kidney Injury

Transplanted MSCs have shown beneficial effects in the setting of kidney injuries, but the kidney-directional homing, for instance, following ischemia-reperfusion injury is poor [43]. Thus, strategies to improve homing of MSCs by genetic modification have also been implemented for kidney repair. MSCs were transfected with plasmids overexpressing Lipocalin-2 (Lcn2) which was shown to offer protection from acute ischemic renal injury, inflammation, and infection [44]. Modified MSCs were injected intravenously in the AKI model induced by cisplatin in rats [45]. There was a significant improvement in renal function and histology upon MSC-Lcn2 injection in these rats through the improved regenerative potential of the modified MSCs and their ability to secrete protective factors such as HGF, IGF-1, FGF, and VEGF.

CXCR4-modulated bone marrow-derived MSCs were also administered by straight perfusion while draining blood from donor kidneys and 24 hours (a time point when SDF-1 level peaked in the transplanted kidney) after transplantation *via* the caudal vein in a rat transplantation model with unilateral nephrectomy [46]. It was shown that MSCs overexpressing the CXCR4-eGFP fusion protein on their cell surface had significantly enhanced homing to the transplanted kidneys with respect to native MSCs. Some homing to other tissues, like the lungs, bone marrow, spleen and liver were also noted, without any significant difference between modified and non-modified MSCs-injected mice. Thus, CXCR4 had beneficial effects on renal cell proliferation and survival, while it reduced pro-inflammatory cytokine levels and reduced infiltration of macrophages, CD3+ T-cells and dendritic cells, hence resulting in amelioration of kidney transplant failure.

In another study, overexpression of nuclear factor erythroid-2 related factor 2 (Nrf2) by recombinant adenovirus in bone marrow-derived MSCs resulted in resistance to apoptosis *in vitro* [47]. Intravenous injection of the modified MSCs in a cisplatin-induced AKI rat model provided even greater improvement in renal function and histology compared to control MSCs. These studies show that genetic modification of MSCs to enhance their homing, and consequently therapeutic function, *in vivo* is feasible. However, studies regarding the stability and properties of the modified MSCs in the long term *in vivo* are much awaited.

4. IMPORTANT DECISIONS BEFORE PERFORMING CELL TRANSPLANTATION

Several factors are critical for the success of cell therapy in patients. Any underlying disease or advanced age of the donor may affect stem cell functionality (proliferation, differentiation, secretory function) and number [48].

Moreover, in preclinical studies, usually, young animals are used to study MSCs' therapeutic efficacy, while in clinical practice, the diseases to be treated can affect both children (in case of hereditary diseases) or elderly (in case of age-related diseases). In the latter case, the therapeutic outcome observed in preclinical models may not be achieved due to microenvironment differences.

The decision of which administration routes to adopt is discussed in the previous chapters and should be adapted to the disease context and target organ. Regarding the frequency of MSC administration, in a systematic review and meta-analysis to analyse the therapeutic efficacy of MSCs against liver disease and the factors involved, Zhao *et al.* showed the single injection of MSCs showed an improvement in albumin, alanine aminotransferase and total bilirubin as early as 2 weeks post-cell injection with respect to multiple injections which showed improvement only after 8 weeks or more if any [49]. Moreover, in some cases, such as in the mouse model of experimental allergic asthma, double intravenous injection of adipose tissue-derived or bone marrow-derived human MSCs, performed during ovalbumin sensitisation and ovalbumin challenge, showed that the double MSC treatment significantly increased infiltration of inflammatory cells in the lungs with respect to the single MSC infusion (during OVA challenge only) [50]. MSC injection was more effective in the latter case when the lung environment was inflammatory, hence showing that both dose and timing of MSC injection are important. All these parameters have to be considered accurately before proceeding with clinical studies.

5. IMPORTANCE OF BIOMARKERS IN HUMAN DISEASE MONITORING

In preclinical studies, it is possible to monitor MSC homing and engraftment in order to correlate with therapeutic outcomes. In patients, this is not always possible. There is thus an urgent need to find biomarkers that can assist in the monitoring of the fate of MSCs *in vivo*.

EVs can provide important insights into the prediction and/or monitoring of pathological processes following MSC administration. This is because EV types can be distinguished on the basis of protein they contain: exosomes contain endosomal proteins; microvesicles contain cytosolic and plasma membrane proteins, and apoptotic bodies contain nuclear, endoplasmic reticulum or Golgi proteins [51]. For instance, the detection of graft-derived exosomes expressing Collagen V was found in the serum of lung transplant recipients 3 months before acute rejection, implying that exosomes with lung associated self-antigens could serve as a non-invasive rejection biomarker [52]. Thus, biomarkers to monitor clinical efficacy response are required for each MSC/disease combination.

CONCLUSION

The impressive technological advances in regenerative medicine have shown the great potential of MSCs application in clinics. However, several issues need better control and further investigations in order to set the pace for the upcoming MSC-based therapy. Modifying the therapeutic cell or its microenvironment to better direct a sufficient quantity of administered cells to the sites of injury is a significant step forward. Cell labelling and tracking technologies allowing to visualise MSCs in the organs of interest have helped in correlating cell homing with therapeutic outcomes. Specific biomarkers, which permit the non-invasive and longitudinal assessment of treatment outcome, will also help in tackling some of the hurdles currently facing the routine use of MSCs in the clinical setting.

LIST OF ABBREVIATIONS

α -SMA	α-smooth muscle actin
AKI	Acute kidney injury
CCL_4	Carbon tetrachloride
CKD	Chronic kidney disease
CMFDA	(5-chloromethylfluorescein diacetate)
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/Caspase 9
CXCR4	Chemokine CXC receptor 4
eGFP	Enhanced green fluorescent protein
EV	Extracellular vesicle
HLA	Human leukocyte antigen
IL	Interleukin
Lcn2	Lipocalin-2
MHC	Major histocompatibility complex
Nrf2	Nuclear factor erythroid-2 related factor 2
MSC	Mesenchymal stromal/stem cell
PDGF	Platelet-derived growth factor
PLGA	Poly(lactic acid-glycolic acid)
SDF-1	Stromal cell-derived factor-1
VEGF	Vascular endothelial *growth* factor

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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.

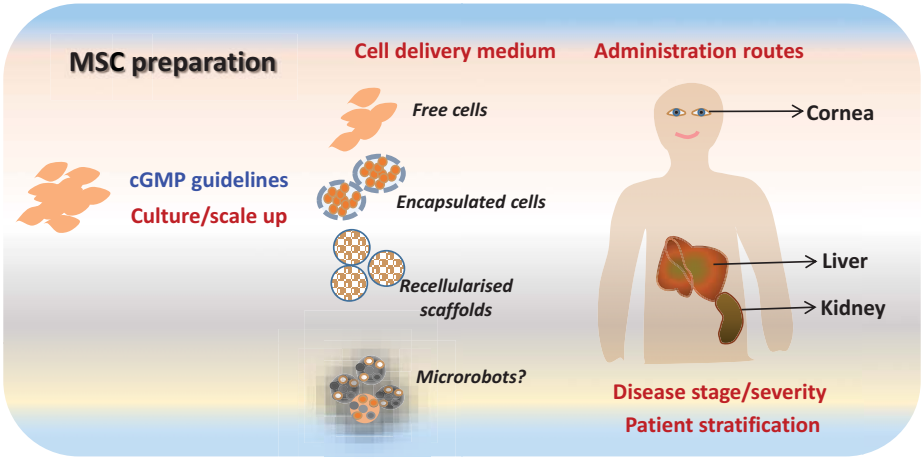

Fig. (1). MSCs delivery in the clinics. Improvements are required at several steps, from MSC preparation to cell administration route. Standardisation of MSC production according to current GMP guidelines is the first step towards getting comparable inter-studies results, followed by the choice of delivery 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].

The safety of human liver-derived MSCs has also been evaluated in a Phase I/II clinical trial in patients affected by Urea Cycle Disorder and Crigler-Najjar syndrome. Liver-derived MSCs were infused intraportally in the patients. One month after cell infusion, the overall incidence rate of adverse events was low, showing tolerability of these MSCs in the patients [9]. Importantly, the human liver MSCs could in part restore metabolic activity in these patients. In another study, intrahepatic administration of human liver-derived MSCs (HLSCs) in pediatric patients with inherited neonatal-onset hyperammonemia was performed for safety study [10]. No adverse events or intra-and extra-hepatic complications were observed during the course of the study. Despite an increase in protein intake, the patients transplanted with HLSCs were metabolically stable. It is important to note that in most of the clinical studies performed, MSCs served as an important bridge therapy whilst awaiting liver transplantation [10].

The search term "mesenchymal stem cells" revealed 1002 clinical studies registered by the FDA, of which 307 have been completed to date (last search date: 03/06/21). Interestingly, 787 are early trials (phase 1 or phase 2), 55 studies progressed to Phase 3, and 4 to Phase 4, of which 2 have been completed with one in the US and one in India (www.clinicaltrials.gov). The latter (*clinicaltrials.gov* NCT04243681) regards the use of autologous CD34+ hematopoietic cells and MSCs in patients with cryptogenic decompensated cirrhosis. Results of the preceding Phase I and Phase II studies have been published, and it was shown that cell infusion through the hepatic artery under fluoroscopic guidance was safe and the patients showed a trend towards improvement of the MELD score and serum albumin, without worsening of clinical parameters [11]. Ongoing clinical studies are recruiting patients for the treatment of liver diseases with varying aetiologies with MSCs. A recently published Phase I/II study in Indonesia (*clinicaltrials.gov* NCT04357600) will use allogeneic MSCs derived from the umbilical cord for intravenous injection in patients with liver cirrhosis. One hundred million cells will be injected per patient and during the 6 months follow-up, with an assessment of liver function, MELD score and Child-Pugh score.

Most clinical studies suggest that MSC therapy is safe in patients with liver disease. However, few studies have shown the therapeutic efficacy of MSCs in improving liver function. Thus, randomized controlled trials are required to increase the number of patients enrolled to confirm the efficacy of MSCs therapy in liver disease [12].

2.2. Ocular Surface

Different routes of administration of MSCs have been investigated in human clinical studies for ocular surface regeneration. The first clinical trial performed

with bone marrow-derived MSCs aimed at analysing the safety and efficacy of these cells in improving corneal epithelial damage in patients affected by limbal stem cells deficiency (*clinicaltrials.gov* NCT01562002) [13]. In this proof-o--concept, randomised, and double-masked pilot trial, MSCs were seeded on the human amniotic membrane, which was topically applied to the damaged eye to promote regeneration. Other clinical studies registered on the www.clinicaltrials.gov website have employed subconjunctival injection of MSCs derived from various sources for corneal damage repair, but hitherto no results have been published (Table **1**) [14]. Despite the fact that restoration of the corneal epithelium has been observed in animal models, further clinical studies are required to state that these MSC delivery routes are safe for the patients, and especially that therapeutic efficacy is achieved.

Table 1. Currently registered clinical trials using MSC-based products for the treatment of corneal diseases.

NCT Number	Title	Status	Conditions	Cells/injection routes	Phases	Locations
NCT04484402	Treatment of Patients With Inflammatory-dystrophic Diseases of the Cornea Using Autologous Stem Cells	Completed	Corneal Ulcer|Corneal Disease|Corneal Dystrophy	MSCs/NA	Phase 1|Phase 2	Belarus
NCT01562002	Safety Study of Stem Cell Transplant to Treat Limbus Insufficiency Syndrome	Completed	Limbus Corneae Insufficiency Syndrome	Bone marrow-derived MSCs/Amniotic Membrane Transplant	Phase 1|Phase 2	Spain
NCT03878628	Treatment With Allogeneic Adipose-derived Mesenchymal Stem Cells in Patients With Aqueous Deficient Dry Eye Disease	Active, not recruiting	Dry Eye|Kerato Conjunctivitis Sicca|Aqueous Tear Deficiency	Adipose tissue-derived MSCs/ NA	Early Phase 1	Denmark
NCT04615455	Mesenchymal Stem Cell Therapy of Dry Eye Disease in Patients With Sjögren's Syndrome	Recruiting	Keratoconjunctivitis Sicca, in Sjogren's Syndrome	Allogeneic Adipose-derived MSCs/ transconjunctival injection	Phase 2	Denmark
NCT04213248	Effect of UMSCs Derived **Exosomes** on Dry Eye in Patients With cGVHD	Recruiting	Dry Eye	Umbilical Mesenchymal Stem Cells derived **Exosomes** /drops, topical application	Phase 1|Phase 2	China

(Table 1) cont.....

NCT Number	Title	Status	Conditions	Cells/injection routes	Phases	Locations
NCT03967275	Subconjunctival Injection of Allogeneic Mesenchymal Stem Cells in Severe Ocular Chemical Burn	Recruiting	Severe Ocular Chemical Burn	Allogeneic bone marrow-derived MSCs /subconjunctival	Observational	France
NCT03237442	Umbilical Cord Mesenchymal Stem Cells Injection for Ocular Corneal Burn	Unknown	Ocular Corneal Burn	human umbilical cord MSCs/ subconjunctival	Phase 1|Phase 2	China
NCT02325843	the Treatment of Human Bone Marrow Mesenchymal Stem Cells in Ocular Corneal Burn	Completed	Chemical Burns	human bone marrow-derived MSCs/ subconjunctival	Phase 2	China

The search terms “cornea” and “mesenchymal stem cells” were used (www.clinicaltrials.gov).,

2.3. Kidney

MSCs isolated from various sources have also been used in patients with kidney diseases in several clinical trials. Cardiac surgery subjects suffering from AKI were treated with allogeneic bone marrow-derived MSCs delivered intravenously to evaluate the safety and efficacy of these cells for the treatment of kidney injury (*clinicaltrials.gov* NCT01602328). In another study, intra-aortic infusion of MSCs was performed in patients with AKI following cardiac surgery, and no adverse events were observed (*clinicaltrials.gov* NCT00733876). Interestingly, a decreased renal inflammation was seen following intra-arterial delivery of autologous adipose tissue-derived MSCs in patients with CKD due to hypoxia, inflammatory injury and human renovascular hypertension (*clinicaltrials.gov* NCT02266394). The safety and efficacy autologous adipose tissue-derived MSCs intravenous injection in CKD patients [15]. No adverse effects were noted, and importantly, in some patients, there was urinary protein excretion, showing some therapeutic effects of administered MSCs. MSCs were also used in clinical trials on patients with focal segmental glomerular sclerosis, diabetic kidney disease and autoimmune disease (refractory systemic lupus erythematosus, lupus nephritis) [16]. Moreover, MSCs were injected in kidney transplant recipients in a Korean Phase I clinical study. Two patients were injected with allogeneic MSCs (1 million cells/kg every other week) for 4 cycles *via* the peripheral vein in the distal arm [17]. No serious adverse effects were found, but renal function gradually decreased between the last cell infusion and the study endpoint. These results suggest that MSC administration in these patients is safe, but further studies are needed to show the long-term therapeutic efficacy of these cells.

Other studies are currently recruiting patients. For instance, Type 2 diabetic

nephropathy-affected patients are being recruited in Shanghai, China, for treatment with umbilical cord-derived MSCs (*clinicaltrials.gov* NCT04216849). A dose of 1.5 x 10^6/kg MSCs will be administered through the peripheral vein at 0, 8, 16, 24 and 32 weeks, with the primary endpoints being measurement of the glomerular filtration rate and urinary albumin creatinine ratio, and the secondary endpoints regarding assessing the levels of HbA1C, plasma insulin, C-peptide and insulin at 48 weeks post-treatment.

On the basis of the results of clinical studies performed hitherto, it can be deduced that MSC administration in patients with kidney diseases is safe, and there are some indications of efficacy. The use of MSCs has been extensively investigated in pre-clinical studies and has given promising results. The beneficial effects now need to be investigated in large scale clinical trials whilst evaluating the optimal MSC dose and injection route as well as the source of MSCs before these cells can be accepted as a revolutionary strategy for kidney regeneration and disease treatment.

3. EXTRACELLULAR VESICLES MOVING INTO CLINICAL TRIALS

EVs are a promising part of the cell-free therapeutic approaches under consideration in clinics. Currently, there are 216 clinical trials registered on www.clinicaltrial.gov website when the search term “exosomes” is applied, of which 48 are in East Asia, 60 in Europe and 73 in the US (last updated on 01/06/21). Some studies have been completed; others are recruiting. Most of these clinical trials are on the feasibility and safety of EV administration for different clinical applications, ranging from chronic diseases to cancer. Interestingly, a randomised, placebo-controlled, phase II/III clinical pilot study has been completed, and results were published [18]. It was shown that intra-arterial and intravenous administration, respectively, of 2 doses of umbilical cord-derived EVs was safe and was accompanied by a significant improvement in estimated glomerular filtration rate, serum creatinine level, blood urea and urinary albumin creatinine ratio in grade III-IV chronic kidney disease patients. On the other hand, no amelioration in kidney function was seen in placebo-treated patients. Thus, bio-products from MSCs are very promising for the treatment of human diseases, but several challenges remain to be addressed for the routine application of EVs.

CONCLUSION

The studies on MSC-based medical treatments have raised hope, but at the same time debates, regarding the controversies around this issue. Variable results in clinical trials have been a matter of discussion, and despite the setbacks, MSC-

based therapy has obtained approval in several countries. Many factors can influence clinical outcomes, and it has become imperative, in order to step further in this important pillar of regenerative medicine, to encourage standardisation of MSC production procedures globally to obtain clear clinical benefits. Studying the outcomes of MSC injection *via* different routes in clinical trials, and identifying the most functional one for the treatment of each organ, is also crucial in paving the way towards a routine application of these stem cells in the clinics.

LIST OF ABBREVIATIONS

AKI Acute kidney injury

GMP Good Manufacturing Practice

CKD Chronic kidney disease

EMA European Medicines Agency

FDA Food and Drug Administration

HBV Hepatitis B virus

HLSCs Human liver stem cell

MELD Model for End-Stage Liver

MSC Mesenchymal stromal/stem cell

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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

(Table 1) cont.....

NCT no.	Title	Status	Conditions	Interventions	Locations
NCT03384433	Allogenic Mesenchymal Stem Cell Derived Exosome in Patients With Acute Ischemic Stroke	Recruiting	Cerebrovascular Disorders	Biological: exosome	Iran
NCT04356300	Exosome of Mesenchymal Stem Cells for Multiple Organ Dysfunction Syndrome After Surgical Repair of Acute Type A Aortic Dissection	Not yet recruiting	Multiple Organ Failure	Biological: Exosome of MSC	–
NCT04544215	A Clinical Study of Mesenchymal Progenitor Cell Exosomes Nebulizer for the Treatment of Pulmonary Infection	Recruiting	Drug-resistant	Biological: Dosage 1 of MPCs-derived exosomes; Dosage 2 of MPCs-derived exosomes; No MPCs-derived exosomes	China
NCT03437759	MSC-Exos Promote Healing of MHs	Active, not recruiting	Macular Holes	Biological: exosomes derived from mesenchymal stem cells (MSC-Exo)	China
NCT04798716	The Use of Exosomes for the Treatment of Acute Respiratory Distress Syndrome or Novel Coronavirus Pneumonia Caused by COVID-19	Not yet recruiting	Covid19|Novel Coronavirus Pneumonia|Acute Respiratory Distress Syndrome	Drug: MSC-exosomes delivered intravenously every other day on an escalating dose: (2:4:8) or (8:4:8) or (8:8:8)	United States
NCT03562715	microRNAs Role in Pre-eclampsia Diagnosis	Completed	Preeclampsia		–
NCT04213248	Effect of UMSCs Derived Exosomes on Dry Eye in Patients With cGVHD	Recruiting	Dry Eye	Drug: Umbilical Mesenchymal Stem Cells derived Exosomes	China
NCT04850469	Study of MSC-Exo on the Therapy for Intensively Ill Children	Not yet recruiting	Sepsis|Critical Illness		China

(Table 1) cont.....

NCT no.	Title	Status	Conditions	Interventions	Locations
NCT04388982	The Safety and the Efficacy Evaluation of Allogenic Adipose MSC-Exos in Patients With Alzheimer's Disease	Recruiting	Alzheimer Disease	Biological: low, mild or high dosage MSCs-Exos administrated for nasal drip	China
NCT04173650	MSC EVs in Dystrophic Epidermolysis Bullosa	Not yet recruiting	Dystrophic Epidermolysis Bullosa	Drug: AGLE 102	_
NCT02138331	Effect of Microvesicles and Exosomes Therapy on Î²-cell Mass in Type I Diabetes Mellitus (T1DM)	Unknown status	Diabetes Mellitus Type 1	Biological: MSC exosomes.	Egypt

3. MSC-BASED THERAPEUTICS FOR COVID-19

The COVID-19 pandemic has dramatically impinged socio-economic and health burden worldwide. Although vaccines have been developed in the past months, it is too early to understand how long protection against SARS-CoV-2 infection will be conferred and if reinfection will not occur. Moreover, the mutation events encountered in the virus since its first report are worrisome. Thus, alternative approaches are needed to combat or halt COVID-19 symptoms, especially the severe ones that require patient hospitalisation for acute lung injuries and acute respiratory distress syndrome. Thus, MSC therapy has been considered by several research groups. The multifactorial mode-of-action of MSCs renders these cells a valid therapeutic option for the treatment of COVID-19 symptoms [8]. MSCs are capable of releasing various growth factors, cytokines (such as prostaglandin E2, granulocyte–macrophage colony-stimulating factor, IL-6, and IL-13) that modulate the lung immune system to fight against the cytokine storm, which activates signalling cascades to recruit immune cells such as humoral B-cells, T-cells, and macrophages to create a proinflammatory environment [9]. Several clinical trials have been registered recently regarding the use of MSCs for the treatment of COVID-19 symptoms. Of the 78 studies registered on the clinicaltrials.gov website, 13 have been completed (Table **2**). Results are available for only one of these studies (*clinicaltrials.gov* NCT04491240), in which the safety and efficiency of aerosol inhalation, twice per day for 10 days, of MSC-derived exosomes, were assessed severe patients hospitalized with COVID-1--associated pneumonia.

Table 2. Completed clinical trials using MSC-based products in COVID-19 patients ((https://www.clinicaltrials.gov)..

NCT no.	Title	Study Results	Conditions	Interventions	Locations
NCT04713878	Mesenchymal Stem Cells Therapy in Patients With COVID-19 Pneumonia	No Results	Coronavirus Disease 2019 (COVID-19) Pneumonia	Mesenchymal stem cells	Turkey
NCT04898088	A Proof of Concept Study for the DNA Repair Driven by the Mesenchymal Stem Cells in Critical COVID-19 Patients	No Results	COVID-19 Pneumonia	Biological: Mesenchymal Stem Cells Transplantation	Turkey
NCT04288102	Treatment With Human Umbilical Cord-derived Mesenchymal Stem Cells for Severe Corona Virus Disease 2019 (COVID-19)	No Results	Corona Virus Disease 2019 (COVID-19)	Biological: UC-MSCs	China
NCT04349631	A Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Autologous Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19	No Results	COVID-19	Drug: HB-adMSCs	United States
NCT04573270	Mesenchymal Stem Cells for the Treatment of COVID-19	No Results	Covid19| Prophylaxis	Biological: PrimePro|Other: Placebo	United States
NCT04355728	Use of UC-MSCs for COVID-19 Patients	No Results	Corona Virus Infection| ARDS| ARDS, Human| Acute Respiratory Distress Syndrome| COVID-19	Biological: Umbilical Cord Mesenchymal Stem Cells + Heparin along with best supportive care.| Other: Vehicle + Heparin along with best supportive care	United States

(Table 2) cont.....

NCT no.	Title	Study Results	Conditions	Interventions	Locations
NCT04522986	An Exploratory Study of ADR-001 in Patients With Severe Pneumonia Caused by SARS-CoV-2 Infection	No Results	Severe Acute Respiratory Syndrome Coronavirus 2	Biological: Mesenchymal stem cell	Japan
NCT04535856	Therapeutic Study to Evaluate the Safety and Efficacy of DW-MSC in COVID-19 Patients	No Results	Covid19|Corona Virus Infection| SAR	Drug: allogeneic mesenchymal stem cell|Other: Placebo	Indonesia
NCT04492501	Investigational Treatments for COVID-19 in Tertiary Care Hospital of Pakistan	No Results	Covid19| Cytokine Release Syndrome| Critical Illness| ARDS	Procedure: Therapeutic Plasma exchange|Biological: Convalescent Plasma|Drug: Tocilizumab|Drug: Remdesivir|Biological: Mesenchymal stem cell therapy	Pakistan
NCT04276987	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes Treating Severe Novel Coronavirus Pneumonia	No Results	Coronavirus	Biological:MSCs-derived exosomes	China
NCT04392778	Clinical Use of Stem Cells for the Treatment of Covid-19	No Results	Covid19| Pneumonia| Multiple Organ Failure| Corona Virus Infection	Biological: MSC Treatment| Biological: Saline Control	Turkey
NCT04491240	Evaluation of Safety and Efficiency of Method of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia.	**Has Results**	Covid19| SARS-CoV-2 PNEUMONIA| COVID-19	Drug: EXO 1 inhalation| Drug: EXO 2 inhalation| Drug: Placebo inhalation	Russia
NCT04400032	Cellular Immuno-Therapy for COVID-19 Acute Respiratory Distress Syndrome	No Results	Acute Respiratory Distress Syndrome| Covid19	Biological:Mesenchymal Stromal Cells	Canada

However, the FDA has recommended against the use of MSCs for the treatment of COVID-19 except in approved clinical trials (https://www.covid19treatment guidelines.nih.gov/cell-based-therapy/, last updated on April 21, 2021). This is because data supporting the use of MSCs in patients with SARS-CoV-2 infection are scarce. To date, only case reports and small, open-label studies have been performed. For instance, a pilot study in China regarded the intravenous administration of MSCs lacking angiotensin-converting enzyme 2 (ACE2, the receptor that SARS-CoV-2 uses for entry into cells) in 10 COVID-19 patients [10]. Seven patients were treated with MSCs and 3 with placebo. It was shown that MSCs could cure or significantly improve the functional outcomes of COVID-19 patients whilst remaining free from COVID-19 infection. The placebo-treated patients on the other hand, had their conditions worsened or stable with severe disease. The results from other studies using umbilical cord-derived MSCs also are promising, but care should be taken when intravenously administering MSCs for the reasons described in the previous chapters for the treatment of other diseases, as well as for the variable levels of highly procoagulant tissue factor (TF/CD142) expressed by MSCs that can worsen the already hypercoagulable procoagulant state of COVID-19 patients with severe illness [8, 11, 12].

CONCLUSION

MSCs are indeed promising in the clinical practice, in most cases acting more like a bridging therapy rather than a definitive one. With the multiplicity of stem cell administration routes available and the possibility of performing multiple injections over time, organ transplantation can be significantly postponed. MSC administration may improve patients' conditions and tissue microenvironment in order to allow a better outcome upon successive surgical intervention. Much hope lies in the MSC secretome, the emergence of new technologies and future high-quality clinical trials clinical studies that will provide insights into the best scenario for the application of MSC-based products effectively for the treatment of a variety of devastating conditions (Fig. **1**). In the near future, we shall also see microrobots at work in clinical studies, as a further step ahead in this multidisciplinary era of regenerative medicine.

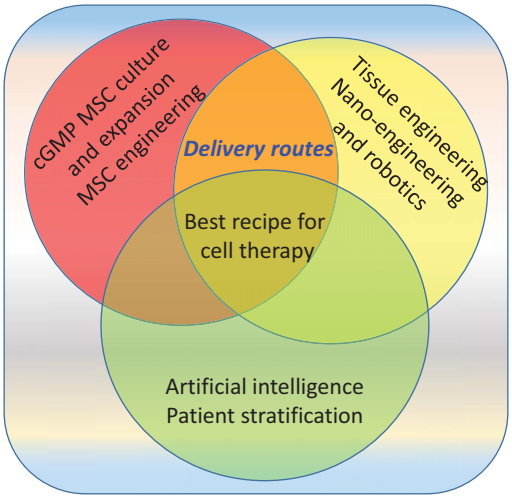

Fig. (1). The best recipe for cell therapy. Future trends in stem cell therapy research regarding the development of an optimal, multidiscipline-based recipe to increase MSC therapeutic efficacy in the clinic.

LIST OF ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
COVID-19	Coronavirus Disease-19
EV	Extracellular vesicle
IL	Interleukin
MSC	Mesenchymal stromal/stem cell

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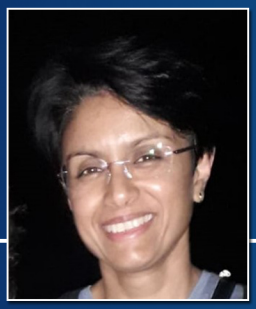

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