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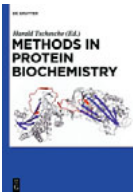

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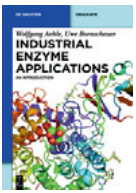

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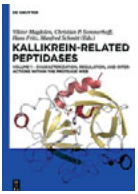

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Stem Cell-Dependent Therapies

Mesenchymal Stem Cells in Chronic Inflammatory Disorders

Edited by
Gerhard Gross and Thomas Häupl

DE GRUYTER

Editors

Gerhard Gross
Helmholtz Centre for
Infection Research (HZI)
Inhoffenstr. 7
38124 Braunschweig
Germany
gerhard.gross@helmholtz-hzi.de

Thomas Häupl
Department of Rheumatology and Clinical
Immunology
Division of Bioinformatics
Charité University Medicine
Charitéplatz 1
10117 Berlin
Germany
thomas.haeupl@charite.de

Cover image: Murine lymphocytes (spleen; blue) adhere to a lipopolysaccharide (LPS) activated mesenchymal stem cell (MSC; green). LPS provokes in MSCs an inflammatory response leading to lymphocyte recruitment. Photo: Manfred Rohde, Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany.

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Preface

During more than 20 years of research with mesenchymal stem cells (MSCs) we were initially investigating MSC-dependent regenerative aspects, and in the last years we turned to the trophic properties of MSCs as well. We were, and still are, intrigued by the tremendous therapeutic options residing in MSCs – and we are not alone. The general interest in this particular stem cell type is also reflected by the fact that currently (June 2013) 326 clinical trials with MSCs are registered in the ClinicalTrials.gov data base. Most of these trials deal with the MSC-dependent intervention of chronic inflammatory and autoimmune disorders.

From a pharmacological perspective, a therapeutic approach involving cells in general, and stem cells in particular, is extremely complex, but provides a completely new armamentum, which will never be achieved by a single molecule or a combination of drugs. In the last decade, in the field of anti-inflammatory therapies we experienced the many advantages of using the organism's own biomolecular repertoire as biological drugs. Compared with small chemicals, these biologicals fit precisely into biological processes by stimulating or interfering with defined molecular pathways. Moreover, the rapidly evolving technology of copying existing biological concepts may ultimately lead to the replacement of organs by the individual's own precursor/stem cell repertoire.

The stem cell-dependent modification of disease conditions provides a new pharmacodynamic potential. The biological concept of pathway activators or inhibitors opens entirely new therapeutic modalities in a flexible and integrative way. The pharmacokinetic restrictions of small molecules and biologicals distributed throughout the organism may be overcome by cellular therapeutic approaches allowing a time- and site-specific drug/factor release precisely adjusted in the dose needed. This vision also depends on the already pre-existing capabilities of such cells, which are involved in the natural process of healing after injury and inflammation. Furthermore, we are learning more and more about the reprogramming of cells and the state of differentiation, which may be used to adapt to disease-specific needs.

We recently developed the exciting notion of bringing together a panel of international experts to share their knowledge and their thoughts regarding the trophic and immunomodulatory capacities of MSCs, to discuss their therapeutic potential for inflammatory disorders and autoimmune diseases, to review the current state of clinical applications, and to balance the benefits and risks of MSCs for the intervention in chronic inflammatory disorders. This book begins with a reflection on the role of MSCs in stem cell biology and in the homeostasis of the organism as well. Then, the current state of the MSC-dependent therapy of inflammatory disorders and autoimmune diseases is investigated such as graft versus host disease, chronic kidney, liver and lung disease, ischemic heart and inflammatory bowel disease, diabetes, osteoarthritis, various rheumatic and neurological disorders, and, lastly, tumors and solid organ transplantation. In addition, the immunoprivileged status of MSCs is ques-

tioned, the role of MSCs in various experimental animal disease models is compared with the corresponding human disorders, a role for MSCs in tumor interventions is envisioned, and, lastly, a systems biology approach for stem cells and inflammation is described.

In spite of the many clinical trials alluded to above, the basic issues for a successful therapy with MSCs remain unresolved. The migration and homing of MSCs are only insufficiently understood, the efficacy and the rational of allogeneic versus autologous MSC-applications have not been entirely worked out, and many problems remain regarding the heterogenous nature of MSCs, cell numbers, time, duration, and frequency of MSC-application for an optimal therapeutic outcome. To overcome some of these issues it may also be essential to define more sophisticated culture conditions which resemble the MSC-niche environment *in vivo*. Nevertheless, MSCs may already be seen as a great hope and as an extremely promising approach not only to tissue regeneration but also to their therapeutic role as pro-angiogenic, antifibrotic, anti-inflammatory, immunosuppressive, and potentially antitumorigenic effectors.

Lastly, we would like to thank the many colleagues and collaborators we have had the privilege to meet, with whom we could cooperate and discuss the various issues of stem cell biology and inflammation. We cannot name all of them, but we would like to mention here the following: Florence Apparailly, Gerd-R. Burmester, Pierre Charbord, Yuti Chernajovsky, Dan and Zulma Gazit, Andreas Grützkau, Andrea Hoffmann, Inge Hollatz, Danny Huylebroeck, Christian Jorgensen, Christian Kaps, Jean Pierre Marie, Hubert Mayer, Andreas Radbruch, Jochen Ringe, Manfred Rohde, Virginia Seiffart, Michael Sittinger, Bruno Stuhlmüller, Przemko Tylzanowski, Kristin Verschueren, Herbert Weich, and Siggi Weiss.

June 2013 Gerhard Gross, Thomas Häupl

Contributing authors

Reza Abdi
Transplantation Research Center
Brigham and Women's and Children's Hospital
Harvard Medical School
221 Longwood Ave
LMRC Bldg
Room 310
02115, Boston, USA
eMail: rabdi@rics.bwh.harvard.edu
Chapter 15 – Corresponding author

Graça Almeida-Porada
Department of Regenerative Medicine
Wake Forest Institute for Regenerative Medicine
391 Technology Way
Winston-Salem NC 27157-1083, USA
eMail: galmeida@wakehealth.edu
Chapter 13 – Corresponding author

Ênio Jose Bassi
Department of Immunology
Institute of Biomedical Science IV
Universidade de São Paulo
Brazil
Chapter 14

Scott A. Bergfeld
Departments of Pediatrics
Biochemistry and Molecular Biology, and Pathology
Keck School of Medicine of the University of Southern California and The Saban Research Institute of Children's Hospital Los Angeles
Los Angeles CA 90027, USA
Chapter 17

Chiara Bocelli-Tyndall
Department of Rheumatology
University Hospital
Basel, Switzerland
Chapter 3

Léo H. Bühler
Surgical Research Unit
University of Geneva
Medical School
Switzerland
Chapter 16

Niels Olsen Saraiva Câmara
Department of Medicine
Division of Nephrology
Universidade Federal de São Paulo – UNIFESP
and Department of Immunology
Institute of Biomedical Science IV
Universidade de Sao Paulo
Rua Prof Lineu Prestes 1730, 05508-900
Sao Paulo, SP, Brazil
eMail: niels@icb.usp.br
Chapter 14 – Corresponding author

Astra I. Chang
University of California
Davis Medical Center
Department of Internal Medicine
Division of Hematology & Oncology, and UC Davis Institute for Regenerative Cures
Sacramento CA 95817, USA
Chapter 18

Danilo Candido de Almeida
Department of Medicine
Division of Nephrology
Universidade Federal de São Paulo – UNIFESP
Brazil
Chapter 14

Pierre Charbord
INSERM U972
Hôpital Paul Brousse
Batiment Lavoisier- Secteur Jaune
12/14 Avenue Paul Vaillant-Couturier
Villejuif 94800, France
eMail: pcharbord@noos.fr
Chapter 1 – Corresponding author

Christian Cordano
Department of Neurosciences
Ophthalmology, Genetics, Rehabilitation and Child Health
University of Genoa
Italy
Chapter 10

Yves A. DeClerck
Departments of Pediatrics
Biochemistry and Molecular Biology, and Pathology
Keck School of Medicine of the University of Southern California and The Saban Research Institute of Children's Hospital Los Angeles
4650 Sunset Blvd, MS #54, Los Angeles, CA 90027, USA
eMail: ydeclerck@chla.usc.edu
Chapter 17 – Corresponding author

James E. Dennis
Benaroya Research Institute at Virginia Mason
1201 Ninth Avenue
Seattle, WA 98101-2795, USA
eMail: jdennis@benaroyaresearch.org
Chapter 11 – Corresponding author

Charles Durand
UPMC UMR7622
Laboratoire de Biologie du Développement
Paris, France
Chapter 1

Carmen Gonelle-Gispert
Surgical Research Unit
University of Geneva
Medical School
CMU- 1
rue Michel-Servet
1211 Geneva 4, Switzerland
eMail: Carmen.Gonelle@unige.ch
Chapter 16 – Corresponding author

Gerhard Gross
Helmholtz Centre for Infection Research (HZI)
Inhoffenstrasse 7
38124 Braunschweig, Germany
eMail: gerhard.gross@helmholtz-hzi.de
Chapter 5 – Corresponding author

Thomas Häupl
Department of Rheumatology and Clinical Immunology
Division of Bioinformatics
Charité University Medicine
Charitéplatz 1
10117 Berlin, Germany
eMail: thomas.haeupl@charite.de
Chapter 19 – Corresponding author

Andrea Hoffmann
Hannover Medical School
Department of Trauma Surgery
Biology of the Musculoskeletal System
Hannover, Germany
Chapter 5

Anthony P. Hollander
School of Cellular and Molecular Medicine
University of Bristol
Bristol BS8 1TD, United Kingdom
Chapter 2

Jens Kastrup
Cardiac Catheterization Laboratory 2014
Cardiology Stem Cell Laboratory
The Heart Centre
Rigshospitalet
Copenhagen University Hospital
9, Blegdamsvej
2100 Copenhagen, Denmark
eMail: jens.kastrup@rh.regionh.dk
Chapter 9 – Corresponding author

Nicole Kerlero de Rosbo
Department of Neurosciences, Ophthalmology, Genetics, Rehabilitation and Child Health
University of Genoa and Advanced Biotechnology Center
University of Genoa
Italy
Chapter 10

Andreas Kurtz
Berlin-Brandenburg Center for Regenerative Therapies
Charité Universitätsmedizin Berlin
Augustenburger Platz 1
13353 Berlin, Germany
and College of Veterinary Medicine
Seoul National University
Seoul, Republic Korea
eMail: andreas.kurtz@charite.de
Chapter 4 – Corresponding author

Sandra Laggies
Helmholtz Centre for Infection Research (HZI)
Braunschweig, Germany
Chapter 5

James D. Lord
Benaroya Research Institute at Virginia Mason
Seattle, WA, USA
Chapter 11

Ciara N. Magee
Transplantation Research Center
Brigham and Women's and Children's Hospital
Harvard Medical School, Boston, MA, USA
Chapter 15

Marwan Mounayar
Transplantation Research Center
Brigham and Women's and Children's Hospital
Harvard Medical School, Boston, MA, USA
Chapter 15

Jan A. Nolta
University of California
Davis Medical Center
Department of Internal Medicine
Division of Hematology & Oncology
UC Davis Institute for Regenerative Cures
Sacramento, CA 95817, USA
Chapter 18

Su-Jun Oh
College of Veterinary Medicine
Seoul National University, Seoul, Republic Korea
Chapter 4

Clarice Silvia Taemi Origassa
Department of Medicine
Division of Nephrology
Universidade Federal de São Paulo – UNIFESP
Brazil
Chapter 14

Antonello Pileggi
Preclinical Cell Processing & Translational Models Program Cell Transplant Center
Diabetes Research Institute, University of Miami
1450 NW 10th Avenue (R-134), Miami,
FL 33136 – USA
eMail: apileggi@med.miami.edu
Chapter 8 – Corresponding author

Christopher D. Porada
Department of Regenerative Medicine
Wake Forest Institute for Regenerative Medicine
Winston-Salem, NC, USA
Chapter 13

Abbas Ali Qayyum
Cardiac Catheterization Laboratory 2014
Cardiology Stem Cell Laboratory
The Heart Centre
Rigshospitalet
Copenhagen, Denmark
Chapter 9

Camillo Ricordi
Cell Transplant Center and Diabetes Research Institute
University of Miami
The Cure Focus Research Alliance
USA
Chapter 8

Olle Ringdén
Division of Therapeutic Immunology, F79 and Center for Allogeneic Stem Cell Transplantation
Dept. of Laboratory Medicine
Karolinska Institutet
Karolinska University Hospital Huddinge
SE-141 86 Stockholm, Sweden
eMail: olle.ringden@ki.se
Chapter 6 – Corresponding author
Chapter 7

Mauricio Rojas
Pulmonary Allergy and Critical Care
McGowan Institute for Regenerative Medicine
University of Pittsburgh Medical Center
USA
Chapter 12

Behnam Sadeghi
Division of Therapeutic Immunology, F79 and
Center for Allogeneic Stem Cell Transplantation
Dept. of Laboratory Medicine
Karolinska Institutet
Karolinska University Hospital Huddinge
SE-141 86 Stockholm, Sweden
eMail: behnam.sadeghi@ki.se
Chapter 6
Chapter 7 – Corresponding author

Virginia Seiffart
Helmholtz Centre for Infection Research (HZI)
Braunschweig, Germany
Chapter 5

Jianming Tan
Fuzhou General Hospital of Xiamen University
Cell and Stem Cell Institute of Xiamen University
P.R. China
Chapter 8

Alan Tyndall
University Department of Rheumatology
Felix Platter Spital
Burgfelderstrasse 101
4012, Basel, Switzerland
eMail: alan.tyndall@fps-basel.ch
Chapter 3 – Corresponding author

Antonio Uccelli
Department of Neurosciences, Rehabilitation,
Ophthalmology, Genetics
Maternal and Child Health
University of Genoa and Advanced
Biotechnology Center
Center of Excellence for Biomedical Research
University of Genoa, Largo Paolo Daneo, 3,
16132 Genoa, Italy
eMail: auccelli@neurologia.unige.it
Chapter 10 – Corresponding author

Daniel J. Weiss
Pulmonary and Critical Care, University of
Vermont
College of Medicine Burlington
VT. 05405 , USA
eMail: daniel.weiss@med.uvm.edu
Chapter 12 – Corresponding author

David C. Wraith
Experimental Pathology
School of Cellular and Molecular Medicine
University of Bristol
Bristol BS8 1TD, United Kingdom
eMail: d.c.wraith@bristol.ac.uk
Chapter 2 – Corresponding author

Jian Wu
University of California
Davis Medical Center
Department of Internal Medicine
Division of Gastroenterology & Hepatology
UC Davis Institute for Regenerative Cures and UC
Davis Comprehensive Cancer Center
Cures, 2921 Stockton Blvd, Suite 1610,
Sacramento, CA 95817, USA
eMail: jdwu@ucdavis.edu
Chapter 18 – Corresponding author

Xiumin Xu
China-US Collaborative Human Cell Transplant
Program Cell Transplant Center
Diabetes Research Institute
University of Miami, USA
Chapter 8

Shang Zhang
School of Cellular and Molecular Medicine
University of Bristol
Bristol BS8 1TD, United Kingdom
Chapter 2

Table of Contents

Charles Durand and Pierre Charbord

1 Mesenchymal stem cells in the context of stem cell biology

Abstract This introductory chapter reviews the constitution of stem cell systems, the different attributes of stem cells, and the interaction between stem cells and the microenvironment (stem cell niches). It then evaluates the specific case of bone marrow mesenchymal stem cells (MSCs).

1.1 Introduction – Definitions

Stem cells are the cells that generate tissues and regenerate tissues after injury. The prototypic adult stem cells are the hematopoietic stem cells (HSCs). It is known since the 1950s that lethally irradiated mice survive if they receive after irradiation bone marrow cells collected from syngeneic donors [1]. Till and McCulloch showed in the 1960s that the bone marrow of the syngeneic donors contained a minor population that, a few days after intra-venous injection, was able to generate colonies visible with the naked eye [2]. These investigators showed by means of chromosomal markers that each colony derived from a single cell, *i.e.* each colony was a clone. The population of clonogenic cells was considered to be the population of stem cells responsible for the survival of irradiated mice, since it was capable to rescue the aplastic recipient with multi-lineage repopulation (radioprotective capacity). In the subsequent years, HSC population characterization was progressively refined leading in the late 1960s to a population positive for the expression of c-kit, Sca-1 and Thy-1 (at a low level) proteins, but negative for the expression of lineage markers of erythroid, myeloid and lymphoid differentiation (Lin) [3]. Since then additional markers have been described [4]. It has been shown that a single stem cell could entirely repopulate the hematopoietic lineages of primary recipients giving rise to chimeric animals; cells of the same phenotype from the reconstituted primary recipient can in turn repopulate secondary irradiated recipients [5]. However, even with the most refined population not all mice transplanted with HSCs can be reconstituted, leading to the notion that stem cells can be defined at the population level, but cannot be yet identified as single cells. Whether this notion is due to still imperfect experimental conditions or due to stochastic fluctuation of markers within the population remains a major problem in stem cell biology. Whatever the explanation, it is critical to mention here that, presently, *bona fide* murine bone marrow HSCs can only be identified retrospectively by transplantation studies. The same statement can be made for human HSCs, whatever their source (bone marrow, placental or peripheral blood).

The experiments reported above clearly indicate that stem cells constitute a minor population of cells (*e.g.* HSCs constitute about 0.005 % of total bone marrow

cells) that share two fundamental properties that distinguish them from other adult cell types. They have, as a population, the ability to self-renew and to give rise to mature cell types. Self-renewal, the capacity of mother cell(s) to give rise to daughter cell(s) with identical attribute(s), is not *per se* a sufficient condition to define the stem cell population (*e.g.* lymphocytes self-renew after antigen introduction to develop a clone that specifically recognizes the antigen). A *bona fide* stem cell population must simultaneously self-renew and commit to (a) specific lineage(s). This can be achieved by two mechanisms: either the mother stem cell divides asymmetrically giving rise to one identical daughter stem cell and to another committed daughter cell (lineage mechanism), or a stem cell divides symmetrically giving rise to two identical daughter stem cells while another stem cell gives rise at the same time to two committed cells (population mechanism) (reviewed in [6–8]). Thus, asymmetric division is a property of stem cells, but is not the sole type of division in a stem cell population. Since expansion of the stem cell population can be achieved only by symmetrical division, stem cell populations use either lineage or population mechanisms to divide depending on the tissue requirements (steady-state versus stress conditions).

In terms of differentiation, a major discrimination is made between totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent stem cells are cells that give rise to all cells of the embryo including the extra-embryonic annexes. Such cells are the fertilized eggs and the cells generated by the subsequent 2–3 divisions. Pluripotent cells give rise to all cells of the organism, but not to cells of the trophoblast. They are found in the inner mass of the blastocyst. Adult tissue stem cells (all stem cells of subsequent stages, including fetal stem cells) are either multipotent or unipotent, depending on the tissues. One example is the skin, containing unipotent stem cells giving rise only to interfollicular epidermis and bulge stem cells giving rise to all epidermal components [9].

Although adult stem cells have been described in most tissues, there are instances where stem cells are not responsible for tissue regeneration after injury. A typical example is that of the liver where regeneration after partial surgical resection is due to proliferation of differentiated hepatocytes, contrarily to regeneration following some toxic injuries that result from the activity of stem cells (reviewed in [10]). Another example is that of the endocrine pancreas where newborn beta cells are generated by division of insulin-positive pre-existing differentiated beta cells [11].

1.2 Embryonic and adult tissue stem cells

Totipotency and pluripotency are very transient properties, existing in the embryo for a few hours to a few days depending on the species. A remarkable feat, achieved first in mice [12] and then in humans [13], has been the generation of cell lines from the inner mass of blastocysts. Pluripotent cells from these lines show extensive self-renewal since lines initiated from single cells can replicate for many generations

with maintenance of their differentiation potential. Pluripotency is evidenced by the generation of animal chimeras following micro-injection of labeled cells into blastocysts further developed into late-stage embryos (reviewed in [14]). Pluripotency is also shown by the induction, using specific protocols, of differentiation into the many lineages of the three germ layers and germ cell lineage, and the development, after injection into mice, of teratomas including neuro-ectodermal, mesodermal and endodermal derivatives (reviewed in [15,16]).

Pluripotent stem cell lines constitute an essential tool to study stem cell attributes. Their study has allowed determining the gene networks and conditions critical for self-renewal, as well as the epigenetic state associated to pluripotency (reviewed in [17]). Embryonic stem cell lines also constitute an exceptional tool to model human diseases and test the efficiency and toxicity of putative novel drugs. Some facets of the stem cell state as defined *in vitro* have been also observed *in vivo* in cells of the inner cell mass, strongly suggesting that these lines are true counterparts of native transient cells of the epiblast blastocyst and not culture creations (reviewed in [18]). However, extrapolation of the transcriptomic and epigenetic landscape to adult tissue stem cells is not warranted because of the differences between the two types of stem cells [19].

Adult tissue stem cells are either multipotent or unipotent. Depending on the tissue, there might be one or several stem cell type(s) per tissue. The diversity of stem cells reflects the tissue diversity. For example, in the brain, stem cells localized in deep tissue areas (subventricular zone of lateral ventricles and subgranular zone of the dentate gyrus) give rise to the three neural lineages, neurons, astrocytes and oligodendrocytes; these neural stem cells generate new neurons and macroglial cells able to migrate following defined pathways to other brain regions such as the olfactory bulb (reviewed in [20]). In addition, the microglial cell component derives from HSCs, which may also be present in the brain as suggested by a recent report [21]. Similarly, two distinct cell types are present in the bone marrow of mammals, HSCs and MSCs, which is a unique situation where two stem cells cooperate to make the specific HSC niche (see below).

1.3 Adult tissue stem cells and progenitors

Adult tissue stem cells are self-renewing. However, even for the prototypic HSC whose self-renewal capacity can be stringently tested *via* consecutive transplantations from one recipient mouse to the other, self-renewal has only been evidenced for a few rounds of transplantations [22, 23]. This may be related to cellular alterations secondary to transplantation stress altering homing and engraftment without affecting stem cell function *per se* [24], but may also be due to other mechanisms, such as reduction of telomere length [25].

Progenitors (constituting part of the transit amplifying compartment) are discriminated from stem cells by the lack of self-renewal ability, which is probably related to

the decreased expression of telomerase, the probability to detect telomerase activity in individual cells being proportional to the probability of self-renewal potential [26]. Moreover, progenitors are committed cells already expressing lineage-specific markers. They may be multipotential or unipotential and give rise to colonies when plated in semi-solid or liquid cultures (each colony being the progeny of the clonogenic colony-forming cell or CFU). They are highly proliferative, being responsible for (multi)lineage amplification.

Quiescence has been usually considered an essential property of adult stem cells (as opposed to embryonic stem cells). It has been extensively studied in bone marrow HSCs. The more recent data obtained *via* computational modeling suggest that bone marrow murine HSCs are dormant, dividing about every 145 days [23]. Stem cell exit from dormancy and active entry into the cell cycle appear to be proportional to the severity of the bone marrow depletion. Such recent data appear to confirm the hypothesis laid down long ago that HSCs would not contribute to the daily turnover of hematopoiesis, being solicited only in cases of stress or injury [27]. The daily production of hematopoietic cells would then be maintained by the proliferative activity of the progenitors, some of which should have self-renewing capacity. This notion puts into question the formal discrimination between stem and progenitor cells.

Distinction between stem cells and progenitors faces major technical problems. Stem cells represent a rare cell population that does not express specific membrane antigens, making positive sorting difficult. However, enrichment can be obtained by using a defined combination of surface markers and/or vital dyes whose expression depends on the cell DNA and RNA content or on the activity of membrane transporters (defining the "side population" of stem cells able to exclude Hoechst 33342). The recently generalized use of genetic lineage tracing helps improve the distinction between stem and progenitor cells since it indicates not only which, but also for how long a specific cell and its progeny are labeled [28, 29]. Stem cells should give rise to a usually multilineage progeny permanently labeled in long-term follow-up, contrarily to progenitors whose progeny is labeled in short-term, but not in long-term follow-up. This method therefore provides information on the *in vivo* behavior of stem cells and progenitors *via* study of their clonal progeny in a nonstress situation.

In hierarchical models, stem cells and progenitors belong to consecutive compartments. Cells within a compartment homogeneously express one or several feature(s) allowing the clear-cut discrimination from the adjacent compartments. Moreover, cell flow from one compartment to the next is unidirectional (corresponding to irreversible differentiation). In such models, stem cells are intrinsically predetermined entities. Such a rigid scheme has been long debated. Early on, a screw model was proposed whereby a distinction was made between "actual" and "potential" stem cells corresponding to amplifying transit cells recovering a self-renewing potential under regenerative conditions (as opposed to steady state) (reviewed in [30, 31]). Recent reports provide data that do not fit in a hierarchical model. It has been shown in lineage tracing studies that labeled clone kinetics (number of clones and number of

cells within clones) fitted best with stochastic models whereby the fate of individual stem cells or progenitors is defined by fixed probabilities, in contrast with the predictable dynamics of the population (reviewed in [7]). Differences between tissues may relate to the role of intrinsic or extrinsic (niche-related) factors in the model. In mammalian intestine the balance between proliferation and differentiation reached in the stem cell population would depend on the presence of niche cells [32]. In interfollicular epidermis this balance would rely on cell-autonomous regulation of a progenitor cell population [33, 34]; how regeneration occurs in case of injury remains unclear. Recent reports also indicate that progenitors may de-differentiate into stem cells [35], which may fit with self-organizing system models in which pathways may be bi-directional [36].

1.4 Adult stem cells and tissue homeostasis

Another way to approach adult stem cell biology is to envision the requirements of the tissues in which stem cells reside. A major distinction has to be made between tissues with high cell turnover rate and others. The former consists in blood, intestine and skin. In these tissues differentiation leads to end-cells with finite lifespan whose compartment has to be constantly replenished (in the hematopoietic system an exception has to be made for macrophages endowed with large proliferation potential and for lymphoid cells that include a subset of memory cells reactivated after re-introduction of the antigen). Under a steady-state condition, the blood, intestine and skin compartments are completely renewed in a matter of days. Under stress (hemorrhage, irradiation, burns...), the daily demand for differentiated cells increases. One understands therefore the requirement of self-renewal capacity for the stem cells at the origin of these tissues and the necessity for the stem cells to be able to shift from lineage to population mechanism.

On the contrary in other tissues, tissue renewal is low or nil, except during development or after injury. For example, bone is renewed in ≈ 10 years (reviewed in [37]) and muscle satellite cells are activated only after traumatism. In these instances, there is no requirement in a steady-state condition for stem cell activation. Although in these tissues stem cells may self-renew, the requirement for self-renewal is less absolute than in tissues with high cell turnover rate.

1.5 Adult stem cell niches

The balance between self-renewal and differentiation is not only controlled by cell autonomous mechanisms involving transcription factors, regulators of the cell cycle, components of the cytoskeleton and micro-RNAs. It is also critically dependent on the dynamic interactions between stem cells and their surrounding microenvironments

or niches. Following the work of Wolf and Trentin on the hemopoietic-inductive microenvironments [38], the concept of a stem cell niche was proposed by Schofield in 1978 and defines the cellular and molecular microenvironment that regulates stem cell functions [39]. Nowadays, stem cell niches are extensively studied both in invertebrate and vertebrate models and have been identified in several tissues such as the skin, hair follicles, intestine, brain, skeletal muscle and bone marrow (reviewed in [40]).

The mechanisms of stem cell regulation by niches have to fit with the mechanisms of stem cell self-renewal (lineage versus population mechanism as defined above). In Drosophila testes or ovaries germinal stem cells are in physical contact with (hub or cap) niche cells (reviewed in [6]). During stem cell division the mitotic spindle is oriented perpendicularly to the niche cell. The daughter cell that maintains its contact with the niche remains a stem cell, while that which loses contact becomes a progenitor. The asymmetry of fate is therefore conferred not by unequal repartition of material between the two daughter cells but by the persistence or absence of contact with the niche cell. A similar model might be applied for mouse intestinal stem cells. A stem cell at the base of the crypt would divide symmetrically to give rise to transit amplifying cells proceeding toward the villus tip. The stem cell that would therefore lose its contact with the niche cell (Paneth cell) would be replaced by a neighboring stem cell that has maintained its contact with the niche cell (reviewed in [7]). These examples illustrate how stem cells and niche cells might interact to ensure tissue maintenance.

Candidate niche cells that should pre-empt the contact with stem cells are of diverse types. They might be differentiated cells belonging to a lineage distinct from that of stem cells (*e.g.* hub or cap cells of Drosophila reproductive organs), cells differentiated from the stem cells (*e.g.* Paneth cells of mouse intestine) or another type of stem cell (bone marrow MSCs). Regardless of their nature or even of the species, niche cells appear to make use of specific signaling pathways such as Wnt, Notch and members of the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) family (reviewed in [8]). Moreover, some cytokines thought to be directly active on stem cells might also be effective by impacting niche cells, as exemplified by the effect of granulocyte-colony-stimulating factor (G-CSF) that downregulates a number of hematopoietic regulators in nestin-positive bone marrow niche cells (reviewed in [40]).

The bone marrow HSC microenvironment is one of the most well characterized stem cell niches. It is composed of stromal cells that derive from MSCs. Historically, cultures of primary bone marrow adherent cells were initially used to study the supporting activity of the bone marrow hematopoietic microenvironment [41]. Then, the establishment of stromal cell lines from hematopoietic tissues represented an exceptional tool to further explore the cross-talk between HSCs and stromal cells and the role of the microenvironment in regulating HSC activities [42]. *In vivo*, murine bone marrow MSC-derived cells including osteoblasts and sinusoidal endothelial and

mural cells have been shown to play a critical role in the maintenance of HSCs in the bone marrow [4, 43, 44]. In contrast, adipocytes were shown to negatively regulate HSC homeostasis [45]. Recent studies have elegantly demonstrated that MSCs themselves are a major component of the bone marrow HSC niche [46]. Using transgenic mice expressing the green fluorescent protein (GFP) under the control of the nestin promoter, these investigators identified a rare population of cells in the BM harboring the potentialities of MSCs to self-renew and differentiate into osteoblasts, adipocytes and chondrocytes. The nestin$^+$ cells are negative for the expression of endothelial markers such as CD31, CD34 and VE-cadherin and are located in perivascular areas and in the parenchyme of the bone marrow. Importantly, nestin$^+$ MSCs colocalize with HSCs and adrenergic nerve fibers that have been shown to also contribute to the regulation of bone marrow HSCs [47]. In addition, functional experiments showed that transplanted HSCs localize near nestin$^+$ cells in the bone marrow and *in vivo* depletion of these cells leads to reduction in HSC numbers [46].

Similar results were obtained with human culture-amplified bone marrow MSCs. Ten weeks after intra-bone injection of labeled MSCs in immune deficient mice, 60 % of the labeled cells were alpha-SM actin$^+$ and located in the vicinity of sinusoids, while 30 % were alkaline phosphatase$^+$ and located in the endosteal region [48]. In 2007, the team of Paolo Bianco reported results obtained after subcutaneous injection of human CD146$^+$ bone marrow colony forming units-fibroblasts (CFU-fs) in immune deficient mice [49]. Four weeks after transplantation the few human cells that retained the expression of CD146 were located on the abluminal side of mouse-derived endothelial cells forming incipient sinusoids. By week 8, foci of hematopoietic cells were clearly associated to the CD146$^+$ peri-sinusoidal cells. Remarkably, implantation of a single CFU-f gave identical results.

1.6 Commitment and differentiation

Two models are used to describe stem cell differentiation. In the first model, stem cells do not express any differentiation markers; they are said to be a blank slate. Commitment is then characterized by the appearance of the first differentiation markers followed by others as differentiation proceeds. In the second model, that of lineage priming, stem cells express lineage markers at low to moderate level. Differentiation is then characterized by the increase of markers specific for the differentiation pathway together with the progressive decline in markers characterizing alternative pathways. Transcriptional noise would account for lineage priming with stochastic oscillatory expression of certain transcripts (reviewed in [50]). States with maximal expression of certain transcripts frozen by external conditions (microenvironment) would characterize committed cells.

Lineage priming was first described in HSCs [51] and confirmed since by many reports (reviewed in [50, 52]). High-throughput population-based studies have sug-

gested that lineage choice in stem cells was governed by transcriptional noise [53]. However, a recent study on individual cells indicates that *bona fide* stem cells, contrarily to progenitors, only sporadically express lineage regulators [54]. Commitment would be characterized by expression of lineage regulators in a discrete and non-coordinated way, the precise identity of the first intervening regulator varying from one cell to the other.

Lineage priming (also called promiscuous expression) has been also described in cells from pluripotent embryonic lines (reviewed in [18, 50]). However, recent data indicate that such cells cultured in the presence of kinase inhibitors exhibit lower expression of genes implicated in ectoderm and mesoderm specification, suggesting that they are in a state close to the blank slate [55].

Taken together these data indicate that stem cells constitute a heterogeneous population where, again, the theoretically simple distinction between stem and progenitor cells is not easily resolved.

1.7 The case for bone marrow MSCs

Contrarily to other types of stem cells, MSCs have been mainly studied in humans and are usually obtained after culture amplification. The reason for this is the clinical potential of these cells that led to skip, unfortunately, in-depth study of their attributes. Moreover, animal models have been only recently available due partly to the development of lineage tracing methods. We will therefore indicate the major attributes of this kind of stem cells in humans and then briefly review the data using mouse models. Clonogenicity, self-renewal ability, phenotype, differentiation potential and lineage priming are the major properties of the population of MSCs either present among culture-amplified cells or among cells found in the bone marrow mononuclear fraction.

Enumeration of MSCs *in vitro* is done in most cases by counting the number of CFU-fs, *i.e.* the number of colonies consisting of more than 50 cells of fibroblastoid appearance [56, 57]. However, other methods have also been used: seeding in methyl cellulose in presence of appropriate growth factors [58, 59], which may yield the human counterpart to murine bone marrow mesenchymal rosettes [46], counting colonies after seeding at limiting dilution [60–62], selecting only high-proliferative potential MSCs containing more than 1000 cells [63, 64].

Replating of CFU-fs may give some indication on the self-renewal capacity of MSCs provided that each mother multipotential colony yields a colony with the same differentiation potential [65, 66]. Studies have shown that this was rarely the case, the situation more frequently found being that a CFU-f gives a CFU-f with lesser differentiation potential or no colony at all. Cogent demonstration of self-renewal has been provided by Sachetti *et al.*, showing that one bone marrow human CFU-f whose progeny consisted in CD146+/CD90+ cells was still containing after ectopic *in vivo*

amplification in the immune-deficient mouse, a very small fraction of CD146+/CD90+ cells capable of yielding, upon secondary plating, one or two fibroblastoid colonies [49]. To further prove a sustained self-renewal capacity, the secondary colonies should have been transplanted to secondary recipients, with final similar results. This difficult experiment has not yet been performed.

Flow cytometry indicates that more than 50 out of 100 studied membrane protein antigens are detectable on culture-amplified cells for about 2 weeks [67]. Among these are a number of adhesion molecules (cell adhesion molecules belonging to the immunoglobulin superfamily, integrins, tetraspanins, surface proteoglycans and the hyaluronate receptor CD44), a few cytokine and morphogen receptors (Wnt, Notch, BMP and tyrosine kinase receptors), enzymes associated to the cell membrane (CD13, CD26, CD73), and some membrane antigens with still uncertain function. Most of these molecules are represented in many tissues. However, certain associations may be relatively specific for MSCs, including CD73, CD90, CD105, CD146, CD200 and CD271 and the glycolipid antigens SSEA-4 and GM2. These latter molecules can be used to isolate a population enriched in MSCs from mononuclear cells of the bone marrow. The enrichment factor is about 300, which indicates that we are still far from obtaining the kind of purity described for HSCs.

MSCs differentiate into osteoblasts, chondrocytes and adipocytes. Another lineage of differentiation appears to be that of vascular smooth muscle cells. Differentiation into that lineage was first described in the 1990s [68]. The number of publications that have confirmed that MSCs have the potential to differentiate into that pathway is so large that it could be integrated in the functional requirements defining MSCs (reviewed in [69]). The neural differentiation program may be intrinsic to certain MSCs that would be neuro-ectodermal, but not mesodermal, in origin (reviewed in [69]). Studies made long ago have shown that most of the connective tissues of the upper part of the body (head, neck and thorax) are of neuro-ectodermal origin (reviewed in [70]). More recent studies have shown that the first wave of murine MSCs was generated by neuro-ectodermal cells, that some of the bone marrow femur MSCs in mouse still issued from the neuro-ectoderm and that neural crest stem cells from mice and humans gave rise to MSCs with osteoblastic, chondrocytic, adipocytic and vascular smooth muscle potential (reviewed in [69]). Many investigators have reported the differentiation of human bone marrow MSCs into other lineages: skeletal and cardiac muscle, neurons and hepatocytes (reviewed in [69]). The use of specific culture conditions (*e.g.* harsh selection of serum batches, induction of specific cellular stress before seeding) may allow the generation of rare, eventually pluripotent, cells present in the bone marrow cell suspension.

Lineage priming is another property of stem cells shared by MSCs [71]. This property may be related to the known plasticity of cells of the mesenchymal lineage, *i.e.* their capacity to adapt to changing microenvironments (reviewed in [69, 72]).

The data obtained from human studies strongly suggest that bone marrow MSCs constitute a *bona fide* stem cell population. Recent data from mouse studies have

largely confirmed these conclusions [46, 73–76]. Bone marrow MSCs were characterized by different markers, using flow cytometry (expression of Sca-1, CD90, CD140a and CD105, lack of expression of CD45, Ter119 and CD31) or transfecting gene promoter located upstream of a reporter protein (leptin receptor/Lepr, intermediate filament nestin, transcription factor COE2/Ebf2 and signaling molecule Myxovirus influenza virus resistance protein 1/Mx1). Cells expressing these molecules were scattered in the bone marrow predominantly in perivascular (Sca-1$^+$/CD140a$^+$, nestin$^+$, leptin receptor$^+$ cells) and endosteal regions (Mx1$^+$, Ebf2$^+$ cells). Labeled cells were clonogenic, yielding high amounts of CFU-Fs (enrichment >1000 for Sca-1$^+$/CD140a$^+$, Mx1$^+$ and Ebf2$^+$ cells), or mesenchymal rosettes (in the case of nestin$^+$ cells). Self-renewal of nestin$^+$ or Ebf2$^+$ cells was evidenced by serial replating and clonogenic cell recovery from ectopic ossicle generated from single colony or mesenchymal rosette implanted subcutaneously in syngeneic mice. Sca-1$^+$/CD140a$^+$, Mx1$^+$ and Ebf2$^+$ cell types generated osteoblasts, chondrocytes and adipocytes *in vitro*. Nestin$^+$ and Ebf2$^+$ cells contributed to the chondrocytic and osteoblastic lineages after transplantation, while Mx1$^+$ cells contributed only to the osteoblastic lineage and were able to repair bone fracture after local implantation at fracture sites. Sca-1$^+$/CD140a$^+$ cells were also able to differentiate into endothelial cells *in vitro*. Finally, Ebf2$^+$ cells were for most part in the G0 phase of the cell cycle and individually expressed adipocytic, osteoblastic and chondrocytic markers, indicative of lineage priming. These data not only confirm the presence of MSC-like cells in the bone marrow of mice, but also allow distinguishing subsets within this compartment with regard to phenotype, differentiation potential and niche formation.

1.8 Clinical prospects

Transplantation of the paradigmatic HSC leads to total reconstitution of the blood system of an aplastic subject after a few days to weeks. The mechanism of reconstitution is well known. The HSC binds to specific sites on the endothelial lining of marrow sinuses, then crosses the endothelial barrier and homes to the bone marrow logettes where clones develop and differentiate. Finally the end-cells find their way back to the marrow sinuses and into the bloodstream. The sites of HSC homing have been described above as niches where HSCs are in direct contact with MSCs and their immediate osteoblastic or vascular smooth muscle progeny. In short, HSC reconstitute the blood system *via* proliferation and differentiation of its progeny and in concert with associated stromal MSC that regulate HSC activity.

This model of regeneration adequate for HSCs can hardly apply to MSCs. It is admitted, albeit controversial, that MSCs can repopulate bone and cartilage when implanted to fractured bone or injured cartilage. However, regeneration by proliferation and differentiation of the progeny cannot explain the clinical results obtained after transplantation of these cells inducing improvement if not cure of immunologi-

cal disorders such as graft-versus-host disease (GvHD), autoimmune diseases (lupus erythematous, Crohn's disease (CD), juvenile diabetes) (reviewed in [77–79]) or of post-irradiation syndrome [80]. Two other major mechanisms appear to be at play in these situations: cell reprogrammation and secretion of "trophic factors". Many experiments have indicated that MSC could be reprogrammed after transfer of transcription factors (reviewed in [81]); more importantly from a physiological viewpoint, cytokines also appear to be implicated in this process [82]. Secretion of cytokines and chemokines by cultured stromal cells and by MSCs is well known. That these molecules are also implicated in the regenerative effect of transplanted MSCs appears increasingly a major mechanism of regeneration. Of particular importance are the anti-apoptotic activity of the growth factors and the anti-inflammatory activity of certain interleukins (reviewed in [79, 83]). Hence the emerging scenario for regeneration by MSCs implanted locally or injected systematically would be the secretion during a crucial time span of molecules active on tissue stem cells of different types or on differentiated cells at the sites of injury.

1.9 Concluding remark

The mandatory confrontation of experimental data with theoretical models discloses the complexity of the stem cell entity. This complexity has led to the proposition that stem cells would constitute a functional entity rather than a defined cell type with a precise molecular signature [84]. Presently, it appears reasonable to keep open the debate and consider that several models may account for stem cell attributes.

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Shang Zhang, Anthony P. Hollander, and David C. Wraith

2 Are mesenchymal stem cells immune privileged?

Abstract Mesenchymal stem cells (MSCs) are multipotent stem cells originally isolated from bone marrow. They have the potential to differentiate into cells of the musculoskeletal system such as osteoblasts, chondrocytes, fibroblasts and adipocytes. As a result, MSCs are a promising cell source for tissue repair and regeneration. The possibility that MSCs enjoy immune privilege suggests that allogeneic cells could be used. This would facilitate the development of stock MSC-derived cell lines as 'off-the-shelf' products. In recent years, MSCs have been shown to possess immunosuppressive properties and they have been shown to be promising immunosuppressants to control graft-versus-host disease (GvHD) and autoimmune diseases. Previous studies showed controversial results about the mechanisms of immunosuppression by MSCs, although this is most likely through activation of MSCs by interferon-γ (IFN-γ) and the subsequent expression of immunosuppressive molecules, such as indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO). In addition, species differences underlying the mechanisms were shown. However, in animal models, in vivo tracking of MSC grafts showed allogeneic MSCs failed to survive for long in comparison with syngeneic cells. MSCs induced the memory type of cluster determinant (CD) 4+ T cells and specific antibodies against them in allogeneic hosts. There is growing evidence that the immunosuppressive properties of MSCs and mechanisms for this immunosuppression are shared by other cells such as fibroblasts, macrophages and epithelial cells, suggesting that the immunosuppressive effect of MSCs is a common phenomenon and that MSCs are not as immune privileged as assumed to be. MSCs have the potential to be used as immunosuppressants because of their powerful suppressive effects but the off-the-shelf use of allogeneic MSCs for tissue repair and regeneration should be questioned.

2.1 Introduction – Definition of mesenchymal stem cells (MSCs)

MSCs were first isolated by Fridenstein *et al*. from bone marrow and described as fibroblast-like, colony forming progenitor cells [1]. They were later found to have the capacity for self-renewal and to be multipotent, having the potential to differentiate into a variety of specialized cells, including osteoblasts, chondrocytes, adipocytes, fibroblasts, skeletal myocytes, and tenocytes [2]. Human MSCs are isolated from bone marrow simply by a plastic adherence method. Besides bone marrow, MSCs were also claimed to exist in other mesenchymal tissues such as adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood and trabecular bone, since cells isolated from these diverse tissues share the differentiation capabilities of MSCs [3]. The International Society for Cell Therapy (ISCT) provided the minimal criteria

required to define human MSCs: these included adherence to plastic, expression of a panel of surface antigens (cluster determinant (CD) $CD105^+$, $CD73^+$, $CD90^+$, $CD45^-$, $CD34^-$, $CD14^-$ or $CD11b^-$, $CD79\alpha^-$ or $CD19^-$), human leukocyte antigen ($HLA\text{-}DR^-$) and possession of multipotent differentiation potential. But these criteria are not universal for MSCs isolated from other species, *e.g.* on murine MSCs, surface antigen expression is not universally the same as human MSCs and is not well characterized [4]. A single specific surface antigen that unequivocally defines MSCs has not been yet defined; as a result, adherence to culture dishes and the trilineage differentiation capacity are the main features used to define MSCs.

MSCs are an attractive research field for scientists because these cells have the potential to regenerate damaged or degenerated tissues and organs, especially skeletal tissues. Therefore MSCs are a promising cell source for tissue engineering. The first tissue-engineered trachea using a patient's own stem cells was successfully transplanted in place of the collapsed trachea in a patient with bronchomalacia [5]. Another potential usage of MSCs was discovered in the last decade. They were found to have the potential to be used as immunosuppressive agents to treat graft-versus-host-disease (GvHD) and autoimmune diseases. The immunosuppressive effect of MSCs on immune cells has been studied extensively. Notably, the administration of MSCs improved the clinical symptoms of GvHD in clinical trials [6–9] and also ameliorated autoimmune conditions such as experimental autoimmune encephalomyelitis (EAE) in animal models [10–12]. In fact, major histocompatibility complex (MHC) matched or mismatched MSCs showed a similar immunosuppressive effect to each other. The immunosuppressive effect of MSCs was unrelated to MHC type, *i.e.* MHC matching was not required, as shown by *in vitro* and *in vivo* studies [7, 13, 14]. Allogeneic MSCs could not elicit proliferation of lymphocytes in co-culture *in vitro* suggesting they lacked immunogenicity [7, 13, 15]. To address the question whether MSCs are immunoprivileged, we would like to review the immunosuppressive properties of MSCs and the possible mechanisms involved.

2.2 The immunosuppressive effect of MSCs on immune cells

The early work on the immunosuppressive effect of MSCs was undertaken on baboon models. The initial idea of those researchers was to test the ability of MSCs to enhance hematopoietic stem cell grafts and the homing ability of MSCs to bone marrow. Surprisingly, researchers found MHC-mismatched MSCs could survive up to 76 days in one recipient [16]. They decided to do further research on the immunological properties of these stem cells. Baboon MSCs could not elicit allogeneic responses from lymphocytes *in vitro* suggesting they lacked immunogenicity. Furthermore, they were found to be able to inhibit the proliferation of lymphocytes stimulated by either allogeneic cells or a nonspecific mitogen. The immunosuppressive capability of baboon MSCs was then demonstrated *in vivo* since a single dose injection of the cells was able

to significantly inhibit skin graft rejection compared to controls, although the grafts were ultimately rejected due to a neutrophilic rather than a lymphocytic infiltrate [15]. The lack of immunogenicity [13] and the immunosuppressive effect was soon confirmed with human MSCs [13, 17]. A typical immunosuppression test involves adding MSCs to mixed lymphocyte reactions (MLR). However, the suppressive effect of MSCs did not depend on the type of stimulation used to drive the proliferation of lymphocytes [15, 17, 18]. The stimulation could be either by a nonspecific mitogen such as phytohemagglutinin (PHA), concanavalin A (ConA) or antibody-coated beads, as well as allogeneic cells such as peripheral blood leukocytes (PBLs) and dendritic cells (DCs). In addition, both MHC-matched and mismatched third party MSCs had immunosuppressive effects on lymphocyte proliferation in a dose response manner. In fact, the immunosuppression by MSCs was observed to be so powerful as to generate an almost complete inhibition of T cell proliferation [17, 19].

MSCs have suppressive effects on a wide range of lymphocytes (Fig. 2.1). They were reported to be able to suppress the proliferation of $CD4^+$ T cells, $CD8^+$ T cells, naïve antigen-specific T cells, memory T cells, and B cells [17, 18, 20–22]. MSCs also have modulatory effects on DCs. DC maturation, migration and antigen presentation were

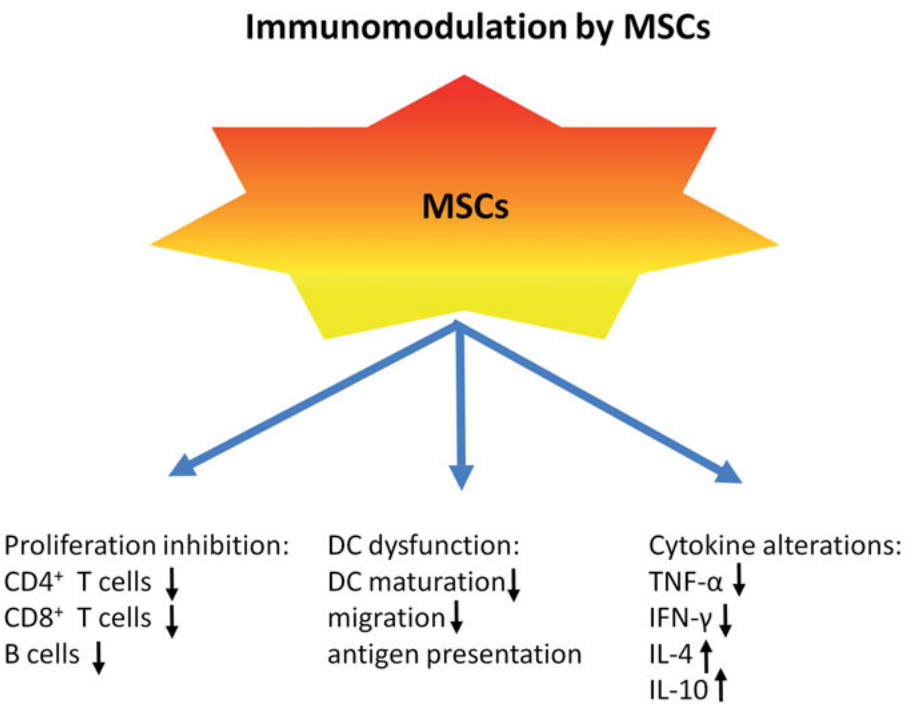

Fig. 2.1: The immunomodulation by MSCs. MSCs have immunomodulatory effects on a wide range of lymphocytes and alter cytokine production.

suppressed by MSCs [23, 24]. MSCs modulate cytokine production by lymphocytes when they were cultivated with different lymphocytes [25]. Tumor necrosis factor-α (TNF-α) production was decreased when MSCs were co-cultured with lipopolysaccharide (LPS) stimulated DC1 cells. Interleukine-10 (IL-10) levels were increased when MSCs were co-cultured with LPS stimulated DC2 cells. Interferon-γ (IFN-γ) production was decreased when MSCs were added to effector T cells undergoing T helper cell 1 (Th1) cell differentiation. MSCs increased IL-4 production in co-culture with effector T cells undergoing Th2 differentiation. In addition, MSCs decreased IFN-γ production in co-culture with natural killer (NK) cells. Thus, MSCs may affect different subsets of lymphocytes and create an anti-inflammatory environment when they are co-cultured with lymphocytes.

2.3 The potential clinical benefits of MSCs as immunosuppressants

Besides the powerful and wide-ranging suppression shown in *in vitro* experiments, *in vivo* experiments showed that MSCs possess beneficial immunomodulatory properties and have promising clinical applications. For example, skin grafts were prolonged by MSCs in a baboon model [15] and a case of severe-acute, steroid-resistant GvHD was cured by third party haploidentical MSCs in a pediatric patient [6]. Since then, numerous *in vivo* studies have been done to study the immunosuppressive properties of MSCs.

GvHD is a common complication following bone marrow transplantation. The lymphocytes in the graft attack the recipient due to the lymphocytes seeing the tissues of the recipient as foreign antigens. Acute steroid-refractory GvHD is incurable and life threatening. Clinical trials have been undertaken to test MSCs as an orphan drug for the treatment of GvHD. In a Phase II clinical trial, 39 of 55 patients with steroid-resistant, severe, acute GvHD responded to the treatment with MSCs [7]. Among them, 28 (78 %) of 36 patients with involvement of the disease in one or two organs had a response compared with 11 (58 %) of 19 patients with the involvement of three organs. In this trial, MSCs from either HLA-identical sibling donors (n = 5), haploidentical donors (n = 18) or third-party HLA-mismatched donors (n = 69) were injected. The response rate was not related to donor HLA-match. The results of a Phase III clinical trial were, however, relatively disappointing when comparing a commercialized off-the-shelf allogeneic MSC product "Prochymal" with placebo [8]. There was no statistical difference between Prochymal and the placebo on the primary endpoints, although Prochymal benefitted subgroups of patients with either steroid-refractory gastrointestinal GvHD or liver GvHD. Further trials are ongoing. Encouragingly, a trial of Prochymal on pediatric patients showed good results. The overall response at day 28, defined as organ improvement of at least one stage without worsening in any other, was 64 % of 59 patients. Overall survival rate (day 100) was 62 % [9]. As

shown on the company's website, Prochymal has been approved by the regulatory authorities of both Canada and New Zealand to be clinically marketed for the treatment of acute (GvHD) in children as the first-in-class stem cell therapy. The fact that the immunosuppressive effect of MSCs is seen both *in vitro* and *in vivo* and appears to be independent of MHC-matching suggests that allogeneic MSCs can be used allogeneically as commercialized off-the-shelf immunosuppressant products.

MSCs have been shown to ameliorate both epitope induced and adoptively transferred experimental autoimmune encephalomyelitis (EAE), the experimental murine equivalent of the disease multiple sclerosis (MS). Intravenously injected green fluorescent protein (GFP) transfected MSCs were found to have entered the central nervous system one month after administration showing the ability of MSCs to mobilize to the inflammatory site [10, 11]. The pathology results showed reduction in the levels of immune cell infiltration in inflammatory sites of MSC-treated EAE mice compared to the nontreated EAE mice [11, 12].

2.4 The mechanisms of immunosuppression by MSCs

As a result of the promising clinical benefits of MSCs as immunosuppressants, much effort has been put into understanding the possible mechanisms of this immunosuppressive effect, in order to guide the clinical applications. However, due to different cell isolation and culture methods, as well as the different assays used to test the immunosuppressive effect, many possible mechanisms have been reported by scientists, and they are often conflicting. Multiple mechanisms may be involved in the suppression process.

A large number of studies have been done to investigate the mechanisms involved in immunosuppression by MSCs. Most of them were performed on either human or murine cells, although for both these species the results have been conflicting. Species differences underlining the mechanisms of immunosuppression by MSCs were indicated [26, 27]. Hence, we have conducted a comprehensive review of articles describing the immunosuppressive properties of human MSCs compared with murine MSCs.

2.5 The mechanisms of immunosuppression by human MSCs

The activation of T cells requires two signals. One signal is through the interaction between T cell receptor and the antigenic epitope presented by MHC molecules. Another signal is through costimulatory molecules. The lack of costimulatory molecules will induce T cell anergy [28]. Human MSCs express MHC-I but not MHC-II molecules on their cell surface, and they are negative for costimulatory molecules CD40, CD40L, B7.1 (CD80), B7.2 (CD86). However, the lack of immunogenicity of human MSCs is not simply due to their lack of MHC-II and costimulatory expression. MHC-II

molecules on the surface of MSCs can be upregulated by IFN-γ but human MSCs still lacked immunogenicity after MHC-II surface expression was upregulated by IFN-γ [13,14]. The lack of immunogenicity is also not simply due to the lack of B7 molecules as human MSCs transduced with B7 molecules and pulsed by IFN-γ still lacked immunogenicity. This suggests that low level expression of MHC molecules and the deficiency of B7.1 and B7.2 costimulatory molecules were not sufficient to induce T cell tolerance. MSCs were not, therefore, behaving as anergy-inducing antigen presenting cells (APCs) [14]. In terms of immunosuppression, pre-incubation of human MSCs with IFN-γ enhanced the suppression, which suggests other mechanisms are involved rather than the lack of MHC and costimulatory B7 molecules [14, 29].

One question that still needs to be answered is whether the immunosuppression effect of MSCs is mediated by other cells such as APCs. The fact that the immunosuppressive effect is seen on antibody-coated bead stimulated lymphocytes suggests that human MSC-mediated immunosuppression does not require the mediation of APCs [17]. Contradictory results were reported; human MSCs inhibited T cell proliferation by altering APC maturation [19]. An increased number of APCs in the suppression assay decreased IFN-γ production. However, in this paper, they did not show that this effect was directly associated with the proliferation of lymphocytes.

The important question as to whether the immunosuppressive effect of human MSCs is contact dependent remains controversial. Human MSC immunosuppression of T cells was reported to be contact independent since MSCs were still immunosuppressive if they were separated from proliferating lymphocytes by a semi-permeable membrane [14, 17, 30]. Supernatant from human MSC/MLR (mixed-lymphocyte reaction) co-culture suppressed lymphocyte proliferation [18, 31]. The contact independent property of the immunosuppressive effect of MSCs suggests this effect is mediated through soluble factors. However, conflicting results showed that immunosuppression was contact dependent, as human MSCs failed to suppress IFN-γ production by stimulated PBLs [32] or the proliferation of T cells [33] when separated by a semi-permeable membrane.

Soluble factors were investigated as the candidates for the immunosuppressive effect of human MSCs. Addition of transforming growth factor-β1 (TGF-β1) or hepatocyte growth factor (HGF) neutralizing antibodies partially restored T cell proliferation suppressed by human MSCs; when those two antibodies were added together, T cell proliferation was restored to levels comparable to the MLR without MSCs [17]. However, this effect was not seen in a later study, where the level of TGF-β1 or HGF remained unchanged in the MLR with or without the addition of human MSCs; anti-TGF-β1 and anti-HGF together did not restore PHA stimulated lymphocyte proliferation suppressed by human MSC (73 ± 7 %; n = 6) compared with no MSC control (66 ± 3 %; n = 2) [18]. Another study also reported that anti-TGF-β was not able to reverse the immunosuppression by human MSCs [32].

Recently, several studies indicated that human leukocyte antigen HLA-G could be involved in the immunosuppression mediated by human MSCs; blocking antibodies

against HLA-G significantly reduced the suppression of lymphocyte proliferation by human MSCs in either MLR or PHA stimulated PBLs cultures [34–36]. Two of these studies found that human MSCs secreted soluble HLA-G (sHLA-G) constitutively [34, 36]. Another study suggested, however, that isolated MSCs alone did not secrete sHLA-G [35]. sHLA-G was secreted by stimulated PBMC and upregulated in co-culture with MSCs. HLA-G was suggested to function synergistically with IL-10. Both anti-IL-10 and anti-HLA-G reduced MSC-mediated suppression of lymphocyte proliferation in MLR significantly [35, 36]. Anti-HLA-G decreased the level of IL-10 in the MLR/MSC co-culture or PHA/PBL/MSC co-culture. An IL-10 neutralizing antibody decreased the level of HLA-G in cultures of MSCs with PBLs, stimulated with PHA [35]. These results suggest that HLA-G and IL-10 regulate the secretion of each other. Selmani *et al*. demonstrated that HLA-G secretion supports the upregulation of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells (Tregs) [36]. However, the role that IL-10 plays in the immunosuppressive effect of human MSCs is controversial. IL-10 was upregulated when lymphocytes were cultured with MSCs; the immunosuppressive effect of human MSCs was partially reversed by blocking IL-10 activities [32]. In another study, human MSC immunosuppression appeared not to depend on IL-10 [30]. IL-10 was increased in the presence of MSCs in the MLR but not in the PHA stimulated cultures. The addition of a neutralizing anti-IL-10 antibody failed to abolish the suppression by MSCs, but further suppressed proliferation of PBLs in the MLR, and had no effect in PBLs stimulated by PHA. MSCs may play different roles too in the suppression of lymphocytes proliferation in MLR and nonspecific antigen stimulated PBLs. We can conclude that IL-10 may play a minor or no role in the suppressive effect of human MSCs depending on the experimental conditions used to test immune suppression.

IFN-γ was indicated as an important mediator for MSC immunosuppression in more recent reports. Many researchers have suggested that the mechanisms involve a negative feedback effect resulting from secretion of IFN-γ induced immunosuppressive molecules, although the exact mechanisms are still under debate. The possible suppressive molecules expressed by IFN-γ activated MSCs include prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO). Among them, PGE2 and IDO were reported to be associated with the immunosuppressive effect of human MSCs [25, 30, 37–39].

PGE2 has been reported to suppress T cell proliferation by different mechanisms. PGE2 synthesis is the result of cyclooxygenase (COX) and prostaglandin E synthase (PGES) activities in response to proinflammatory cytokines such as IFN-γ and TNF-α [40, 41]. PGE2 binds to E-prostanoid receptor (EP receptor) EP1-4; EP2 and EP4 are the main receptor subtypes to mediate the action of PGE2 in human and murine $CD4^+$ T cells [42, 43]. The activation of EP receptors leads to downstream cyclic adenosine monophosphate (cAMP) production; cAMP then exerts anti-proliferative activities through multiple mechanisms [43]. Immunosuppression by MSCs was reported to involve the secretion of PGE2. Human MSCs co-cultured with PHA stimulated PBLs to produce high levels of PGE2; PGE2 inhibitors partially reversed the suppression

of PBLs co-cultured with human MSCs [25]. Another paper showed the COX inhibitor indomethacin only marginally restored PHA-induced proliferation of PBLs suppressed by MSCs but not proliferation of MLRs suppressed by MSCs [30]. On the other hand, indomethacin failed to restore proliferation of MLR suppressed by MSCs in one report, although anti-IFN-γ recovered proliferation of MLR suppressed by MSCs entirely [38]. Other molecules stimulated by IFN-γ might be involved rather than PGE2.

IDO is a molecule that has an immunosuppressive effect on T cell proliferation. IDO is an enzyme that metabolizes tryptophan to generate kynurenine. The depletion of the essential amino acid tryptophan inhibits T cell proliferation. The production of IDO mainly depends on the stimulation by IFN-γ [44]. IDO was first reported to inhibit microbes as a host defense response [45].

In 1998, Munn *et al*. found IDO was crucial to maternal tolerance in pregnant mice [46]. Many types of cells can express IDO [47]. Recent findings have shown that dendritic cells and macrophages can both be stimulated to express IDO and thus exhibit regulatory function [44, 48, 49]. Meisel *et al*. reported that human MSCs express IDO upon stimulation with IFN-γ. Proliferation of T cells was largely restored in the MSC/PBL culture by the addition of tryptophan [37]. Another study suggested that immunosuppression by human MSCs was not entirely due to IFN-γ driven IDO production. MSCs from an IFN-γ receptor deficient patient were still immunosuppressive. IDO inhibitor largely reversed the proliferation of lymphocytes under the suppression by wild type MSCs but did not have any effect on IFN-γ deficient MSCs. Medium conditioned by MSC/MLR inhibited proliferation of PBMC. The inhibition was partially reversed by the depletion of insulin-like growth factor binding protein (IGFBP) from the conditioned medium. The authors suggested IGFBP played a role in the immunosuppressive effects of MSCs [31]. In another report, both human MSCs and fibroblasts exerted immunosuppressive properties and the immunosuppression partially functioned through IFN-γ induced IDO expression since the IDO inhibitor 1-methyl-tryptophan (1-MT) only partially restored the proliferation of MLR suppressed by human MSCs, although anti-IFN-γ restored the proliferation entirely [38]. Human MSCs were reported to express different types of toll-like receptors (TLRs). TLR3 and TLR4 ligands polyinosinic:polycytidylic acid (poly (I:C)) or LPS could upregulate the surface expression of TLR3 and TLR4 and enhance both IDO activity and the immunosuppressive properties of human MSCs. IDO1 but not IDO2 appears to be responsible for TLR-induced tryptophan degradation in MSC since the IDO1 inhibitor 1-L-MT (1 mM) but not the IDO2 inhibitor 1-D-MT (1 mM) inhibited poly (I:C)-induced tryptophan degradation in MSCs. The IDO1 inhibitor 1-L-MT (1 mM) abrogated human MSCs immunosuppression on MLR in poly (I:C) and LPS pretreated co-cultures. The authors further suggested protein kinase R (PKR), IFN-β and signal transducer and activator of transcription 1 (STAT1) were involved in the TLR-IDO1 pathway. Specific inhibitors and siRNA knockdown approaches were used to prove this signaling pathway [39].

2.6 Immunosuppression by murine MSCs and the species difference underlying the mechanisms of immunosuppression by MSCs

Several studies investigated similar candidates possibly involved in the mechanisms of immunosuppression by murine MSCs. The immunosuppressive effects of murine MSCs were seen on antibody-coated bead stimulated lymphocytes implying that the immunosuppression by murine MSCs does not require the mediation of APCs [19, 20]. Whether apoptosis or proliferative arrest are involved in the mechanisms of immunosuppression by murine MSCs remains controversial [19, 20]. Conflicting results have also been seen on whether the immunosuppressive effect of murine MSCs is contact dependent. Murine MSCs exhibited immunosuppression in a contact independent manner; both MSCs in a transwell system or the culture supernatants from the immunosuppression assay were as immunosuppressive as MSCs in direct contact [50, 51]. However, other results were reported showing that immunosuppression by murine MSCs is contact dependent [20].

Soluble factor TGF-β1 was expressed constitutively by murine MSCs at a low level but similar amounts of exogenously added TGF-β1 were not able to suppress the proliferation of murine lymphocytes [21]. Exogenous HGF or IL-10 were also unable to suppress lymphocyte proliferation. Murine MSC immunosuppression was not dependent on $CD4^+/CD25^+$ Tregs because $CD25^+$ depleted and $CD25^+$ replete T cell populations were equally inhibited by murine MSCs [20]. Expansion of the $CD4^+CD25^+FOXP3^+$ regulatory T cell subset was seen among lymphocytes stimulated with anti-CD3/CD28; however, the proportion of cells expanded remained the same with or without MSCs co-culture [22]. $CD4^+CD25^+$ Treg cells appear not to account for the immunosuppression mediated by murine MSCs, although the involvement of inducible Treg cells was not excluded in these studies.

IFN-γ has been implicated as an important mediator for murine MSC immunosuppression. When murine MSCs were combined with stimulated lymphocytes derived from an IFN-$\gamma^{-/-}$ mouse, the immunosuppression was largely abrogated compared with murine MSCs cultured with similarly stimulated wild type lymphocytes [22, 52]. IFN-γ plays a critical role in mediating murine MSC immunosuppression. The B7.H1 co-inhibitory surface molecule was induced on murine MSCs by INF-γ. An IFN-γ neutralizing antibody blocked the upregulation of B7.H1; the immunosuppression displayed by B7.$H1^{-/-}$ MSCs was also partially abrogated [22]. Besides B7.H1 upregulation, downstream consequences of IFN-γ activation on murine MSCs were reported to include upregulation of PGE2, IDO, and NO [21, 26, 50, 53, 54].

The importance of PGE2 in immunosuppression by murine MSCs was indicated by several reports. Indomethacin decreased the amount of PGE2 and partially reversed the proliferation of murine splenocytes in co-culture with MSCs from a C3H10T½ murine MSC cell line (C3 MSC) [50]. The immunosuppressive properties of murine MSCs were suggested to be attributable to IL-6-activated pathways through PGE2

upregulation. Indomethacin decreased the level of IL-6 and PGE2 in the ConA treated splenocyte/MSC co-cultures. Anti-IL-6 or indomethacin partially reversed suppression of splenocyte proliferation in the co-culture with C3 MSCs. Knocking down IL-6 partially abrogated the immunosuppression mediated by MSCs [55].

English *et al*. reported that both PGE2 and IDO were involved in murine MSC immunosuppression since inhibition of either PGE2 or IDO significantly reversed the suppression of lymphocyte proliferation by murine MSC [21]. However, IDO was not involved in the immunosuppressive effects of murine MSCs in other reports [26, 53].

Another possible downstream signal through which INF-γ mediates suppression is NO. NO is synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). iNOS activity is inducible by LPS, dsRNA or proinflammatory cytokines such as IL-1, TNF-α and IFN-γ [56–58]. NO mediates its effects through different pathways, including the reaction with transition metals to increase intracellular cyclic guanosine monophosphate (cGMP), the s-nitrosylation reaction with target proteins and the reaction with superoxide to form peroxynitrite. These activities lead to inhibition of mitochondrial respiration, protein synthesis inhibition or over-expression, DNA damage and cellular or tissue damage [58, 59].

Sato *et al*. were the first to report that the immunosuppressive effect of murine MSC was through NO-mediated inhibition of STAT5 phosphorylation. Both iNOS and COX inhibitors but not the IDO inhibitor reversed the suppression of ConA stimulated T cells [53]. Later, Ren *et al*. demonstrated murine MSC immunosuppression was through the proinflammatory cytokines and NO [54]. Murine MSCs have no suppressive effect on anti-CD3 stimulated $CD4^+$ and $CD8^+$ T cell blasts in the absence of cytokines, indicating that MSCs require a T cell cytokine to be immunosuppressive. The combination of cytokines IFN-γ and one of TNF-α, IL-1α or IL-1β addition with MSCs but not either one of these cytokines alone suppressed the proliferation of T cell blasts. Immunosuppression mediated by MSCs was blocked by an anti-IFN-γ antibody alone, or the combinations of three antibodies including anti-TNF-α, anti-IL-1α and anti-IL-1β. INF-γ is required but on its own is not sufficient and the synergistic effect of TNF-α, IL-1α or IL-1β is also required for the immunosuppression to be exerted by MSCs. This paper also found that a PGE2 blocker, anti-IL-10, anti-TGF-β, or IDO blocker had no effect on the immunosuppression of murine MSCs. However, iNOS activity and NO production both correlated with the immunosuppression by MSCs; the iNOS inhibitor L-NMMA abrogated MSCs mediated immunosuppression. Furthermore, iNOS-deficient MSCs lack immunosuppressive capability. The authors further demonstrated that immunosuppression by murine MSCs was associated with a panel of chemokines such as (C-X-C) ligand 9 (CXCL9) and CXCL10 thought to mobilize T cells adjacent to MSC. Those chemokines were produced and upregulated by murine MSCs in the presence of proinflammatory cytokines. Anti-(C-X-C) receptor 3 (CXCR3) or anti-(C-C) receptor 5 (CCR5) antibodies partially abrogated the immunosuppression by murine MSCs.

The same group reported the species difference between mouse and human or other primates in terms of the mechanisms of MSC-mediated immunosuppression. They reported that immunosuppression mediated by murine MSCs acted through pro-inflammatory cytokine driven NO production but that the immunosuppression associated with human MSCs was through IDO and was unrelated to NO [26]. The iNOS inhibitor had no effect on the immunosuppression of human MSCs. However, the IDO inhibitor 1-MT abrogated the immunosuppression of human MSCs whereas 1-MT did not have this effect on murine MSCs. They demonstrated that the immunosuppression of both human MSCs and murine MSCs was triggered by proinflammatory cytokines. The final downstream activated pathway was, however, IDO for human but NO for murine MSCs. Chemokines were involved in the immunosuppression of MSCs from both species. The species difference that exists between human MSCs and murine MSCs was reported by another paper. Under the treatment with proinflammatory cytokines, human MSCs but not murine MSCs produced IDO and as a result exhibited antibacterial, antiprotozoal and antiviral effects [27]. In contrast, iNOS activity was detected in murine MSCs but not human MSCs under the same conditions. The growth of Toxoplasma gondii parasites was inhibited by the iNOS activity of murine MSCs.

To conclude, IFN-γ plays a crucial role in activating MSCs in response to activated lymphocytes. IFN-γ and other proinflammatory cytokines including TNF-α, IL-1α, IL-1β and IL-6 may work synergistically to stimulate MSCs to secrete an anti-proliferative molecule, IDO in the case of human MSCs or NO in the case of murine MSCs. The effects of other inhibitory molecules cannot be excluded at this point since conflicting results were reported (Fig. 2.2).

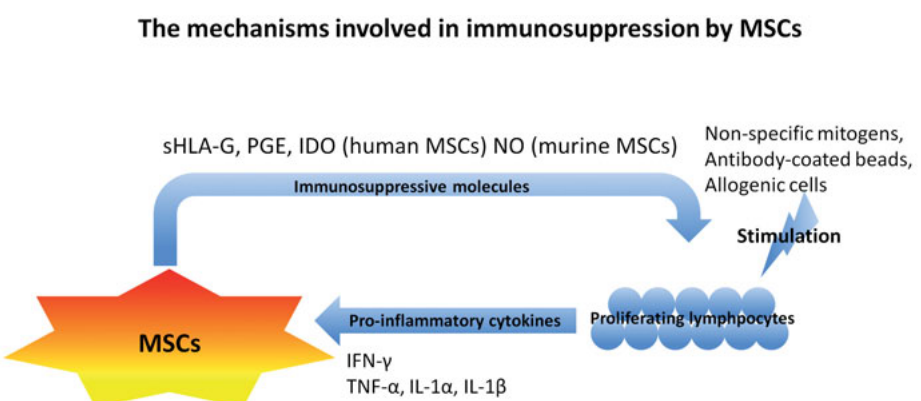

Fig. 2.2: The possible mechanisms involved in immunosuppression by MSCs. IFN-γ activated MSCs secrete immunosuppressive molecules as a negative feedback mechanism to regulate T cell proliferation.

2.7 Immunosuppression mediated by fibroblasts

Fibroblasts have also been reported to possess immunosuppressive properties. In the early 1990s, human gingival fibroblasts were reported to have IFN-γ-dependent immunosuppressive effects on PHA or allogeneic APC stimulated T cells [60]. Jones *et al*. reported that the anti-T cell proliferative effects of human MSCs were shared by all stromal cells including fibroblasts and chondrocytes [33]. In mechanistic terms, the immunosuppressive effects of human fibroblasts and MSCs were comparable and both functioned partially through IFN-γ induced IDO production [38]. It has been confirmed in another study that human MSCs and fibroblasts share the immunosuppressive effects but the authors did not find a role for IDO production [61]. The accepted, standard method to identify MSCs is to use a panel of surface markers. However, fibroblasts share a wide range of surface markers with MSCs [33, 38, 61]. Fibroblasts exist everywhere in the body; they have the capacity to synthesize and maintain the extracellular matrix [62]. MSCs are essentially multipotent fibroblasts. It is not surprising that fibroblasts from elsewhere are functionally similar in terms of immunosuppression. In fact, fibroblasts isolated from sites of the body other than bone marrow share the multipotent differentiation capabilities with MSCs to various extents as previously reported [33, 38].

2.8 The mechanisms of the immunosuppressive effect of MSCs are shared with other nonstromal cells

The immunosuppressive effect of MSCs and its mechanisms are not only shared with fibroblasts but also shared with other cells such as macrophages and dendritic cells. IFN-γ and CD40L activated mature human DCs were able to suppress T cell proliferation through IDO-mediated tryptophan depletion [48]. IFN-γ-mediated NO production was shown for murine macrophages [56, 59, 63]. iNOS activity and NO production by murine lung epithelial cells was detected and shown to be elevated by the combination of IL-1β, TNF-α, and IFN-γ [64]. The immunosuppressive effect and its mechanisms are thus not specifically endowed to MSCs. The negative feedback effect that is clearly triggered by proinflammatory cytokines is a major response shared by other cell types.

2.9 How long can MSCs survive *in vivo*?

MSCs were demonstrated to be immunosuppressive and have shown benefits in the treatment of GvHD in clinical trials and the treatment of autoimmune diseases in experimental models. Before these cells become a frontline treatment for such diseases, however, it is important to ask how long MSCs can survive after injection and to what extent they are able to migrate into target tissues? Erythropoietin (EPO) secret-

ing engineered MSCs were used to study whether murine MSCs can survive in an allogeneic recipient [65]. Hematocrit (HCT) was monitored as the reporter for the survival of MSCs. However, HCT only rose transiently and rapidly declined to a baseline value in MHC-mismatched recipients. In contrast, HCT remained at a high level for more than 200 days when using syngeneic grafts. In another study, allogeneic MSCs pre-infused mice more rapidly rejected carboxyfluorescein succinimidyl ester (CFSE) labeled allogeneic splenocytes from the same strain of donor mice, compared with syngeneic MSCs pre-infused mice or PBS pre-infused controls, which indicates that the infusion of allogeneic MSCs is able to trigger a memory T cell response [66]. Zangi *et al*. reported luciferase labeled allogeneic MSCs or fibroblasts were both rejected by recipients within 14 days, although MSCs survived longer than fibroblasts *in vivo*. MSCs or fibroblasts grafts were rejected much faster in re-challenged mice suggesting that allogeneic MSCs were able to induce immunological memory *in vivo*: memory phenotype CD4+ cells were markedly enhanced following the infusion of allogeneic MSCs compared with syngeneic control cells [67]. Moloney *et al*. reported that engineered GFP expressing allogeneic rat MSCs were gradually rejected by recipient rats within 42 days. In addition, host immunosuppression significantly improved the survival of allogeneic rat MSCs [68]. In a long-term study, MSCs were injected into the infarct site in a rat model of myocardial infarction [69]. MSCs were shown to differentiate into myogenic and endothelial cells. However, allogeneic MSCs were rejected after 5 weeks. The serum of allogeneic MSC recipients contained specific antibodies that reacted with differentiated but not undifferentiated allogeneic MSCs. This indicates that the immune privilege of MSCs may be only limited to a short term when the cells still remain in an undifferentiated state. The differentiation of MSCs may alter antigen expression and as a result immune rejection is provoked; alternatively, the immunosuppressive properties of MSCs may be lost as cells differentiate. Conflicting results were reported. Allogeneic human MSCs were able to engraft in bone marrow stroma and skin; and produce clinical benefits in osteogenesis imperfecta patients after 4 to 6 weeks [70]. Allogeneic MSCs in one recipient could survive up to 76 days in a baboon model [16]. However, because an *in vitro* species difference exists between human and murine MSCs, not all the animal model data is necessarily relevant to human. More human data are needed to draw clear conclusions as to whether allogeneic human MSCs and tissues derived from them will survive.

2.10 Conclusion and discussion

In conclusion, MSCs have proven to be immunosuppressive for T cell driven immune responses including cell proliferation and cytokine secretion. The mechanisms have been extensively investigated although results are conflicting. Many researchers have suggested that the mechanisms involve IFN-γ activated immunosuppressive molecules in a negative feedback mechanism to regulate the T cell proliferation and cytokine secre-

tion. The possible suppressive molecules secreted by INF-γ activated MSCs include IDO, PGE2 and NO. Therefore, INF-γ pretreated MSCs may be better immunosuppressants for clinical application. However, these reported mechanisms do not belong uniquely to MSCs. Other cells express similar suppressive molecules in response to proinflammatory cytokines; these include macrophages, DCs, epithelial cells and some tumor cells. In addition, skin fibroblasts and fibroblasts isolated from elsewhere have proven to be as immunosuppressive as MSCs. Those facts suggest that the immunosuppressive effect is not specifically endowed to MSCs. Nevertheless, this should not prevent MSCs being investigated as potential immunosuppressants; indeed, the administration of MSCs improved the clinical symptoms of GvHD in clinical trials [6–9] and also ameliorated autoimmune conditions such as EAE in animal models [10–12]. The fact that the immunosuppressive effect of MSCs is observed in MHC-mismatched recipients led to the hope that MSCs could be used as off-the-shelf products for more convenient and effective use both for immunosuppression and tissue regeneration. Furthermore, there is some evidence that MSCs are still immunosuppressive even after differentiation [14, 29, 71]. Taken together, if MSCs were immunoprivileged and the immunoprivilege was maintained long-term following differentiation, then tissue engineered tissue or organs derived from MSCs could be applied as off-the-shelf products. However, the fact that MHC-mismatched MSCs do not survive for long in recipients suggests that MHC-mismatched MSCs might only be used as short-term immunosuppressive drugs alone. Off-the-shelf allogeneic MSCs may, therefore, find application as immunosuppressive drugs but they are less likely to be useful for tissue engineering. Despite their lack of immunogenicity and immunosuppressive properties *in vitro*, the *in vivo* response of the immune system against MSCs is more profound. Memory $CD4^+$ T cells and specific antibodies were detected in hosts after the infusion of allogeneic MSCs [67, 69]. MSCs may be immunosuppressive, but this does not provide sufficient immunoprivilege for allogeneic MSCs to be useful for tissue engineering.

The immune system is balanced by central and peripheral tolerance. Articles reviewed indicate DCs, macrophages, MSCs and other specialized cells including fibroblasts all play their roles in the peripheral tolerance to defend our body against the possibly overactive responses, immune pathology, and unexpected autoimmune phenomena. During the inflammatory response, not only immune cells play an important role, "nonimmune" components such as fibroblasts, epithelial cells, MSCs are also doing their job. We suggest that the immunosuppressive properties of fibroblasts should be given special attention in future studies because these cells are distributed all over the body. IFN-γ was considered as a proinflammatory cytokine involved in immune responses. As reviewed in this article, however, IFN-γ and other proinflammatory cytokines also plays a key role in the immune regulation by activating a variety of cells to produce immunoregulatory molecules, although this activation may have other functions such as defending the host body against the invasion of microbes [27]. How the immune system manages to balance the inflammatory response with immune regulation has always been an important topic, IFN-γ acti-

vated molecules are important targets to regulate the balance of the immune system between overactivation and hyporesponse.

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Alan Tyndall and Chiara Bocelli-Tyndall

3 Mesenchymal stem cell therapies for autoimmune diseases

Abstract Mesenchymal stem cells, more appropriately called multipotent mesenchymal stromal cells (MSCs), have been suggested as therapy in a wide variety of autoimmune diseases (AD) for at least a decade. The early observations that MSCs could inhibit the mixed lymphocyte reaction (MLR) led quickly to a concept of immunoprivileged infused MSCs "homing" to sites of tissue distress where a paracrine and / or cell-cell contact event occurs resulting in resolution and healing. This appeared to be substantiated by early clinical observations in acute graft-versus-host disease (GvHD). Despite subsequent suggestive data from thousands of published *in vitro* phenomena, hundreds of animal models and dozens of clinical case reports and small series, MSCs are not yet registered for the treatment of any autoimmune disease at the time of writing (December 2012). This delay could have several causes including lack of standardization in MSCs definition and therapeutic product description, choice of inappropriate clinical outcomes and clinical trial designs. It could also be that MSCs as currently applied have little clinically relevant effect on autoimmune disease. These issues require urgent clarification by way of prospective randomized double-blind clinical trials.

3.1 Introduction

The mesenchymal stromal progenitor cell has been described for over 30 years [1], but only reached clinical attention following a publication of a Phase I feasibility and safety study in 23 patients in full remission following treatment for various hematological malignancies [2]. Autologous bone marrow derived mesenchymal progenitor cells were expanded *ex vivo* and given back *via* intravenous infusion (IVI). No adverse effects were noted. Five years later, in 2000, the first published results of MSCs used therapeutically suggested that they were again safe and feasible and had the potential for hematopoietic stem cell (HSCs) graft enhancement [3]. Autologous *ex vivo* expanded bone marrow derived MSCs were given to 28 breast cancer patients receiving an autologous HSC transplant.

Several reports followed suggesting that MSCs could inhibit the mixed lymphocyte response, an effect which was independent of the MHC complex, since third party MSCs were equally effective [4]. Subsequent *in vitro* studies suggested that MSCs may avoid immune surveillance, to be so called "immunoprivileged" and to possess anti-inflammatory and anti-proliferative properties [4, 5]. The Karolinska group reported in 2004 the first case of severe acute graft-versus-host disease (GvHD) responding to allogeneic MSCs [6], which was followed by an escalation of *in vitro*, animal model

and clinical studies involving MSCs. The immune modulatory, anti-inflammatory, anti-fibrotic and tissue protective properties were studied. The first animal model of autoimmune disease, experimental allergic encephalomyelitis (EAE) was positive, even though MSCs were not detected in the central nervous system of the mice, but rather in lymph glands and spleen [7]. An immune modulation *via* the secondary lymph organ effect(s) was postulated.

The past decade has witnessed an explosion of publications of *in vitro* and animal model observations supporting a potential role for MSCs in human disease, particularly AD (reviewed in [8]). Not all have been positive *e.g.* worsening of murine arthritis [9] or increased autoantibodies in murine systemic lupus erythematosus (SLE) [10], but these reports have been either ignored or re-explained. Many paracrine factors and cell-cell contact mechanisms have been proposed, ranging from factors employed by the placenta to avoid rejection *e.g.* IDO [11], soluble HLA-G [12] to mechanisms involved in immune tolerance such as Treg recruitment and activation [13], IL-10, TGF-β, hepatocyte growth factor (HGF) [5], DC maturation arrest [14] and antigen presentation without costimulation [15] have been proposed. These are summarized in Figure 3.1, with arrows showing both MSCs' influence on target immune competent cells and in some cases the reverse, so called "cross-talk".

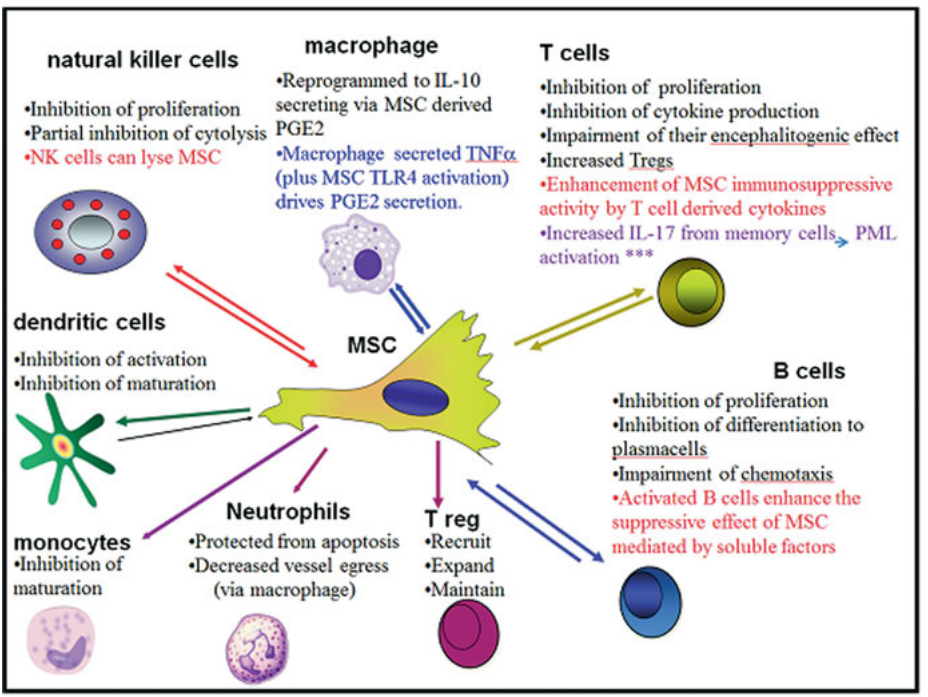

Fig. 3.1: Interaction of immune cells with MSCs.

An elegant example is that of the Prockop group which showed in a murine ischemic brain injury model that human MSCs were able to upregulate survival signals in neurons which in turn were able to induce anti-inflammatory genes in the MSCs [16]. Previous work from this group had shown that MSCs are able to rescue stressed cells *in vitro via* mitochondria exchange [17], and more recently this two-way information exchange has been confirmed by another group who showed that microparticle exchange was an important mechanism [18]. The Prockop group also showed that MSCs survival *in vivo* was limited to 7 days, the same as in a SCID mouse, suggesting a nonimmunological basis for the short engraftment. Similar clinical findings were recently published; 18 patients who had received MSCs for acute GvHD treatment and later died from various causes were examined at autopsy. No donor MSCs were found later than 50 days post treatment, but also no ectopic tissue or tumors were detected [19]. This may be a problem if MSCs are expected to engraft and differentiate into functional tissue. However, if a "hit and run" effect on an unwanted immune reaction is desired, then short-term engraftment may be an advantage regarding potential malignant transformation of MSCs *in vivo* [20]. However, no consistent mechanism for the immunomodulatory effect of MSCs has emerged as being dominant, and some reports are contradictory, one group being unable to confirm the secretion of HCF or TGF beta from MSCs [21].

In addition, mechanisms belonging to basic tissue injury responses and beyond the innate and adaptive immune systems may be operative. One group has shown that the "immune modulation" in a murine model of myocardial infarction was due to intravenous MSCs' microembolic injury to the lung causing release of TNF stimulated Gene 6 protein (TSP6), which has an anti-inflammatory activity [22].

The first human AD results were reported in 2007 by an Iranian group. Ten multiple sclerosis (MS) cases received intrathecal allogeneic umbilical cord MSCs with mixed results. Subsequently over 20 small series have been published of various AD, also with mixed results (Tab. 3.1). Two large randomized prospective trials (Crohn's disease (CD) and acute GvHD) were reported as not reaching their primary endpoints and were never published.

On this background of encouraging but at times confusing preclinical data and suggestive uncontrolled clinical data, it is time to review the current strategies and define what is established and what needs further clarification.

3.2 Autoimmune disease

Autoimmune diseases are a heterogeneous collection of disorders in which single or multi-organ involvement results in dysfunction thought to be due to an aberrant immune reaction against self-structures. Although the adaptive immune system has been the focus of attention in the past, recently the role of the innate immune system is being appreciated [23], with a distinction between "autoimmune", *e.g.* SLE,

Table 3.1: Studies using MSCs in patients with chronic inflammatory diseases.

Autoimmune Disease	Patient Number	MSCs source	Route	Outcome	Reference
multiple sclerosis	10	allogeneic bone marrow	intrathecal	mixed	[67]
multiple sclerosis	1	allogeneic umbilical cord	intravenous	improved	[68]
multiple sclerosis	3	allogeneic and autologous fat	mixed IVI & intrathecal	improved clinic but not MRI.	[69]
multiple sclerosis	10	autologous bone marrow	intrathecal	improved clinic but not MRI.	[70]
multiple sclerosis	15	autologous bone marrow	intrathecal plus IVI in 5	some improved	[71]
multiple sclerosis	10	autologous bone marrow	intravenous	Minor visual improvement only	[72]
Crohn's fistulae	14	autologous fat	intra fistula	71 % closure	[73]
Crohn's fistulae	10	autologous fat	intra fistula	100 % closure (30 % partial)	[74]
Crohn's	10	autologous bone marrow	intravenous	some improved	[32]
Crohn's /& ulcer. colitis	4 3	allogeneic umbilical cord	intravenous (1 mill. /kg)	improved (never iTNFa)	[75]
scleroderma – digital ulcers	2	autol. blood & bm MNC	local	improved	[76]
scleroderma	5	allogeneic bone marrow	intravenous	some improvement variably durable	[77]
scleroderma – ischemia	1	autologous bone marrow	intravenous	improved ischemia (later died)	[78]
SLE nephritis	15	allogeneic bone marrow	intravenous	improved SLEDAI & proteinura	[79]
SLE nephritis	16	allogeneic umbilical cord	intravenous	improved SLEDAI & proteinura	[80]
SLE	2	autologous bone marrow	intravenous	no clinical change (increased Tregs)	[81]
SLE alveolar bleed	4	allogeneic umbilical cord	intravenous	improved	[82]
SLE heterogenous	87	allogeneic BM / umbilical cord	intravenous	most improved	[83]
SLE cytopenia	35	allogeneic BM / umbilical cord	intravenous	most improved	[84]
Type II diabetes	10	allogeneic placental	intraveneous (×3)	all improved (at 3 months)	[85]

and "auto-inflammatory" *e.g.* Still's disease, being drawn [24]. To date no agent or therapeutic strategy has been able to fully eliminate either disorder, with the possible exception of immune ablation and autologous HSC transplantation [25]. This is most likely related to the fact that autoreactive antigens and immune competent cells remain even if the clinical manifestations of the disease are suppressed.

As seen in Figure 3.1, MSCs have the potential to influence most components of both the innate and adaptive immune systems. It is therefore unlikely that one or more infusions or applications of MSCs will completely and permanently reset autoimmunity or auto-inflammatory diseases, given the short engraftment [19]. However a short duration "hit and run" effect may offer a clinically useful strategy, particularly if the reported low toxicity is confirmed with long-term follow-up.

3.3 Mesenchymal stem cells (MSCs)

Since Friedenstein's initial description of "fibroblast precursors" in 1974, thousands of papers have been published using the term "mesenchymal stem cell", probably encompassing various disparate stromal cell precursor cell types. The true stemness of the cell has not been established, and in order to standardize the literature, a consensus was published in 2005 [26] with the suggestion to call the cells multipotent mesenchymal stromal cells, retaining the acronym "MSCs". Most groups use this basic definition but often with significant variations.

Independent of cell heterogeneity, it would appear from *in vitro* results that MSCs share with other stromal cells an ability to inhibit lymphocyte proliferation. In fact in one study the most potent anti-proliferative stromal cell was the fully differentiated human chondrocyte when compared with skin fibroblasts, bone marrow derived MSCs and dedifferentiated chondrocytes [27].

Another important issue concerning application of MSCs to human AD is the choice of autologous versus allogeneic cell products. *In vitro* data suggest that MSCs derived from patients with AD are not normal with respect to certain specific functions. In rheumatoid arthritis patients, bone marrow derived MSCs showed defective hematopoietic support function [28], as they did in scleroderma patients in whom also MSCs had impaired differentiation potential and early senescence as well [29]. However, several groups have confirmed that the *in vitro* anti-proliferative potential of MSCs derived from AD patients is equal to that of healthy controls over a wide range of AD including RA, Sjögren's disease, SLE [30], scleroderma [31] and Crohn's disease [32].

However, the choice of autologous versus allogeneic may also be influenced by pragmatic issues such as availability of a standardized, approved product (allogeneic from bone marrow, fat or umbilical cord/ placenta) versus autologous requiring designated GMP facilities and subject to inter-individual variability of product phenotype [15].

3.3.1 Animal models

The clinical impact of any intervention in autoimmune disease does not automatically imply an immunosuppressive effect. Many manifestations of autoimmune diseases may relate to vascular, inflammatory or fibrotic complications of uncontrolled autoimmune processes. A typical example is the use of endothelin receptor antagonists in scleroderma vascular manifestations. Therefore models of AD should also include tissue injury models as well.

An immunosuppressive effect of MSCs *in vivo* was first suggested in a baboon model, where infusion of *ex vivo* expanded donor or third-party MSCs delayed the time to rejection of MHC incompatible skin grafts [33]. MSCs also downregulated bleomycin induced lung inflammation and fibrosis in murine models, if given early (but not late) after disease induction [34]. This effect was achieved through the reduction of inflammation mediated by IL-1R antagonist secreted by MSCs and capable of antagonizing IL-1a secreting T cells and TNF-a producing macrophages [35]. Similar results were obtained by the infusion of MSCs in an acute lung injury murine model leading to a decreased production of pro-inflammatory cytokines and increased levels of IL-10 [36] and in a murine hepatic fibrosis model (carbon tetrachloride induced) using an MSC line bearing the fetal liver kinase-1 (FLK1) marker [37]. In all these studies, the protective effect of MSCs on lung cells occurred despite limited levels of engraftment in the target organ or transdifferentiation.

Similarly, it was shown that MSCs-derived conditioned medium is enriched with many chemokines able to reverse fulminant hepatic failure through the inhibition of liver infiltration by leukocytes and subsequent death of hepatocytes [38]. Tissue protective effects were also seen in a rat kidney model of ischemia/ reperfusion injury in which syngeneic MSCs were used. Fibroblasts alone did not do this. These effects were not mediated by MSCs' transdifferentiation but, in contrast, by bystander mechanisms including the inhibition of pro-inflammatory cytokines and an anti-apoptotic effect on target cells [39]. In another study it was demonstrated that the reno-protective effect of MSCs was mediated by the mitogenic and pro-survival insulin growth factor-1 (IGF-1) produced by the MSCs [40]. Recently a vasculotropic effect of infused MSCs in the kidney [41] was shown which could be relevant to SSc. It is important to remember that the final phenotype of autoimmune disease expression is mostly a combination of immune mediated inflammation, vascular occlusion and fibrosis, all potentially modulated by MSCs.

Evidence supporting the paracrine hypothesis for MSCs-mediated effect on target tissues was provided also by studies demonstrating that MSCs modified with the pro-survival gene Akt1 can rescue ischemic cardiomyocytes and restore ventricular function [42]. Restoration of retinal function and substantial delay of retinal degeneration through inhibition of photoreceptor apoptosis was obtained through subretinal transplantation of MSCs [43]. Inhibition of apoptosis, prolonged survival and proangiogenic effects were also detected in hypoxic endothelial cells upon exposure to IL-6

and VEGF rich MSCs-derived conditioned medium [44]. Neuroprotective effects were observed also by MSCs infusion in animal models of stroke [45]. Cerebral ischemia is often a major issue in severe autoimmune diseases such as SLE and vasculitis. It is noteworthy that common paracrine mechanisms independent from transdifferentiation appear to support the therapeutic plasticity of MSCs for a wide range of experimental diseases.

Based on their immunomodulatory features together with tissue-protective properties and, possibly, some capacity of transdifferentiating, MSCs represented a seemingly ideal strategy to treat autoimmune disorders. Experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS), was the first AD model in which the therapeutic potential of MSCs was addressed. The intravenous administration of syngeneic MSCs resulted in both clinical and histological improvement. The response was dependent on time of MSCs treatment, the earlier the better, and was associated with the induction of tolerance toward the immunizing myelin antigen MOG (myelin oligodendrocyte glycoprotein) [7]. In another paper, similar amelioration was obtained with human MSCs in a PLP (proteolipid protein)-induced model of EAE that showed engraftment of MSCs in mouse CNS but with limited evidence of transdifferentiation into neural cells [46]. Several other studies confirmed the beneficial effect of MSCs injected systemically [47, 48], intraperitoneally [49] or locally inside the CNS [48] in EAE murine models, suggesting that MSCs not only exert a potent inhibition of the autoimmune attack to the CNS but are also endowed with significant neuroprotective effects despite limited evidence of CNS infiltration [50]. It is noteworthy to emphasize that also neural stem cells [51] and, more recently, human embryonic stem cell derived neuronal precursor cells [52], displayed a striking beneficial effect upon administration in EAE-affected mice through bystander mechanisms leading to immunomodulation of autoreactivity and neuroprotection.

Similar effects have been observed in experimental models of rheumatoid arthritis (RA) where MSCs alone [53, 54] or genetically modified overexpressing IL-10 [55] prevented tissue destruction and suppressed the autoimmune response against collagen II. However not all models were positive, possibly explained by the use of the murine MSCs cell line C3H10T½ (C3) [9].

In an experimental model of diabetes induced in mice by streptozotocin it was observed that MSCs promote endogenous repair of pancreatic islets and renal glomeruli [56]. Similarly, co-infusion of MSCs and bone marrow cells, following sublethal irradiation, inhibited proliferation of pancreatic β-cell-specific T cells isolated from the pancreas of diabetic mice and restored insulin and glucose levels through the induction of regeneration of recipient derived pancreatic β-cells in the absence of transdifferentiation [57]. The immunosuppressive effect of MSCs on T cells was exploited also in a multi-organ autoimmunity mouse model where MSCs homed to the mesenteric lymph nodes significantly improving the autoimmune enteropathy [58].

However, despite the plethora of animal model data (over 600 publications in Pubmed in 2012 alone), the clinical utility of MSCs in autoimmune disease will only be

clarified by well-designed prospective, randomized and where possible also double-blind clinical trials.

3.4 Results of MSCs clinical trials

The initial acute GvHD case published in The Lancet [6] triggered enthusiastic clinical application of MSCs in other situations of tissue injury and inflammation such as AD. A recent European review of noncellular therapy (excluding conventional HSC transplants) showed that in 1,010 surveyed cases 11 % were for AD, 55 % being autologous (Fig. 3.2) [59].

In addition, in *clinical trials.gov*, over 280 clinical trials involving MSCs are registered, of which around 50 target various AD, mostly diabetes mellitus (n=23), MS (n=11) and Crohn's disease (n=11). However, solid published data are few, being mainly case reports and small series (Tab. 3.1). It is difficult to interpret these results due to a large heterogeneity of MSCs sources, doses, routes of delivery, AD types, outcome measures assessed and length of follow-up.

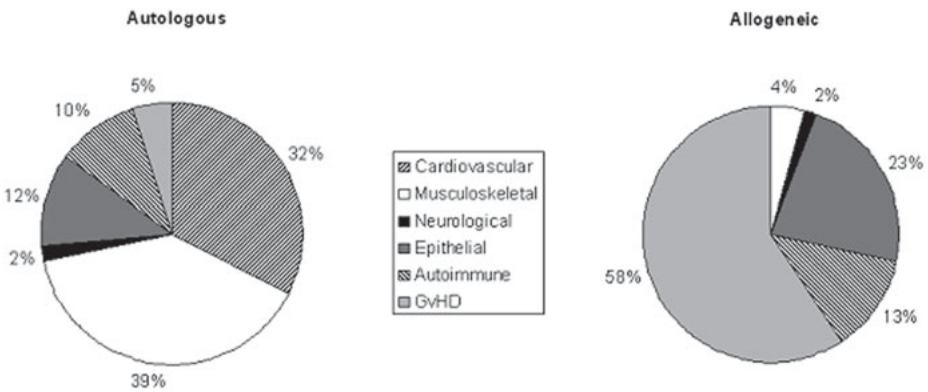

Fig. 3.2: Application of cellular and engineered tissue therapy in Europe 2010 (excluding HSC transplantation for conventional indications). From: Martin et al. The survey on cellular and engineered tissue therapies in Europe in 2010. Tissue Engineering Part A 2012; 18: 2268–79. By courtesy).

In many of the case series, other potentially immune modulating agents such as glucocorticoids and / or cyclophosphamide were given at the same time as the MSCs, confusing the interpretation of the outcome. In others, it is impossible from the data presented to determine if conventional therapy had been exhausted before application of the experimental MSCs treatment.

However, the major confounding factor is the heterogeneity of the clinical manifestation and pathophysiology of the AD in the patient groups tested *i.e.* ischemia *versus*

inflammatory *versus* fibrotic features. The only way to clarify this will be through adequately powered prospective randomized clinical trials, ideally also double blinded. In addition, patient selection should be as homogeneous as possible, given the wide spectrum of clinical manifestations in diseases such as SLE and scleroderma. Two such trials, acute GvHD [60] and Crohn's disease, failed to reach their primary endpoints but were never published. However, in the acute GvHD glucocorticoid unresponsive subgroup with combined liver, skin and gastrointestinal manifestations, significant responses were seen. In the Crohn's disease protocol the company Osiris considered that the failure to reach the primary endpoint could have been due to study "design flaw", especially relating to patient-based subjective outcome parameters. High expectations of stem cell therapy unconsciously may bias reporting. With approval from the FDA, the study was reopened for further enrolment (reviewed in [61]).

3.5 Safety of MSCs

So far no treatment-related deaths have been directly attributed to MSCs toxicity in humans in published clinical studies or registry data. Potential reduced tumor surveillance has been suggested by some murine models of melanoma [62], and recruitment of MSCs from bone marrow and fat to enhance the tumor "niche" [63]. In addition, MSCs have been seen to mutate into sarcoma cells in a murine model [20]. Recent concern over aneuploidy karyotypic changes in cultured MSCs which had already been infused into humans in an acute GvHD study were later attributed to donor related factors, rather than culture induced changes [64]. Cell culture contamination may also give false positive results for apparent malignant transformation in cultures of MSCs [65]. However, one proven case of malignant transformation of donor neural stem cells in a case of ataxia telangiectasia [66] is a reminder that stem and progenitor cell therapy is a complex and poorly understood new therapeutic area requiring high-quality clinical studies and long-term well-maintained patent registries to understand the true "benefit / risk" of the therapy.

3.6 Conclusion

MSCs have been the focus of intense basic and clinical research over the past decade because of their easy availability, apparent low acute toxicity and their pleiomorphic anti-inflammatory, antiproliferative and tissue protective properties.

It seems that a short-term "hit -and-run" effect is the most likely mode of action, rather than a long-term engraftment and tissue differentiation.

Despite copious *in vitro* and animal model work supporting their potential for treating disease, prospective randomized and ideally double-blind clinical trials are needed to confirm their efficacy.

A certain reporting bias of positive results exists, not only relating to *in vitro* and animal model studies, but also the uncontrolled clinical trials and case reports. In a center capable of treating over 100 SLE patients, one must ask why the early encouraging results and low acute toxicity has not led yet to definitive randomized studies.

In addition, although acute toxicity appears to be minimal, long-term follow-up is required, especially relating to tumor surveillance and ectopic tissue formation. Large prospective registries and interdisciplinary collaboration are to be encouraged.

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Andreas Kurtz and Su-Jun Oh

4 Mesenchymal stem cells in osteoarthritis and rheumatic disease

Abstract Rheumatic diseases are a formative and increasing health burden causing morbidity and severe pain in musculoskeletal body parts. Rheumatoid arthritis and osteoarthritis are rheumatic diseases affecting the joints through different pathologic mechanisms. In rheumatoid arthritis (RA), autoimmunity towards cartilagieous components causes local inflammation and subsequent severe joint damage, while in osteoarthritis (OA), wear and tear of the joints leads to destruction of the articular cartilage with secondary inflammation. Mesenchymal stem cells (MSCs) have several features that are potentially beneficial in RA and OA and that have been explored in clinical studies in animals and humans. The immune modulating properties may slow disease progression in RA by suppressing autoimmune responses and reduction of inflammation. The capability of MSCs to differentiate into chondrocytes and bone together with the abilities to produce extracellular matrix and to modulate the local microenvironment can be explored in both RA and OA for the repair of damaged cartilage. Moreover, resident MSCs in joint tissues participate in the maintenance of tissue homeostasis. In this chapter, the state of use of MSCs for the treatment of rheumatic joint diseases is described and possible future directions towards to full exploitation of MSCs for the treatment of RA and OA are developed.

4.1 Introduction – Rheumatic diseases

Rheumatism or rheumatic diseases are nonspecific terms for medical conditions affecting the joints and connective tissues. The more than 100 rheumatic diseases are currently recognized include ankylosing spondylitis, pain in several musculoskeletal body parts such as the back, neck, shoulders, wrists, legs, ankles and hip, tendinitis, capsulitis, rheumatoid arthritis and osteoarthritis. Although these common disorders differ epidemiologically, they are all characterized by chronic-intermittent pain and difficult to treat. Here, we will focus on rheumatoid arthritis and osteoarthritis, the two most common rheumatic diseases, which are also most intensively studied regarding therapeutic intervention using MSCs.

4.2 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) was introduced already in the 1850s [1]. It is a serious long-term disease with dominant extra-articular features, limited treatment options, and poor outcomes. Rheumatoid arthritis most often strikes between ages 30 and 40, and

50 % of the risk for development of rheumatoid arthritis is attributable to genetic factors. Smoking is the main environmental risk. In industrialized countries, rheumatoid arthritis affects 0.5–1.0 % of adults, with 5–50 per 100 000 new cases annually. About 75 % of those affected are women. Uncontrolled active rheumatoid arthritis causes joint damage primarily of the small diarthrodial joints of the hands and feet, disability, decreased quality of life, and cardiovascular and other comorbidities. Rheumatoid arthritis is characterized by chronic systemic inflammation and autoantibodies. It presents with persistent largely T cell mediated synovial inflammation and associated destruction of the joints, damage to the synovial membrane and underlying cartilage and bone. In the initial stages of RA, autoimmunity is induced to collagen-rich joint components followed by the evolution of a destructive inflammatory process [2–4]. Auto-antibodies are commonly produced against rheumatoid factor and anti-cyclic citrullinated peptide antibody. Progression of the autoimmune response leads to aberrant infiltration of Th1 and Th17 helper cells into the joint tissues [5–7], the release of proinflammatory cytokines and chemokines, which promote infiltration of macrophages, neutrophils and fibroblast-like synoviocytes (FLSs) resulting in the expression of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-17 and IL-1 as well as destructive enzymes including matrix metalloproteinases (MMPs) [8, 13]. These inflammatory changes of the joint milieu eventually result in destruction of joints [2, 8]. Furthermore, reduced numbers and function or regulatory T cells contribute in the immune imbalance in RA [9–12].

High levels of these proinflammatory cytokines are present in the synovial fluid and the serum of patients with rheumatoid arthritis. The observation that these cytokines play a role in the pathogenesis of this disease has led to the use of highly effective biologic therapies for RA, for instance, TNF-α inhibitors and B-cell-depleting therapies. The current pharmacological management of rheumatoid arthritis involves early intervention with synthetic disease modifying anti-rheumatic drugs (DMARDs). If inflammation cannot be adequately suppressed by these means, biologic DMARDs targeting the proinflammatory cytokine TNF-α are employed or alternatively, biologics of a different mechanism of action class can be used. Despite all those treatments, concerns about side effects and long-term treatments based on biologics exist [14], while there are still approximately 20–40 % of patients with inadequate responses to anti-TNF. Moreover, anti-inflammatory therapies have no apparent effect on the regeneration of cartilage and bone and none reaches long-term drug-free remission. Another potential target for the treatment of inflammatory arthritis are the FLSs, which are resident cells of synovial joints, involved in pannus formation, and are key players in the destruction of cartilage and bone in RA joints [15]. FLSs contribute to disease progression through stimulation of both inflammation and tissue damage [16].

4.3 Osteoarthritis (OA)

Osteoarthritis (OA) is the most prevalent of the chronic diseases affecting the elderly, commonly the result of joint wear and tear, inappropriate loading, trauma, biochemical changes and genetic background [17–23]. Major known risk factors for OA are age, obesity, gender, ethnicity, physical activity, previous joint injury, and genetics [24]. At the age of 65 years, a population majority demonstrates radiographic signs of the disease in at least one joint. All articular joints can be affected but most frequently involved are the knee, hip, hand, spine, and foot, while the wrist, shoulder, and ankle are relatively spared. Its clinical manifestations include joint pain and impairment to movement, affecting surrounding tissues with local inflammation. The affected articular cartilage is a unique avascular, aneural connective tissue at the surface ends of long bones, consisting of chondrocytes, which synthesize, assemble and organize an extensive extracellular matrix [25–28]. The dominant macromolecule of this matrix, aggrecan, is extensively coated with glycosaminoglycan chains. The osmotic gradient formed within the polymer results in a gel-like state, which is kept in its form by a network of collagen type II and enables the tissue to resist compressive loading [29, 30]. Additional ECM molecules include hyaluronan, link protein, small leucine-rich proteoglycans, cell-surface proteoglycans, fibril-associated collagens with interrupted triple helices (FACIT collagens) and glycoproteins. Besides regulating the macromolecular structures, these provide signaling cues for chondrocyte arrangements, activity during development and in the maintenance of tissue homoeostasis [29, 31, 32].

OA is characterized by a disturbance of the homeostasis in articular cartilage, synovial membrane and subchondral bone. Destruction of articular cartilage stimulates synovial lining cells and articular chondrocytes within diseased cartilage to synthesize and secrete proteolytic enzymes: matrix metalloproteinase, aggrecanase, proinflammatory cytokines and mediators such as nitric oxide and prostaglandins, which degrade the cartilaginous matrix [33, 34]. Under physiological conditions, the homoeostasis of healthy articular cartilage and integrity of its extracellular matrix is maintained by a finely tuned dynamic equilibrium of synthesis and degradation. It is maintained by anabolic growth factors and cytokines including transforming growth factors (TGFs) and bone morphogenetic proteins (BMPs), and catabolic cytokines such as interleukins and TNF-α. In addition, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) act downstream of gene activation to modulate protein levels within the matrix [17–23]. Imbalances between synthesis and degradation cause progressive loss of the macromolecular matrix components and the degradation of the cartilage tissue. The loss of cell-matrix-mediated signal transduction leads to an increase in cellular proliferation and reversion to synthesis of epiphyseal cartilage akin to endochondral ossification. With continued pathological assault, catabolic factors dominate the anabolic mechanisms and the matrix functionally deteriorates [19, 35, 36].

Although the main pathologic feature of OA is loss of articular cartilage, the involvement of other joint tissues makes it a disease of the whole joint [37]. Joints with OA show new formation of cartilage and bone on the joint margins (osteophytes), sclerosis of the subchondral bone, and in most patients bone marrow lesions and synovial activation. The two latter structural changes are correlated with the severity of symptoms [38–41]. Osteoarthritis in the knees can be caused by meniscal defects. The synovium plays pivotal roles during the natural course of meniscal healing and contains MSCs with higher ability of proliferation and superiority in chondrogenesis than MSCs from other sources [42, 43].

One of the major problems of damaged cartilage is its inability to repair itself, even when the underlying disease process is eliminated. In the rare cases of spontaneous healing of articular cartilage, a long-lasting functional regeneration of the joint is likely never achieved [44]. The low capacity to self-repair following injury is a consequence of the avascular nature of the articular cartilage and low proliferative capacity of chondrocytes [45, 46]. Adequate therapies to treat OA with relief of symptoms and joint regeneration are not yet available. Treatment of pain with Duloxetine, the most studied serotonin–norepinephrine reuptake inhibitor class of drugs with regard to osteoarthritis is either provided alone or added to therapy with background nonsteroidal anti-inflammatory drugs. Biological treatments include inhibition of IL-1, which stimulates production of matrix metalloproteases and reduces production of aggrecan and other matrix constituents. Blockage of TNF-α evidently showed no conclusive effect [47, 48], and application of monoclonal antibodies against nerve growth factor (NGF) showed in preliminary studies only efficacy in pain management.

The ultimate therapeutic solution currently is joint arthroplasty, accompanied by systemic and local analgesic treatment. Both symptoms and structural damage are treated by so-called disease modifying OA drugs (DMOADs), such as strontium ranelate, hyaluronic acid and glucosamine, providing some symptomatic relief but unable to restore joint function [49]. Strontium ranelate has been shown to not only reduce bone turnover, but to stimulate cartilage formation *in vitro* [50, 51]. Intra-articular injection of fibroblast growth factor-18 (FGF-18) and bone morphogenic protein-7 (BMP-7) [52], and of autologous platelet-rich plasma are currently being tested in OA. FGF-18 and BMP-7 in animal models of osteoarthritis showed increased chondrogenesis and cartilage repair after intra-articular injection [53]. Surgical procedures to stimulate self-repair of the joint by Pridie drilling or microfractures, or to reconstruct it using biological autografts for mosaicplasty are mostly effective for focal cartilage defects and show limited benefit in OA. Arthroscopic drilling and abrasion of the articular surface to penetrate the vascularized subchondral bone leads to infiltration with undifferentiated mesenchymal cells that eventually differentiate and synthesize a fibrocartilage tissue. Whilst temporarily offering some functional respite, with greatest efficacy seen with smaller-sized defects, the tissue is biomechanically suboptimal and eventually fails [54, 55]. Finally, autologeous chondrocyte transplantation (ACI) is frequently used on OA with best results in focal lesions [56].

4.4 MSCs in healthy and rheumatic joint tissues

MSCs, which are present as resident cells in healthy joint cartilage, are most likely involved in maintaining tissue homeostasis [57, 58]. These resident MSCs have been isolated in various periarticular tissues including synovium, synovial fluid, cartilage, intra-articular fat and periosteum [57, 59–62]. In arthritic joints in RA and OA, the number and characteristics of MSCs change in synovial fluid and in articular cartilage, perhaps as a result of increased TNF-α and IFN-γ. Increased numbers and changed characteristics may reflect a role for MSCs in re-establishment of homeostasis and joint regeneration [58, 59, 63]. MSCs cultured from arthritic synovial fluid are likely derived from damaged joint structures and show the same phenotype as bone marrow-derived MSCs. Similarly, MSCs isolated from articular cartilage of osteoarthritic patients are increased in numbers compared with cartilage from normal subjects, with similar characteristics including chondrogenic differentiation potential [58, 64]. This finding supports the potential for intrinsic cartilage repair in diseased joints and a possible role for MSCs in the pathophysiology of osteoarthritis. Moreover, chondrogenic progenitor cells migrate to cartilaginous defects in diseased joints at late stages of arthritis to form reparative tissue [65]. Some studies suggest that the chondrogenic and adipogenic differentiation potential of MSCs isolated from tissues in osteoarthritic joints may be reduced, but not the osteogenic potential [66]. This altered differentiation potential may reflect disturbed homeostasis and in turn account for the increased bone density and generalized loss of cartilage in OA. Other authors have demonstrated arthritis-associated changes in the characteristics of MSCs populations in the bone marrow [67], raising questions as to whether the bone marrow niche may have a more significant role in the pathophysiology of joint disorders. The presence and differentiation potential of MSCs in osteoarthritic joints with its potential function in maintaining homeostasis and promoting repair is another major justification for the use of MSCs in arthritic rheumatic disease.

In RA, progressive joint destruction is mediated by T cells and fibroblast-like synoviocytes (FLSs) with relevant contribution to pathogenesis from the bone marrow's abnormal hemopoietic and stromal cells in early disease. Reduced hematopoietic activity and stromal cell function in RA derived bone marrow is accompanied by elevated TNF-α secretion by stromal cells. Stromal and hemopoietic function was restored following TNF-α suppression [68]. MSCs from bone marrow migrate to the joint space environment prior to the onset of acute inflammation in a mouse model of RA, which also appears to be TNF-α dependent. Prevention of this migration by TNF-α suppression delayed the onset of arthritis [69]. Supporting this pathogenic model are the increased numbers of progenitor cells within the bone marrow microenvironment at the onset of disease [67]. The reduced osteoblastic differentiation potential of these cells was associated with periarticular bone loss. Hence, alterations in features of bone marrow-derived stroma cells may also promote the subsequent osteopenia in established RA. This progressive osteopenia and bone loss

is accompanied by enhanced osteoclastic activity in juxta-articular skeletal tissues. Bone marrow-derived and intra-articular adipose tissue derived MSCs from OA and RA patients are of comparable phenotype, with similar osteoblastic differentiation capacities [70, 71]. MSCs derived from intra-articular adipose tissue demonstrated similar phenotype in both inflammatory and degenerate joints with effective chondrogenesis and matrix production capacities. These characteristics were inhibited by exposure to TNF-α [70].

4.5 Application of MSCs in rheumatic diseases

Marrow nucleated cells are being used routinely in regenerative orthopedics [72]. The knee microfracture surgery [73] is based on releasing marrow cells into cartilage lesions to initiate fibrocartilage repair in osteochondral defects. Although [73] the MSCs proportion in these marrow preparations is low, between 1 in 10,000–1,000,000 of the nucleated cells [74], bone marrow nucleated cells implanted into degenerated human peripheral joints have shown some promise for joint repair [75]. Culture expansion of MSCs allows the provision of defined and preconditioned therapeutic cell populations to the joints [76, 77]. Expanded MSCs are usually transferred with growth factors to allow for continued survival and engraftment to the damaged tissue, either as cell suspensions [78] or within supportive bioscaffolds [79–81].

MSCs appear to be specifically suited for the treatments of complex joint tissue defects as they are highly expandable while maintaining high plasticity and differentiation potential into a variety of connective tissues including cartilage, bone, fat, tendon, ligament and marrow stroma [61, 82–86] (Fig. 4.1). Similarly, MSCs were shown to inhibit proliferation of fibroblast-like synoviocytes [106]. Moreover, MSCs have immunomodulatory and anti-inflammatory properties that make them especially applicable in the therapy of autoimmune diseases through their inhibition of cytotoxic T cell proliferation. Similarly, MSCs were shown to inhibit proliferation of fibroblast-like synoviocytes [106]. In addition, MSCs show low immunogenicity due to low levels of MHC and costimulatory molecules together with expression of molecules associated with immune privilege, which justifies allogeneic application. Interestingly, one study showed that the immune-related molecules B7-H3/CD276 and HLA-E, which are expressed in undifferentiated MSCs, remain expressed in the differentiated progeny [87] of umbilical cord-derived MSCs (UCMSCs). This suggests that also after the acquisition of a mature phenotype, MSCs-derived chondrocytes, adipocytes and osteocytes maintain their immune privilege. Finally, MSCs are efficient producers of extracellular matrix and modulators of the local microenvironment, which may support the repair of tissue damage, migration and differentiation of tissue specific cells. Their responsiveness to chemotactic cues and their mobility allow the cells to migrate to diseased organs where they may serve as vehicles presenting or locally releasing proteins with therapeutic effects [88].

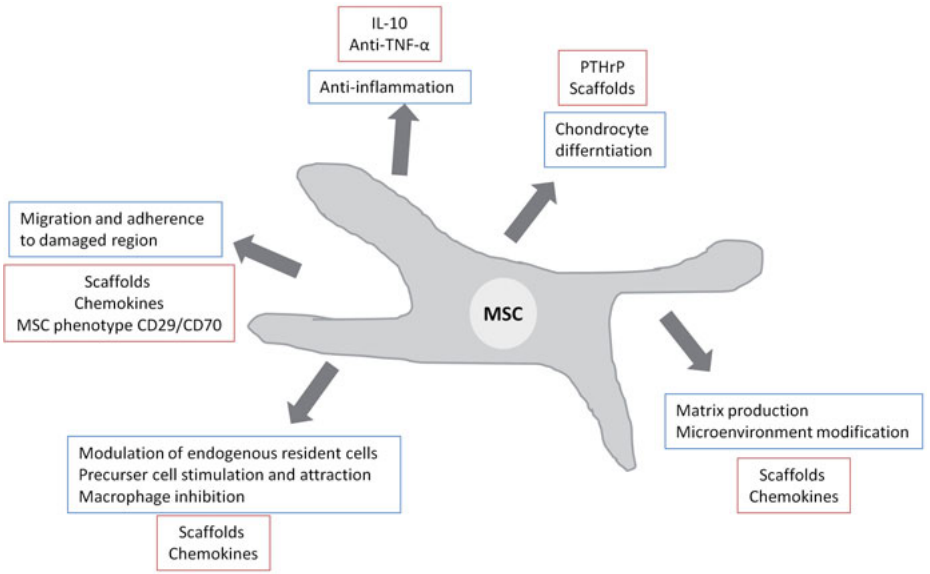

Fig. 4.1: Potential characteristics of MSCs that can be explored for tissue regeneration and treatment of rheumatic joint diseases. Possible means that are currently explored to improve these functions are provided (red boxes).

Pittenger [86] demonstrated the multipotent differentiation of MSCs into three cell lineages of adipocytes, osteoblasts and chondrocytes, making these cells particularly interesting for joint cartilage and bone regeneration. In contrast to mature chondrocytes, MSCs are easily expandable, making them an attractive cell source for autologous and allogeneic cell therapies [89, 90]. Upon differentiation into prechondrocytes and the subsequent production of cartilage-specific ECM molecules such as aggrecan and collagen type II, the MSCs obtain a rounded cellular morphology characteristic of chondrocytes [72, 91]. A possible obstacle for the use of *in vitro* expanded MSCs has been posited because *in vitro* chondrogenesis of MSCs follows a rapid differentiation program analogous to that seen during fracture callus wound healing [72, 92]. The production of soluble factors by articular chondrocytes *in vivo* may decrease hypertrophic differentiation of transplanted MSCs as shown in *in vitro* co-culture experiments, which identified parathyroid hormone-related protein (PTHrP) as a possible signaling molecule [93, 94]. Prevention of endochondral ossification by transplanted chondrogenic MSCs may be possible by using or inducing these factors in a therapeutic setting.

Table 4.1: Experimental animal studies using MSCs for RA and OA models.

Species	MSC	Treatment	Follow-up	Results	Reference
Caprine	BMMSC	OA, intra-articular (i.a.) MSC	–	Improvement	[107]
Donkey	BMMSC	OA, i.a. MSC + HA	6 months	Improvement	[33]
Cynomolgus	BMMSC	OA, i.a. MSC + acellular dermal matrix	24 weeks	Improvement	[108]
	BMMSC	OA, i.a. clonal chondrogenic MSC / MSC	12 weeks	chMSC>MSC	[109]
Porcine	BMMSC	OA, i.a. MSC+ PLA coated PGA +dexamethasone implant	6 months	Improvement	[110]
	BMMSC	OA, i.a. MSC + PCL electrospun nanofibers	6 months	Improvement	[111]
	BMMSC	OA, i.a. MSC + HA, 3xweekly intervals	12 months	Improvement	[112]
Dog	ADMSC	OA, i.a. MSC	6 months	Improvement	[104]
Rabbit	BMMSC	OA, i.a. MSC + HA implant	12 weeks	Improvement	[113]
	BMMSC	OA, i.a. MSC + OPF with GMP	12 weeks	Improvement	[114]
	BMMSC, hUCB stem cells	OA, i.a. MSC +PLA (i.a.)	–	Improvement	[115]
	BMMSC	OA, i.a. MSC	12 weeks	Improvement	[116]
	BMMSC	OA, i.a. MSC collagen gel implant	–	Improvement	[117]
	BMMSC	OA, i.a. MSC + HA + gelatin	4 months	Improvement	[118]
	BMMSC	OA, i.a. MSC + HA + gelatin	12 weeks	Improvement	[119]
	BMMSC	OA, i.a. alginate encapsulated MSC	6 months	Cell-independent improvement	[120]
	BMMSC, ADMSC, syMSC, muscle-derived MSC	OA, i.a. MSC + collagen gel implant	12 weeks	Improvement	[121]
	BMMSC	OA, i.a. MSC + PCL	6 months	Improvement	[122]
	UCMSC, BBMSC, fibroblasts	OA, i.a. cells + PLA	12 weeks	MSC/PLA>MSC> fibroblasts	[115]

	ADMSC	OA, i.a. MSC + PLGA + chitosan	12 weeks	Improvement	[123]
	ADMSC	OA, i.a. MSC	12 weeks	Protective	[124]
	Synovial MSC	OA, trochlear groove MSC injection	24 weeks	Improvement, cell attachment	[125]
	Infrapatellar MSC	OA, i.a. MSC	20 weeks	Improvement	[126]
Mouse	Lapine BMMSC	OA, i.a. MSC + fibrin hydrogel	4 weeks	Improvement	[127]
	ADMSC	RA, i.v. 3x MSC	42 days	Improvement	[128]
	BBMSC (IL-10 transduced)	RA, systemic weekly intravenous (i.v.)	6 weeks	Suppress immune response	[129]
	BMMSC	RA, intraperitoneal (i.p.)	42 days	Improvement	[97]
	BMMSC	RA, systemic, i.v. + TNF-α	–	No benefit	[130]
	Human UCMSC	RA, i.a. MSC +/- TNF-α	28 days	No benefit for MSC alone	[131]
	Human gingival MSC	RA, systemic i.v.	60 days	Improvement	[101]
	BMMSC	OA, i.a. MSC + HA/PEG +/–TGF-β3	2 weeks	HA>PEG	[132]
	ADMSC	OA, i.a. MSC	8 weeks	Macrophage interaction	[133]
	Goat BMSC	OA, i.a. MSC + PEODA		Improvement	[134]
Mouse (SCID)	Human BMMSC	OA, i.a. MSC	4 weeks	Improvement	[135]
	Human BMMSC	OA, i.a. MSC cultured on FM coated TGF-β3 releasing PGLA microspheres	3 weeks	Improvement	[136]
Rat	Murine BMMSC	OA, i.a. MSC + PLGA/ NHA	12 weeks	Improvement	[137]
	Synovial MSC	OA, i.a. MSC	12 weeks	Improvement	[138]
Guinea pigs	BMMSC	OA, i.a. MSC + HA / HA	5 weeks	MSC+HA>HA	[139]

Abbreviations: ADMSCs (adipose-derived MSCs), BBMSCs (blood and bone marrow-derived MSCs), BMMSCs (bone marrow-derived MSCs), FM (fibronectin matrix), GMP (gel microparticles), HA (hyaluronic acid), hUCB stem cells (human-derived umbilical cord blood stem cells), NHA (nano-hydroxyapatite), OA (osteoarthritis), OPF (Oligo(polyethylene glycol) fumarate), PCL (polycaprolactone), PEG (plyethyleneglycol), PEODA (Photopolymerizablepoly(ethyleneoxide)diacrylate), PGA (polyglycolicacid), PLA (polyactic acid), PLGA (poly-lactide-coglycolide), RA (rheumatoid arthritis), syMSCs (synovium-derived MSCs).

4.6 MSCs application in animals

The application of MSCs in rheumatic diseases has been studied extensively in animal models with encouraging results [4, 95–100]. Based on these, MSCs derived from bone marrow, adipose tissues, synovium, infrapatellar fat pad, gingival, umbilical cord tissue and blood were used for clinical studies in patients with rheumatoid arthritis, osteoarthritis, systemic sclerosis, inflammatory myopathies and others [95, 101] (Tab. 4.1). In addition, applications of mainly adipose-derived MSCs (ADMSCs) are routinely applied in dogs and horses with rheumatoid arthritis and osteoarthritis [102–105].

To date, experience with the use of MSCs in the treatment of RA is limited to a few cases, with controversial results from preclinical models [4, 96–99, 106, 107]. In an inflammatory mouse model of collagen induced arthritis (CIA), single dose systemic bone marrow-derived MSCs (BMMSCs) injection demonstrated reduced T cell responses, prevented the occurrence of severe joint damage and decreased the serum concentration of TNF-α, but MSCs were not detectable in the treated joints. However, no benefit and reversal of the immunosuppressive properties of BMMSCs by TNF-α supplementation was also shown [96, 97]. In another study, no clinical or histological benefit was seen after injection of human umbilical cord-derived MSCs (UCMSCs) into the articular cartilage in a mouse model of collagen-induced arthritis. In contrast, progression of arthritis after MSCs injection was accelerated in the presence of TNF-α. Co-injection of UBMSCs and TNF-α inhibitor reduced the disease symptoms and decreased expression of CD90, HLA-G, and IL-10 in MSCs, indicating that inhibition of TNF-α decreases cartilage destruction by modulating the immunogenicity of MSCs and making co-injection a potentially more effective therapy for ameliorating the disease [107]. UCMSCs were also used in a rat model of adjuvant RA and compared with umbilical cord-derived hematopoietic stem cells. At 34 days post cell injection, the clinical signs improved most significantly in the MSCs group, associated with reduced TNF-α, IL-1 and IFN-γ serum levels, increased IL-10 expression, lower joint inflammation and extensive fibrosis [108].

A protective and therapeutic role for human adipose-derived MSCs (ADMSCs) was shown in a murine CIA model [109]. Here, the incidence and severity of arthritis was reduced through the inhibition of the production of proinflammatory mediators, increased production of anti-inflammatory cytokine IL-10 and reducing antigen-specific T cell expansion. Another study used a murine CIA model for treatment with systemic weekly infusions of murine BMMSCs transduced to express increased IL-10, or with unmodified MSCs. A more pronounced decrease in serum IL-6 and anti-collagen II antibodies, and an overall T cell proliferative response inhibition was observed in transduced MSCs as compared to unmodified MSCs [110], as MSCs suppressed immune responses in autoimmune arthritis as well as modulate cytokine production.

There is evidence that ADMSCs and synovial-derived MSCs (syMSCs) may represent a more reliable and potent source of cells for application in RA and OA, although

the relative efficacy of different sources of cells cannot be assessed from the available data [90]. Aspects for determining therapeutic cell sources include accessibility and clinical feasibility, which promoted the search for alternatives to BMMSCs. Synovial autologous MSCs have several advantages over other MSCs, including superior chondrocytic differentiation potential [111, 112]. In one study, gingival-derived mesenchymal stem cells (GMSCs) were injected systemically in a CIA mouse model resulting in a significant decrease in the severity of arthritis and pathology scores, and downregulated inflammatory cytokines IFN-γ and IL-17A. Infusion of GMSCs resulted in an increase in regulatory T cells early in spleen and LN and later in synovial fluid. When $FOXP3^+$ Tregs were depleted, the GMSCs effect was moderately reversed while pretreatment of GMSCs with CD39 or CD73 inhibitors significantly reversed the protective effect of GMSCs, indicating that the MSCs phenotype is relevant for the therapeutic effects [100].

Despite the positive experimental data reported in some animal models of inflammatory joint disease, it is unclear whether success with some of these treatment models for discreet cartilage defects involves chondrogenic differentiation of progenitor cells.

In OA, MSCs should be advantageous for joint repair by generating new cartilage, by releasing factors that stimulate cartilage formation by resident chondrocytes or other cells in the joint, and by inhibition of joint inflammation (Fig. 41). The first reported application of MSCs in OA was in a caprine menisectomy model [115]. Stifle joints with induced OA that were treated with MSCs showed regeneration of meniscal tissue and diminished articular cartilage damage. The MSCs were detectable in the soft tissues of the joints but not in articular cartilage, making it unlikely that these cells contributed directly to cartilage maintenance or repair. On the other hand, MSCs release a diversity of cytokines and growth factors, which stimulate chondrocyte proliferation and matrix synthesis [116]. The infrapatellar fat pad has been tested as a source for therapeutic MSCs in an experimental rabbit model of OA by direct intraarticular injection of a single cell dosage. Radiological assessment confirmed development of OA changes after 20 weeks; rabbits receiving MSCs showed lower degrees of cartilage degeneration, osteophyte formation, and subchondral sclerosis than the control group [113]. Hypertrophic differentiation of transplanted MSCs leading to endochondral ossification is a possible risk for MSCs treatments in rheumatic joint diseases. This risk may be reduced when using MSCs derived from synovial tissue as these may have enhanced chondrogenic potential and reduced tendency to hypertrophic differentiation in comparison with bone marrow-derived MSCs [164, 165]. This restricted potency may be a developmental heritage as initiation of joint formation proceeds from a thin layer of flattened mesenchymal cells, which are morphologically distinct from those in the surrounding mesenchymal condensation. This subset of MSCs proliferates and differentiates into the tissues found within the synovial joint. MSCs directly derived from these tissues provide possibly a higher capacity for chondrogenic repair and reduced ossification potential. This developmental view provides

valuable insights into the generation of chondrocytes from MSCs with a phenotype closest to that of articular cartilage, which would also assemble an ECM with optimal biomechanical properties [72, 166].

Intra-articular injection of synovium MSCs in a rat model of a massive meniscal defect enhanced meniscal regeneration. The injected MSCs adhered to the lesion, differentiated into meniscal cells directly and promoted meniscal regeneration without mobilization to distant organs [117]. The scaffold-free local adherence of transplanted MSCs was studied in another model in which synovial MSCs in suspension were injected on full-thickness osteochondral defect in the trochlear groove of the femurs in rabbits. The result demonstrated that only 10 minutes post transplantation 60 % of the cells adhered to the defect and most attached to the defect at 1 day. The cartilage defect improved at 24 weeks [118]. Remarkably, intra-articular injection of green fluorescent protein (GFP)-labeled BMMSCs plus hyaluronic acid in donkeys with different degrees of induced osteoarthritis also resulted in significant clinical and radiological improvement [33]. Moreover, GFP-labeled cells were found in the surface and interior of the articular cartilage in treated joints, indicating their participation in the reparative process.

Expanded MSCs are polyclonal, showing variable differentiation potential, which possibly results in variable therapeutic capacity and outcomes. A study evaluating the repair potential of selected chondrogenic clonal and nonselected blood and bone marrow-derived MSCs (BBMSCs) by delivering them into the injured cartilage site in an OA model in *Cynomolgus* monkeys demonstrated that the abrasions of articular cartilage and histological scores were significantly improved and repaired at 12 weeks by MSCs-based treatment, particularly in the selected clonal MSCs-treated group [123].

The majority of autologous chondrocyte or MSCs treatments involve direct injection of MSCs or autologous cells into a joint or defect [119]. Most of these studies show retention of only a small portion of injected cells attached to the cartilage defects, integrating and participation in tissue formation. Consequently, observed clinical effects may be mainly due to paracrine conditioning effects.

Whether MSCs protect against cartilage loss or contribute to cartilage repair by trophic factors in an OA joint is not yet clear. Although it is expected that the primary effect of stem cell treatment occurs through tissue-specific differentiation in OA, most data suggest that the therapeutic potential of MSCs is strongly dependent on paracrine effects [157, 158]. Predominantly activated macrophages, which release proinflammatory factors like IL-1, complement factors and damage-associated molecular pattern molecules (DAMPs) in OA associated synovitis and the thickened lining layer [159, 160] may be chronically stimulated by fragments of damaged cartilage. Indeed, selective elimination of synovial macrophages prior to induction of experimental murine OA prevented synovial activation, cartilage destruction and osteophytes [161]. Related to this, adipose-derived MSCs (ADSC) injected in OA rabbit knee joints protected against development of cartilaginous and meniscal damage [157], in line with results from a murine OA model in which injected ADSCs closely

interacted with synovial macrophages and inhibited ligament damage and development of joint destruction at later time points [162]. It remains to be investigated whether MSCs in these settings also stimulate macrophages to produce elevated levels of growth factors.

Furthermore, intra-articular injection of MSCs as a suspension may lead to their attachment to nontarget areas and the delivery of uncommitted MSCs to cartilaginous lesions and result in not reproducibly and satisfactorily regenerated tissue, but rather induces fibro-cartilage formation or endochondral ossification. As most of the intra-articular injected MSCs may adhere to the synovial tissue, this may increase the risk of adverse effects, such as synovial proliferation [118]. The presence of CD73, CD90 and CD29 on the surface of MSCs is considered to be associated with enhanced chondrogenic capacity by mediating cell-cell and cell-matrix interaction [120, 121]. As the expression for example of CD29 in MSCs changes with time of cell cultivation, this parameter may critically impact on adhesion of transplanted MSCs to the cartilage lesion tissue, again indicating phenotype profiles of MSCs that may directly influence therapeutic outcome [121–123].

Since cartilage can be generated by MSCs, attempts to engineer articular cartilage in animal models using chondrocytes in combination with different scaffolds have been made [114]. These newer strategies involve the use of scaffolds to support engraftment and matrix formation of implanted MSCs populations within the cartilage. A longer retention time of transplanted MSCs may also improve conditioning of the local environment, attraction of native progenitors and immunomodulatory efficacy [79, 85, 124–126]. To this end, retention and encapsulation strategies aimed at the phenotypic tailoring and maintenance of MSCs at the site of injury have been proposed [125, 127–133], including functionalizing of scaffold with peptides, antibodies, and the introduction of nanostructured guidance cues, hydrogels such as collagen-based membranes, hyaluronic acid (HA), fibrin, alginate and various synthetic materials such as polylactide-coglycolide (PGLA) fleece [134]. Injection of MSCs together with hyaluronic acid appears to be advantageous. A porcine partial-thickness cartilage defect in the medial femoral condyle was treated by direct intra-articular injection of autologous BMMSCs suspended in HA. MSCs were injected three times at weekly intervals and morphological and histological analysis at 6 and 12 months showed improved cartilage healing [167]. Umbilical cord MSCs (UCMSCs) are of generally higher proliferative capacity and are exposed to fewer environmental and ageing related risk factors than MSCs from adult donors. In a comparative study using chondrocytes, BMMSCs and fibroblasts from rabbit, and human UCBMSCs were embedded in polylactic acid (PLA) matrices [169]. The cell/PLA mixtures were transplanted into full-thickness defects in the femoral trochlear grooves of both knees in rabbits and analyzed 6 weeks and 12 weeks after implantation. The results of this study indicated that full-thickness cartilage defects treated with chondrocyte or autologous BMMSCs transplantation were repaired with hyaline-like cartilage tissue, and repair was significantly better than in tissues treated with fibro-

blasts and human UBMSCs. It was also indicated that repaired tissues treated with MSCs appeared to have better cell arrangement, subchondral bone remodeling, and integration with surrounding cartilage than did repaired tissues generated by chondrocyte implantation. The synthetic polypeptide, water-soluble poly(L-glutamic acid)(PLGA) together with chitosan (CHI) has been used to realize a biocompatible and biodegradable cartilaginous scaffold for autologous ADMSCs for chondrogenic induction *in vitro*. Transplantation of the ADMSCs scaffold constructs to repair full-thickness articular cartilage defects in the rabbit femur trochlea resulted in coverage of articular defects with newly-formed cartilage and integration of the regenerated cartilage with surrounding native cartilage and subchondral bone at 12 weeks post-implantation. The newly generated cartilage showed similar extracellular matrix deposition, glucoseaminoglycan, type II collagen and biomechanical properties as in the native one at 12 weeks post-implantation [135].

Hydrogels form a network of water-insoluble super-absorbent polymer chains that are utilized as vehicles for cell delivery and encapsulation for cartilage repair. HA is also a major component of the cartilaginous extracellular matrix and additionally supports chondrogenic differentiation. Intra-articular transplantation of human BMMSCs suspended in hyaluronic acid in the knee joints of spontaneous osteoarthritis in guinea pigs: at 5 weeks post transplantation, partial cartilage repair was noted in the HA-MSCs group but not in the HA alone group. In this study, migration, differentiation, and proliferation of MSCs in the HA-MSCs group was demonstrated together with type II collagen accumulation around both residual chondrocytes and transplanted MSCs in the OA cartilage [168]. The assessment of BMMSCs encapsulated in HA or HA-gelatin-based scaffold demonstrated formation of repair tissue in a rabbit model [136–138]. The additional supplementation of hydrogels transforming growth factor- β3 (TGF-β3) further increased collagen type II and aggrecan deposition [139, 140]. The efficacy of MSC-containing fibrin hydrogels to repair cartilage has been investigated using several animal models and BMMSCs. Interestingly, the repair of equine full-thickness cartilage defects using MSCs in fibrin hydrogels demonstrated the support of chondral repair, yet poor cell retention with a time-reduced efficacy when compared to controls [105, 137]. Alginate-encapsulation of chondrocytes and MSCs promotes chondrogenesis *in vitro*. Application of alginate-encapsulated MSCs in comparison with chondrocyte-differentiated MSCs in a rabbit model of bilateral full-thickness cartilage defects resulted in improved glucoseaminoglycan composition and hyaline-like cartilage regeneration in the transplanted sites at 6 months post treatment, with no obvious difference between the alginate group and the MSCs-chondrocyte group [141]. Further improvements of hydrogels for MSC-triggered cartilage reconstruction include improvements of the mechanical properties on the macrostructural level through composite engineering and the combination with antibodies, growth factors or peptides to provide guides for cell migration or differential specificity [142–144]. Studies on the integrin binding RGD peptide and fibronectin have shown that the anticipated effects on *in vitro* upregulation of SOX-9, aggrecan and

collagen type II in MSCs during chondrogenesis are dependent on peptide density, suggesting that controlled RGD-release can induce production of higher amounts of matrix by encapsulated MSCs [142, 145, 146].

Another approach currently being investigated is the targeted binding of MSCs to cartilage surfaces through cell-bound antibodies, yet these approaches are only at an early investigative phase [147]. The use of natural matrices is an alternative to synthetic products. In one study, acellular dermal matrix (ADM) was loaded with clonal chondrogenic BMMSCs *in vitro*, the MSCs-ADM grafts transplanted in an experimental, genetically close to human model of knee joint cartilage defect in *Cynomolgus* monkeys. Articular cartilage defects were considerably improved and repaired in particular by the MSCs-ADM-treated monkeys [148]. When considering mechanical and structural enhancements of scaffolds towards the 3D environment of cartilage on the nano- and microscales, nanotopographical cues and peptide-enhanced nanostructures are being developed. Electrospinning demonstrated the feasibility of electrospun biodegradable polycaprolactone (PCL) nanofiber scaffolds to maintain and support MSCs. Human BMMSCs were seeded in the scaffold and applied in a swine model of full-thickness cartilage defects [149]. The scaffolds promoted cartilage repair, regenerated hyaline cartilage-like tissue and restored a smooth cartilage surface, while the chondrocyte-seeded or acellular constructs produced mostly fibrocartilage-like tissue with a discontinuous superficial cartilage contour. It is noteworthy that no immune reaction was observed in this model. As further improvement in nanostructuring and layering of scaffolds become available, steering of MSCs' potency and function specifically into the tissue-desired direction may be feasible [150]. In another approach the direct cultivation of MSCs in microcarrier scaffolds was used to promote MSCs differentiation and matrix production, and to allow *in vitro* formation of transplantable, preformed cell-matrix units. When MSCs were cultured on CultiSpher-S gelatin microcarrier beads, expanded in spin culture, and ectopically implanted in rats, bead-expanded MSCs retained their multipotency, and bone tissue formation. Moreover, fibronectin coating of TGF-β3-releasing PLGA microspheres to generate an injectable MSCs delivery vehicle resulted in the formation of histologically resembling, collagen II and aggrecan containing cartilage in SCID mice [151, 152].

Microfractures are frequently used to induce a healing response in articular cartilage defects. Penetration of the subchondral bone leads to blood clot formation and allows multipotent mesenchymal cells to access the defect. This recruitment of endogenous MSCs leads to cartilaginous repair tissue formation. Enforced stimulation of endogenous resident MSCs from the bone marrow, synovium or cartilage [64] to induce migration to the injury site, differentiation and the joint repair processes in a controlled fashion without exogenous provision of therapeutic cells is an attractive alternative strategy [79]. These *in situ* tissue engineering strategies may be based on cell-free implants consisting of matrices for local factor delivery for progenitor cell recruitment and differentiation. If the endogenous MSCs are recruited into chondroinductive matrix, new cartilage may be formed. It has been shown that synovial fluid

and serum contain chemoattractors for the recruitment of MSCs *in vitro* and these as well as chemokines, platelet-rich plasma and bone morphogenetic proteins, may be explored for MSCs recruitment [153–156]. In combination with controlled release or scaffold-fixated chondrogenic inducers such as matrix molecules TGF-β and bone morphogenetic proteins with fibroblast growth factor family members and insulin-like growth factor-1 (IGF-1) MSC recruitment and site specific differentiation can be combined and enhanced [79].

Evidence on the long term efficacy of MSCs in the treatment of rheumatic joint diseases is lacking or incomplete. In veterinary medicine, application of MSCs has been available since 2003 and is used as a treatment option in dogs, cats, and horses. ADSC injection intra-articularly in hips and elbows has resulted in improved scores for lameness, pain, and range of motion compared with control dogs [103, 163]. In this randomized, blinded, placebo controlled clinical trial, dogs with chronic osteoarthritis of the coxofemoral and humeroradial joints were used. Recently, even animals such as a tiger and a leopard have been treated for arthritis with MSCs.

4.7 Clinical studies in humans

The treatment of osteoarthritis with MSCs is an attractive and active field of research. Currently at least 23 clinical trials are planned or ongoing, about half of them using allogeneic MSCs [34, 95, 170] (www.clinicaltrials.gov). Cells sources are bone marrow, adipose tissue and umbilical cord and injected with or without additional microfractures or scaffolds, *e.g.* HA and a proteinized collagen matrix hydroxyapatite paste. In the case of rheumatoid arthritis, at least 2 trials to assess safety are currently ongoing. Several groups reported on clinical application of using MSCs with for the treatment of established RA and OA (Tab. 4.2). There were significant improvements postoperatively in pain scores, Tegner activity scores and visual analog scale (VAS) pain scores in a cohort of 25 randomized patients given intra-articular injections of infrapatellar fat pad-derived ipMSCs, coupled with arthroscopic debridement [171]. Although initially promising, final follow-up pain scores at 16 months showed similar outcomes between the treatment and control groups. Hence, despite promising early efficacy, long-term benefits are currently not clear. In a second study by the same group, clinical imaging results were presented of 18 patients who received intra-articular injections of autologous ipMSCs in platelet-rich plasma for the treatment of knee osteoarthritis. The results showed significantly decreased OA-scores, improved Lysholm and VAS scores and an improved whole-organ magnetic resonance imaging (MRI) score at final follow-up at 24 months. Interestingly, improvements in clinical and MRI results were positively related to the number of stem cells injected [172]. Results were also reported on two case series with 4 and 6 patients who received intra-articular injection of BMMSCs with modest effects at 6 months follow-up and no adverse effects at 12 months [173].

A case study of 2 patients receiving ADMSCs together with hyaluronic acid, dexamethasone and platelet-rich plasma showed good outcomes after 3 months, with subjective pain, functional status and cartilage thickness improved. Furthermore, there have been several studies on human patients reporting encouraging outcomes within 6 months of intra-articular injection of autologous BMMSCs for regeneration of knee cartilage. Improvements in both VAS scores and range of motion, and significant articular cartilage growth and meniscus regeneration were shown. Case reports of percutaneously implanted autologous BMMSCs showed successfully regeneration of articular and meniscus cartilage in human knee [43]. The effectiveness of the approach needs to be studied involving more patients, however, a large safety study with more than 300 patients showed fewer complications than would normally be associated with surgical intervention [174]. A three year follow-up in a subset of these patients showed improvement in over 50 % of the reporting patients. In the treatment of a full-thickness defect in the articular cartilage of a human knee, BMMSCs were expanded using platelet-rich plasma and embedded within a collagen gel before being surgically grafted intra-articularly resulting in clinical improvement [175]. The comparison of BMMSCs injection in a one-step technique in repairing osteochondral lesions of the knee with outcomes obtained with autologous chondrocyte implantation in similar lesions showed high similarities [176]. In a study that used a combination of BMMSCs with fibrin glue for intra-articular injection in a full-thickness cartilage defect, improvement was demonstrated over the 1-year follow-up, although only three of five cases resulted in complete defect fill [177]. In a further study, which involved 50 patients with mild to moderate osteoarthritis knee, the groups received arthroscopic debridement alone or injection of BMMSCs concentrate along with the arthroscopic debridement. Follow-up showed improved osteoarthritis outcome scores and quality of life [178]. In summary, as double-blind controlled studies in humans have not yet been published, the long-term efficacy in human OA patients is still not known.

In one of the first clinical studies in humans of the efficacy of systemic infusion of autologous ADMSCs, 10 patients with autoimmune tissue damage due to conditions including RA and polymyositis were treated [90, 179]. In addition to systemic infusion, some patients received additional local intra-articular cell transplantation. Although this study represented only case-study data, high expansion efficiency for the ADMSCs was demonstrated with no loss in potency, safety profile, migration and homing characteristics. Despite the positive experimental data reported in murine models of inflammatory joint disease, there are currently no systematic and controlled clinical study data available demonstrating successful treatment of RA in humans. Although the systemic use of MSCs to treat autoimmune or inflammatory joint disease is, in principle, a possibility, its development into a therapeutic tool is likely to take time. The success of anti-TNF-α treatments in recent years suggests that the immunomodulatory properties of MSCs are a potentially therapeutic mode of action, in addition to their ability for matrix production and chrondrogenic dif-

ferentiation. Furthermore, this established treatment would shift the focus for MSCs-based approaches towards those patients who do not or only poorly respond to anti-TGF treatments.

In the treatment of cartilage defects, degenerate or otherwise, autologous cells have been used since the mid-1990s with variable results [119, 180], and although there are many reports of successful outcomes, the clinical application of these cells remains limited by the number of questions still surrounding their use.

In a small number of patients, positive effects on symptoms and no adverse effects were reported. However, these studies are still of limited scientific scope and did not evaluate structural changes. Therefore, a conclusive evaluation of the efficacy of MSCs transplantation for articular cartilage regeneration in human joints is not yet possible. It is furthermore too early to conclude on the mode of action and most effective mode of cell provision into the rheumatic joint to fully explore the multiple regenerative assets that characterize MSCs.

4.8 Risks and benefits of MSCs treatments in rheumatic diseases

The application of MSCs in rheumatic diseases offers exceptional promise for patients. These include their participation in new cartilage formation and matrix deposition, in the stimulation of residual cells and regenerative modulation of the microenvironment and well as the suppression of inflammation.

However, before entering into large scale clinical application associated risks must be carefully considered [44, 181]. *In vitro* cultivation and modification of MSCs before grafting may modify cellular characteristics such as differentiation or proliferative potential, or the cells may become vehicles for potentially immunogenic antigens or infectious agents [182]. The use of cultured MSCs in patients therefore needs the strict application of relevant rules for medicinal products to exclude potential for tumorigenesis or transmission of harmful agents. There are several reports on the unfavorable effects of culture expanded MSCs in the context of tumor alterations and malignant transformation. In particular, some evidence suggests that MSCs modulate development and progression of tumors. MSCs have been shown to migrate to tumor sites and support tumor cell proliferation, invasion, and metastasis [183–186]. In the context of OA, this aspect has most likely only relevance with regards to its association with age and hence age-related increased genetic burden and increased cancer risks, which may be transferred to autologous MSCs and promoted during *in vitro* expansion. Moreover, MSCs preparations are variable in quality and present a heterogenous cell composition, requiring the need for the development of application specific standards for MSCs as well as access to culture protocols and clinical study data [187, 188].

MSCs are highly migratory cells and may differentiate at their migratory endpoint into ectopic tissues. Indeed, endochondral ossification, generation of fat and fibrous tissue formation has been observed in preclinical settings. For example, when allo-

Table 4.2: Experimental human clinical studies using MSCs for treatment of OA and RA.

MSC	Treatment	No. of patients	Follow-up	Results	Reference
ipMSC	Concentrated injection + PRP	25 ipMSCs 25 only PRP	16.5 months	Improvement	[183]
ADMSC	Concentrated injection + HA + PRP+ dexamethasone	2	3 months	Improvement	[191]
	Injection	3	3–13 months	Improvement	[192]
BMC	BMC injection after debridement	25 BMC 25 Debride-ment alone	–	Improvement	[190]
BMMSC	Cultured BMMSCs injection	6	12 months	Improvement	[193]
	Cultured BMMSCs on collagen scaffold	2	31 months	Improvement	[194]
	Cultured BMMSCs injection	4	12 months	Improvement	[185]
	Cultured BMMSCs on collagen gel + periosteum	12 BMSCs 12 Controls	16 months	Improvement	[195]
	Cultured BMMSCs w/ total ankle arthroplasty	3	2–24months	Improvement	[196]
	Cultured BMMSCs injection + 10 % platelet lysate	1	24 weeks	Improvement	[43]
	Cultured BMMSCs/UC-MSCs(Allogeneic) injection	4	6–23months	Improvement	[197]
	Cultured BMMSCs injection(Allogeneic)	4	12–18 months		[198]
	Cultured BMMSCs injection	2	–		[199]
	Cultured BMMSCs injection	2	8 months		[200]
UCMSC	Cultured UCMSCs injection	16	3–28 months		[201]

Abbreviations: BMC (bone-marrow concentrate), BMMSCs (bone marrow-derived MSCs), HA (hyaluronic acid), ipMSCs (infrapatellar fat pad-derived MSCs), OA (osteoarthritis), PRP (platelet-rich plasma), RA (rheumatoid arthritis), UCMSCs (umbil""ical cord-derived MSCs).

geneic ADMSCs were injected intra-articularly in a canine model of OA, the cells were detectable at the joint but also at ectopic sites such as the thymus and the gastrointestinal tract several weeks post grafting [189]. Since osteogenesis is the prevalent route of MSCs development, the formation of calcification at unwanted sites has been shown [190]. This risk may be reduced by local application of MSCs in rheumatic joint diseases and the addition of supportive measures for cell retention. However,

at the same time any possible therapeutic effect by systemic delivery would be lost. Immunogenicity of MSCs is a possible risk factor and has been extensively studied especially for allogeneic application. MSCs have been reported to be hypoimmunogenic due to the downregulation of MHC class II and costimulatory molecules and thus appear appropriate for allogeneic transplantation [191, 192]. In addition, the cells prevent T cell responses directly and through modulation of dendritic cell function, and produce immunosuppressive cytokines. These phenotypic characteristics were maintained even after chondrogenic differentiation according to one study [87]. To avoid allogeneic rejection and inflammation risks, the use of autologous cells, especially with multiple treatment circles, may be a safe choice.

Treatment of human rheumatoid diseases with MSCs is still in its infancy. Although it bears enormous potential for new treatments, it is necessary to be aware of the possible safety and efficacy risks and to carefully balance these risks with the benefits in accordingly designed clinical studies. This includes the establishment of standardized protocols for expansion and application of the cells in controlled experimental settings.

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Andrea Hoffmann, Virginia Seiffart, Sandra Laggies and Gerhard Gross

5 Mesenchymal stem cells in enthesis formation and repair

Abstract The regeneration of tendon and ligament injuries or joint replacements after degeneration by wear and tear, trauma or inflammation still is a major clinical challenge to orthpaedic medicine. Tendons and ligaments are poorly vascularized, heal slowly and lead to the formation of fibrous, scarry tissue lacking the original flexibility and biomechanical properties. Tendon-to-bone healing involves complex biological activities between nonhomogenous soft and hard tissues and, moreover, inflammatory disorders often target sites of tendon-bone-insertions. The latter may lead to severe enthesopathies associated with substantial bone erosion and undesirable new bone formation. We will here review recent progress in the management of musculoskeletal disorders of the joints specifically focusing on stem cell-dependent approaches for the repair of tendon-to-bone junctions.

5.1 Introduction

The attachment site of tendon or ligaments to bone (enthesis, tendon-to-bone junctions, osteotendinous junctions) serves as an anchor to allow musculoskeletal movements. Entheses are able to resist high stress concentrations but are sensitive to overuse injuries and to inflammatory conditions as well. For example, chronic inflammatory disorders may cause severe enthesopathies. Moreover, the osteoarthritis-mediated abnormalities in ligament, tendon, bone and enthesis are comparable to those observed under inflammatory conditions such as *e.g.* psoriatic arthritis [1]. In addition, acute trauma and aging may also cause the destruction of the enthesis and the detachment of tendon from bone.

The rotator cuff tendons are a prominent example and especially sensitive to damage of the enthesis. So-called rotator cuff disease increases with age and is in general caused by degeneration of the tendon, rather than injuries from sports or trauma. Here, four muscles surround the humeral head at the rotator cuff. When the tendons are injured, the rotator cuff bursa supplying gliding material for free motion may become inflamed. The rotator cuff tendons generally tear off at their insertion site. In addition, enthesis disorders of the Achilles tendon, of the ligaments of the knee and of the flexor tendons of the hand cause severe problems. Therefore, surgical interventions for the repair of tendon-to-bone junctions are routine, however, only 20 to 60 % of the surgical interventions heal [2].

The integration of healing tendon or ligament into bone after surgical reconstruction is, therefore, an issue of considerable importance. So far, quite a number of differ-

ent approaches have been devised which should lead to better healing rates. Several reviews have competently dealt with these problems *e.g.* [3–5]. Here we will focus on the stem cell-mediated healing degenerated bone and tendon/ligament matrix by wear and tear and by chronic inflammatory disorders as well.

5.2 Structure of the tendon-to-bone junction

Tendons are part of the musculotendinous system in the body. They connect muscles to bone while ligaments link bone to bone. Both tendon and ligaments transmit the forces developed by muscle contractions across joints, stabilize these or produce motion. Tendons originate in muscle (musculotendinous junction, MTJ) and insert into bone at a tendon-to-bone junction (enthesis; osteotendinous junction, OTJ). Early pioneering work by Biermann and co-workers distinguishes two different forms

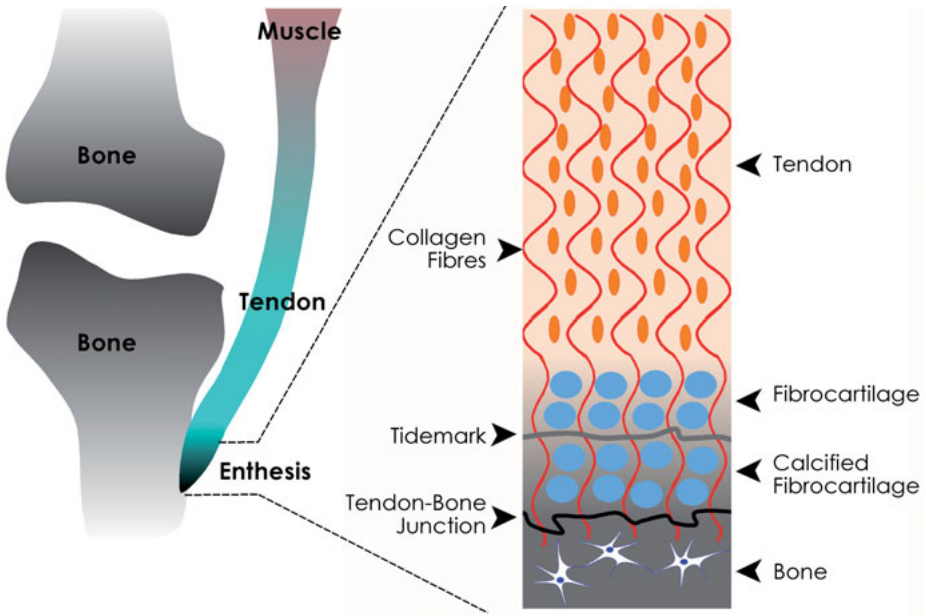

Fig. 5.1a: Schematic structure of a tendon-bone insertion with a direct, fibrocartilaginous interface (enthesis, osteotendinous junction, tendon-to-bone junction). Fibrous connective tendon-tissue attaches the tendon to the bone. The tendon matrix is composed of collagen fiber bundles, in general aligned with the long axis of the tendon consisting predominantly of type I collagen with lesser amounts of other collagen types and, in addition, proteoglycans and other glycoproteins. Tendon tissue also harbors the cellular components of the tendon, tenoblasts and tenocytes (zone 1). The fibrocartilaginous tendon-bone insertion (enthesis) shows a gradual transition from tendon via fibrocartilage (and mineralized fibrocartilage) to bone. Uncalcified fibrocartilage is separated from calcified/mineralized fibrocartilage by a tidemark. The drawing is adapted from [85].

of the OTJ, diaphysial-periosteal and chondral-apophyseal attachments, according to its site of long bone attachment [6] but more recent work by Benjamin and co-workers introduced broader terminologies for the entire musculoskeletal system. These authors classify entheses as being either of the fibrous (indirect) or fibrocartilaginous (direct) type, depending on the character of the tissue at the tendon/ligament-bone interface *e.g.* [7].

The fibrocartilaginous (direct) type of enthesis is composed of four zones: a dense fibrous connective tissue tendon or ligament zone, uncalcified fibrocartilage, mineralized fibrocartilage, and bone (Fig. 5.1a). The outer border of calcification is indicated by a basophilic tidemark which separates fibrocartilage and calcified fibrocartilage, similar to the tidemark found in articular cartilage. In contrast, a fibrous (indirect) enthesis lacks both fibrocartilage intermediate zones.

The specialized structure of the tendon-to-bone junction prevents collagen fiber bending, fraying, shearing, and failing [8]. At the bony insertion site, elastic and soft tendon/ligament anchors to hard bone, materials of rather diverse physical properties. Therefore, structures adjacent to the enthesis are involved in the prevention of injury or destruction due to mechanical loading forming an entire ''enthesis organ''. This term has been coined to point out that additional structures adjacent to an enthesis are responsible for stress dissipation at the attachment site [9]. For example, the Achilles enthesis organ consists of a tendon insertion associated with a complex of adjacent fibrocartilages, a bursa and a fat-pad. 14 such complex enthesis organs have been described in the organism [10].

5.3 Enthesis resident T cells are involved in enthesopathies provoking inflammation and bone remodeling

As pointed out above, entheses are subjected to considerable wear and tear. Therefore, enthesopathies may develop in response to overuse conditions such as tennis elbow and jumper's knee. But enthesopathies are also associated with osteoarthritis and inflammatory disorders such as psoriatic arthritis, rheumatoid arthritis (RA) or ankylosing spondylitis [11, 12].

Inflammatory enthesopathies are not only focused on the tendon insertion site. Closer examination by magnetic resonance imaging (MRI) has revealed that adjacent tissues are also affected, hard and soft tissue and the synovium as well. In psoriatic arthritis it has been proposed that an enthesitis is the primary disorder triggering secondary inflammatory pathologies like synovitis and osteitis through the release of proinflammatory factors [13, 14]. Spondyloarthropathies are a family of inflammatory rheumatic diseases that cause arthritic bone erosion and, in addition, substantial new bone formation. The most common form of this disorder is ankylosing spondylitis, which eventually leads to immobility of the spine due to ongoing bone formation and fusion of the vertebrae. Several investigations noted the involvement of the

IL-23-receptor in ankylosing spondylitis but also in inflammatory disorders such as *e.g.* inflammatory bowel disease (IBD) [15, 16]. Therefore, the systemic release of IL-23 in the organism seems to be central for the pathogenesis of ankylosing spondylitis [17–19]. The systemic IL-23 release may be mediated by several conditions such as pathogens in the gut [20], by an unfolded protein response (UPR) of the misfolded leukocyte antigen HLA-B27 [21] and by repetitive biomechanical stress [22].

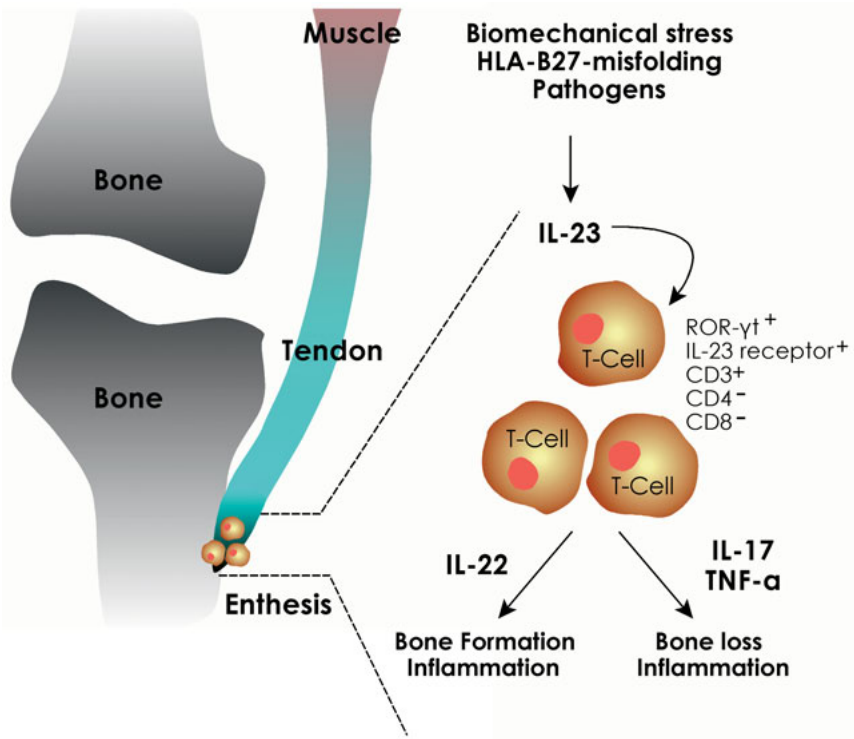

Fig. 5.1b: The systemic release of IL-23 triggers an inflammatory response and bone remodeling *via* enthesis-resident T cells. The systemic release of IL-23 may be initiated in the organism by a variety of conditions. IL-23 activates enthesis resident ROR-γt$^+$ CD3$^+$ CD4$^-$ CD8$^-$ T cells to secrete factors which promote local inflammation and bone erosion, including IL-22, TNF-α and IL-17 [23]. As a response to IL-23-dependent IL-22 release, factors are synthesized which stimulate osteoprogenitor proliferation and differentiation [23]. In ankylosing spondylitis this eventually leads to local bone growth and fusion of the vertebrae.

Until recently it was unclear how systemic IL-23 production may be involved in enthesitis. In an elegant study it was demonstrated that the reason for the collateral damage at an inflamed enthesis may be due to a previously unidentified population of enthesis-resident T cells [23]. It was already a surprise that tendon-to-bone junc-

tions are subjected to immune surveillance by this particular subpopulation of T-lymphocytes. In spondyloarthropathy, IL-23 promotes specific entheseal inflammation by acting on these enthesis-resident ROR-γt$^+$, CD3$^+$, CD4$^-$, CD8$^-$ T cells which express IL-23-receptors (Fig. 5.1b). As pointed out before, IL-23 may be released in the organism under a variety of conditions, including biomechanical stress and pathological changes of the gut microflora. In enthesis-resident T cells, systemic IL-23 leads to the release of proinflammatory cytokines such as IL-22, IL-17 and TNF-α (Fig. 5.1b). Interestingly, the IL-23-dependent release of IL-22 causes the secretion of growth factors resulting in local bone growth and bone remodeling [23]. These findings explain the characteristic symptoms of spondyloarthropathy with extensive bone formation in the presence of inflammatory bone erosion.

The latter may also explain that enthesitis in general and psoriatic arthritis in particular causes not only bone erosion but also the formation of bony elements (osteophytes) [11]. Based on the study described above [23], an anti-IL-23, anti-IL-22 and anti-IL-17 therapy of spondyloarthropathy in particular and enthesopathies in general may ameliorate the pathological conditions. Interestingly, the enthesis-resident T cell population also has the capacity to react to environmental stress which activates the innate immunity. A new and exciting question would be as to whether or not MSCs may not only be effective for the regenerative therapy of tendon-bone attachment sites but also be beneficial due to their immunomodulatory and immunosuppressive properties to interfere with activated T cells (see below).

The repair of damaged or degenerated entheses is, therefore, an important issue. The integration of healing tendon or ligament into bone is under active investigation since the insertion of tendon grafts into bone is often not satisfactory and re-tears frequently arise. In general, the zonal concept of a tendon-to-bone attachment site (Fig. 5.1a) is not re-established in repaired entheses. The poor results seen in tendon-to-bone healing may, therefore, be due to the reduced capacity for stress dissipation at the soft to hard tissue interface.

5.4 Biomaterials and growth factor-dependent regeneration of tendon-to-bone junctions

The challenge for the orthopedic surgeon is to restore the graded structure of an enthesis. Simple strategies clamp the tendon matrix to the bone but many biomaterials such as polyglycolic acid sheets have been successfully used to enhance rotator cuff repair and regeneration [24]. The development of novel biomaterials with biomimetic properties is, therefore, one center of research to achieve increased rates of tendon-to-bone healing.

Several approaches use hydroxyapatite to stimulate enthesis repair. Hydroxyapatite is a chemical compound related to bone and it has been used for tendon-to-bone healing in solid or soluble form. For example, an interface was generated when the

patellar tendon was attached to a hydroxyapatite coated implant. When this hydroxyapatite coated implant was supplemented with autologous cancellous bone or marrow grafts an indirect-like insertion was observed at six weeks after implantation [25]. At twelve weeks, the interface was observed to be a layered neo-enthesis, whose morphology was similar to a normal direct tendon insertion. Without these marrow grafts, however, only collagenous fibrous tissue developed. Hydroxyapatite biomaterials may also be combined with biological factors for enthesis repair. Recently, it has been demonstrated that the delivery of TGF-β_3 (transforming growth factor-beta 3) for rotator cuff repair in an injectable calcium-phosphate matrix increased bone formation, collagen organization and reduced scar formation in the healed enthesis [26]. Magnesium-based bone adhesive may also improve tendon-to-bone healing. This has been documented in a rabbit anterior cruciate ligament reconstruction model based on histological and biomechanical testing at six weeks [27]. Without any doubt, the further development of novel biomaterials will have a great impact on the modalities of enthesis repair.

5.5 Biomechanical stimulation for enthesis repair

The regeneration of an enthesis also depends on biomechanical parameters. Mechanical loading correlated well with the osseous ingrowth of tendon tissue [28, 29]. There are now many approaches to enhance tendon-to-bone repair by applying mechanical stress.

For example, electrical stimulation, pulsed electrical magnetic fields (PEMFs), and low intensity pulsed ultrasound (LIPUS) have successfully been used for the acceleration of tendon-to-bone healing [30]. Also, mechanical stimulations such as extracorporeal shock wave therapy, or ESWT, was able to treat delayed tendon-to-bone injury in an animal model [31]. These results emphasize the role for biomechanical loading in the treatment of tendon disorders but further clinical evaluation is essential.

5.6 Mesenchymal stem cells (MSCs)

As pointed out above, we want to focus in this review on the role of cellular strategies to support tendon-to-bone healing. Very attractive cells for the latter objective are mesenchymal stem cells (MSCs) which, in general, can be isolated from the patient him- or herself by minimally invasive procedures and propagated without major problems. MSCs are adult multipotent stem cells that are capable of differentiation into a number of mesenchymal cell lineages, at least into bone-forming osteoblasts, cartilage-forming chondrocytes and fat-containing adipocytes. MSCs were first recognized by Friedenstein and colleagues, who identified in the bone marrow an adher-

ent, fibroblast-like cell population that could regenerate normal bone *in vivo* [32]. Moreover, MSCs are known to extend their developmental capacity by the secretion of cytokines and growth factors which establish a local regenerative microenvironment. These trophic activities of MSCs are due to secreted bioactive factors with angiogenic, anti-apoptotic and anti-scarring properties [33]. In addition, MSCs secrete factors which are immunomodulatory and immunosuppressive [34] (see below).

MSCs were also termed "marrow stromal cells". In the bone marrow, MSCs are found at endosteal and perivascular locations [35, 36]. Recently, a crucial study documented that in the bone marrow MSCs may form niches together with hematopoietic stem cells and nerve fibers [37]. Interestingly, already 20 years previously this particular niche was monitored as an anatomical unit and at that time termed "neuroreticular complex" [38]. However, it should be emphasized that MSCs are not only present in the bone marrow since they obviously are a subpopulation of human perivascular cells in general [39] and, therefore, can also be isolated from other organs and tissues.

5.7 Stem cell-dependent approaches for repair of osteotendinous junctions

As mentioned above, the fibrocartilage transition zone of direct osteotendinous junctions dissipates the stress concentration at the soft (tendon) to hard (bone) tissue interface. The regeneration of the fibrocartilage zone in osteotendinous junctions is therefore an important goal. Unfortunately, tendon-to-bone healing rarely leads to the formation of a fibrocartilage interface. Moreover, tendon healing results in far stiffer tendon due to extensive scar formation. Such a scarred tendon is stronger than a normal tendon but it is functionally inferior and prone to re-injury. Scar formation not only has an effect on the tendon midsubstance but also on the tendon-to-bone attachment sites. So, the anti-scarring and the tissue-forming capacity of MSCs may both be exploited to have an impact on enhanced tendon regeneration. Cell types other than MSCs have also been investigated for optimizing tendon-to-bone healing, in particular periosteal cells and chondrocytes. Most of these reports emphasize an acceleration of tendon-to-bone repair by these cell-dependent strategies, however, MSCs seem to be the best cellular source [40–48].

Lim *et al*. reported that tendon grafts coated with MSCs enhance the rate and the quality of osteointegration in anterior cruciate ligament reconstruction in a rabbit model. While control reconstructions showed scar tissue spanning the entire tendon-bone interface, MSCs-coating of tendon grafts resulted in a fibrocartilage interface resembling the enthesis of normal anterior cruciate ligament insertions. These MSC-enhanced tendon grafts had a significantly higher failure load and stiffness after eight weeks [40]. Synovial MSCs have been successfully used to improve bone-tendon regeneration in a rat model when Achilles tendon grafts were inserted into a bone

tunnel [44]. Similarly, it was observed in a rabbit model of hallucis longus tendons repair that MSCs were able to re-establish the formation of a fibrocartilaginous tendon-bone interface [49]. However, the fibrocartilage-like tissue did not form within the entire tendon-bone interface. The authors argued that the nonuniform distribution of the bone marrow-derived MSCs at the tendon-to-bone junction and the lack of biomechanical forces interfered with differentiation.

In contrast, there are also other reports indicating that a stem cell-dependent repair does not necessarily improve the regeneration of an enthesis. For example, applying bone marrow-derived MSCs in fibrin glue to a rat model of rotator cuff disease did not improve the structure, composition, or strength of the healing tendon attachment site despite evidence that the MSCs were present and metabolically active [45]. It may be, however, that the study was completed too early since the stem cell mediated repair of tendon-to-bone insertions takes a considerable time. This at least has been suggested in a recent investigation [48].

The latter study represents an encouraging report on the reconstruction of a destroyed rat Achilles tendon enthesis with a healing rate of 50 %, similar to human surgical outcomes [48]. The authors of this study also evaluated the effect of depositing chondrocytes in comparison with MSCs during the initial repair (see below). Interestingly, injection of MSCs significantly improved healing and the load-to-failure conditions after 45 days and generated an entire new enthesis. These results document that a stem cell therapy is an efficient procedure for reconstructing degenerated or destroyed entheses, since all morphological and biomechanical properties investigated were similar to those of native entheses [48].

Chondrocytes are in general part of a fibrocartilage enthesis and produce type II collagen, playing an important role for anchorage and stress dissipation (Fig. 5.1). Therefore, it was a reasonable strategy to also assess the potential of chondrocytes to generate a fibrocartilage transition zone during tendon-to-bone healing. A recent study nicely documents that not only chondrocytes but also the interposition of autologous articular cartilage tissue in a goat partial patellectomy repair model resulted in more fibrocartilage formation [47]. Despite this promising report, one cannot entirely exclude that surgical routine integration of entire cartilage may be subjected to donor site morbidity in a certain number of cases. Chondrocytes may, therefore, be a better choice to restore the fibrocartilage zone at a tendon-to-bone junction as previously proposed [50].

This capacity of chondrocytes for tendon-to-bone healing was also investigated in the Nourissat study and compared to MSC injection [48]. They found that chondrocytes improved the healing rate, but, unfortunately, did not lead to the formation of an ordered fibrocartilage zone [48]. Only MSC-injections lead to the development of an organized enthesis with columnar chondrocytes comparable with a native enthesis 45 days after surgery. In conclusion, MSCs seem superior to chondrocytes in producing a direct osteotendinous junction with the desired fibrocartilage interface during tendon-to-bone healing [48].

5.8 Stem cell-dependent delivery of growth factors

To improve rates of enthesis regeneration, biological factors have been applied for tendon-to-bone healing: members of the TGF-β/BMP-family of growth factors, in particular BMP-2 (bone morphogenetic protein-2) or TGF-β1 (transforming growth factor-beta1) [51–53]. To enhance tendon-bone integration of anterior cruciate ligament grafts, BMP-2 was also supplied by an adenoviral vector [54]. Interestingly, BMP-12 seemed to be able to promote the formation of a fibrocartilage insertion in a rat model [55] and in a sheep infraspinatus repair model also [56]. Therefore, all these factors may also be combined with a stem cell strategy in order to enhance long-term and sustained factor delivery.

For example, MSCs may differentiate *in vitro* and *in vivo* into tendon-like cells by the plasmid-mediated recombinant expression of the intracellular signaling factor Smad-8 in combination with BMP-2 [57]. Bone formation was not observed in spite of the presence of active BMP-2 indicative for a tendon-tissue-specific interference with BMP signaling-mediated osteogenic differentiation. Such a tendon-tissue-dependent interference with BMP signaling could indeed be demonstrated in another study [58]. Based on these observations, we developed the notion that higher expression levels of BMP-2 in the presence of Smad-8 might lead to concurrent bone and tendon/ligament formation and the concomitant development of tendon-bone interfaces.

We could show that tendon-bone insertions may form spontaneously when adult stem cells become endowed with both a tenogenic and bony capacity [59]. The viral vector-dependent modification of murine MSC-like cells (C3H10T½) with constitutively-active Smad-8 and BMP-2 led to the spontaneous formation of fibrocartilaginous tendon-bone junctions after heterotopic implantations into murine muscles (Fig. 5.2a–c). The chondrogenic nature of the osteotendinous junction was substantiated with *in situ* hybridizations specific for collagen II. Smad-8-modified cells were located in tendon-like structures which is indicative for a direct contribution of Smad-8-modified stem cells to tendon development. Primary bone marrow-derived human MSC showed similar results to those obtained for the murine stem cell line (C3H10T½), however, the time needed for the primary human MSC-dependent development of tendon-to-bone structures was considerably longer. Another major difference was that the heterotopic transplantation of primary human MSCs modified with viral expression vectors to express Smad-8/ BMP-2 resulted in tendon-to-bone junctions without fibrocartilage elements [59].

The lack of fibrocartilage intermediates in tendon-to-bone attachment sites derived from modified primary human MSCs could be due to the lower chondrogenic capacity of BMP-2 in this cell type. In primary human MSCs, BMP-2 has a distinctly lower chondrogenic competence than *e.g.* TGF-β_3. Future studies have to show whether other members of the TGF-β-family may provide the missing chondrogenic capacity to restore the fibrocartilage interface in tendon-to-bone junctions derived from primary human bone marrow MSCs. In addition, it has recently been observed

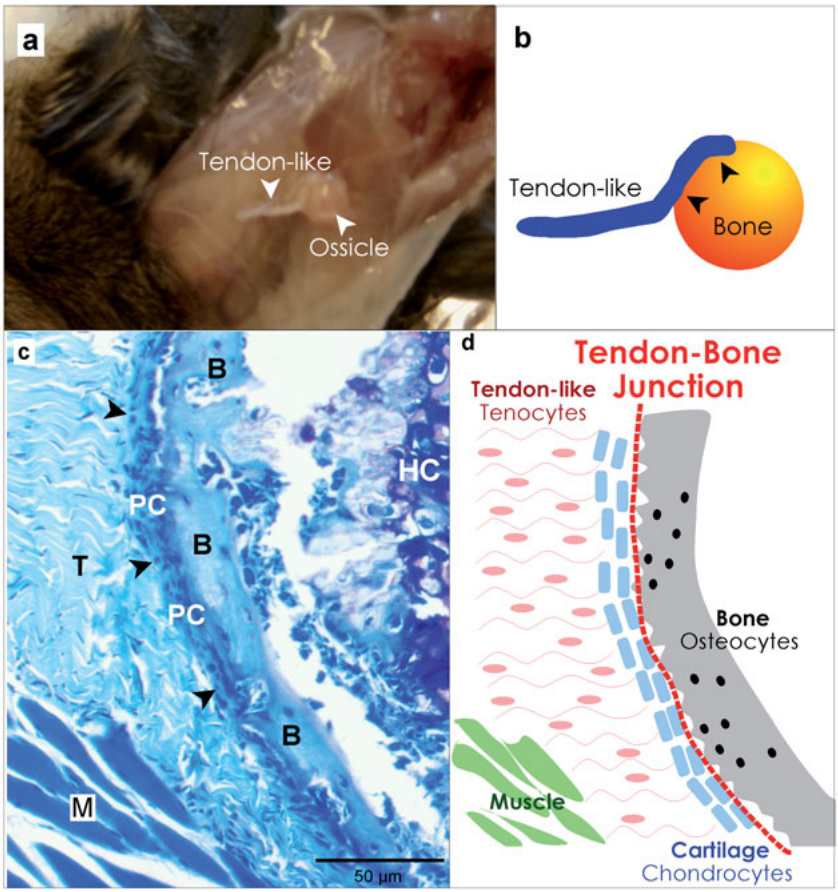

Fig. 5.2: Heterotopic intramuscular transplantation of adenovirally modified mesenchymal progenitors (C3H10T½) expressing Smad-8 and BMP-2 results in tendon-bone-insertions. Murine mesenchymal progenitors C3H10T½ have been infected with recombinant adenoviral particles mediating the expression of BMP-2 and Smad-8. One day before transplantation, modified stem cells were seeded on a collagen sponge (Duragen). For heterotopic transplantation of modified stem cells into mice, an intramuscular pocket was formed in the thigh muscle and filled with the collagen sponge containing the cells. The sponge was inserted and positioned near the muscle belly. Skin was sutured and four weeks after implantation mice were sacrificed. The explants were embedded in paraffin, sectioned and histologically characterized. Further details are as described in [59]. (a) Tendon and bone formation of Smad-8/ BMP-2-modified progenitors (C3H10T½) after heterotopic intramuscular implantation of modified stem cells on a collagen sponge (8 weeks). The schematic drawing indicates the potential formation of a tendon-bone interface (black arrowheads). (b) Fibrocartilage osteotendinous junctions (OTJ)-like structure formed by implanted mesenchymal progenitors (4 weeks). The toluidine blue-positive, chondrocyte-like cells between tendinous and bony tissue are indicated (black arrowheads). (c) Schematic drawing of (b) indicating the position of the OTJ. B, bone; HC, hypertrophic chondrocytes; M, muscle; PC, proliferating chondrocytes; S, collagen sponge; T, tendon-like structures. (From [59], copyright: 2010, G. Gross. This material has been reproduced with permission of John Wiley & Sons, Inc.)

that stem cells derived from tendon may spontaneously develop osteotendinous junctions if they interact with bony calvariae. The type of the OTJs has not been described more closely but it seems that chondrocytes are not present [58].

All these data indicate a remarkable competence of adult stem cells to form osteotendinous junctions once they possess both a tenogenic and an osteogenic capacity. These observations support the notion that tendon-to-bone insertion is a process which is primarily driven by the local activity of growth factors and signaling mediators. This process is further shaped and maintained by mechanical loading as suggested by Moffat and colleagues [60]. So, the overall parallel orientation of new tendons generated by heterotopic implantations of modified MSCs with actively contracting muscles implies that mechanical loading exerted by the microenvironment does play a major role in the formation of tendinous structures. Moreover, extended implantation times (two months vs. one month) resulted in tendon matrix formation containing substantial amounts of adipogenic appendages [59]. This may also be attributed to the reduced mechanical loading conditions at the heterotopic sites of MSC-dependent tendon formation. Nevertheless, these observations may eventually contribute to the establishment of stem cell-dependent regenerative therapies involving tendon/ligaments and the insertion of tendon grafts at bony attachment sites.

5.9 Stem cell-dependent delivery of tenogenic transcription factors

Unfortunately, only few transcription factors have been described as specifically being expressed in tendons. Recent experiments document that the homeodomain transcription factor *Mohawk* influences tendon morphogenesis [61]. In Drosophila, tendon differentiation relies upon the transcription factor *stripe*, an early growth response (Egr)-like transcription factor. Its vertebrate homologues *Egr1* and *Egr2*/*Krox20* seem also to exert an influence on tendon formation [62]. *Scleraxis* (*Scx*) is still the only transcription factor displaying an expression pattern restricted mainly to tendons and ligaments [63, 64]. However, *Scx* is not necessary for tenocyte specification, since tendon progenitor cells are present and many tendons still form in *Scx*–/– mutant mice [65]. So far, only a few reports describe a role for *Scx* in tendon healing. A recent study describes that adenoviral-mediated *Scx*-modifications of bone marrow-derived MSCs improves healing of tendon-to-bone insertion site after rotator cuff repair [66].The authors postulate that *Scx*-modified MSCs can augment rotator cuff healing especially at early time points. The authors emphasize, however, that further studies are needed to determine the efficacy of this strategy. The recent finding that the transcription factor *Scx* upregulates BMP-4 expression in tendon cells at their insertion site during embryonic development [67] could support the observation that *Scx* may indeed simulate stem cell-dependent formation of tendon-bone insertions [66].

5.10 Stem cell-dependent delivery of matrix metalloproteinases

Recent studies demonstrated a potentially critical role of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in the pathophysiology of rotator cuff tears. Some investigators argue that the catabolic MMPs negatively affect tendon-to-bone integration. So, interference with the activity of MMPs has been documented to increase the healing of tendon in a bone tunnel. Load to failure analyses also showed improvements [68]. In addition, the local delivery of an MMP-inhibitor after surgical repair of the rotator could improve healing at the tendon-to-bone surface interface [69]. The latter study contrasts in some respects with another interesting investigation of the same group [70].

Gulotta *et al*. developed the hypothesis that additional signals are required to increase the effectiveness of an adult stem cell therapy for tendon to bone healing. They asked the question of whether or not recombinant expression of the membrane type 1 matrix metalloproteinase may increase repair of osteotendinous junctions [70]. The membrane type 1 matrix metalloproteinase (MT1-MMP, also called MMP-14) is a membrane-bound matrix metalloproteinase which is expressed in tendon *anlagen* and stimulates endochondral ossification during embryonic development [71]. Therefore, MT1-MMP might improve healing by stimulating the transition from unmineralized to mineralized fibrocartilage. Indeed, marrow-derived MSCs modified to express MT1-MMP by an adenoviral expression system augmented rotator cuff healing at four weeks by the presence of more fibrocartilage at the insertion site and, concomitantly, improved its biomechanical strength after repair [70]. A positive role for MT1-MMP in tendon-to-bone integration may also be involved in the Smad-8/ BMP-2-modified MSC-system which has been described above and which is able to form *de novo* heterotopic osteotendinous junctions [59]. These Smad-8/ BMP-2-modified MSCs do express MT1-MMP quite well (unpublished observation).

5.11 Trophic activities of MSCs in enthesis repair

Mesenchymal stem cells have the ability to differentiate in various lineages and, in addition, they function as trophic mediators secreting a variety of cytokines and growth factors. This helps to recruit MSCs to sites of injury and to subsequently mediate many therapeutic effects in tissue regeneration. Among the secreted factors are angiogenic and neurotrophic factors, factors which suppress immune recognition and/or the expansion of B and T cells and also provide a microenvironment for hematopoietic stem cell (HSC) maintenance [72, 73].

Considering MSCs' immunomodulatory and immunosuppressive actions, once activated by inflammatory signaling pathways, MSCs are able to recruit lymphocytes into a cellular complex interfering with lymphocyte proliferation and secretion of inflammatory cytokines [74–76]. MSCs are able to suppress T cell functions, decrease

the production of IFN-γ, TNF-α and increase anti-inflammatory IL-10 and IL-4 secretion [77–79]. In addition, MSC-dependent immunomodulation involves factors which are released in MSC-T-cell interaction such as inducible nictric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), heme oxygenase (HO-1), transforming growth factor-β (TGF-β) and soluble HLA-G5 [78, 80–82].

As pointed out above, enthesis resident T cells are involved in inflammatory disorders which cause severe enthesopathies (see above). This leads to the notion that MSCs may not only be used to induce a tenogenic developmental program or the formation of functional entheses but, in addition, that they are valuable entities to exert anti-inflammatory and protective activities against activated enthesis resident T cells.

5.12 Outlook

The use of mesenchymal stem cells has advanced significantly in the last several years. There are now many reports which provide evidence that a stem cell therapy may also be a modality for the treatment of various forms of tendon disorders including the repair of tendon-to-bone junctions. The tendon/ligament-bone attachment sites are susceptible to injury and, unfortunately, the current regenerative technologies fail to restore the function and the anatomic structure of tendon/ligament entheses. To re-establish the biomechanical properties of tendon/ligament attachment sites, a full regeneration of this structure is required. In a study characterizing the biomechanical properties of tendon/ligament-to-bone interfaces, Moffat *et al*. suggest the construction of a multiphased scaffold to support the establishment of distinct and continuous tissue regions observed at the native interface [60]. Results of such a biomimetic strategy have already been reported [83, 84].

The establishment of stem cell-dependent regeneration of tendon/ligament-bone interfaces might be considered as another critical step in the regeneration of functional entheses. Several studies now document that MSCs seem to be very effective in regenerating entheses so that the tendon firmly integrates into bone and forms a fibrocartilage interface as well. The ability of adult stem cells to form tendon/ligament attachment sites if they possess both a distinct tendinous/ligamentous and osteogenic capacity may be important especially in combination with the biomimetic modalities suggested before to efficiently reconstruct soft tissue-to-bone interfaces.

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Olle Ringdén and Behnam Sadeghi

6 Mesenchymal stem cells for clinical/therapeutic interventions of graft-versus-host disease

Abstract Mesenchymal stem cells (MSCs) differentiate into several tissues of mesenchymal origin. MSCs express HLA class I, but do not express HLA class II on the cell surface. MSCs have little immunogenicity and are not extensively lysed by allogeneic cytotoxicT- cells (CTLs) or natural killer (NK) cells. MSCs inhibit alloantigen-inducd T-cell activation in vitro in mixed lymphocyte cultures (MLCs) and CTLs. Such an effect was observed regardless of major histocompatibility system (MHC) incompatibility between MSCs and responder or stimulatory cells in MLCs. MSC inhibition of CTL-mediated lysis is caused by soluble factors. Epstein-Barr virus (EBV) and cytomegalovirus (CMV) induced proliferation and interferon-γ (IFN-γ) production in vitro were not affected by third party MSCs in contrast to CTLs to acells (FMCs) Decidual stromal cells (DSCs) from placenta of maternal origin also inhibiCs. DSCs FMCs suppressed the production of IFN-γ and IL-17 and stimulated section of IL-10. FMDSC inhibition is contact-dependent in contrast to MSCs.

Because MSCs have immunomodulatory effects, we introduced them to treat therapy-resistant acute graft-versus-host disease (GvHD), a life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT). MSCs completely reversed severe acute GvHD, especially in children, but unfortunately not in all patients. Following our initial clinical studies, MSCs have been published for treatment of acute GvHD in 190 patients, and for chronic GvHD in 61 patients. The complete response rate was reported to be 52% and 26% in the two groups, respectively. A prospective randomized study for treatment of steroid-refractory acute GvHD was also performed by th siris Company in the U.S.A Following this, MSCs have been registered for treatment of steroid-refractory acute GvHD in children in Canada and New Zealand.

6.1 Clinical graft-versus-host disease

GvHD is a major obstacle after HSCT [1]. In experimental animals, severe skin lesions, wasting and diarrhea was seen in mice receiving bone marrow from allogeneic animals which was not observed in recipients of grafts from syngeneic animals. T cells from the donor graft are responsible for triggering GvHD and are activated by recipient MHC antigens [2]. In humans, the MHC consists of the HLA class I and class II antigens. Antigen-presenting cells (APCs) present MHC antigens to CD4+ helper T cells that recognize antigens with HLA class II molecules [3]. IL-1 produced by monocytes stimulates CD4 cells, which release IL-2 with a subsequent activation of CD8+ so-called cytotoxic T cells. The CD8+ cells react with MHC class I targets. Macrophages

and NK cells are also activated. MHC class II expression is enhanced by IFN-γ which further activates T cells and NK cells.

In humans, targets for acute GvHD are skin, gut and liver. Severity of acute GvHD is graded from 0 to IV [4, 5]. GvHD grade 0 is a limited skin rash and grade IV is a life-threatening disorder, often involving skin, gut and liver. Skin GvHD presents as a maculopapular skin rash and with epidermal loss. Gastrointestinal GvHD presents with diarrhea, abdominal pain and in severe cases with hemorrhages. Acute GvHD in the liver is detected with increased bilirubin and sometimes elevated liver enzymes. Acute GvHD of clinical significance grades II–IV occurs in 35–40 % of recipients of grafts from HLA-identical sibling donors. Using unrelated donors, this risk is slightly increased. GvHD is accompanied by a severe immunological deficiency and infections by bacteria, fungi and virus are frequent and may be fatal [5, 6]. MHC disparity is a major risk factor for acute GvHD. Additional risk factors include a female donor to a male recipient, treatment with granulocyte colony-stimulating factor (G-CSF), older age, white/black versus Asian/Hispanic race, among others [7–9]. Acute GvHD can be prevented in experimental animals and in humans by T cell depletion of the graft.

Most often, unmanipulated bone marrow or peripheral blood stem cell grafts are infused and the patients are treated with prophylactic immunosuppression to prevent GvHD. Most commonly, a calcineurin inhibitor is combined with a short course of methotrexate [10.

First-line treatment for acute GvHD includes prednisolone (1–2 mg/kg/day), which is tapered dependent on response. The outcome is dismal for patients with steroid-refractory acute GvHD. Several immunosuppressive drugs have been tried in such patients including antithymocyte globulin (ATG), monoclonal antibodies against the T cell receptor, IL-2 receptor antibodies, antitumor necrosis factor-α (TNF-α) antibodies, recombinant human IL-1 receptor antibodies, psoralene with ultraviolet light (PUVA), thalidomide, denileukin diftotoxin, methotrexate, rapamycin, mycophenolate mofetil, pentostatin, alefacept, and more. The wide variety of immunosuppressive agents used is due to the poor outcome when second line therapy is introduced. We were the first to use MSCs for life-threatening acute GvHD [11, 12].

6.2 Chronic graft-versus-host disease

In general, chronic GvHD appears three months or later after HSCT [5, 13]. Chronic GvHD resembles several autoimmune disorders. Clinical manifestations of chronic GvHD include skin disease, sicca syndrome, keratoconjunctivitis, mucositis, strictures in the esophagus and vagina, malabsorption, wasting, liver disease with elevated liver enzymes, obstructive bronchiolitis, neuropathy and myositis. Chronic GvHD of the skin may look like lichen ruber planus. If untreated and in severe cases, scleroderma may appear. A rare but severe complication is contractures of tendons. Obstructive bronchiolitis is a severe condition, which is difficult to treat and in the

long run the only curative treatment may be a lung transplant. In most patients with chronic GvHD, the disease is mild and the only symptom may be sicca syndrome with a dry mouth and dry eyes. However, for the few patients with severe diseases, the disorder may be extremely disabling with involvement of several organs. Patients with chronic GvHD often suffer from gram-positive bacteria with sinuitis and pneumonia due to impaired immune function [14]. Chronic GvHD occurs in between 20 and 50 % of HSCT patients. Reactivation of herpes viruses, especially CMV, is common. Classification is limited or extensive based on organs involved [15] or mild, moderate or severe, based on the judgment of the treating physician [16]. The National Institutes of Health (NIH) have developed a consensus development project to measure the various organ involvements during chronic GvHD [17]. Like acute GvHD, first-line treatment for the chronic disease is steroids. This can be combined with a calcineurin inhibitor [18]. For patients who do not respond to this therapy, a variety of immunosuppressive drugs have been used [5]. Second-line therapies for chronic GvHD that have been used are thalidomide, 1 Gy total lymph node irradiation, mycophenolate mofetil, rapamune, extracorporeal PUVA, anti-B-cell antibodies, imatinib and also MSCs [5, 13, 19].

6.3 Rationale to use mesenchymal stromal cells for treatment of GvHD

MSCs have immunosuppressive effects and inhibit T cell alloreactivity in mixed lymphocyte cultures (MLC) [20, 21]. They were also found to prolong skin allograft survival in baboons. T cell mitogenic responses are also inhibited by MSCs. MLC was constantly inhibited by MSCs in high concentrations (5–50 %), but was variably inhibitory or stimulatory of MLC when used at low concentrations (0.01 % to 1 %) [21]. MSCs were also found to suppress MLC after differentiation to osteocytes, chondrocytes and adipocytes [22]. After stimulation with IFN-γ, MSC inhibition of MLC was potentiated. MSC also inhibited the development of cytotoxic T cells (CTLs), but did not inhibit NK cell mediated lysis of K562 [23]. Inhibition by MLC was most probably caused by a soluble factor, because MSCs inhibited response in MLC also when the cell populations were separated by a transwell membrane. Maccario and co-workers showed that MSCs increased CD4+, CD25+, FOXP3 regulatory T cells (Tregs) and activated T cells [24]. To induce Tregs, MSC secretion by HLA-G was required [25]. Factors that are involved in MSC-mediated immune inhibition are IFN-γ, IL-1β, transforming growth factor-β (TGF-β), indoleamine 2,3-deoxygenase (IDO), IL-6, IL-10, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), nitric oxide (NO), hemoxygenase-1 (HO-1) and HLA-G [26–30]. IDO is up-regulated by MSCs and IDO depletes tryptophan which induces the metabolit kynurenine which depletes T cells.

MSCs also affect dendritic cells (DCs). Among other things, surface expression of class II molecules, CD11c, CD83 and co-stimulatory molecule and IL-12 production

are decreased which impair the antigen-presenting capacity of dendritic cells. MSCs also inhibit the dendritic cell production of TNF-α [30, 31]. We reported that MSCs stimulated IgG secretion [32]. B-cell proliferation was reported to be inhibited by high concentrations of MSCs (1:1) [33]. After macrophages were co-cultured with MSCs *in vitro,* they expressed high levels of IL-10 and IL-6, low levels of IL-12 and TNF-α and showed a high level of phagocytic activity [34]. These activated macrophages may have a significant role in tissue repair. Furthermore, MSCs attract proinflammatory M1 macrophages which have antimicrobial activities [35]. MSCs can further repolarize these macrophages into anti-inflammatory M2 macrophages that promote wound healing [36]. Wang and co-workers also showed that chemokine monocyte chemoattractant protein-1 (MCP-1) in cerebral ischemic tissue promotes migration of infused MSCs to the site of injury [37]. It seems that MSCs are mobilized towards the site of damage during tissue injury. There is a bidirectional interaction between MSCs and inflammatory cells. MSCs can release several growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), angiopoetin-1 (Ang-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), adrenomedullin and stromal cell-derived factor (SDF). They are generated by proinflammatory stimuli, such as TNF-α, lipopolysaccharide (LPS) and hypoxia in a nuclear factor kappaB (NFκB)-dependent manner [38]. MSCs' effect on wound healing is due to the secretion of cytokines and growth factors and is attributed to inhibition of inflammation and angiogenesis as suggested by a rat corneal damage study [39]. Of course, the exact mechanism by which MSCs promote tissue healing and regeneration may be different for different types of injuries and toxicity. Stromal cell-derived factor-1α (SDF-1α) and CXCR4 interactions are involved in the migration of MSCs to target tissue [40].

Some studies show that MSCs need to be licensed to become immunosuppressive. Thus, in some studies, MSCs needed to be pretreated with IFN-γ, sometimes together with TNF-α or IL-1 to induce optimal immunosuppression [41]. Mycophenolate mofetil was shown to enhance the immunosuppressive effect of MSCs by promoting the survival of heart allografts in a mouse model [42]. In contrast, cyclosporine diminished the immunosuppressive effect by MSCs. This discrepancy may be due to that cyclosporine inhibits IFN-γ production which is not inhibited by mycophenolate mofetil. If MSCs are treated with IFN-γ, MHC class II are upregulated and immune inhibition is enhanced [22].

Nitric oxide in high concentration can inhibit immune responses. Murine MSCs express inducible NO upon stimulation with IFN-γ in combination with TNF-α or IL-1 [43]. There is a species difference in MSC-mediated immunosuppression. Mouse MSCs uses NO, whereas human and monkey MSCs use IDO as their immunosuppressive molecule.

It seems that MSCs need both contact dependent and independent interactions. A direct cell contact is required between MSCs and CD4+ T cells to achieve maximal reduction of FOXP3 Tregs [44].

Thus, MSCs seem to have a dual role in affecting acute and chronic GvHD. MSCs induce monocytes and macrophages which start a wound healing process. At the site of injury, MSCs themselves seem to have an immunomodulatory effect and decrease alloreactivity. These two properties seem to be the reason why MSCs have shown dramatic effects on some patients with severe acute GvHD [11, 12, 45].

6.4 Experience of MSCs in clinical acute graft-versus-host disease

In our *in vitro* experiments we found that MSCs effectively decreased alloreactivity in MLC [21–23]. We also know that MSCs could prolong skin allograft survival in a baboon model by a few days [20]. We also know that it was safe to infuse MSCs to patients [46]. With this background I (OR) decided to treat a 6-year old boy with grade IV acute GvHD with abdominal pain and watery hemorrhagic diarrhea who, apart from cyclosporine, had been treated with high-dose prednisolone, several pulses of methylprednisolone, psoralene and ultraviolet light (PUVA) treatment, extracorporeal PUVA, infliximab, daclizumab, mycophenolate mofetil and methotrexate. Despite all these attempts, GvHD progressed. In this situation, I decided to try MSCs. It was my idea, decision and responsibility. The boy with acute lymphoblastic leukemia (AML) had received HSCT from an unrelated donor. I aspirated bone marrow from the boy's mother and after three weeks of culture, 90×10^6 MSCs were harvested. 60×10^6 MSCs were infused on day 73 (Fig. 6.1) [11]. After one week, stool normalized and after two weeks he could start eating. Bilirubin declined within a couple of weeks. Due to presence of minimal residual disease, cyclosporine was discontinued. By day 150, diarrhea, abdominal pain reappeared and bilirubin increased dramatically. A second dose of MSCs (30×10^6) was infused, which had been stored frozen in liquid nitrogen. This time it took a little longer before stool and bilirubin normalized (Fig. 6.1).

Eight patients with steroid-refractory acute GvHD were included in our initial Phase I compassionate use trial [12. In one of the patients, we could demonstrate MSC donor DNA in colon and lymph node, the target organs for GvHD in this patient. Among these, six patients had complete response, out of which one developed CMV gastroenteritis and died. Two patients did not respond at all and died from progressive GvHD. Survival among these eight patients was compared to 16 control patients with colonoscopy-verified gastrointestinal therapy-resistant grades III and IV acute GvHD, and the MSC group had a statistically significantly better survival ($p<0.03$). This promising trial encouraged a larger multicenter Phase II study, which included 55 patients from five centers [45].

In the multicenter study, all patients received bone marrow-derived MSCs expanded in fetal calf serum (FCS). The doses infused to the patients ranged from 0.4 to 9.0×10^6 cells/kg from five HLA-identical sibling donors, 18 haploidentical donors and 69 unrelated HLA-mismatched donors. MSCs were given from one infusion in 27 patients, two infusions in 22 patients, four infusions in three patients and one patient

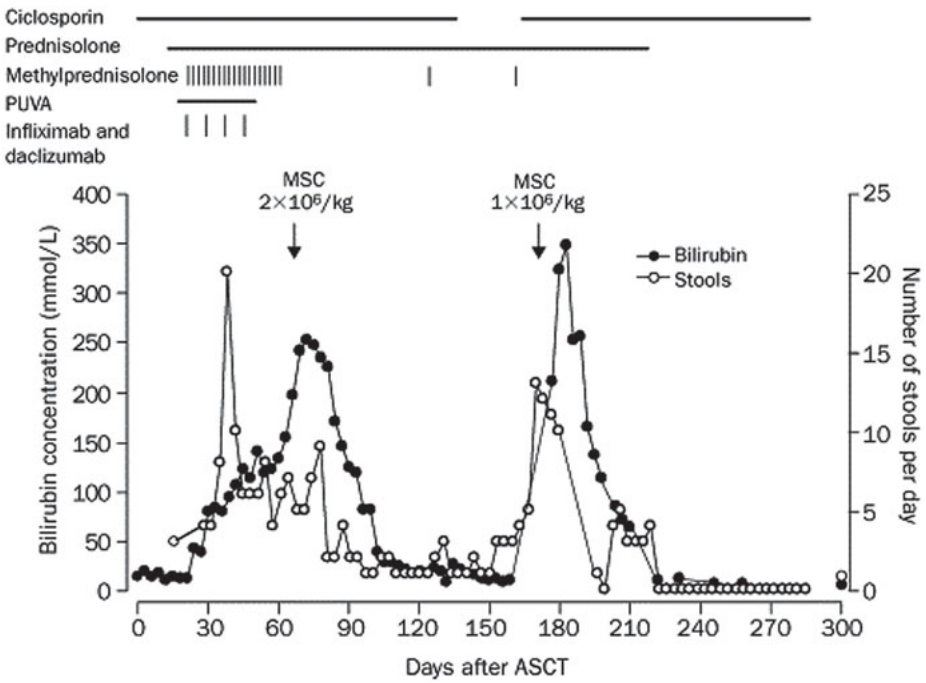

Fig. 6.1: Clinical course and immunosuppression of the patient ↓ = mesenchymal stem-cell transplantation. ASCT = allogeneic stem-cell transplantation. MSC = mesenchymal stem cells. (From [11], with permission from The Lancet)

received four infusions and one received five infusions. The MSCs were from passage 1 to 2 in 56 infusions and from passage 2 to 4 in 36 infusions. Complete response was seen in 55 % of the patients and children tended to have a better response, being 68 % as compared to 43 % in adults. Complete or partial response was seen in 2/5 of those receiving MSCs from HLA-identical sibling donors, 9/13 when haploidentical MSCs were used and 27/37 using third party MSCs. Complete responders had a 2-year survival of 52 % which was significantly better than 16 % for partial and nonresponder patients ($p<0.02$).

Following these studies, there have been 19 reports and altogether 190 patients treated for acute GvHD, most of them being refractory to steroids (Tab. 6.1). They have all been summarized previously [47].

The MSC dose ranged from 0.4 to 9.0 × 10^6/kg and the number of doses ranged from 1 up to 21. Complete response was reported in 98/190 (52 %), partial response in 44 (23 %) and nonresponse in 48 (25 %) of the patients. The uniform experience is that in no case have any side effects of infusion by MSCs been reported. There is also no report of ectopic tissue formation. In an autopsy study of 18 patients who were treated with MSCs, we found no signs of any ectopic tissue formation or malignant tumors of MSC donor origin [48].

Tab. 6.1: Mesenchymal stromal cells for treatment of acute graft-versus-host disease. A summary of published studies (from 21 publications) overall and according to expansion medium.

	No. of patients	MSC dose × 10^6/kg, range	No. and range of doses	Complete response	Partial response	Nonresponders
Summary, all patients	190	0.4–9.0	1–21	98 (52 %)	44 (23 %)	48 (25 %)
Dependent on expansion medium						
Fetal calf serum	134	0.4–9.0	1–21	80 (60 %)	29 (21 %)	25 (19 %)
Platelet lysate medium	24	0.6–2.8	1–5	6 (25 %)	3 (13 %)	15 (62 %)
Human AB-serum (all adults)	13	0.5–2.9	1–4	1 (8 %)	8 (62 %)	4 (30 %)

Abbreviations: CR, complete response; PR, partial response; NR, nonresponse; UC, umbilical cord; FMC, fetal membrane cells; NR, not reported

Among the confirmatory reports of MSCs for steroid-refractory acute GvHD, Fang and co-workers used adipose tissue-derived MSCs and reported that 5/6 patients had a complete response [49].

Among 13 adult patients who were given platelet lysate medium-expanded MSCs, 2/13 adult patients had complete response with partial response in five [50].

Ho and co-workers treated three adult patients with MSCs, around 1×10^6 cells/kg, with two complete responders and one nonresponder, who died shortly after infusion [51]. Müller *et al*. reported on two children with complete response after treatment with MSCs [52]. Kebriae and co-workers reported on a prospective randomized study where patients with grade II–IV acute GvHD were randomized to receive Prochymal® MSCs at either 2 or 8×10^6 cells/kg [53]. Median age of the adult patients included in the study was 52 years. MSCs were given for acute GvHD grade II (n = 21), grade III (n = 8) and grade IV (n = 2). There was no difference in response rate between those receiving 2 or 8×10^6 MSCs/kg. Initial response to Prochymal® therapy was 94 % with a complete response seen in 77 % of the patients. Twelve children with therapy-resistant grades III and IV acute GvHD were included in a Prochymal® study [54]. Two children were treated with 8×10^6 MSCs/kg and the remaining ten were treated with 2×10^6 MSCs/kg twice a week for four weeks. The age of the children ranged from 0.4 to 15 years of age. Complete response was observed in 7 (58 %), partial response in 2 (17 %) and mixed responses were seen in 3 (25 %) in this pediatric cohort. Complete resolution of gastrointestinal GvHD was observed in 9 (75 %) of the patients. At 100 days, survival was 58 % after Prochymal® therapy.

Osiris Therapeutics, Inc. also performed a prospective double-blind placebo-controlled Phase III study for grades II–IV acute GvHD (Tab. 6.2) [55]. The primary endpoint was complete response at 28 days after infusion of Prochymal®. The patients were randomized to Prochymal® versus placebo 2:1 among 192 patients included in

the trial. Complete response at 28 days did not differ between the Prochymal® and placebo groups, most suffering from GvHD of the skin. Among patients with GvHD of the liver, complete response was seen in 75 % in the Prochymal® arm, as opposed to 47 % in the placebo group (p = 0.026). Durable complete liver response was reported in 29 % and 5 % in the two groups, respectively (p = 0.046). Patients treated for gastrointestinal acute GvHD treated with Prochymal® had a complete response rate of 88 % compared to 64 % in the placebo arm (p = 0.018). This prospective randomized study included 28 children. Children receiving Prochymal® had a complete response rate of 86 % as opposed to 57 % in those treated with placebo (p = 0.094). Based on these three studies using Prochymal® for acute GvHD, Prochymal® has been registered in Canada and New Zealand for treatment of steroid-refractory acute GvHD in children.

Nine children with severe acute steroid-refractory GvHD were treated with platelet lysate-expanded MSCs [56]. The median MSC dose was 1.2 × (range 0.7–2.8) × 10^6 MSCs/kg. Three of the children had complete response and two had a partial response and four were nonresponders.

Tab. 6.2: Osiris studies of MSC Prochymal Phase III acute GvHD trial, protocol 265, for grades II–IV acute GvHD (cf. [55]).

Endpoint	No. of patients	Prochymal	Placebo	P-value
CR for 28 days	192	45 %	46 %	ns
Response of liver GvHD	61	76 %	47 %	0.026
Durable complete liver response	61	29 %	5 %	0.046
Gastrointestinal GvHD	71	88 %	64 %	0.018
Children, response rate	28	86 %	57 %	0.094

In 134 cases, the MSCs were expanded in FCS and in those patients complete response was seen in 60 % (Tab. 6.1). In three studies, MSCs were expanded in platelet lysate [50, 56, 57]. A complete response rate was 25 % (6/24) with a partial response in 13 % and 62 % were nonresponders (Tab. 6.1). Three patients with acute GvHD grades II and seven with grades III–IV were treated with MSCs expanded in autologous serum. Complete response was noted in one patient, partial response in six, whereas three were nonresponders (Tab. 6.1) [58]. Arima and co-workers expanded bone marrow-derived MSCs in donor serum. They injected MSCs intra-arterially to the mesentery artery in three patients with steroid-refractory acute gastrointestinal GvHD with partial response in two and no response in one [59]. Among 13 adult patients treated with MSCs expanded in human serum, there was only one complete response and the overall response rate was 70 % (Tab. 6.1). It is not possible to compare here which expansion medium is optimal, because these are different patient populations regarding severity of GvHD and there were mainly adult patients receiving MSCs expanded in platelet lysate and

human AB-serum. Among the 114 patients receiving MSCs expanded in FCS, a majority were children, who have a better response to MCS therapy.

Some studies with few patients included have also been reported. Thus, Müller and co-workers reported on two children with acute GvHD after treatment with MSCs did not progress to chronic GvHD [52]. He also reported on a child who developed hemophagocytosis and suffered from three-lineage failure, both which resolved after MSC therapy. Muroi reported on two patients treated with MSCs who did not respond [60]. Lim *et al.* reported on an 18-year old woman with severe acute GvHD with diarrhea and jaundice, who did not respond to corticosteroids, tacrolimus and mycophenolate mofetil. She was given MSCs, 2×10^6/kg at two doses and had a partial response. Liver function normalized, she had no diarrhea, but colonoscopy showed multiple ulcers in the entire colon [61]. Sato and co-workers reported on a patient with severe acute GvHD with persistent bloody diarrhea, abdominal cramps and peritonitis with free gas suggesting colon perforation. After treatment of MSCs from the bone marrow of the same donor who had given hematopoietic peripheral blood stem cells, abdominal free air disappeared, but diarrhea and abdominal pain did not completely disappear. The patient was discharged, but later died of septic shock. We also reported that peritonitis due to colon perforation and abdominal defense was reversed twice by infusion of MSCs in a 64-year-old woman [62].

Wu and co-workers also reported on two children with severe steroid-refractory acute GvHD who responded to umbilical cord blood-derived MSCs, expanded in fetal calf serum and given at a dose of 3.3 and 8×10^6 cells/kg to the two patients, respectively [63]. In a recent study, Chen *et al.* reported on two patients with grade II, five patients with grade III and twelve patients with grade IV acute GvHD who received a total of 58 infusions of cord-derived MSCs at a dose ranging from 0.6 to 7.0×10^6 cells/kg. The median dose was 2.1×10^6 cells/kg [64]. Seven patients received one infusion, two patients received two infusions and ten patients received three or more infusions. Complete response was seen in eleven patients, four had partial response and four did not respond. Eleven of the patients survived, six died from acute GvHD, one from infection and one from leukemic relapse.

There are only sparse reports regarding long-term outcome in patients treated for acute GvHD with MSCs.

We followed 31 patients treated with MSCs for acute GvHD (n = 23) and hemorrhagic cystitis (n = 8) between 2002 and 2007 [65]. Among patients who received MSCs from passage 1–2, the survival was 75 % as opposed to 21 % for those receiving MSCs from passage 3–4 ($p<0.01$). However, long-term survival was poor. Another study showed that MSC-treated patients more often died from invasive fungal infection compared to controls with severe acute GvHD and not treated with MSCs [66].

6.5 Treatment of acute GvHD with stromal cells from alternate sources, adipose tissue-derived, umbilical cord blood-derived or fetal membrane-derived stromal cells

Harvesting of the bone marrow is necessary to develop bone marrow-derived MSCs. Therefore, alternative sources have been thought of, such as adipose from plastic surgery, umbilical cord or placental tissue (Tab. 6.3).

Tab. 6.3: Treatment of acute graft-versus-host disease with stromal cells from alternate sources, adipose tissue, umbilical cord or fetal membrane cells from placenta.

Study Author, year [ref.]	Source of stromal cells	No. of patients	Age, median (range)	MSC dose × 10^6/kg	Doses	CR	PR	NR
Fang 2007 [49]	Adipose	6	40 (22–49)	1–2	1–2	5	–	1
Fang 2007 [67]	Adipose	2	12, 15	1–2	1–2	2	–	–
Wu 2011 [63]	UC	2	4, 6	3.3–8	1–3	2	–	–
Chen 2012 [64]	UC	19	NR*	0.6–7.2	1–>3	11	4	4
Ringdén 2013 [70]	FMC	8	57 (1–64)	0.9–2.8	1–2	2	4	2

* Abbreviations: CR, complete response; PR, partial response; NR, nonresponse; UC, umbilical cord; FMC, fetal membrane cells; NR, not reported

Fang *et al.* used adipose tissue-derived MSCs in eight patients [49, 67]. Most of the patients had complete response to this therapy and there was only one nonresponder (Tab. 6.3). There were two studies including altogether 21 patients who were treated with stromal cells derived from umbilical cord [63, 64]. The first study included two children who both responded to stromal cell therapy for their GvHD. In the second study, the age of the patients is not known. These patients received from one up to more than three doses of stromal cells and most of the patients responded (Tab. 6.3).

The advantages of using placenta-derived MSCs are an unlimited supply, no need for invasive procedures and no ethical consideration. Human placenta-derived MSCs have multilineage differentiation potential and inhibit MLC and mitogenic lymphocyte proliferation [68]. We found that decidual stromal cells (DSCs) from the decidua being of maternal origin had the strongest inhibition in MLC, compared to bone marrow-derived MSCs, stromal cells from umbilical cord and placental Willi cells [69]. The DSCs were positive for MSC markers, negative for hematopoietic markers, expressed HLA class I, but not HLA class II. DSCs expressed high levels of adhesion molecule CD49d and CD54. Stromal cells from cord and placenta induced IL-17 secretion, but not DSCs. DSCs needed contact with stimulator and responder cells in MLR, in contrast to bone marrow-derived MSCs (Erkers T, Nava S, Yosef J, Ringden O, Kaipe H. Decidual stromal cells promote regulatory T cells and suppress alloreactivity in a cell-contact-dependent manner. Stem Cells and Development, in press 2013). In

MLC, DSCs increased the frequency of CD4+ CD25 high FOXP3+ Tregs (T-reg) and augmented the intensity of CD25 expression of CD4+ T cells. By blocking the activity of IFN-γ, IDO, prostaglandin E2, and PD-L1 the possibility of DSCs to inhibit in MLC was exhibited. Naturalization of IDO also reduced the frequency of T-regs.

We also used DSCs for treatment of severe steroid-refractory grades III-IV acute GvHD [70]. Median age in these patients was 57 years (range 10 months – 64 years). Two patients had complete response, four had partial response with an overall response rate of 75 % and two were nonresponders. None of these patients had any acute side effects of DSC infusion. However, a deteriorating patient with acute GvHD grade IV where palliative care had started experienced seizures after infusion of DSCs. This is the only patient among 443 treated with stromal cells for GvHD where an acute side effect has been reported. It is probable that the seizures were due to the patients' poor condition, rather than the infusion of DSCs.

6.6 Mesenchymal stromal cells for treatment of chronic graft-versus-host disease

Chronic GvHD resembles autoimmune disorders and therefore, treatment of this disorder may be also relevant for autoimmune disorders, such as Crohn's disease (CD), ulcerative colitis (UC), rheumatoid arthritis (RA), multiple sclerosis (MS), system lupus erythematosis (SLE), *etc*. We were the first to use MSCs for the treatment of chronic GvHD in a 27-year old male with chronic myeloid leukemia. He developed extensive chronic GvHD of the skin with lichenoid changes all over his body and a slight increase in liver enzymes. He received cyclosporine and prednisolone and was given 1×10^6 MSCs/kg on day +153 after transplant when ALT was 3.61 μkat and AST 0.9 μkat/l. The lichenoid skin changes did not improve, but the liver enzymes declined [12. This case may therefore be judged as a partial response.

Subsequently, anecdotal reports of MSCs for the treatment of chronic GvHD are increasing [71].

Fang reported on one patient treated with adipose tissue-derived MSCs for chronic GvHD who had a complete response [49]. Müller *et al*. treated three patients with chronic GvHD, one of whom had slight improvement [52]. Two patients did not respond and one of them died of Epstein–Barr virus post-transplant lymphoproliferative disorder (EBV-PTLD). EBV-PTLD was also the cause of death in our patient treated with MSCs for chronic GvHD [12. The third patient died from chronic GvHD. Subsequently, Zhang and co-workers treated twelve patients with chronic GvHD [72]. Three patients had complete response and could discontinue all immunosuppressive drugs. Six patients had partial response and three did not respond (Tab. 6.4). In the twelve patients, he saw complete resolution in the skin (3/12), obstructive bronchiolitis (1/3), joints (1/5), liver (3/10), mucositis, sicca syndrome of the mouth (4/12), and eye (2/7).

Zhou treated four patients with sclerodermatous chronic GvHD with platelet lysate-expanded MSCs, 10 to 20 × 10^6 cells/kg from 4 up to 8 doses per patient [73]. The MSCs were given by intra bone marrow injection. Symptoms gradually improved in all four patients. They also studied the ratio of helper T cell lymphocytes (Th1 cells to Th2 cells) which was dramatically reversed, with an increase in Th1 and a decrease in Th2.

A larger study using MSCs for chronic GvHD constituted 19 patients with refractory chronic GvHD who were treated with 1 to 5 doses of 0.2 to 1.4 cells/kg [74]. Response was seen in 14/19 (74 %) of the patients according to NIH criteria. For chronic GvHD of the skin, response rate was 78 %. One out of three patients with sclerodermatous chronic GvHD had a partial response. Higher cumulative responses were noted in oral mucosa, the gastrointestinal tract and the liver, where it was about 90 %. A patient with obstructive bronchiolitis did not respond and subsequently died from invasive fungal infection. In this report, clinical improvement of chronic GvHD was accompanied by an increased ratio of CD5+, CD19+/CD5– CD19+ B-cells and CD8+, CD28–/CD8+ CD28+ T cells. Two-year survival was 78 %.

A pediatric study included five patients with chronic GvHD, two with overlap syndrome – *i.e.*, acute GvHD progressing to chronic disease [56]. Four children received one dose of around 1 × 10^6 MSCs/kg. One had complete response of skin and mucosa. Three patients had partial response, reported in skin, mucosa, mucosa and liver, and skin and liver which was temporary. One of these children received four doses. One child had no response of chronic GvHD in skin.

Lim and co-workers reported on a 46-year old man with AML who underwent HSCT and developed skin rash, tongue erosion and ocular sicca 122 days after transplantation. In addition, he developed progressive jaundice. He received two doses of 1 × 10^6 MSCs/kg, bilirubin dropped and liver enzymes normalized after a second dose of MSCs. Following a third dose of 0.5 × 10^6 MSCs/kg, GvHD of skin, eye and oral cavity showed regression, but no complete response [61].

Eight adult patients were treated with MSCs, ranging from 0.2 to 1.2 × 10^6 cells/kg with five patients given one dose, two patients given two doses, two treated with three doses and one was given four doses [58]. One patient with sicca in the mouth and slight thrombocytopenia had a complete response. Three patients had partial response, all had chronic GvHD of the gastrointestinal tract, one in addition had sicca in mouth and eyes with exanthema and the third patient also had musculoskeletal involvement. In this series, there were four nonresponders of whom two died from GvHD and toxicity, respectively.

More recently, Hermann and co-workers reported on seven patients treated with MSCs for chronic GvHD given from 2 up to 11 doses with a median of 7 [75]. He saw complete response in two patients, one with skin manifestations and one with oral mucositis. Four patients had partial response with the following resolutions, one of mucositis, two with keratoconjunctivitis sicca and one of the skin. Three of those patients also had obstructive bronchiolitis that did not improve at all. A patient with

Table 6.4: Mesenchymal stromal cells used for treatment of chronic graft-versus-host disease according to published studies (n = 10).

Study Author, year [ref.]	Source of stromal cells	Expansion medium	Cell dose × 10^6/kg	Range of doses	No. of patients	CR	PR	NR
Overall outcome			0.2–20	1–11	61	16 (26 %)	29 (48 %)	16 (26 %)
Ringdén 2006 [12]	BM	FCS	0.6	1	1		1	
Fang 2007 [49]	Adipose	FCS			1	1		
Muller 2008 [52]	BM	FCS	0.4–3	1–3	3		1	2
Zhang 2009 [72]			1–2.1	1–3	12	3	6	3
Zhou 2010 [73]	BM	Plt-L	10–20	4–8	4	4	–	–
Weng 2010 [74]	BM	FCS	0.2–1.4	1–5	19	4	10	5
Lucchini 2010 [56]	BM	Plt-L	0.7–2.8	1–5	5	1	3	1
Lim 2010 [61]	BM	FCS	0.5–1	3	1		1	
Perez-Simon 2011 [58]	BM	AB-serum	0.3–3.7	1–3	8	1	3	4
Herman 2012 [75]	BM	FCS		2–11	7	2	4	1

Abbreviations: CR, complete response; PR, partial response; NR, nonresponders; BM, bone marrow; FCS, fetal calf serum; Plt-L, platelet lysate;

chronic GvHD of the liver did not respond at all despite 11 doses and died from liver failure. Three patients with obstructive bronchiolitis all died of respiratory failure, two with chest infections.

In the overall response rate among 61 patients published and treated for chronic GvHD, the complete response rate was 26 %, with partial response in 48 % and no response in 26 % of cases (Tab. 6.4).

6.7 Clinical trials of prophylaxis with mesenchymal stromal cells for graft-versus-host disease

MSCs may be given together with the graft to enhance engraftment and/or for the prevention of graft failure and GvHD. The rationale for these indications are that MSCs produce several growth factors of importance for hematopoietic stem cells in addition to their immunosuppressive effects [76]. The first safety study of co-transplantation of MSCs at the time of HSCT was performed by Lazarus *et al.* using MSCs from the HSCT donor. This study showed that it was safe to infuse MSCs and that there was a relatively low probability of grades II–IV acute GvHD, being 28 % (Tab. 6.5). We treated eight patients with MSCs to enhance engraftment and for previous graft failure with promising results from this preliminary study. Among other things, we saw that a patient with severe aplastic anemia and retransplantation had good recovery and

Table 6.5: Mesenchymal stromal cells used as prophylaxis for graft-versus-host disease or to promote engraftment.

Study Author, year [ref.]	No. of patients	MSC dose × 10^6/kg	Outcome, findings
Lazarus 2005 [46]	46	1–5	Safe, GvHD II-IV 28 %
Le Blanc 2007 [77]	8	1	Safe
Ball 2007 [79]	14	1–3.3	Faster leukocyte recovery. Trend for less graft failure vs. historic controls.
Macmillan 2009 [78]	11	0.06–5	75 % of cord blood grafts had platelet engraftment.
Gonzalo-Daganzo 2009 [80]			
Baron 2010 [81]	20	1–2	Less GvHD and infectious death. TRM 10 %.
Bernardo 2011 [87]	13	1–3.9	Less GvHD vs. retrospective controls. No death by GvHD.
Liu 2011 [82]	27 vs. 25 randomized controls	0.3–0.5	Faster platelet engraftment.
Kuzmina 2012 [83]	19 vs. 18 randomized controls	0.9–1.3	Acute GvHD II+, 5 % vs. 39 %
Berglund 2012 [84]	7	1	Reduced survival vs. other recipients of cord blood transplants

Abbreviations: TRM, transplant related mortality

resolution of Henoch–Schönlein purpura [77]. Macmillan *et al*. infused MSCs together with cord blood transplants in an attempt to speed up hematopoietic recovery [78]. Among eight evaluable patients, all achieved neutrophil engraftment at a median of 19 days. Ball and co-workers did co-transplantation of MSCs with haploidentical HSCT transplants in children [79]. Compared to retrospective controls, they saw less rejection of the haploidentical HSCT and a faster engraftment of platelets. Gonzalo-Daganzo and co-workers also performed a pilot study with third party MSCs combined with cord blood transplants [80]. In a randomized Phase II study, 55 patients undergoing haploidentical HSCT were randomized to co-infusion with MSCs, 3–5 × 10^5 cells/kg or no cells [80]. The MSCs group had faster time to reach more than 50 × 10^9 platelets/L, 22 days as opposed to 28 days in the controls ($p = 0.04$).

The concentrations of SDF-1α, thrombopoietin and IL-11 were elevated in the MSC group compared with the controls. Acute GvHD and survival did not differ between the two groups. Baron and co-workers did co-transplantation of MSCs at the time of HSCT in patients undergoing nonmyeloablative conditioning [81]. The probability of acute GvHD grades II–IV was 45 % in the MSC group as compared to 56 % in retrospective controls. Death from GvHD or infection was 10 % in the MSC group as opposed to 31 % in the con-

trols. A randomized study showed that the MSC patients had faster platelet engraftment [82]. In a randomized prophylactic study, MSCs were given at a dose of around 1×10^6 cells/kg at the time of blood count recovery [83]. Acute GvHD grades II–IV developed in 5 % of the MSC-patients as opposed to 39 % in the controls ($p = 0.002$). Overall mortality was 5 % and 17 % in the two groups, respectively. In a retrospective study from our unit, MSCs, 1×10^6/kg, were given at the time of transplantation to seven recipients of cord blood transplants [84]. These patients had a decreased overall survival in multivariate analysis. The causes of death were relapse in three cases and infection in three patients.

6.8 Discussion on clinical use of mesenchymal stem cells

MSCs have created a lot of interest in HSCT because they support hematopoiesis and have immunomodulatory properties and were shown to reverse life-threatening steroid-resistant severe acute GvHD [11, 12, 45, 49, 50, 52–61]. Including the Prochymal® Phase III study, there are 382 patients reported to have been treated for acute GvHD (Tabs. 6.1 and 6.2). Among the 190 published patients, a complete response rate of 50 % is encouraging and only 25 % did not respond at all (Tab. 6.1). However, some of these patients were at an early stage and it is unclear how many of those may have responded to more conventional immunosuppressive therapy. There may also be a bias with more successful cases reported. There are probably many more patients treated and it is less likely that those with a poor outcome are reported to the same extent as those with a favorable outcome. Although the prospective randomized Prochymal® study did not show any difference in the primary endpoint, CR at 28 days (Tab. 6.2), it was encouraging to see that response of the liver and gastrointestinal tract was better in the Prochymal® compared to the placebo arm [55]. There is a lack of prospective randomized studies of MSCs for severe acute GvHD. One reason is that each center has few cases. Another reason is the new regulatory issues on the use of stem cells within the European Union. Due to this, a planned prospective randomized study for steroid-refractory acute GvHD giving MSCs or placebo in a double-blind fashion only recruited patients from our center. There were only 20 patients included and the study required 90 patients to be conclusive. Now a new randomized study for the treatment of steroid-refractory acute GvHD will start in Holland and Italy.

Despite responses to MSC therapy, long-term survival has been poor [65]. This study suggested that low passage MSCs may have an advantage versus higher passages. This may be a problem using bone marrow-derived MSCs, because it can sometimes take several passages before there are enough MSCs to be used for transplantation. The Osiris study used MSCs in passage 5. This may thus not be the optimal passage number. Because half of the patients with steroid-refractory acute GvHD do not respond, there is need for improvements in this therapy. A cell-dose of around 1 to 2×10^6 cells/kg seems sufficient and a prospective study comparing 2×10^6/kg with 8×10^6/kg did not show any difference in response rate [53]. In some patients, it

seems like one dose is sufficient, but in others repeated doses may be required. Therefore, one probably should be open to giving several doses not just to responders, but maybe also to nonresponders.

An important issue is to find out why some patients respond and others do not. This does not seem to be due to HLA-compatibility, because one study showed that the response rate was the same whether HLA-identical, haploidentical or third party MSCs were used [45].

The fate of MSCs after infusion has also been discussed [76]. After infusion, they first home to the lung and thereafter to the spleen and liver. Thereafter, they seem to be distributed to almost all organs. In cases of tissue toxicity, they seem to home to damaged organs [12. Even though MSCs show very low immunogenicity compared to lymphocytes [22], it is likely that they are rejected. In the xeno situation it has been demonstrated that MSCs are rejected [85]. Therefore, it seems probable that also allogeneic MSCs are eventually rejected. However, it seems as they can exert their effects because HLA-mismatched third party MSCs are as effective as HLA-identical MSCs in the treatment of acute GvHD [45]. If not, autologous or HLA-identical disease would be proven to be more effective than allogeneic MSCs. The dramatic and positive effects seen in some patients with severe grade IV acute GvHD may be due to two mechanisms. Much focus has been on the immunosuppressive effect by MSCs on alloreactive T cells [76]. MSCs not only affect alloreactive T cells, but also dendritic cells and more or less suppress the whole immune system. This immunomodulatory effect may be utilized and may be local at the site of injury during acute GvHD in the gastrointestinal tract, the liver or the skin. However, a much more dramatic effect may be the wound healing where a completely damaged gastrointestinal tract can heal within a week after infusion of MSCs [11, 12]. This may be due to that MSCs home to the spleen and activate CD11b+ cells, which subsequently heal the damaged tissue. It was demonstrated that splenectomy prevented the MSCs from healing experimental enterocolitis. It was also demonstrated that bone marrow stromal cells prevented experimental colitis that required host CD11b+ cells [86]. Thus, there is an important cross-talk between MSCs and the innate immune system.

6.9 How should we best utilize MSC treatment of GvHD?

More than half of the patients with steroid-refractory severe grades III–IV acute GvHD seem to respond, although improved survival is only seen in those patients who have a complete response [45]. When MSCs are given at an earlier stage for grade II acute GvHD, the complete response rate is above 90 % [53]. Even if many of those patients may have responded to conventional therapy, it may be advantageous to treat with MSCs upfront at grade II acute GvHD, because MSCs do not have any side effects, in contrast to immunosuppressive drugs, which apart from an increased risk of infections have diabetogenic effects, nephrotoxicity, hepatotoxicity, neurotoxicity and

allergic reactions. Bernardo and co-workers treated cord blood transplant recipients at the time of transplant and also at grade II acute GvHD with MSCs. With this policy they saw no patient who developed grades III and IV acute GvHD and there was no death attributed to acute GvHD, compared to 26 % in historic controls ($p = 0.05$) [87]. Thus, early treatment for grade II acute GvHD seems today to be the best option, because there is no way to settle which patient will be a responder and who will be a nonresponder in patients with grades III–IV acute GvHD. Some patients who did not need MSCs may be treated, but there seems to be little to lose. Even if MSCs seem to suppress the immune system profoundly *in vitro*, there seems to be few, if any, side effects. Acute toxicity has not been reported, and there is no ectopic tissue formation [48]. Even if MSCs affect alloreactive CTLs, they did not seem to affect CTLs against viruses such as CMV or EBV [88]. We saw a significantly higher incidence of death by invasive fungal infection among patients with severe acute GvHD treated with MSCs, as opposed to patients with severe acute GvHD and not treated with MSCs [66]. MSCs induce IDO which depletes tryptophan which in turn induces accumulation of kynurenine which decreases T cell cytotoxicity. IDO plays an important role in limiting the inflammatory response to fungi [89]. By inducing Tregs and inhibit Th17, IDO and kynurenine contribute to provide the host with an immune mechanism against fungi. It is possible that IDO induction by MSCs may result in an over-stimulation of the inflammatory response, favoring invasive fungal infection. Patients with severe acute GvHD have a damaged gastrointestinal tract, are treated with heavy immunosuppression and often with antibiotics, all paving the way for fungal colonization. All these mechanisms taken together may explain the high incidence of death from invasive fungal infection. Further studies are needed to find out if MSCs interact with immunity against fungi.

Many patients, especially with severe gastrointestinal acute GVHD, suffer from hemorrhages. We have seen that hemorrhages stop and are controlled after treatment with MSCs or DSCs [11, 12, 62, 70]. Stromal cells may also heal hemorrhagic cystitis and major gastrointestinal hemorrhages in patients without GVHD [62, 90]. Apart from wound healing MSCs have profound effects on coagulation, which are the mechanisms behind this effect [91, 92]. Thus MSCs or DSCs may be used to treat severe hemorrhages.

Fetal calf serum-expanded bone marrow-derived MSCs are the most commonly used (Tab. 6.1). Because of concerns using bovine products, platelet lysate medium and human serum have also been successfully used to expand MSCs (Tab. 6.1). So far, there are only few patients treated with platelet lysate medium MSCs for acute GvHD ($n = 24$) and MSCs expanded in human serum ($n = 13$). Although the response rate seems to be better for MSCs expanded in fetal calf serum, such a conclusion cannot be drawn, because of different responses in children compared to adults and also different responses in early disease grade II as opposed to grades III–IV. To evaluate properly if one expansion medium is superior to the other, a prospective randomized study is required.

Bone marrow as a source of MSCs has also been challenged in a few studies (Tab. 6.3). Adipose tissue left over from plastic surgery has been one such source [49]. There is only a small number of patients treated, but outcomes so far have been most promising. Umbilical cord-derived MSCs has also been employed [63, 64], also with promising results. The placenta and the fetal membranes function as an immunological barrier between the mother and the developing fetus during pregnancy. Stromal cells isolated from various parts of the placenta tissue, including amnion, chorion, decidua and umbilical cord have shown immunosuppressive capacities [69]. These cells are assessable without any invasive procedure and with few ethical considerations as the placenta is discarded after delivery. Therefore, placental tissue may provide a valuable source for stromal cells to be used for therapy [70]. Also here promising results were seen. The complete response rate may seem lower than alternate tissues, but it should be considered that the median age among the patients treated with DSCs was 57 years of age, whereas using the other sources mainly younger patients were treated.

MSCs are also increasingly used for chronic GvHD (Tab. 6.4). Today there are 61 patients reported and the complete response rate is 25 % with a partial response rate of 48 %. Also here it is possible that many more patients are treated and those with poor responses are less often reported as compared to those with responses. Also here it seems as if early treatment and patients with mild chronic GvHD are more likely to respond, compared to those with more severe and disabling chronic GvHD. For instance, it seems that there are few, if any, patients with obstructive bronchiolitis who have shown any long-lasting response. Also here bone marrow-derived MSCs expanded in fetal calf serum are the most commonly used, with a few patients treated with adipose tissue-derived MSCs and a few patients receiving bone marrow-derived MSCs expanded in platelet lysate medium or human serum (Tab. 6.4). To really evaluate the effect on chronic GvHD, there is a need for prospective randomized studies. To fully evaluate the effects of MSCs with regard to side effects such as infections and other toxic side effects that may be induced by other immunosuppressive therapies, MSCs should probably be compared with other immunosuppressive drugs used for treatment of chronic GvHD such as pentostatin, mycophenolate mofetil, extracorporeal PUVA or anti-B-cell antibodies [5, 13, 19].

Although MSCs have a promising effect for the treatment of acute and chronic GvHD, there is a need for additional prospective randomized studies to establish their efficacy compared to other immunosuppressive therapies. Further research is also needed to optimize MSC therapy. It may be important to optimize the homing of the MSCs by manipulating molecules and receptors which are crucial. We also should optimize the source of cells and we also need to do comparative randomized studies with different sources of MSCs. Early passage seems to be beneficial and therefore techniques to optimize expansion in early passage need to be explored.

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Behnam Sadeghi and Olle Ringdén

7 Mesenchymal stem cells for graft-versus-host disease in experimental animal models

Abstract Using MSCs for the treatment of experimental models of GvHD provide a platform to study the underlying mechanisms and is a unique opportunity to optimize treatment modality. As in humans, MSCs can be isolated from several tissues of mice. However, isolation techniques and expansion methods are not as simple as in humans. Contradictory to clinical experiences, the majority of MSC therapy in mice models of acute GvHD are concentrated on prevention rather than treatment of established GvHD. Experimental results noticeably vary from no effect up to 80% improvement in survival. Incongruity of findings in anials studies is greatre than that observed in human practice. Essentially, the effectiveness of MSC therapy in animal practice is mainly dependent on the MSC cell dose, time of infusion and donor/recipients disparity. To improve the efficacy of MSCs on GvHD few reports applied genetic manipulated MSCs as an alternative approach. The findings are promiing, however, the subject needs to be explored more in the future. In this chapter, we summarize the experimental experience using MSCs for acute and chronic GvHD.

7.1 Introduction – Experimental models of graft-versus-host disease (GvHD)

Hematopoietic stem cell transplantation (HSCT) has revolutionized our knowledge about stem cell biology and introduced a curative treatment for both malignant and nonmalignant diseases as well as different metabolic disorders [1–4]. HSCT is an excellent example of transitional research that began with laboratory animal investigations and was finally employed as a successful clinical treatment [5–7]. Currently, allogeneic HSCT is used worldwide and many patients benefit from this impressive therapeutic approach. Like other medical techniques, HSCT has drawbacks and complications. Among them is GvHD which is the main hampering complication related to allogeneic HSCT. Clinical signs of GvHD have been reported since the first experimental [8] and clinical practice [4, 9].

Experimental medicine, particularly animal models of HSCT, has vastly improved our knowledge concerning transplantation biology and related complications. Establishment and improvement of HSCT was primarily based on experiments in dogs, mice and rats [10–12]. Several of these models are still valid and important in exploring different aspects of HSCT and GvHD [13, 14]. Choosing an animal model for HSCT or GvHD studies depends on feasibility, facilities and experiences as well as research purposes. Some animal models *e.g.* canine and rat models are mainly important in the

development of clinically conditioning regimen and immunosuppressant evaluation. While mouse models, beside these aims, are mainly used for immunobiology studies and mechanistic assessments. Additionally, mice are especially privileged compared to other animals, among the reasons are: development of well-characterized inbred strains, knock-out and transgenic mouse, lower cost and availability of reagents. Therefore mice are the most utilized model for investigating HSCT and GvHD research [13, 15].

There are several mouse models for induction of acute and/or chronic GvHD [13–15]. According to the project plan, study aims and of course resources and facilities, any of these models could be applied. It should be remembered that none of the animal models and particularly mouse models represent a full picture of human GvHD. Based on the animal model selected, different parts of the immune system and/or relevant mechanisms will be responsible or will be involved in the development of GVHD. Consequently, in any of the chosen models part of the manifestations related to the clinical setting may be highlighted or underscored. In the majority of mouse models, inbred animals are used as donor and/or recipient, meaning that the pattern of GvHD that arises is not fully comparable with the clinical setting in which human beings are outbred. Additionally, in almost all clinical HSCTs immunosuppressive medication starts before or immediately after graft infusion, while in none of the standard mouse models of GvHD immunosuppressive drugs are applied, unless indicated or for any particular reason. Based on our laboratory experiences there are significant differences in the pattern and outcome of GvHD in mice when immunosuppressive drugs are given or not (unpublished data).

Reciprocally, experimental animals provide a great opportunity for mechanistic evaluations. For example, we can produce various inbred animals lacking or overexpressing one or all MHC class I or II antigens as well as tissue-specific or general expression of particular antigens on tissue versus the hematopoietic system [16]. Essentially, most of our knowledge in immunobiology and pathogenesis of GvHD originates from this strategy and approach.

In general, GvHD in mice can be directed against MHC class I, class II (or both) or minor histocompatibility antigens (mHA). Nevertheless, the differences in minor HA in those that are different in MHC class I or II should not be ignored. Although in any adoptive immune response the contribution of immune cells, including CD4+ and CD8+, are necessary. However, if the mouse GvHD developed against MHC class I or class II the main effector cells would be CD8+ or CD4+ T lymphocytes, respectively. Accordingly, the severity of GvHD and outcome would be a subject of MHC (major and minor) disparity between donors versus recipients. Obviously the intensity of conditioning also has an important role.

Acute GvHD in mice can be induced via intravenous infusion of bone marrow cells (either T cell depleted or crude) supplemented with varying numbers of donor lymphocytes (either from spleen or lymph node) into conditioned total body irradiation (TBI) or busulfan combined with cyclophosphamide (Bu-Cy) treated recipi-

ents. Since the number of T cells in the bone marrow (BM) of mice is lower than that observed in human BM, adding T cells from an extra source *e.g.* spleen cell or lymph node is mandatory. The donor bone marrow provides stem cells that allow hematopoietic reconstitution while T cells promote engraftment and prevent graft rejection.

7.2 Immunobiology of experimental GvHD

Essentially, graft-versus-host disease can be considered as an exaggerated immune response to an unlimited resource of antigen. It is believed that GvHD is initiated by the conditioning regimen [17]. Acute GvHD is manifested by damage to the skin, liver and the gastrointestinal tract mainly through Th1 pathway, whereas chronic GvHD is more similar to an autoimmune syndrome (Th2 pathway), for example, scleroderma-like skin disease, salivary and lacrimal gland involvement (sicca syndrome) [18]. Billingham formulated [19] three basic elements for developing GvHD. First, the graft should contain immune competent cells, mostly donor T lymphocyte [20, 21]. Second; the recipient must be immuncompromised *i.e.* not able to reject transplanted cells and third, the recipient tissues must present antigens that are not expressed by the donor's cells [22]. Along with these fundamental requirements other variables play important roles in the pattern and/or severity of GvHD *e.g.* intensity of conditioning [23], degree of major or minor histocompatibility disparity [24, 25], immune status of host and donor, cytokine gene polymorphisms and killer immunoglobulin receptors (KIR), family of natural killer (NK) cells [26] and environmental factors [27]. However, to simplify the mechanisms underlying GvHD the consequences of events are summarized in three different but related phases [28] (Fig. 7.1).

1. Tissue damage attributable to conditioning
2. Donor T cells activation
3. Immune based host tissue damage

In this hypothesis, the conditioning regimen induces tissue damage and releases inflammatory cytokines. Consequently, residual host (and donor) antigen presenting cells (APCs) capture the released antigens and differentiate into mature APCs. The matured APCs then present the host alloantigens to the donor T cells which initiate the second phase of GvHD (donor T cell activation). Finally, activated donor T cells migrate to and invade different organs (target tissues) causing organ damage that is expressed as clinical manifestations of GvHD. In this simple procedure several immunological variables including different cytokines, chemokine and gene polymorphism and nonimmunological factors *e.g.* GI bacterial flora and environmental conditions [27, 29] will change the fate of allo-HSCT. Remarkably, the role of some mediators like IFN-γ, IL-2 is paradoxical and the outcome of GvHD is dependent on the donor/host polymorphism, serum level and timing of flare-up after allo-HSCT.

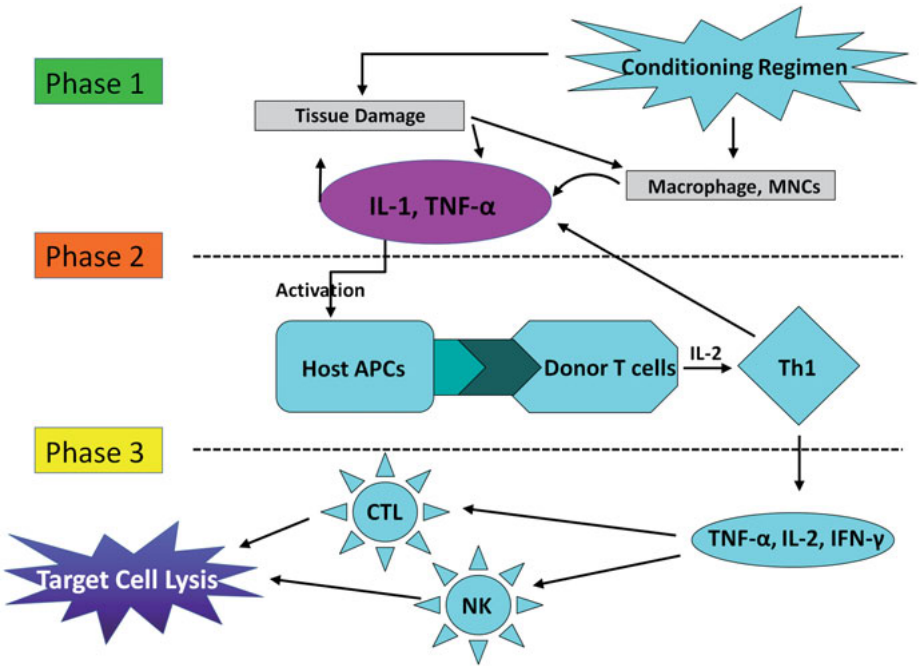

Fig. 7.1: Schematic map of GvHD; pretransplant conditioning regimen induces tissue damage and also promotes host residual immune cells to secret proinflammatory cytokines (Phase 1). Host (and donor) antigen presenting cells (APCs) acquire and process released antigen then present and stimulate donor T cells (Phase 2). Activated donor T cells migrate to periphery. Subsequently, activated donor T cells induce tissue damage through cell- or cytokine-mediated cytotoxicity (Phase 3). MNCs= mononuclear cells, IL-1= interleukin 1, TNF-α= tumor necrosis factor-alpha, APCs= antigen presenting cells, IL-2= interleukin 2, Th1= T helper 1, IFN-γ= interferon gamma, CTL= cytotoxic T lymphocyte, NK= natural killer cells.

7.3 Mesenchymal stromal cells in mice

Since the first report about the presence of a distinct population of fibroblast-like cells in the bone marrow [30, 31], several groups have shown different features as well as various functions of these cells. Many years later these cells were named "mesenchymal stem cells (MSCs)" [32]. The cells were originally different and discernible from the majority of hematopoietic stem and progenitor cells due to their adherence to plastic and also phenotype characteristics (fibroblast-like appearance) in culture [30, 33, 34]. Plastic adherence is a key element in isolating and expanding MSCs; however, it is not an exclusive criterion [35]. Additionally, other criteria including surface markers and transdifferentiation potential of MSCs into trilineage (osteoblast, chondrocyte and adipocyte) have been included in isolating protocols [36, 37]. Owing to the vast heterogeneity in constitution, the International Society for Cellular Therapy proposed

the use of "multipotent mesenchymal stromal cell" (MSC) [38]. Nevertheless, in the murine system the isolation and expansion of MSCs is not as simple as in a human setting [35]. There are several substantial species-specific differences between mouse and human MSCs. Following is a summary list of these differences:

1. Without using growth factor or co-culture with hematopoietic cell, isolation and expansion of mouse bone marrow MSCs, especially in early passage, is difficult. Mouse BM-MSCs are massively contaminated with hematopoietic cells [33, 39, 40]. This could be due to the limited number of inhabitant MSCs progenitors in the mouse BM, difficulty in the releasing of or complicated growth networking with hematopoietic cells in mice [33].
2. Not all of the markers or cell-surface receptors, which are defined well in human MSCs, has a counterpart in mouse MSCs.
3. There are significant differences in the yield, growth potential, differentiation capacity and cell-surface markers among MSCs originating from different mice strains [35, 41].
4. Murine MSCs can obtain chromosomal changes following *in vitro* cultures. It has been shown that they have a tendency to transform into a malignant cell [42–44]. With a lesser tendency few reports [45] showed the malignant transformation of human MSCs, seemingly mouse MSCs have a higher predisposition for spontaneous malignant transformation [42].

To isolate and expand mouse BM-derived MSCs (BM-MSCs) several factors should be considered including strain, animal age, utilized media and method [41]. Like human MSCs, mouse MSCs were first isolated from the bone marrow [30, 34, 46], however it can potentially be isolated from all tissues [47] *e.g.* fat [48], kidney [49], compact bone, bone epiphysis [50] and even fetal membrane (Behnam Sadeghi, Olle Ringden unpublished data). In this chapter we mainly concentrate on BM-MSCs from mice.

Due to difficulties in extracting and culturing murine BM-MSCs several methods have been developed including magnetic isolation, enzymatic isolation and/or applying special media and culture techniques [41, 51, 52]. Therefore, to find a common method is a big challenge in the isolation and expansion of mouse MSCs. It should be optimized based on the laboratory setting and research purpose. One of the reasons contributing to the complications and difficulties in isolating mouse MSCs is their low frequency in the mouse BM. It has been estimated that the frequency of mouse BM stromal progenitor cells is about 1 in 100,000 to 1 in 1,000,000 BM nucleated cells, which is extremely low [35]. Although in most of the reports plastic adherence and fibroblast-like appearance are the first distinguishing criteria, however, defining cell-surface markers and biologic properties should be added to inclusion criteria. Because of the substantial discrepancy between different strains there is still no common consensus on the unique pattern of cell-surface markers for mouse MSCs. This discrepancy originates from the strain differences, tissue origin of MSC, isolation method, different passage and finally materials used [35, 41].

However, to standardize research among scientists in the field the minimum criteria, beside adherence and trilineage differentiation, should be optimized among conflicting reports. Therefore, from the review of literature we can consider that mouse MSCs should not express hematopoietic and lineage markers including CD11b, CD31, CD45 and CD117 while they do express CD44, CD29, CD105 and CD106. Additionally, the expression of other markers like CD34, CD90, Sca-1 is not consistent and relates to strains, passage number or the isolation method used [41, 47, 51–53].

7.4 Mesenchymal stromal cells and mouse models of graft-versus-host disease

Mesenchymal stromal cells are considered as multipotent cells [54] which are able to differentiate into mesenchymal lineages including adipocyte, osteocytes chondrocyte and myocytes [55]. There are reports showing that MSCs also can differentiate into tissues of ectodermal (such as neurons) [56] and endodermal origin, such as hepatocytes [57]. Beside this multipotential capacity, MSCs are known as strong immune suppressor cells. Due to the *in vitro* and *in vivo* immune suppressor function of MSCs, it has been assumed that these cells might be effective in some inflammatory disorders, *e.g.* autoimmune diseases [58], organ transplant [59] and GvHD [60–62]. The immunomodulatory effects of MSCs are done through various mechanisms including cell contact and/or secreting soluble mediators [63].

The reports of applying MSCs in animal models of GvHD can be categorized into two main groups: inter- or intra-species studies. This means that either human MSCs (xenogeneic source) or animal MSCs (auto- or allogeneic source) are infused into the recipient animals. Before exploring available data, we have to be sure whether human MSCs will survive in an animal (xenogeneic) host, how efficient they will be, and whether changes in function might happen in a xenosetting. From a review of the literature it seems that MSCs works across species barriers [64]. Apart from the GvHD model, effectiveness of MSCs administration has been evaluated in several animal models of inflammatory, regenerative or metabolic disorders [65–67]. A majority of these reports present promising results. However, it is not clear if these are due to rejection of negative data or they are really true findings; an issue that needs to be explored in the future. In the following, we mainly concentrate on the experimental data concerning the effect of MSCs in animal models of GvHD (Tab. 7.1).

In vitro immune suppressor assay has shown that MSCs decrease T cell proliferation following allogeneic or mitogenic stimulation [63, 68, 69]. Moreover, both allogeneic and syngeneic MSCs were able to suppress proliferation of responder cells meaning that the effect is independent of MHC matching [70]. The immune suppressor capacity of BM-MSCs is comparable to conventional immunosuppressive drugs which are used in HSCT practice [68]. It means that MSCs potentially could be used instead of immune suppressive drugs to prevent GvHD. Considering these data, Chung *et al.*

Table 7.1: Mouse mesenchymal stromal cells used for the treatment of experimental acute GvHD.

References	Donor	Recipient	Graft	MSC Source	Cell dose × 10^6	Infusion time	Outcome
Chung *et al.* 2004 [68]	C3H/he (Kk)	BALB/c (Kd)	10×10^6 BM ± 5×10^6 SP cells	BM – Donor	0.1	Day 0	Improved survival in the group that received just BM not in the group that received BM + SP cells
Sudres *et al.* 2006 [70]	C57BL/6 (Kb)	BALB/c (Kd)	3×10^6 BM 5×10^5 T cells	BM – Donor	0.5–4	Day 0	No effect on GvHD, by any dose
Yanez *et al.* 2006 [63]	C57BL/6 (Kb)	B6D2F1 (Kb/d)	10×10^6 BM 20×10^6 SP cells	Adipose-derived – Recipient	0.05 (repeated)	Days 0, +7 and +14 Days 14, +21 and +28	Significant survival improvement following infusion at days 0, +7 and +14
Li *et al.* 2008 [72]	C57BL/6 (Kb)	BALB/c (Kd)	20×10^5 splenocyte	Compact bone – Donor	0.02–2	Day 0 or +3	Cotransfusion of higher dose of MSC (>1×10^6) delayed GvHD mortality
Badillo *et al.* 2008 [71]	C57BL/6 (Kb)	C57BL/6 × BALB/c (Kb/d)	10×10^6 BM 30×10^6 SP cells	BM – Donor	0.15–1 (single) 0.05 (repeated)	Days 0 or +2 or +10 or +21 Days 0, +7 and +14	No effect on GvHD (prevention or treatment) with any dose/time points
Polchert *et al.* 2008 [73]	BALB/c (Kd)	C57BL/6 (Kb)	N/A	BM – Donor	0.1–0.5	Day 0 or +2 or +20 or +30	Improved survival following infusion at day +2 or +20
Joo *et al.* 2010 [77]	C3H/he (Kk)	BALB/c (Kd)	5×10^6 BM 1×10^6 SP cells	C3H10T1/2 (MSC-Cell line) – Donor	0.5–2	Day 0	Improved survival and lower histopathologic damage following MSC infusion (>1×10^6)
Min *et al.* 2007 [62]	C57BL/6 (Kb)	B6D2F1 (Kb/d)	10×10^6 TCD-BM 20×10^6 SP cells	IL-10 transduced BM-MSC – Donor	2	Day +1	Improved survival and lower clinical manifestation of GvHD following IL-10 transduced MSC infusion
Chen *et al.* 2012 [75]	C57BL/6 (Kb)	BALB/c (Kd)	5×10^6 BM 2×10^6 SP cells	CXCR4 transduced BM-MSC – Donor	0.2 (repeated)	Days +1 and +7	Improved survival and lower histopathologic damage following CXCR4 transduced MSC infusion

Abbreviations BM= bone marrow, SP= spleen cells, TCD= T cell depleted, N/A= not accessible

applied donor-derived BM-MSCs in the fully mismatched mouse model of GvHD [68]. The survival rate and clinical score of GvHD was better in those animals that were transplanted with donor BM cell (without SP cell as source of T cell) and received BM-MSC, while those that received BM and SP cells with BM-MSCs did not get any benefit as compared to the control [68]. Based on their report it seems that BM-MSCs might promote BM engraftment and maybe have some beneficial effect in preventing or delaying mild GvHD, especially when there is a low dose of donor T cells. Extrapolating these data to a moderate or severe GvHD should be done carefully, because mouse BM contains few T cells and in the presented data MSCs did not prevent GvHD in animals that received both BM and spleen (SP) cells. Therefore, it seems that MSCs were not as effective as expected from the clinical data.

Sudres *et al*. applied donor source of MSCs in a mismatched mouse model of GvHD (B6 → Bal). First they have shown that murine MSCs inhibit T cell proliferation *in vitro*. Despite this immunosuppressive property, the infusion of MSCs did not show any protective effect on the fate and histological features of GvHD [70]. Even escalating dose of MSCs had limited or no effect on histopathological damage related to GvHD. One important issue in this paper is the immunosuppressive properties of isolated MSC. The significant inhibitory response in mixed lymphocyte reaction (MLR) was seen when high numbers of MSCs were applied to the culture (ratio of MSC/CD3; 8 : 1 and 4 : 1). This is different from all other reports regarding *in vitro* inhibitory effect of MSCs. Usually allogeneic or mitogenic stimulated T cells will be suppressed by adding MSCs at the ratio of 1 : 10 or 2 : 10 (MSC/T cells) while in the present report the ratio was the completely opposite, which causes uncertainty about the functionality of isolated MSCs. Another possibility of lack of response could be due to the infusion time which in their report was the same day as the day of transplantation [70].

Badillo *et al*. addressed that MCSs may be able to treat ongoing GvHD, but were not effective in preventing GvHD [71]. Therefore, they used donor-derived BM-MSCs both at early and repeatedly at late phase of GvHD. GvHD was induced by infusing 10×10^6 BM and 30×10^6 spleen cell from B6 donor to B6 × Balb/c (F1) recipient mice following 900 cGy irradiation (parent to F1 model). Based on their report, donor-derived MSCs neither prevented GvHD nor were able to treat ongoing GvHD [71]. In their report infusion of various cell doses of MSCs (1.5×10^5 up to 1×10^6 cell/mouse) did not show any beneficial effect [71]. They also have shown that IFN-γ will increase the expression level of MHC-I and also induce MHC-II expression on the MSCs' surface.

In contrast to the above mentioned reports, Li *et al*. have shown that donor-derived MSC delayed the development of GvHD [72]. In their paper they used a fully mismatched mouse model for GvHD. Sublethally irradiated Balb/c mice were transplanted with 20×10^6 splenocyte only from C57BL/6 (B6) donor. Experimental GvHD groups received additional graded dose of MSCs from 2×10^4 up to 2×10^6. They found that high dose (>1×10^6 MSCs) can postpone GvHD-related mortality as well as decrease clinical manifestations and histopathological grade of GvHD in recipient animals. The protective effect of MSCs was seen only when MSCs were infused at day 0

but not later (day+3). Through *ex vivo* and *in vivo* assays they have shown that MSCs decreased DC maturation and migration, increased the frequency of naive T cells in the spleen and somehow increased the expression of FOXP3 [72]. The observed effect of MSCs was elucidated through direct (cell to cell) and indirect (mediator release) contact. Although these are valued findings, it is difficult to compare them with previous reports. First, they just used donor splenocyte to induce GvHD, while having bone marrow cells (as a source of stem cell) might change the migratory pattern and engraftment of infused MSC and alter the fate of GvHD. Second, the recipient mice in this study underwent nonmyeloablative conditioning. Low intensity conditioning will affect the cytokine storm and inflammatory milieu following conditioning therefore the outcome might be different from other repots in which myeloablative regimens were used. Third, the MSCs were isolated from compact bone not the usual bone marrow mononuclear cells. How this affects the function and efficacy of MSCs needs to be explored.

As mentioned earlier, adipose tissue is another source of MSCs. In an attempt to evaluate the therapeutic effect of adipose-derived MSCs (Ad-MSCs) on GvHD, Yanez *et al*. administered recipient-derived Ad-MSC in the haploidentical (parent to F1) model of GvHD [63]. Recipient B6D2F1 mice were lethally irradiated by 1100 cGy and then transplanted with 10×10^6 BM and 20×10^6 Sp cells from C57BL/6 donor. In the experimental groups, 5×10^4 Ad-MSCs cells were repeatedly infused either at days 0, +7 and +14 (group 3) or days +14, +21 and +28 (group 4). In contrast to the previous negative findings, they showed that repeated infusion of Ad-MSCs starting at the beginning of GvHD (group 3) decreased the mortality and intensity of GvHD [63] while late infusion (group 4) of Ad-MSCs did not protect the animals. It is important to note that timing as well as dose of infused MSCs are significant factors affecting the outcome. In contrast to the report by Sudres *et al*., in which MSCs were only able to inhibit the lymphocyte expansion by the ratio of 8 : 1 [70], in the present study Ad-MSCs suppressed mitogen-induced lymphocyte proliferation by a ratio of 1 : 20 and above.

Considering the discrepancy among mouse studies Polchert *et al*. hypothesized that MSCs should be properly activated to show their efficacy [73]. A full mismatched mouse model including Balb/C and C57BL/6 mice were used as donor and recipients, respectively. Bone marrow-derived MSCs (1×10^5) from the donor were infused at days 0 or +2 (preventive), +20 (ongoing) or +30 (established GvHD) in a fully mismatched model of GvHD. MSC infusion significantly decreased the severity and mortality of GvHD when applied as preventive (day+2) or treatment of ongoing (day +20) GvHD but not at day 0 and +30 (established GvHD) [73].

Higher MSCs cell doses (5×10^5) did not have an additive effect on prevention of GvHD when the infusion time was day+2, but did dramatically increase the survival of GvHD animals when the infusion time was day +20. They speculated that the higher serum level of IFN-γ at days +2 and +20 will activate MSCs and elicit a therapeutic effect. Therefore, they used IFN-γ knockout (KO) mice as source of donor T cells. In this situation, the infusion of MSCs was not effective, at any time points. Based on

this observation it seems that MSCs' function is dependent on the serum level of IFN-γ [73]. Interferon gamma has a paradoxical influence on the fate of GvHD. It has been shown that the splenocytes from IFN-γ knockout (KO) mice induce more severe GvHD with higher mortality as compared to using wild donor [74]. This synergistic effect needs to be further explored. They have also shown that *ex-vivo* stimulated MSCs with high (not low) concentration of IFN-γ will decrease the intensity of GvHD. Unfortunately in this report the ratio of MSCs and donor splenocyte was not mentioned.

Given all the successes and failures in using mouse MSCs for prevention/treatment of GvHD, some groups introduced engineered MSCs as a new approach to optimize their efficacy [62, 75]. Min *et al*. used IL-10 transduced mouse MSCs in a haploidentical (parent to F1) mouse model of GvHD [62]. First they evaluated the effect of different cell doses as well as single or repeated unmanipulated MSCs infusion on prevention of GvHD. Donor source of BM-MSC from 5×10^5 to 2×10^6 cells/mouse were infused either at day +1 or days +1, +3 and +5 following allo-HSCT. Remarkably, the survival and clinical score of GvHD was worse as compared to control animals [62]. To increase the immune suppressive function of MSC, they developed transduced MSC overexpressing IL-10. These cells were able to secrete a high level of IL-10 . While *in vitro* immune suppressive function of genetically engineered MSCs was not much amplified, they significantly decreased GvHD-related mortality [62]. In line with the better survival in recipient animals, administration of MSCs did improve clinical manifestations of GvHD. Not all of the inflammatory cytokines declined following infusion of IL-10-engineered MSCs [62]. In another group of GvHD animals they infused exogenous IL-10 (without infusing MSCs) to mimic the same strategy. Interestingly, no improvement in GvHD was observed. This means that exogenous IL-10 does not work and there are other underlying mechanisms involved.

Defects in the migratory pattern and homing of MSCs to the proper place at a correct time were the reasons why Chen *et al*. decided to transduce mouse MSCs with the CXCR4 gene [75]. They speculated that CXCR4-overexpressed MSCs migrate more effectively to the injured sites and have higher engraftment capacity. GvHD was induced in a fully mismatched donor/recipient pair *via* infusion of 5×10^6 BM with 2×10^6 SP cells. Donor-derived MSCs were transduced with CXCR4 gene using lentiviral vector. Experimental groups received 2×10^5 (10 % of donor spleen cells) CXCR4 + enhanced GFP (EGFP) or EGFP (empty vector alone) bearing MSCs at days +1 and +7 following allogeneic HSCT. Considering infusion time, it seems that MSCs administration was considered as a preventive approach. *In vitro* assay showed that migration capacity of CXCR4-overexpressed MSCs improved more than EGFP transduced cells. Although short-term homing (24 hours after infusion) of CXCR4-MSCs to the BM and SP cells was higher, no data regarding long-term survival and homing was presented. They found that adoptive transfer of CXCR4 overexpressed MSCs increased survival and decreased GvHD clinical and histological score in GvHD animals [75].

Another part of experimental studies concerning MSCs involve the application of human MSCs to laboratory animals [76–79]. Although this approach may give valu-

able information, we should keep in mind that in the xenosituation not all of the mediators, cell contact and also surrounding milieu are similar to that of the MSCs. Theoretically and practically these differences might change the outcome and therefore any conclusion and extrapolation should be made with caution [80].

The effect of human umbilical cord blood-derived MSCs (*hu*UCB-MSCs) on GvHD was evaluated in a xenomouse model by Tisato *et al*. [76]. Twenty million human peripheral blood mononuclear cells (*hu*PBMC) were infused to the mildly (250 rad) irradiated NOD-SCID mice as model of GvHD. At different time points, single or repeated doses of *in vitro* expanded *hu*UCB-MSCs were infused to the recipient animals. The results indicate that single dose injection of *hu*UCB-MSC, co-infused with the graft, does not protect against GvHD. Repeated doses of *hu*UCB-MSCs started at the time of transplantation and continued for four weeks decrease the intensity of GvHD. Additionally, when the GvHD was established, *hu*UCB-MSC did not have any therapeutic effect [76]. Interestingly, they showed that when labeled *hu*UCB-MSCs were infused with or without *hu*PBMC (GvHD) the trafficking and migration pattern were different. It was shown that T cell proliferation significantly decreased if MSC/T cell ratio reached 10 % or above. An essential drawback in these types of experiments is that the NOD-SCID mice are not a good representative of a real transplantation setting. NOD-SCID mice already lack main immune components cells while in an HSCT setting we compromise the recipient immune system by the conditioning regimen up to a level that it does not reject the donor graft. Therefore, conditioning related toxicity and the presence of host residual immune cells will make a big difference between this model and the real HSCT and GvHD setting. Another important issue in this paper was that they did not infuse any stem cells along with PBMC. We know that SC cells have cross-talk with and close relations to the MSCs which might affect their function and migration pattern. Otherwise, if we consider this model as an *in vivo* mixed lymphocyte reaction, with unlimited source of allogeneic stimulator, the interpretation may be more representative.

In a later study, Jeon *et al*. used C57BL/6 (B6) mice as donor and BALB.B mice as recipient. These mice are matched for major histocompatibility antigens but differ at minor histocompatibility antigens. Thus this model is closer to the human setting than the previously described models [81]. Based on their report *hc*-MSCs, which were harvested by a different method, strongly suppressed both allogeneic and OKT3 stimulated human PBMC proliferation (*in vitro*). However, they did not inhibit and even increased mouse T cell proliferation *in vitro* [81]. Infusion of *hc*-MSCs to the recipient mice either at day 0 (prevention approach) or day +7 (treatment approach) did not improve survival or morbidity related to GvHD. In fact, experimental animals had lower survival compared to the control group [81]. They concluded that maybe human MSC could not exert an immunomodulatory function in a xenogeneic system. Based on our lab experiences and other reports [64] this is not always the case. Human MSCs may not be effective in treating mouse GvHD. However, as an immunomodulatory cell it inhibits xenogeneic MLR. Beside the lab-to-lab differences, the opposite observation

by this group could be partly due to the MSC harvesting method (subfractionation culturing method) [82]. Although the method used by these investigators may give good yield and functional cells for human trials, it cannot be excluded that during harvesting some clones, which are effective across species barriers, will be deleted. They also mentioned that there were "some differences in differentiation capability" [82].

As a conclusion it is obvious that the success rate of MSC administration for prevention or treatment of GvHD is higher in clinical (human) practice as compared to experimental (mouse) studies (Tabs. 7.1 and 6.5). The contradictory reports in the mouse studies are more common that in the human reports. Some points that we should remember when interpreting data from MSCs and mouse GvHD are; donor and recipient combination and used MHC disparity level, conditioning regimen both intensity and type, graft composition (reflected by the ratio of donor T cell in the graft), GvHD pattern (reflecting donor T cell activation pattern in the recipient body), MSCs cell dose, infusion time, isolation method and source (both strain and tissue of origin). Beside all of these considerations we should be aware that in none of the mouse models of GvHD any immunosuppressive drugs are applied. This makes a major difference with the clinical setting in which immunosuppressive therapy with calcineurine inhibitors starts before stem cell transplantation. The majority of successful reports of using MSCs in clinical practice employed MSCs in treating ongoing GvHD [61, 83] rather than preventing GvHD [84–86]. While in most existing reports using MSCs in mouse models, the approach was preventing rather than treating GvHD (Tab. 7.1). We should also emphasize that almost all the mouse models of GvHD run in inbred animals in an isolated and very clean environment which is not applicable to outbreed humans.

Lastly, an important issue related to the immunosuppressive properties of MSCs is if their effect (or side effect) on the systemic immunosuppression may lead to tumor growth or relapse. This is an important issue in the field of HSCT. Those reports indicating efficacy of MSCs on prevention or treatment of GvHD in mice should address their effect on tumor growth as well. Overcoming GvHD is of course the main target. However, in a majority of HSCT patients relapse is a counterbalance factor that should be considered for any therapeutic approach. This was yet not addressed in any of the presented reports. Some reports show a relationship between MSCs and tumor growth [87, 88]. This is an important topic that should be explored in more detail in the future in the context of MSC treatment or prevention of GvHD.

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Antonello Pileggi, Xiumin Xu, Jianming Tan and Camillo Ricordi

8 Mesenchymal stem cells and organ transplantation: initial clinical results

Abstract The widespread application of organ transplantation to the large population of patients with end-stage organ failure who are in dire need of its benefits is currently being hampered by a number of challenges. Amongst them are the *shortage of organs for transplantation* and the need for *lie-long immunosuppression* with the associated untoward side effects. The field of regenerative medicine is rapidly evolving, thus creating exciting opportunities toward the development of novel therapeutic protocols aimed at ameliorating, reducing, modifying, correcting and curing medical conditions. Mesenchymal stromal (stem) cells (MSCs) are appealing for inclusion in organ transplantation protocols because of the increasing body of evidence in support of their beneficial properties both in tissue repair and for the modulation of immunity. Herein, we review the encouraging results of the recent clinical trials on the use of MSCs in organ transplantation.

8.1 Introduction

Transplantation of cells, tissues and organs is performed to restore *functio laesa* (impaired function) due to genetic defect, toxicity, trauma or inflammation. Over the last four decades, the remarkable progress in organ recovery, preservation and transplant techniques along with the availability of efficient immunotherapeutic agents has contributed to making organ transplantation a viable therapeutic option for patients requiring functional restoration of renal, hepatic, cardiac, intestinal, endocrine pancreatic, small bowel, lung, cornea, and more recently even composite grafts such as limb and face tissues. Nonetheless, several challenges are currently limiting the widespread application of organ transplantation to the large population of patients with end-stage organ failure who would benefit from it. Amongst them are the *shortage of organs for transplantation* and the need for *lifelong immunosuppression* with its associated untoward side effects. Different approaches have been proposed to overcome such limitations that may allow achieving high degrees of success measured as high rates of engraftment, long-lasting graft survival and function. Ultimately, the goal to achieve is immune tolerance.

The field of regenerative medicine is rapidly evolving, providing exciting new opportunities toward the development of therapeutic protocols aimed at ameliorating, reducing, modifying, correcting and curing medical conditions. In the context of organ transplantation, we are witnessing a growing interest in the use of cell therapies to favor the success rate and to establish permanent graft acceptance without the need for lifelong immunosuppressive therapy. In particular, mesenchymal stromal

(stem) cells (MSCs) are appealing for inclusion because of the increasing body of evidence in support of their beneficial properties both in tissue repair and for the modulation of immunity [1].

The clinical application of MSCs in organ transplantation is very recent [2–6] with only few reports available in the medical literature assessing the impact of MSCs on clinical outcomes in relatively small numbers of transplant recipients who received solid organ transplantation and with short-term follow-up. Nonetheless, the results have been thus far quite encouraging, showing positive effects of the use of MSCs therapy. Herein we will review and discuss the results of these seminal clinical trials.

8.2 Rationale for the use of MSCs in organ transplantation

Transplantation of organs and tissues has become a common clinical therapeutic option to restore end-stage organ failure worldwide. The significant progresses recorded over the last four decades in organ recovery and transplant techniques, immunobiology and immunotherapy have helped in expanding the indication and increasing the longevity of transplanted tissues, while reducing side effects. Nonetheless, there are several drawbacks and challenges currently limiting the transplantation field.

8.2.1 Shortage of donor organs for transplantation

Despite the steady increase in cadaveric organ donation for transplantation in recent years, the source of transplantable organs is insufficient to fulfill the high demand and the death rates in the waiting lists for transplantation remain unfortunately high. The majority of organs are currently recovered from heart-beating donors following *cerebral death*. In order to expand the donor pool, utilization of *marginal donor* organs (*i.e.*, recovered from elderly individuals) has been proposed, though concerns about delayed or partial function, as well as longevity of the graft remain a concern. Another approach which has been proposed is the use of donation after cardiac death (*non-heart-beating donors*, NHBD) and has been implemented in selected Centers, though logistic reasons may limit the implementation of interventions fast enough to minimize the consequences of warm ischemic damage on the organs to be transplanted.

The use of *living donors*, generally siblings or related to the recipient, is becoming increasingly common in recent years, not only for kidneys (living donor kidney transplant, LDKT) [7] but also for segmental liver [8] and pancreas [9] in experienced Centers. This approach makes it possible to perform the transplant as an elective surgical procedure allowing adequate time to implement targeted interventions (*i.e.*, to induce donor-specific hyporesponsiveness) [10, 11].

8.2.2 Ischemia-reperfusion injury

It has been recognized that the fate of transplanted tissues greatly depends on variables such as the stress and hypoxic conditions endured following donor decease (cerebral or cardiac), duration and type of management in the intensive care unit (ICU), warm and cold ischemia during and after organ recovery, and duration of organ preservation. The changes induced by anoxia in the endothelium of vascular structures and organ parenchyma result in cellular death, activation of stress-induced signal transduction pathways that affect the organ functionality while increasing immunogenicity by inducing the elevation of expression of major histocompatibility complex molecules (MHC), integrins and proinflammatory mediators, which all contribute to a cascade of events that promote innate immunity and may amplify the severity of adaptive immune responses, compromising the fate of the graft. It has been recognized that organs obtained from cadaveric donors with extended ischemic preservation times are more prone to acute and chronic rejection episodes, which lead to progressive graft failure faster than organs obtained from healthy, living donors that have endured minimal ischemic insults [12, 13].

The cytoprotective and immunomodulatory properties of MSCs make them appealing for the modulation and mitigation of ischemia-reperfusion injury in transplanted organs. Indeed, a body of experimental evidence strongly supports the ability of MSCs to preferential homing at the site of injury, regardless of the route of administration [14, 15]. Moreover, the therapeutic potential of MSC inoculum in organ ischemia reperfusion models has been recognized [16, 17]. Benefits of MSC treatment would include rescue of marginal donor organs, reduction of the activation of innate immune responses that can injure the tissue leading to chronic and progressive fibrosis, as well as the potential to reduce 'danger signals' and, in turn, favor immune tolerance induction protocols.

8.2.3 Chronic immunosuppression

Prevention of immune rejection of transplanted tissues is achieved by the means of lifelong immunosuppression. Current protocols rely mainly on an 'induction' treatment, which is generally implemented with lymphodepleting agents that consists of a biologic agent [such as rabbit anti-lymphocyte globulin (rATG), anti-CD25 antibody (targeting the interleukin-2 receptor) , anti-CD52 antibody (campath-1H, alemtuzumab), anti-CD3 antibody, amongst others], which may be combined with triple maintenance immunosuppression: inhibitors of calcineurin (CNI: cyclosporine A, CsA; and tacrolimus), inhibitors of the molecular target of Rapamycin (mTOR: sirolimus and everolimus), inhibitors of purine / pyrimidine synthesis (such as mycophenolate mofetil, MMF; mycophenolic acid, MPA; azathioprine, AZA), and steroids. Novel agents and biologics are emerging that target co-stimulatory molecules

(*i.e.*, anti-LFA-1; CTLA4Ig, etc.), B cells (*i.e.*, anti-CD20, rituximab), T cell trafficking (FTY720, fingolimod), amongst others.

Unfortunately, the use of immunosuppressive agents is associated with a plethora of untoward side effects that affect the quality of life and life expectancy of transplanted patients. Immunosuppressed individuals are more prone to develop opportunistic infections that are more severe than in subjects with a healthy immune system. Prophylactic antibiotics (to prevent *Pneumocystis Carinii* infection) and antivirals (to prevent *de novo* or reactivation of cytomegalovirus, CMV; Epstein–Barr virus, EBV; JC polyomavirus, BK virus, etc.) are commonly part of the standard of treatment of transplant recipients. The severity of infection may impose the need to reduce immunosuppression increasing the risk of compromising graft survival.

Chronic immunosuppression (CNI alone or in combination with mTOR inhibitors or MMF/MPA) is also associated with organ toxicity. Progressive renal dysfunction requiring dialysis and kidney transplantation may develop over time in nonuremic patients receiving chronic immunosuppression. Similarly, beta-cell dysfunction may occur after years of immunosuppression, requiring introduction of exogenous insulin (post-transplant diabetes mellitus). Reduction of immunosuppression, such as CNI dose, has been recognized to positively influence the longevity of renal grafts.

The 'Holy Grail' of transplant immunobiology is the achievement of permanent acceptance of transplanted tissues without the need for lifelong anti-rejection therapy. While the benefits of achieving such a goal will become apparent, based on the issues discussed herein, indefinite acceptance of transplanted organs has been attained sporadically or in limited patient cohorts, with limited reproducibility thus far [10, 11, 18–21]. Harnessing the immunomodulatory properties of MSCs products may be of assistance in redirecting the immune system toward regulatory circuits that may synergize with protocols aimed at the induction of immune tolerance enhancing efficacy and its achievement more reproducibly. Indeed, several experimental studies support the ability of MSCs to modulate the function of T cells, B cells, NK cells, dendritic cells, monocytes and to synergize in inducing Treg and other 'regulatory' phenotypes [22–24].

8.3 Considerations regarding the choice of the clinical protocols

At the present time, there is no consensus in the scientific community on what is the best source of MSCs for any given therapeutic application, neither on the most appropriate route(s) and schedule of administration, nor on the optimal concomitant therapy that could provide the highest degree of efficacy in improving transplantation outcome. The increasing interest on the use of MSCs in solid organ transplantation has prompted multiple discussions in different professional venues aimed at promoting its translation from bench-to-bedside in the most efficient manner. In recent

years, the *Mesenchymal Stem Cell in Solid Organ Transplantation* (MiSOT) Study Group has been a very active international consortium of organ transplant-focused translational researchers and clinicians with thoughtful discussions on critical endpoints, safety, efficacy and high standards that should be aimed at for the successful implementation of MSCs in solid organ transplantation. Their work has been summarized in position statements that are contributing to the progress in the field [22–24].

8.3.1 Definition, identity and product release criteria for human MSCs preparations

MSCs are heterogeneous stromal cell populations of possible perycytic origin that are generally enriched by undergoing several expansion cycles in defined culture conditions based on adherence to plastic and expression of specific phenotypic markers [25]. The techniques utilized for the isolation (*i.e.*, enzymatic, non-enzymatic), culture conditions (*i.e.*, defined media, the use of human or animal serum, serum-free media, or human platelet extract, oxygen levels, *etc.*), enrichment protocol(s) (*i.e.*, flow cytometry sorting based on specific surface cell markers, *etc.*) and assessment of MSCs have not yet been standardized and may vary depending on the source of tissue and on the processing laboratory. At the present time, the consensus on MSCs product identity and release are based on the 2006 position statement of the *International Society for Cellular Therapy* (ISCT) suggesting the need to meet three criteria: (1) adherence to plastic; (2) specific surface antigen by flow cytometry (≥95 % of the MSCs population must express CD105, CD73 and CD90; cells must lack expression ≤2 % positive of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II); and (3) multipotent differentiation (must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions) [26]. Another important quality step consists in the exclusion of chromosomal aberrations that may occur *in vitro* (generally after multiple culture passages) and may result in a hypothetical heightened risk of malignancy. It is conceivable that the criteria may change in the near future, as new and more sophisticated analytical approaches will provide more precise assessment of cell subset comprised in the MSCs product.

8.3.2 Source of human MSCs

MSCs may be *autologous* (that is, obtained from the patient's own tissues or from a human leukocyte antigen [HLA]-identical sibling), *allogeneic* (that is, obtained from another individual) who, in the case of organ transplantation could be the same donor of the tissue/organ transplanted ('*donor-specific*', haploidentical), or from an indifferent ('*third-party*', HLA-mismatched) donor (Fig. 8.1). Seminal clinical studies performed by LeBlanc *et al.* in recipients of hematopoietic stem cell (HSC) transplant

experiencing severe graft-versus-host disease (GvHD) refractory to conventional steroid treatment demonstrated the efficacy of bone marrow-derived MSCs (BM-MSCs) in ameliorating clinical outcome in a high proportion of patients irrespective of the HLA matching of the MSC donors [27, 28]. Similarly, *in vitro* suppression of transplant recipient immune responses using donor-specific MSCs has been demonstrated [29]. Considering the need to isolate and expanding MSCs in adequate numbers prior to inoculum, in the case of cadaveric solid organ transplantation it would be practical to favor the use of either autologous MSCs that could be obtained from the prospective recipient and cryopreserved until a cadaveric organ becomes available, or of third-party (off-the-shelf) allogeneic MSCs. In the case of living donors, the option of isolating donor-specific MSCs also becomes available.

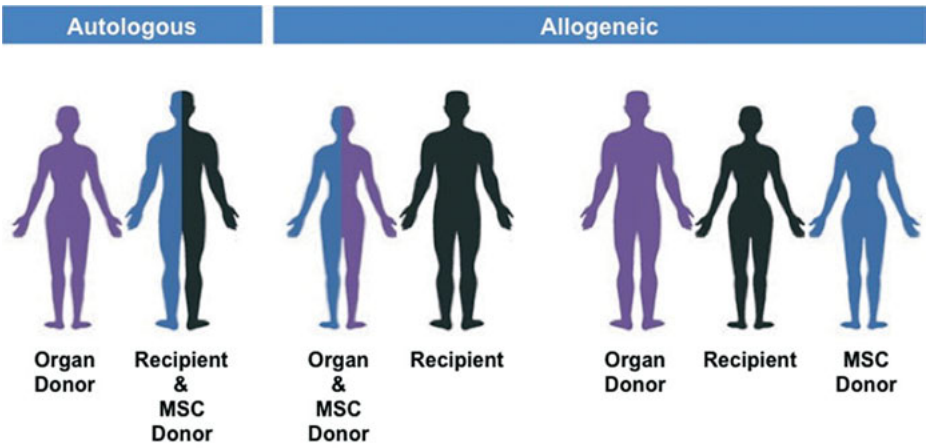

Fig. 8.1: Donor:recipient MSC transplant combinations.

Cells with comparable phenotype and differentiation potential *in vitro* that are compatible with current 'MSC' definition have been obtained from the bone marrow, adipose tissue, umbilical cord tissue and blood, the perirenal fat tissue, amongst other tissues [30, 31]. Depending on the conditions utilized for MSC isolation (*i.e.*, enrichment based on defined surface markers, media formulation, low oxygen culture, *etc.*), slightly different cell products have been developed by academic/hospital institutions or industry. Due to the wide variability in tissue processing, cell isolation and expansion, as well as assessment protocols amongst laboratories, side-by-side comparisons are not definitive yet [31, 32]. Nonetheless, the immunomodulatory and tissue repair properties of putative MSCs seem to be relatively comparable regardless to the different sources they are obtained from.

Another aspect that has not been fully elucidated is the potential impact of chronic medical conditions on donor and/or recipient MSCs microenvironment, which may

influence the efficacy and potency of the cellular product [33]. For instance, experimental data seem to suggest that MSCs obtained from individuals with diabetes (a dysmetabolic, inflammatory condition) may display reduced replicative rates *in vitro* and altered reparative potential in the experimental models of ischemia when compared to MSCs of healthy donors [34, 35]. In addition, MSCs obtained from autoimmune diabetes-prone mice may have impaired immunomodulatory potential when compared to non-diabetic-prone donors [36]. It remains to be determined whether MSC functional impairment in chronically ill individuals may be reversible under appropriate conditions (*i.e.*, in culture and/or *in vivo*). Individuals with end-stage renal disease (ESRD) and uremia display proinflammatory profound alterations and immune cell dysfunction [33, 37, 38], though recent studies seem to suggest that MSCs obtained from adipose tissue [39] or bone marrow [40] of uremic patients retains *in vitro* potency and immunomodulatory effects.

The regulatory framework for cell manufacturing and interindividual variability in final cell product yields and quality may also introduce additional hurdles (high costs to establish and maintain dedicated cGMP facilities and personnel, *etc.*) for a widespread application of autologous and donor-specific MSCs in the clinical setting. For this reason, availability of consortia relying on 'centralized' or 'regional' cell processing facilities may represent a viable alternative toward maximizing efficiency while containing operational costs. In addition, availability of off-the-shelf, 'standardized' products (*i.e.*, through a regional or commercial partner) may provide assistance in overcoming some of the logistic limitations.

8.3.3 Potential interactions between MSCs and concomitant therapy

The use of concomitant therapy may affect the viability, potency and efficacy of MSCs products after inoculum in the immunosuppressed recipient. Thus, there is a recognized need to determine potential synergies or competitions in order to design combinatorial regimens that exploit the beneficial effects of MSCs in the most efficient manner in the clinical settings. Interactions between MSCs and immunotherapy have been identified in the experimental setting, raising the concern over inhibitors of calcineurin (*i.e.*, cyclosporine A, CsA; or tacrolimus) and of mTOR (*i.e.*, sirolimus and everolimus), but apparently not cell cycle inhibitors such as mycophenolate mofetil (MMF) and its active metabolite mychophenolic acid (MPA), may interfere with the immunomodulatory properties of MSCs [2, 41–44]. The use of biologics such as rATG, anti-CD25 monoclonal antibody, and/or anti-CD52 antibody, amongst others, is common practice as induction regimen for organ transplant recipients. The effects of the interaction between MSCs and rATG have been evaluated by Perico *et al.* [2] who demonstrated that rATG binds to human MSCs in a dose-dependent fashion (*i.e.*, at concentrations of 5.0 and 0.5 μg that are comparable to those achieved in transplant recipients after the first and last injection, respectively). They also showed minimal

rATG binding to human MSCs and impairment of their ability to suppress mixed lymphocyte reactions *in vitro* when exposed to serum collected on day 7 and 14 from renal transplant recipients treated. Interestingly, addition of cyclosporine, MMF or steroids to the MSCs cultures did not appear to affect their ability to suppress T cell responses to mitogenic stimulation with anti-CD3/CD28 antibodies *in vitro*, with rather synergy observed when MMF was used in the assay [2]. The authors used these preliminary data to design the timing of MSC inoculum on day 7 in their clinical trial to minimize possible interference with immunotherapy (see below) [2]. Franquesa *et al*. further evaluated the interaction between rATG on MSCs *in vitro* confirming the dose-dependent binding but also the negative effect on MSC viability, impairment of immunomodulatory properties and, importantly, the fact that they were susceptible to be lysed by cytokine-activated CD8+ cytotoxic cells and NKT cells [45]. The observation that MSC inoculum may allow avoidance of biologic treatment at the time of induction in clinical transplant recipients without negatively affecting patient and graft outcomes (see below) [3] opens new therapeutic opportunities for the definition of novel immunomodulatory protocols that combine cellular and pharmacologic agents to achieve synergistic results and hopefully toward the optimization of approaches for the induction of immune tolerance.

8.3.4 Safety of MSCs-based treatments

The multipotency and ability to modulate immune responses of MSCs are properties that the transplant community aims to harness. However, the very same qualities may represent a safety threat for transplant recipients. While there are no clinical data supporting the development of neoplasms directly related to the use of MSCs inoculum, this aspect should not be underestimated, particularly when considering the use of multipotent stem cell inoculum in immunodepressed individuals who have increased propensity to develop tumors. Hypothetically, the immunomodulatory properties of MSCs also may result in enhanced immunosuppression, particularly when combined with immunotherapy, which may result in higher risk for viral infections (*e.g.*, *de novo* or reactivation), lymhoprolipherative disease and progressive multifocal leukoencephalopathy that could be life-threatening. Appropriate prophylaxis, close monitoring and careful assessment of the immune and viral status of the recipients may be of assistance in implementing prompt interventions to minimize risks for the patients.

A recent meta-analysis on MSCs clinical studies on 1,012 participants with different clinical conditions provided a reassuring result on the safety of MSCs in humans [46]. Nonetheless, the heterogeneity of the medical conditions and protocol utilized in each trial included in the analysis should suggest caution. It is imperative to carry out a thorough, extended monitoring of patients enrolled in ongoing clinical transplant trials which will be instrumental towards assessing the long-term safety of MSCs therapy in the clinical settings.

8.4 Clinical MSCs and solid organ transplantation trials

Following the seminal clinical trials by LeBlanc *et al*. demonstrating successful treatment of GvHD in recipients of hematopoietic stem cells [27, 28], the growing interest for the implementation of MSCs also in 'solid organ' (as opposed to 'hematological') transplantation has been recorded in the transplant community [22–24]. The number of clinical trials underway or already registered with ClinicalTrials.gov is increasing (Tab. 8.1), with a renewed interest that has followed the promising results of the first published series of MSCs and organ transplantation trials between the years 2011 and 2013 [2–6].

8.4.1 Autologous MSCs in the induction phase with standard immunosuppression

The use of MSCs in solid organ transplantation was first reported as pilot safety and clinical feasibility study of autologous BM-MSCs in two patients with end-stage renal disease (ESRD) receiving living donor kidney transplantation (LDKT) by Perico *et al*. [2]. In their study, the patients received autologous MSC inoculum intravenously (1.7×10^6 cells and 2.0×10^6 cells per kg body weight, respectively) one week after Living Donor Kidney Transplantation (LDKT) under standard immunosuppressive therapy based on induction with anti-CD25 antibody (basiliximab, 20 mg intravenously pre-transplant and day 4), fractioned rATG (0.5 mg/kg daily from day 0 to day 6), and maintenance with CNI (CsA; target trough blood levels 300–400 ng/ml for the first week, then 100–150 ng/ml at month 5 post-transplantation), MMF (target plasma trough MPA levels of 0.5 to 1.5 μg/ml), and a short-course of steroids (500, 250 and 125 mg methylprednisolone for first three days and then 75 mg of oral prednisone progressively tapered and weaned by one week post-transplant). Overall the trial demonstrated the feasibility and safety of MSC inoculum with only a transient increase in serum creatinine after MSCs inoculum that was self-limited and without sequel. Both patients showed good graft function at the 12-month follow-up. An interesting finding of this trial was the observed increase in the frequency of a T cell regulatory (Treg) population ($CD4^+CD25^{high}FoxP3^+CD127^-$) that was paralleled by marked reduction of a memory T cell population ($CD45RO^+RA^-CD8^+$), when compared to historical controls receiving LRDKT with the similar immunosuppressive regimen but without MSCs inoculum. A note of caution, despite the interesting preliminary mechanistic observations, the small sample size of this pilot trial and the lack of concomitant controls preclude any possible generalizations at the present time.

Table 8.1: Registered clinical trials of MSCs in solid organ transplantation (ClinicalTrials.gov).

NCT*	Title	Site	Settings	Type of MSCs	MSCs Inoculum	Type of Study	Start Date
00646724	Cotransplantation of islet and mesenchymal stem cell in Type 1 diabetic patients	Fuzhou, China	Islet transplant	UC-MSCs	1–2 × 10^6/kg bw simultaneous islet and MSCs transplantation via hepatic portal vein	Interventional Safety/Efficacy Single Group Assignment Open Label	Jan 2008
00658073	Induction therapy with autologous mesenchymal stem cells for kidney allografts	Fuzhou, China	Living donor kidney transplant	Autologous BM-MSCs	1–2 × 10^6/kg bw, IV at reperfusion and day14	Interventional Randomized Safety/Efficacy Parallel Assignment Open Label	Mar 2008
00659620	Mesenchymal stem cell transplantation in the treatment of chronic allograft nephropathy	Fuzhou, China	Chronic kidney rejection	Autologous BM-MSCs	1–2 × 10^6/kg bw, IV once a week for 4 weeks (total 4 injections)	Interventional Safety/Efficacy Single Group Assignment Open Label	May 2008
00734396	Mesenchymal stem cells and subclinical rejection	Leiden, Netherland	Kidney rejection	Autologous BM-MSCs	10^6/kg bw, IV 7 days apart (total 2 injections)	Interventional Non-Randomized Safety/Efficacy Single Group Assignment Open Label	Feb 2009
00752479	Mesenchymal stem cells under basiliximab/low dose RATG to induce renal transplant tolerance	Bergamo, Italy	Living donor kidney transplant	Autologous BM-MSCs	2 × 10^6/kg bw IV.	Interventional Randomized Safety/Efficacy Parallel Assignment Open Label	May 2008

NCT*	Title	Site	Settings	Type of MSCs	MSCs Inoculum	Type of Study	Start Date
01429038	Mesenchymal stem cells after renal or liver transplantation	Liege, Belgium	Liver or kidney transplant	Allogeneic (Third-party) BM-MSCs	1.3-3.0 × 10^6/kg bw day 3±2	Interventional Non-Randomized Safety/Efficacy Parallel Assignment Open Label	Feb 2012
01668576	Properties of mesenchymal stem cells in lung transplant candidates	Atlanta, GA, USA	Lung transplant	Autologous BM-MSCs	In vitro assessment only	Observational Cohort Cross-sectional	Aug 2012
01690247	Human mesenchymal stem cells induce liver transplant tolerance	Beijing, China	Liver transplant	UC-MSCs	10^6/kg bw IV once every 4 weeks day 0 to 12 weeks	Interventional Randomized Safety/Efficacy Parallel Assignment Open Label	Feb 2012

* Abbreviations: BM: bone marrow; bw: body weight; IV: intravenous; MSCs: mesenchymal stromal (stem) cell; UC: umbilical cord;

8.4.2 Autologous MSCs in the induction phase with avoidance of biologics at induction and reduced maintenance immunosuppression

From February 2008 to May 2009, we performed the first large-scale prospective, open-label, randomized clinical trial on 159 patients with ESRD that was aimed at comparing the risk benefit profile of autologous BM-MSC infusion vs. anti-CD25 antibody (basiliximab) induction therapy for LDKT (Tan *et al.*) [3]. All patients received the same treatment with MMF and corticosteroids. The control group received anti-CD25 antibody and standard dose CNI (either CsA or tacrolimus). In our trial, instead of anti-CD25 treatment the patients in the experimental arms received MSCs inoculum ($1–2 \times 10^6$/kg intravenously at the time of reperfusion and on day 14 post-transplant) with either standard or reduced dose CNI (80 % of standard dose) [47]. Reduction of CNI dose is considered a highly desirable goal in organ transplant recipients to prevent organ toxicity, including nephrotoxicity [47, 48]. The primary outcome of the study was the incidence of biopsy-confirmed acute rejection and estimated glomerular filtration rate (eGFR) within the first year. The secondary outcome of our study was one-year patient and graft survival and incidence of adverse events, including opportunistic infections.

Overall, our results support the safety and efficacy of MSC therapy in solid organ transplantation. We observed that autologous MSCs could replace anti-CD25 blockade in this transplant patient population. When compared to the control group, patients receiving MSCs inoculum displayed lower frequency of and less severe biopsy-confirmed acute rejection in the first semester post-transplant, none of which was steroid-resistant requiring rATG (vs. 7.8 % in the control group). Faster recovery of renal graft function during the first month post-transplant was observed in the patients receiving MSCs treatment, which may suggest a possible faster recovery from ischemia-reperfusion injury that has been recognized as a risk factor for graft failure and acute rejection [49, 50]. Recipients of MSCs also displayed fewer adverse events and significantly lower incidence of opportunistic infections than controls. Notably, despite avoidance of anti-CD25 blockade and/or reduction of CNI dose, graft function and patient safety were not compromised in our study with patients in all groups displaying comparable graft function at one year. Rather, the inclusion of MSC treatment to the protocol was associated with tangible clinical benefits, such as the ability of lowering immunosuppression, improving graft function and reducing acute rejection episodes and frequency of opportunistic infections, all of which are common challenges in the management of transplant recipients. Particularly, this is an encouraging result considering opportunistic infections occurring mostly in the first two trimesters post-transplant are associated with the highest mortality rate in kidney transplant recipients in China [51]. Interestingly, the low rates of opportunistic infections in our trial were observed not only in the patients receiving low CNI dose, as expected, but also, albeit to a lesser degree (in a statistically significant manner), in those receiving standard CNI dose which was unexpected. Of note, a recent study

described antibiotic activity for human MSCs [9] possibly through the production of cathelicidin CAP-18/ LL-37 [52].

Similar beneficial effects of concomitant cellular therapies allowing reduction of maintenance immunosuppression were observed in a previous trial we performed in recipients of renal allografts from cadaveric and living-related donors treated with whole or fractionated donor-specific BM cell transplantation [53]. In order to achieve improved renal allograft function to a degree comparable to that we observed in our MSCs trial [3], clinical protocols relying on profound lymphodepletion of the recipients with anti-CD52 antibody induction (in absence of cellular therapy), but with a trade-off of heightened risk of severe opportunistic infections, even in low-risk subjects such as those receiving live donor kidney transplantation (LDKT) [54]. Thus, collectively the results of our clinical trial are very encouraging for the use of cell-based therapies to improve patient and graft outcomes in solid organ transplantation. Extended follow-up will be of assistance in assessing the long-term safety of autologous MSCs in transplant recipients, while more in-depth studies will be needed to help understand the mechanisms underlying the beneficial impact of cellular therapy in this patient population.

8.4.3 Allogeneic MSCs in the induction phase

A nonrandomized, pilot clinical trial of donor-specific, BM-MSC transplantation in twelve consecutive uremic individuals undergoing LDKT (6 experimental and 6 controls) was recently reported by Peng *et al.* [4]. Aim of the study was to assess the safety and efficacy of donor-derived BM-MSCs in LRDKT to reduce CNI (tacrolimus) dose and improve transplantation outcome. All patients received induction with cyclophosphamide (Cytoxan; 200 mg/day) and methylprednisolone (750, 500, 250, and 250 mg/day) from days 0 to 3. Maintenance immunosuppression included MMF (1 g/day) and prednisone (30 mg/day from day 4 and then tapered by 5 mg every week to the maintenance dose of 15 mg/day thereafter). Tacrolimus was started on day 4; the control group received standard dose (0.07–0.08 mg/kg/day), whereas patients in the experimental group received low dose (0.04–0.05 mg/kg) along with two infusions of donor BM-MSCs: 5×10^6 cells directly into the renal allograft artery at the time of kidney reperfusion, and 2×10^6 cells/kg intravenously one month later. Overall, the study demonstrated the feasibility and safety of the protocol, including direct MSC injection into the renal artery that was uneventful and may have potentially contributed to modulating the intragraft post-ischemia-reperfusion inflammatory response. The group of patients in the MSCs and low-tacrolimus dose maintained stable graft function during the one-year follow-up period. They also displayed more peripheral memory ($CD27^+$) B cells in the experimental group than controls at 3 months, while other mechanistic endpoints (*i.e.*, lymphocyte phenotype, intracellular cytokine expression, one-way mixed lymphocyte responses *in vitro*, chimerism, *etc.*) did not

differ statistically amongst study groups. Despite the demonstration of safety and feasibility, the observation of the ability of maintaining good graft function with lower tacrolimus doses in the MSC group, and the commendable attempt to a mechanistic analytical approach, the small sample size suggests caution with the interpretation and generalizations of the results of this interesting pilot study at the present time.

Another pilot and feasibility clinical trial on the use of donor BM-MSCs in seven HLA mismatched LDKT recipients has been reported by Lee *et al.* [6]. All patients received conventional immunosuppressants based on induction with rATG (for a total of 8–10 days at 1.5 mg/kg daily) and maintenance with CNI, MMF and steroids. On the day of kidney transplantation, donor BM-MSC (1×10^6 cell/kg) was directly injected into the bone marrow of the recipient's right iliac bone, based on previous study suggesting good engraftment achieved following intra-bone marrow hematopoietic cell inoculum in humans [55]. The inoculum was uneventful. No graft failure was recorded, though biopsy-proven acute rejections were observed in 3 recipients during the follow-up period controlled well with steroid pulse therapy. Acute antibody-mediated rejection was observed at day 9 after the transplantation in one patient who was treated by intravenous immunoglobulin (IVIG) and plasmapheresis; acute cellular rejection was observed at 43 days and 613 days of post-transplantation, responsive to steroids; another acute cellular rejection episode was detected on the protocol biopsy at 12 months after the transplantation; two cases of borderline change observed in two patients not associated with clinical signs of rejection and not require any additional treatment. A significant reduction of donor-specific lymphocyte and mitogen-induced T cell proliferation were observed in two patients. No chimerism was detected at any time. Donor-specific lymphocyte or T cell proliferation and Treg priming responses were observed in some patients. Collectively, the study suggests that intra-BM administration of MSCs could be technically performed, though the impact of allogeneic BM-MSCs on clinical LDKT survival was not dramatic as a possible consequence of the limited sample size.

8.4.4 Autologous MSCs for the treatment of biopsy-proven subclinical rejection, progressive renal interstitial fibrosis and tubular atrophy

A pilot trial of autologous bone marrow-derived MSCs to treat acute rejection episodes and renal interstitial fibrosis and tubular atrophy (IF/TA) in six (out of 15 screened) recipients of fully HLA mismatched LDKT has been recently reported by Reinders *et al* [5]. By protocol, patients displaying rejection or increased IF/TA at the 6-month protocol biopsy (compared to the previous one done at 4 weeks) received 10^6 cells/kg twice intravenously one week apart. After MSC inoculum, patients were monitored clinically and immunologically for 24 months. Immunosuppression was based on anti-CD25 antibody (basiliximab) induction and standard triple-drug maintenance based on CNI (tacrolimus or CsA), MMF and prednisone. Maintenance doses were not

changed in the six patients. Valganciclovir prophylaxis was administered for 3 months (except for a cytomegalovirus-negative donor:recipient status). The MSC inoculum was uneventful and well tolerated by all study subjects. Three study subjects experienced opportunistic viral infection after MSCs inoculum: one patient developed BK virus-associated nephropathy 21 weeks after MSCs infusion that resolved without reduction of immunosuppression; another patient *de novo* CMV infection 2 weeks after MSCs infusion (6 months after prophylactic valganciclovir discontinuation) that resolved without reduction of immunosuppression; one other patient displayed a low-grade CMV viral load persisted in the months after MSCs infusion, despite reduction of clinical immune suppression. In two subjects with allograft rejection (Banff 1A with mild interstitial fibrosis/tubular atrophy (IF/TA) and Banff 1B, respectively) biopsy performed subsequent to MSCs inoculum demonstrated resolution of tubulitis without IF/TA. The authors also described reduced *in vitro* leukocyte proliferative responses 12 weeks after MSCs inoculum. Although a limited sample size, the results of this pilot trial are quite encouraging as it suggests for the first time a potential direct effect of MSCs therapy in promoting resolution of the features of rejection in clinical allogeneic renal grafts (Tab. 8.2).

Table 8.2: Beneficial effects associated with MSCs therapy in recent clinical organ transplantation trials.

Observed Benefit	Settings	Type of MSCs	Time of inoculum*	Ref.
Induction of Treg Inhibition of memory T cells	LDKT	Autologous BM-MSCs	1 week	[2]
Alternative to anti-CD25 blockade Reduction of maintenance CNI dose Early graft function Reduced acute rejection Reduced opportunistic infections	LDKT	Autologous BM-MSCs	Day 0 and 2 week	[2]
Reduction of maintenance CNI dose Transient increase of memory B cells	LDKT	Donor-specific, allogeneic BM-MSCs	Day 0 and 1 month	[4]
Reduced tubulitis, interstitial fibrosis/tubular atrophy	LDKT	Autologous BM-MSCs	6 months	[5]
Reduced MLR responses	LDKT	Donor-specific, allogeneic BM-MSCs	Day 0	[6]

* Abbreviations: BM: bone marrow; CNI: calcineurin inhibitor(s); LDKT: living donor kidney transplant; MSCs: mesenchymal stromal (stem) cell

8.5 Future perspectives

The ultimate goal of organ transplantation is to ameliorate patient and graft survival following end-stage organ disease. The immunomodulatory properties of MSCs are appealing to promote tissue repair (*i.e.*, ischemia-reperfusion) and to reduce immunosuppression therapy. Therefore, MSC administration could lead to improving engraftment and longevity of functionality, while minimizing the side effects of current antirejection protocols. In addition, harnessing MSC-mediated immunomodulatory function may ultimately favor the induction of immune tolerance. We are living in very exciting times with the implementation of novel clinical trials aimed at establishing safety, feasibility and efficacy of MSCs use to improve solid organ transplant outcomes [2–6, 23]. A number of issues remain to be addressed by concerted translational research approaches, particularly related to the identification of the most suitable source for transplantable MSCs products, standardization of cell processing and product release, route of administration and optimization of concomitant therapy to be used to maximize efficacy for organ transplantation and for any other clinical application by and large. There are several emerging new protocols for the isolation and utilization of different cell products for regenerative and immune therapy applications [56]. The premises are quite promising justifying cautious optimism for the immediate future.

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Abbas Ali Qayyum and Jens Kastrup

9 Stem cell therapy in patients with ischemic heart disease

Abstract Ischemic heart disease (IHD) is one of the leading causes of death worldwide and characterised by the formation of atherosclerosis in the coronary arteries reducing the quality of life of the patients with chest pain and/or dyspnea.

IHD can result in acute myocardial infarction (AMI), chronic ischemic heart disease (CIHD) and heart failure. Common for all these conditions is lost and/or dysfunctional cardiomyocytes and endothelial cells. Current conventional therapies cannot replace the dysfunctional and lost cardiomyocytes and endothelial cells. Cell-based regenerative therapies using cells harvested from different tissue sources could be a new treatment of patients with IHD. At the moment, autologous stem cell treatment is used more often than allogeneic. Adipose tissue from the abdomen is an attractive source for harvesting mesenchymal stem cells (MSCs). It contains 300 times more MSC-like cells than bone marrow which has traditionally been the source of stem clls. Pre-treatment before delivery of the cells into the heart may ensure the effectiveness of the delivered progenitor cells.

Many preclinical and clinical studies have shown encouraging results. Still, many factors remain to be elaborated such as timing of the delivered cells, route of delivery, dose of the cells, cell type *etc*. On-going trials will give answers to many of the questions andlp makinge cell-based therapy an established treatment in patients with IHD in near future.

9.1 Introduction

Ischemic heart disease (IHD) is one of the leading causes of death worldwide. According to the World Health Organization more than 7 million people die each year from IHD. Due to the development of atherosclerosis in young age and the manifestation later on, the lifetime risk for IHD increases with age and the risk for IHD after 40 years is 49 % for men and 32 % for women [1]. Moreover, the increasing aging population in the developed countries will become a burden to the health care sector in near future if the prevention and treatment of IHD is not improved.

The heart is an essential organ pumping blood around supplying all organs in the body with nutrients and oxygen, and carrying away waste products and carbon dioxide. The blood supply to the heart itself is through the coronary arteries. IHD is characterized by the formation of atherosclerosis in the coronary arteries, which slowly reduces the blood supply to the heart muscle. This can give rise to chest pain and dyspnea on exertion, in cold weather or in emotional stress situations. Further-

more, this can result in acute myocardial infarction (AMI), chronic ischemic heart disease (CIHD), heart failure and sudden cardiac death.

After a myocardial infarction up to one billion cardiomyocytes are lost due to apoptosis and necrosis, which are then replaced by fibroblasts and visualized through advanced imaging techniques as scar tissue [2] (Fig. 9.1). This rapid formation of scar tissue prevents myocardial rupture and saves the life of the affected patient. However, the ventricle wall is now thin and the ability to contract is reduced, leading to heart failure and reduced organ perfusion with increased morbidity and mortality.

Until now, the cells in the heart were thought terminally differentiated. However, cardiac progenitor cells (CPCs) in the heart have been shown to be able to differentiate into endothelial cells and cardiomyocytes [3-8]. Nevertheless, following a myocardial infarction, the amount of CPCs may not be sufficient to repair the damage caused by the occluded blood vessel. This is probably due to the low number of CPCs in the heart and destruction of CPCs in the infarcted area. Moreover, the ability of the CPCs *in vivo* to proliferate and differentiate may decrease with age [9, 10].

Current medical and interventional strategies cannot replace the lost cardiomyocytes even though these therapies have reduced the overall mortality of patients with IHD. While for some patients the symptoms can be handled thorough medication and lifestyle changes, there remains a group of patients having severe daily symptoms with chest pain and dyspnea, and reduced quality of life.

To treat this group of patients, a lot of work is being done by researchers to find new treatment options. Cell-based therapies have emerged as a novel potential therapy for treatment of patients with IHD. Inside the different potentially clinically useful cell lines, mesenchymal stem cells (MSCs) are a promising source for repair-

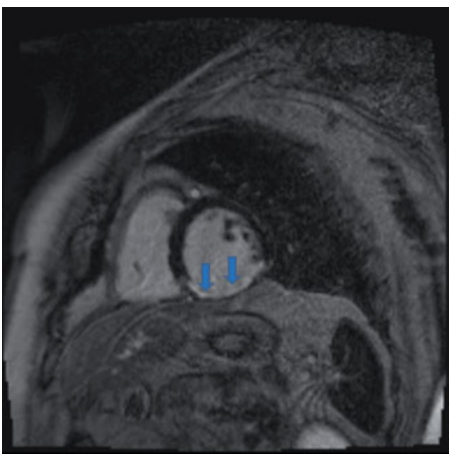

Fig. 9.1: Cardiac magnetic resonance imaging showing scar tissue. The blue arrows indicate the fibrotic scar tissue in the left ventricle in a patient with previous myocardial infarction. In this region, the myocardial wall is thin and the contractility is reduced.

ing the myocardial damage including replacement of cardiomyocytes and endothelial cells, which are lost or dysfunctional due to myocardial infarction and IHD.

9.2 Cell type and source for clinical therapy

Several clinical studies have been conducted for the treatment of patients with IHD using different types of cells *e.g.* mononuclear cells, $CD34^+$ cells, $CD133^+$ cells, MSCs *etc.* [11]. No consensus exists between researchers about the best cell type for the treatment of IHD at the moment. Whether these cell linages are related to each other has to be elucidated as well.

Traditionally, cells obtained from bone marrow have been widely used for regenerative cardiac purposes. The presence of MSCs in all tissues of the body indicates their importance. At the time of birth, MSCs can be isolated from the umbilical cord blood. Storage of these and later on autologous usage have long perspectives and will probably be overtaken by allogeneic MSCs therapy.

Most of the patients with IHD have a body mass index above average. This makes adipose tissue from the abdomen an attractive source for harvesting MSCs, which contains 300 times more MSCs-like cells than bone marrow [12]. Furthermore, cells isolated from adipose tissue may also grow faster than bone marrow-derived cells [13]. Having in mind that only a small amount of the harvested material from bone marrow represents MSCs compared to obtaining the MSCs analogue adipose-derived stem cells (ADSCs) from adipose tissue, means that adipose tissue is an attractive source of cells. Up to 1% of adipose cells are estimated to be ADSC, while MSCs represent only 0.001–0.002% of cells in bone marrow [13].

The primarily obtained cells from the adipose tissue (adipose tissue-derived cells (ADC)) also called stromal vascular fraction can be used immediately after purification or the ADSCs can be isolated and culture expanded (Fig. 9.2). A comparable study in mice with AMI showed that no differences may exist between cultured human ADSCs and freshly isolated human ADCs in improving the heart function [14]. However, the effect of MSCs may be dose dependent [11, 15]. And after culturing the isolated ADSCs and bone marrow-derived MSCs, a several fold increase in total cell counts can be reached (Fig. 9.3).

At the moment, autologous stem cells are used most frequently for the treatment of patients with IHD. The properties and proliferation rate of the obtained cells may differ from patient to patient. For investigating the proliferative potential of bone marrow-derived MSCs, a study recruited 51 patients undergoing elective coronary artery bypass grafting (CABG) due to CIHD [16]. Surprisingly, the proliferation of MSCs was higher in patients with diabetes mellitus, in patients receiving steroid treatment, known chronic obstructive pulmonary disease (COPD), renal failure and impaired left ventricular ejection fraction (LVEF). Whether higher proliferation of MSCs is correlated to increased action of these stem cells is an interesting issue to be answered.

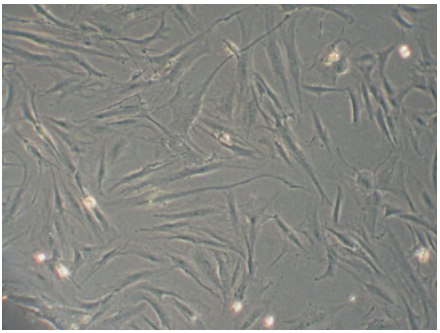

Fig. 9.2: Adipose-derived stem cells. Culture expanded adipose-derived stem cells (ADSCs) visualized through light microscope. These ADSCs are obtained from a patient known with ischemic heart disease and will be delivered to the patient intramyocardially after culture expansion.

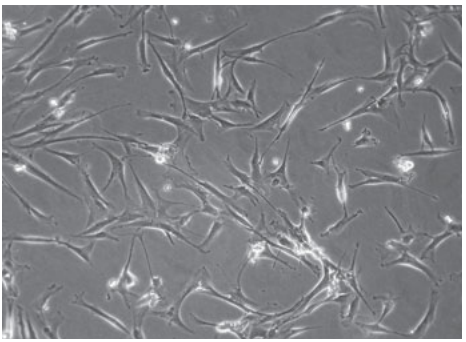

Fig. 9.3: Bone marrow derived mesenchymal stem cells. Culture expanded mesenchymal stem cells (MSCs) visualized through light microscope. Traditionally, MSCs have been obtained from the bone marrow and used for autologous delivery in patients with heart disease.

9.3 Mechanisms behind regeneration of damaged myocardium

The mechanisms for replacement of dysfunctional cardiomyocytes, fibroblasts and angiogenesis in the damaged myocardium are subjected to intense investigations. In patients with myocardial perfusion abnormalities due to CIHD, the formation of blood vessels is one way to enhance the perfusion in the ischemic region [17, 18], while in patients with infarct the fibroblasts have to be replaced by cardiomyocytes (Fig. 9.4). The main intention of this regeneration is to increase supply of oxygen and nutrients in the area with diminished blood flow and to restore the function of the damaged/dysfunctional myocardium. Reducing the acute endogenous inflammatory response leading to necrosis and apoptosis of cardiomyocytes and endothelial cells may be the main target of interest in patients with AMI.

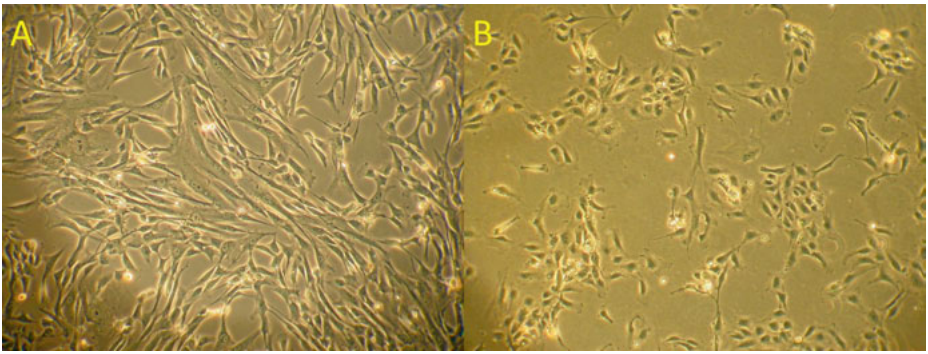

Fig. 9.4: Cardiomyocytes and fibroblasts. Cardiomyocytes (A) and fibroblasts (B) visualized through light microscope. Cardiomyocytes are contractile cells. These cells are spindle-formed and replaced by fibroblasts after a myocardial infarction forming scar tissue. The scar tissue is necessary to prevent myocardial rupture in patients with acute myocardial infarction. However, these cells are noncontractible and can lead to heart failure.

Animal studies indicate that MSCs are capable of differentiating into vascular and cardiac cell lineages [19-26]. However, the mechanism behind this is not yet fully elucidated. The proliferative capacity of stem cells may decrease with age [9, 10] even though this is controversial [27, 28]. MSCs obtained from animals have been shown to be able to differentiate into endothelial cells and cardiomyocytes [4-6] but this may not be the truth or at least not the whole mechanism by which they exert their effect. MSCs secrete a great amount of growth factors and cytokines by which they mediate endogenous regeneration *via* activation of resident CPCs or trapping of circulating stem cells. Moreover, the cytokines may lead to anti-inflammation, anti-apoptosis and anti-remodeling [29]. The secretion of cytokines and growth factors [30] may stimulate CPCs in the heart [5] to differentiate into cardiomyocytes and enhance vessel formation. CPCs need nutrients, growth factors and a stimulating micromilieu. MSCs can live in an inflammatory milieu and exert an immunomodulatory effect, which may protect CPCs so that they can exert their effect.

Several growth factors are upregulated and especially the important vascular endothelial growth factor (VEGF) expression is increased when oxygen delivery is decreased, *e.g.* in patients presented with AMI and after exercise, myocardial and skeletal muscle VEGF expression is raised, respectively [17]. However, myocardial biopsies obtained from patients known to have CIHD undergoing CABG did not show an upregulation in VEGF in areas with reduced perfusion due to coronary artery disease [31]. Nevertheless, VEGF play a critical and essential role in the formation of vessels [32]. Additionally, the secretion of several different growth factors by MSCs is increased in hypoxic tissue [33].

Different pretreatment regimens have shown that these MSCs survive better after delivery to target area in the heart than untreated. A group of researchers genetically modified MSCs to overexpress the anti-apoptotic gene *BCL2* [34]. BCL2 protein has been found overexpressed in B-cell lymphoma and is a main regulator in the pathway to inhibit cell death. The genetically modified MSCs had significantly higher survival compared to control group at day 4, week 3 and week 6 after intramyocardial (IM) delivery in an AMI rat model. Furthermore, this led to greater capillary density and reduced infarct size.

In another study bone marrow-derived MSCs were pretreated with VEGF [35]. Culturing MSCs may induce cellular stress which can be determined by measuring the expression of the proteins p16INK, p21 and p19ARF. MSCs at passage 10 were treated with VEGF-A165 for 24 hours, which increased cell proliferation and reduced p16INK, p21 but not p19ARF compared to untreated MSCs at passage 2–3. Furthermore, this study showed in an AMI mice model that, co-injection of MSCs and VEGF increase engraftment of MSCs compared to MSCs injected alone by 2.1 and 2.9 fold after one day and after one week, respectively.

Moreover, some interesting findings have been done by a research group who pretreated MSCs with a cocktail of growth factors [36]. Rat bone marrow-derived MSCs pretreated with fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) showed better survival after exposure to 0.5 % hypoxia for 30 hours in *in vitro* conditions compared to untreated MSCs ($P < 0.05$). Cardiomyocytes exposed to hypoxia for 24 hours in culture with the pretreated MSCs increased the survival of cardiomyocytes compared to cardiomyocytes exposed to hypoxia alone. Furthermore, these pretreated MSCs were injected into the border zone of an AMI rat model after 7 days. The histological analysis of the rat heart 7 weeks after the delivery showed that the infarct area was significantly reduced in rats receiving pretreated MSCs compared to untreated MSCs ($p < 0.05$). No significant difference in secretion of insulin-like growth factor-1 and hepatocyte growth factor was found between pretreated and untreated MSCs.

Although, preclinical studies using VEGF demonstrated promising results in improving perfusion and formation of blood vessels [18], IM injections of adenovirus carrying VEGF-121 did not increase exercise capacity in 17 patients with CIHD [37] and IM injections of plasmid VEGF-A165 did not show improvement in stress-induced myocardial perfusion in 70 patients with CIHD [38].

Furthermore, the effect of IM-delivered VEGF-A165 plasmid followed by granulocyte colony stimulating factor (G-CSF) was investigated in patients with CIHD [39]. Sixteen patients with proved reversible myocardial ischemia visualized on single photon emission computerized tomography were included into the study. One week after IM injections of plasmid VEGF-A165, the patients received subcutaneous injections of granulocyte colony-stimulating factor (G-CSF) for 6 days. Despite a 10-fold increase in circulating $CD34^+$ stem cells, no changes in myocardial perfusion or clinical symptoms were seen compared to a control group.

Pretreatment of MSCs before the delivery to increase the secretion of growth factors may be another approach in clinical studies. VEGF-A165 is probably one of the most active isoforms [17]. VEGF stimulation may induce differentiation of MSCs into endothelial linage and the micromilieu around the MSCs may stimulate them to secrete growth factors for the use of vessel formation and cardiomyogenesis. This approach is being tested in a clinical Phase II trial enrolling patients with severe CIHD [40] after encouraging results from an open label study [15, 41].

9.4 Preclinical experience with stem cells for IHD

Many stem cell studies in small and large animals have been conducted before and along with clinical trials to assess the risk of these new cell-based therapies and to predict the feasibility and efficacy.

A large meta-analysis of preclinical studies including 52 publications with more than 800 animals showed a significant increase in LVEF of 7.5 % compared to controls ($P < 0.001$) [42]. This meta-analysis included both studies of AMI ($n = 23$) and CIHD ($n = 29$) animal model. These animals received mononuclear cells, skeletal myoblasts, MSCs, endothelial progenitor cells, somatic cells, cardiosphere-derived cells, embryonic stem cells, hematopoietic progenitor cells or ADSCs. Different routes for cell delivery were also used. The animals receiving higher number of cells, animal models of CIHD, animals with left anterior descending (LAD) infarction and greater degree of involved myocardium showed greater benefit from cell treatment. Interestingly, the subanalysis showed a larger benefit in animals treated with MSCs compared to bone marrow-derived mononuclear cells. No significant differences in death were seen in animals treated with cells compared to controls.

The largest study included in the meta-analysis [42] was published in 2009 [43]. Forty-seven sheep with AMI were treated with allogeneic MSCs. The sheep received IM 25–450 million MSCs. This randomized, dose-escalating, placebo controlled study indicated that IM delivery of MSCs may influence post-AMI LV remodeling in a dose-dependent manner.

9.5 Cell-based therapy in patients with IHD

Several Phase I and Phase II studies have demonstrated that stem cells delivered into the heart in patients with myocardial infarction are safe and feasible. A large meta-analysis of clinical studies including 50 publications with more than 2600 patients with AMI and CIHD enrolled and treated with bone marrow-derived cells showed a significant increase in LVEF of 3.96 % compared to controls ($P < 0.00001$) [11]. A threshold of 40×10^6 cells was associated with improved LVEF and reduced infarct size, while treatment under this value did not reveal any benefit in these parameters.

The improvement in LVEF and infarct size was seen regardless of the type of IHD (AMI vs. CIHD). No differences were seen in patients with AMI receiving cells < 7 days vs. 7–30 days after AMI. The increase in LVEF was significantly greater in patients who received bone marrow-derived cells with heparin compared to patients who received bone marrow-derived cells without heparin (P = 0.002). The improvements were not associated to the treated vessel territories and persisted for more than 12 months. Interestingly, the patients treated with bone marrow cells showed a reduced incidence of death and recurrent AMI.

IM injections of bone marrow-derived mononuclear cells along with CABG in patients with CIHD have also been shown to significantly improve LVEF by 5.40 % (P < 0.009) from baseline to follow-up (3 to 6 months). This meta-analysis of 6 studies including 179 patients undergoing CABG showed no difference in cardiovascular events between the patients receiving cells compared to those who did not [44].

A meta-analysis of 29 randomized clinical trials with more than 1800 patients with AMI alone showed that intracoronary (IC)-delivered bone marrow-derived stem cells improved LVEF significantly. However, the stem cells mobilized after G-CSF treatment did not show a significant improvement [45], suggesting that endogenous mechanisms to increase the number of circulating stem cells may not be sufficient and exogenously delivered stem cells are necessary for the increase in LVEF [46].

A total of 10 clinical trials with 422 patients with CIHD were included in a meta-analysis for the assessment of the effect of bone marrow-derived stem cells and circulating progenitor cells used along with standard revascularization therapy. This meta-analysis showed a significant increase in LVEF in patients who received cells compared to controls at 3 and 6 months follow-up [47].

A lot has been discussed regarding stem cells obtained from different patients. A meta-analysis including 10 randomized clinical trials with more than 800 patients with AMI showed a significant increase in LVEF of 3.79 % in patients receiving bone marrow-derived cells compared to a control group (P < 0.001). Surprisingly, this meta-analysis showed cell therapy being significantly more effective in patients with diabetes, in aging patients and in females [27].

The timing of delivered stem cells has also been a subject for discussion in patients with AMI. This was evaluated in a meta-analysis including 7 trials with 660 patients who received IC-delivered bone marrow mononuclear cells. Stem cells delivered between 4 and 7 days after the AMI were associated with significant improvement in LVEF compared to stem cells delivered within 24 hours after AMI (P = 0.01) [48]. This suggests that the acute inflammatory response as a result of the AMI must diminish before exogenously delivered stem cells can survive.

These meta-analyses included patients with acute and chronic myocardial ischemia, different doses of cells, delivery routes and different types of cells. At the moment no consensus exists regarding the influence of these factors on the clinical outcome.

9.6 MSCs in patients with IHD

Tables 9.1 and 9.2 summarize the published studies using MSCs for patients known with AMI and CIHD, respectively. The first clinical trial using MSCs for IHD was published in 2004 [49]. Since then, several Phase I and II trials have been published. To date, the largest study included 69 patients with AMI who were treated with IC MSCs infusion [49]. This randomized, placebo controlled study treated 34 patients with 48-60 $\times 10^9$ MSCs. Compared to the control group, the 3 months follow-up data showed that it was safe and there was a significant increase in LVEF which persisted at 6 months follow-up ($P = 0.01$). Furthermore, perfusion defects measured by positron emission tomography ($P = 0.001$) and NOGA ($P = 0.01$) at 3 months follow-up decreased significantly.

An interesting study was published in 2009 [59]. This randomized double-blinded, placebo-controlled study enrolled 53 patients with AMI who received intravenous (IV) doses of 0.5, 1.6 or 5 million allogeneic MSCs per kg bodyweight. Patients who received MSCs infusion had significantly fewer episodes of arrhythmia ($P = 0.025$) at 6 months follow-up and forced expiratory volume in 1 second (FEV1) was significantly higher in the group treated with MSCs ($P = 0.01$). A dose-dependent effect was not observed. No effect on LVEF between groups was observed. However, in patients with anterior AMI, LVEF increased significantly compared to baseline ($P = 0.0436$). This observation was not found significant in the control group.

The first large study injecting VEGF-stimulated MSCs IM in patients with CIHD was published in 2011 [15]. A total of 31 patients were treated with an average of 21.5 million VEGF-stimulated MSCs. At 6 months follow-up, this study showed a significant reduction in angina attacks and symptoms ($P < 0.001$), increase in exercise capacity ($P < 0.001$) and LVEF measured by magnetic resonance imaging ($P < 0.001$) compared to baseline. This study showed a trend towards improved outcome with increasing number of cells injected. Increase in exercise capacity and reduction in clinical symptoms persisted at 1 year follow-up ($P < 0.001$) [41].

The first study comparing allogeneic versus autologous MSCs obtained from bone marrow and delivered IM in 30 patients with CIHD found no differences in serious adverse events at 30 days follow-up and at 1 year follow-up [58]. This dose-escalating study found in patients who received autologous MSCs a significant increase in 6 minute walk test at 6 and 12 months follow-up compared to baseline while this was not the case for patients receiving allogeneic MSCs. However, both allogeneic and autologous MSCs reduced infarct size measured with computed tomography scan at 13 months follow-up compared to baseline by 31.61 % ($P < 0.001$) and 34.93 % ($P < 0.001$), respectively. No statistically significant increase in LVEF was seen in the groups. Interestingly, inverse dose-response effect on LVEF, LV systolic volume and infarct size was found for patients receiving 20×10^6 vs. 200×10^6 MSCs.

Due to the vulnerable myocardium, no studies have yet treated patients with AMI and IM injections of MSCs (Tab. 9.1). Furthermore, no studies have been published

Table 9.1: Studies using mesenchymal stem cells in patients with acute myocardial infarction.

Reference	No. of patients	Follow-up months	Design	Dose	Delivery route	Outcome	Year
Chen et al. [49]	69	3	RPCT	48–60 × 10^9	IC	Improved LVEF, LV dimensions, perfusion and wall motion	2004
Katritsis et al. [50]	22	4	Open label	1–2 × 10^6	IC	Improved wall motion and perfusion	2005
Hare et al. [59]	53	6	RPCT	0.5/1.6/5 × 10^6 /Kg	IV	Increased FEV1. Not arrhythmogenic	2009
Yang et al. [51]	16	6	Open label	10 × 10^6	IC	Safe and feasible	2010
Houtgraaf et al. [52]	14	6	RPCT	20 × 10^6	IC	Improved perfusion and scar tissue	2012

FEV1: forced expiratory volume in 1 second – IC: intracoronary – IV: intravenous – LV: left ventricular – LVEF: left ventricular ejection fraction – RPCT: randomized placebo-controlled trial.

Table 9.2: Studies using mesenchymal stem cells in patients with chronic ischemic heart disease.

Reference	No. of patients	Follow-up months	Design	Dose	Delivery route	Outcome	Year
Chen et al. [53]	22	6	Open label	5 × 10^6	IC	Increased LVEF and exercise capacity. Reduced symptoms	2006
Mohyeddin-Bonab et al. [54]	8	6	Open label	5.55 × 10^6	IC/IM	Increased LVEF, reduced symptoms and infarct size	2007
Katritsis et al. [55]	5	16–36	Open label	1.3–2.8 × 10^6	IC	Not arrhythmogenic	2007
Williams et al. [56]	4	12	Open label	100/200 × 10^6	IM	Reduced LV dimensions and infarct size	2011
Friis et al. [15]	31	6	Open label	21.5 × 10^6	IM	Increased LVEF and exercise capacity. Reduced symptoms.	2011
Lasala et al. [57]	10	6	Open label	7.5 × 10^6	IC	Increased LVEF	2011
Hare et al. [58]	30	1	ROL	20/100/200 × 10^6	IM	Safe and feasible	2012
Mesoblast	60	12	RPCT	25/75/150 × 10^6	IM	Safe and feasible	UP *

IC: intracoronary – IM: intramyocardial – LV: left ventricular – LVEF: left ventricular ejection fraction – ROL: randomized open label study – RPCT: randomized placebo controlled trial – UP: unpublished data.
* Perin E. A Phase II dose-escalation study of allogeneic mesenchymal precurser cells in patients with ischemic and non-ischemic heart faliure. Presented at: American Heart Association, Scientific session 2011. Orlando, Florida. 14 November 2011.

using IV MSCs in patients with CIHD (Tab. 9.2). The published studies using MSCs in patients with CIHD have mainly been open label studies.

The pro-arrhythmic effect of MSCs has been a potential concern for researchers using stem cell therapy in patients with IHD [60]. However, the Prochymal trial showed a reduced incidence of ventricular arrhythmia compared to the control group [59] and a smaller trial could not detect any arrhythmic effect of MSCs [55].

The improved LVEF may not only be attributed to transdifferentiation of the injected stem cells alone but also to the paracrine effects of them as well. MSCs may contribute to angiogenesis by improving perfusion in the ischemic region, differentiate into cardiomyocytes replacing scar tissue, reduce inflammation and prevent apoptosis (Fig. 9.5). Surrogate markers for improvement according to stem cell therapy such as LVEF have until now been used for evaluation in most of the studies published. However, ongoing trials may have different approaches for the assessment of a clinical relevant effect of the therapy [40].

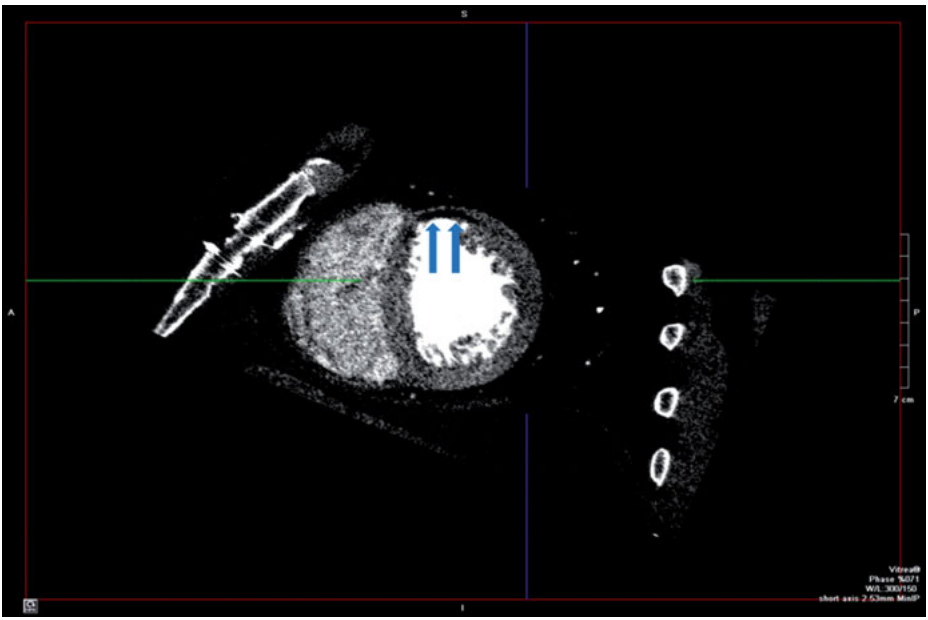

Fig. 9.5: Ischemic region visualized through cardiac computed tomography. The blue arrows indicate the ischemic region identified in the anterior region of left ventricle in a patient with ischemic heart disease. This region may be the target region for stem cell therapy.

9.7 Ongoing clinical trials using MSCs

Several other studies are ongoing using MSCs in patients with AMI and CIHD. Table 9.3 shows the 6 trials registered on clinicaltrials.gov (October 2012) using MSCs in patients with AMI. The tendency towards individualized treatment is reflected in the use of a dose which is based on the bodyweight of the patient. One study is recruiting patients with AMI for IM injections of MSCs (NCT01394432). A similar study has been published including 12 patients with AMI and LVEF < 45 % treated with IM-delivered bone marrow-derived mononuclear cells [61]. The injections were performed into the border zone of the infarcted myocardium 17.5 ± 0.8 days after primary revascularization. Compared to a matched control group, there was a significant increase in LVEF of 7.9 % ($p = 0.001$) at 6 months follow-up.

Table 9.4 collects the 9 trials registered on clinicaltrials.gov using MSCs in patients with CIHD. Two of these studies are being conducted at our center, The Heart Centre, Rigshospitalet, Copenhagen, Denmark. Based on our experiences with the first in-man trial using VEGF-A165 stimulated MSCs in patients with CIHD [15], we are at the moment conducting 2 studies using culture expanded MSCs. In the MSC-HF trial [62], MSCs are obtained from bone marrow and delivered IM in patients with heart failure (NCT00644410). In contrast, the MyStromalCell trial [40] use MSCs isolated from abdominal adipose tissue in patients with CIHD and preserved LVEF, and before IM delivery they are stimulated for one week with VEGF-A165 (NCT01449032).

9.8 Cell delivery and engraftment

The method of delivery may be essential for the success of therapy. The cells have to survive and integrate into the region of interest. The most frequently used methods are IM and IC delivery of the cells. However, IV route for delivery of cells has been used as well.

With IM, the cells are injected into the region of interest in the myocardium by a needle. This enables the cells to be delivered directly into a hypoperfused area in the heart or in the border zone of an infarcted area. Epicardial IM injections are often used in animal models. However, it can only be used in patients undergoing thoracotomy *e.g.* during CABG.

Endocardial IM delivery of cells can be done with the NOGA XP system (Biological Delivery System, Cordis, Johnson & Johnson, US) [38, 63]. The simultaneous electromechanical mapping of the left ventricle by the NOGA XP system showing myocardial viability ensures that the cells are injected into the area of interest [64] (Fig. 9.6).

IM delivery of MSCs may be superior to IC. In a canine AMI model, IM delivery of MSCs resulted in higher cell retention, significantly reduction in myocardial ischemia and increased LVEF compared to IC delivery with MSCs delivered 7 days after AMI and evaluated after 21 days [65].

Table 9.3: Trials registered on clinicaltrials.gov using mesenchymal stem cells in patients with acute myocardial infarction.

Condition	No. of patients	Dose (10^6 per kg bw)	Source	Outcome	Delivery route	LVEF	Status	Phase	Study design	Follow-up months	Reference
AMI	80	1	Autologous	LVEF by SPECT	IC	–	Completed	II/III	ROL	6	NCT01392105
AMI	50	–	Autologous	LVEF by MRI	IM	< 50 %	Recruiting	III	RCT	12	NCT01394432
AMI [59]	48	0.5 1.6 5.0	Allogeneic	Safety	IV	> 30 %	Completed	I	ROL	6	NCT00114452
AMI	20	–	Allogeneic	Safety	IV	30–50 %	Not yet recruiting	I/II	RCT	6	NCT00883727
AMI	135	1	Autologous	LVEF by MRI	IC	< 45 %	Not yet recruiting	III	Open label	13	NCT01652209
AMI	220	–	Allogeneic	LVESV	IV	20–45 %	Not yet recruiting	II	RCT	–	NCT00877903

AMI: acute myocardial infarction – IC: intracoronary – IM: intramyocardial – IV: intravenous – kg bw: kilogram bodyweight – LVEF: left ventricular ejection fraction – LVESV: left ventricular end-systolic volume – MRI: magnetic resonance imaging – RCT: randomized double-blinded placebo-controlled study – ROL: randomized open label study – SPECT: single photon emission computed tomography.

Table 9.4: Trials registered on clinicaltrials.gov using mesenchymal stem cells in patients with chronic ischemic heart disease.

Condition	No. of patients	Dose(10^6)	Source	Outcome	Delivery route	LVEF	Status	Phase	Study design	Follow-up months	NCT
CIHD [15, 41]	31	–	Autologous	Myocardial perfusion by SPECT	IM	–	Completed	I/II	Open label	–	NCT00260338
CIHD	45	20 200	Autologous	Safety	IM	15–50 %	Completed	I/II	RCT	6	NCT00587990
CIHD [58]	30	20 100 200	Autologous vs. Allogeneic	Safety	IM	20–50 %	Completed	I/II	ROL	1	NCT01087996
CIHD	60	–	Autologous	Exercise test	IM	> 40 %	Recruiting	II	RCT	6	NCT01449032
CIHD	60	20 – 40	Autologous	LVEF	IM	< 45 %	Recruiting	I/II	RCT	6	NCT00644410
CIHD	10	60	Autologous	Safety	IM	< 35 %	Recruiting	I/II	Open label	1	NCT01076920
CIHD [73]	60	100 200	Autologous	Safety	IM	≤ 50 %	Recruiting	I/II	RCT	1	NCT00768066
CIHD	60	5 – 1000	Autologous	LVEF by MRI	IM	15–45 %	Recruiting	II	RCT	12	NCT00418418
CIHD	24	–	Autologous	Safety	IM	≤ 50 %	Recruiting	I	Open label	6	NCT01557543

AMI: acute myocardial infarction – CIHD: chronic ischemic heart disease – IC: intracoronary – IM: intramyocardial – IVUS: intravascular ultrasound – LVEF: left ventricular ejection fraction – MRI: magnetic resonance imaging – RCT: randomized double-blinded placebo-controlled study – ROL: randomized open label study – SPECT: single photon emission computed tomography.

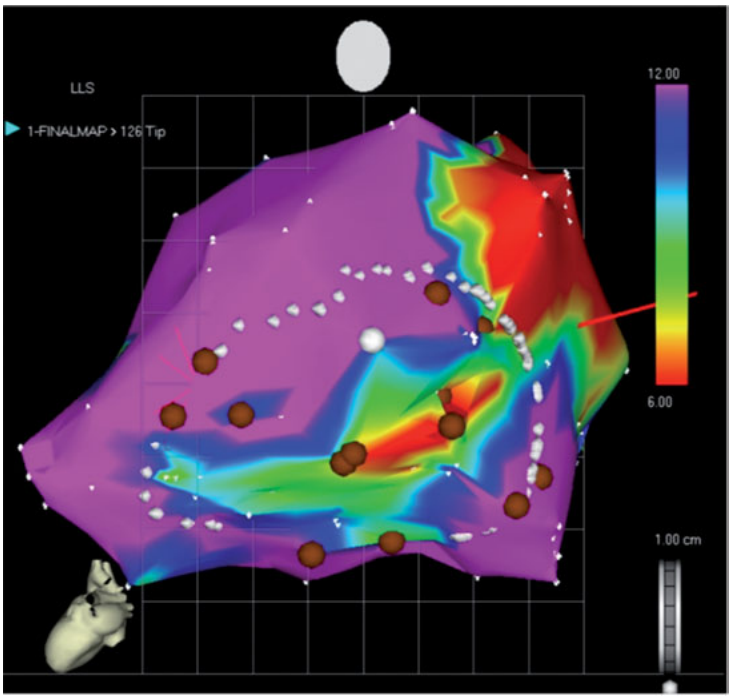

Fig. 9.6: Simultaneous electromechanical mapping of left ventricle. Brown dots in the ischemic region identified by the NOGA XP system (Biological Delivery System, Cordis, Johnson & Johnson, US) indicate the sites of intramyocardial delivered stem cells.

IC-delivered cells inherently need an artery with blood flow reaching the area of interest for the cells to be delivered to the region. IC delivery may result in cells lost to systemic circulation. However, IC-delivered cells cause less myocardial damage compared to IM [66]. Although IM-delivered cells can be injected into the area of interest, it needs expensive machinery, trained personnel and takes more time compared to IC.

IV-delivered cells are less frequently used in clinical trials and only a small amount ends up in the heart while most of the cells are found in lungs, spleen and liver [67]. However, the Prochymal trial showed significantly increased LVEF in the group with anterior infarction who received IV allogeneic MSCs [59]. Additionally, in a CIHD animal model no differences were observed between IC and IM-delivered indium-oxid labeled MSCs assessed by gamma-emission counting [68].

The IV-delivered MSCs may have an ability to home into sites of inflammation and thereby they reach the ischemic region of the heart in patients with AMI [69]. Nevertheless, the use of IV delivery may not cause complications including damage to the heart where the injections are made when cells are delivered IM. Another approach to ensure a successive delivery of cells may be using scaffolds as biomimetic platforms, which are being made for MSCs to increase cellular ingrowth [70].

There is not much knowledge about the homing of the cells in the heart after delivery. Therefore, it would be interesting to track the delivered stem cells noninvasively in patients. The most common cell-labeling agents are fluorodeoxyglucose (FDG), technetium and indium. However, these agents have short half-lives, which only enable tracking of the cells for 24 hours (FDG and technetium) or a few days (indium). Iron oxide labeling of cells enables tracking of the cells for many weeks with magnetic resonance imaging [71]. Upcoming studies may elucidate the fate of the delivered stem cells in clinical settings. However, cell division, apoptosis, uptake by macrophages *etc*. may still create some questions.

9.9 Perspectives

IHD may be a composition of different types of reduced myocardial perfusion ranging from decreased supply of oxygen to acute terminated blood supply with apoptosis, necrosis and infarction. Targeting all these situations may need different approaches. Currently some researchers suggest that MSCs have the optimal properties for cardiac repair in both acute and chronic ischemic states. Common for all these patients are that regeneration of the damaged myocardium by endogenous mechanisms is not sufficient [5]. In spite of the many promising results in clinical stem cell trials, some questions still remain to be answered such as the optimal route of cell delivery, number of cells, the cell type and selection of patients. The use of heparin has also been debated [11, 18] among several other parameters which may influence the preparation of the cells.

It is probably so that a single dose of MSCs may not be enough, and how should patients with more than one ischemic region to treat be dealt with? Answers to all these issues are still lacking and may also depend on how expensive the treatment is. However, the cost of manufacturing MSCs will be reduced when autologous and allogeneic MSCs are more widely used and with improved cultivation methods in *e.g.* bioreactors [59, 72].

Although no consensus exists regarding the optimal timing, method of delivery, preparation of MSCs *etc*. many lessons can be learned from preclinical studies, even though the human systemic blood pool, the vasculature, the hemodynamic/physiology and cellular mechanisms are different. MSCs being superior to mononuclear cells in preclinical studies [42] is encouraging news for clinical researchers.

Cardiac function may be improved through several routes including paracrine signaling through which angiogenesis and cardiomyogenesis are stimulated. Reduction in fibrosis, remodeling and apoptosis are seen besides activation of CPCs and immunomodulation. Cardiomyocytes and endothelial cells are generated either by transdifferentiation of MSCs or by the effect of cytokines secreted from MSCs on resident or circulating stem cells [13]. However, we still need a better method to evaluate the clinical and myocardial effect of the therapy besides measurement of LVEF. Myo-

cardial perfusion through advanced imaging modalities may be a better parameter for evaluating the effect of therapy.

9.10 Conclusion

A lot research has been done and more has to be done yet before MSCs therapy can be implemented as standard treatment in the health sector. The implementation will revolutionize the treatment of patients with established IHD.

Allogeneic MSC therapy is in pipeline and the first steps towards allogeneic MSC therapy have been taken [58, 59]. This will remove concerns about heterogeneous stem cells with differing properties and different results *in vivo*. Furthermore, individualized therapy with the dose dependent on bodyweight of the patient will be more widely used.

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Christian Cordano, Nicole Kerlero de Rosbo, and Antonio Uccelli

10 Mesenchymal stem cells as a strategy for the treatment of multiple sclerosis and other diseases of the central nervous system

Abstract Mesenchymal stem cells (MSCs) are increasingly being considered as an alternative approach to therapy of neurodegenerative diseases of the central nervous system (CNS), in which irreversible neural damage results in permanent neurological impairment. In such diseases, neuroinflammation is often associated with the neurodegenerative process. MSCs are particular good candidates for cell therapy in virtue of their neuroprotective and neuroregenerative properties, but also because of their strong immunomodulatory effect. Diseases spanning all defined categories of CNS degenerative disorders have now been investigatedat pre-clinical levels, substantiating clinical translation in a number of diseases for which Phase I and Phase II clinical trials are now ongoing or anticipated, such as multiple sclerosis. While there is an urgent need for further research, some general features of the effect of MSCs in neurodegenerative diseases have emergApart for from diseases where neuramage are is well focalized and might benefit to a greater extent from local administration, the intravenous route of delivery is generally sufficient for a full effect, probably due to their well-recognized ability to home to sites of tissue damage and to exert their action to a great extent through paracrine mechanisms. Trough pre-clinical studies in various animal models of neurodegenerative diseases, MSCs have been shown to exert a neuroprotective effect by modulating inflammatory processes, releasing neurotrophic factors, promoting neuronal survival and maturation, and inducing the differentiation of neural progenitor cells towards the various neural lineages. How these general features have translated in the different diseases is addressed in thisw of preclpreclinical and clinical studies of MSCs therapy in CNS degeneration.

10.1 Introduction

Neurodegenerative diseases of the central nervous system (CNS) are often associated with considerable irreversible disability consequent to irreversible neurodegeneration. At present, while available drugs might alleviate some of the symptoms, none can reverse neural damage and repair CNS tissue. Consequently, alternative therapeutic approaches are being sought, a major one of which is cell replacement, in particular through stem cell transplantation. Mesenchymal stem cells (MSCs) are at the vanguard in the development of such an approach, not only by virtue of their tissue-repair potential, but also of their immunomodulatory capacity which can have a potentially great impact on tissue repair by downgrading the inflammation often

associated with neurodegeneration. While MSCs have been isolated from several tissues, bone marrow-derived MSCs (hereafter referred to as MSCs) are the best characterized and their effect in neurodegenerative diseases has been extensively studied in preclinical *in vitro* and *in vivo* settings. They will be the focus of this chapter.

10.2 MSCs transplantation for neurological diseases: why, which, and how

While embryonic stem cells, which are multipotential, retaining the ability to differentiate into cell types of all lineages, might be considered as the first choice for stem-cell therapy, considerations such as available source, ethical issues, and national legislation [1], as well as risks associated with their unlimited self-renewal and high proliferation rate [2], have impeded their study and translation into clinical practice. Accordingly, adult stem cells have been increasingly considered for translational therapy and, of these, MSCs hold high promise. Indeed, MSCs, which are phenotypically identified by the expression of variable levels of stromal markers together with the lack of hematopoietic markers [3], can be easily isolated from adult tissues and their isolation and expansion are not ethically restricted. MSCs have been shown to have strong immunomodulatory activity, an important feature in diseases where neurodegeneration is associated and/or enhanced by neuroinflammation. Thus, MSCs can suppress T cell proliferation *in vitro* [4, 5] and *in vivo* [6, 7], and modify effector functions of T and B cells, dendritic cells and cells of the innate immune system, *via* juxtacrine and/or paracrine mechanisms, with release of soluble factors produced constitutively by MSCs or through cross-talk with target cells [8, 9]. *In vitro* studies suggest that MSCs could exert a strong neuroprotective effect in neurological diseases through their paracrine release of neuroprotective factors, leading to the rescue of neurons from apoptosis and promotion of their long-term survival and maturation [10, 11]; other studies have shown that MSCs can induce the differentiation of neural progenitor cells towards the oligodendroglial or neuronal lineages [12, 13]. *In vivo* studies suggest that the neuroprotective effect of MSCs is likely related to release of neuroprotective factors, rather than to CNS engraftment, which is rarely consistent, and transdifferentiation [14]. Preclinical studies have used xenogeneic, allogeneic and syngeneic MSCs and the source has not apparently been a major factor in the efficacy of MSCs in animal models [15]. However, while allogeneic MSCs have been used in clinical studies, their ability to escape immune rejection has not always been confirmed [16, 17] and autologous MSCs are used for most clinical studies, also to reduce the risk of transmission of infectious diseases. Recent studies indicating that MSCs isolated from patient bone marrow display a normal phenotype and are apparently fully functional *in vitro* [18], reinforce the rationale for the use of autologous MSCs. While an intrathecal route of MSC administration might be considered as likely to be more effective for cell therapy of a CNS disease, either to promote engraftment or for

local release of immunomodulating/neuroprotective molecules, preclinical studies [19] suggest that in general intrathecal transplantation is not superior to intravenous administration of MSCs (but see below); this lack of difference in efficacy favors intravenous administration as it also abrogates the need for an invasive procedure that can be associated with significant side effects.

In view of its promising neuroprotective and regenerative potential, MSC therapy has now been investigated through preclinical studies for a wide spectrum of neurodegenerative CNS diseases, including primary degenerative, traumatic, and inflammatory diseases, which have led to its translation in clinical studies for some of these diseases. In the following sections, we shall review the rationale for, and state of the art of, the use of MSCs in preclinical and clinical studies of the relevant categories of neurodegenerative diseases.

10.3 Vascular diseases: ischemic stroke

Ischemic stroke is characterized by the permanent or transient occlusion of a blood vessel due to a thrombus or embolus, which, when not lethal, often results in permanent neurological impairment due to progressive neuronal cell death through necrosis or apoptosis [20] associated with subsequent neuroinflammation.

10.3.1 Preclinical studies

Since 2000, some reports have highlighted promising results obtained in the development of MSC-based therapy for stroke, with different groups showing a remarkable neuroprotective effect in the rodent stroke model. The model most used for preclinical studies is that induced by middle cerebral artery occlusion (MCAO). In a seminal study, Li *et al*. described transplantation of non-hematopoietic cells from adult mouse bone marrow into the striatum four days after embolic MCAO and observed that functional recovery was significantly improved in transplanted mice monitored for 28 days as compared with untreated mice [21]. In 2001, Chen *et al*. used laser-scanning confocal microscopy to investigate the fate of 5-bromo-2'-deoxyuridine-labeled rat MSCs injected i.v. into rats 1 or 7 days after stroke induction; they demonstrated that the MSCs survive and home to the ipsilateral ischemic hemisphere, an observation accompanied by reduced neurological functional deficits in the treated rats monitored for 35 days [22]. In a subsequent study, the same group tested the effect of human MSCs (hMSCs) injected i.v. one day after stroke induction in rats [23]. The treatment resulted in a significant recovery of neurological function in rats monitored for 7 and 14 days; at histological level, a few (1 to 5%) hMSCs expressing proteins characteristic of brain parenchymal cells were observed in the CNS and there was a decrease in apoptotic cells in the ischemic boundary zone accompanied by

an increase in brain-derived neurotrophic factor and nerve growth factor in cerebral tissue, as well as an increase in proliferation of endogenous cells in the subventricular zone (SVZ). In their study, Zhao *et al*. showed that grafting of hMSCs into the ischemic brain one week after stroke induction ameliorated neurological deficits in the treated rats; histological analysis revealed the expression of markers for astrocytes, oligodendroglia, and neurons by the transplanted cells [24]. Based on their observation that angiogenesis was associated with improved neurological recovery from stroke, Chen *et al*. tested the hypothesis that intravenous infusion of hMSCs (one day after stroke induction) promotes vascular endothelial growth factor (VEGF) secretion; they showed an increase of endogenous levels of VEGF and its receptor, VEGFR2, associated with treatment with MSCs, suggesting that administration of MSCs provides a microenvironment capable of activating endogenous restorative mechanisms of ischemic brain [25]. Subsequent studies have attempted to improve the neuroprotective effect of MSCs through genetic modification. Thus, Nomura *et al*. described a reduction in lesion size, as monitored by MRI, and improved functional outcome in rats injected i.v. with hMSCs or with brain-derived neurotrophic factor gene-modified hMSCs 6 hours after middle cerebral artery occlusion, albeit with a greater effect in the group treated with brain-derived neurotrophic factor–hMSCs [26]. In a similar study, Horita *et al*. showed, through monitoring of lesion volume, that intravenous administration of hMSCs transfected with the glial cell line-derived neurotrophic factor gene led to increased recovery from ischemia as compared to untransfected hMSCs [27]. Liu *et al*. tested the hypothesis that angiogenic placental growth factor (PlGF) could increase the therapeutic benefits of MSCs in MCAO. Injection of hMSCs or hMSCs transfected with a PlGF gene (PlGF-hMSCs) i.v. into rats 3 hours after MCAO reduced lesion volume, induced angiogenesis and elicited functional improvement compared with the control group, but the effect was greater in PlGF-hMSCs-treated rats [28]. A concomitant increase in PlGF was observed in the infarcted hemisphere in both treatment groups, albeit greater in the PlGF-hMSCs-treated group, supporting the hypothesis that PlGF contributes to neuroprotection and angiogenesis in cerebral ischemia. However, the actual protective effect of P1GF on neurons in situ remains to be demonstrated. That i.v.-injected MSCs exert their therapeutic effect in MCAO possibly through angiogenesis of damaged ischemic brain lesions was confirmed by Komatsu *et al*. who also showed the long-term effect of MSC therapy with a time window covering at least a one-month period after MCAO [29].

In a recent study, Xin *et al*. postulated that MSCs might exert their beneficial effect in stroke through communication with target cells *via* release of exosome containing microRNA (miRNA). They observed that i.v. treatment of rats with MSCs after MCAO significantly increased the levels of miR-133b, a miRNA expressed in midbrain dopaminergic neurons and shown to promote functional recovery in Parkinson's disease and spinal cord injury, in the ipsilateral hemisphere [30]. Using miR-133b inhibitors, they confirmed the possibility that miR-133b was transferred from MSCs through *in vitro* studies on primary neurons cultured in the presence of exosome-enriched super-

natant fractions from MSCs exposed to brain extracts from rats 72 hours post-MCAO and that it was responsible for the significant increase in neurite branch number and total neurite length observed in the neuronal cultures. These data provided evidence that the neuroprotective effect of MSCs in stroke could occur through exosome-mediated transfer of gene regulators such as miRNAs.

Van Velthoven *et al*. demonstrated the neuroprotective effect of MSCs in ischemia, showing that local administration of MSCs could restore functional cortical rewiring [31]. As shown by retrograde labeling, infusion of MSCs into the ipsilesional hemisphere of mice at days 3 and 10 after neonatal hypoxic-ischemic (HI) brain damage reduced HI-induced contralesional axonal rewiring, while increasing axonal connectivity in the ipsilesional hemisphere. Moreover, the reduction in contralesional remodeling after MSC treatment was positively related to motor performance, suggesting that MSCs represent a promising therapeutic strategy to normalize ipsilesional motor tract routing and improve motor function following HI [31].

Recently, Scheibe *et al*. raised the question of possible deleterious effects of MSCs on the immune response in stroke in view of the stroke-induced immunodepression associated with cerebral ischemia, which predisposes to bacterial infections with increased mortality. Despite their immunosuppressive effects *in vitro*, transplantation of MSCs in mice after MCAO did not affect the serum levels of relevant inflammatory cytokines, suggesting that safety concerns for MSCs transplantation in stroke are likely to be unwarranted [32].

10.3.2 Clinical studies

A first small trial involving only five patients with cerebral infarcts within the middle cerebral arterial territory who were treated with i.v. infusion of culture-expanded autologous MSCs (the trial included 25 control patients who did not receive MSCs) was published in 2005 [33]. The treatment was performed four weeks after stroke, and patients were monitored for one year. This study showed no adverse cell-related, serological, or imaging-defined effects. The Barthel index (an ordinal scale used to measure performance in activities of daily living) and modified Rankin score (a commonly used scale for measuring the degree of disability or dependence in the daily activities of people who have suffered a stroke or other causes of neurological disability, the most widely used clinical outcome measure for stroke clinical trials) improved in the MSCs group during the follow-up period, but these data should be interpreted with extreme caution in view of the very small group of patients treated, the heterogeneity of stroke outcomes in general, and the short follow-up period. The same group evaluated the long-term safety and efficacy of autologous i.v.-administered MSCs in a larger population, through an open-label, observer-blinded clinical trial in which 52 patients with severe infarct in the middle cerebral artery territory were randomized in two groups, one in which patients (n = 16) received autologous *ex vivo*-cultured MSCs

five weeks after the stroke and one in which patients (n = 36) did not receive MSCs; patients were followed for up to five years [34]. This work confirmed safety of treatment during long-term follow-up; functional recovery, measured by modified Rankin scoring, was more frequently observed in the MSC-treated group than in the control group. As shown by neuroimaging analysis, functional improvement observed in the MSC-treated group was most consistent when the subventricular zone (SVZ) was less infarcted, suggesting a possible effect of the MSCs on endogenous neural progenitor/stem cells located in that region.

Honmou *et al*. performed their feasibility and safety study of MSCs treatment for chronic stroke using autologous hMSCs expanded in autologous serum, rather than fetal calf serum used in previous studies. Such procedure removes the risk of humoral immune response to fetal calf serum proteins in the recipient and, most importantly, it allows more rapid expansion of less highly differentiated and transcriptionally stable hMSCs. In this Phase I study (12 patients), the cells were delivered at the chronic stage of the disease, several weeks or months (36–133 days) after the stroke occurred [35]. The reason for treating at chronic stage is based on the possibility that MSCs continue to migrate selectively into damaged brain with accumulation of injected cells into ischemic lesions even after the attenuation of the blood–brain dysfunction (about 1 month after ischemic onset) [29]. Although the study was unblinded and uncontrolled so that conclusions cannot be made on possible therapeutic benefits, it provides evidence supporting the feasibility and safety of delivering autologous hMSCs, cultured in autologous human serum, in stroke patients followed for up to one year. A controlled, but non-randomized, study of the safety, feasibility, and efficacy of autologous MSC transplantation in chronic stroke (lasting 3 months to 1 year) was conducted on a small cohort divided into control and treatment groups (n = 6) matched for age, disease severity, time of stroke onset, and lesion size [36]; in contrast to previous studies, the autologous MSCs were expanded in serum-free medium. The patients were followed for 24 weeks. While this study confirmed the feasibility and safety of autologous MSC transplantation in stroke patients, definite conclusions cannot be reached on the potential therapeutic efficacy of MSCs in chronic stroke in view of the small number of patients in control or treatment groups, and other parameters including factors that might affect recovery from stroke. Obviously, also because the heterogeneity of stroke outcomes makes it difficult to assess treatment in general, clinical trials with larger well-defined cohorts and parameters are necessary to evaluate the efficacy of MSC transplantation for the treatment of stroke.

10.4 Trauma spinal cord injury

Spinal cord injury (SCI) refers to any injury to the spinal cord caused by trauma instead of disease. The initial mechanical damage is compounded by ischemia, release of toxic chemicals from disrupted neural membranes, and electrolyte shifts

which trigger a secondary injury that harms or kills neurons and oligodendrocytes. Studies focused on neuroprotection or axonal regeneration, to stimulate and guide axonal growth or boost remyelination, have turned to cell therapy with various cell types including MSCs, in particular in view of their ability to secrete factors able to support axonal growth, promote angiogenesis and remyelination, downregulate possible spinal cord inflammation and protect from apoptotic cell death such as demonstrated for oligodendrocytes in SCI [37].

10.4.1 Preclinical studies

Studies of the effect of MSCs in animal models of SCI, mostly contusion and transection models, have exploited the well-known trophism of MSCs for sites of tissue damage, hence avoiding direct injection into spinal cord tissue and thereby further damage. MSC transplantation has been conducted through systemic i.v. injection or into the cerebrospinal fluid by lumbar puncture (LP). A study that compared both routes of delivery indicated that, while the proportion of engrafted cells was generally low, it appeared to be greater upon LP delivery, and was associated with decreased host immune response and increased signs of tissue repair [38]. While the majority of studies have been conducted with administration of MSCs at the acute injury phase, and have shown functional improvement, a number of animal studies of MSC transplantation at the chronic phase (6–12 weeks after injury) have also been reported. They show increased functional recovery, suggesting that MSC-based therapy may be beneficial in either acute or chronic phase, albeit with different results (the effect is thought to be much greater in the acute/subacute phase) and acting through different mechanisms depending on the phase at which transplantation is performed (anti-inflammatory/neuroprotection in the acute phase, neuroregeneration in the subacute phase, and neural cell replacement in the chronic phase).

In their attempt to study the mechanisms of action underlying the improved functional recovery following i.v. injection of MSCs in adult rats after spinal cord compression injury, which they confirmed through two complementary locomotor tests, Quertainmont *et al*. used BrdU immunohistochemistry to track MSC fate and assessed changes in cytokine/trophic factor expression three days after MSC transplantation [39]. They did not detect significant MSC engraftment at any time, as reported previously, suggesting that the beneficial effect was associated with neuroprotective factors produced by MSCs, rather than attributable to their differentiation. In this context, functional improvement, which could be observed as early as three days after MSC infusion, was accompanied by an increase in levels of nerve growth factor (NGF) in the injured tissue. While the increase in NGF expression could be due to endogenous cells being stimulated by the MSCs, they showed that MSCs themselves secrete this factor *in vitro*. Functional recovery could not be associated with an increase in axonal regrowth, but they observed significant tissue sparing with increased vascularization

in MSC-treated rats, which they suggest as being due to the strong neuroprotective and repair-inducing effect of NGF [39].

10.4.2 Clinical studies

A few clinical studies for MSC transplantation in SCI have been reported. Of these, only two used *in vitro*-expanded MSCs, whereas the others used mononuclear stem cell preparations, which themselves contain MSCs, but also contain hematopoietic cells at different stages of differentiation and hematopoietic stem cells. In a case report [40], the patient was treated 13 days after SCI (acute phase) with a dose of 3.1×10^7 autologous MSCs injected through LP. The patient was followed for six months and showed an improvement in both motor and sensory scores during the first three months; the motor score improvement continued throughout the whole follow-up.

In the second study [41], 25 patients (15 acute and 10 chronic) were treated with a dose of 1×10^6 autologous MSCs by LP administration and followed for three months. While safe, with no adverse events recorded, the treatment did not result in improvement of neurological scores; however, a beneficial, albeit subjective, effect in quality-of-life scores was reported.

On the basis of these encouraging results on the safety of MSC transplantation, two Phase I studies are currently ongoing and two Phase II studies are set to start (according to clinicaltrials.gov).

10.5 Extrapyramidal diseases

Extrapyramidal diseases span a wide clinical spectrum characterized by neurodegeneration of the basal ganglia. They include Parkinson's disease, Huntington's disease and multiple system atrophy for which therapy through MSC transplantation is being considered.

10.5.1 Parkinson's disease (PD)

Motor impairment in PD results from the death of dopaminergic neurons in the substantia nigra, whose cause is unknown. Pathological hallmarks of PD are Lewy bodies, which are inclusions of accumulated α-synuclein (α-SYN), in neurons, and paucity and/or decreased activity of dopamine produced in midbrain dopaminergic neurons. While early motor symptoms can be controlled by levodopa and dopamine agonists, continued loss of dopaminergic neurons results in inexorable progression of motor deficit. Since 2005 [42], an increasing number of reports have highlighted promising results obtained in the development of MSC-based therapy for PD, with evidence of reduced death of dopamine neurons upon exposure to MSCs either *in vitro* or *in vivo* [43, 44].

10.5.2 Preclinical studies

Two main types of PD models have been developed to understand the pathogenesis and test potential therapeutic approaches: the neurotoxic models induced by environmental or synthetic neurotoxins and the genetic models whereby mice are genetically engineered to express a mutated PD-relevant transgene or for such a gene to be knocked out. None of the models is by itself an overall model for PD; for example, the genetic models do not show the neuronal degeneration associated with PD but some of them show Lewy bodies-like inclusions, whereas the neurotoxic models, such as that induced by 6-hydroxydopamine (6-OHDA), show dopaminergic neuron degeneration but not Lewy bodies-like inclusions; some, but not all, also show neuroinflammation, which has been implicated in the pathophysiology of PD [104].

Kim *et al.*, using a rat PD model induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in which evidence of inflammation in dopaminergic neuronal death has been documented, described one of the possible underlying mechanisms for the neuroprotective effect of MSCs on dopaminergic neurons as anti-inflammatory actions mediated by the modulation of microglial activation [45]. However, most studies of the beneficial effect of MSCs in PD models have focused on potential cell replacement. Possible transdifferentiation of MSCs to dopaminergic neurons under particular stimuli has been shown *in vitro* [46, 47]. Although the functionality of such cells is debated [48], further studies have focused on the possibility of directly grafting into the damaged nervous system *in vitro* differentiated MSCs expressing neuronal markers including those characteristic for dopaminergic neurons; encouraging results showed that intrastriatal injection of these induced neural MSCs could improve symptoms of 6-OHDA-induced PD, with the grafted cells expressing the dopaminergic neuron marker tyrosine hydroxylase (TH) surviving in the striatum for long periods post-transplantation [49–51].

Dezawa *et al.* showed that cells that expressed the dopaminergic neuronal markers TH and the dopamine transporter (DAT) and had the ability to release dopamine could be induced from MSCs through genetic engineering *via* a Notch1 intracellular domain-containing plasmid and appropriate cytokine stimulation; intrastriatal implantation of these cells resulted in improved motor function in rats with 6-OHDA-induced PD [52]. In a very recent follow-up study, the same group showed in hemiparkinsonian macaques that engraftment of A9 dopaminergic neuron-like cells induced in a similar way from autologous MSCs leads to long-term survival of the cells and improvement of motor function; using positron emission tomography coupled with labeled CFT, a ligand for DAT, the group demonstrated that a portion of the transplanted differentiated cells remained in the grafted area for at least seven months [53]. The increased numbers of TH+ terminals observed in the brain of MSC-treated monkeys suggested the formation of new synaptic connections between the engrafted cells and host tissues, further supporting the potential of MSC-mediated therapeutic approach in restoring motor function in PD [53]. Both *in vitro* and *in vivo*

studies have shown that MSCs may have neuroprotective effects on dopaminergic neurons affected in PD not only through differentiation and replacement of damaged cells, but also in a paracrine fashion through the secretion of trophic factors [54 43, 44]. The possibility to enhance such a paracrine-mediated neuroprotective effect has also been tested through transplantation of MSCs genetically engineered to express neurotrophic factors, in particular glial cell-line derived neurotrophic factor (GDNF), the most potent neurotrophic factor for dopaminergic neurons. Intrastriatal transplants of such GDNF-transduced MSCs in 6-OHDA-lesioned rats suggest that such an approach is effective in stimulating recovery of damaged dopaminergic neurons and might lead to further improvement of motor deficit [55–57].

10.5.3 Clinical studies

Results of a pilot open-label uncontrolled clinical study involving seven PD-affected patients were reported in 2010 [58]. Patients received a single dose of autologous MSCs transplanted in the sublateral ventricular zone through stereotaxic surgery and were followed for 10 to 36 months. This study indicated that the unilateral transplantation of autologous MSCs into the sublateral ventricular zone is apparently safe. However, while some marginal improvement was reported in several of the patients, including a decrease in ''off''/''on'' periods in Unified Parkinson's Disease Rating Scale and/or some reduction in drug dosage, the very small number of patients and the uncontrolled nature of the study do not allow the elaboration of useful conclusions on the possible efficacy of such a treatment in human PD [58].

10.5.4 Huntington's disease (HD)

HD is a neurological disorder caused by a genetic mutation in the IT15 gene, which results in the production of a mutant huntingtin (*htt*) protein with an abnormally long polyglutamine repeat that confers a toxic gain of function on the protein. The mutated protein aggregates in striatal medium spiny neurons, as well as neurons in other regions of the neuraxis, causing progressive cell death, *via* as yet unclear mechanisms, and accompanying declines in cognitive, motor, and psychiatric functions. The possibility that stem-cell transplantation could be beneficial to HD patients has already been demonstrated through the study of Bachoud-Lévi *et al*. who observed motor and cognitive improvements, albeit transient, in three of five HD patients transplanted intrathecally with human fetal neural stem cells [59]. However, although the field of MSC treatment in HD is still in its infancy, a number of preclinical studies in relevant HD models have demonstrated that MSCs could be of therapeutic value in the human disease.

10.5.5 Preclinical studies

Animal models of HD fall into two broad categories, genetic and nongenetic. Nongenetic models typically induce cell death either by excitotoxic mechanisms (intrastriatal administration of quinolinic acid (QA)) or by disruption of mitochondrial machinery (systemic administration of 3-nitropropionic acid), which are both mechanisms of degeneration seen in HD brain. Murine genetic models include transgenic mice, where expression of the polyglutamine-encoding portion of the mutant human *htt* gene randomly inserted into the mouse genome is driven by different promoters, and knock-in mice, where such a portion is inserted in the mouse *htt* gene locus, resulting in expression through the mouse *htt* promoter and thereby spatially and temporally accurate production of the mutant protein. The presence of the mutant *htt* RNA/htt protein must be taken into consideration in HD treatment as it results in continual damage of the brain microenvironment.

Snyder *et al*. used a genetic HD mouse model treated with injection of hMSCs into the dentate gyrus to test the hypothesis that MSC transplantation could lead to increased neurogenesis from endogenous cells. As expected from previous studies, there was no engraftment of MSCs, but BrdU labeling indicated that hMSC grafting was associated with proliferation and differentiation of endogenous neural cells in HD striatum. An increase in not BrdU-labeled βIII tubulin$^+$ neurons in the striatum suggested that other, non-stem, cells had differentiated towards the neuronal lineage, and/or that hMSCs induced increased neuronal survival. This increase in neurogenesis was associated with decreased striatum atrophy, both probably exerted through persistent increases in neurotrophic factors [60]. Unfortunately, the possible improvement in neurological impairment was not addressed in this study. In contrast, Lescaudron *et al*. reported that transplantation of syngeneic MSCs in the QA-damaged striatum of HD rat significantly reduced working memory deficits, probably through trophic effects on neuronal progenitors by MSCs [61]. Bantubungi *et al*. followed the fate of undifferentiated MSCs grafted into the striatum following QA-induced degeneration and showed that the pathological environment favored MSC engraftment and proliferation, with local delivery of factors such as stem-cell factor (SCF), possibly by the grafted stem cells themselves, increasing their clinical potency; indeed, SCF, which was strongly upregulated within host cells in the damaged striatum, was able to activate the SCF receptor c-kit and its signaling pathway and to promote the migration and proliferation of MSCs *in vitro* [62]. Because striatal atrophy in HD models is accompanied by subsequent enlargement of the lateral ventricles [63, 64], reduction in lateral ventricle volume has been used to monitor repair. In their study of QA-induced striatal degeneration in rats transplanted with syngeneic MSCs in the damaged striatum, Amin *et al*. reported significant reduction in striatal atrophy that was accompanied by a significant reversion of lateral ventricle volume to that approaching the normal contralateral ventricle; these data suggest that MSCs could treat microanatomical defects in motor disorders of HD [63]. Damage-induced trophism of MSCs, highly important in diseases

that are not necessarily associated with a single lesion, has also been demonstrated in preclinical studies. A recent study used high-resolution MRI to follow *in vivo* the migration of superparamagnetic iron oxide-labeled MSCs transplanted into the brain of QA-lesioned rats. MRI data confirmed by histological analysis indicated that some transplanted MSCs had migrated a great distance along the internal capsule toward the striatal QA-induced lesion; no migration of transplanted MSCs to the striatum was observed in the absence of QA lesioning [65].

10.6 Multiple system atrophy (MSA)

MSA, a sporadic, adult-onset neurodegenerative disease, results in two major motor presentations, parkinsonism (MSA-P) and cerebellar ataxia (MSA-C). It is often considered as a glial dysfunction-related pathology due to the neuropathological presence of glial cytoplasmic inclusions containing primarily α-synuclein (α-SYN). Other pathological hallmarks of the disease are selective neuronal loss and gliosis in cerebellum, olivary nuclei, pyramidal fibers, basal ganglia, intermediolateral column, and Onuf's nucleus. As yet, the effect of MSC treatment in MSA has been poorly studied with few, albeit promising, preclinical data to support clinical translation.

10.6.1 Preclinical studies

Two main types of animal models have been developed in the attempt to reproduce relevant clinical and pathological features of MSA: neurotoxin-based models (6-OHDA or QA injection) to induce the degeneration of nigral and striatal neurons and transgenic models that reproduce the cytopathological hallmark of MSA, that is oligodendroglial accumulation of insoluble α-SYN that results in oligodendrocyte dysfunction with myelin loss and axonal atrophy [66].

Recent studies on the effect of MSCs in MSA performed on both types of models indicate that MSCs might represent a feasible therapeutic approach for MSA. Park *et al.* [67] showed that intravenous injection of hMSCs promoted the survival of cells expressing the neuronal markers TH and NeuN, and modulated inflammation and gliosis in brain of mice with double-toxin-induced MSA, with a coincident improvement in motor behavior. Stemberg *et al.* [68] investigated the effects of murine MSC transplantation in aged transgenic (PLP)-α-SYN mice (αSYN expressed under the control of the oligodendrocyte-specific proteolipid protein (PLP) promoter). There was no improvement in the survival rate and in the behavioral tests in the MSC-treated group, probably due to the advanced age (18 months) of the animals resulting in neurodegeneration at a "point-of-no-return" stage. However, the intravenous administration of MSCs led to a rescue of dopaminergic neurons, evidenced by an increased number of TH-positive cells in the MSC-treated group, which was not related to a

decrease in α-SYN concentration in midbrain-brainstem lysates. Again, the increase in TH neurons was likely associated with downregulation of inflammation and gliosis in MSC-treated mice, as shown by the inverse correlation with proinflammatory cytokine levels [68].

10.6.2 Clinical studies

In 2008, Lee *et al*. [69] investigated the effect of MSCs in 11 MSA-P patients in a proof-of-concept study. The administration protocol included an intra-arterial injection followed by a monthly intravenous injection over a three-month period (at 30, 60, and 90 days after the initial intra-arterial injection). The MSC-treated patients showed improved clinical score and increased glucose metabolism in frontal and cerebellar grey matter as assessed by positron emission tomography (PET) scan during the 12-month follow-up. In a recent second study that followed the same administration protocol, Lee *et al*. [70] improved the trial reliability by a double-blindness protocol and, despite the small number of enrolled MSA-C patients, met their primary endpoint, showing a reduction trend in disability progression with positive secondary endpoints in PET glucose metabolism, cortical density and cognition score. However, there were some concerns about the safety of the intra-arterial procedure, with a number of patients developing ischemic lesions visible on diffusion-weighted brain magnetic resonance imaging [69, 70]; one subject also developed a transient neurological deficit after the intra-arterial procedure [70]. In addition, the reduced long-term efficacy of the procedure suggests the need for further treatments. Nevertheless, this study opens the way for further clinical trials that will need to (i) validate or develop another delivery procedure, (ii) evaluate the effectiveness of MSC treatment in MSA-P patients, (iii) assess the durability of MSCs' effects (*e.g.*, by understanding if additional injections are required and/or useful/efficient), and (iv) enroll a higher number of patients to increase reliability of the results. Obviously, these studies must be supported by further preclinical studies aimed at understanding the precise mechanism of action of MSCs in MSA.

10.7 CNS demyelinating diseases: multiple sclerosis

MS is a chronic demyelinating disease of the CNS in which progressive phases (primary or secondary) are associated with irreversible neurological impairment consequent to a threshold of irreversible chronic axonal loss attributable to the absence of myelin-derived trophic support, being reached. While immunomodulating drugs can control the recurrent episodes of inflammatory demyelination in the relapsing-remitting phase of the disease, they have little or no beneficial effect in progressive MS and there is at present no therapy that can induce CNS repair in MS. MSCs are

prime candidates in strategies that aim at enhancing the neuroprotective/neuroregenerative function of endogenous cells or supplementing them, through transplantation of adult stem cells.

10.7.1 Preclinical studies

A number of animal models for MS have been established, of which most rely on the autoimmune response to myelin proteins, or peptides thereof, upon active immunization or adoptive transfer by autoreactive myelin-specific T cells. Two main forms of this experimental autoimmune encephalomyelitis (EAE) model have been used to evaluate the therapeutic effect of MSC administration: relapsing-remitting EAE induced with proteolipid protein (PLP) peptide 139–151 and chronic progressive EAE induced with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55. In a seminal study published in 2005, Zappia *et al*. demonstrated that administration of syngeneic murine MSCs led to decreased severity of chronic progressive EAE and a reduction in demyelination and CNS infiltration by T cells, B cells and macrophages [6]. Tracking of GFP-labeled MSCs to lymphoid organs, where they interact with activated T cells and DCs suggested that the beneficial effect of MSCs in EAE is mediated through induction of peripheral T-cell tolerance to the immunizing antigen [6]. The immunomodulating activity of MSCs on encephalitogenic T cells was confirmed in relapsing-remitting EAE adoptively transferred with PLP139-151-specific T cells that had been previously exposed to MSCs *in vitro*, which resulted in a milder disease with fewer relapses than that induced by untreated encephalitogenic T cells [7]. In PLP139-151-induced EAE, which is associated with a significant antibody response likely important in demyelination, Gerdoni *et al*. also demonstrated that MSC treatment could inhibit antigen-specific B-cell response [7]. A number of studies have now addressed the mechanisms that underlie the immunomodulatory effect of MSCs in EAE. Rafei *et al*. proposed that MSCs block IL-17-driven inflammation and cellular infiltration of the CNS through their secretion of the antagonist form of CCL2 as ligand for CCR2, which is expressed by pathogenic Th17 CD4+ T cells and whose interaction with CCL2 is essential for EAE development [71]. On the basis of *in vitro* observations, studies have addressed the role of indolamine-2,3-dioxygenase 1 (IDO)-mediated tryptophan catabolism, a major immunosuppressive effector pathway in the suppression of antigen-specific T-cell responses by MSCs in EAE. Contrasting results were obtained in two studies, with MSC transplantation ameliorating relapsing-remitting [72], but not chronic EAE [73]. As supported by *in vitro* studies that demonstrated the immunomodulatory and neuroprotective effect of MSCs, their *in vivo* therapeutic effect in EAE is likely mediated mostly through paracrine mechanisms. Indeed, data on engraftment and/or transdifferentiation in EAE are inconsistent, involving at best only small proportions of transplanted MSCs [6, 7, 74]. In contrast, the neuroprotective effect of MSC transplantation has been clearly demonstrated in both relapsing-remitting and

chronic types of EAE, reducing demyelination and/or promoting remyelination and/or reducing axonal damage, regardless of the route of administration [6, 7, 13, 75–77]. It has been attributed to a combination of suppression of the autoimmune response and induction of proliferation or enhanced differentiation of endogenous progenitor cells, potentially related to MSCs' release of soluble factors such as brain-derived nerve factor [78] or hepatocyte growth factor which, together with its primary receptor cMet (expressed on both immune and neural lineage cells) was shown to be critical in MSC-stimulated recovery, neural cell development and remyelination, promoting functional recovery in chronic EAE [79]. A possible neuroprotective mechanism for MSCs in EAE was proposed by Lanza *et al.* who showed that MSCs abrogate the increase in oxidative stress-associated proteins in neurons exposed to H_2O_2 *in vitro*, and also exert a potent antioxidant effect *in vivo*, demonstrated by the remarkable reduction in CNS levels/activities of antioxidant molecules involved in the defense against EAE-induced oxidative stress and tissue damage [80].

The possibility to enhance the neuroprotective effect of MSCs in EAE has been recently explored through their *in vitro* differentiation towards neural progenitors prior to transplantation [72, 81, 82], or by engineering them to express, and thereby secrete, neuroprotective molecules to synergize MSC-mediated immunosuppression and neuroprotection [83]. However, the results of these studies are somewhat contradictory and further investigations are necessary.

10.7.2 Clinical studies

The consistent beneficial effect of MSC treatment shown for both relapsing-remitting and chronic MS models has prompted its clinical translation in the human disease. To date, four small open-label studies [84–87] have been reported, providing preliminary data on the safety of clinical application of MSCs in MS. All have been conducted with autologous MSCs, in doses of $1–2 \times 10^6$ MSCs per kg of body weight that are considerably lower than those of the preclinical studies, which are not presently translatable to human treatment as it is almost impossible to recover and *in vitro* expand human MSCs from a standard BM aspiration to equivalent quantities. Although data are only available for short-term periods, it appears that MSC treatment is well tolerated and generally safe for MS, albeit with the caveat that intrathecal injection that was used in three of the four studies could be associated with mild to severe adverse events resulting from the invasive procedure [84–86]. Most importantly, none of the ten patients treated with MSCs through i.v. injection experienced a significant adverse event through the seven-month follow-up [87]. While the primary aim of the four clinical studies published so far was to assess safety and tolerability, the open-label Phase IIa proof-of-concept study conducted by Connick *et al.* also aimed at assessing efficacy of MSC treatment by measuring visual parameters in ten patients with secondary progressive MS and visual pathway involvement who had received MSCs i.v. They

observed improvement in visual acuity and visual evoked response latency with an increase in optic nerve area [87]. The other three studies report anecdotal improvement in some clinical and radiological outcomes [84–86]. Obviously, large and long-term controlled clinical studies are needed to clearly assess efficacy along with safety. In this context, an international multicenter clinical trial was recently started in order to better define the safety and the efficacy of an established MSC-treatment protocol on a large cohort of patients [88]. This Phase II trial, guided by the International Mesenchymal Stem Cells Transplantation (IMSCT) study group that includes scientists and clinicians from several centers in Europe, Canada, and Australia, is to be conducted as a randomized, double-blind, cross-over study of i.v.-treatment with autologous MSCs compared with suspension media in MS patients with active disease. Establishing safety of the treatment and its efficacy are the primary objectives of the study to be assessed by clinical evaluation and frequent MRI scans. Secondary objectives include efficacy of treatment evaluated by cumulative MRI activity and brain atrophy, evidence of remyelination measured by magnetization transfer ratio, effect on clinical parameters, visual functions, neuropsychological tests, and immunological responses [89].

10.8 Motor neuron diseases: amyotrophic lateral sclerosis (ALS)

ALS is a relentless degenerative disease involving upper and lower motoneurons; loss of ventral horn motoneurons and corticospinal degeneration are typical neuropathological aspects of the disease and are associated with an inflammatory response characterized by microgliosis and limited T-cell infiltration at sites of neurodegeneration. Most ALS cases are sporadic and of unclear cause, but a small proportion are hereditary, caused by genetic mutations, the most common of which occur in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1). ALS is uncurable, with death occurring typically within five years of diagnosis. Stem-cell therapy, in particular through MSCs, which offers promise of neuroprotection, immunomodulation and possible neuroregeneration, is being increasingly considered for ALS, with a need for safe and effective cellular treatments.

10.8.1 Preclinical studies

Animal models for ALS are genetic, established in rodents *via* transgenes for ALS-associated mutant genes. The most widely used is the SOD1-G93A mouse transgenic for mutant human superoxide dismutase 1 (SOD1), which mimics both sporadic and familial ALS, with progressive hind limb weakness leading to paralysis and death [90]. Preclinical studies to evaluate safety, effectiveness and disease-altering properties of both murine MSCs and hMSCs have been conducted in this model. In the first pioneer-

ing experiment, Zhao *et al.* [91] showed that i.v. injection of hMSCs in pre-symptomatic SOD1-G93A mice led to a significant delay in disease onset and progression, and an increased average lifespan. They reported engraftment of the injected MSCs, which survived for over 20 weeks, migrated into the brain parenchyma and spinal cord, and differentiated into glial cells. Vercelli *et al.* [92] reported similar results in these mice upon transplantation of hMSCs directly into the lumbar spinal cord, with decreased astrogliosis and microglial activation and higher motoneuron counts. Suzuki *et al.* [93] addressed the loss of neuromuscular connections in ALS through direct transplantation into SOD1-G93A rat muscle of hMSCs engineered to express and secrete GDNF, which has been shown to protect motor neurons in a number of different models and is necessary for normal neuromuscular development. The treatment, which increased the survival of the recipient rats, reduced denervation of neuromuscular junctions, provided neurotrophic support through secretion of GDNF and other MSC-intrinsic growth factors, and significantly improved both survival and functioning of motor neurons, but had no effect on glial cell activation.

While these preclinical studies have demonstrated an undeniable benefit from MSC therapy, they have all been performed on asymptomatic animals. Uccelli *et al.* studied the effect of treating SOD1-G93A mice with i.v.-MSCs injection after onset of clinical symptoms. As seen with presymptomatic mice, MSC injection significantly slowed disease progression and improved motor function in ALS mice with ongoing disease. The beneficial effect was linked to reduced oxidative stress and inhibition of glutamatergic excitotoxicity in the spinal cord of treated mice, as well as a reduction in activated microglia and astroglia [94].

10.8.2 Clinical studies

A few small Phase I clinical trials using autologous MSCs have been conducted in ALS, which have proven the feasibility and safety of the procedure [84, 95, 96]. In two of these studies, reported by Mazzini *et al.* [95, 96], nine and ten ALS patients, respectively, were transplanted by intraspinal injection with autologous MSCs suspended in autologous cerebrospinal fluid. There was no immediate or delayed transplant-related toxicity, nor were there signs of toxicity or abnormal growth of the cells; clinical, laboratory, and radiographic evaluations showed no serious adverse events consequent to the transplant. These data represented the first demonstration that focal transplantation of MSCs into the CNS is a safe procedure, as confirmed by long-term observation (up to 9 years) of the patients [97]. However, although a slower disease progression was reported for some patients, no clear clinical benefits were detected [97]. A possible slowdown in ALS progression was also reported in a third study conducted by Karussis *et al.* [84] who analyzed safety and efficacy of intrathecal, or intrathecal plus intravenous, transplantation of autologous MSCs in 19 patients. Thus, while the mean clinical score (ALS Functional Rating Scale) deteriorated slightly during the two

months preceding MSCs injection, it remained stable during the six-month follow-up. Obviously, in addition to further preclinical studies that address mechanisms of action of MSCs in ongoing-disease models, larger trials with long-term follow-up are needed to test the efficacy of the treatment adequately, and at least two Phase II clinical trials are currently enrolling, according to clinicaltrials.gov website.

10.9 Dementia: Alzheimer's disease (AD)

AD, the most common form of degenerative dementia, is generally sporadic, but can be genetic caused by dominant mutations of amyloid precursor protein (APP) or presenilin 1 or 2 (PS1-PS2) genes [98]. Neuronal dysfunction and death are associated to accumulation of amyloid β (Aβ) in neurons and extracellularly (amyloid plaques), neurofibrillary tangles (intraneuronal thick strands composed of hyperphosphorylated protein tau), and neuronal granulovacuolar degeneration, mostly seen in the pyramidal layer of the hippocampus.

10.9.1 Preclinical studies

Both genetic, mostly APP/PS1 double-transgenic, and interventional (direct injection of aggregated Aβ in the hippocampal dentate gyrus) mouse AD models have been used in preclinical studies of the effect of MSCs on AD. The seminal study, conducted by Lee *et al*. [99] showed that intracerebral injection of MSCs in mice with Aβ-induced AD reduced accumulation of Aβ in brain tissue. Such a decrease was apparently due to an increase in activated microglia and their acquisition of a phagocyte-like phenotype, suggesting that, in this model, MSC treatment enhances microglial phagocytosis, which in turn prevents and/or destroys Aβ deposits. The same group [100] later showed similar results with the same protocol of MSCs in the genetic APP/PS1 double-transgenic mouse model. Interestingly, Aβ clearance was associated with restoration of defective microglial function, as evidenced by increased Aβ-degrading factors, decreased inflammatory responses, and elevation of alternatively activated microglial markers. The treated mice showed a decrease in hyperphosphorylated tau, a necessary component of Aβ-induced cognitive dysfunction, in the brain, together with improved cognitive function [100].

This group has also studied xenogeneic human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) for their effect in AD models [101]; they showed that hUCB-MSCs, which reduced murine hippocampal neuron apoptosis induced by Aβ treatment *in vitro* through paracrine mechanisms, downregulated markers of glial activation, oxidative stress, and apoptosis levels in AD mouse brain and restored learning/memory function *in vivo*; intracerebral transplantation of hUCB-MSCs in APP/PS1 double-transgenic mice significantly improved spatial learning and reduced memory

decline, a cognitive amelioration that was associated with dramatic reductions in Aβ deposition, β-secretase 1 levels, and tau hyperphosphorylation [102]. Reversal of disease-associated microglial neuroinflammation, as evidenced by decreased microglia-induced proinflammatory cytokines, elevated alternatively activated microglia, and increased anti-inflammatory cytokines, similar to that seen upon transplantation of murine MSCs, was observed. These results are in line with the recent *in vitro* observation that CX3Cl1 released by MSCs can alternatively activate microglia to acquire a neuroprotective phenotype [9].

10.9.2 Clinical studies

MSC treatment in AD is in its infancy with one clinical trial completed, albeit with results as yet undisclosed, and only one trial currently ongoing to evaluate the safety and efficacy of hUCB-MSC transplantation in AD patients, according to clinicaltrials.gov.

10.10 Concluding remarks

This overview of preclinical and clinical studies of MSC treatment in neurological diseases of the CNS suggests that, despite the complexities of the various diseases for which MSCs have been proposed as a possible alternative therapy, common features based on their neuroprotective and immunomodulatory action have emerged which indicate that MSCs could offer some benefit in all neurological diseases (Fig. 10.1). However, it must be noted that this approach is still mostly in its infancy for a number of diseases; the paucity of preclinical data in certain diseases raises the need for caution in interpretation and an urgent need for further, more disease-adapted research. In addition, while neuroinflammation is likely to be a successful target for MSC therapy, as demonstrated in several neurological diseases where neurodegeneration is associated with inflammation, or is even a consequence of inflammation, such as MS for example, it might be overoptimistic, in the current state of knowledge, to expect considerable neural repair from MSCs alone. Again, further research into their mode of action and/or the possibility to enhance their beneficial effect and/or their neuroprotective/neuroregenerative properties, either alone or in synergy with other cell types, is urgently needed. Additional aspects must be taken into consideration, in particular the possibility that downregulating inflammation should be approached with the proviso that neuroinflammation has also been shown to be essential for recovery in certain diseases such as ALS for example [103], and might necessitate the definition and monitoring of specific windows of therapeutic opportunity. Nevertheless, and despite possible risks [2] that need to be thoroughly evaluated for each disease through large clinical trials, all available data indicate that MSCs can

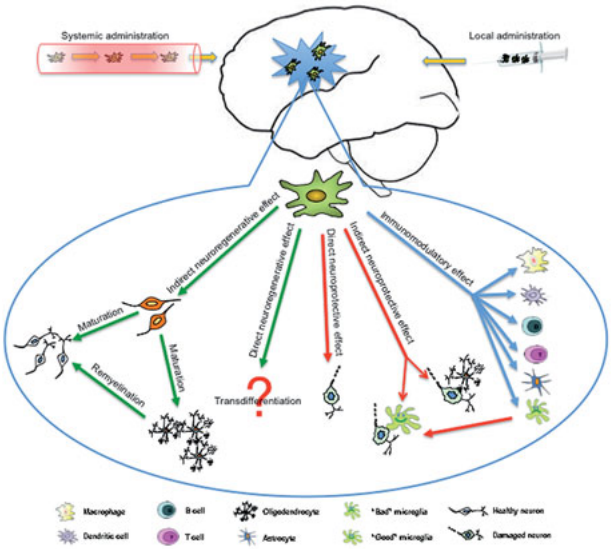

Fig. 10.1: Possible effects of MSC administration for therapy of CNS degenerative diseases. MSCs can have three general types of effects in CNS neurodegenerative diseases, immunomodulation, neuroprotection, and neuroregeneration, which can be interdependent. Thus, in neural damage associated with neuroinflammation, the immunomodulatory effects of MSCs on inflammatory cells are likely to have a profound impact on neurodegeneration by inducing their shift from a pro- to an anti-inflammatory profile, thereby decreasing neural cell damage, and their production of neuroprotective factors. Neuroprotective effects of MSCs can be direct, with their secretion of neuroprotective factors, such as BDNF, GDNF, *etc*., or indirect through a mediator, *e.g.* effect of MSCs on oligodendrocytes inducing their secretion of neurotrophic factor(s). While the direct neuroregenerative effect of MSCs is still controversial, with engraftment and transdifferentiation not consistently demonstrated, their indirect effect through induction of neural progenitor maturation and differentiation has been reproducibly demonstrated.

prevent neural damage and/or restore neural tissue and represent great hope for neurodegenerative conditions that are until now considered as irreversible.

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James E. Dennis and James D. Lord

11 Mesenchymal stem cells for the treatment of inflammatory bowel disease

Abstract Inflammatory bowel disease (IBD) is an incurable immune disorder affecting the gastrointestinal (GI) tract that causes high morbidity, and some mortality. The etiology of IBD is complicated, but clearly centers around the barrier function of the GI tract and the complicated interplay between the GI tract and its associated immune cells. There are a variety of therapeutic treatments that are directed at various aspects of the immune system been have met with limited success. Mesenchmal Stem Ccells (MSCs), with their demonstrable immune-regulatory features, are being studied as a therapeutic modality for the treatment of IBD. This review outlines the most prominent cellular players in the immune regulation of GI barrier function, covers the immunologic basis for MSCs-based IBD therapy, outlines how cell delivery, homing, and engraftment may impact outcomes in IBD disease models, and examines the results of MSCs-based clinical trials for IBD. Finally, several obstacles to effective use of MSCs for IBD treatment are discussed and alternative delivery mechanisms are presented as means to improve MSCs engraftment, which, it is hypothesized, would increase MSCs' therapeutic efficacy.

11.1 Introduction

Inflammatory bowel disease (IBD) is characterized by a state of chronic inflammation of the GI tract that is thought to arise as a result of an overexuberant response of the gut mucosa to foreign antigens. IBD encompasses the two diseases, ulcerative colitis (UC) and Crohn's disease (CD), which are similar in clinical presentation, but differ in their anatomical distribution. Ulcerative colitis, first described in 1859 [1], diffusely involves a continuous segment of the colonic mucosa contiguous with the rectum, while CD, first described in 1932 [2], is a transmural inflammatory disease that can affect any location in the entire GI tract, often in a patchy distribution [3]. UC presents symptomatically with bloody diarrhea, abdominal cramping and the passage of pus and mucus. The clinical presentation of CD is similar to UC, although patients may also present with perianal or abdominal abscesses, fistulas, or strictures, with the latter causing symptoms of bowel obstruction [3].

In addition to symptoms, there are serious long-term consequences of IBD. UC patients can have life-threatening complications, such as toxic megacolon, GI perforations and uncontrolled bleeding [4] that require surgery, and most patients who have CD for 20 years or more will require surgery [5]. All IBD patients have a higher risk of GI tract cancers [6] that increases with the duration of the disease [7]. In addition, 25 % of IBD patients exhibit extraintestinal inflammatory complications that

can involve the eyes, joints, skin, liver, or other sites. While some of these respond to treatment of the underlying IBD, others, such as sclerosing cholangitis or ankylosing spondylitis, run an independent course [3].

There is no medical cure for IBD, but there are a range of treatments which target various aspects of the inflammatory process. Mild to moderate colonic IBD, and particularly UC, can be treated with orally or rectally administered 5-aminosalicylate drugs, which are minimally absorbed by the GI tract and thus provide a localized anti-inflammatory effect. A variety of systemic anti-inflammatories, including corticosteroioids and immunosuppressants, are used for moderate to severe disease, depending on whether the condition is in remission (See Baumgart and Sandborn, 2007, for a detailed review of current and developing treatments [3].) More specific treatments being developed for IBD seek to target specific steps in the inflammatory cascade that occurs in IBD.

11.2 Immunology and intestinal barrier function

Chronic inflammation of the GI tract causes impaired barrier function and tissue destruction in IBD, hence mediators of inflammation are the primary target for IBD therapeutics. The GI tract constitutes the largest lymphoid organ in the body, which is not surprising when one considers that its function is to selectively absorb nutrients from an environment rich in foreign food antigens as well as potentially pathogenic bacterial fauna. The gut is therefore capable of mounting an aggressive response to these potential pathogens. At the same time, this massive collection of immune cells must be tightly regulated so that nutrient antigens are tolerated and to ensure that inflammatory responses are tempered to minimize damage to intestinal epithelial cells (IECs). The regulation of this immune response involves a complex array of signals among multiple cell types. The following is a simplified outline of some of the key components that are potential targets of therapeutic intervention.

The IECs consist of several cell types that have roles in nutrient absorption, barrier function, innate immunity, and immune signaling (Fig. 11.1). To serve a barrier function, IECs are connected by tight junctions and produce a protective mucous layer that insulates the deeper structures of the intestinal mucosa from the gut luminal contents. Specialized IECs, called Paneth cells, secrete antimicrobial peptides (defensins) into this mucous layer. Other IECs can secrete proinflammatory hormones, called cytokines [8] and, in turn, respond to various inflammatory signaling molecules. For example, IECs have receptors to interleukin IL-22 [9, 10], which both stimulates IEC proliferation and upregulates their production of antimicrobial peptides [11]. Goblet cells respond to the cytokine IL-6 by producing intestinal trefoil factor 3 (TFF3), which is anti-apoptotic [12]. Specialized antigen-presenting dendritic cells (DCs) are also present in the gut epithelium and extend pseudopods past the IECs, into the gut lumen, to detect and respond to antigens present in the luminal

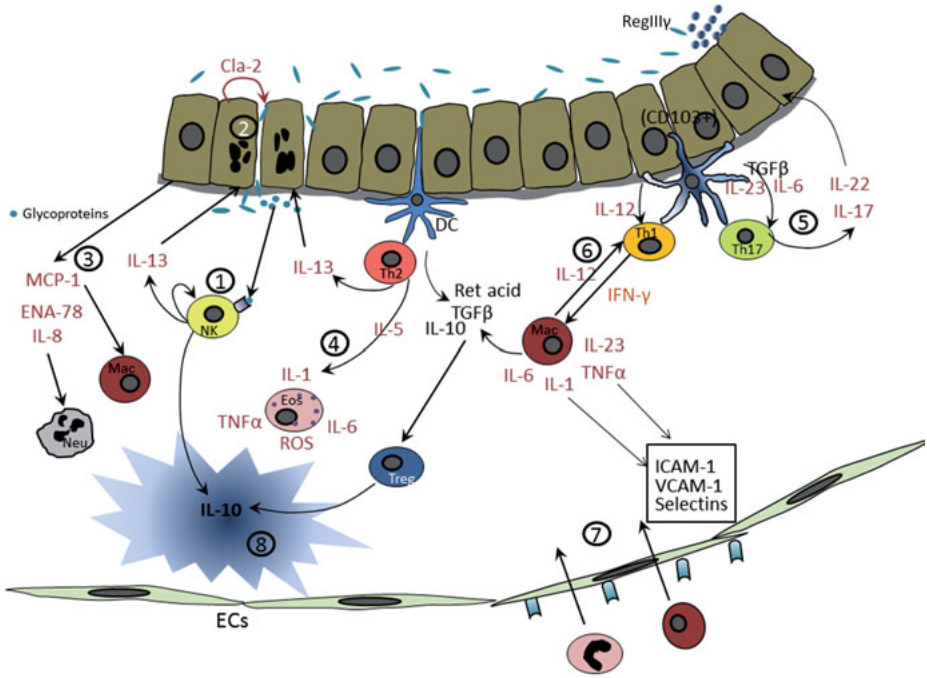

Fig. 11.1: Immunologic cells and pathways in inflammatory bowel disease. A disruption of the normal immune regulation of the gut tract is the centerpiece of the pathogenesis of IBD. Proinflammatory signaling initiates with bacterial and autoantigens that stimulate dendritic cells (DCs) and natural killer cells (NK). Glycolipid antigens stimulate NK cells (1) to upregulate IL-13 receptor (IL-13Rα2) which, in turn, binds autoproduced IL-13 that upregulates NK proinflammatory signaling. IL-13 also upregulates claudin-2 (Cla-2) in epithelial cells (2), which permeabilizes the epithelial cell junctions [127]. Activated endothelial cells produce epithelial neutrophil-activating peptide (ENA-78), MCP-1 and IL-8; MCP-1 recruits macrophages, and perhaps MSCs, and IL-8 recruits neutrophils (3). Dendritic cell-stimulated Th2 cells produce IL-13 and IL-5, which attracts and activates eosinophils (4); the Th2 pathway is characteristic of UC. There are at least two subsets of DCs, a CD103+ subset that can induce Th17 and Th1 cell responses [128] and also promote the induction of epithelial production of the antimicrobial peptide RegIIIγ *via* the production of IL-13 and IL-22, and CX8CR+CD11b+CD11c+ DCs (also positive for CD70) that also induce a Th17 response [129]. The Th17 pathway is stimulated by DC production of IL-6, TGF-β, and IL-23 which, in turn, stimulates the production of IL-22 and IL-17 (5). The Th1 pathway is induced by the production of IL-12 which, in turn, stimulates Th1 cells to produce IFN-γ that simulates a proinflammatory response in macrophages (6). Cytokine production, especially that of TNF-α and IL-1, upregulates the expression of cell adhesion molecules in endothelial cells (7) and chemotactic signals, such as IL-8 and MCP-1, attract leukocytes. Homeostasis of gut inflammation is regulated predominantly through two cytokines, IL-10 and TGF-β, which are produced by several cell types. DCs and epithelial cells produce TGF-β, which induces the expression of Treg cells which, in turn, produce IL-10. IL-10 downregulates inflammation by reducing proinflammatory cytokine production in macrophages, reducing antigen-presenting capacity in DCs and macrophages, and induces the production of soluble antagonists of IL-1 and TNF-α (8).

space [13]. Directly beneath the IEC basement membrane lies the gut-associated lymphoid tissue, where an array of lymphocytes dictates the intricate balance between proinflammatory and anti-inflammatory forces. Central mediators of this balance are regulatory T cells (Tregs).

Tregs are a small subset of CD4+ T cells which constitutively express CD25 and FOXP3 [14, 15] but, upon activation by their cognate antigens, inhibit rather than stimulate an immune response. Mice engineered to lack Tregs develop spontaneous intestinal inflammation [16, 17]. Similarly, humans born with mutations in the FOXP3 gene generate no Tregs, and develop a multiorgan inflammatory syndrome, called IPEX, including a fatal enteropathy resembling severe IBD [18–20]. While there are systemically circulating FOXP3+ "natural" Tregs (nTregs) that arise from the thymus, there are also locally-induced Tregs that have been shown to arise in response to orally-delivered antigens [21], which in turn stimulate Treg formation via dendritic cells [22]. Furthermore, upon activation in the presence of TGF-β, effector CD4+ T cells that are initially FOXP3-negative can be stimulated to express FOXP3, at least transiently, to become "induced" Tregs (iTregs), although whether they share the immunoregulatory properties of nTregs *in vivo* is controversial [23].

Tregs produce several factors that are able to modulate the immune response, such as IL-10. IL-10 inhibits proinflammatory cytokine production by monocytes and macrophages, reduces the antigen-presenting capacity of monocytes and dendritic cells, decreases TNF-α production [24] and also stimulates the production of soluble antagonists to IL-1β and TNF-α [25]. The importance of IL-10 to intestinal immunoregulation is revealed by the fact that genetically IL-10-deficient (IL-10 −/−) mice are extremely prone to developing colitis resembling human IBD [26, 27]. IL-10 modulates inflammatory tissue damage by inhibiting MMP2 and MMP9 production by monocyte/macrophages via the inhibition of prostaglandin E2 and cyclooxygenase 2 production [28]. Another key immunomodulatory factor produced by Tregs is TGF-β, which promotes FOXP3 expression by activated CD4+ T cells [29]. In the presence of IL-6, TGF-β also promotes CD4+ T cell differentiation into IL-17A-secreting Th17 cells [30] which, in turn, produce IL-22 which promotes IEC barrier function, as noted above. While Th17 cells have been implicated in some models of autoimmunity [31], and are present in increased numbers in IBD mucosa [32], pharmacological blockade of IL-17A was recently shown to have a negative impact on CD [33], suggesting that Th17 cells may be more beneficial than pathogenic in IBD. Finally, Tregs have been shown to express cytotoxic molecules that induce apoptosis in effector cells [34, 35].

In IBD, this balance between proinflammatory and anti-inflammatory signaling is lost and chronic inflammatory conditions are produced. While the antigens driving inflammation in IBD are undefined, several lines of evidence suggest a key role for the gut microflora. For example, antibiotics have a salutary benefit in CD (but not UC), while probiotics can benefit patients with UC (but not CD) [36]. The gut microbiome of IBD patients differs substantially from that of healthy individuals [37], although whether this is a cause or effect of inflammation remains to be seen.

Finally, most individuals with CD develop circulating antibodies to antigens from normal gut flora, such as *Saccharomyces cerevesiae* or *E. coli*, which are not seen in other people [38–40].

Swedish identical twin concordance studies have demonstrated that IBD is also heavily influenced by genetics [41]. Genetic background testing has shown that there are several susceptibility loci, but the lineage is multifactorial and complex [42]. To date, over 100 genetic polymorphisms have been associated with IBD, many of which show an overlap between CD and UC [43–45]. Many of these polymorphisms implicate genes of the immune system in IBD pathogenesis. Indeed, efforts to treat IBD have generally been directed at the various cellular and molecular components of the inflammatory cascades that contribute to the dysregulation of inflammation. Examples of these treatments include: unsuccessful trials of recombinant IL-10 or IL-11 cytokines, as well as successful trials of antibodies to the cytokines TNF-α, IL-12/IL-23, and to integrins involved in leukocyte homing [3].

11.3 Cell-based treatments for IBD

11.3.1 Hematopoietic cell transplantation

Hematopoietic stem cell transplantation (HSCT) was recognized as a possible treatment modality for IBD based on observations of disease remission in CD or UC patients undergoing allogeneic HSCT for cancer. The earliest observation of IBD remission after HSCT was reported by Drakos *et al.*, in 1993 [46], which was followed by reports on another 24 patients between 1996 and 2007 (reviewed by Garcia-Bosch *et al.* [47]). Of these 25 patients, 22 achieved clinical remission of IBD for a mean follow-up of 20 months and several patients remained in remission for many years [48–52]. More recently, Phase I and Phase II studies on the use of autologous HSCT to treat moderate to severe refractory CD patients have been conducted. In one Phase I study on autologous HSCT after myeloablation, 11 of 12 patients were shown to be in remission at a median follow-up of 18.5 months [53], while in a different Phase I/II study, 3 out of 4 patients showed complete clinical remission at a median follow-up of 16.5 months [54]. In a recent study on 3 patients with severe refractory CD, two patients who received HSCT were shown to be in remission at 5 and 6 years post-transplantation [55]. Finally, in a recent Phase I/II study in 24 patients with CD, all showed a significant decline in their CD activity index (CDAI) to below 150 following HSCT after nonmyeloablative conditioning, and 9 of the 24 patients remained disease free after 5 years [56].

These studies show that autologous HSCT is capable of inducing at least temporary remission in a majority of CD patients, and extended remission in some, but is not a cure for most. Allogeneic HSCT may carry a greater chance for a cure, as it replaces the genetically at-risk host immune system with presumably less predis-

posed donor cells, but it remains a highly morbid procedure with significant mortality risks, and thus would provide equipoise only for the most severe, refractory cases of CD. A major limitation to the use of allogeneic HSCT is graft-versus-host disease (GvHD), where donor immune cells react against the host tissue as foreign. While the risk of GvHD can be mitigated by depletion of mature lymphocytes from donor grafts, its predominant manifestation in the GI tract could be difficult to distinguish from recurrent IBD. Furthermore, if cells outside the hematopoietic system, such as IECs, intrinsically predispose CD patients to develop intestinal inflammation, there could be a disproportionately high incidence of GI GvHD in this cohort.

The mechanism of action of autologous HSCT treatment of CD is not clear, although it is hypothesized to be the result of re-setting the randomly-determined antigen specificity of the adaptive immune system by repopulating the immune system while under a noninflammatory "cease fire" after myeloablation [56]. Alternatively, HSCT could alter the character of the immune system towards a more tolerizing phenotype. One successful study of autologous HSCT for CD showed a significant increase in the number of circulating Treg cells in post-HSCT patients compared to pre-HSCT [56]. Indeed, a role for Tregs in supporting gut immune homeostasis following HSCT has come from studies correlating larger frequencies of Treg cells with a lower incidence of GvHD [57–61], although other studies have refuted this finding [62–66].

11.4 T regulatory cells (Tregs)

Another option as a cellular treatment for IBD is the infusion of Treg cells. Since Tregs play such a prominent role in the modulation of inflammation in the GI tract, it has been hypothesized that Treg infusion would be a potential treatment or cure for IBD. Intestinal inflammation in murine models of IBD has been prevented by infusion of CD4+CD45RB^{low} [67] or CD4+CD25+ (>95 % FOXP3+) Tregs [68]. Even after intestinal inflammation was established in murine IBD models, adoptive transplantation of CD4$^+$CD25$^+$ Treg cells was also able to reverse established intestinal inflammation in murine models of IBD mediated by T cells [69], *Helicobacter hepaticus* [70], or *Leishmania major* [71]. In the latter case, this disease reversal was mediated by IL-10, TGF-β and CTLA4, as antibodies to each of these molecules abrogated the protective effect of CD4$^+$CD25$^+$ T cells.

Another study of a murine colitis model used Treg transplantation in combination with rapamycin treatment as a means to increase the number of Tregs in the gut. The rapamycin treatment alone reduced the degree of inflammation, and the infusion of rapamycin-treated, culture-expanded Tregs completely prevented the development of colitis [72]. The inclusion of rapamycin in this study is based on results showing that rapamycin inhibits lymphocyte proliferation, partially inhibits the differentiation of CD4$^+$ T cells and is able to increase the relative number of Tregs [73, 74]. However, a

randomized controlled trial of everolimus, a derivative of rapamycin, showed a lack of clinical efficacy in the treatment of human CD [75].

Nonetheless, these successes in mice have recently prompted the first trial of Treg-based therapy in human IBD. A recent Phase I open label trial in 20 patients with treatment-refractory CD infused a single dose of *in vitro*-expanded CD4+ T cell clones capable of making IL-10 in response to ovalbumin [76]. Roughly half of these cells expressed the Treg markers FOXP3 or CD25, and many expressed potential inhibitory mechanisms of Tregs, such as CTLA4, CD39, or Granzyme B. This small study showed clinical benefit in a subset of patients (n=8) that received the lowest dose of Tregs (10^6 cells) although objective measures of inflammation (C reactive protein and fecal calprotectin) were less clear, and disease activity scores reverted to baseline in most recipients by 12 weeks after infusion. Paradoxically, benefit was least evident in patients who received larger Treg doses (10^7–10^9 cells), and was associated with a smaller fraction of FOXP3+ Tregs in the peripheral blood, suggesting that it is the quality, not quantity, of infused Tregs that leads to improvement.

Such a suggestion is perhaps not unexpected, when one considers that IBD is not associated with a dearth of Tregs, as is seen in IPEX, but rather with a paradoxically increased FOXP3+ fraction of T cells in the inflamed mucosa [77, 78]. An explanation offered for this paradox is that the excess FOXP3+ cells seen in IBD may simply be activated effector T cells in a TGF-β-rich environment becoming iTregs, which may lack some of the regulatory properties of thymically-derived nTregs [79]. However, nTregs and iTregs can be differentiated by the nuclear factor Helios, expressed exclusively in nTregs [80], and in both intestinal mucosa and peripheral blood, the FOXP3+ cells contain just as large a fraction of Helios+ nTregs in IBD patients as in people without IBD (Fig. 11.2). Furthermore, the Tregs of IBD patients appear to have normal *in vitro* inhibitory function, regardless of whether they are isolated from the intestinal lamina propria [81], the blood [82], or the mesenteric lymph nodes [83, 84].

11.5 Mesenchymal stem cells (MSCs)

11.5.1 Immunologic basis for MSCs and IBD

Various sources of mesenchymal stem cells (MSCs) have been tested as a treatment modality for inflammation-related diseases, such as IBD [85, 86–88], GvHD [89–91], rheumatoid arthritis (RA) [92, 93], type I diabetes [94, 95], and multiple sclerosis (MS) [96, 97]. The effectiveness of MSCs for these conditions is ascribed to the various immunomodulatory capabilities of MSCs, most of which are outlined in earlier chapters of this book. With respect to MSC-based treatments for IBD, it is useful to highlight the immunomodulatory effects that have the potential to impact the microenvironment of the GI tract in IBD, as was illustrated in Figure 11.1. As described in earlier chapters, MSCs have the ability to modulate immune cells via the secretion of

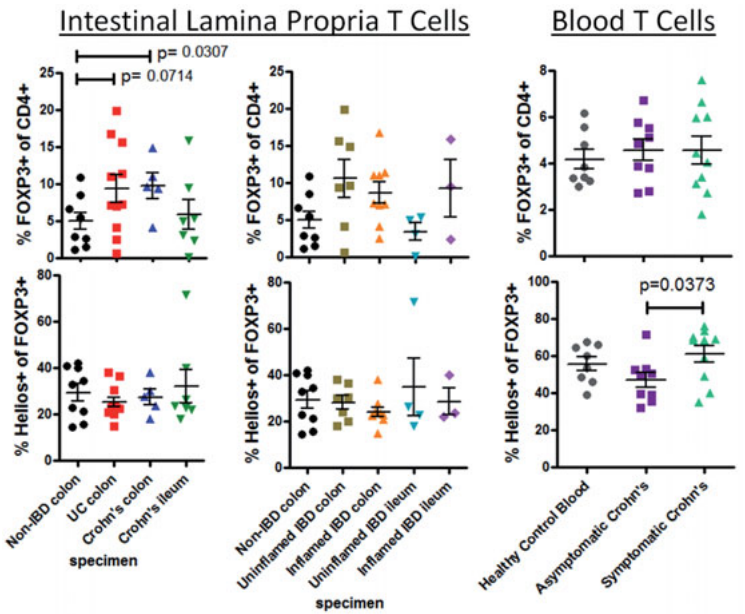

Fig. 11.2: Increased FOXP3+ CD4+ T cells in IBD mucosa are not Helios-negative iTregs. The percent of CD4+ T cells expressing FOXP3 with or without Helios was quantified by flow cytometry in the inflamed or noninflamed colonic or ileal lamina propria cells of a cohort of patients who underwent surgical resection of this tissue for IBD or another indication. Similar analyses were performed on the peripheral blood mononuclear cells of a separate cohort consisting of CD patients who were or were not in remission, or age and sex matched healthy control subjects.

various soluble factors such as TGF-β [98], prostaglandin E2 (PGE2) [99], indoleamine 2,3-dioxigenase (IDO) [100], nitric oxide (NO) [90], and TSG-6 [101], and also through direct cell contact in the absence of soluble factors [102]. The target cells for these various immunomodulatory effects include nearly every immune cell found in the IBD gut environment. For example, MSCs have been shown, *in vitro*, to decrease the secretion of TNF-α in mature DCs and to increase the secretion of IL-10 in mature DC2 cells [99]. MSCs have also been shown to inhibit the secretion of IFN-γ by Th1 and NK cells and, when MSCs were co-cultured with peripheral blood mononuclear cells, there was an increase in the percentage of Treg cells [99].

However, although there is strong evidence that MSCs have these modulatory effects on immune cells, many of the studies are in strictly defined mouse models while others were conducted *in vitro*, so it still needs to be determined if these reported immunomodulatory effects are active in the more complicated microenvironment of the inflamed gut. As a step towards the clinical application of MSCs for IBD, human-derived MSCs have been tested in animal models of IBD. Gonzalez *et al.* [103] examined the effect of adipose-derived human MSCs in trinitrobenzene sulfonic acid (TNBS)-induced colitis and showed that human MSC-treated mice significantly increased

survival and abrogated the clinical signs of colitis (*i.e.*, weight loss and diarrhea). In addition, they showed that hMSC treatment upregulated IL-10 production, reduced the level of inflammatory cytokine production, and increased the number of Tregs.

11.6 MSC homing and engraftment

The homing and engraftment of MSCs is dependent on a number of different factors including: immunoprivileged state of MSCs (reviewed in Chapter 2), the route of delivery (direct, venous or arterial), the homing signal(s) emanating from the site of the lesion, the chemokine receptors on the MSCs, and the ability of MSCs to bind, pass through the endothelial layer (diapedesis), and engraft (attach and survive).

As indicated in Chapter 2, MSCs are considered to be immunoprivileged and many studies indicate that they can fly under the immune radar due, in part, to their lack of expression of any costimulatory molecules or class II major histocompatibility complex (MHC), and their expression of only low levels of class I MHC. This privileged status is arguable, with some studies indicating that MSCs can be recognized by the immune system, especially after they begin to differentiate. In a study of MSCs injected in cardiac tissue, Huang *et al.* showed that MSCs induced to differentiate increased the expression of MHC Ia and MHC II and reduced their expression of MHC Ib [104]; both MHC Ia and MHC II expression is immunostimulatory while MHC Ib expression is immunosuppressive. Even if it is possible for MSCs to avoid recognition by the adaptive immune system, there is still the possibility of immune recognition by the innate immune system. For example, a recent study demonstrated that both autogenic and allogeneic transplanted MSCs are recognized by complement in serum and can be damaged by complement-mediated attack [105], although it was noted that the allogeneically transplanted MSCs were more susceptible to complement-mediated attack. This raises the possibility that much of the inefficiency of MSC engraftment, to date, may be accounted for because of MSC death caused by the innate immune system or by the adaptive immune system if the injected MSCs start to express differentiation markers. At the time of this review, no study we are aware of has looked at ways to avoid recognition by the adaptive immune system as a way to boost engraftment.

The method of stem cell delivery can have a significant impact on outcome. Direct delivery is the simplest of the approaches because it is not dependent on systemic circulation, chemotactic signaling or diapedesis. However, as previously indicated, direct injection is not practical in patients where disease is widespread. Still, even directly-injected MSCs need to be immunoprivileged in order to survive long-term, and the MSCs need to bind to the lesion and engraft without being washed out. Even in studies where MSCs are directly injected, a vast majority of the cells have been shown to not take up permanent residence in the injected tissue. For example, in a rat myocardial infarction model, only 0.3–3.5 % of the MSCs were detected by Y-chro-

mosome qPCR analysis 6 weeks post-injection, and these numbers do not account for any MSC proliferation that might have occurred post-engraftment [106]. In another study, green fluorescent protein (GFP)-expressing MSCs injected into rat hearts and quantified by pPCR one month later showed only 5.1 % of the injected signal was still present and, again, these data did not account for any post-injection increase in the number of MSCs due to proliferation [107]. The general assumption is that in order to be effective, MSCs need to be delivered in sufficient numbers to the affected organ(s), and the cells need to survive and engraft, at least if there is to be long-term efficacy. However, this does not preclude the possibility that studies, to date, have had little or no MSC engraftment but could still show a therapeutic effect. In fact, few studies clearly defined the exact mechanism for positive therapeutic effects that have been observed.

For systemically injected MSCs, it has been demonstrated that MSCs first accumulate in the lungs, then the liver, and later become more widely dispersed throughout the body [108]. Quantification of MSC engraftment after systemic injection into baboons showed only 0.1–2.0 % engraftment at 9 and 21 months post-infusion, based on qPCR of GFP-transduced MSCs in multiple organs [109]. Similarly, it was shown by qPCR quantification of human cells injected into SCID/NOD mice that 83 % of the injected cells accumulated in the lungs after 5 minutes, and were cleared of the lungs with a half-life of about 24 hr. Interestingly, only 0.04 % of the injected dose could be detected in 6 organs at 48 hr post-injection and by 96 hr post-injection the amount was only 0.01 % [101]. It has been speculated that the systemic injection of MSCs may simply produce a short-term downregulation of inflammation via soluble factors which may be sufficient to break the cycle of chronic inflammation. It remains a distinct possibility that significant long-term MSC engraftment has not been a significant factor in any of the reported positive therapeutic outcomes. In the study by Lee *et al.* [101], it was demonstrated that only trace numbers of cells were retained within the lungs or other tissues, yet there was still a demonstrable therapeutic effect on post-infarct heart function. Interestingly, they showed that entrapped MSCs upregulated TSG-6 expression, a known regulator of inflammation [110], and that when MSCs were treated with TSG-6 siRNA there was no therapeutic effect on heart function. These results show that, at least in some cases, the therapeutic effect of injected MSCs could be a result of the systemic, and possibly transient, production of immunomodulatory factors. Still, the general supposition is that if the level of MSC engraftment can be increased and cells can be effectively delivered to the target organ there would be an increased likelihood of long-term efficacy, and several groups are applying various methods to try to increase MSC engraftment efficiency.

One method used to increase cell engraftment has been to coat cells with specific molecules that can increase binding, and subsequent engraftment, to organs of interest. For example, Sackstein *et al.* [111], showed that the CD44 receptor found on MSCs could be chemically modified to produce the E-selectin/L-selectin ligand and those modified MSCs preferentially engrafted into bone marrow. However, this methodology

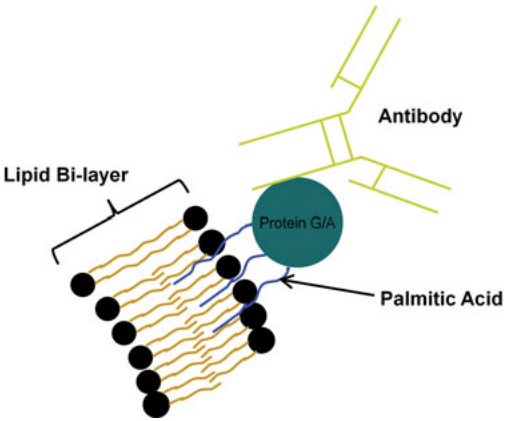

Fig. 11.3: Cell "painting" technology. Cells are first coated with palmitated protein A or protein G which intercalates into the cell lipid bi-layers. After washing, the cells are then incubated with antibody, whose Fc region binds to protein A or G.

is limited by the need to modify pre-existing cell surface receptors to produce ligands of interest. An alternative methodology to direct MSC binding to tissues was developed where MSCs are "painted" by a two-step process, first with palmitated protein G or protein A and, second, with an antibody or antibodies of choice (Fig. 11.3) [112]. Building on this technology, Ko *et al*. demonstrated that MSCs painted with antibodies to ICAM-1 were able to bind to activated endothelial cells and resist detachment by shear forces equivalent to arterial flow [113]. Using this technology, Ko *et al.*, tested the efficacy of MSC treatment, with or without precoating with antibodies, in a mouse model of IBD [114]. In this study, Kaplan–Meier plots of survival post-treatment with dextran sulfate sodium showed that mice injected with MSCs coated with antibodies to MadCAM or VCAM-1 had survival rates significantly higher than mice injected with non-coated MSCs (Fig. 11.4). As a possible mechanism of action, it was shown that there was an increase in the percentage of Tregs in the total T cell population in the MSC-injected groups, with the highest percentages found in the mice injected with antibody-coated MSCs.

The route of systemic delivery also has a significant impact on cell distribution. Several studies have shown that MSCs accumulate in the lungs immediately after intravenous injection [101, 108]. The study by Gao *et al.* [108] also showed that the injection of a vasodilator (nitroprusside) decreased the number of MSCs captured in the lung by about 40 %, which resulted in increased numbers of cells found in other organs. Intra-arterial delivery shows a cell distribution pattern significantly different from that of intravenous delivery. For example, in a study tracking injected MSCs for the treatment of brain tumors, 7 or 9 mice injected intra-arterially with luciferase-expressing MSCs showed a positive bioluminescence signal in brain tissue, while none of the 12 mice that were injected intravenously with luciferase-expressing MSCs

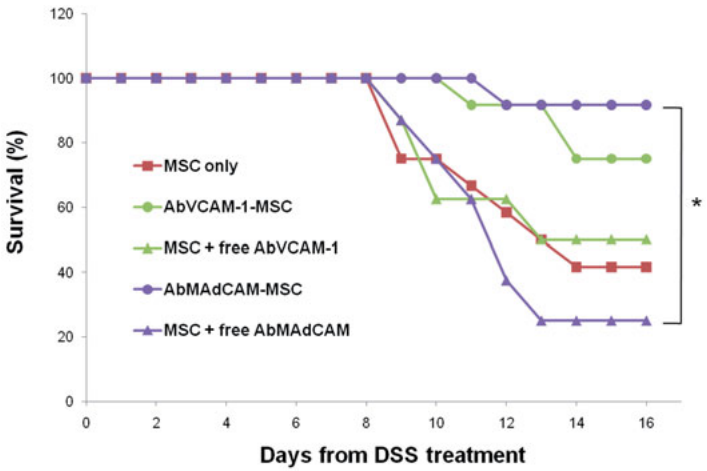

Fig. 11.4: Kaplan-Meier plot of post-dextran sulfate sodium (DSS) survival in mice injected with mesenchymal stem cells (MSCs), with or without cell targeting. Mice given 5 % DSS in their drinking water and injected intravenously, on day 2 post-DSS, with vehicle (phosphate buffered saline), MSCs only, or MSCs coated with antibodies of VCAM-1, MadCAM, or isotype control antibody. Reprinted with permission from Ko *et al.*, 2010 [114].

showed a positive bioluminescence signal [115]. Interestingly, this study also showed a gradual decrease in bioluminescence, and Di-I labeling as detected by immunofluorescence, over a period of 14 days, which is another indicator that few, if any, MSCs permanently engraft.

In summary, several methods are available to enhance MSC binding to specific organs, either by chemical modification of endogenous cell surface receptors [111] or by coating MSCs with antibodies [112]. In addition, intra-arterial injection appears to be a more effective method of introducing MSCs systemically than intravenous injection, primarily due to entrapment in lung tissue after intravenous injection. However, long-term engraftment of MSCs remains elusive. While MSCs have been demonstrated to have features that render them less likely to be detected and eliminated via the adaptive immune system, this immunoprivileged state can disappear as MSCs enter specific differentiation pathways and begin to express class II MHC. In addition, there is evidence that MSCs may not be able to avoid detection by the adaptive immune system [105].

11.7 MSC clinical trials

Three Phase I clinical trials have been conducted using hMSCs for the treatment of CD [116, 117] or for fistulizing disease [118], which encompasses some CD patients. In

the earliest study [118], 4 patients were injected with adipose-derived hMSCs and 8 fistulas were tracked over time. 6 of the 8 fistulas showed complete closure while the remaining 2 showed partial closure; no adverse events were noted. In another study, 10 patients received 1–2 × 10^6 cells/kg body weight intravenously, two times, 7 days apart. At 6 weeks follow-up, 3 patients showed significant reduction in CDAI scores (of ≥ 70 points), while disease worsened in another 3 patients to the point of requiring surgery [117]. In another study, local injections of MSCs were studied in 10 patients with fistulizing CD [116]. The patients' bone marrow MSCs were expanded, frozen until needed, and freshly thawed cells were injected 2–5 times at 4-week intervals with a median of 2.0 × 10^7 hMSCs in a track along the length of the fistula. Complete closure was observed in 7 of the patients and partial closure was observed in 3, and the CDAI's were reduced by at least 150 points in all patients at one year follow-up. The number of Tregs in mucosal biopsies was quantified and showed a significant increase (nearly 3 times) from pretreatment to 12 month follow-up. Based on these Phase I trials, hMSCs from bone marrow or adipose tissue are well tolerated, but only the locally-injected cells showed any significant therapeutic effect.

A Phase II trial using adipose-derived MSCs for the treatment of complex perianal fistulas [119] was conducted based on the results of the Phase I described above [118]. In this study, 49 patients were enrolled, 14 of whom had CD. The patients were randomly assigned to receive either a direct injection of 2.0 × 10^7 adipose-derived MSCs into the wall of the fistulized GI tract along with fibrin glue, or just fibrin glue alone. If healing was not seen by eight weeks, subjects received either another 4.0 × 10^7 MSCs plus glue, or glue alone, as originally randomized. In the overall study, it was demonstrated that significantly more patients receiving adipose-derived MSC treatment healed (71 %) compared to controls (16 %) ($\rho < 0.001$). Within the CD subgroup there were similar results, although the results did not reach statistical significance ($\rho = 0.10$) due to a small sample size. A subsequent open label study of these adipose-derived MSCs, administered as above, on another 24 CD patients with complex perianal fistulas, but inactive luminal inflammation (CDAI<200) [120] reported reduced fistulas in 69.2 %, complete closure of the injected fistula in 56.3 %, and complete closure of all fistulas in 30 % of patients after 24 weeks of follow-up. These results resemble the clinical efficacy that anti-TNF therapies have demonstrated in clinical trials [121, 122], although the latter were not conducted in patients without luminal inflammation, and thus may not be directly comparable with this MSC data.

The results from these studies indicate that hMSCs, either from bone marrow or from adipose tissue, can effectively treat fistulas when injected locally into the lesion. It is less clear whether systemic treatment is effective. One Phase I study of systemically infused MSCs had as many patients increase the severity of their disease as those whose symptoms improved [117]. A different open label Phase II pilot was conducted using bone marrow-derived hMSCs (Prochymal™, Remestemcel-L) on 9 patients with refractory CD. The patients were injected, intravenously, with either low (2.0 × 10^6 cells/kg) or high (8.0 × 10^6 cells/kg) dose of Prochymal™ mesenchymal stem cells

[123]. There was mean decrease in CDAI scores of 105 ($\rho = 0.004$) and a significant increase in quality of life (IBDQ) scores by day 28 ($\rho = 0.008$) with clinical remission in 3 of the 9 patients. A Phase III clinical trial of these cells commenced in May 2007, but as of this writing, has not completed enrollment. Similarly, a Phase II randomized controlled trial of another commercially developed bone marrow-derived hMSCs (Multistem®) based on multipotent adult progenitor cells [124] commenced enrollment of UC patients in February, 2011, but has yet to conclude.

An important issue to be addressed is the type of IBD being studied and the possible mechanism of the cell therapeutic effect. Most of the aforementioned studies were focused on fistulizing CD, which is a subset of the overall CD population. What is encouraging for the general treatment of CD is that some of the studies showed positive assessment of CD in general (CDAI and IBDQ scores) and not just repair of the fistulas. More encouraging were the results showing an increase in circulating and mucosal Tregs [116], which indicates a possible mechanism of action on the inflammatory process. Still, these studies have been limited in the number of patients being tested and thus do not address the wide heterogeneity of CD. Furthermore, these human clinical studies are limited in their ability to dissect out the mechanism(s) of action.

One of the primary issues to be addressed is the fate of the injected MSCs, which is simply not possible in human trials. Clearly, it would be advantageous if the hMSCs became engrafted into the tissue in order to effect a long-term repair, although there is the possibility that even a transient treatment, if it effectively quiets the inflammation, may be sufficient to break the cycle of the disease to at least bring the patient into a state of remission. The direct injection therapy has its limitations in that it is only practical for patients with focal lesions along the GI tract. Systemic delivery is the only practical means of treating most patients with IBD, so a means for more effective delivery, and, ideally, long-term engraftment, may be required in order to obtain efficacious results in human trials.

11.8 Summary and future directions

Because of the lack of a curative treatment for UC and CD, there is a clear need for the development of more effective long-term therapeutics to address these severely debilitating and sometimes fatal diseases. Cellular therapies are a rational approach for effecting a long-term solution, based on the assumption that engraftment of these therapeutic cells would make a systemic shift in the immune-homeostasis of the patient towards a more anti-inflammatory direction. HSCTs, Tregs and MSCs have each been tested as possible cell therapies for IBD, and each of the cell sources have shown some positive effects in early studies. The use of HSCTs and Treg cells as treatments has to be weighed against the risks to the patient that result from the conditioning regimen and the additional risks of GvHD for HSCT. In very severe cases of CD where a patient's life is in danger, the benefit of HSC transplantation may be

warranted. Treg transplantation may also carry unknown risks associated with the overall downregulation of the immune system these cells are known to mediate. The first clinical application of Treg infusion has, ironically, been for the prevention of a complication of HSCT, namely GvHD [125]. As an open label study, this small pilot trial was difficult to interpret for clinical efficacy, but no increase in infections, mortality, or early cancer relapses were seen among the 23 recipients.

One major advantage of the use of MSCs may be their safety profile, as there have been few reports to date of serious adverse reactions among recipients. Furthermore, unlike HSCT, therapy with MSCs requires no toxic conditioning. Only a few studies have been conducted on the use of MSCs for the treatment of IBD and the results have been encouraging, particularly for fistulizing CD, but inconclusive. These human studies are complicated by the diversity of IBD itself which includes both UC and CD patients with varying degrees of disease severity and differing responses to currently available treatments, which limits the number of patients that can be enrolled in these studies, and adds to the variability of the results. Another variable is the stock of MSCs that is being used for these studies. Currently, the only standard for the preparation of MSCs is the flow cytometric analysis of various cell surface markers and confirmation that MSCs differentiate down the osteogenic, chondrogenic and adipogenic pathways. These are useful criteria, but none of these assays addresses the potential for these cells to modulate the immune system and it is already known that there is a high degree of variability in the absolute level of expression that human MSCs have for cytokines and growth factors [126]. Some metric for evaluating the potency of MSC preparations for modulating the immune system might prove advantageous for increasing the efficacy of the treatment.

Another major issue is long-term engraftment. As discussed earlier, few studies have effectively tracked the fate of injected MSCs, and those that have, have shown that very few MSCs survive long term. While most studies indicate that MSCs can avoid immune rejection because of their minimal expression of MHC class II molecules and costimulatory molecules, there is evidence that MSCs are recognized by the innate immune system [105] and some studies have indicated that MSCs are rejected after they express an end-stage phenotype. The phenotypic commitment of delivered MSCs is, in most cases, completely unknown. Whether MSCs retain the immuno suppressive phenotype observed *in vivo* or instead differentiate to express cell surface molecules recognized as foreign by the recipient immune system is a difficult question to ask since any MSCs that express these foreign antigens would be specifically eliminated.

Efficient delivery of MSCs is another question that has not been fully addressed. While direct injection into a lesion may be appropriate for a focal fistula, in cases limited to mucosal inflammation or involving large tracts of the intestine, local injection is not feasible. Systemic injections are problematic in that MSCs are known to accumulate, at least temporarily, in the lungs, and few studies have shown clear evidence of MSC homing and engraftment. Arterial injections have been shown to be

a more effective means of avoiding lung entrapment, and can give MSCs a greater chance of getting to target tissue(s), yet such a route of administration would require invasive angiography, and would still not obviate the need for MSCs to bind and undergo diapedesis in order to engraft. Methods to promote binding to activated epithelium have shown promise in animal models [111, 114], but no studies have been conducted in humans.

In summary, MSCs have been shown to be effective modulators of the immune system in *in vitro* studies and are being tested as possible therapeutics for several immune conditions, such as GvHD and IBD. While several studies have shown positive effects with MSC treatment, all are very preliminary (Phase I or II) and far from definitive. Advances in the characterization of the immune-regulatory potency of therapeutic MSCs may help increase efficacy, as would improvements in MSC delivery and survival.

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Daniel J. Weiss and Mauricio Rojas

12 Mesenchymal stem cells in chronic lung diseases: COPD and lung fibrosis

Abstract Recent advances in understanding the potential roles of stem cells and cell therapies in lung biology and diseases, and progress in ex vivo lung bioengineering offer new promise for a range of lung disease.. These include both chronic fibrotic and chronic obstructive lung diseases, devastating conditions that are increasing in prevalence worldwide and that have no cure except lung transplantatin.. In this chapter we will review current approaches for cell-based therapies for pulmonary fibrosis and for chronic obstructive pulmonary disease (CO.

12.1 Introduction

Many chronic lung diseases, including chronic obstructive lung disease (COPD) and idiopathic pulmonary fibrosis (IPF) are increasing in prevalence. While available treatments may lessen the severity of symptoms, both COPD and IPF are relentlessly progressive leading to death from respiratory failure. Lung transplantation is the only curative approach; however, there is a significant shortage of suitable donor lungs and many on waiting lists die before a lung becomes available. Further, lung transplantation requires lifelong immunosuppression and 5 year mortality after transplantation is approximately 50 %. Lung transplantation is also not a realistic option for patients in many parts of the world. New therapeutic approaches are thus desperately needed.

Approaches utilizing cell-based therapies for lung diseases have progressed rapidly in recent years. Systemic or intratracheal administration of different stem and progenitor cell types including endothelial progenitor cells, amniotic fluid cells, and others have been demonstrated to have efficacy in different preclinical models of lung diseases [1–3]. However, the majority of available preclinical data has focused on investigation of mesenchymal stem (stromal) cells (MSCs) derived primarily from bone marrow but also from adipose and cord blood or placental tissues. A steadily increasing number of articles demonstrate efficacy of either systemic or intratracheal MSC administration in a growing spectrum of lung injury models in mice and in a slowly growing number of clinical investigations in lung diseases [1–8]. This includes mouse models of acute lung injury and bacterial lung infection [9–20], asthma [21–33], bronchiolitis obliterans [34], bronchopulmonary dysplasia [35–42], COPD [43–52], ischemia re-perfusion injury [53–55], obstructive sleep apnea [56, 57], postinflammatory lung fibrosis [58–68], pulmonary hypertension [69–74], sepsis and burns [75–81], and other critical illness or autoimmune-related lung injuries including hemorrhagic shock, lupus, pancreatitis, silicosis, and ventilator-induced lung injury [82–87]. MSCs

have also been demonstrated to have efficacy in models of primary and metastatic lung cancers [88–98]. MSCs administration has also been demonstrated to alleviate inflammation and injury produced by intratracheal instillation of either endotoxin or of gram-negative bacteria in human lung explants [99, 100].

The mechanisms by which MSCs might alleviate inflammation and injury are not completely understood and, as in other organ systems, likely involve multiple pathways including release of soluble mediators and/or microsomal particles as well as cell-cell contact. Importantly, the mechanisms of MSCs actions are different in different lung diseases. For example, available information demonstrates that MSCs alleviate endotoxin-induced acute lung injury in mouse models through release of soluble anti-inflammatory, antibacterial, and angiogenic substances, including IL-10, angiopoietin 1 (Ang-1), keratinocyte growth factor (KGF), LL-37, and others [10, 13, 15, 18, 20]. The MSCs also influence macrophages to acquire an anti-inflammatory M2 phenotype. In contrast, MSC administration in mouse models of asthma (allergic airways inflammation) ameliorates both airways hyper-responsiveness and lung inflammation through effects on antigen-specific T lymphocytes and by upregulating T-regulatory cells [23–25, 27]. Release of different soluble mediators, including TGF-β or adiponectin, may be involved [25, 32]. Other mechanisms have been proposed for MSC actions in different lung disease injury models (Fig. 12.1). As such, as MSCs-

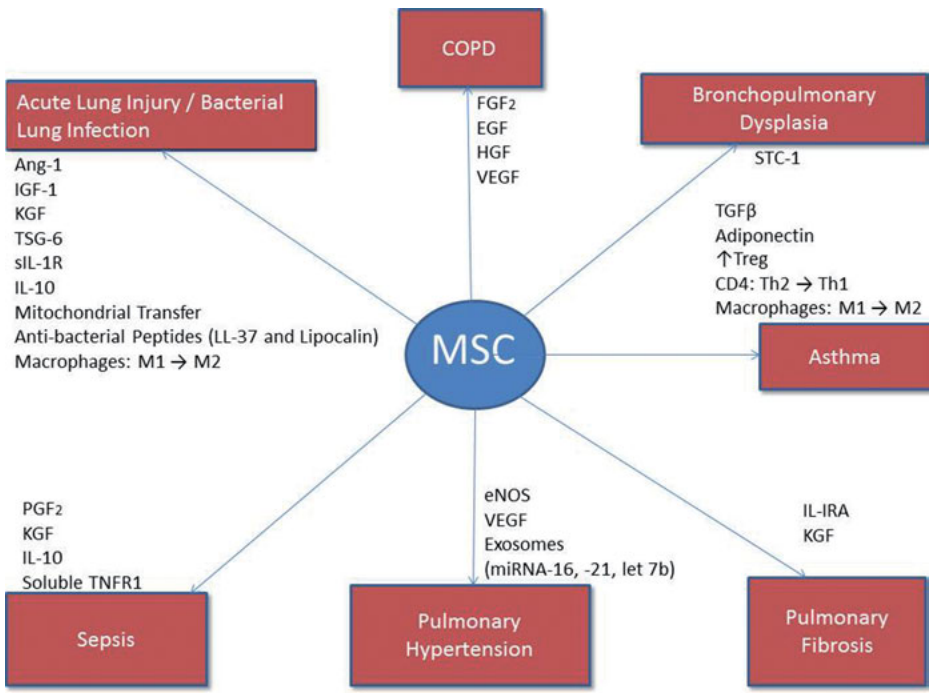

Fig. 12.1: Schematic of MSCs actions in different preclinical lung injury models.

based therapies are developed for lung diseases, the specific disease pathogenesis in the context of the known actions of the MSCs must be carefully considered.

Nonetheless, a growing number of clinical investigations of MSC-based therapy in different lung diseases including COPD and IPF are occurring [101]. In the following sections, the rationale for potential MSC effects, available preclinical data, and considerations of clinical trials of MSCs in COPD and IPF will be considered.

12.2 Idiopathic pulmonary fibrosis

The interstitial lung diseases comprise a group of diffuse pulmonary parenchymal diseases that are classified together because of similar clinical, radiologic, physiologic, and/or pathologic manifestations. Idiopathic pulmonary fibrosis or IPF is the most common and most severe form of idiopathic interstitial pneumonia. It is often relentlessly progressive, leading to death from respiratory failure within 2–5 years of diagnosis in the majority of instances [102]. IPF is characterized by the inability of normal repair of the epithelial cell injury resulting in the activation and expansion of the fibroblast/myofibroblast population with exaggerated deposition of extracellular matrix with destruction of the lung architecture. Classically IPF shows in filtrates on chest radiographic imaging and when biopsy is performed it is defined by the presence of microscopic honeycombing and fibroblastic foci.

The occurrence of IPF increases in both prevalence and incidence in the sixth decade of life [103, 104]. Symptoms typically occur at age 50 to 70 years, and most patients are > 60 years of age at the time of clinical presentation [105]. The annual incidence of IPF appears to be rising and is estimated at 5–16 per 100,000 individuals [105]. IPF is more common in men, and the prevalence rises dramatically with age [106]. IPF is sufficiently uncommon under the age of 50 as to mandate an exhaustive search for a different etiology of the lung fibrosis such as an underlying connective tissue disease or occult environmental exposure in young patients, particularly women. In contrast, pulmonary fibrosis in patients over the age of 70 is significantly more likely to be classified as IPF[106]. Risk factors for IPF include a history of cigarette smoking, male gender and age. The precise contribution of genetic transmission to IPF is difficult to ascertain because of the late onset of disease presentation, but it appears to be responsible for 5% of IPF cases. Further, most cases of IPF are diagnosed late in disease progression with irreversible destructive fibrotic changes in the lung parenchyma. While there may be smoldering lung inflammation, attempts to counter this have proven ineffective as have any other therapeutic strategies investigated to date. As such, it is difficult to predict the rate of progression in individual patients and also to design clinical investigations of new agents. Large numbers of patients must be studied to obtain appropriate power for adequate statistical analyses of outcome measures. At present, there are no available methods of diagnosing IPF patients early in disease progression before clinical symptoms occur.

The pathogenesis of IPF has long been felt to reflect abnormal proliferation of local lung fibroblasts. However, in recent years, data has suggested that circulating bone marrow-derived fibrocytes can be recruited to the lung following injury and can potentially contribute to the burden of fibroblast accumulation and tissue remodeling [107–111]. In addition, fibrocytes have been identified in the blood of patients with IPF [108] and might be useful as a clinical marker for disease progression. Fibrocytes express stem and leukocyte cell markers such as CD45 and CD34, traffic to the lungs in response to CXCL12 in a bleomycin injury murine model, and can produce type I collagen [109, 110]. High levels of circulating fibrocytes have been associated with age-related susceptibility to lung fibrosis in a mouse bleomycin model and poor prognosis in IPF. Further, increasing evidence suggests a primary role for alveolar epithelium in the pathogenesis of IPF. A combination of environmental, genetic, and age-related factors appear to coalesce to create an alveolar epithelium that is susceptible to injury from either unknown endogenous factors or exogenous insults such as viral infection or microaspiration. The idea that constant injury drives exhaustion of reparative local endogenous lung epithelial progenitor cell with progressive accumulation of senescent cells has also been postulated to contribute to development of lung fibrosis [112].

As IPF is predominant in older patients, it is important to consider effects of aging in the lung and mechanistic links with the pathogenesis of IPF in developing cell therapy-based strategies. Aging may also play a role in COPD pathogenesis and will comparably guide development of cell-based therapy approaches for COPD. Aging is a natural process characterized by progressive functional impairment and reduced capacity to respond appropriately to environmental stimuli and injury [113]. Like any other organ, the lungs also age. Physiological lung aging is associated with several anatomic (enlargement of alveoli without alveolar wall destruction, reduced surface area for gas exchange, and loss of alveolar attachments supporting peripheral airways, often referred to as “senile emphysema”) and functional changes (reduced elastic recoil and increased gas trapping) that result in a progressive decrease in expiratory flow rates with age in otherwise healthy people. On the other hand, epidemiological studies indicate that aging is associated with an increased incidence of two common chronic respiratory diseases; chronic obstructive pulmonary disease or COPD and IPF. Interestingly, although COPD and IPF are distinct disease entities, they share some similarities. Both occur later in life, both are punctuated by episodes of “exacerbations” that are often of unclear origin, and both are characterized by enhanced deposition of collagen and fibrosis (although, admittedly, this occurs in different locations in each disease, in the small airways in patients with COPD and in the lung parenchyma in IPF). Last, and interestingly, both conditions can coexist in the same patient [114]. It is plausible; therefore, that abnormal regulation of the mechanisms of normal aging may contribute to the pathobiology of both COPD and IPF [114, 115].

Our understanding of the biology of the aging lung has advanced remarkably, although the molecular mechanisms linking aging to IPF or to COPD, remain unclear. Cellular senescence, oxidative stress, abnormal shortening of telomeres, apoptosis,

and epigenetic changes affecting gene expression have been proposed to contribute to the aging process and aging-associated diseases which are very similar to the mechanism implicated on the pathophysiology of IPF. Animal studies also support the link between aging and susceptibility to fibrosis by demonstrating an increased vulnerability of the aged lung to injury. For example, bleomycin-induced lung injury, a commonly used model in rodents, or lung infection with murine gamma herpes virus 68 cause severe progressive pulmonary fibrosis only in naturally aged wild type mice when compared to young mice [116–119]. These observations, in some way, challenge the traditional concept that progressive fibrosis is the result of chronic injury.

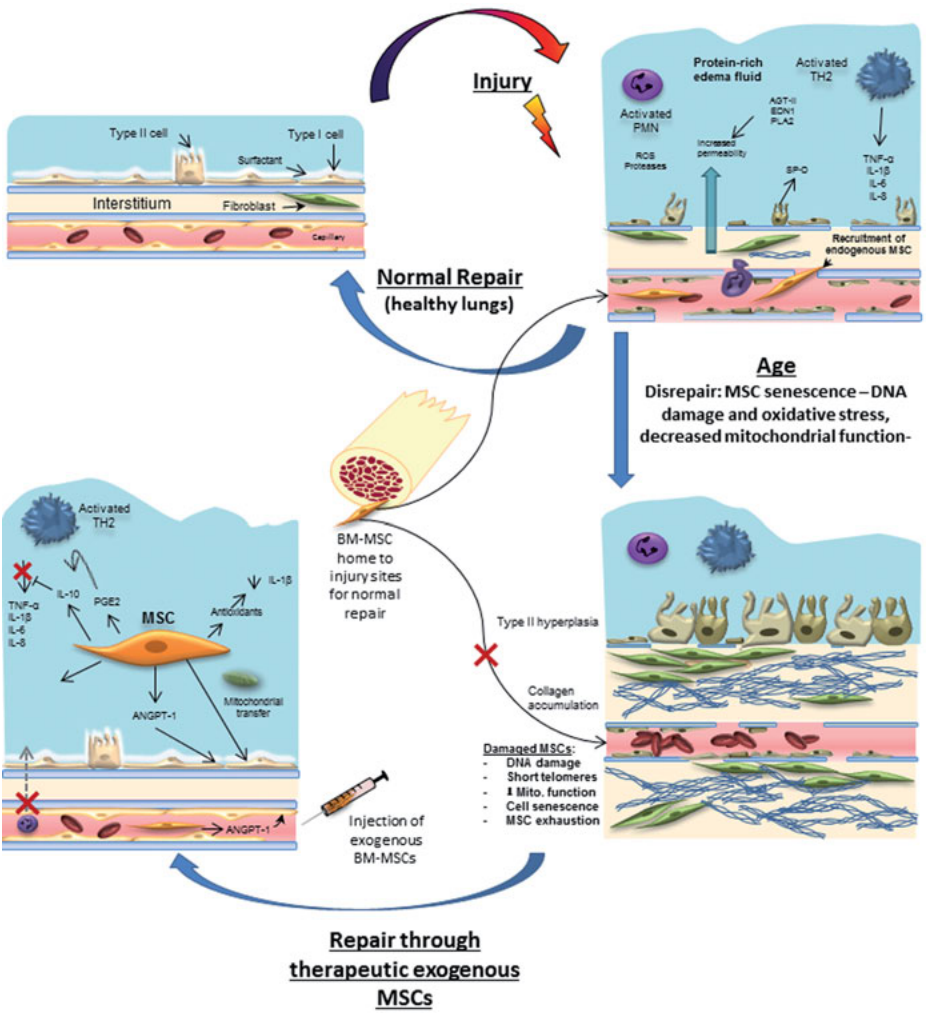

Fig.12.2: Potential effects of aging on MSCs repair of injured lungs.

What chronic injury leads to is an accelerated aging by shorting telomeres, increasing DNA damage diminishing mitochondria function of the stem cells with a consequent depletion and inability to repair. At this point, a single injury will result in progressive fibrosis. While the cellular and molecular mechanisms of physiological aging in the lung are still not well-understood [120–123], oxidative stress, cellular and immunosenescence, as well as changes in a number of anti-aging molecules and in the extracellular matrix are thought to be key mechanisms for the increased susceptibility to lung fibrosis. (Fig. 12.2) In particular, oxidative stress, resulting from a systemic imbalance among the lung's antioxidant systems (*e.g.*, superoxide dismutases, glutathione) and reactive oxygen species (ROS) results in the generation of excess free radicals that overwhelm cellular antioxidant defenses. For example, generation of reactive oxygen species have been suggested to be linked to increased cellular oxidative stress and apoptosis of alveolar epithelial cells [124]. Moreover, there is evidence suggesting that ROS increase the release of TGF-β from alveolar epithelial cells and can directly activate TGF-β in cell-free systems by disrupting its interaction with latency-associated peptide (LAP) [124.]. However, whether excessive oxidative stress associated with aging increases the risk of developing IPF, or the oxidative stress occurring in IPF is simply the result of the pathophysiology of the disease, is unknown [125].

12.3 MSCs and animal models of fibrotic lung disorders

Animal models for lung fibrosis have been difficult to develop and there is no one model that mimics the clinical presentation of IPF [126]. One commonly utilized model in mice and other animals is administration of the chemotherapeutic agent bleomycin, most commonly by intratracheal instillation but also by either systemic or intraperitoneal route [127]. Bleomycin can cause pulmonary fibrosis in patients [128, 129] and when administered to mice, pathogenesis occurs in 3 stages. An initial cytotoxicity leads to apoptosis and necrosis of the alveolar epithelial cells followed by an inflammatory phase characterized by infiltration of neutrophils and macrophages and elevation of proinflammatory cytokines including IL-1β and TNF-α in the lung microenvironment which peaks at day 7. Levels of the profibrotic cytokine TGF-β are also increased and contribute to an aberrant repair and remodeling process resulting in enhanced deposition of collagen and other extracellular matrix (ECM) proteins beginning at 1–2 weeks. The fibrosis together with impaired re-epithelialization of the alveolar wall is a hallmark of the fibrotic process. However, if mice survive the initial inflammatory and subsequent fibrotic periods, the fibrosis will gradually resolve over several months. As such, while not completely mimicking clinical IPF, nonetheless, valuable information on the pathogenesis of lung fibrosis has been obtained using this model.

A number of studies have investigated anti-inflammatory and antifibrotic effects of syngeneic MSCs administration in mouse, rat, or pig models of bleomycin-induced

Table 12.1: Preclinical studies of MSC in models of lung fibrosis.

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls
Pulmonary Fibrosis							
Ortiz 2003 [58]	Mouse bleomycin IV MSCs immediately or 7 days after bleomycin	Mouse BM Plastic adherent CD11b, 34, 45-depleted	No	Syn	– Decreased histologic and inflammatory injury – Decreased hydroxyproline, MMP2, MMP9 content	None specified (soluble mediators)	No
Rojas 2005 [59]	Mouse bleomycin IV MSCs immediately after bleomycin	Mouse BM Plastic adherent CD11b, CD45-depleted	No	Syn	– Increased osteopontin – Decreased histologic injury and lung fibrosis – Decreased inflammatory cytokines	None specified (soluble mediators)	No
Ortiz 2007 [60]	Mouse bleomycin IV MSCs immediately after bleomycin	Mouse BM Plastic adherent CD11b, 34, 45-depleted	No	Syn	– Decreased systemic inflammation (serum IL1β, IFNγ, IL-6, MIP1α, KC	Release of IL-1RA	No

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls
Zhao 2008 [61]	Rat bleomycin IV MSCs 12 hrs after bleomycin	Rat BM plastic adherent mononuclear cells isolated from Percoll gradient of total BM	No	Syn	– Decreased histologic injury – Decreased hydroxyproline, laminin, hyaluronan – Decreased TGF-β, PDFG-A, PDGF-B, IGF mRNA	None specified (soluble mediators)	No
Aguilar 2009 [62]	Mouse bleomycin IV MSCs 8 hrs and 3 days after bleomycin	Mouse BM Texas (Tulane) MSCs Core Mouse HSCs	Lentivirus transduced to express KGF	Syn	– MSCs: Decreased lung collagen mRNA and protein, no change in histologic injury – HSCs: decreased collagen, histo injury, aSMA, TNFa, CCL-2, CCL-9 – HSCs: increased type 2 cell proliferation	KGF secretion	No
Kumamoto 2009 [63]	Mouse bleomycin IV MSCs 3 days after bleomycin	Mouse BM Plastic adherent for 2 hours or 9 days CD11b, 31, 45-depleted	No	Syn	– Decreased histologic injury and hydroxyproline – Decreased inflammatory cells	None specified (soluble mediators)	No

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls
Moodley 2009 [64]	SCID mouse bleomycin IV MSCs 24 hrs after bleomycin	Human umbilical cord (Wharton's jelly) Plastic adherent	No	Xeno	– Decreased histologic injury – Decreased hydroxyproline, type 1 collagen mRNA – Increased MMP2, decreased TIMP-2	None specified (soluble mediators)	Primary human lung fibroblasts – No effect on bleomycin induced collagen mRNA or MMP-2 expression – other endpoints not clarified
Cargnoni 2010 [65]	Mouse bleomycin IV, IP, or IT cells 15 min after bleomycin	BALB/c mouse placenta	No	Allo and Xeno	– Improved body weight (allo and xeno, IP and IT) – Decreased histologic fibrosis at D14 but not D9 (allo IV, IP, IT)	None specified (soluble mediators)	Allogeneic Mouse BCF fetal membrane-derived cells – No effects on lung inflammation at 9 or 14 days (IP or IT)

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls
		Human term placenta amnion and chorion-derived stromal cells human term placenta-derived epithelial cells used as a mix of 50 % hAMCS + hCMSCs and 50 % hAECs	No	Allo and Xeno	– No change in lung inflammation at D9 or 14 (allo IV) – Decreased tissue neutrophils on D14 (allo IP, IT) – No change in lung inflammation D3,7,9,14 (Xeno IP,IT) – Decreased severity of histologic injury at D14 (Xeno IP, IT) – Decreased tissue neutrophils on D14 (Xeno IP but not IT) – Increased lung inflammation at D14 in control mice (Xeno IP, IT)	None specified (soluble mediators)	– Decreased lung fibrosis at D14 (IP or IT) – Stimulated inflammation in PBS control mice at D9 IIP and IT) and D14 (IT)

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls
Lee 2010 [67]	Rat bleomycin IV MSCs 4 days after bleomycin	Rat BM Plastic adherent	No	Syn	– Decreased neutrophilic lung inflammation – Decreased BALF proinflammatory cytokines, nitrite, nitrate – Decreased collagen accumulation	None specified (soluble mediators)	No
Cabral 2011 [66]	Pig IT bleo BMDCs IV on D90	BM mononuclear cells	No	Syn	– Euthenasia 180 days – HRCT dec fibrosis – No change in histology	None	No
Saito 2011 [68]	Mouse IT Bleo IV MSCs 24 hr after bleo	Mouse BM	Lentiviral transduction to express CCL2 inhibitor (7ND)	Syn	– Improved survival – Dec histologic injury – Dec lung collagen – Dec BAL inflammatory cells – Dec BAL IL-1β, IL-6 – 7ND-expressing MSCs more effective than MSCs	Soluble mediator activating CCL2 pathways	No

lung fibrosis [58–68] (summarized in Tab. 12.1). Notably, MSC administration by either systemic or intratracheal route during the acute bleomycin-induced inflammatory stage ameliorate the acute inflammation and are protective against subsequent development of fibrotic changes. These effects occur in the absence of what appears to be any substantive engraftment of the MSCs in the lung and available data so far implicates release of soluble anti-inflammatory mediators as contributing to the MSCs effects. Mediators suggested as playing roles include IL-1 receptor antagonist (IL1RN) and KGF [60, 62]. Comparable effects have also been observed following administration of allogeneic or xenogeneic MSCs (human) following bleomycin-induced acute lung inflammation in immunodeficient SCID mice [65].

However, while MSCs have been shown consistently to attenuate inflammation in numerous experimental models of injury, it is important to recognize that the time window is a critical factor in optimizing the protective effect of MSC transplantation. Administration of MSCs or a population of bone marrow-derived mononuclear cells that contained MSCs at time intervals longer than 7 days after bleomycin administration had no effect on established fibrotic changes in either mouse or pig lungs [58]. Further, using a different model of lung fibrosis induced by radiation exposure in rodents, MSCs administration at time points at which established fibrotic changes were present, were detected in the interstitium as myofibroblasts suggesting that fibroblastic differentiation of MSCs occurred in response to mediators produced in the injured tissue [130, 131]. These data suggest that MSCs administration in the setting of an established or ongoing fibrotic response may worsen the disease process and augment scarring in injured tissue rather than reversing it. Analogous data demonstrates that systemically administered MSCs can contribute to the connective stromal tissue in breast and ovarian cancers [132–139]. As such, available data only supports a potential ameliorating effect of MSC administration in fibrotic lung diseases if administered early in the disease course during active inflammation. At present, there is no data to support an ameliorating effect of MSCs on established lung fibrosis. Thus, careful consideration must be given to clinical investigations of MSCs in fibrotic lung diseases.

To obtain additional insights into the mechanisms by which MSCs might confer protection against bleomycin-induced lung injury, we felt that a thorough characterization of the local and systemic response to MSCs infusion would be necessary. We studied this by administering bleomycin to busulfan treated mice to induce a myelosuppression, and to mice with a normal, intact bone marrow. A subgroup of mice within each group received an additional infusion of GFP^+ MSCs 6 h after bleomycin-treatment. Our results showed that the MSC infusion conferred a substantial survival benefit in myelosuppressed bleomycin-treated mice. Morphometric analysis of the lung at day 14 revealed that the MSCs infusion protected against bleomycin-induced lung injury. Engraftment of MSCs in the lung was quantified at day 14, and the intensity of GFP staining in the lung was greatest in myelosuppressed animals that received MSCs compared to mice that had an intact bone marrow. We found mRNA levels of Th1 cytokines (IL-2, IL-1β, IFN-γ) were significantly decreased in the lung 14 days after

bleomycin, and IL-4 expression was also upregulated. MSC infusion also increased circulating levels of G-CSF and GM-CSF at day 14.

To determine the most appropriated MSCs to be used as a possible therapy for chronic diseases, age of the MSCs is an important element to consider. MSCs from elderly people have different morphology, increased production of ROS and oxidative damage [140], DNA-methylation changes affecting cell differentiation [141], slower proliferation rate in culture [142, 143], shorter telomeres [143] and a large proportion stain positive for senescence-associated beta-galactosidase [144]. Aging mice are characterized by a senescence-related increase in fibrocyte mobilization (and a parallel decrease in MSCs) and higher serum levels of CXCL12. Conboy and colleagues showed that an old mouse with declining organ stem cell capacity had a rejuvenation of aged muscle and liver progenitor cells when it was surgically joined with a young mouse in order to share circulatory systems [145]. Several studies since have demonstrated that both physiological aging and pathologic senescence can affect these functions. In a rat model for cardiomyopathy, human MSCs from aged donors did not perform as well as the ones from young donors [146]. MSCs from old donors fail to differentiate *in vitro* into neuroectodermal cells [147]. In one case, the administration of stem cells from young mice restored cardiac angiogenesis in senescent mice when stem cells from old mice failed to do so [148]. The superiority of very young B-MSCs can be explained by several aspects of their biology [149]. Briefly, MSCs of fetal origin express the pluripotency stem cell markers (Oct-4, Nanog, Rex-1, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81), have longer telomeres and greater telomerase activity and express more human telomerase reverse transcriptase. Fetal MSCs were also more readily expandable and senesced later in culture than their adult counterparts [149, 150].

MSCs from old mice are further characterized by a quiescent state with low metabolic activity and are primarily in the G0 phase of the cell cycle. This quiescent state is maintained by both extrinsic and intrinsic mechanisms and has been postulated to be a way of preserving their long-term proliferative potential and genomic integrity. The problem that arises is that quiescent MSCs escape DNA damage checkpoints and several repair pathways that are cell cycle dependent and as a result the accumulation of DNA damage during aging, ultimately leading to rapid stem cell depletion or exhaustion. DNA repair capacity reduces with aging [151–156].

We have demonstrated the importance of the integrity of the genome of B-MSCs in protecting against bleomycin-induced fibrosis and modifying the lifespan of mice. Using parabiosis, pairing young WT with an Ercc1-/Δ deficient mouse that has diminished ability to repair DNA breaks, results in a decrease on lung fibrosis and more than doubling the lifespan of Ercc1-/Δ mice in direct correlation with maintenance of body weight. Recently, our observation was confirmed in separate studies using Ercc1 KO mice which demonstrated that lifespan of mice lacking Ercc1 increases only by the infusion of young WT B-MSCs. Lifespan was not changed by infusion of natural old B-MSCs, or murine embryonic fibroblasts as control. These results suggest that B-MSCs can have a direct effect on the lifespan of mice [157].

The extracellular matrix (ECM) provides structural support by serving as a scaffold for cells, and as such the ECM maintains normal tissue homeostasis and mediates the repair response following injury. Tension applied through collagen fibrils at the ECM-cell interface might lead to protein synthesis, cell mitotic activity and changes in gene expression via activation of MAPK phospho-relay systems [158, 159]. Collagen and elastin, the main proteins of ECM, form the scaffold of the alveolar structure and determine the mechanical properties of lung parenchyma. Collagen represents 15–20 % of the total dry weight of the pulmonary tissue with type I and type III collagen adding up to 90 % of the total amount. Another protein, fibronectin, forms fibrils that are connected to other matrix components and has been implicated in cell adhesion, migration, epithelial–mesenchymal transition, phagocytosis, and cell growth.

The composition of ECM changes during aging and these alterations undoubtedly might contribute to the determination of the fate of MSCs. In general in the aging connective tissue, collagen type I content of ECM is increased whereas collagen type III content is decreased along with elastin fibers. The proteoglycan content also appears to decrease with age. The exact mechanism of how the age-dependent changes in ECM components affect lung repair is still unclear, although it is known that fibronectin expression increases in clinical and experimental models of fibrosis [117, 121, 160–162], which in turn affects the morphology of MSCs and increases the expression of α-SMA on MSCs suggesting a possible association with the disrepair process.

Taken together, these observations indicate that aging leads to changes in the expression of TGF-β and extracellular matrix composition that might have important implications in the lung repair process by driving the phenotype of the cells which are interacting, including MSCs [163].

12.4 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD), including chronic bronchitis and emphysema, is the third leading cause of death in the United States, resulting in over 126,000 or one in every 20 deaths in 2005 and affects millions worldwide [164]. Further, COPD is increasing in prevalence, and actuarial projections suggest that COPD will be the third leading cause of death worldwide by the year 2020 [165, 166]. COPD also has significant economic impact in health care expenditure and in illness-related decreased productivity. The pathology of COPD is heterogeneous and can include both destructive emphysematous changes and thickened bronchiolar walls with variable luminal mucus occlusion. Disease pathogenesis also includes both chronic pulmonary and systemic inflammation. Thus, potential anti-inflammatory effects of systemically administered MSCs may decrease inflammation resulting in improved lung function and quality of life.

Currently utilized preclinical models of COPD include both intratracheal instillation of destructive enzymes such as papain or elastase or exposure to cigarette

smoke (reviewed in [167]). Notably, while these models can produce acute inflammation (elastase, papain) that resolves after several days or chronic on-going smoldering inflammation (cigarette smoke exposure), it takes several weeks or even months, notably with cigarette smoke exposure, to develop characteristic emphysematous changes in the lung. Several transgenic mouse models are also available in which specific gene deletions or overexpression can lead to emphysematous changes in lung parenchyma either manifest at birth or that develop over time [168, 169]. While none of these fully mimic the pathologic and temporal aspects of COPD in humans, they nonetheless provide useful information.

Investigations of MSC-based cell therapy in several animal models of COPD have suggested potential efficacy. The majority of these have utilized intratracheal elastase or papain instillation in which syngeneic MSCs of bone marrow, adipose, or lung origin have been administered at times ranging from 2 hours to 2 weeks after injury [43, 45–49] (summarized in Tab. 12.2). In each of these cases, MSC administration resulted in improved histologic changes and decreased inflammation. While engraftment of the MSCs as type 2 alveolar epithelial cells was proposed as a potential mechanism of MSC action [46], most subsequent studies have not found any significant engraftment and release of soluble mediators such as hepatocyte growth factor (HGF), EGF, or VEGF has been proposed to mediate MSC actions [43, 44, 47, 49]. Two recent studies have found parallel protective effects of MSCs following repeated sequential administration in mice or rats previously exposed to cigarette smoke or cigarette smoke extract [50, 52]. These latter two studies are particularly informative as the MSCs were given well into the course of the injury and during exposure. MSCs have also been investigated for the ability to provide a stroma for tissue repair [51] and also for repair of tissue planes following experimental lung volume reduction surgery in rats [44].

These promising results need to be more fully explored but provide a basis for considering clinical investigation of MSCs in patients with COPD. The ostensible goal is to reduce chronic pulmonary and systemic inflammation and improve lung function and/or quality of life. Slowing of disease progression is also suggested by the available preclinical data. Notably, the first ever multicenter, double-blind, placebo-controlled Phase II trial of PROCHYMAL^{TM} (Osiris Therapeutics Inc., Columbia MD) for patients with moderate-severe COPD (FEV1/FVC <0.70, 30 % < FEV1 < 70 %) has been completed [101]. The trial involved 62 patients in 6 participating US sites. The primary and secondary goals were, respectively, to determine safety of MSC infusions in patients with lung disease and to assess the potential efficacy of MSCs for decreasing the chronic inflammation and associated symptoms. Importantly, the trial demonstrated safety in an older population of significantly affected COPD patients with a number of co-morbidities. No infusional toxicity or clinical suggestion of significant microemboli was observed in multiple infusions of either study drug or of vehicle control (4 infusions per patient for a total of 248 infusions). No serious adverse events attributable to the infusions were observed over a subsequent two year follow-up and no serious attributable changes were observed in a range of safety assessments.

Table 12.2: Preclinical studies of MSCs in COPD.

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls? COPD
Shigemura 2006a [43]	Rat elastase following lung volume reduction surgery Direct topical application of ASC-seeded polyglycolic acid at time of surgery	Rat adipose Plastic adherent	No	Syn	– Improved histologic repair	HGF secretion	No
Shigemura 2006b [44]	Rat elastase IV ASCs 1 wk after elastase	Rat adipose Plastic adherent	No	Syn	– Decreased apoptosis, improved histologic repair – Improved gas exchange and exercise tolerance	HGF secretion	No
Yuhgetsu 2006 [45]	Rabbit elastase IT MSCs 24 after elastase	Rat BM mono-nuclear cells from Ficoll gradient of total BM	No	Syn	– Improved histology and lung function – Decreased BALF fluid inflammation – Decreased tissue MMP2 and MMP9 expression	None specified (soluble mediators)	No
Zhen 2008 [46]	Rat papain +/– 7.5 Gy TBI IV MSCs after papain (timing not specified) P2–P3 4 x 106 MSCs/rat Male MSCs into female rats	Rat BM Plastic adherent mono-nucelar cells from Percoll gradient	No	Syn	– Decreased histologic injury – Decreased alveolar cell apoptosis	Engraftment as type 2 alveolar epithelial cells	No

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls? COPD
Katsha 2011 [49]	Mouse IT elastase IT MSCs 14 days after elastase	Mouse BM Plastic adherent	No	Syn	– Decreased histologic injury – Decreased IL1β – Transient increase in lung EGF, HGF, and secretory leukocyte protein inhibitor	None clarified (soluble mediators)	No
Zhen 2010 [47]	Rat papain IV MSCs 2 hr after papain	Rat BM Plastic adherent mononucelar cells from Percoll gradient	No	Syn	– Decreased histologic injury – Partial restoration of VEGF expression in lung homogenates	TNF-α-stimulated VEGF secretion by MSCs	No
Hoffman 2011 [48]	Mouse IT elastase	Mouse BM (Tulane) Lung MSCs from primary explant cultures Flow characterization? Adipo/chondro/osteo	No	Syn	– Higher retention of L-MSCs in lung – Dec histo injury (MLI) at 22-28 days	Non-postulated	No
Katsha 2011 [49]	Mouse IT elastase IT MSCs 14 days after injury 5 x 10E5 cells/mouse Passage 5	Mouse BM MSCs Pos CD 73, 90, 105 Neg CD 11b, 45 Adipo/osteo	si EGF treated MSCs	Syn	– Dec histo injury at days 7,14,21 after MSCs – Dec BAL IL-1b at days3 and 5 after MSCs – Dec IL-1b mRNA in lung homgenates at days 1,3,5,7 after MSCs – Inc EGF, HGF, and SLPI mRNA at various time points after MSCs	EGF production by MSCs induces SLPI in cultured MLE-12 cells	BLKCL4 lung fibroblasts – Don't mimic effects

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls? COPD
Schweitzer 2011 [50]	Mouse cigarette smoke for 4 months IV ASCs every other week for 3rd and 4th months of CS exposure 5 x 10E5 cell/infusion Passage <3	Human or mouse adipose plastic adherent	No	Syn or Xenogeneic	– Decreased histologic injury and caspase activation – Decreased BALF inflammation – Decreased CS-induced MAPK signal transuction – Decreased weight loss – Decreased BM suppression – Dec lung homogenate caspase 3, p38	None specified	No
	NOD-SCID mouse (for xenogeneic) human ASCs 3 days after VEGFR blockade	Human or mouse adipose plastic adherent	No	Syn or Xeno	– Human ASCs decreased histologic injury and caspase 3 activation following VEGFR blockade – Emboli if used > 5x 105 MSCs or if >passage 3 – hASC CM inc wound repair in cultured lung endothelial cells dec with water soluble C extract	None specified	No
		– MAPK phos, JNK, AKT – Dec CS infused BM function – Dec VEFGR blockade-induced air space enlargement					

Injury Model	Experimental model, route and timing of treatment	MSCs Source	MSCs Modified?	Syn Allo or Xeno	Outcome compared to injury effects	Potential Mechanisms of MSCs actions	Cell Controls? COPD
Ingenito 2012 [51]	Sheep IT elastase 5 doses over 20 wks L-MSCs in fibinogen/fibrin/ poly-L-lysine scaffolds 5–10 x 10E6 cells/scaffold Passage 5	Sheep lung-derived MSCs pos S100A4 neg fibrillin 1, CD45, aSMA, col I adipo/ chrondo/osteo	No	Syn	– inc lung tissue mass and perfusion – inc histo cellularity, cell retention, ECM – improved lung mechanics	Paracrine effects Inc epithelial proliferation in in vitro co-culture expts	No
Kim 2012 [52]	Mouse lung fibroblasts In vitro CSE exposure Rats In vivo cigarette smoke 8-wk rat, 5d/wk, 6 months IV MSCs or CM 2x week IV for 5 weeks starting at week 8 Euthanasia 3 weeks later	Rat BM Plastic adherent CM P3-P5 From 90 % confluent cells	Yes (ISCT)	Xeno Syn	– In Vitro CSE Exposure – Dec in CSE induced capsase 3, p53, p21, p27, Akt, p-Akt expression – Inc in CSE-induced dec'd ECM expression and collagen gel contraction – Dec in CSE-induced COX-2 and PGE synthase 2 expression – PI3K inhibitor partially reversed the effects – In vivo CS Exposure restored fibroblast proliferation, inc Akt	FGF2 release by MSCs	Conditioned media from RF2-6 cells, HFASMCS, NHLFs – Didn't mimic effects

However, no improvement in efficacy outcomes including pulmonary functions, 6 minute walk evaluation, quality of life questionnaires, or physician's global assessment were observed. Notably, a significant decrease in the circulating C-reactive protein (CRP), commonly elevated in COPD patients, was observed for the initial study period in MSCs treated vs. vehicle-treated patients. While losing significance over time, this trend continued for the duration of the 2 year observation period. Thus, while failing to demonstrate efficacy, this trial importantly provides a firm basis for safety of MSC use in patients with COPD and other chronic lung diseases and also provides a potential mechanistic clue of *in vivo* MSC effects. Further larger-scale trials will be necessary to more fully examine potential effects of MSCs on these and other clinical assessments in this patient population.

Other factors may have influenced potential MSC efficacy in the COPD trial. For example, the dosing and treatment schedules utilized were empirically based on data from MSC trials in other diseases and may not be effective in chronic lung diseases. Further, MSCs may not be effective in decreasing the full spectrum of pathophysiology that contributes to the clinical manifestations of COPD, including chronic progressive structural tissue damage. As more is learned about the effects and mechanisms of MSC actions, disease choice becomes more important. Chronic persistent lung diseases with low level or smoldering inflammation, such as COPD or IPF, may not be the best therapeutic targets for MSCs intervention [5]. More acute diseases of lung inflammation, for example the acute respiratory distress syndrome (ARDS) or bronchopulmonary dysplasia, may be more amenable to the intense, short-lived, anti-inflammatory effects of administered MSCs [6–8]. Similarly, chronic immune-based inflammatory lung diseases, such as severe steroid-refractory asthma, may also be better targets [25, 27]. To this end, clinical trials of MSCs for ARDS and for septic shock are currently in development in the United States and in Canada, respectively. These demonstrate growing efforts towards carefully conducted closely regulated clinical trials of cell therapies for lung diseases in Europe, Brazil, and Australia as well as the United States and Canada. Nonetheless, given the substantial human and economic burdens of COPD and IPF and the compelling need for new therapies, further investigations of MSC therapies are warranted.

12.5 Conclusions and future directions

Cell therapy approaches for lung diseases including COPD and IPF continue to evolve at a rapid pace. Mesenchymal stem cells have actions that might be beneficial in both diseases. However, MSC have different mechanisms to ameliorate inflammation and injury in different lung diseases pathogeneses. As such, further understanding of the range of MSC actions in IPF and COPD must be better understood in order to continue to develop rational approaches for clinical investigations. Nonetheless, cell-based therapies offer potential hope for these two devastating and incurable diseases. Other

factors inherent in these diseases, notably aging, also may have significant impact on the use of MSCs for cell therapy approaches.

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Christopher D. Porada and Graça Almeida-Porada

13 Mesenchymal stem cells as therapeutics for liver repair and regeneration

Abstract Given the shortage of available donors and the ever-increasing number of patients with late-stage liver disease/failure, new approaches for repairing the liver that can eliminate the need for transplanting a partial or complete human liver to cure the patient are urgently needed. The use of cell therapy as a means of repairing/repopulating the liver has several advantages over whole organ transplantation, but requires a donor cell type that can fulfill a fairly extensive list of criteria. Mesenchymal stem cells (MSCs) are readily available and easy to isolate, have the ability to be expanded extensively *in vitro* to generate clinically relevant cell numbers, and appear to harbor the ability to give rise to hepatocyte-like cells. In addition, MSCs secrete a myriad of factors that can stimulate the regeneration of endogenous parenchymal cells, induce fibrous matrix degradation, and modulate inflammation/immunity. This chapter highlights some of the promising preclinical studies that have led us and others to conclude that MSCs represent one of the most promising cells for repairing/regenerating the diseased liver, and presents the early results of several clinical trials exploring the potential of these multifaceted cellular therapeutics.

13.1 Introduction

The liver of animals and human is unique among organs in that it has the ability to intrinsically regulate its own growth to maintain a constant mass relative to the organism's body weight [1–3]. Under normal conditions, and even following such traumatic injury as 2/3 hepatectomy, the entire liver is regenerated solely through proliferation of liver-resident hepatocytes, which appear to only need to undergo 1 or 2 divisions to achieve complete restoration of the liver's mass and functionality [1–6]. Despite this remarkable intrinsic capacity for self-repair/regeneration, however, a wide variety of diseases, including cirrhosis, unresectable hepatic malignancy, ischemia, metabolic/hereditary and autoimmune disorders, and viral- or drug/toxin-induced hepatitis, can disrupt the ability of the liver to adequately repair itself, resulting in encephalopathy, coagulopathy, functional renal failure, and metabolic alterations, which collectively lead to a life-threatening situation for which liver transplantation is the only definitive therapy [7–10].

Given the current marked inadequacy of available livers for transplant, thousands of patients are awaiting liver transplantation. This shortage has led to the emergence of potential guiding principles in determining organ allocation priorities, and to the development of techniques for either reducing the size or for splitting liver grafts. Moreover, in order to increase the donor supply, adult-to-child, or adult-to-adult living

donor liver transplantation was introduced into clinical practice [11]. This practice now represents a significant proportion of the liver transplants performed each year, and its use has helped to combat, to some degree, the shortage of donor organs. Sadly, despite these efforts to maximize the use of potential donor organs, the increasing demand for organs still greatly exceeds the available supply. Currently, over 16,000 people in the United States are on the waiting list to receive a life-saving liver transplant, yet only about 6000 transplantations are performed annually. Combining this shortage of donor livers with the nearly 9000 new cases that are added to the list each year, the sobering reality is that roughly 1400 people will die each year, in the US alone, awaiting a liver transplant [12]. Furthermore, even when a patient is fortunate enough to find a compatible donor and receive a liver transplant, several factors can still thwart the ultimate success of this procedure, such as operative damage, immune rejection towards the new organ, relapse of the pre-existing liver disease, and lifelong side effects due to immunosuppression [13, 14]. Moreover, after liver transplantation, several long-term morbidities can arise, such as cardiovascular and retinal complications, lymphoproliferative disorders, and chronic renal failure [14–16]. There is thus an urgent need to develop new approaches for repairing the liver that can eliminate the need for transplanting a partial or complete human liver to cure the patient.

13.2 Cell therapy for liver disease

The transplantation of cells rather than an entire or partial liver as a means of repairing/repopulating the liver [1, 3, 6, 17–25] would have several inherent advantages. The first of these is the far less invasive nature of transplanting cells as opposed to replacing an entire organ. This would result in significantly less morbidity and far lower cost to the patient. Secondly, it is reasonable to presume that suspensions of an individual cell type may appear less immunogenic to the recipient's immune system than an intact solid organ, since solid organs contain a wide variety of cells, many of which are immunogenic and could thus contribute to and/or trigger an immune response and rejection of the transplanted organ [5]. Another advantage to using cells rather than organ transplant is that, in some instances, when time allows and no underlying genetic disease in present, autologously-derived cells could be employed, eliminating the need for costly immunosuppression and its associated high morbidity. In addition, the cells to be transplanted could be expanded *in vitro* to ensure an optimal number of cells. Even in the case of an underlying genetic disease, the patient's own cells could be manipulated *ex vivo* to correct the defect using gene transfer [26]. An additional advantage of working with cells rather than an intact organ is that a predetermined mixture of cells of a specific lineage at different stages of differentiation could be prepared and transplanted to ensure that rapid, perhaps short-term, engraftment would be obtained to quickly supply the patient with the requisite hepatic function. At the same time, more primitive stem/progenitor cells of the same lineage could be transplanted, thus

providing a source of long-term engrafting cells that would ideally mediate lifelong correction of the patient. This ability to transplant multiple cells at varying stages of development/differentiation could ultimately circumvent one of the major inherent difficulties with using cellular therapy to treat liver disease, namely, the fact that the patient needs to constantly have a certain critical mass of functioning hepatocytes to maintain the basic metabolic requirements for the patient's survival [1–3, 5].

13.3 The ideal cell for liver regeneration

Hepatocytes are responsible for carrying out many of the vital functions attributed to the liver, including converting food into energy in the form of glycogen, filtering waste products from the blood, metabolizing drugs, and removing/inactivating toxins. Their ability to perform all of these functions, coupled with the fact that they comprise the majority of the liver's mass, would appear to make hepatocytes a logical choice for cellular therapy within the liver in place of organ transplantation. Unfortunately, the ability to harvest human hepatocytes for transplant is limited by the same problem as clinical liver transplantation, namely that the number of people in need of a transplant will always far exceed the number of available donor organs. In addition, human hepatocytes are large and fragile, making them difficult to isolate to high purity without inducing damage [27, 28]. Moreover, differentiated hepatocytes cannot be efficiently expanded in culture [29], making it unlikely that sufficient numbers of hepatocytes will be available for repopulating the liver of an adult human patient. Further confounding the situation is the repeated observation that, for reasons that are not well understood, in most instances, only a small percentage of hepatocytes actually durably engraft within the liver following transplantation [30–35].

Hepatic stem/progenitor cells (HpSCs) could represent an alternative to the use of hepatocytes, given their intrinsic ability to extensively expand, differentiate into all mature liver cells, and reconstitute the liver when transplanted, with minimal immunogenicity [36–38]. Indeed, the potential of these cells has been demonstrated in mice, and studies in humans have confirmed their presence and regenerative ability in the setting of hepatitis, cirrhosis or inborn metabolic disorders [39–41]. Still, the ability to identify cells derived from nonhepatic sources that could be used as cell therapeutics would open new avenues in the treatment of liver disease.

The first property of a cell that would be ideally suited for liver repopulation would likely be its ready availability and ease of isolation. The ultimate cell to be used for liver regeneration would be one which could be readily harvested from an adult or, ideally, from the patient to be treated, in a minimally invasive fashion. In addition to ease of isolation, an ideal cell for liver regeneration should have the ability to be easily expanded *in vitro*, ensuring that adequate numbers of cells could be generated to mediate repopulation of a significant portion of the recipient's liver. The ability to be expanded *in vitro* would also allow that the tissue sample to be collected from the patient/donor could

be fairly small, and yet still provide adequate cell numbers for transplant, thus reducing the invasiveness and the risks of the cell collection procedure. Moreover, the ability to expand the cells *in vitro* would allow the use of genetic modification, if needed, to permanently correct any underlying genetic defect within the patient's cells. The cells could then subsequently be selected *in vitro* and further expanded to ensure that all cells to be transplanted had been genetically corrected and would thus be therapeutic.

Looking beyond characteristics that facilitate isolation and *in vitro* manipulation, cells to be used for liver regeneration would need to have several biological characteristics to ensure that they were capable of restoring liver function following transplantation. The first of these is the ability to give rise *in vivo* to fully functional hepatocytes and to carry out all the myriad of the hepatocyte's tasks, which frequently change as a function of hepatocyte developmental/differentiation state [21]. The cells also need to rapidly and efficiently home to the liver upon infusion, and engraft at high levels within the damaged regions of the recipient's liver, integrating into the native cytoarchitecture, and thus ensuring help quickly arrives at the locations that need it most.

Perhaps one of the most challenging characteristics that will need to be met in an ideal cell type for liver regeneration is that once the transplanted cells have engrafted, they need to compete effectively over time with the host's endogenous pool of hepatocytes [5, 21] to ensure that a meaningful fraction of the liver's mass is gradually replaced with healthy, curative donor-derived hepatocytes. Unfortunately, in most injuries and metabolic disorders, with the exception of fumarylacetoacetate hydrolase (FAH) deficiency (a model of hereditary tyrosinemia type I) [42, 43], the transplanted donor cells have no real advantage over the host's own hepatocytes, which are also stimulated to divide by injury/disease. As a result, the majority of liver repopulation is mediated by the endogenous hepatocyte pool, resulting in very low levels of engraftment [30–35, 44, 45]. Very recent work [46] has now demonstrated that it is possible to pharmacologically induce a state of FAH deficiency in normal mice. By engineering transplanted cells to lack homogentistic acid dioxygenase (HGD), they could be rendered resistant to FAH inhibition, and thus given a selective growth advantage upon transplantation. This approach proved safe/nontoxic in mice, and resulted in an impressive 20–100-fold increase in the levels of donor-derived hepatocytes at 4–6 weeks after transplant, suggesting it holds great promise for enhancing donor-derived liver repopulation in a variety of settings in which the donor cells would not possess a proliferative/survival advantage. An overview summarizing some of the properties an ideal cell for liver regeneration should possess is presented in Figure 13.1.

13.4 Mesenchymal stem cells (MSCs) as cellular therapeutics

The presence within the bone marrow (BM) stromal compartment of a population of unique microenvironmental cells was first suggested by Friedenstein over 30 years ago [47, 48]. It was, however, the pioneering work of Caplan that established the

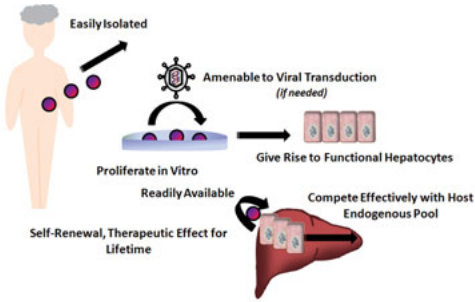

Fig. 13.1: Properties of an ideal cell for liver regeneration.

concept of the mesengenic process, driven by mesenchymal stem cells (MSCs) [49, 50]. Like many rare stem cell populations, MSCs cannot be isolated to absolute purity, although numerous culture methods and purification procedures such as plastic adherence, Ficoll gradient centrifugation, or cell-sorting using surface markers have all been used to enrich for BM-derived MSCs, with each laboratory preferring its own method of isolation. This makes the comparison of results obtained by various laboratories very difficult, since each lab is likely studying somewhat different cell populations despite the fact that all of these cells have collectively been referred to as MSCs. To facilitate comparison of results from different groups and encourage the use of a better-defined population of MSCs, the International Society for Cellular Therapy has stated that MSCs should have several characteristics, aside from simple adherence to plastic. They must express CD105, CD73, and CD90, but not express CD45, CD34, CD14, CD11b, CD79, or CD19 and HLA-DR surface markers. Furthermore, they must be able to differentiate into the various mesenchymal cell types found within the bone marrow, *i.e.* bone, cartilage, and fat [51].

Over the past several years, studies have provided compelling evidence that MSCs' differentiative capacity far exceeds that originally reported by Friedenstein. Indeed, *in vitro* and *in vivo* transplantation studies have now shown that MSCs have the capacity to differentiate not only into mesodermally-derived cell types such as bone [52], cartilage [53], tendon [54], muscle [55], cardiomyocytes [56–61], endothelial cells [62–64] and adipose tissue [65], but, even more remarkably, can also give rise to cells derived from ectoderm (neurons and glia [66–71]) and endoderm (pancreatic beta cells [72–74] and albumin-producing hepatocyte-like cells [75–79]). This extraordinary multipotentiality has generated a great deal of interest in applying MSCs to tissue repair/regeneration as well as cell therapy approaches for a variety of diseases/injuries. Specifically, the findings on hepatocytic potential suggested that MSCs could serve as cells for repairing the injured or failing liver.

Importantly, although MSCs constitute a very small percentage of the nucleated cells present in the BM, between 0.001 and 0.01 %, they can be expanded exponentially while maintaining their original phenotype and differentiative potential [80].

Indeed, Bruder *et al*. demonstrated that human BM-derived MSCs can readily be propagated *in vitro* until passage 38±4 before senescing [81]. Moreover, by plating these cells at a low density and consistently passaging them before they have reached confluence, it is possible to accelerate their growth rate and increase their expansion capacity [82]. This ability to be expanded extensively *in vitro* has two important implications for their use in cellular-based liver therapies. The first of these is that a very small BM aspirate could be taken from the patient and adequate cells obtained for transplantation through extensive expansion *in vitro* following isolation. Secondly, by virtue of their ability to be expanded in culture without loss of *in vivo* potential, MSCs could be harvested from the patient's own marrow even if the liver disease present was the result of an underlying genetic defect, since MSCs can readily be genetically manipulated *in vitro*, with both viral and nonviral vectors [83–87]. Following genetic manipulation, a pure population of genetically corrected autologous MSCs could then be propagated to generate sufficient numbers of cells to achieve meaningful levels of engraftment following transplantation.

In addition to their broad differentiative potential, MSCs also appear to possess the intrinsic ability to home to sites of injury following systemic infusion. Importantly, from the standpoint of developing a clinically viable and safe cell-based therapeutic, MSCs appear to selectively engraft and differentiate into tissue-specific cells that are missing or defective due to the disease in question, while contributing very little, if at all, to normal/healthy tissue [88–91]. For the past several years, scientists have attempted to elucidate the mechanism by which MSCs are selectively attracted to sites of injury. During pathological conditions, several cytokines/chemokines are produced, which will stimulate MSCs to express: (1) integrins, by which MSCs will bind to endothelial cells, and (2) cytokine/chemokine/complement receptors, by which MSCs will migrate towards the inflammatory site. This complex network of signaling allows MSCs to establish cell-cell contact and mediate rolling with endothelial cells. Additionally, they also transmigrate into the extracellular matrix by interacting with integrins and fibronectin stimulated by MSCs-secreted ligands. Despite these insights, more information is required for a complete understanding of this process and harnessing it to enhance MSCs engraftment after transplantation [92].

Although MSCs are still most often isolated from BM, more recently, MSCs have also been identified in, and isolated from, several other tissues including cord blood (CB-MSCs), cord matrix (hWJSCs), amniotic fluid (AF-MSCs), placenta, adipose tissue (AT-MSCs), brain, liver, lung, and kidney [93–95]. The presence of these cells in several organs/tissues raises the possibility that they could have a crucial function in organ homeostasis, and/or repairing the tissue, and suggests that MSCs isolated from these tissues may have a unique transcriptional or proteomic signature that renders these cells biased in terms of homing or differentiation towards the organ of origin. Indeed, differences exist in the cytokine/chemokine molecules produced by MSCs from various sources and in their differentiative capabilities [96, 97]. For example, fetal liver MSCs (FL-MSCs) exhibit much more rapid growth kinetics than BM-MSCs, due,

at least in part, to a greater abundance of transcripts involved in cell cycle regulation, DNA repair and chromatin regulation. In addition, FL-MSCs have longer telomeres, likely due to greater telomerase activity than adult sources of MSCs. As a result, these cells are more expandable and they senesce later in culture [98]. More importantly from the standpoint of clinical utility, FL-MSCs also exhibit reduced immunogenicity and an enhanced ability to inhibit T cell proliferation when compared to BM-MSCs [99], perhaps due to enhanced expression of HLA-G1 [100].

Unfortunately, despite the promise of offering MSCs primed for repair of specific tissues, the inherent difficulty in obtaining organ-specific MSCs such as those derived from liver, will likely preclude their widespread use in a clinical setting. Ideally, for cellular therapies, one would like a readily available source of cells that could be used as off-the-shelf therapeutics. MSCs are present in significant numbers in discardable tissues such as fat, cord blood, placenta and amniotic fluid, and these MSCs have the ability to be expanded and frozen without loss of viability or differentiative potential, making MSCs from these tissues an attractive option.

13.5 MSCs for treating liver disease

13.5.1 In vitro models to study MSCs hepatic differentiation

It could be argued that the best way to study the differentiative potential of MSCs is to perform *in vitro* studies that allow the creation of a carefully controlled microenvironment, enabling the researcher to definitively establish the true multipotential capability of MSCs at the single cell level, or at the level of a clonally-derived population. Accordingly, several protocols have now been developed that demonstrate the ability of both murine and human BM-MSCs to differentiate *in vitro* into hepatocyte-like cells [76, 78, 101–106]. These MSCs-derived hepatocytes exhibit the same morphology and antigenic profile as native hepatocytes, and they appear to be functional, based upon uptake of low-density lipoprotein, urea production, and glycogen storage. Whether these cells exhibit polarity like native hepatocytes has yet to be unequivocally demonstrated, however.

These initial findings with BM-MSCs have now been extended to include MSCs derived from adipose tissue, amniotic fluid, CB, and Wharton's jelly, with adipose-derived MSCs showing the greatest propensity to differentiate *in vitro* into what appear, by all current metrics, to be functional hepatocytes [107–109]. It was initially hypothesized that CB-MSCs might harbor an innate capacity to differentiate into hepatocytes, since they constitutively express early as well as more mature hepatic markers and functions [110]. However, after several studies, it became clear that CB-MSCs differentiate only partially, displaying early and some mature hepatic markers/functions, but lacking the expression of other proteins that are critical for liver development [105, 110]. While this discovery initially reduced the enthusiasm for the use of CB-MSCs

as therapeutics for liver disease, it is important to realize that the immature nature of the hepatocytes they form could still enable them to treat disorders such as metabolic liver disease, in which generation of fully functional mature hepatocytes is not required, as long as the transplanted cells produce adequate levels of the missing/defective enzyme for correction. This limited differentiative capacity does, however, likely preclude their use for treating conditions such as acute hepatic failure.

Two groups have thus far analyzed the *in vitro* differentiation of human Wharton's jelly stem cells (WJSCs). Zhang *et al*. applied a one-step protocol with HGF and FGF-4 and found that, after 21 days, cells expressed albumin, alpha-fetoprotein (AFP), and cytokeratin 18 (CK-18) [111]. In other studies, Lin *et al*. induced differentiation of the cells by co-culturing them with murine liver tissue previously treated with thioacetamide (TAA), a chemical used to induce chronic fibrosis. Only two days after induction, hWJSCs expressed hepatic markers, providing evidence that, with the appropriate stimuli, hWJSCs can very rapidly reprogram to adopt a hepatocytic fate. AF-MSCs were also tested for their ability to differentiate *in vitro* into hepatocytes. The differentiation process employed by these investigators consisted of two steps: first, the MSCs were treated for 1 week with EGF and FGF to commence induction along the hepatic lineage; and second, a maturation step, during which the cells were treated with dexamethasone and oncostatin-M for 2 weeks. The MSCs-derived hepatocyte-like cells obtained at the end of this induction expressed several hepatic markers/functions, including albumin production, uptake of low density lipoproteins, glycogen storage, and urea production [112, 113].

Collectively, the results of these *in vitro* studies provide compelling evidence that MSCs derived from a variety of sources all possess the ability to give rise to what appear to be functional hepatocytes, albeit at varying levels. If complete functional differentiation could be achieved, MSCs could represent viable cellular therapeutics for treating liver disease, and thus provide a much-needed alternative to whole or partial liver transplantation.

13.5.2 In vivo models to study MSCs as cellular therapies for liver disease/injury

Despite all the knowledge that can be gained from performing *in vitro* studies, they are inherently limited by the need to supply all of the requisite factors to observe the desired differentiation/reprogramming. This becomes problematic when one wishes to discover/investigate novel properties of MSCs, since, in most cases, these factors are not yet known. Adding to this problem is the lack of suitable assays to rigorously establish that the "hepatocyte-like cells" generated *in vitro* are, in fact, bona fide hepatocytes that perform all of their required physiologic functions. For this reason, scientists are forced to resort to *in vivo* transplantation studies, in the hope that the mediators/factors present within the microenvironment of the target organ can coax the transplanted MSCs to reprogram towards the desired cellular fate. Performing studies *in vivo* also

ensures that all of the appropriate cues are present to influence migration/homing of MSCs to the tissue/organ in question; an essential issue to consider if the ultimate goal is to develop therapies using MSCs. Transplantation *in vivo* also provides the opportunity to examine the ability of the MSCs-derived cells to seamlessly integrate into the existing cytoarchitecture and adopt appropriate behavioral characteristics. Ideally, studies of this nature should be performed with human MSCs and their derivatives, to ensure the clinical translatability of the results obtained. Due to the ethical and practical issues, however, investigators can only test the ability of human stem cells to engraft/differentiate within a xenogeneic setting, using suitable small or large animals as recipients.

Based on the promising *in vitro* findings discussed above, MSCs have now been tested in a wide variety of liver injury/disease model systems for their ability to generate hepatocytes and correct these liver defects. Using MSCs isolated from a variety of mouse, rat, and human tissues, investigators have now provided evidence that MSCs can mediate varying degrees of correction/repair of the liver following a variety of mechanical and drug/toxin-induced injuries, including partial hepatectomy, treatment with the toxin CCl_4, injury induced by allyl-alcohol, high lipid diet, treatment with 2-AAF, and even pathogenic infection [21, 77, 103, 108–110, 114–135].

While the results of these studies have clearly highlighted the great promise of MSCs for treating liver injury/disease, they have also, unfortunately, been confounded by the problem of each group of investigators using MSCs defined in different ways ranging from specific antigen profile to simple plastic adherence. Nevertheless, looking at these studies collectively, it is clear that MSCs are able to exert beneficial effects in a wide range of injuries and disease states within the liver. Another major issue which has complicated interpretation of the data generated from these studies in liver, as well as those conducted looking at the potential of MSCs to mediate repair in other organ systems, is the observation that a therapeutic benefit is often observed in the absence of any evidence of long-term engraftment of the transplanted MSCs within the damaged organ. These findings led to a great deal of debate as to whether MSCs can actually generate hepatocytes, and sparked additional studies that have now shown that MSCs can also mediate tissue repair by acting as "trophic factories", releasing specific cytokines and growth factors that modulate the activity of tissue-specific cells, suppress local inflammation, and inhibit fibrosis and apoptosis, thereby facilitating endogenous tissue regeneration [26, 136, 137].

Adding to the complexity of the functions/effects of MSCs, it was recently discovered that MSCs can transfer mitochondria or mitochondrial DNA to cells that have been damaged by ischemia and reperfusion. By transferring mitochondria or mitochondrial DNA, MSCs can rescue the cells that have nonfunctional mitochondria, rescuing these cells and enabling regeneration of the tissue [138]. In recent years, it was also shown that MSCs express an array of miRNAs, small noncoding RNA's that are involved in regulation of gene pathways controlling such processes as stem cell differentiation, hematopoiesis, immune response, neurogenesis, stress responses, and the development of skeletal and cardiac tissue [139–142]. These regulatory miRNAs have

now been shown to be present inside exosomes/microvesicles that are secreted by MSCs, which are then transferred to neighboring cells to regulate their activities [143]. Following secretion of the exosomes, the miRNAs contained therein can enter the injured cell and induce differentiation and/or production of soluble mediators, and stimulate cell-cycle re-entry, providing yet another means by which MSCs can communicate with injured cells to promote tissue regeneration. Indeed, a recent study has shown that the injection of purified MSCs-derived exosomes alone was able to alleviate liver fibrosis induced by CCl_4 [144].

Upon arrival at the site of injury, MSCs also fulfill another vital function, which is to modulate the inflammatory microenvironment present within the damaged/diseased tissue by releasing soluble factors and by cell-cell contact. MSCs are known to inhibit proliferation and maturation of cytotoxic T cells, helper T cells, B cells, dendritic cells, and NK cells, as well as to inhibit NK-mediated cytotoxicity. These broad-ranging actions enable MSCs to interfere with each component of the adaptive immune system. MSCs can also stimulate the production of Tregs, which can further dampen the immune response. By dampening the ongoing inflammation and/or aberrant immune reaction present within the damaged/diseased tissue, MSCs facilitate the process of repair/recovery, further adding to their potential in regenerative medicine [92].

Looking specifically at the liver, recent studies have shown that MSCs can provide significant therapeutic benefit during acute hepatic failure by releasing chemotactic cytokines that reduce leukocyte infiltrates and hepatocyte death and increase hepatocyte proliferation [145, 146]. For example, Tsai *et al*. recently showed that the direct injection of MSCs into rats with CCl_4-induced liver fibrosis resulted in a significant reduction in the liver fibrosis. However, although MSCs engrafted, they did not differentiate into albumin-producing cells, but secreted cytokines that promoted liver regeneration and thereby restored liver function [131]. In addition, other studies have now revealed an additional property of MSCs that may make them ideally suited for treatment of liver diseases involving fibrosis: the ability to enhance fibrous matrix degradation, likely through the induction of metalloproteinases and inhibition of Dlk1 [17, 119, 137, 147–152]. Moreover, other researchers have found that MSCs are able to prevent liver fibrosis by suppressing the function of activated hepatic stellate cells, inducing their apoptotic death and diminishing collagen synthesis [153]. Studies like those by Lin *et al*. have shown that MSCs may utilize multiple mechanisms to exert their effects, both engrafting and differentiating into albumin-producing cells, and producing metalloproteinases that significantly reduced the collagen deposits in a rat model of chronic liver fibrosis [154]. An overview of the multiple mechanisms by which MSCs may exert a therapeutic benefit in liver disease/injury appears in Figure 13.2. However, these promising results must be interpreted carefully, because other studies have suggested that under different conditions, transplanted MSCs may actually contribute to the myofibroblast pool, enhancing the fibrotic process within the liver [17, 155–158]. This has led to the current feeling within the field that the effect

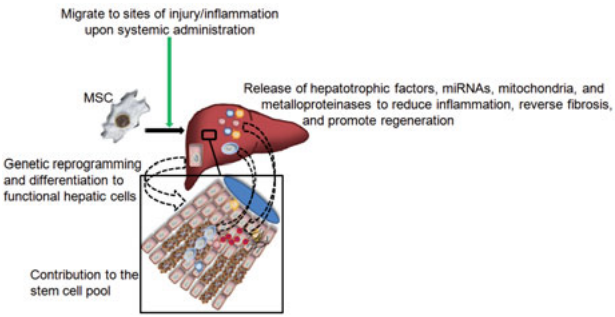

Fig. 13.2: Mesenchymal stem cells (MSCs) for liver regeneration.

of MSCs will probably vary with the nature of the liver injury/disease that is being treated, the specific experimental model in which the therapy is being tested, and perhaps even the time frame of MSCs application, such that MSCs could be beneficial if administered at certain stages of disease progression and harmful if administered at other stages. Thus, it appears that the therapeutic potential of MSCs may have to be investigated for each specific disease/injury to be treated to delineate the optimal time frame and population to be administered to achieve the desired effect, ensuring they provide benefit rather than harm.

13.6 The fetal sheep model

The aforementioned studies exploring the therapeutic properties of MSCs all used model systems generated by inducing an external stress, such as chemical- or radiation-induced injury or by depleting a specific cell type in the recipient, *e.g.*, partial hepatectomy. While these studies have provided compelling evidence that MSCs represent valuable cellular therapeutics for liver disease, they have also revealed that whether MSCs will exert beneficial or harmful effects is dictated largely by the presence of activated cells and the microenvironment within the injured or diseased organ at the time of transplantation. In addition, it also seems that the mechanism by which the transplanted MSCs produce their effects may also vary, depending upon the injury/disease state in question. This variability in outcome has made it very hard to even begin to dissect the molecular pathways by which MSCs are able, at least in some situations, to undergo reprogramming to produce functional hepatocytes. To fully exploit the marked therapeutic potential of MSCs, a thorough understanding of the mechanisms that control the cell fate and their efficient application to drive differentiation towards the hepatocytic lineage are needed; such an understanding will require the appropriate model system.

The ideal experimental model would allow transplantation of human MSCs, which could engraft and differentiate/reprogram under normal physiological condi-

tions, and be exposed to all necessary stimuli/support to evaluate the full potential of the cells in question without using an injury or genetic defect to bias which cell types will be formed following transplantation. Additionally, such a model should allow the generation of a broad spectrum of differentiation states of the donor-derived cells in the desired tissue at adequate levels to enable delineation of the mechanisms by which they were generated. Irrespective of the source of donor cells and mechanisms involved in reprogramming, however, the first key step for proper function is for the cells to reach the target organ. During fetal life, a series of carefully regulated migratory processes, likely employing the circulatory system, ensure that adequate numbers of appropriate stem/progenitor cells reach the target tissues/organs when needed. Under the permissive milieu of the target tissue, the entering stem cells are induced to proliferate and differentiate to produce the required type(s) of cells.

With these permissive aspects of the developing early gestational-age fetus in mind, we hypothesized [159–162] that the ideal way to evaluate the full hepatic potential of human stem cell populations would be to transplant these cells into healthy recipients during the fetal period in which the liver is rapidly proliferating and differentiating. Given their large size, long lifespan, and the high degree of physiologic, developmental, and immunologic similarity they share with humans [163–175], we chose sheep as a model system. By performing the transplant during the period of the "pre-immune" stage of development, we showed it is possible to achieve significant engraftment of allogeneic sheep cells and xenogeneic human cells within the fetal sheep, in the absence of any conditioning [159, 161, 162, 176–179], to create a lifelong chimera [180].

To investigate the *in vivo* differentiative potential of human MSCs in the absence of injury/selective pressure, we clonally derived several adult human BM-MSCs populations by sorting with an antibody against Stro-I [181]. We found that by selecting for Stro-1+CD45-GlyA- BM cells, we can reliably obtain a population that is highly enriched, both phenotypically and functionally, for MSCs. We therefore used this selected population for all of our studies to examine human MSCs differentiative potential.

To rigorously test whether MSCs could generate significant numbers of hepatocytes *in vivo*, we examined the ability of clonally-derived human MSCs from adult BM to generate functional albumin-producing hepatocytes *in vivo* following transplantation into fetal sheep recipients, comparing two routes of administration, intraperitoneal (IP) and intrahepatic (IH) [75]. Human hepatocytes formed after transplantation of BM-MSCs into fetal sheep were then identified by hepatocyte paraffin 1 (HEPAR-I) staining, coupled with human-specific fluorescence in situ hybridization. Our results showed that, although MSCs efficiently generated significant numbers of hepatocytes by both routes of administration, the IH injection resulted in a 5-fold increase in the number of hepatocytes generated, when compared to the IP route (12.5 % ± 3.5 % versus 2.6 % ± 0.4 %) [75]. A representative HEPAR-1-stained section from the liver of a sheep transplanted in utero with human BM-MSCs is shown in Figure 13.3. In addition

to higher levels of hepatocytes, the animals that received an IH injection also exhibited a widespread distribution of donor-derived (human) hepatocytes throughout the liver parenchyma, while those receiving an IP injection exhibited a preferential periportal distribution of human hepatocytes that produced higher amounts of albumin (please see [75]). This is in agreement with previous studies which demonstrated that localization of the hepatocyte within the liver is strictly associated with the levels of synthesis of certain plasma proteins, such that hepatocytes localized in the periportal area of the liver produce higher levels of albumin, compared to hepatocytes situated in other lobular zones [182–184].

In other studies, we evaluated the ability of MSCs derived from the fetal kidney to form hepatic cells *in vivo* and *in vitro* [185]. Like their BM counterparts, these cells gave rise to significant numbers of human albumin-producing hepatocyte-like cells upon *in utero* transplantation into fetal sheep. Furthermore, after culture in specific inducing media, cells with hepatocyte-like morphology and phenotype were obtained, suggesting that metanephric-derived MSCs could also serve as a source of cells with hepatic repopulating ability. Similar results were also obtained in the fetal sheep model, using a novel, adherent MSCs-like cell population isolated from umbilical cord blood, which the authors termed unrestricted somatic stem cells, or "USSC" [186]. This cord blood-derived MSCs population gave rise to albumin-producing human parenchymal hepatic cells at levels of >20 % in the recipient liver, in the absence of any injury or genetic defect.

Collectively, these studies provided compelling evidence that MSCs represent a valuable source of cells for liver repair and regeneration and demonstrate that, by altering the site of injection, the efficiency of hepatocyte formation can be dramatically altered, the generation of hepatocytes occurs in different hepatic zones, and the resultant hepatocytes exhibit differing functionality, just like their naturally-occurring counterparts. These results are highly relevant for designing a potential cellular therapy for liver regeneration, as depending on whether the overall goal of the therapy is to provide hepatocytes to restore the liver architecture or to achieve normal levels of a secreted therapeutic protein into the circulation, different routes of injection would likely be needed. However, a transplantation approach combining both administration routes may be needed to achieve functional repopulation of the liver.

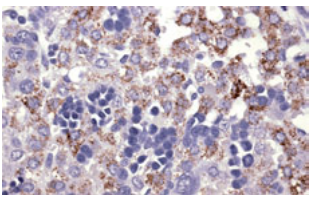

Fig. 13.3: Formation of functional hepatocyte-like cells (stained brown with HEPAR-1) after transplantation of human BM-MSCs into fetal sheep.

A key aspect to assessing the utility of MSCs therapy for regenerative medicine for the liver, and for other organs as well, is the mechanism whereby the transplanted cells replace/repopulate the recipient liver [75]. Indeed, there has been a great deal of controversy about the mechanism by which MSCs reprogram and differentiate into other cell lineages, such as hepatocytes. While the aforementioned *in vitro* studies have unequivocally shown that MSCs can, under the appropriate conditions, reprogram to generate what appear to be functional hepatocytes [107–109, 117, 187–189], it appears that, *in vivo*, cell fusion could be one of the mechanisms by which MSCs appear to give rise to hepatocytes, rather than true reprogramming/ transdifferentiation [190–192].

To begin delineating the mechanism(s) of hepatocyte formation following transplantation of human MSCs, we performed studies in the fetal sheep model [193], which we felt would be ideal for this analysis, given the robust generation of human-derived hepatocytes. We labeled human BM-MSCs with CFSE, which irreversible stains the plasma membrane [194, 195], or DiD, which labels all cell membranes, membrane-derived vesicles, and intracellular organelles such as mitochondria [196–198]. Consequently, fetal sheep were IP injected with either CSFE-positive MSCs alone or CFSE-positive MSCs in combination with DiD-positive MSCs. Confocal microscopic analysis for the presence of CFSE+ or DiD+ cells revealed that the transplanted cells initially appeared in the liver at 25h post-transplant, and their numbers then increased, reaching a maximum at 40h post-transplant. At all time points after transplantation, 95 % of the CFSE+ or DiD+ cells were also positive for Ki67, indicating that the higher levels of the cells observed at later time points were likely due to the proliferation of the initial MSCs that engrafted in the liver and not a result of more cells engrafting in the organ. These studies have important clinical implications, since they suggest that, independently of the low initial MSCs engraftment into a certain tissue, the real contribution of the cell to that tissue depends not only upon the initial engraftment levels, but also on the tissue's intrinsic proliferative capacity. Following engraftment of transplanted cells into the liver, hepatoblasts were generated that, due to their intrinsic proliferative capacity [199], continued proliferating and further contributing to the chimeric tissue [200].

We next examined the timeline of MSCs differentiation into organ-specific cell types in the liver, identifying differentiation of the transplanted cells by their simultaneous positivity for CFSE or DiD and α-fetoprotein, since hepatocytes acquire the expression of this protein during normal fetal liver development [201, 202]. At 25h post-transplant, cells that were positive for CFSE or DiD were already expressing α-fetoprotein, indicating that the transplanted MSCs were not only present in the tissue at this first time point of analysis, but they had already differentiated into a hepatocyte-like phenotype. A representative section of the liver from a sheep transplanted *in utero* with human BM-MSCs is shown in Figure 13.4, demonstrating the presence, at only 25 hours post-transplant, of alpha-fetoprotein positive DiD and CFSE-labeled cells. These results thus showed, for the first time, that transplanted MSCs engraft within the recipient liver, proliferate, and rapidly commence hepato-

cytic differentiation. By next performing fluorescence *in situ* hybridization (FISH) using a human- and a sheep-specific probe, coupled with confocal microscopy for the CFSE or DiD labels, we were able to conclusively demonstrate that the transplanted human MSCs are able to generate significant numbers of functional hepatocytes in the absence of cellular fusion or membrane vesicle/organelle transfer [75, 193], independent of fusion, and by true reprogramming/ transdifferentiation [193] suggesting that these cells do in fact possess a degree of true plasticity. In fact, we observed a sequential differentiation program, in which cells gradually expressed markers of differentiation, from the most undifferentiated cell to the mature fully differentiated cell type in the organ in question. The lack of fusion as a requirement for liver repopulation was in stark contrast to numerous studies in which the transplanted stem cells' differentiative capacity was restricted to cells of the liver through drug- or genetically-induced lesions/deficiencies [190–192]. Collectively, the results of our studies in the fetal sheep model and those in injury/disease models suggest that the means by which the transplanted MSCs contribute to the recipient liver is dependent on the model system employed, such that an animal model in which proliferation of endogenous hepatocytes has been arrested, such as those using chemical-induced

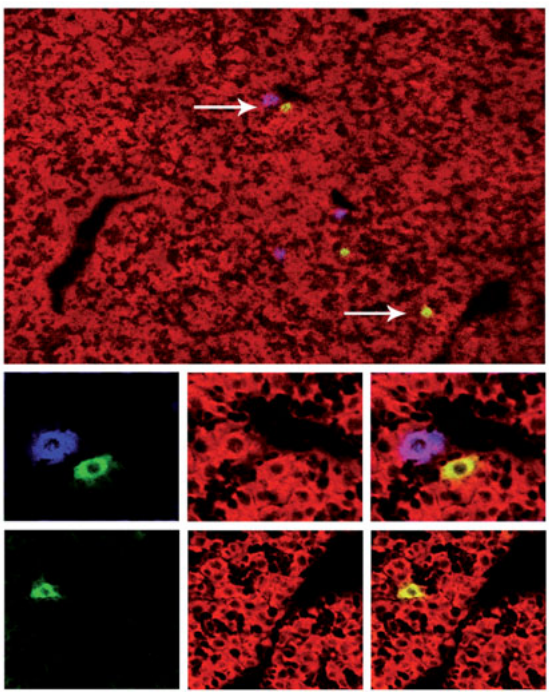

Fig. 13.4: Presence of alpha-fetoprotein-expressing (AFP; red), DiD^+ (blue; appears pink when co-localized with AFP) and $CFSE^+$ (green; appears yellow when co-localized with AFP) cells in the liver of sheep at 25 hours after *in utero* transplantation with human BM-MSCs.

injury, will require replication of the transplanted cells and therefore, favor transdifferentiation of the transplanted MSCs. On the other hand, in an animal model that promotes proliferation of endogenous and MSCs-derived hepatocytes, both mechanisms are possible, but fusion seems to be favored.

Despite the significance of the findings in these fetal models, it is important to note that, even when using an optimal route of injection, the overall levels of liver engraftment may still be too low to achieve cure in many clinical situations. While the fetus has long been presumed to be immuno-naïve, recent studies in mice have suggested that this may not be the case, since syngeneic cells engraft at higher levels than allogeneic cells of the same phenotype following *in utero* transplantation [203]. Thus, it is possible that some rudimentary immune surveillance exists within the fetus and limited the levels of engraftment within the liver. MSCs are well known for their immune-evading and immunomodulatory properties, but studies in murine and swine models have provided evidence that MSCs are not completely invisible to the recipient's immune system, nor immune-inert. Indeed, upon *in vivo* administration, MSCs are able to trigger immune responses, resulting in rejection of the transplanted cells [204–208]. Based on these prior studies, we hypothesized that further reducing the immunogenicity of the MSCs prior to transplant might enable us to achieve even higher levels of engraftment and hepatocyte generation, both in this "pre-immune" fetal model and, perhaps, even in recipients with a more developed/mature immune system, as would be seen in a clinical setting.

To test this hypothesis, we genetically modified human MSCs to stably express unique short region (US) proteins from the ubiquitously prevalent human cytomegalovirus (HCMV), which are known to exert potent immunomodulatory/immune-evading properties *via* reducing cytotoxic T cell recognition, and compared the immunogenicity and immunomodulatory properties of these "US-MSCs" to unmodified MSCs and to MSCs transduced with an empty control vector (MSCs-E). Our results revealed that MSCs expressing US6 (MSCs-US6) and US11 (MSCs-US11) exhibited the most pronounced reduction in HLA-I expression and, accordingly, induced the lowest level of human or sheep PBMNC proliferation in mixed lymphocyte reactions. Moreover, expression of US11 additionally protected MSCs from NK cytotoxic effects [209]. Based on these promising *in vitro* results, we transplanted MSCs-US6, MSCs-US11 and MSCs-E, *via* the IP route, into fetal sheep recipients. Tissues were collected at 60 days post-transplant and analyzed by qPCR and immunofluorescence for engraftment and hepatocytic differentiation of the transplanted cells. These analyses revealed that expression of either the US6 or the US11 HCMV protein on the transplanted MSCs led to significantly enhanced levels of liver engraftment compared to those seen with MSCs-E. These results clearly show that by enhancing the immuno-evasive properties of MSCs, the levels of engraftment and hepatocyte generation can be significantly increased, even in the context of a fetal recipient whose immune system is presumed to be largely immature. As such, this approach would be expected to produce an even more pronounced effect in adult, immunocompetent recipients in need of regenerative liver therapy.

13.7 Clinical trials using MSCs for liver regeneration

The less than satisfactory clinical results obtained thus far with hepatocyte transplantation led to a great deal of interest in trying to apply the successes seen with MSCs in animal models to the treatment of human patients with liver disease/injury. Regrettably, while the use of bone marrow-derived cells for treating cardiac disease has advanced a great deal in the last few years, the clinical use of these cells for repair/regeneration within the liver is still in its relative infancy. Nevertheless, 12 clinical trials using either MSCs or bone marrow-derived cell populations containing MSCs in patients with liver disease have been reported to date, one of which was an imaging study demonstrating the ability to track [111In]-oxine-labeled MSCs *in vivo* following infusion into patients with advanced cirrhosis [210]. Of the remaining 11 trials, 6 employed BM mononuclear cells, which likely contained not only MSCs, but also hematopoietic cells and endothelial progenitors, making interpretation of the data difficult. In the first of these studies [211], nine patients with cirrhosis were treated by peripheral vein infusion of autologous whole, unselected BM cells. 24 week follow-up revealed some improvement in the Child–Pugh score. The score is based on measurements of 5 clinical parameters: bilirubin, serum albumin, INR (prothrombin time), the presence/absence of ascites, and the degree of hepatic encephalopathy and its responsiveness to medication. Biopsy at this same time point also provided evidence of an increase in hepatocyte turnover. Importantly, however, no control arm was included. Similar improvements were reported by Lyra *et al*. [212], in a clinical trial involving 10 patients with chronic liver disease, but again, no control arm was included. However, a more recent randomized, controlled study by this same group [213] has now confirmed these findings, but has shown that the benefit only persisted for 90 days post-infusion. Similar success was also reported in patients with alcoholic liver cirrhosis [214] who experienced improved serum albumin levels, INR, and decreased Child-Pugh scores following infusion of autologous BM cells, compared to the control group, throughout the 24 week period of monitoring. In contrast to these successes were two recent clinical trials conducted by Nikeghbalian *et al*. [215], and Couto *et al*.[216], the former of which showed autologous BM mononuclear cells produced no benefit in patients with decompensated cirrhosis [215]. The results of the latter trial by Couto and colleagues [216] were even more troubling. In this trial, despite the use of autologous cells, one patient developed an unexplained graft-versus-host-like phenomenon, and another developed hepatocellular carcinoma. Since this trial only involved 8 patients and lacked a control group, one must be careful not to overanalyze these results. Nevertheless, these unexpected adverse events highlight the need for careful monitoring and vigilance, even when employing autologous cells.

In addition to the trials using unfractionated BM cells, 5 trials have also been conducted using MSCs derived from either BM or cord blood. The first of these, conducted by Mohamadnejad and colleagues in Iran [217], tested the safety and efficacy

of BM-MSCs in patients with cirrhosis. Infusion of BM-derived MSCs *via* a peripheral vein was well tolerated, and had a definite therapeutic effect, since the quality of life of all 4 patients was improved at 12 months post-infusion, and the MELD scores for 2 of the 4 patients improved significantly during the course of the trial. Unfortunately, there was no control arm to the study, and only 4 patients were tested. Similar results of improved model for end-stage liver disease (MELD) scores, albumin levels and INR were reported by Kharaziha *et al*. [218] in another uncontrolled trial infusing BM-MSCs into 8 patients with end-stage liver disease. To-date, only 3 appropriately controlled clinical trials have been conducted with MSCs. The first of these, performed by Zhang *et al*. [219] in China, involved 30 MSCs-treated patients and 15 controls, and showed that only patients receiving cord blood-derived MSCs experienced a significant improvement in MELD scores, albumin levels, bilurubin levels, and a reduction in ascites volume, during a 1-year follow-up period. The last two trials with MSCs were both performed in Egypt, and were unique in that they tested the therapeutic potential of MSCs that had been predifferentiated towards the hepatic lineage. In the first of these trials [220], BM-MSCs infusion resulted in improved MELD scores, albumin levels, bilurubin levels, and INR, but curiously, no differences were seen between the patients receiving undifferentiated MSCs and those who received MSCs that had been predifferentiated towards the hepatic lineage. In the second trial [221], only BM-derived "hepatocyte-like cells" were tested in 40 patients with end-stage liver failure due to hepatitis C (20 patients received cells, 20 were controls). Again, statistically significant improvements in all clinical measures of hepatic function were only observed in the group receiving the MSCs-derived cells.

Collectively, these studies provide hope that MSCs may prove to be a valuable resource for cell-based therapies for liver disease. However, the results of these studies must be interpreted with some trepidation, given the limited number of patients enrolled in each trial, the lack, in many cases, of appropriate controls, and the occurrence of adverse events. Furthermore, since the cells in these trials were autologously derived, there was no way for the investigators to assess the actual engraftment, persistence, or differentiative potential of the transplanted cells, leaving the mechanism responsible for the observed clinical improvements open to speculation.

13.8 Summary/Conclusions:

Liver insufficiency and failure is a life-threatening condition for which organ transplantation is the only definitive therapy. The current shortage of donor organs available for transplant and the severe morbidity and mortality associated with this procedure highlights the dire need for alternatives to whole or partial liver transplantation. While hepatocyte transplantation represents an option, the limited availability of donor livers and the inability to maintain and expand hepatocytes in culture precludes this option from becoming clinically viable. MSCs offer several advantages

such as: extensive expansion *in vitro*, multipotent differentiative capacity, the ability to selectively and efficiently migrate to sites of injury following systemic infusion, potent immunomodulatory and trophic properties, and the ease with which they can be genetically modified, making it possible to use autologous cells, even in the case of underlying genetic disease. MSCs can be isolated from a wide range of human tissues and, despite subtle differences, they all share the same beneficial characteristics, making MSCs transplantation a promising approach for liver repair/regeneration. Unfortunately, the lack of standardized protocols for isolating this somewhat elusive and likely heterogeneous cell population and the use of widely different injury/disease models has made the interpretation of these results rather challenging, and has left the critical question of the mechanism(s) whereby these cells mediate their beneficial effects largely unresolved. Despite these issues, the limited number of human clinical trials performed to-date have certainly given cause for a reserved optimism as to the future promise of this approach to treating liver diseases.

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Danilo Candido de Almeida, Clarice Silvia Taemi Origassa,
Ênio Jose Bassi and Niels Olsen Saraiva Câmara

14 Mesenchymal stem cells attenuate renal fibrosis

Abstract The progressive impairment of kidney function is characterized by the loss of nephrons, which causes a decrease in the glomerular filtration rate, leading to severe kidney disease. This process is associated with the excessive accumulation of extracellular matrix proteins (collagen type I-IV) and structural changes, leading to a collapse of the kidney parenchyma and the consequent loss of renal functionality. Currently, dialysis and renal transplantation are the most effective treatments available for end-stage kidney disease. Mesenchymal stem cells (MSCs) possess great trophic multipotentiality and actually represent an innovative and affordable treatment for acute and chronic kidney diseases. MSCs, through the proteolytic function of MMPs and the regulation of the MMP/TIMP balance, can remodel the fibrotic area and, together with their ability to promote the expression of renoprotective (HGF, BMP-7, VEGF and HO-1) and immunosuppressive (PGE-2, IDO, iNOS and HLA-G5) molecules, may stimulate angiogenesis and endogenous renal cell spreading, restoring kidney function. However, these cellular and molecular pathways still remain poorly understood. In conclusion, MSC-based therapy could potentially be applied in clinical practice to treat renal fibrosis; nevertheless, future studies should be performed to investigate the precise mechanisms and possible side effects of MSC treatment.

14.1 Introduction – Kidney function

The kidney is a special organ essential to the urinary system with important regulatory roles in the maintenance of homeostasis. In humans, the kidneys are paired organs situated in the abdominal cavity, more specifically in the retroperitoneal space. The kidney is surrounded by a very thin and bright fibroelastic membrane, called the renal capsule. This membrane adheres to renal surfaces and blood vessels in a concave space, designated as the hilum. The hilum is a plexus formed by the renal artery, the renal vein, lymph vessels, nerves and the ureter, which expands inside the renal sinus, forming the pelvis structure (Fig. 14.1a) [1]. Macroscopically, the kidney parenchyma is divided into two major structures: the cortex and the medulla. The cortex is composed of the glomeruli, as well as the proximal and distal tubules, and the medullary compartment contains the loop of Henle and collecting duct, which are connected to the renal calyx and pelvis (Fig. 14.1a) [1, 2].

The functional unit of the kidney is called a nephron, which is composed of many structures, such as arteries, veins, arterioles, capillaries, glomeruli, Bowman's capsule, the loop of Henle, specialized tubules (proximal cortical and distal) and the collecting duct. This framework participates in the reabsorption and secre-

tion process of various molecules, such as ions, carbohydrates and amino acids (Fig. 14.1b) [2].

Functionally, the kidneys work as a "natural filter of the blood", removing metabolites (such as urea, creatinine, ammonium and uric acid), which are directed to the urinary bladder. Moreover, the kidney participates in whole-body homeostasis by acting in the regulation of electrolytes, extracellular fluid volume (water reabsorption), in the maintenance of the acid-base balance (in the reabsorbtion of bicarbonate and excretion of hydrogen ions), and in the regulation of blood pressure (sodium chloride absorption). This system can also produce hormones, including calcitriol and erythropoietin, and enzymes, such as renin. In this context, the kidney can

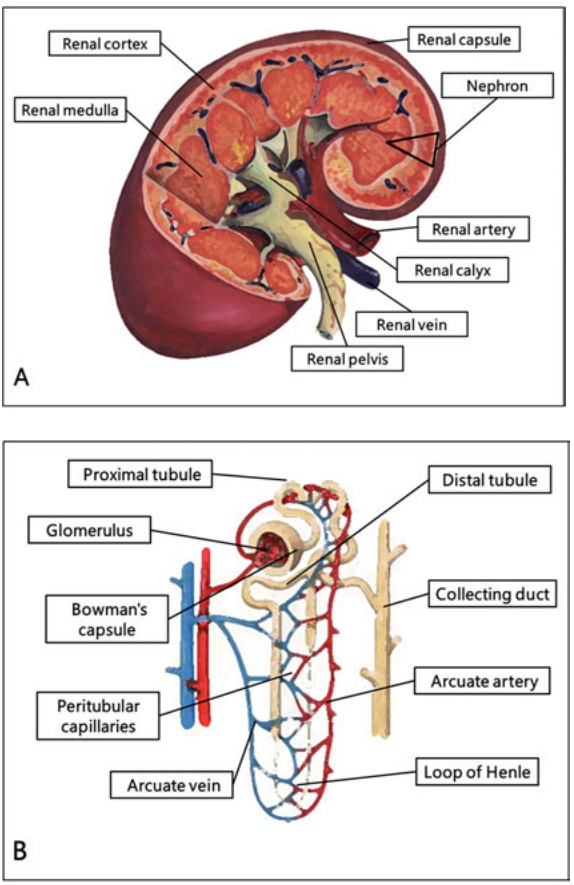

Fig. 14.1: Diagram of renal section showing renal structures (a). An overview of the nephron and its structures (b) [Images modified from EmeCeDesigns, 2012, access in http://www.biologyreference.com/Ho-La/Kidney.html#b, Photo by: Ken Pitts Science Page, 2011, acess in http://kenpitts.net/bio/human_anat/kidney_nephron.gif].

accomplish some homeostatic functions in concert with other organs, promoting the interaction between various endocrine hormones, such as renin, angiotensin II, aldosterone, antidiuretic hormone, and atrial natriuretic peptide, among others [1, 2, 3].

14.2 Kidney dysfunction and chronic kidney disease (CDK)

The gradual impairment of kidney function is normally characterized by a progressive and irreversible decline of its large number of functional units. The loss of nephrons causes the impairment of the glomerular filtration rate, which leads to acute and chronic kidney disease (CDK) [3, 4].

Currently, renal failure has a prominent impact on clinical practice and presents as a great public health problem in many countries. Renal injury affects approximately 500 million people worldwide, and its prevalence ranges from 5–10 % of all hospitalized patients; involving 30–50 % of patients in intensive care units (ICU) [5, 6]. Kidney injury may progress to chronic disease, and chronic disease can also be established gradually without reaching or passing through an acute phase. Despite advances in the establishment of new treatments, the rate of mortality and morbidity among patients with renal injury remains high and is estimated to be approximately 21–70 % [7–9].

The state of renal dysfunction is not considered a unique clinical entity, but a multiplicity of associated events, which cover several pathologies with distinct morphological and functional characteristics [10]. In addition, renal injury may be associated with other factors, such as decreased renal perfusion (42 %), major surgery (18 %), exposure to radio-contrast agents (12 %), aminoglycosides administration (7 %) and comorbidities (diabetes, hypertension and anemia) [5, 11].

In this sense, the number of people diagnosed with CKD has increased regularly all over the world, and a significant proportion of these patients progress towards end-stage renal disease (ESRD). The incidence of CKD is higher in patients with type 2 diabetes and elderly people; this disease is characterized by high levels of serum creatinine and a lower glomerular filtration rate. This defective function decreases the kidneys' ability to excrete waste products, allowing the loss of protein or red blood cells into the urine [12–14].

The physiopathology of CKD is defined by an accelerated loss of specialized cells, such as tubular epithelial cells, endothelial cells and pericytes. A continuous decline in renal function is associated with the excessive accumulation of extracellular matrix proteins (collagen type IV and collagens type I and III) and structural changes within all renal compartments (vascular, glomerular and interstitial tubule) [15].

Fibroblasts play a major role in the development of renal fibrosis by participating in intense collagen synthesis and matrix stabilization. Nevertheless, other cell types inserted into the kidney milieu, such as myofibroblasts, can become activated and produce abnormal levels of collagen, which causes the maintenance of

a fibroblast phenotype. This activation may be related to a variety of stimuli, such as the migration of inflammatory cells into the renal parenchyma, an inappropriate immune response and abnormal activity of the renin angiotensin system (Fig. 14.2) [12, 16]. A process called epithelial-to-mesenchymal transition (EMT) was proposed as a major mechanism contributing to the development of CKD. In this event, epithelial cells lose their intercellular contacts and polarity and undergo cytoskeleton reshaping by expressing proteins specific to mesenchymal cells, such as fibroblast-specific protein-1 (FSP-1), α smooth muscle actin (α-SMA), fibronectin and collagen I and collagen III [15, 17].

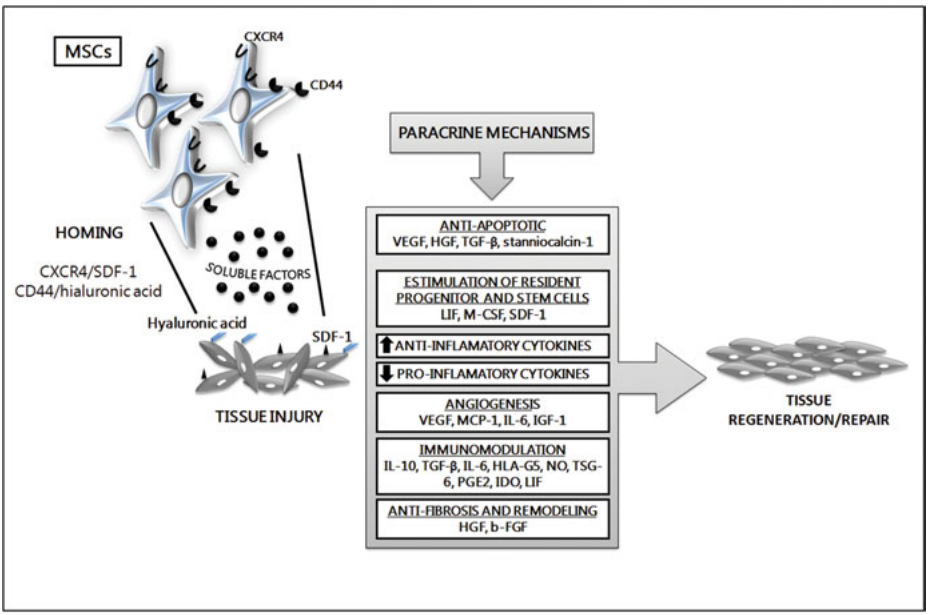

Fig. 14.2: Homing and mechanisms of action of MSCs in tissue repair and regeneration. MSCs are able to home to injured tissues and restore local function after their *in vivo* injection. This property could be attributed to the secretion of growth factors and chemokines, as well as the expression of extracellular matrix receptors on the surface of the MSCs, resulting in interactions, such as the CXCR-4/SDF-1 and CD44/hyaluronic acid interaction. These cells can promote the regeneration/repair of injured tissue through the secretion of several molecules in a paracrine mechanism, which is associated with anti-apoptotic, proangiogenic, immunomodulatory and antifibrotic properties.

14.2.1 Molecular and cellular interaction in renal fibrosis

Some cell interactions during renal remodeling are controlled by the release of chemokines, cytokines, and growth factors. In this context, angiotensin II has an impor-

tant role. This molecule stimulates the production of chemokines (such as MCP-1), promoting the migration of inflammatory cells and directly inducing collagen synthesis through the activation of several transcriptional pathways: endothelin, PAI-1, PDGF, TGF-β and EGF [18]. In addition, the activation of PDGF or EGF receptors stimulates the MAP/ERK kinase signaling concomitant with the synthesis of the transcription factor AP1, which is closely related with renal fibrosis.

TGF-β is predicted to be a key molecule involved in the fibrotic process. This molecule activates a family of intracellular signal transducers called the Smads. There are many types of Smad proteins; however, only two are relevant to fibrosis: Smad3 and Smad7. Smad3 is predicted to be more fibrogenic, while Smad7 might be more protective. Thus, the nuclear translocation of kinases and Smads and their interactions with DNA-binding sites, including that of the gene encoding activating protein 1, leads to the regulation of DNA synthesis, cellular proliferation, and fibrogenesis [16].

Moreover, the activation of the nuclear transcription factor kappa B (NFkB) appears to play an important role in the synthesis of chemokines and cytokines by kidney cells. In addition, peroxisome proliferator activated receptors (PPARs) are also involved in the regulation of cell cycling and extracellular matrix (ECM) processing in response to injury signals from renal cells [16].

In contrast, other molecular interactions can inhibit the fibrotic initiation signal. For example, AT1 receptor antagonists, angiotensin converting enzyme (ACE) inhibitors, ETA/B, epidermal growth factor receptor (EGFR) receptor antagonists and inhibitors of MAP/ERK kinase phosphorylation, may all prevent the activation of collagen type I gene in the renal vessels, glomeruli and renal cortex, decreasing the formation of fibrosis [15, 16].

The shift in the regulation and relative expression between pro- and antifibrotic members of the TGF-β/BMPs superfamily is another very important factor to be considered in the development of renal fibrosis [17].

After kidney injury, the dynamic interactions of a number of different cell types (endothelial and tubular cells), as well as endogenous and/or inflammatory infiltrating cells (monocytes, macrophages and T lymphocytes), with cytokines and chemokines, will determine whether the process will result in the organized tissue repair and remodeling or the onset of fibrosis (Fig. 14.2) [18]. As a consequence, these resident and inflammatory cells become activated *via* the TGF-β/Smad pathway, stimulating mesangial cells, fibroblasts and epithelial cells (after the epithelial-mesenchymal transition) to produce large amounts of extracellular matrix. This continuous deposition of matrix molecules results in fibrous scarring, which can distort the architecture of the renal tissues, leading to a collapse of the renal parenchyma (tubular atrophy, loss of podocytes and depletion of capillarity) and the consequent loss of renal functionality (Fig. 14.2) [19].

14.3 Mesenchymal stem cells (MSCs): Definition and basic features

Mesenchymal stem cells (MSCs) are multipotent and nonhematopoietic cells capable of self-renewal and generate different cell lines (osteoblasts, adipocytes and chondrocytes). Based on their ability to adhere to plastic surfaces or other components of the extracellular matrix, these cells can be isolated from various postnatal tissues, such as bone marrow, placenta, umbilical cord, tooth pulp, skin and adipose tissue [20].

Morphologically, human MSCs are fusiform cells with the ability to generate fibroblastic colony-forming units (CFU-fs) on plastic surfaces during their early growth stages in culture. Importantly, these cells have to present a specific phenotype: they have to be negative for hematopoietic and endothelial surface markers CD14, CD45, CD31, CD34, CD133 and positive for CD105, CD166, CD54, CD90, CD55, CD13, CD73, Stro-1 and CD44 surface molecules [21]. In general, there are three criteria for the characterization of MSCs: adherence to plastic surfaces; potential to differentiate into osteoblasts, adipocytes and chondrocytes; and expression of some stem cell-related surface antigens.

Thus, MSCs have great potential in the field of cellular therapy, as they can be isolated from many sources and then used to generate several cell lineages using specific *in vitro* conditions of differentiation. Moreover, these cells can provide a therapeutic effect in experimental models of a wide range of inflammatory and autoimmune diseases by promoting the regeneration and repair of injured tissues through many mechanisms, as shown in Figure 14.3.

14.3.1 Therapeutic potential of MSCs and their mechanisms of action in the repair/regeneration of tissue injury

Some therapeutic mechanisms have been proposed to explain the benefits achieved by treatments with MSCs in several experimental models of tissue injury, inflammatory diseases and autoimmune diseases. First, it has been hypothesized that these cells could differentiate and give rise to new cells in the injured tissues through a process called “transdifferentiation”. In addition, these cells also could fuse with resident cells in the target tissue in a process described as fusion, which is not well understood. However, more recently, several studies proposed another hypothesis that MSCs can repair tissues without engaging in fusion or transdifferentiation in a paracrine mechanism of regeneration/repair through the secretion of a range of soluble factors. Interestingly, MSCs can produce and secrete a broad variety of cytokines, chemokines and growth factors, which may potentially be involved in tissue repair and regeneration (Fig. 14.3) [22].

After *in vivo* injection, these cells are able to migrate to injured tissues and restore their resident function. This property could be attributed to the secretion of growth factors and chemokines, as well as the expression of extracellular matrix recep-

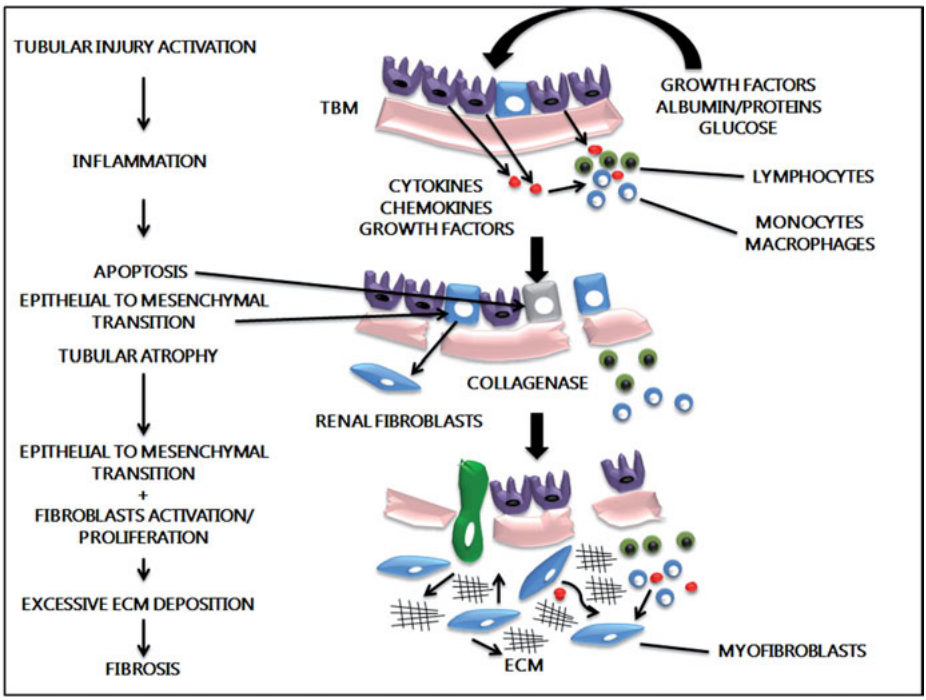

Fig. 14.3: Myofibroblastic differentiation and production of cytokines and extracellular matrix. Activated fibroblasts or myofibroblasts are derived from several different sources, including resident stromal fibroblasts, epithelial cells (epithelial-mesenchymal transition (EMT)), and bone marrow-derived fibrocytes. Myofibroblasts synthesize and deposit ECM components, which mainly include collagen type I and smaller amounts of collagen type III, fibronectin, elastin, laminin, proteoglycan and glycosaminoglycan, and they release various cytokines and mediators, which stimulate myofibroblasts in a paracrine manner. Infiltrating inflammatory cells, parenchymal cells and other cells also release cytokines and mediators.

tors on the surface of the MSCs. Stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, are crucial for the homing and migration of multiple stem cell types. In this context, a study demonstrated that the interaction of SDF-1/CXCR4 can mediate bone marrow-derived mesenchymal stem cell (BMSC) migration to sites of myocardial infarction and restore organ function [23]. Interestingly, CXCR4 expression in MSCs was increased under conditions of hypoxia, and SDF-1 induced MSC migration in a dose-dependent manner [23]. In injured kidneys, an important mechanism of MSC homing is the interaction between hyaluronic acid, which has increased expression in the damaged kidney, and its receptor CD44, which is expressed on the MSC surface (Fig. 14.3). In this sense, when MSCs were isolated from mice lacking the CD44 molecule, they were unable migrate to damaged kidneys; and furthermore, these cells CD44 KO also lost their therapeutic efficacy *in vivo* in a model of acute tubular injury induced by glycerol [24].

In an injury, the blood supply and neovascularization are very important for tissue recovery. Therefore, the secretion of proangiogenic factors by MSCs is very important for ischemic tissue recovery. Several proangiogenic molecules have been characterized in the conditioned medium of MSCs, including MCP-1 (monocyte chemotactic protein-1), VEGF (vascular endothelial growth factor), IL-6, angiopoietin and bFGF (basic fibroblast growth factor) (Fig. 14.3).

Soluble factors that enhance endothelial cell growth and survival, such as VEGF, hepatocyte growth factor (HGF), GM-CSF and stanniocalcin-1, are all involved with angiogenic and anti-apoptotic properties. VEGF, a molecule secreted by MSCs, is upregulated under conditions of hypoxia *in vitro* [25]. Moreover, mice with ischemic hind limbs demonstrated a marked improvement in perfusion when treated with adipose-derived human MSCs, suggesting that the delivery of MSCs may enhance angiogenesis and cardiovascular protection, which are both regulated by hypoxia [25]. Additionally, MSCs can express several molecules involved in the biogenesis of the extracellular matrix, suggesting that transplanted MSCs can inhibit fibrosis [26].

Furthermore, MSCs appear to have a major advantage over many other cells type used for cellular therapy, these cells can exert an immunomodulatory effect on several cells of the immune system. MSCs promote the suppression of the immune response by inhibiting the maturation of dendritic cells and suppressing the function of T lymphocytes, B lymphocytes and NK cells, as previously reported [27]. MSCs are capable of suppressing the proliferation of both CD4+ and CD8+ T cells [28]. Recently, many factors produced by MSCs that can promote lymphocyte suppression, such as TGF-β, HGF, inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxigenase (IDO), PGE2, HLA-G5 and IL-10, were characterized as possible molecules responsible for this immunomodulation in several studies [27]. MSCs may also regulate the immune response, inhibiting proliferation and immunoglobulin production by B lymphocytes [29]. Likewise, MSCs can inhibit the differentiation, maturation and activation of dendritic cells (DCs), generating immature DCs. MSCs can change the secretory cytokine profile of DCs by stimulating regulatory cytokines such as IL-10 (TH2 cytokine) and inhibit proinflammatory cytokines' release, such as IFN-γ, IL-12 and TNF-α (TH1 cytokines) [30].

The immunomodulatory property of MSCs has been successfully used in animal models for several autoimmune and inflammatory diseases, such as graft-versus-host disease (GvHD), autoimmune diabetes, rheumatoid arthritis (RA), multiple sclerosis (MS) and lupus erythematosus, among others. Thus, the therapeutic application of MSCs is a promising concept in regenerative medicine for the possible treatment of several diseases and tissue injuries.

14.4 MSCs and kidney diseases

Currently, dialysis and renal transplantation are the most effective treatments available for end-stage kidney diseases. Alternative strategies, such as pharmaco-based therapies or therapies with growth factors, have been explored in animal models to enhance renal recovery and survival, but all have failed to show any substantial improvement in medical practice [31, 32].

Stem cells, however, have great plasticity, and these cells may migrate to sites of injury and differentiate into specialized cells or promote regeneration by cross-talking with endogenous tissue-specific cells. The use of stem cells will also allow a greater understanding of the mechanisms involved in repair, including renal cell interactions and the release of soluble factors. In this sense, stem cell therapies have gained a special focus in nephrology, mainly for their potential in the development of more promising and effective therapeutic strategies.

In light of evidence that MSCs possess a great trophic multipotentiality, these cells have been used in several protocols for various human and nonhuman diseases (GvHD, multiple sclerosis (MS), osteogenesis imperfecta and Crohn's disease (CD)) and represent an innovative and affordable treatment for acute and chronic kidney diseases [33]. In 2004, Mogiri and colleagues were the first group to demonstrate that MSCs possess renoprotective properties that promote the improvement of renal recovery. The authors demonstrated that MSCs migrated to the damaged tissue and restored kidney structure and function with a significant increase in the proliferation index in the renal parenchyma [34].

Other studies have identified some benefits for MSC-based treatments of renal diseases. These works demonstrated that animals which received infusions of MSCs presented an improvement in renal function (low serum creatinine and urea), a higher cell proliferation rate (high positivity for PCNA) and an increase in the expression level of anti-inflammatory cytokines (IL-4) [35]. In addition, a recent study demonstrated that rodents treated with MSCs, presented low index of IL-1β, IL-6 and TNF transcripts, indicating a modulation of the inflammatory profile of the Th1 to Th2 response [36].

14.4.1 MSCs have a prominent antifibrotic effect in distinct models of experimental chronic kidney diseases

The precise participation of MSCs in the prevention of fibrotic processes is still unclear and is being explored by many researchers. Some believe that these cells could be effective in reversing fibrosis, while others claim that MSCs could not have any properties in this context. However, based on a large amount of evidence from experimental models for kidney diseases, we propose that MSCs can be useful in preventing and attenuating renal fibrosis.

A pioneering study using a model of chronic kidney disease with close similarities to human disease (a mouse that lacked the α3-chain of type IV collagen) revealed that animals treated weekly with MSCs presented a reduced indicators of renal fibrosis. MSC-treated mice showed improvement in fibrosis status associated with a decrease in macrophage infiltration (a hallmark of CDK) and the α-SMA-level. Moreover, after MSC injection, an enhancement of BMP-7 and VEGF levels was detected together with an increase in peritubular capillaries, suggesting that the maintenance of interstitial vasculature can be associated with less interstitial fibrosis [37].

An elegant work performed in a remnant chronic kidney model (5/6 nephrectomy or Nx) showed that MSCs can effectively attenuate renal fibrosis through tissue remodeling and immunosuppressive activity. This study demonstrated an increase in renal function (creatinine, urea or proteinuria levels) that was followed by a decrease in proinflammatory cytokines (IFN-γ, IL-6 and TNF-α) and an increase in anti-inflammatory molecules, such as IL-4 and IL-10 [38]. Surprisingly, after MSC infusion, a global improvement in renal protective parameters (HGF, HO-1 and BCL-2/Bad ratio) was observed concomitantly with a marked decrease in the renal fibrosis index assessed by Sirius red, Masson, α-SMA and fibroblast-specific protein-1 (FSP-1) expression. In addition, molecules involved in the initiation and maintenance of renal fibrosis, such as vimentin, fibronectin, TGF-β, Smad-3 and collagen 1–3, were all downregulated in animals treated with MSCs [38].

A second study by the same group, using an experimental model of interstitial fibrosis (unilateral severe ischemia-reperfusion of 6 weeks), observed that MSC infusion can promote a substantial reduction in the fibrosis score, which was verified by the downregulation of profibrotic-related molecules, such as collagen-1, vimentin, connective tissue growth factor (CGF) and FSP-1. In addition, the contralateral kidney conserved its peculiar morphology, and Sirius red and Masson staining were weakly observed in MSC-treated mice [39]. To investigate whether MSCs could stop or reverse fibrosis that had already been established, the authors performed an infusion of MSCs after 6 weeks of unilateral severe ischemia. Interestingly, in the MSC group, the researchers detected a recovery in the functional parameters, an upregulation of renoprotective molecules (IL-10 and BMP-7) and less interstitial fibrosis with low expression of type I collagen, vimentin and FSP-1 [39].

To uncover the mechanism involved in the MSC-mediated antifibrotic effect, another investigation utilizing a left ureteral obstruction kidney model observed that the epithelial-to-mesenchymal transition (EMT), a hallmark of fibrosis, can be modulated to interrupt the progression of fibrosis [40]. A significant shift in the E-cadherin/α-SMA ratio, a marker of EMT, was demonstrated. In this study, the E-cadherin levels became absent in the injured group and had their index restored after MSC infusion. Furthermore, the Masson score, as well as the α-SMA, collagen and FSP-1 proteins levels, were all reduced in renal tissues after MSC treatment [40].

Finally, to demonstrate the potential clinical applicability of MSCs in preventing renal fibrosis, two studies using a secondary model of chronic kidney disease

(diabetic nephropathy and chronic aristolochic acid nephropathy) reported results with considerable similarities to the findings of the basic studies that were previously mentioned. MSC therapy effectively prevented renal injury (creatinine and urea) and promoted decreases in collagen, TGF-β and α-SMA accumulation in addition to an increase in renal HGF, E-cadherin and BMP-7 expression [41, 42].

14.4.2 Mechanisms related to MSCs prevent renal fibrosis

Although the specific process by which MSCs can stop, reduce or reverse renal fibrosis is not understood, studies have suggested that MSCs may produce extra-cellular matrix-remodeling molecules, which will recast the affected area and promote functional improvement.

In this context, MSCs can synthesize and secrete multiple matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) membrane type-1 matrix metalloproteinase (MT1-MMP) , MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, TIMP-1, TIMP-2. TIMP-3 and TIMP-4), which can act in a synergistic remodeling of the fibrotic area by replacing damaged with new functional tissue. Hence, the appropriate balance between MMP/TIMP may be a determining factor in the functional recovery of the affected area [43–45].

The participation of MSCs in the modulation of the MMP/TIMP balance was evidenced in experimental models of infarcted hearts. In the first study, the authors demonstrated that the TIMP2/MMP2 and TIMP3/MMP9 ratios can be altered after MSC treatment [46]. Additionally, another work showed that conditioned medium from MSCs (CM-MSC) decreased the viability of collagen secretion by cardiac fibroblasts. Furthermore, CM-MSC increased the levels of MMP2, MMP-9 and MT1-MMP in cardiac fibroblasts, which occurred in conjunction with HGF expression and a reduction in TIMP-2 activity. When MSCs were injected in a rat model of postischemic heart failure, they promoted a significant decrease in ventricular fibrosis (lack of Sirius red stain), as well as an upregulation of HGF and an improvement in cardiac function (increase in cardiac wall thickness and ventricular ejection fraction) [47].

Thus, based on these experimental findings, we believe that MSCs can recognize damage signals and migrate to sites of injury. Through the proteolytic function of MMPs, as well as the regulation of the MMP/TIMP balance, MSC could promote the remodeling of the fibratic area. Together with the induction of the expression of renoprotective (HGF, BMP-7, VEGF and HO-1) and immunosuppressive (PGE-2, IDO, iNOS and HLA-G5) molecules, MSCs also may stimulate angiogenesis and the spreading of endogenous renal cells, thus restoring kidney function (Fig. 14.4).

14.5 Final considerations

As previously discussed, several factors may be responsible for the antifibrotic regulatory effect of MSCs. However, due to the complexity of the fibrosis milieu, it is suggested that the combination of synergism/antagonism among different cells types, cell-to-cell contacts, microenvironment interactions and the secretion of soluble factors should all be considered when attempting to understand the process as a whole. In addition, MSCs can possess species- and tissues-specific features, which could cause distinct *in vivo* effects. Furthermore, the importance of long-term evaluation (months to years) should also be considered, primarily due to the potential risks that are associated with the systemic transplantation of MSCs.

In summary, MSCs can orchestrate the production of several molecules with distinct functions; this includes molecules with proangiogenic (VEGF, HGF, IGF-1 and TGF-β), antifibrotic (b-FGF and HGF) or immunoregulatory (PGE-2, IDO, iNOS and

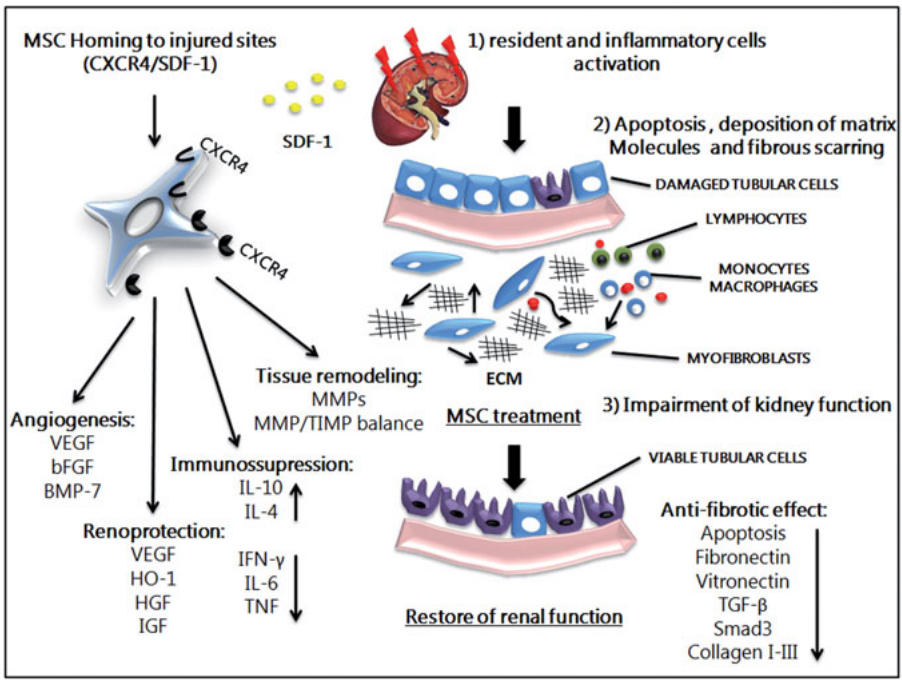

Fig. 14.4: Mechanism associated with the antifibrotic effect of MSCs in chronic kidney disease. MSC can recognize damage signals and migrate to sites of injury. Briefly, kidney fibrotic status can be characterized by three main steps: (1) activation of resident inflammatory cells; (2) apoptosis, deposition of matrix molecules and fibrosis scarring; and (3) impairment of kidney functions. Then, MSCs, through several mechanisms (proangiogenic, renoprotection, immunosuppression and tissue remodeling), may remodel the fibrotic area, decrease the apoptosis index and stimulate angiogenesis and endogenous renal cell spreading, thus restoring kidney function.

HLA-G5) properties, as well as activators of progenitor endogenous cells (SCF, LIF and SDF-1). These properties are predicted to characterize the principal mechanisms responsible in the promotion of kidney repair (Figs. 14.3 and 14.4). However, a precise understanding of these molecular pathways, as well as the role of small regulatory molecules such as transcription factors and miRNAs, remains poorly elucidated. In conclusion, experimental evidence suggests that MSC therapy could be potentially applied in clinical practice to treat renal fibrosis; nevertheless, future studies should be performed to investigate the precise mechanisms and possible side effects associated with MSC treatment.

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Marwan Mounayar, Ciara N. Magee, Reza Abdi

15 Immunomodulation by mesenchymal stem cells – a potential therapeutic strategy for type 1 diabetes

Abstract The incidence of type 1 diabetes continues to rise, such that the development of an effective treatment to prevent or reverse the disease becomes ever more critical. Various forms of immunotherapy have been investigated in multiple clinical trials without success; much effort is now focused on the development of novel immunomodulatory strategies to tackle the disease, including the use of mesenchymal stem cell (MSC)-based therapy. MSCs have attracted overwhelming interest due to their powerful immunomodulatory properties which span both the innate and adaptive immune system, making them ideally suited for the treatment of immune-mediated disorders. In the context of diabetes, systemic infusion of MSCs suppresses beta cell-specific auto-reactive T cells, while their cotransplantation with islet cells provides the allograft with an immunoprivileged and nurturing microenvironment. This chapter introduces MSCs and their mechanisms of immunomodulation, and how these properties have been exploited in the context of autoimmune diabetes.

15.1 Introduction

Mesenchymal stem cells (MSCs) were first identified in studies led by Friedenstein and colleagues [1, 2], wherein the low-density culture of bone marrow cells on plastic culture dishes led to the identification of plastic-adherent, colony-forming unit fibroblasts [3]. Initial studies demonstrated the ability of MSCs to differentiate into various cell lineages, including bone, cartilage, and adipose tissue, both *in viv*o and *in vitro* [4]. However, subsequent studies have shown that MSCs are a heterogenous population of cells with varying levels of multipotency. In fact, only a small proportion of cells among MSCs have been shown to be capable of self-renewal [5]. Some investigators have therefore proposed using the term "multipotent mesenchymal stromal cells" instead of mesenchymal stem cells [6, 7]. In the bone marrow, MSCs are a rare population of cells that comprise only 0.001–0.01 % of total nucleated cells [8]. Although most of the original MSC studies were based on bone marrow-derived MSCs, they have now been described in almost all tissues, including muscle, fat, kidney, pancreas, umbilical cord blood and peripheral blood [9]. The exact functions of MSCs in various tissues remain unclear; however, their function appears to be specific to the tissue from which they are derived: for example, bone marrow MSCs are believed to play a crucial role in the support and regulation of hematopoietic stem cells [10].

To date, there is no single marker available to characterize MSCs [5]. Therefore, the identification and phenotypic characterization of MSCs is currently based upon a suggested panel of positive and negative markers, in addition to their ability to differ-

entiate into cells of the mesenchymal lineages. According to the International Society of Cellular Therapy (ISCT), human MSCs are defined as plastic-adherent cells lacking the expression of hematopoietic stem cell markers (CD34), monocyte/macrophage markers (CD11c or CD14), B cell markers (CD79a or CD19) and class II MHC, but which express CD73, CD90 and CD105. Moreover, they should display trilineage differentiation capacity *in vitro*, possessing the ability to differentiate into bone, cartilage and fat cells [11].

15.2 Mechanisms of immunomodulation

The immunomodulatory properties of MSCs have been shown to be effective across a wide spectrum of immune cells [12]. They were initially shown to suppress the proliferation of mitogens, CD3 / CD28, and alloantigen-stimulated T lymphocytes [13, 14]. They are now also known to downregulate the activation markers CD25, CD38, and CD69 on phytohaemagglutinin (PHA)-stimulated T lymphocytes [14], and to suppress the proliferation of both $CD4^+$ and $CD8^+$ T cells; this suppression is non-MHC-restricted and occurs irrespective of the donor source of MSCs. Transwell culture systems have shown that the suppressive functions of MSCs are both contact-dependent and contact-independent [15].

A number of potential suppressive mechanisms exist: soluble factors such as transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), hepatic growth factor (HGF) and IL-10 secreted by MSCs have all been found to suppress T cell-mediated antigen responses *in vitro* [16]; furthermore, the induction of indolamine 2,3-dioxygenase (IDO) in MSCs by interferon-gamma (IFN-γ) has also been shown to inhibit T cell proliferation, thought to be due to the enhanced conversion of tryptophan to kynurenin by indolamine 2,3-dioxygenase, with consequent depletion of tryptophan [17]. Other suppressive mechanisms reported in the literature include increased expression of inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) [18, 19].

MSCs have further been shown to modulate T cells by increasing the number of CD4+CD25+FOXP3+ regulatory T cells (Tregs) [20]. Activated T cells induced the production of HLA-G5 by MSCs, which, in turn, led to the secretion of IL-10 by dendritic cells and an increase in Tregs. T cell inhibition has been also reported to occur through ligation of the MSC-expressed programmed death ligand-1 (PD-L1) with its receptor programmed death-1 (PD-1) on T cells [21].

MSCs regulate the humoral arm of the immune system by inhibiting both the proliferation of B cells and their expression of chemokine receptors [21, 22]. In a murine model of systemic lupus erythematosis (SLE), MSCs were shown to inhibit both B cell proliferation and IgG secretion [23].

MSCs are also capable of indirectly regulating lymphocytes by modulating cells of the innate immune system, thereby limiting the capacity of antigen-presenting cells

to present specific antigens. MSCs were shown to inhibit the maturation of monocytes into dendritic cells (DC) [24, 25]; furthermore, co-culture of mature DC with MSCs led to the downregulation of class II MHC, CD11c, CD83, CD80, CD86 and IL-12 [25].

MSCs were initially believed to evade natural killer (NK) cytotoxicity due to their expression of class I MHC; however, subsequent studies have demonstrated that MSCs are indeed susceptible to NK cell-mediated lysis [26, 27]. Nevertheless, MSCs were shown to be capable of suppressing both the cytotoxicity of NK cells and their production of IFN-γ [20, 28–30]. Interestingly, the presence of IFN-γ actually promoted the protection of MSCs from NK cytotoxicity, a desirable characteristic in the context of autoimmunity [28]. We have shown that MSCs were partially protected from NK cell cytotoxicity by constitutive expression of a natural inhibitor of Granzyme B, the serine protease inhibitor PI-9; indeed, MSCs were further protected from Granzyme B-mediated NK killing when PI-9 expression was enhanced [30].

However, it has also been shown that the milieu in which the MSCs exist has the ability to influence their capacity to regulate: Waterman *et al*. recently reported that distinct TLR ligation directed MSCs towards differing phenotypes: TLR4 ligation polarized MSCs toward a proinflammatory phenotype, whereas TLR3 ligation shifted the cells towards an immunosuppressive phenotype [31].

15.3 MSC therapy for type 1 diabetes (T1D)

15.3.1 Why does MSC therapy hold value in T1D?

T1D is an immune mediated disease in which autoimmune responses against the beta cells of pancreas result in the development of diabetes. T1D has become a major public health issue worldwide. The incidence of T1D has been rising steadily, rendering it one of the most challenging global health problems of the 21st century. Although T1D prevention studies using immunosuppressants have yielded promising results, they are limited by the inadequacy of monotherapy and serious morbidity associated with lifelong immunosuppression, which have underscored the need to consider alternative strategies. Diabetes is a leading cause of end-stage in renal disease and other end organ damages such as blindness. When taken together with the costs associated with the treatment of these complications, development of safe and effective therapies for T1D would represent a remarkable accomplishment due to lack of effective therapy. Given the autoimmune nature of T1D, most of the strategies rely on immunosuppressive or antigen-specific therapies. The main problem with these strategies has been the idea that a monotherapy would address various forms of immunoregulatory defects which may exist in a complex disease such as T1D. Like other complex autoimmune diseases, T1D is caused by a combination of immune cell dysfunction (including T cell, NK cells, B cells, and dendritic cells), as well as the perpetuating cascades of inflammatory cytokines resulting in an abrogation in maintaining periph-

eral and central tolerance. The immunomodulatory effects of MSCs, particularly their immunoregulatory interactions with virtually all types of immune cells, make them particularly interesting candidate cells for the treatment of T1D. Furthermore, MSCs exert generalized anti-inflammatory effects that could be highly important in maintaining peripheral tolerance which is impaired in T1D. While the plasticity of MSC has generated much interest in tissue (*i.e.*, islet) regeneration, their immunomodulatory capacity has more immediate clinical implications. Hence, most of the recent trails have focused on their immunomodulatory capacity to reduce the burden of immune-mediated diseases.

15.3.2 Preclinical studies to prevent and reverse T1D

The efficacy of MSCs therapy for the prevention and reversal of autoimmune diabetes has been repeatedly shown in a variety of murine models of the disease. There are several animal models to examine the efficacy of therapy before testing in humans. Nonobese diabetic (NOD) mice, which are the most commonly-used mice, are an autoimmune model which shares many features of autoimmune diabetes in human. These mice develop diabetes over time manifested by the infiltration of immune cells to the pancreas and destruction of insulin producing beta cells. Diabetes could also be produced by injecting streptozotocin into mice, which to large extent selectively destroys the pancreas. However, this model is not a perfect replica of T1D.

Intravenous delivery of human bone marrow-derived MSCs was initially shown to improve hyperglycemia in streptozocin (STZ)-induced diabetic NOD/SCID mice, increasing the numbers of pancreatic beta cells and, consequently, levels of insulin secretion [32]. A subsequent study by Urban *et al*. highlighted the effectiveness of a combined regimen of bone marrow cells and MSCs in the suppression of autoreactive T cells and enhanced regeneration of intrinsic beta cells [33]. Importantly, a single injection of MSCs efficiently suppressed the proliferation of beta cell-specific autoreactive T cells. Similarly, Ezquer *et al*. demonstrated that systemic delivery of MSCs to STZ-induced diabetic C57BL/6 mice significantly reduced their hyperglycemia and increased the number of morphologically normal islets [34]. We have shown that the injection of fully MHC-mismatched Balb/c MSCs prevented and reversed diabetes in NOD mice, a murine model of spontaneous autoimmune diabetes [35]. Surprisingly, autologous NOD-derived MSCs not only failed to treat diabetes, but also resulted in the formation of tumors, an adverse outcome not seen with MSCs derived from other strains of mice. In a subsequent study, we demonstrated the effectiveness of congenic nonobese resistance (NOR)-derived MSCs in reversing diabetes in NOD mice without subsequent tumor development [36]. The NOR are diabetic resistant mice which are about 85 % homologous to the spontaneously diabetic NOD strain and, in many respects, are analogous to nondiabetic siblings of T1D patients. Importantly, our data indicate that the NOR MSC (semi-allogeneic donor strain) were more effective than

BALB/c MSC (complete allogeneic donor strain) in treating diabetes in NOD host. We believe this superiority resulted from the eventual rejection of the allogeneic BALB/c MSCs. This work highlights the importance of considering the source of MSCs when seeking to determine the optimal MSC-based therapy.

15.3.3 MSC implications in islet cell transplantation

Various approaches designed to harness the potent immunosuppressive properties of MSCs for use in islet transplantation have been undertaken, as it is believed that the infusion of MSCs at the time of islet transplantation provides a protective, immune-privileged environment for the allogeneic islets by suppressing alloreactive T cells. Moreover, MSCs are believed to improve islet allograft function and survival by improving revascularization and ameliorating engraftment. The successful use of MSCs in this manner could reduce, or even eliminate, the need for immunosuppressive drugs, thereby minimizing the adverse sequelae associated with currently available immunosuppression.

Figliuzzi *et al*. reported that co-infusion of MSCs with syngeneic islet grafts reduced the number of islet cells required to achieve normoglycemia in STZ-induced diabetic Lewis rats [37]. In their study, Ito *et al*. co-transplanted Lewis rat-derived MSCs and islets into STZ-induced diabetic NOD SCID mice. All mice recipients of both islets and MSCs achieved normoglycemia, whereas mice treated with Lewis islets alone failed to reverse hyperglycemia in 70 percent of cases [38]. Similar results were observed when streptozocin-derived C57BL/6 mice were co-transplanted with syngeneic kidney-derived MSCs and islets. The majority of mice receiving the co-transplanted grafts reverted to normoglycemia, as compared to less than half of the mice receiving islet transplantation only [39]. The improved engraftment was attributed to the effect of MSCs in improving revascularization of the graft. Using a model of syngeneic marginal islet mass transplantation, Sordi *et al*. reported an increase in the proportion of hyperglycemia reversal when islets were co-transplanted with pancreas-derived MSCs rather than in isolation [40]. Furthermore, the lag time to achieving normoglycemia was shortened when MSCs were administered in conjunction with islet transplantation. This effect was also attributed to the supportive function of MSCs in enhancing vascularization rather than the differentiation of MSCs into pancreatic beta cells. Berman *et al*. published similar co-transplantation outcomes in a nonhuman primate model of diabetes: co-transplantation of islets and MSCs into STZ-induced diabetic cynomolgous monkeys resulted in better engraftment and graft function than islet transplantation alone [41]. Ding and colleagues also reported significant prolongation of islet allograft survival in a mouse model of transplantation upon co-administration of syngeneic MSCs, an effect attributed to cleavage of the interleukin-2 receptor on T cells by MSC-secreted metalloproteases [42].

15.3.4 MSCs and clinical trials for T1D

The powerful immunomodulatory effects exerted by MSCs on both the innate and adaptive immune system make them ideally suited to reprogram a hostile immune system. To date, preclinical studies have provided a proof of concept for the effectiveness of MSC therapy in various animal models of type 1 diabetes. The number of currently registered clinical trials (clinicaltrials.gov; see Tab. 15.1) investigating the use of MSC therapy in the context of type 1 diabetes indicates the widespread interest in

Table 15.1: Table listing the ongoing clinical trials employing MSCs as therapeutic modality in autoimmune diabetes (clinicaltrials.org).

Trial name	MSC source	MSC therapy combined with	Identifier	Sponsor
Umbilical cord mesenchymal stem cells infusion for initial type 1 diabetes mellitus	Umbilical cord MSC	–	NCT01219465	Qingdao University
Treatment of patients with newly onset of type 1 diabetes with mesenchymal stem cells	Bone marrow MSC	–	NCT01068951	Uppsala University Hospital
Umbilical mesenchymal stem cells and mononuclear cells Infusion in type 1 diabetes mellitus	Umbilical cord MSC	Bone marrow mononuclear cells	NCT01374854	Fuzhou General Hospital
Autologous transplantation of mesenchymal stem cells for treatment of patients with onset of type 1 diabetes	Bone marrow MSC	–	NCT01157403	Third Military Medical University
PROCHYMAL® (human adult stem cells) for the treatment of recently diagnosed type 1 diabetes mellitus (T1DM)	Bone marrow MSC	–	NCT00690066	Osiris Therapeutics
Stem cell therapy for type 1 diabetes mellitus	Umbilical cord MSC	Bone marrow mononuclear cells	NCT01143168	Cellonis Biotechnology Co. Ltd.
safety and efficacy of mesenchymal stem cells in newly-diagnosed type 1 diabetic patients	Bone marrow MSC	–	NCT01322789	University of Sao Paulo
Cotransplantation of islet and mesenchymal stem cell in type 1 diabetic patients	Bone marrow MSC	Islets	NCT00646724	Fuzhou General Hospital
Human menstrual blood-derived mesenchymal stem cells transplantation in treating type 1 diabetic patients	Menstrual blood-derived MSC	–	NCT01496339	S-Evans Biosciences Co., Ltd.

translating these exciting preclinical data into an effective and safe clinical therapy; we await the outcome of these studies with great interest.

15.4 Safety of MSC therapy

To date, millions of MSCs have been administered to hundreds of patients, the vast majority of whom have not experienced any serious adverse sequelae. The few initial reports on tumor formation following MSCs therapy were later determined to be caused by MSC contamination with immortalized cancer cell lines, indicating that tumor formation was not a risk of the therapy *per se* [43]. However, this highlights the absolute need to ensure stringent handling of MSCs to minimize the risk of cross contamination with cancer cells, as well as the importance of developing new screening tests to assess MSCs' chromosomal stability and monitor tumorigenicity. Further preclinical studies are required to address such clinically relevant questions as dosing frequency, trafficking and survival of MSCs, and would also provide an opportunity to identify synergistic immunosuppressive strategies.

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Carmen Gonelle-Gispert and Léo H. Bühler

16 Fibrogenic potential of human multipotent mesenchymal stem cells in inflammatory environments

Abstract The progressive impairment of kidney function is characterized by the loss of nephrons, which causes a decrease in the glomerular filtration rate, leading to severe kidney disease. This process is associated with the excessive accumulation of extracellular matrix proteins (collagen type I-IV) and structural changes, leading to a collapse of the kidney parenchyma and the consequent loss of renal functionality. Currently, dialysis and renal transplantation are the most effective treatments available for end-stage kidney disease. Mesenchymal stem cells (MSCs) possess great trophic multipotentiality and actually represent an innovative and affordable treatment for acute and chronic kidney diseases. MSCs, through the proteolytic function of MMPs and the regulation of the MMP/TIMP balance, can remodel the fibrotic area and, together with their ability to promote the expression of renoprotective (HGF, BMP-7, VEGF and HO-1) and immunosuppressive (PGE-2, IDO, iNOS and HLA-G5) molecules, may stimulate angiogenesis and endogenous renal cell spreading, restoring kidney function. However, these cellular and molecular pathways still remain poorly understood. In conclusion, MSC-based therapy could potentially be applied in clinical practice to treat renal fibrosis; nevertheless, future studies should be performed to investigate the precise mechanisms and possible side effects of MSC treatment.

16.1 Introduction

Multipotent mesenchymal stromal cells (MSCs) take part in the formation and regeneration of connective tissues in the body such us bone, muscle, cartilage and fat. MSCs were originally isolated first from bone marrow [1, 2] where they reside in the bone-lining compartment and support bone growth. A functional role was further attributed to MSCs in the differentiation of hematopoietic stem cells (HSCs) [3–6], further confirmed by studies where co-transplantation improves outcome of bone marrow transplantation MSCs after myeloablative treatment [7]. Since then, MSCs have been isolated from almost all tissues and have been suggested to occupy a perivascular niche similar to pericytes [8–10]. It is still controversial whether MSCs isolated from different tissues fulfill the same physiological roles or are committed to other more tissues specific functions.

One important field of MSCs research concerns the transdifferentiation potential of MSCs, also called, plasticity. MSCs have been tested for their potential to differentiate across germinal boundaries into various epithelial cells as well as neurons.

Studies in experimental animal models of degenerative diseases investigating the cell fate of transplanted MSCs demonstrated rather limited engraftment and developmental plasticity. New findings suggest that the beneficial effects observed may result from the capacity of MSCs to modify tissue environment and decrease inflammatory as well as immune reactions. In chronic and acute liver diseases, most studies revealed that only low numbers of MSCs engraft and transdifferentiate into hepatocytes. As liver fibrosis may evolve into cirrhosis, a potentially life-threatening disease, it is of special interest to keep in mind that MSCs may contribute to fibrosis together with stellate cells and portal fibroblasts.

Subsequently, it became more evident that MSCs are involved in wound healing processes, contributing to myofibroblast and fibroblast population [11]. Whether this is due to local or circulating MSCs remains controversial. Systemically injected MSCs were also found in sites of inflammation and diseased tissues, such as tumors, where they may contribute to tumor growth. Many efforts are ongoing to identify specific molecular mechanisms mobilizing and guiding MSCs to sites of injury, which will help to develop strategies to enhance MSCs homing and engraftment to sites of injury. Genetically manipulated MSCs, expressing therapeutic factors, may then be used for the specific targeting of tumors or other tissue injuries [12–19].

16.2 Fibrogenic potential in *ex vivo* expanded MSCs

There still remains a fundamental problem in MSCs biology, which is the inability to prospectively isolate MSCs from tissues. Therefore, the characterization of these cells in an unmanipulated state is still lacking and we can only speculate that cells with similar characteristics exist, as such, *in vivo*. MSCs once isolated constitute a fast growing cell population, heterogeneous in many aspects, reflecting maybe differences related to isolation and culture proceedings. MSCs display no unique marker for a prospective isolation. After expansion they are identified by a set of surface antigens which are also expressed by fibroblasts [20]. However, MSCs are distinguished from fibroblasts by their trilineage differentiation potential. Clonal expansion of MSCs demonstrated clearly that *in vitro* clonal progenies also display different lineage commitments and differentiation potential [21].

For clinical applications, large amounts of MSCs are necessary. Injections of 1–10 × 10^6 MSCs per kilogram of body weight might be carried out several times which means that as many as 1×10^9 hMSCs could be required for one patient. To achieve such cell numbers, extensive and rapid cell culture is required and represents a critical issue in cell therapies when using autologous cells. MSCs present at low frequency in the bone marrow (1/10,000) and are isolated from a bulk of mononuclear cells through their property of adhesion on plastic culture plates. Independently of tissue origin, the primary culture of MSCs is heterogenic in terms of shape (spindle-shaped or flat) and size (20–30 μm). During early passaging MSCs are spindle-shaped but adopt after

few passages an enlarged and flattened phenotype accompanied by senescence with a cell cycle arrest in the G1 phase. The default pathway for most MSCs population, in culture, is osteogenesis [2, 22]. MSCs' development into osteoblasts can be conducted by surfaces coated with extra cellular matrix (ECM) proteins like collagen I, vitronectin, fibronectin and laminin as demonstrated by increased alkaline phosphatase activity and higher amounts of mineralized matrix and calcium [23–28]. Further passaging induces a gradual loss of the adipocyte differentiation potential [22].

Expansion of MSCs under various culture conditions allowed to observe that α-smooth muscle actin (α-SMA) expression increases in culture progressively over time, suggesting the appearance of profibrogenic MFs. As in fibroblasts, addition of transforming growth factor β (TGF-β) to MSCs cultures induces the contractile phenotype. Expansion of MSCs on extensible surfaces, composed of high-extension silicon rubber, coated with collagen type I, without passaging, preserves the three-lineage differentiation potential and decreases the development of MSCs into profibrogenic myofibroblasts [29]. *In vitro*, the contractile phenotype of MSCs is influenced also by the mechanical property of substrates, such as the stiffness. MSCs treated with TGF-β and cultured on a soft substrate (a thick layer of collagen; 500 μm) fail to induce a contractile phenotype in MSCs compared to cells cultured on a stiff substrate (a thin layer of collagen; microns) [30]. Platelet-derived growth factor (PDGF-BB) is a potent mitogen for mesenchymal cells, and currently used in MSCs culture to increase growth rates. MSCs express abundant cell-surface PDGF receptor-α but also PDGF receptor-β. Using PDGF-BB in culture also decreases α-SMA expression whereas culturing MSCs with PDGF-AA increases α-SMA expression, demonstrating that the contractile phenotype of MSCs is modulated *via* two distinct PDGF-BB receptor pathways [31, 32]. Therefore, under several culture conditions α-SMA expression can be induced. Precautions should be taken to avoid an undesired contractile phenotype due to inappropriate extensive *ex vivo* culturing.

16.3 Evidence of MSCs infiltration into tumor stroma

Solid tumors are composed of tumor cells and a nontumor component, the tumor stroma. This tumor stroma is composed of different mesenchymal cell types, secreting cytokines, growth factors, angiogenic factors and proteolytic enzymes which actively contribute to the proliferative and invasive behavior of the tumor cells.

Several studies revealed the presence of MSCs in tumor stroma [33]. Surprisingly, α-SMA-expressing MSCs with gene expression profiles similar to cancer associated fibroblasts (CAFs) have recently been identified in an elegant mouse model of inflammation-induced gastric dysplasia. In this model, bone marrow from transgenic mice expressing red fluorescent (RFP) α-SMA and a green (eGFP) fluorescent collagen I1α, showed that 20 % of the cancer-associated myofibroblasts (CAFs) originated from bone marrow-resident MSCs. Moreover, they discovered that in parallel to disease

progression, the number of MFs derived from MSCs increases in the bone marrow where they contribute to the normal stem cell niche; suggesting further that MSCs may be recruited into tissues where cell growth and differentiation is disturbed only following bone marrow remodeling. The recruitment of MFs into the tumor stroma was dependent of TGF-β and SDF1-α [34]. Further, a malignant progression of bone marrow-derived MSCs was described in a setting of chronic inflammation of gastric mucosa by *Helicobacter* leading to a new concept in which epithelial cancer may originate from mesenchymal bone marrow cells [35]. In humans, mesenchymal cells with comparable characteristics to bone marrow-derived MSCs were isolated from pediatric tumors. These cells showed similar inhibitory effects on NK cells *in vitro*, suggesting that immunosuppressive behavior exerted by MSCs facilitate immune evasion of tumors cells [36]. Similarly, in mice, bioluminescent imaging revealed that expanded hMSCs also engraft and remain detectable at inflammatory sites after injection [12, 33, 37–40]. It is, however, not clear whether MSCs display antitumor functions or whether they co-opt to promote tumor growth [33]. MSCs secrete proangiogenic factors [41] which may contribute to tumor angiogenesis [42–44]. However, MSCs may also participate in tumor growth by increasing angiogenesis by differentiation into smooth muscle cells. In a setup where weakly metastatic human breast carcinoma cells were mixed to MSCs, it was demonstrated that MSCs increases the metastatic potency of cancer cells [45].

MSCs homing to tumor and their eventual participation in tumor growth and carcinogenesis are dependent on many factors such as *in vitro* expansion, route of administration and the molecular characteristics of the tumor cells. MSCs, once present in the tumor microenvironment, take part in a complex interplay with the tumor cells and may evolve into myofibroblasts through tumor secreted factors and cytokines, such as TGF-β. Interaction with other tissue derived stromal cells may also play important roles.

16.4 Controversies regarding therapeutic benefits of bone marrow-derived MSCs in liver fibrosis

MSCs are extensively studied in animal models for the treatment of acute or chronic liver injury. In chronic liver insults, the lasting activation of tissue repair mechanisms leads to an excessive deposition of extracellular matrix (ECM) components. Mainly implicated in the ECM production are perisinusoidal hepatic stellate cells (HeSCs) and portal fibroblasts.

Some studies analyzing diseased livers identified contractile α-SMA positive myofibroblasts of bone marrow origin. In humans, studies transplanting sex-mismatched bone marrow or liver allowed to observe that bone marrow cells contribute to scar formation and liver fibrosis. Male patients transplanted with livers from female donors showed that 7 to 22% of the liver fibrosis-related myofibroblasts contained

the Y chromosome. The liver of a female patient developing hepatitis C-induced cirrhosis 10 years after having received a male bone marrow transplant showed that 12 % of myofibroblasts contained the Y chromosome and could be considered to be of extrahepatic origin [46]. Homing of MSCs residing in the bone marrow into the injured liver was shown to be dependent upon sphingosine 1-phosphate gradient and sphingosine 1-phosphate receptor type 3 [47]. In irradiated mice, transplantation of sex-mismatched bone marrow demonstrated that the contribution of bone marrow to parenchymal regeneration in a cirrhotic liver was very low (0.6 % of hepatocytes). However, the contribution of bone marrow cells to the hepatic stellate cell pool and myofibroblast population was high and reached 68 % and 70 % respectively [48].

Many studies have been conducted to analyze the contribution of *ex vivo* expanded MSCs to liver tissue regeneration. Engraftment and differentiation into injured liver of systemically injected MSCs appeared to occur only to a minor extent and several studies failed to measure significant effects. Transaminases levels, and liver fibrosis area remained unchanged between controls and rats transplanted with rat bone marrow-derived MSCs [49]. Similar negative results were reported with human cord blood-derived MSCs transplanted into a cirrhotic rat liver [50]. Perturbingly, several studies revealed not only an insignificant rate of transdifferentiation into hepatocyte-like cells but revealed the presence of α-SMA - expressing MSCs in the liver parenchyma. Three different protocols were used in this study, inducing an acute and chronic injury, combining irradiation and liver injury through carbon tetrachloride (CCl4). Livers from mice with acute injury revealed the presence of few myofibroblasts whereas chronic injured livers displayed higher numbers of myofibroblast-like cells expressing α-SMA [51].

However, several other investigators observed beneficial effects and reported that MSCs decrease the progression of liver fibrosis. Numerous studies in animal models demonstrated that MSCs have a beneficial effect in liver fibrosis induced by diethylnitrosamine (DEN) or CCl4 [52–58]. Systemic allotransplantation of bone marrow-derived MSCs into BALB/c mice with CCl4-induced liver fibrosis showed a reduced level of the fibrosis marker hydroxyprolin in sera as well as reduced hepatic necrosis [54]. Further beneficial effects of MSCs were also observed in rats with chronic liver injury induced by CCl4 [59, 60]. MSCs derived from various other tissues exerted antifibrotic effects, like human umbilical cord-derived MSCs [61] and human placenta-derived MSCs [21]. More recently, *in vitro* predifferentiated hepatocyte-like cells increased beneficial effects in the treatment of experimental liver fibrosis [62, 63]. Engraftment of MSCs into host's liver occurred at low frequency supposing that the beneficial effects of MSCs occurred through modifying the tissue environment and by decreasing inflammatory as well as immune reactions rather than by transdifferentiation. MSCs expressing high levels of matrix metalloproteinases (MMPs) and an increased degradation of ECM could be a possible mechanism contributing to the antifibrotic effects observed [64–66]. Further, increased levels of anti-inflammatory cytokines such as IL-10 and decreased levels of proinflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor – α (TNF-α) and TGF-β may account for the beneficial effects [67].

Explaining the discrepancies between the beneficial antifibrotic effects and the contribution of MSCs to fibrogenic tissue remains complicated. Depending on the animal model (strains of mice), type of liver injury, the nature and number of MSCs injected and injection site (systemic versus intraportal) MSCs may modulate inflammation or infiltrate into the injured liver and become profibrogenic. In animal models including irradiation or bone morrow-transplantation pretreatments, the inflammatory condition of the animals may contribute to a profibrogenic outcome of MSCs. More generally, developing strategies inhibiting bone marrow-derived MSCs from contributing to liver fibrosis could help to reduce progression of fibrosis and may represent a new therapeutic axis.

16.5 Limited contribution of MSCs to liver regeneration in acute liver injury

The genetic modification leading to transdifferentiation into hepatocytes still remains enigmatic but interestingly, a recent study showed that overexpression of hepatic lineage-specific transcription factors GATA4, HNF1-α and FOXA-3, and the inactivation of $p19^{Arf}$ in mouse tip-tail fibroblasts, directly induces their transdifferentiation into functional hepatocyte-like (iHep) cells [68]. Differentiation of MSCs into hepatocyte-like cells or toward hepatocytes expressing alpha-foetoprotein (α-FP) and albumin as well as other proteins functionally related to hepatocyte has been demonstrated *in vitro* [69–74]. Factors like fibroblast growth factor 4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M, which are key molecules in embryonic liver development, were found to induce hepatospecific gene expression in MSCs. Downregulation of Notch and Wnt signaling pathways occur during transdifferentiation and might be implicated in the phenomenon [75, 76]. Given the fact that MSCs showed a greater developmental plasticity than initially thought, studies investigating the potential of MSCs to differentiate into hepatocytes *in vivo* were performed.

Liver regeneration is a pathophysiological process in which hepatocyte replication is initiated after loss of liver mass due to chemical or viral injury or resection of liver lobes. Hepatocyte replication is a multistep process divided into priming phase, proliferative phase and growth termination. The priming phase is initiated by cytokines, such as TNF-α and IL-6 but also by nutritional and hormonal signals. The second phase is the replicative response, dependent on several growth factors like HGF, epidermal growth factor (EGF), TGF-β. Macroscopically, during this process hepatocytes become hypertrophic and show transient steatosis. Then, when the liver has restored its initial mass, the termination of all replication is dependent on TGF-β1 and all signalling events induced by remodelling of ECM.

Several studies have been conducted to investigate the possibility of MSCs physically participating in the regeneration process. A common animal model of liver regeneration used is the 2/3 hepatectomy, which triggers quiescent hepatocytes to

become proliferative until restoration of liver mass and metabolic capacity. Further, acute injury models such as a single dose of CCl4 or allyl alcohol (AA) are also used to induce liver regeneration in rodents. In this context, injection of *ex vivo* expanded MSCs either systemically or into the liver or spleen showed unsatisfactory results with low levels of engraftment. Injection of preconditioned MSCs into immunodeficient Pfp/Rag2 $^{-/-}$mice liver, in which regeneration was temporarily inhibited through treatment with propanolol hydrochloride, led to engraftment of human albumin expressing cells around portal veins. However, the proportion of transplanted cells was evaluated at 1% of the total liver mass [78]. Bone marrow-derived MSCs and hepatocytes from rats were compared for their ability to engraft in a rat model of acute liver injury induced by CCl4 or AA. In such a setting, engraftment of hepatocytes was detected demonstrating the validity of the experimental model. The largest clusters of hepatocytes were detected in rats treated with AA and retrorzine, which impairs hepatocyte proliferation. None of these conditions allowed the engraftment of MSCs [78–80]. In a model of acute liver injury, where MSCs were injected into the parenchyma of AA-treated rat liver, transdifferentiation of MSCs into hepatocyte-like cells could be observed. However, the transdifferentiation of MSCs was limited to the site of injection suggesting that differentiation occurred without structural integration into the liver tissue [81].

Despite low levels of engraftment and differentiation of MSCs into hepatocytes, some beneficial effects could be observed in an acute model of injury. In a rat model of fulminant hepatic failure induced by D-galactosamine, infusion of conditioned medium of MSCs decreased levels of apoptosis and enhanced liver regeneration [82]. The observed effects might be attributed to a decrease in systemic inflammatory cytokine since decreased levels of IL-6 and TNF-α were measured. Further, several genes known to be upregulated during hepatocyte replication were increased suggesting a

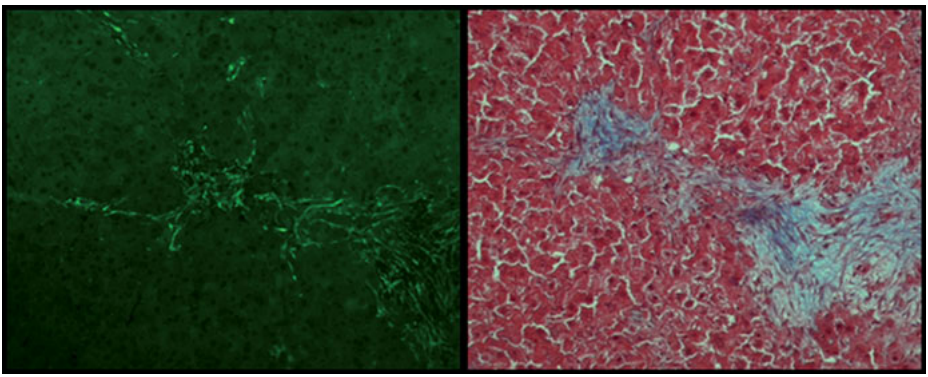

Fig. 16.1: Liver section showing engraftment of human bone marrow-derived MSCs into regenerating mouse liver. Left panel: Specific identification of human MSCs by immunofluorescence staining against α-SMA (green). Right panel: Consecutive liver section colored against collagen (blue).

direct effect on hepatocytes. However, specific mediators contributing to this anti-apoptotic and regenerating effect have still to be identified. In summary, engraftment of MSCs and differentiation of MSCs into hepatocytes in animal models of hepatic regeneration is limited. To circumvent the difficulties of MSCs to cross endothelial walls and to enter into the liver parenchym, MSCs were directly injected into the liver parenchym. In a mouse model of liver regeneration induced by 2/3 hepatectomy, human MSCs injected into the hepatic parenchyma, 2 days after hepatectomy, expressed α-SMA, displaying a profibrogenic phenotype. α-SMA positive cells merged with collagen deposition and in some animals the collagen fibers organized into broad fibrous *septa* (see Fig. 16.1) [83]. Mechanisms leading to this outcome might be the presence of increased levels of inflammatory cytokines as well as TGF-β during the regenerating process. Effectively, in such a setting, local increased inflammation due to injection may also favor the evolvement of MSCs toward myofibroblasts.

16.6 Conclusion

MSCs adopting a contractile phenotype have been identified in tumor stroma and in injured and fibrotic liver. MSCs engraftment and their potential interrelationship with these tissues has only been characterized to a limited extend. More work needs to be done to uncover the mechanisms involved in the transformation of MSCs to myofibroblasts and to characterize their precise role in the pathogenesis of progressive tumors and liver fibrosis. First ongoing clinical trials using MSCs for the treatment of liver diseases should include well-defined endpoints allowing to evaluate the risk of transplanted MSCs to contribute to liver fibrosis.

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Scott A. Bergfeld and Yves A. DeClerck

17 Mesenchymal stem cells and the tumor microenvironment

Abstract The contribution of bone marrow-derived cells to cancer progression has now been well recognized. However, much of the attention has been on the role of hematopoietic stem cell (HSC)-derived cells and much less focus has been on mesenchymal stem cells (MSCs). In this chapter, we review our current knowledge of the contribution of MSCs and their lineage derivatives to cancer progression. MSCs have multiple functions in cancer progression, some being antitumorigenic and most being protumorigenic. This dual role is now supported by recent suggestions that – like immune cells – MSCs may be "polarized" and educated by tumor cells. Tumor cells and MSCs communicate with each other by various mechanisms that either require cell-cell contact or the secretion of soluble factors or exosomes released by tumor cells and MSCs. MSCs not only play a role in the microenvironment of the bone marrow but are also recruited by tumor cells. When present in tumors they can become tumor-associated fibroblasts or adipocytes and contribute to cancer progression. The ability of MSCs to colonize tumors has led to the investigation of their potential as delivery vectors, a function that is discussed in the next chapter. How MSCs actually contribute to progression in human cancer is a question that has not been fully answered and deserves much attention. Recent reports have demonstrated that MSCs can be identified and isolated from fresh human tumor specimens but the demonstration that they actively contribute to cancer progression in patients is still missing.

17.1 Introduction

The contribution of hematopoietic stem cells (HSCs) to cancer initiation and progression has been the focus of much attention over the last two decades, as the role of bone marrow-derived endothelial progenitor cells and innate and non-innate immune cells in tumor vascularization and inflammation has been well recognized [1, 2]. However, much less attention has been paid to mesenchymal stem cells (MSCs) and to their critical role in the bone marrow niche and in cancer metastasis. There is now clear evidence that MSCs also play an important contributory role in cancer progression not only in the bone marrow microenvironment but also in the microenvironment of the primary tumor where these cells are recruited and home.

The presence of MSCs in cancer tissue was first demonstrated in the bone marrow of patients with multiple myeloma, where these cells were shown to promote the growth of tumor cells through direct contact and through the expression of chemokines and growth factors like interleukin-1β (IL-1β), IL-6, granulocyte macrophage colony stimulating factor (GM-CSF), G-CSF, stem cell factor (SCF) and tumor necrosis

factor-α (TNF-α) [3]. Our laboratory was among the first research groups to point to the growth-promoting activity of these cells on metastatic neuroblastoma cells, in part by their ability to secrete IL-6 when in the presence of neuroblastoma cells [4]. Similar roles have since been reported in ovarian cancer, melanoma, and malignant B cells [5–8]. It was later suggested in animal models that these cells could promote metastasis by their ability to produce large amounts of CCL5 (Rantes), a chemokine that stimulates tumor cell motility and metastasis [9]. It is thus now evident that MSCs are not innocent bystanders in malignant tissues. They can stimulate or inhibit tumor cell proliferation, promote survival and drug resistance, be a niche for tumor-initiating cells, modulate the immune system and contribute to angiogenesis. In the early 2010's *in vivo* animal studies suggested that MSCs are not only resident in the bone marrow but are actively recruited by tumor tissues through the production of a large variety of chemoattractive molecules, cytokines and growth factors. When homed to a tumor, MSCs have the capability to differentiate into tumor-associated fibroblasts (TAF) or other mesenchymal tumor-associated cells including adipocytes, myofibroblasts or osteoblasts [10]. MSCs recruited by tumors are not only phenotypically but also functionally different from normal MSCs not associated with tumors. It thus raises the fundamental questions of how these cells are educated by tumor cells, of what mechanism orients their behavior toward an anti- or a protumorigenic activity, and of how tumor cells and MSCs communicate with each other and with other stromal cells in the TME. Recent evidence suggests that MSCs can not only capture exosomes released from tumor cells but also produce exosomes that are taken by tumor cells. The important question – whether MSCs can be a therapeutic target in cancer therapy – has not yet been fully answered. This question has two aspects. On one side, because of their high tropism for tumor cells and their high ability to be genetically engineered, MSCs have been considered as ideal vehicles to deliver antitumor proteins and antitumor agents into primary tumors. On the other side, the dual function of MSCs as anti- and protumorigenic cells suggests that it may not always be beneficial to prevent their recruitment by tumors and block their activity, and calls for caution in using these cells as therapeutic vehicles in cancer. The use of MSCs as vehicles for tumor delivery is not further elaborated in this chapter as it is discussed in the next chapter.

In this chapter, after reviewing the tumor microenvironment, we discuss some of the critical aspects of the role and the function of MSCs in cancer initiation and progression. Our discussion is focused on some of the most critical and unanswered questions: How do we define MSCs? Are MSCs polarized like T cells and macrophages in tumors? How are MSCs educated by tumor cells? How do tumor cells and MSCs communicate with each other? Are MSCs actively recruited by tumors? What evidence do we have that these cells play an active role in human cancer?

17.2 The tumor microenvironment and its role in cancer initiation and progression

It is now well recognized that tumor cells do not act alone but in close concert with normal cells present in the tumor tissue [2]. The environment of a tumor, known as the tumor microenvironment (TME), has 3 major components. A first component consists of extracellular matrix (ECM) proteins and proteoglycans that are present in variable amounts and variable combinations in tumor tissue. Under physiological conditions these molecules play a fundamental role in the organization and function of specific organs. The central dogma was that ECM proteins present a barrier against invasion [11] and thus were primarily antitumorigenic. It is now well appreciated that they can have a protumorigenic function. For example, the presence of a collagen-rich ECM (known as desmoplastic reaction) in several subtypes of cancers of the breast and pancreas plays an important role in promoting not only the growth of cancer cells but also cancer initiation [12, 13]. Stiffness of the ECM is also a factor promoting growth [14]. The second component of the TME consists of a variety of secreted soluble factors and microvesicles (including exosomes) that either remain in solution or are trapped in the ECM. These molecules and vesicles act as ligands and delivery shuttles mediating the communication between tumor cells and stromal cells in a two-way direction. The third component of the TME is made of normal cells that either migrate from neighboring tissues (resident cells) or are recruited to tumors from the bone marrow (bone marrow-derived cells). Fibroblasts, myofibroblasts, pericytes, adipocytes, macrophages and endothelial cells typically come from adjacent tissues. Hematopoietic cells such as polymorphonuclear neutrophils, mast cells, eosinophils, monocytes, T and B lymphocytes, dendritic cells and NKT cells are typical examples of bone marrow-derived cells. However it is now apparent that nonhematopoietic cells like endothelial cells or fibroblasts can also be recruited from stem cells derived from the bone marrow, including angioblasts and MSCs which are the focus of this book.

It is important to emphasize that the function and role of the TME in cancer are dynamic and subject to significant change over time. The TME can play a neutral role or be a friend or adversary of tumor cells, and this role can shift as the cancer progresses. The currently accepted dogma is that in the early stages of cancer progression, the dominant function of the TME is to limit the growth and invasion of malignantly transformed cells and to initiate an immune response aimed at eliminating transformed cells. For example, the basement membrane plays a critical role not only in limiting the invasion of cancer cells but also in promoting a nonmalignant phenotype [15] and providing a barrier against local invasion [16]. Cells of the innate and adaptive immune system like macrophages, Tregs, NKT cells and cytotoxic T cells act to suppress tumor cells, and produce soluble factors like interferon, IL-1 and IL-2 that have an immunostimulatory function. In contrast, as cancer cells overcome or escape the immune system and other barriers of the TME, the TME undergoes significant phenotypic changes that convert its function from tumor suppressive toward tumor

promoting. Macrophages become polarized and secrete inflammatory cytokines that promote the proliferation, survival and metastatic behavior of cancer cells [17]. TAF produce proteins that are immunosuppressive [18], bone marrow-derived cells are recruited and contribute to vasculogenesis and to the production of cytokines and chemokines such as IL-6, IL-8, IL-10, transforming growth factor (TGF-β), and vascular endothelial growth factor (VEGF) that promote, rather than inhibit, the proliferation and survival of tumor cells. This simplified view of the role of the TME in cancer progression needs to be taken with some caution, however, as the reality is more complex than initially assumed [19]. For example, there is now preliminary but convincing evidence that early changes in the microenvironment of a tissue can promote or even precede the development of malignant cells. Early changes in the stroma of normal breast tissue characterized by a downregulation of the CD36 protein have been shown to promote the malignant transformation of mammary epithelial cells [13], the recruitment of VEGFR1 positive bone marrow-derived cells and the deposition of a fibronectin-rich ECM, and to precede the homing of metastatic cells [20]. Thus there is evidence that even at early stages of cancer initiation or metastasis, the TME can have a protumorigenic function.

17.3 How do we define MSCs in cancer?

MSCs express a number of surface markers that provide targets for identification by *ex vivo* staining (Tab. 17.1). As these markers are shared by other types of immune cells, a panel of staining targets is typically required to pinpoint MSCs within human or mouse tissues [21]. An exception to this rule is the antigen STRO-1, which can be used to reliably identify multipotent stromal cells in human bone marrow [22]. It is important to note that while these markers can identify native MSCs, such as those present in bone marrow, their expression pattern will change when MSCs are recruited to peripheral tissue and differentiate into mature stromal elements.

The fate of MSCs within tumor tissues can vary depending on the tumor type. MSCs exposed to conditioned media from glioblastoma multiforme mainly undergo pericytic differentiation and promote vascular stability [23]. Angiogenic factors produced by tumors can stimulate expression of endothelial markers in MSCs, though this differentiation effect occurs only rarely *in vitro* and not at all *in vivo* [24, 25]. MSCs may also contribute to a population of multipotent stromal cells which undergo osteogenic differentiation in prostate cancer, leading to calcification of the tumor endothelium [26]. Additionally, changes in the tumor microenvironment experienced by MSCs can have dramatic effects on their differentiation potential within the same tumor type. MSCs isolated from primary mammary carcinoma show adipocytic differentiation while those isolated from metastatic lesions in the lungs show osteogenic differentiation [27]. Aside from pericytes and, infrequently, osteoblasts and adipocytes, the most common phenotype generated by tumor-engrafted MSCs is the fibroblast. These

Table 17.1: MSCs markers. Most accepted markers characteristic of MSCs in mouse and human.

Mouse	Human
CD44 (Hyaluronic acid cell adhesion molecule)	CD29 (Integrin B1)
CD73 (5'-Nucleotidase)	CD44 (Hyaluronic acid cell adhesion molecule)
CD90 (Thy-1)	CD73 (5'-Nucleotidase)
CD105 (Endoglin)	CD90 (Thy-1)
CD106 (Vascular cell adhesion molecule)	CD105 (Endoglin)
Sca-1 (Stem cell antigen-1)	CD106 (Vascular cell adhesion molecule)
Nestin	CD117 (c-Kit)
	CD146 (Melanoma cell adhesion molecule)
	CD166 (Activated leukocyte cell adhesion molecule)
	CD271 (Low-affinity nerve growth factor receptor)
	STRO-1 (Stromal cell antigen-1)

cells, dubbed "carcinoma-associated fibroblasts" (CAF) or TAF, are primarily localized to the tumor stroma and display a number of protumorigenic functions [28, 29]. Within the tumor microenvironment, TAF are defined by their production of growth factors such as hepatocyte growth factor (HGF), epithelial growth factor (EGF), IL-6, or ECM remodeling proteins like tenascin-c, thrombospondin-1 or stromelysin-1. They also produce activated fibroblast marker expression such as fibroblast specific protein (FSP) or fibroblast activated protein (FAP), and myofibroblastic differentiation marked by α-smooth muscle actin [30]. Both FSP1, a calmodulin family member, and FAP, an integral membrane gelatinase, are upregulated in the reactive stroma of tumors and fibrotic tissues. While the majority of TAF are derived from local, tissue-resident fibroblasts, bone marrow-derived MSCs have been found to generate at least 20 % of this cell population [31].

17.4 What are the roles of MSCs in cancer progression?

MSCs have multiple roles and functions in cancer progression that are pro- as well as antitumorigenic (Tab. 17.2). It is important to point out that most of the studies that have examined the function of MSCs in cancer were done either *ex vivo* on MSCs that were harvested from the bone marrow of nontumor bearing mice or from the bone marrow of normal individuals, or *in vivo* in immunodeficient mice, conditions that were substantially different from the original environment in which these cells were present. These studies may also not always have addressed the dynamic nature of the interaction between tumor cells and stromal cells during various steps of tumor progression. Reports showing conflicting results and opposite functions thus need to be examined within such context.

Table 17.2: Pro and anti-tumorigenic functions of MSC.

Pro-tumorigenic		Anti-tumorigenic	
CAF differentiation		Contact dependent growth inhibition	– Akt inhibition – MAPK inhibition – Cell Fusion
Angiogenic Stimulation	– VEGF, bFGF, TGFb, IL-8, IL-6, angiopoeitin	Vascular disruption	– ROS generation – Caspase-3 cleavage
Immunosuppression	– IL-6, IL-10, TGF-b, PGE2, iNOS, IDO, HGF	Drug Sensitization	– ERK1/2 inhibition
Contact independent growth stimulation	– IL-6, CCL5, TGFb, EGF, BMP2, SDF-1, CXCL7 – ADAM12 – Estrogen Receptor – ERK1/2 – Exosomes	Pro-apoptotic stimulation	– PARP-1 cleavage – Caspase-3 cleavage – p21 upregulation – Caspase-3,8 upregulation – Bax upregulation – Bcl-2 downregulation – Microvesicles – Cell fusion
Contact dependent growth stimulation	– Cell fusion – Cancer stem cell renewal		
Anti-apoptotic stimulation	– STAT3 – MAPK – PI3K/Akt – antioxidant enzymes – tumor autophagy – Cell fusion – Survivin		
Vascular stability	– Endothelial/pericyte differentiation		
Invasive/Metastatic stimulation	– CCL5, NRG1, IL-6, VEGF, TGFb, SDF-1 – CXCR2, CXCR3 – EMT activation – Estrogen receptor – ADAM10 – MMP1,3,13		
Drug resistance	– IL-6, PGE2, GDF15 – STAT3 – Fatty acids		

17.4.1 Effect of MSCs on tumor cell proliferation

Many reports point to a positive contribution of MSCs to the proliferation of a variety of tumor cells. Carcinoma-associated MSCs (CA-MSCs) present in ovarian cancer tumors, have a normal morphologic appearance and a normal karyotype, are nontumorigenic, and have the capability to differentiate into adipose tissue, cartilage and bone. They have an expression profile distinct from that of MSCs from healthy individuals, including increased expression of bone morphogenic protein BMP-2, -4, and -6. When combined with tumor cells *in vivo*, CA-MSCs promote tumor growth more effectively than control MSCs. This effect can be mimicked by BMP2 treatment *in vitro*, while inhibiting BMP signaling *in vitro* and *in vivo* partly abrogates MSCs-promoted tumor growth [32]. In breast cancer cells, MSCs increase the efficiency of primary mammosphere formation in normal and malignant breast cells and decrease E-cadherin expression, a biologic event associated with breast cancer progression [33]. Much of the proliferative effect of MSCs seems to be related to their ability to promote the growth of tumor initiating (stem) cells, which is consistent with MSCs playing a role in the "tumor niche". In osteosarcoma, the subcutaneous co-implantation of rat osteosarcoma with rat MSCs isolated from the bone marrow results in a higher incidence of tumor formation and tumor growth rate than when osteosarcoma cells are injected alone [34]. However there are almost as many reports that MSCs also inhibits the proliferation of tumor cells [35]. For example, MSCs inhibit the growth of human glioma cell lines and patient-derived primary glioma cells *in vitro*. Co-administration of MSCs and glioma cells *in vivo* reduces tumor volume and vascular density, an effect that is not observed when glioma cells are injected with immortalized normal human astrocytes [36].

One potential explanation for this discrepancy is that like macrophages, MSCs can become polarized toward two different phenotypes. It has been reported that MSCs express two types of toll-like receptors (TLR), TLR3 and TLR4 [37]. TLR3 activation inhibits MSCs differentiation into bone and fat and stimulates the secretion of protumorigenic and immunosuppressive cytokines, whereas TLR4 activation in MSCs stimulates the production of other antitumorigenic inflammatory cytokines. Using a nomenclature reminiscent of the one used in the polarization of the immune system, these authors suggest that TLR3-mediated activation promotes the development of MSC2 cells whereas TLR4-mediated activation orients the cells toward an MSC1 antitumor behavior. Further characterization of these cells, and the demonstration that they can be identified *in vivo*, are still forthcoming.

17.4.2 MSCs promote survival

There is a limited amount of published data examining the effect of MSCs on tumor cell survival. These data consistently support the concept that MSCs promote the sur-

vival of cancer cells by protecting them from spontaneous and stress-induced apoptosis. Our laboratory provided evidence that MSCs protect neuroblastoma cells from drug-induced apoptosis and that this effect is in part mediated by IL-6, which is overexpressed by MSCs when in the presence of tumor cells [4, 38]. Downstream of IL-6 is the STAT3-dependent expression of many survival proteins like Bcl-XL, Bcl2, Mcl1 and survivin [39]. Another recent study points to reactive oxygen species (ROS) as an additional pathway by which MSCs provide a protective environment for tumor cells by affecting their metabolism. MSCs-derived staniocalcin-1 (STC-1) promotes the survival of lung cancer cells by uncoupling oxidative phosphorylation, reducing intracellular ROS, and shifting their metabolism towards a more glycolytic metabolic profile consistent with the Warburg effect [40].

17.4.3 MSCs are proangiogenic

The concept that MSCs could promote angiogenesis came from early non-cancer related studies showing that local implantation of MSCs induces a neovascular response in injured and ischemic tissues that promotes their revascularization and repair [41]. MSCs treatment post-stroke promotes angiogenesis and vascular stabilization, which is at least partially mediated by VEGF/FLK1 and Ang1/Tie2 [42]. MSCs contribute to angiogenesis by a number of different mechanisms. They are a source of several angiogenic factors such as VEGF, platelet-derived growth factor (PDGF) and placental growth factor (PlGF), induced by bone morphogenic protein-2 (BMP-2) [25,43,44]. These growth factors are known to recruit endothelial progenitor cells and stimulate their growth and differentiation into mature endothelial cells (EC). MSCs also contribute to pericytes along the tumor vasculature. Grafted MSCs integrate into tumor vessel walls and express pericyte markers, α-smooth muscle actin, neuron-glia 2, and PDGF receptor-β (PDGFR-β) but not endothelial cell markers, indicating that these cells act as pericytes within tumors [45]. However, the effect of MSCs on angiogenesis is not always one that stimulates vascularization. Experiments have shown that injection of MSCs into tumors like B16 melanoma reduces rather than increases vessel density. Other studies have shown that when co-injected in matrigel at high numbers with EC, MSCs intercalate between EC, establish Cx43-based intercellular gap junctional communication with EC, and increase the production of ROS that leads to EC apoptosis and capillary degeneration [46].

17.4.4 MSCs have an immunosuppressive function

Among the first evidence that MSCs have an immunosuppressive function was the observation that, in mixed lymphocyte cultures, murine MSCs inhibit the proliferation of allogeneic lymphocytes mediated by CD8+ regulatory cells [47]. These authors

observed that MSCs expressing human BMP-2 are not rejected when implanted in allogeneic immunocompetent mice and, importantly, that the subcutaneous injection of B16 melanoma cells leads to tumor growth in allogeneic recipients only when MSCs are co-injected. MSCs exhibit extensive anti-proliferative properties against lymphocytes under different conditions [48] and stimulate the early formation of tumors in mouse adenocarcinoma cells (Renca) implanted in syngeneic mice [49]. The mechanisms behind the immunosuppressive effect of MSCs are complex. MSCs express TLR which enhance their immunosuppressive phenotype. Immunosuppression mediated by TLR is dependent on the production of kynurenines by the tryptophan-degrading enzyme indoleamine-2,3-dioxygenase-1 (IDO-1). Induction of IDO-1 by TLR involves an autocrine IFN-β signaling loop, which is dependent on protein kinase R (PKR) but independent of IFN-γ [50]. MSCs are also a source of several soluble immunosuppressive factors, such as IL-10, TGF-β and prostaglandin E2 (PGE2). Expression of these factors is in part mediated by galectin-1 and galectin-3 which are constitutively expressed and secreted by human bone marrow MSCs [51]. Inhibition of galectin-1 and galectin-3 gene expression with small interfering RNAs abrogates the suppressive effect of MSCs on allogeneic T cells [52].

Another immunosuppressive mechanism is through fibroblast activation protein-α (FAP-α), which is expressed by MSCs-derived TAF. FAP-α attenuates the cellular response to cytokines like interferon and TNF-α. Depletion of FAP-α in fibroblasts in mice results in the formation of hypoxic necrosis in tumor cells and stromal cells that is caused by IFN-γ and TNFα [18,53].

17.4.5 MSCs promote epithelial to mesenchymal transition

MSCs promote the acquisition by malignant epithelial cells of a motile mesenchymal phenotype (epithelial to mesenchymal transition or EMT) that promotes invasion and metastasis. The mechanisms involved are complex. In direct co-culture with breast cancer cells, MSCs increase the expression of several oncogenes such as *ncoA4* and *fos*, proto-oncogenes such as *fyn* and *jun*, and genes involved in invasion (*mmp-11*), angiogenesis (*vegf),* and survival (*ifgr1, bcl-2*) while simultaneously downregulating genes associated with proliferation (*Ki67*, *mybl2*), as well as reducing the pool of ATP. Co-culture of MSCs with tumor cells is also associated with the expression by tumor cells of N-cadherin, vimentin, twist and snail, and with the downregulation of E-cadherin, all markers of EMT [54]. Other studies have shown that tumor cells produce IL-1β which upregulates PGE2 production by MSCs. PGE2 in turn acts in an autocrine manner to upregulate the expression of several cytokines by MSCs and in a paracrine manner to activate, in concert with cytokines, β-catenin in MSCs and to promote EMT [55].

MSCs differentiate into osteoblasts, adipocytes and fibroblasts that interact with tumor cells. It is important to emphasize that being multipotent, MSCs are not static cells but can differentiate into a large variety of stromal cells such as osteoblasts,

chondroblasts, adipocytes, muscle cells and even neural cells [4]. Their function in the tumor microenvironment needs thus to be considered within this context. The contribution of these MSCs-derived cells to cancer progression has been investigated over the last 10 years. The function of osteoblasts in forming the HSC niche has been well recognized [56]. This niche is in fact competed for by tumor cells. For example, human prostate cancer cells directly compete with HSC for occupancy of the mouse HSC niche and increasing the niche size with agents like parathyroid hormone promotes metastasis. In contrast, decreasing the niche size compromises metastatic dissemination. Disseminated PCa cells can be mobilized out of the niche and be returned to the circulation by HSC mobilization protocols. Once in the niche, tumor cells reduce HSC numbers by driving their terminal differentiation. Thus the osteoblastic niche which maintains the pool of HSCs can also serve as a metastatic niche, promoting the homing of circulating tumor cells [57]. Osteoblasts also play a critical function in bone metastasis. They are stimulated by parathyroid hormone related peptide (PTHrP) produced by many tumor cells and, as a result, increase their production of the receptor activator of NFkB ligand (RANKL), a strong inducer of osteoclast maturation and activation [58]. Other MSCs-derived cells that have been the focus of recent interest are adipocytes. Animal studies have shown that leukemia cells migrate into adipose tissue within ten days after implantation and that *in vitro*, murine leukemia cells migrate towards adipose tissue explants and 3T3-L1 adipocytes or adipocyte conditioned media. Migration is mediated by stromal-derived factor-1α (SDF-1α) (CXCL12) [59]. In addition, adipose tissue explants protect leukemia cells against the cytotoxic effect of daunorubicin and vincristine. Cancer associated adipocytes (CAA) also undergo a dynamic exchange of metabolites. Specifically, CAA release fatty acids through lipolysis which are then transferred to cancer cells and used for energy production through β-oxidation. The abundant availability of lipids from adipocytes in the tumor microenvironment supports tumor progression and uncontrolled growth [60]. Adipocytes also secrete several hormones like endotrophin, a cleavage product of the COL6α3 chain that augments fibrosis, angiogenesis, and inflammation through recruitment of macrophages and endothelial cells, or leptin that enhances tumor cell proliferation [56, 61]. CAA are also a source of proteases, including MMP-11, and proinflammatory cytokines, such as IL-6 and IL-1β, that play a key role in the acquisition of a proinvasive phenotype by tumor cells [62]. Finally, it should be mentioned that MSCs can also differentiate into TAF that express specific proteins like S100 calcium binding protein A4 (S100A4; FSP-1) and FAP whose function in cancer has been previously discussed. These TAF promote skin tumor development by producing monocyte chemotactic protein-1, which maintains chronic inflammation and has an immunosuppressive effect [18, 63]. Much less is known of the role of myofibroblasts in tumors, although recent evidence suggests that these cells are involved in the production of collagen and the formation of a highly cross-linked collagen matrix that stimulates tumor cell proliferation, migration and chemoresistance [64].

17.5 How do tumor cells communicate with MSCs?

Communication between tumor cells and MSCs has two consequences: it promotes their recruitment (an aspect discussed in the next section) and educates MSCs toward an anti- or protumorigenic behavior (an aspect discussed in this section). In the presence of tumor cells, MSCs produce soluble factors and chemokines in the tumor microenvironment that affect tumor cells and other stromal cells in a paracrine manner but also affect MSCs in an autocrine manner. The communication between tumor cells and MSCs is a reciprocal two-way communication (Fig. 17.1). Tumor cells communicate with MSCs by two major mechanisms, one that is cell-cell contact-dependent, mediated by adhesion molecules, and one that is independent of cell-cell contact and

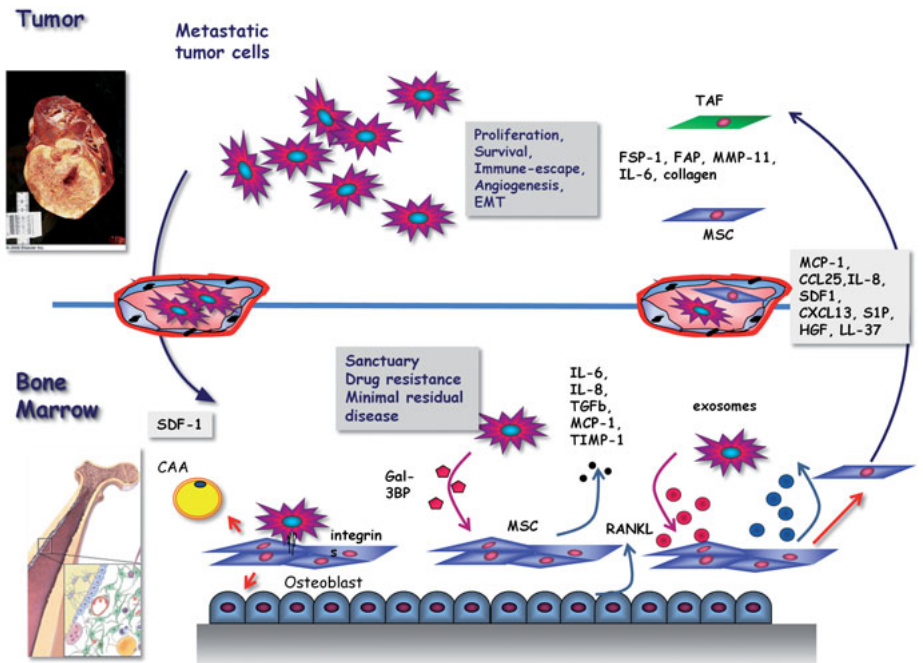

Fig. 17.1: Reciprocal interactions between tumor cells and MSC in the bone marrow and the tumor. Circulating tumor cells are attracted into the bone marrow niche by several cytokines like SDF-1. When in the bone marrow, tumor cells find a sanctuary and interact with MSC *via* integrin-dependent mechanisms, *via* the production of soluble factors like Gal-3BP and *via* exosomes. MSC respond by producing cytokines including IL-6, IL-8, MCP-1, growth factors like TGF-β and VEGF as well as exosomes. MSC are also recruited by primary tumors through the production by tumor cells of factors such as MCP-1, SDF-1, CCL-25, IL-8, CXCL13, S1P, HGF, LL-37 that attract MSC. When in tumors, MSC can become TAF expressing FSP-1, FAP, MMP-11, IL-6 and collagen that affect tumor cell proliferation, survival, immune-escape, angiogenesis, EMT and the formation of a desmoplastic stiff matrix. The bone marrow is thus not only a site of metastasis but also a "remote-controller" of the primary tumor.

is primarily mediated by the production of soluble factors and subcellular vesicles by tumor cells.

Evidence supporting the involvement of a cell-cell adhesion mechanism was first obtained from studies demonstrating that adhesion of normal and malignant B cells, including myeloma cells, to MSCs promotes their survival and proliferation [65, 66]. Adhesion-independent mechanisms also play an important role. Tumor cells produce several soluble factors that activate specific pathways in MSCs. Among those is galectin-3 binding protein (Gal-3BP), a multimeric glycoprotein secreted by neuroblastoma cells and present in the tumor stroma, which interacts with galectin-3 present at the cell surface of MSCs to activate the Ras/Raf/MEK/ERK pathway [51]. Activation for this pathway has several effects. It stimulates the expression of the protumorigenic cytokine IL-6 by MSCs [51,67] and the production of the immunosuppressive factor TGF-β [52,68]. TGF-β and BMP are also directly produced by many tumor cells and are present in the bone marrow microenvironment where they are released upon degradation of the bone matrix by tumor cell-stimulated osteoclasts [69]. BMP promotes the differentiation of MSCs by coordinating an increase of α-smooth muscle actin and a decrease in gelsolin [70]. TNF-α, which is also produced by tumor cells but not by MSCs, serves as an additional pathway for MSCs activation [71]. MSCs also communicate with tumor cells and other normal cells in the TME. MSCs, for example, secrete IL-6, CCL2/MCP-1 and TIMP-1. IL-6 not only promotes tumor cell proliferation [72] and survival [38] but also osteoclast maturation and activation [4]. CCL2 promotes the migration of breast cancer cells [73].

It has been recently suggested that exosomes could be critical communicators between tumor cells and stromal cells including MSCs. Exosomes are extracellular vesicles of 30–100 μM in diameter that are released by cells from endosomes. They contain a large variety of structural and regulatory proteins in addition to coding and noncoding miRNA and DNA [12]. They were initially reported to play a key role in the communication between immune cells and their cellular targets [36]. They are produced by all normal and malignant cells and they have been recently shown to be a mechanism by which tumor cells educate the bone marrow and drive the formation of a premetastatic niche in distant organs by bone marrow derived-cells [74]. Exosomes are produced by MSCs [75, 76] and the release of growth factor-containing exosomes by MSCs is, for example, responsible for the ability of these cells to improve renal function in rodents with kidney failure. The mechanism involves a horizontal transfer of insulin-like growth factor (IGF-1) receptor mRNA from MSCs-derived exosomes to tubular cells which potentiates tubular cell sensitivity to locally produced IGF-1 [77]. In a very recent and elegant study, Roccaro *et al*. demonstrated that MSCs from the bone marrow of myeloma patients release exosomes that are transferred to myeloma cells, thereby resulting in modulation of tumor growth *in vivo*. They found that these exosomes contain miRNAs whose content differ from normal MSCs, with a lower content of the tumor suppressor miR-15a. In addition, myeloma-associated MSCs produce exosomes that have higher levels of oncogenic proteins, cytokines,

and adhesion molecules compared to exosomes from MSCs derived from normal bone marrow. Importantly, whereas MSCs-derived exosomes from patients with myeloma promote myeloma tumor growth, MSCs-derived exosomes from normal bone marrow inhibit myeloma cell proliferation. These studies demonstrate that exosomes represent a unique mechanism of communication between MSCs and cancer cells. Because tumor cells also secrete exosomes, it is likely that they also play an important role in communicating with MSCs and in altering their behavior and educating them. However, at this point, this aspect has not been fully explored.

In summary, the mechanisms by which tumor cells and MSCs interact and communicate are multiple and complex. They involve adhesion-dependent and -independent mechanisms. Whereas the main focus over the last 10 years has been on adhesion molecules and soluble factors, an emerging new mechanism of communication between tumor cells and the microenvironment has been the release of exosomes by tumor cells and stromal cells. These microvesicles are likely to play a critical role not only in the reciprocal communication between tumor cells and MSCs but also in the communication between MSCs and other stromal cells in the microenvironment.

17.6 Are MSCs recruited by tumor cells?

Experiments in mice transplanted with fluorescent protein-labeled bone marrow cells have clearly demonstrated that bone marrow-derived HSC expressing VEGFR1 or VEGFR2 are actively recruited in primary tumors and/or at metastatic sites where they contribute to the formation of a premetastatic niche (VEGFR1 positive cells) or to the tumor vascular system (VEGFR2 positive cells) [78]. However, similar evidence that bone marrow-derived MSCs are actively recruited by tumor cells has been lacking in part because of the absence of clear markers defining MSCs and difficulties in transplanting these cells. Evidence that MSCs can leave the bone marrow and home to distant tissues was initially derived from experiments in injured organs [79]. Tumor cells secrete many chemoattractants that promote the migration of MSCs [29] and MSCs express receptors of all four chemokine subfamilies: CC, CXC, CX(3)C, and C. Dose-dependent migration of MSCs by chemokines like CCL2 /MCP1, CCL25 (TECK), CXCL8 (IL8), CXCL12 /SDF1-α, and CXCL13 (BCA1), has been demonstrated in *in vitro* chemotaxis assay [80,81]. Sphingosine 1 phosphate (S1P), a bioactive lipid that acts via G-protein-coupled receptors, also exerts strong chemoattraction on MSCs through MMP-mediated signaling events and the RhoA/ROCK and MEK1/ERK intracellular pathways [82]. HGF could be another mechanism as MSCs express its receptor c-Met [83]. MSCs migrate towards apoptotic, but not vital or necrotic, neuronal and cardiac cells in injured tissue. HGF, which is expressed by the apoptotic cells only, interacts with c-Met which is expressed by MSCs. Blocking HGF bioactivity resulted in significant reduction of MSCs migration. Because many tumors express HGF, it could be one of the mechanisms attracting MSCs as it has been shown in glioma [36]. Under

hypoxic conditions breast cancer cells secrete high levels of IL-6, which serves to activate and attract MSCs. IL-6 acts in a paracrine fashion on MSCs, stimulating the activation of both STAT3 and MAPK signaling pathways to enhance migratory potential and cell survival [84]. TNF-α is another factor. In myocardial infarct it is capable of potentiating MSCs migration as well as inhibiting MSCs migration as an indirect consequence of OPG induction, which might result in a suboptimal recruitment of circulating MSCs [85]. LL-37 (leucine, leucine-37), the C-terminal peptide of human cationic antimicrobial protein 18 which is present in many tumors, also stimulates the migration of MSCs. LL-37 facilitates ovarian tumor progression through recruitment of MSCs to serve as proangiogenic factor-expressing tumor stromal cells [86].

Irradiated tissues and irradiated tumors also are potent chemoattractants for MSCs. Irradiated 4T1 cells have increased expression of cytokines like MCP-1, TGF-β1, VEGF and PDGF-BB. Interestingly, the chemokine receptor CCR2 is upregulated in MSCs exposed to irradiated tumor cells and inhibition of CCR2 leads to decrease of MSCs migration *in vitro* [87]. Thus not only the production of a chemoattractant by tumor cells but also the expression of its correspondent receptor in MSCs are altered by radiation therapy. Interestingly, there is recent evidence that the production of some of these chemokines is controlled by miRNA. Using microarray and bioinformatics approaches, Lu *et al.* identified six miRNAs with differential expression in damaged liver tissue. They found that miR-27b could directly interact with the 3'UTR of SDF-1α to suppress SDF-1α protein expression compared to normal C57BL/6 murine liver tissue [88].

These studies, however, have been predominantly done *in vitro*. Some evidence that bone marrow-derived MSCs can be recruited by tumors comes from experiments in mice demonstrating that MSCs acquire a TAF phenotype following exposure to or systemic recruitment into adenocarcinoma xenograft models including breast, pancreatic, and ovarian cancer. These cells can be recognized by the expression of FSP1 and FAP, the expression of markers phenotypically associated with aggressiveness, such as tenascin-c, thrombospondin-1, and stromelysin-1, and the production of protumorigenic growth factors including HGF, EGF, and IL-6 [89].

The question whether MSCs from the bone marrow are released and recruited by primary tumors has, however, not been entirely elucidated. Several laboratories, including ours, have shown that MSCs injected in the tail vein of mice can be found in primary tumors. However, the number of these cells is generally small and *in vivo* kinetics studies have shown that viable donor MSCs injected i.v. in mice are present in the lungs up to a maximum of 24 h after infusion, after which they disappear [90].

Direct evidence of MSCs recruitment by human tumors is lacking so far. However there is some indirect evidence. MSCs have been isolated and expanded *in vitro* from several fresh, enzymatically digested tumor tissues. MSCs-like cells from human esophageal carcinoma (hEC- MSCs) and adjacent noncancerous tissues (hECN- MSCs) have been isolated. These cells express several MSCs markers such as CD13, CD29, CD44 and CD105 [39]. Other groups have shown that MSCs can be isolated as CD29+,

HLA+ and CD105+ cells from gastric tumors not only from tumoral tissue but also from adjacent nontumoral tissue [91, 92]. A recent study in 15 patients with non-small cell lung cancer (NSCLC) demonstrated that MSCs can be isolated not only from tumor tissue but also from corresponding normal lung tissue. These MSCs were characterized and selected according to their mesenchymal multilineage differentiation capability. When compared to MSCs from normal tissue, tumor-derived MSCs showed accelerated growth kinetics and reduced sensitivity to cisplatin [93].

The question whether, under pathological conditions of cancer progression, MSCs are actively recruited by tumor cells and are educated into TAF, CAA and other tumor-associated mesenchymal cells has not been entirely resolved. Recent studies suggest that MSCs are recruited, but these preliminary studies await important confirmation and validation in larger numbers of tumors. Whether MSCs also are recruited by normal tissue and contribute to the formation of a premetastatic niche is another interesting question that has not been explored so far.

17.7 Can we target MSCs in human cancer?

This last question remains presently unanswered. Animal experiments indicate, however, that MSCs can be targeted *in vivo* with drugs like imatinib (Gleevec) that blocks PDGFR-mediated signaling. Mice xenografted with KM12 cells and MSCs developed rapidly growing tumors. Treatment of these tumor-bearing mice with imatinib increased survival significantly. Moreover, the ability of MSCs to migrate to tumor stroma was impaired by imatinib and the number of MSCs surviving in the tumor microenvironment was significantly decreased [94]. Blocking TGF-β may be another approach to inhibit MSCs. TGF-β signaling is essential for differentiation of human BM-MSCs to TAF in the TME and their protumorigenic effects. Thus, blocking the TGF-β/Smad pathway may have an anti-MSCs effect in addition to an overall immunostimulatory effect. Small molecule inhibitors of TGF-β are currently in clinical trials [95,96]. Zoledronic acid (ZA), a nitrogen-containing bisphosphonate approved by the FDA for patients with bone metastasis, significantly reduces activation of AKT and ERK in MSCs, along with their production of cytokines like IL-6 and RANTES and their ability to migrate, suggesting that the antitumor effect of ZA may include a direct effect on MSCs [97]. Cox-2 inhibitors have also been shown to inhibit osteogenesis in MSCs, suggesting that under inflammatory conditions they may inhibit the formation of the osteoblastic niche. Accordingly, the osteogenic potential of MSCs is inhibited and delayed by treatment with high-dose nonsteroid anti-inflammatory drugs [98,99].

17.8 Conclusion

There is now significant experimental evidence that MSCs contribute to cancer progression and that most of this contribution is directed toward a tumor promoting effect. As our understanding of the mechanisms of communication between MSCs and tumor cells and the recruitment of MSCs by tumors increases, our ability to identify approaches to target MSCs in cancer will increase. Such knowledge will identify pathways that become activated upon contact between tumor cells and MSCs which could be targeted for therapy. We therefore should not only look at these cells as delivery vectors but also as targets for intervention.

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Astra I. Chang, Jan A. Nolta, and Jian Wu

18 Mesenchymal stem cells as a carrier for tumor-targeting therapeutics

Abstract Current chemotherapy is not tumor-selective and gives rise to severe adverse effects for patients. Mesenchymal stem cells (MSCs) exhibit a unique tumor-homing property and could be used as a drug carrier for targeting tumor therapy. The tumor-homing property of MSCs depends on the hypoxia and inflammatory status in the tumor, and is modulated by factors such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α) or other cytokines released from tumors. MSCs may be isolated from umbilical cord blood or adipose tissues, and are readily engineered for carrying therapeutics, such as oncolytic adenovirus, specific cytotoxic molecules, nucleotides, prodrugs cytokines or antibodies, or to produce therapeutic molecules within a tumor site. The most promising therapeutics include blockers for VEGF, prodrugs (*e.g.* ganciclovir), oncolytic adenovirus, thymidine kinase, and pro-apoptotic "TRAIL". The efficacy of these bio-engineered MSCs has been evaluated in animal models of pulmonary, breast, gastrointestinal, and pancreatic cancer xenografts grown in immune-deficient mice, and their safety has been shown in some early phase human trials, but they have yet not been moved to later phase clinical application. Although these novel approaches are promising, MSCs may have some risks for cancer patients since MSCs are found to be immunosuppressive in tumor sites, are pro-angiogenic, and in some cases may promote tumor growth. Therefore, whether bio-engineered MSCs will be a useful therapeutic vehicle depends on the property of the specially engineered cell population, tumor types and locations, as well as the time and route of administration of MSCs-based therapeutics. This chapter discusses approaches to utilize MSCs' tumor-homing properties for improving current cancer therapy.

18.1 Introduction

Mesenchymal stem cells (MSCs) are a promising cell therapy in a wide variety of tissue injuries and disorders, whether acting directly, as in repair of bone, tendon and cartilage; indirectly as an immune modulator or revascularizing agent; or as a biocarrier for drugs, peptides, proteins, or other gene products. Preclinical studies in neurodegenerative diseases [1], cardiovascular diseases [2, 3], autoimmune diseases [4, 5] and others have allowed for this promise to be quickly brought to fruition as clinical trials testing the utility of MSCs for targeting diverse diseases are currently ongoing. This chapter provides an overview of how MSCs are therapeutically useful in targeting malignant tumors. Various cell intrinsic and environmentally responsive properties make MSCs highly attractive for therapeutic uses, especially in the realm

of cancer. These properties are described in detail in this chapter along with how MSCs could be utilized or manipulated to suppress tumor growth and prevent deadly metastases.

18.2 Enhanced angiogenesis as a target for tumor therapy

A key feature of malignancy is uncontrolled cell division. For a tumor to grow beyond a diameter of 1 to 2 mm, cancer cells need to interact with and gain support from stromal tissue including vascularization. In an early investigation describing the tumor as a wound that does not heal, it is pointed out that the dense firm nature of many solid tumors is due largely to collagenous stroma (Fig. 18.1), which in some cases may account for more than ninety percent of the total tumor mass [6]. Due to the hypoxic environment surrounding the growing mass, tumors often behave like wounds that activate the intrinsic healing response and induce the surrounding stroma to attract inflammatory cells, fibroblasts, and angiogenic cells, similar to some of the events in physiological wound healing. In this way, the tumor also becomes vascularized *via* angiogenesis, which plays an essential role in tumor growth and metastasis [7, 8]. Therefore, it appears that anti-angiogenesis should be an excellent target for cancer therapeutics and indeed has been a major arena for drug development in the past decade. However, there have been some major shortcomings and

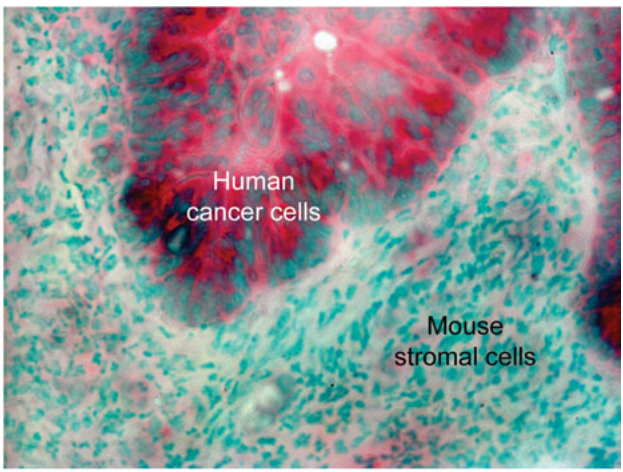

Fig. 18.1: Stromal involvement in a malignant tumor. Shown here is a representative section of xenografted human colon cancer cells (red) that have developed to a large subcutaneous tumor in a mucopolysaccharidosis type VII (MPSVII) mouse model demonstrating that a large amount of non-cancer cells (non-red) may be part of a solid tumor. Histochemical staining was based on the presence (red – human cancer cells) or absence (non-red – mouse stromal cells) of glucoronidase. 200X.

how to deliver effective therapeutics selectively to tumor parenchyma and to avoid affecting normal cells is a long-term effort.

Under physiological conditions, angiogenesis is a tightly controlled process balanced by proangiogenic and angiostatic factors, as well as cell-cell and cell-extracellular matrix interactions. Vascular endothelial growth factor (VEGF)-A was identified as the primary proangiogenic factor during the 1990s [9], with basic fibroblast growth factor as a close second [10]. Oncogenic mutations resulting in the increased Ras expression also lead to the upregulation of VEGF-dependent angiogenesis [11]. VEGF-A is known to increase vascular leakage, and is therefore a vascular permeability factor, playing important roles in the inflammatory process [7, 12]. Rather than well-organized structures, tumor neovasculature is malformed with tortuous and leaky vessels, due to little adhesion between endothelial cells [13, 14]. The subsequent leakage is due to high levels of VEGF-A released by tumor cells and surrounding fibroblasts stimulated by numerous growth factors such as epidermal growth factor (EGF), TGF-α, TGF-β, keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and hypoxia inducible factor-1 (HIF-1) [7]. The leaky tumor vasculature allows fibrinogen molecules in the plasma to come in to contact with cancer cells and form large fibrin strand bundles, which may further develop into an immense collagenous stroma that helps form or reinforce tumor stroma.

In addition, VEGF-A collaborates with cytokines, such as interleukin-4 (IL-4) and IL-10, in the conversion of M2-polarized macrophages into tumor-associated macrophages (TAM), which promote immunosuppression and tissue remodeling to allow for invasion and metastasis [15]. At the same time, many types of cancer cells continuously release high quantities of the mitogen PDGF, which may attract more mesenchymal cells, such as fibroblasts, macrophages, smooth muscle cells and endothelial cells, to the surroundings of the tumor. Some of these key molecules within tumor angiogenesis may prove to be useful in engineering MSCs for anti-angiogenic therapeutics in cancer therapy.

18.3 Why current therapies are not effective enough

Cancer was responsible for one in every eight deaths worldwide in 2011. Moreover, incidences of certain cancer types are increasing. For example, in the United States breast cancer is the most common and second most lethal type in women. In Korea, a similar scenario arose in 2002 and has remained, with breast cancer becoming the most prevalent type of malignancy in women. Certainly there is a great need to better understand the oncogenesis of these cancers and to develop better therapeutics, which is precisely what physicians and scientists hope to achieve with the use of MSCs.

Radiation and chemotherapy remain the major treatment options for patients who are contraindicative for surgical resection. Nonetheless, these therapies increase dis-

comfort and morbidity, and may be ineffective against tumor-initiating/cancer stem cells, yet cause toxicity or killing of normal cells. Tumor-initiating cancer stem cells are now thought to be the culprits of metastasis, which are responsible for the vast majority of oncogenic fatalities [16]. However, it is important to note that there are currently few pharmaceutics on the market to target these cells. Similarly, in most cases the high incidence of mortality in patients with pulmonary malignancies (whether lung cancer or pulmonary metastatic diseases) is due to a lack of ability to deliver targeted therapeutics. On the other hand, a wide range of pharmaceutics therapeutically target tumor angiogenesis, which is an excellent example of how current therapies, although they are the best available, may still be insufficiently effective.

18.3.1 Shortcomings of current anti-angiogenic pharmaceuticals

As described above, antitumor angiogenesis is an intuitive target for cancer therapeutics, and this therapy has been used for various types of malignancies [17]. It is clear that VEGF-A plays a key role in angiogenesis and has been a primary target for anti-angiogenic therapies due to its abnormally high expression in most human malignancies and association with poor prognoses [18]. FDA-regulated Phase IV clinical trials continue even after therapeutics reach the market. Although preclinical studies aiming to inhibit the VEGF-A pathway have demonstrated decreased tumor growth and have moved to clinical application, recent clinical observations are demonstrative of the limited efficacy of these therapies. For example, bevacizumab (commercially known as Avastin®), a recombinant humanized monoclonal antibody specifically targeting VEGF-A, is a current standard of care; but it is among pharmaceuticals that have led to increased morbidity but have not increased overall patient survival. Ranibizumab, also targeting VEGF-A, is a monoclonal antibody. Ramucirumab is a monoclonal antibody against the VEGFR-2 receptor, whose primary ligand is VEGF-A. Aflibercept is an anti-angiogenic agent designed to target both VEGF-A and -B as well as PDGF. Aflibercept has been shown to inhibit VEGF-induced angiogenesis in preclinical laboratory and animal models, and promotes progression-free survival in Phase III clinical trials; but again it does not positively affect overall survival [14]. Moreover, targeting the angiogenic cascade are several tyrosine kinase small-molecule inhibitors, such as ramucirumab, ranibizumab, sunitinib and pazopanib [14]. With such a multitude of various antitumor angiogenic agents and the lack of extension of overall survival, it is unfortunately clear that these agents are not as effective as had been anticipated [19].

It is now suggested that anti-angiogenic agents may actually stimulate or potentiate invasive and metastatic properties [20]. Cancer stem cell enrichment within a tumor is driven by the Wnt signaling pathway activated *via* the Akt/β-catenin pathway, which is stimulated by hypoxia-inducible factor 1α (HIF-1α) during hypoxia caused by anti-VEGF agent treatment (Fig. 18.2). Hypoxia is also a potent inducer of the epi-

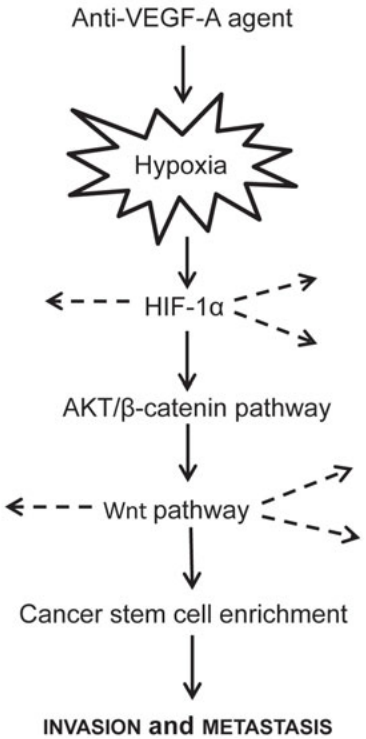

Fig. 18.2: Anti-VEGF-A therapeutics may induce hypoxia. A schematic flow chart illustrates the possible unwanted consequences of anti-VEGF-A which may induce hypoxia within a tumor, ultimately resulting in an enrichment of cancer stem cells that are believed to be more invasively aggressive and metastatic.

thelial-mesenchymal transition (EMT), a sort of transdifferentiation of cancer cells with an epithelial-like character, in which they acquire a motile, more mesenchymal-like phenotype [21]. EMT is thought to be the initiation of metastasis and is initiated by upregulation of metalloproteinase release by tumor cells. Matrix metalloproteinases (MMPs), acting to disrupt the basement membrane, may activate HIF-1α, and promote intravasation [22, 23]. Inhibition of these enzymes has been considered as an anti-metastatic target, and MMP inhibitors could be delivered tumor-specifically with the use of MSCs. Although VEGF-D is physiologically involved in lymphangiogenesis along with VEGF-C, higher levels of VEGF-D expression have been observed with anti-angiogenic therapy; and are now thought to be predictive of resistance to anti-angiogenic agents [24]. The role of VEGF-D in promoting tumor angiogenesis is not currently known, however it appears to be involved in the process especially in the absence of VEGF-A, for example, following the removal of VEGF-A by anti-angiogenic agents [24]. Yet another angiogenic player is placental growth factor (PlGF) [25].

Although PlGF expression may not be augmented in all tumors, several studies now implicate that PlGF is so abundant in the angiogenic switch in neoplastic cells, that it has quickly become a prognostic marker in some cancers [14, 26]. Furthermore, angiopoietin may also be involved in tumor angiogenesis since its receptor, Tie-2, is overexpressed in tumor vasculature, which is also associated with poor prognoses [27] and is one of the targets of early tumor therapeutic engineered MSCs [28].

Thus, one of the primary problems in targeting angiogenesis as an anticancer therapeutic is that there exists tremendous redundancy in the process. With conventional therapeutics patients may find themselves with the arduous task of having to be administered a large multitude of various drugs to target the critical process of angiogenesis in tumor growth, let alone other processes in addition, such as proliferation, cell cycle progression, apoptosis, migration, invasion and metastasis.

18.4 Why mesenchymal stem cells would be useful for tumor targeting

18.4.1 The tumor-homing properties of MSCs

Physiologically, MSCs are thought to contribute to the maintenance of stromal and connective tissues in organs remote from the bone marrow – a function that gives purpose to their highly proliferative attribute. In wounds where tissue damage is being repaired and cell turnover is thus increased, MSCs may be engrafted in and become part of the tissue [20]. This property probably explains Wagner's observations in patients with another type of chronic inflammatory disorder, epidermolysis bullosa. These patients received allogeneic bone marrow transplants and showed similar engraftments of the allogeneic cells in blistering areas of the epidermis [29]. This property also in part explains the strong tropism of MSCs to tumors due to their high resemblance to wounds. The innate ability and actions of MSCs to home to sites of hypoxia and inflammation [30], including tumors, have been extensively investigated by many groups. We transduced MSCs to constitutively express green fluorescent protein (GFP) to track migration to the tumor bed following intravenous (tail-vein) injection in an immune deficient mouse xenograft model of pancreatic cancer. MSCs migrated to metastatic tumors as demonstrated by the localization of these green cells in tumor parenchyma (Fig. 18.3).

The precise mechanisms through which MSCs are recruited to sites of inflammation and hypoxia are not fully understood. Nonetheless, several pathways have been implicated to play roles in the enhanced migratory signaling of MSCs trafficking. Some of the postulated mechanisms are equally responsible for the recruitment process in hypoxic states as well as in the inflammatory process. Tumors exhibit both hypoxia and release many similar cytokines as are released in the inflammatory process in areas of injury/wounds although the complex interplay between MSCs

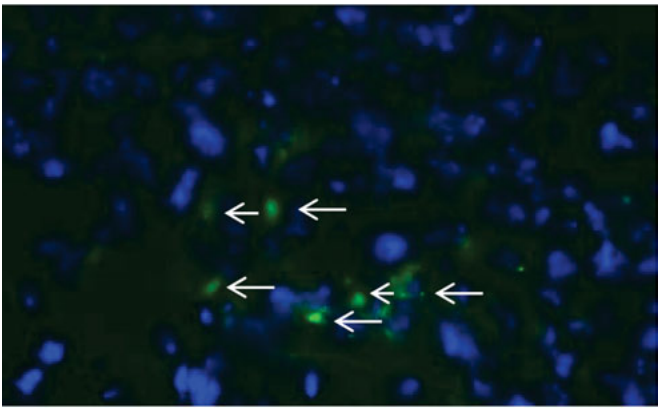

Fig. 18.3: Mesenchymal stem cell (MSCs) home to the tumor bed. A representative section of a xenografted human breast cancer (MDA-MB-231) tumor in a mouse model in which human mesenchymal stem cells (MSCs) expressing GFP (green) were administered by tail-vein injection and migrated intrinsically to the tumor site. The visualization of MSCs in the xenograft tissue indicates the tumor-homing character of these MSCs. 200X.

and tumors through various cytokines is not yet fully understood. The schematic illustration in Figure 18.4 highlights some of the plausible signaling mechanisms leading to the homing of MSCs to the tumor environment. MSCs trafficking towards hypoxic regions is enhanced by chemoattractants such as IL-6, monocyte chemotactic protein-1 (MCP-1), PDGF and VEGF-A (which act synergistically), and insulin-like growth factor-1 (IGF-1), which are released from areas of injury and inflammation, as well as tumor cells. Secretion of IL-6 from cancer cells is especially upregulated by hypoxia, which may occur as the tumor outgrows its vascularity, or as a consequence of anti-angiogenic therapies as discussed earlier. IL-6 is a cytokine, which normally plays a role in the immune response and inflammation, in part as a result of hypoxic conditions, and acts in a paracrine fashion to recruit and activate MSCs. MSCs recruitment and activation is achieved through the upregulation of the STAT3 and MAPK signaling pathways, both of which enhance MSCs migration as well as their survivability. Thus, both the STAT3 and MAPK pathways play critical roles in the ability of the MSCs to adapt to the hypoxic environment [31].

Both TNF-α and IL-1β released from cancer cells activate V-CAM-1 on the surface of MSCs helping slow the migration of MSCs. Once in an area of hypoxia, the hypoxia itself stimulates HIF-1α in MSCs and appears to mitigate the activity of GTPases, for example decreasing the active form of the GTPase RhoA, which further results in a slow-down of MSCs migration once within the hypoxic environment [32]. However, MSCs may not have arrived at their oncogenic location just yet. The hypoxic environment furthermore activates upregulation of membrane type 1 matrix metalloprotease (MT1-MMP) in MSCs. Activated HIF-1α enters the nucleus and binds to its regulatory element on the 3BP2 promoter (P3BP2). MT1-MMP acts in concert with HIF-1α

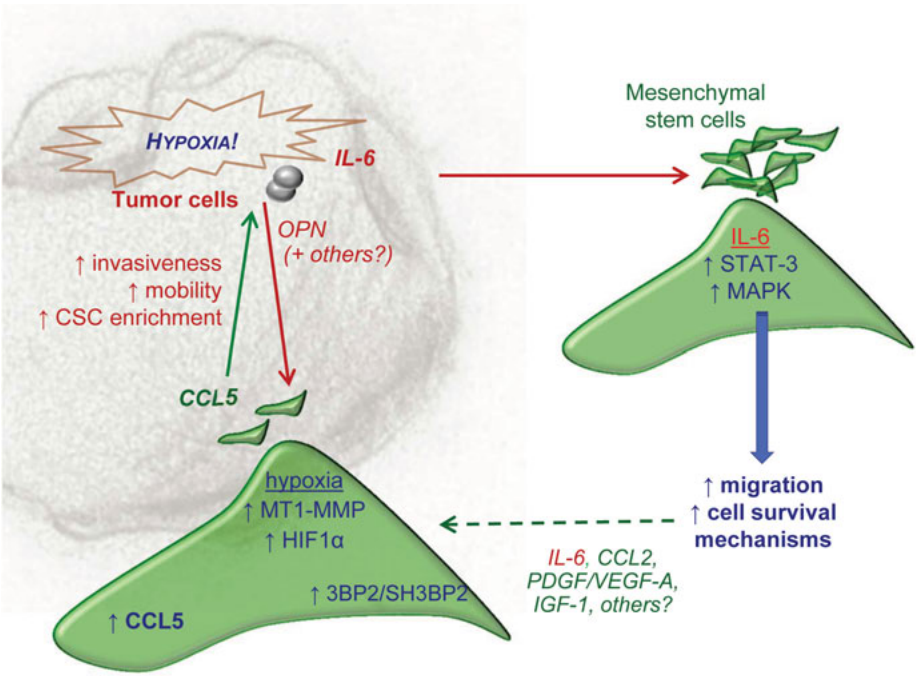

Fig. 18.4: Schematic illustration of the mechanisms leading to the migration of mesenchymal stem cells (MSCs) towards the hypoxic environment within a malignant tumor. Under hypoxic stress, some tumor cells will release IL-6 which will act on distant MSCs, activating the STAT-3 and MAPK signaling pathways which prime the MSCs for migration and increase cell survival mechanisms they will need within the hypoxic tumor environment. The MSCs migrate towards the tumor (green dashed line) following the cues of inflammatory molecules and chemoattractants released from the tumor site, such as IL-6, MCP-1, PDGF, VEGF-A, IGF-1, or others. Cytokines within the hypoxia environment stimulate MSCs to increase their expression of the matrix metalloproteinase (MT1-MMP) and hypoxia inducible factor-1 alpha (HIF-1α). HIF-1α will enter into the nucleus and bind to the P3BP2 promoter to induce genes for enhanced mobility and migration in both MSCs and cancer cells. Together with osteopontin (OPN) and possibly other molecules released from tumor cells, HIF-1α will additionally act within MSCs to increase expression of the chemokine CCL5. CCL5 released by MSCs acts on cancer cells to increase invasive actions, mobility, proliferation and enrichment of cancer stem cells (CSC) and may also act as a gradient that migratory cancer cells follow towards the MSCs and vasculature through which they may metastasize. The signaling molecules incorporated here are by no means comprehensive and much of the precise mechanisms remain to be elucidated. Red – molecules released by or actions stimulated within cancer cells. Green – molecules released by or actions of mesenchymal stem cells. Blue – molecules acting on or gene expression increases or actions stimulated in both mesenchymal stem and cancer cells. Acronyms (approximately clockwise): IL-6 = interleukin 6, STAT-3 = signal transducer and activation of transcription 3, MAPK = mitogen-activated protein kinase, PDGF = platelet-derived growth factor, VEGF-A = vascular endothelial growth factor A, IGF-1 = insulin-like growth factor 1, MT1-MMP = membrane type 1 matrix metalloproteinase, HIF1α = hypoxia inducible factor 1 alpha, OPN = osteopontin.

to promote the upregulation of 3BP2 expression. Although not fully characterized in MSCs, 3BP2, similar to IL-6, is known to play an endogenous role as an immune response adaptor protein that regulates the differentiation of leukocytes and activates their motility. 3BP2 is similarly capable of stimulating MSCs migration, demonstrating a consistent mechanism of 3BP2 action on the role of motility in leukocytes as well as MSCs [33]. It is plausible that MSCs here are switching from an analogous "having taken the water way to the area of their destination" to now "walking the remainder of the way".

Understanding these mechanisms is important to fully take advantage of, while not disturbing, the tumor-homing property of MSCs, in the most efficient manner. These MSCs may be used as vehicles for exosomal delivery of pharmaceuticals, or engineered to express suicide-inducing transgenes of gene products that will halt metastatic communication between tumor-initiating cancer cells and the metastatic niche.

18.4.2 MSCs as a diagnostic tool

One technique with promising clinical utility is currently being developed, which involves MSCs labeled with biocompatible superparamagnetic iron oxide nanoparticles to track the homing of the MSCs to primary tumors as well as to multiple metastatic pulmonary tumors, at very low cell numbers [34]. The nanoparticles generate a local magnetic field perturbation exhibited as a localized hypointensity at a cellular level using magnetic resonance imaging. This application in humans would have great value in detecting possible mini- or micro-metastases that would otherwise be clinically undetectable. Given the high mortality rate due to metastases of tumor cells, MSCs for such diagnostic as well as therapeutic uses are promising for clinical applications. Identified micro-metastases may be operable, however for other types of tumors, there is a clear lack of therapeutics that can directly target them such as pulmonary malignancies (whether lung cancer or pulmonary metastatic diseases) resulting in high incidences of mortalities and poor survival. Therefore, MSCs as a biocarrier to deliver targeted therapies to pulmonary tumors in addition to detection-oriented nanoparticles would be very valuable.

18.4.3 Antitumor effects of unmanipulated MSCs

It has been observed that MSCs homing to the tumor bed may cause growth inhibition and abolishment. This has been demonstrated in a Kaposi's sarcoma murine model in which bone marrow-derived MSCs were administered intravenously, homed to the tumor, and retarded growth [35]. Growth of breast carcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D), and osteosarcoma (MG-63) cells has been inhibited

by extracts from MSCs isolated from Wharton's jelly where all three cancer cell lines exhibited cell shrinkage, apoptotic blebbing and vacuolations, as well as inhibition of migration [36]. In another study, Wharton's Jelly-derived MSCs were shown to also cause regression of mammary carcinomas in a rat model after intratumoral injection. Similarly, nonengineered human umbilical cord-derived MSCs were administered intravenously in a xenografted rat model of human breast carcinoma (MDA-MB-231), and homed to lung metastases where a reduction in tumor burden was subsequently observed [37]. How these MSCs are acting is not understood although it has been postulated that the MSCs isolated from human umbilical cord blood secrete the molecule dickkopf (DKK1), which is a negative regulator of Wnt signaling [38]. The canonical Wnt/β-catenin pathway, which is critical in development, is critical in tumorigenesis, thus secretion of DKK1 results in a suppression of the Wnt pathway, in turn inhibits cancer cell growth. In addition, co-culture of glioma cells with MSCs reduced PDGF release from glioma cells, which may be responsible for the suppression of angiogenesis [39].

All of these antitumor effects by unmanipulated MSCs must be interpreted carefully. The efficacy appears to be strongly dependent on cancer type as well as the source of MSCs. Bone marrow-derived MSCs may have a negative effect on certain sarcomas, but also have the undesirable opposite effects on carcinomas, including participation in the formation of the tumor microenvironment and metastatic niche, promotion of tumor growth and aiding in metastases. However, MSCs isolated from various human umbilical cord tissue or human umbilical cord blood may have suppressive effects on some types of carcinomas, such as breast and ovarian. Further investigations remain to reveal how cancer type-specific these effects are and whether the mechanism of action is mediated directly through cell-cell contact communication, *via* various secreted signaling molecules, or possibly by exosomal communications.

18.4.4 Vesicular communication of MSCs: How MSCs can be used as a drug-delivery vehicle

Exosomes have been identified as vesicular carriers for intercellular communication and are increasingly being found to play vital roles in the information transfer between cells. Exosomes are 40–100nm diameter vesicles (with a density in sucrose of 1.13-1.19g/ml and sedimentation at 100,000g) having a similar topology as a cell and containing a wide array of biologically active molecules. They are formed through the fusion of multivesicular endosomes with the plasma membrane, and released by most cell types [40]. MSCs, sometimes described as ambulatory cells, are of no exception. Time-lapse video recording of MSCs reveals that MSCs are highly active in culture and will crawl right up against neighboring cells as they travel along, appearing to probe them. This includes cancer cells, as demonstrated *in vitro* in Figure 18.5. In most cases cells will leave small exosomal vesicles that MSCs may pick up or MSCs

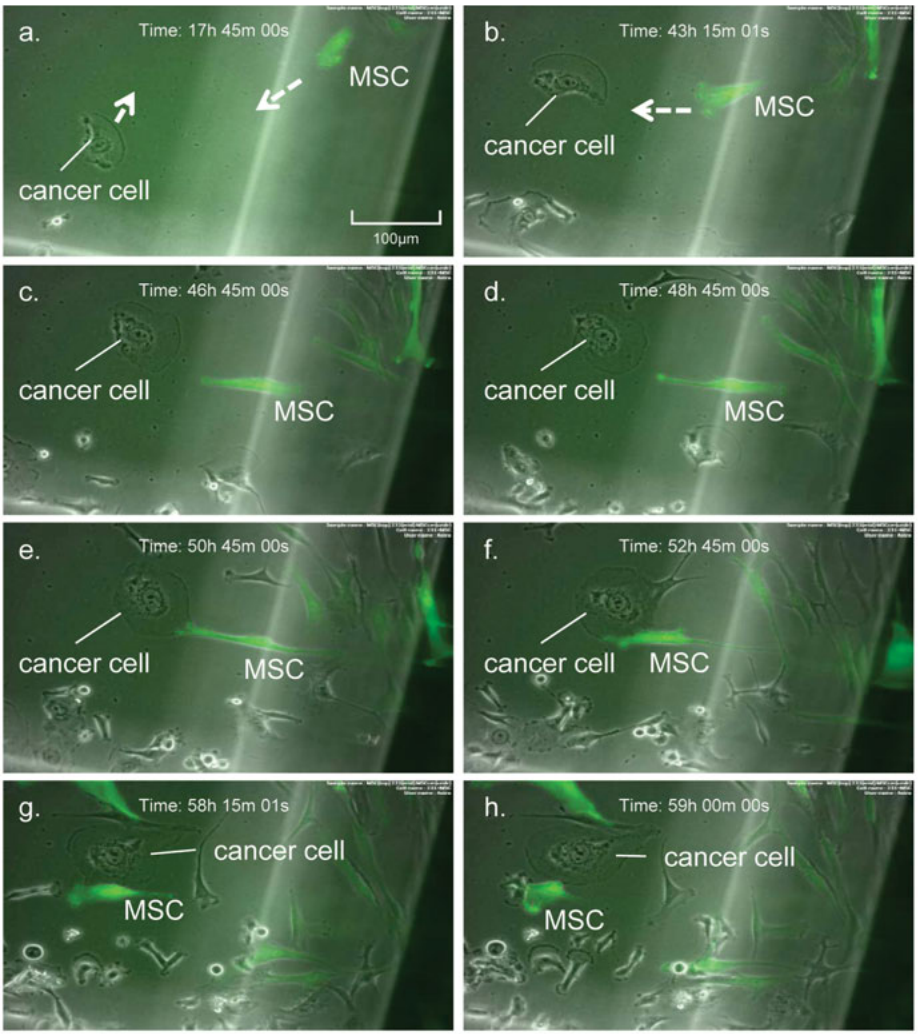

Fig. 18.5: Mesenchymal stem cells (MSCs) migrate towards cancer cells and communicate *via* direct contact. Shown here are still images of a time-lapse video recording of an under-agar assay in which breast cancer cells (MDA-MB-231) attract MSCs (green). As with most cells, MSCs will directly probe the cancer cells possibly sending and picking up signaling exosomes, *etc*.

may deliver their own exosomal packets to other cells. This may be one vital way in which MSCs are able to play their ambulatory role. For example, in the kidney, MSCs will protect against acute tubular injury *via* the horizontal transfer of mRNA to tubular epithelial cells by exosomal delivery, which confers to the tubular epithelial cells apoptotic resistance and functional recovery [41]. Similarly, exosomes secreted

by MSCs have been shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [42].

Isolated exosomes from bone marrow-derived MSCs have also been shown to favor tumor growth and angiogenesis [43]. Certainly unknown factors in the tumor stimulate the release of such exosomes for delivery of needed factors for the continued dominant growth and survival of cancer cells. In a xenograft model of breast cancer, it was recently revealed that cancer-associated fibroblasts (CAF) secrete exosomes which potently stimulated protrusive activity and motility of breast cancer cells (MDA-MB-231) and that this activity is dependent on the exosomal protein CD81 [44]. But what if scientists were able to manipulate this tumor environmental exosomal release such that rather than the pro-survival growth factors, mRNA or other molecules, the exosomes delivered by MSCs instead contained potent anti-survival, anti-invasive, or anti-metastatic molecules, perhaps a silencing of key molecules such as exosomal CD81. Such manipulation of MSCs-delivered exosomes as drug-delivery vehicles is already being explored for therapeutic treatment of other diseases.

Current application of exosomes is hampered by drug loading strategies, which are currently being optimized as our understanding and characterization of exosomes increases. Thus, these exosomes may soon reach their enormous therapeutic potential. The two different strategies being developed involve *in vivo* loading during the intracellular biogenesis of the exosomes, or *in vitro* loading of isolated, purified exosomes [45]. Exosomes are an advantageous alternative to currently used liposomes since, like liposomes they are able to deliver their contents across the cytoplasmic membrane and provide a barrier to premature elimination. But unlike liposomes, exosomes are naturally occurring, less toxic, and better tolerated. They also have intrinsic homing ability conferred by the presence of specific ligands on their surfaces that interact with complimentary receptors on their targeted cell recipients. These membrane ligands are amenable to manipulation *in vitro* as are their contents, thus allowing the loading of therapeutic agents for tissue-specific homing. In the case of cancer, tumor-specific homing is enhanced by the exosomes being delivered by MSCs, which will naturally home to the tumor bed including metastatic ones that may not otherwise be detected. Furthermore, exosomes secreted by MSCs have the added advantage for this use in that they are immunologically inert.

18.5 MSCs as a gene product-delivering vehicle

18.5.1 Genetically modified MSCs for therapeutic delivery

Various different vectors have been used in studies to deliver gene silencing (*e.g.* siRNA) and gene-directed enzyme/prodrug therapies. Most often viral vectors such as adenoviruses [46], adeno-associated virus or lentiviruses have and are being utilized in clinical trials. But these viral vectors have been unsuccessful in transducing

tumors with effective levels of therapeutic genes, due to various reasons including the inability of the vector to penetrate the tumor mass or to reach distant metastasizing cancer cells. By taking advantage of the intrinsic migratory and communicative properties of MSCs to cancer cells this major obstacle of effectively delivering therapeutic genes can be overcome. MSCs are most often isolated from bone marrow, although other sources such as umbilical cord and placenta are quickly becoming viable options, expanded and genetically modified *in vitro*. In fact, the accessibility to genetic modification and expansion capability make MSCs ideal vehicles for tumor-targeted gene therapies, prodrugs, and cytokines or chemokines. For example, rather than patients having to take lengthy broad-acting chemotherapy infusions, in the future they might be administered a single set of engineered MSCs expressing various anti-angiogenic molecules for tumor suppression. Various methods to introduce these genes into MSCs have been successfully used, including viral transduction using adenovirus (especially oncolytic adenovirus, described below), measles virus, retroviruses, lentiviruses, or by OriP/Epstein–Barr virus nuclear antigen (EBNA)-based episomal plasmids, or recently transposon-based gene vectors. Studeny *et al.* performed one of the first applications of MSCs as a delivery vehicle in which they transduced MSCs with adenoviral vectors to introduce expression of interferon-β (IFN-β) [47]. The transduced MSCs were injected intravenously to mice with established melanoma xenografts and resulted in an inhibition of tumor growth as well as prolonged survival. Since this study, several other genes, such as TRAIL or cytokines, have been transfected or transduced into MSCs of different sources to treat a variety of cancer types.

18.5.2 Potential for MSCs-delivered anti-angiogenic therapies

Despite potential inadequacies in targeting angiogenesis as a single process in tumor growth and spread, halting tumor angiogenesis is a critical approach in ceasing cancer progression. As described earlier, tumors achieve angiogenesis in part by acting as a nonhealing wound including the recruitment of inflammatory cells that create a sort of smoldering inflammation that may promote malignancies. Various inflammatory factors play important roles to either promote or inhibit tumor angiogenesis, and many other aspects of tumor growth, cancer progression and metastasis [48]. It therefore follows that engineered MSCs targeting inflammation may be an excellent therapeutic option that can halt several processes at once. For example, proinflammatory interleukin-12 (IL-12) [49] has been demonstrated to have strong antitumor and anti-angiogenic effects [50]. However, systemic administration of IL-12 is also associated with severe toxicity [51]. To solve this problem, MSCs presents an ideal vehicle for delivery of the cytokine to the tumor site. In the study by Ryu *et al.*, glioma-targeting MSCs derived from umbilical cord blood were engineered to secrete a modified form of IL-12 having a higher T-cell helper 1 (Th1) and antitumor immunity

potency. At seven days post-treatment, significantly decreased tumor blood vessels as well as increased apoptotic cells were demonstrated in mice bearing intracranial gliomas xenograft compared to those treated with PBS- or unengineered MSCs [49]. The MSCs-delivered anti-angiogenic therapeutics may be enhanced with additional genes such as semaphorin 3A, under tumor-triggered expressional control, which help relieve the hypoxic pressure that may trigger epithelial-mesenchymal transition (EMT) and other metastatic events [52].

18.5.3 MSCs-mediated tumor-homing of oncolytic adenovirus enhances tumor therapy

Ideally, an oncolytic virus would selectively target malignant cells, infecting them and self-amplify by replicating within cancer cells, ultimately killing them. Oncolytic adenovirus has been shown to be effective in suppressing tumor growth, even completely eradicating colon xenografts after intratumoral injection [53]. Nevertheless, as previously discussed, intratumoral injection would not be suitable for many tumor types and distant or multiple metastatic sites. Furthermore, the distribution of adenoviral infection within the tumor would not be even. Intravenous adenoviral administration could lead to high levels of liver infection and toxicity, and cause a strong immune response to eliminate the virus. Yet another setback is that adenovirus does not have tumor-specific tropism. Despite this, oncolytic viruses continue to be pursued by some companies taking them into clinical trials [54]. Therefore, these oncolytic adenoviruses need a cancer-preferential carrier to reach tumor sites. Clearly, MSCs with their innate tumor-homing feature would be an ideal carrier for recombinant oncolytic adenoviral vector to reach the primary tumor and any distant metastatic sites.

Yong *et al.* employed human bone morrow-derived MSCs labeled with green fluorescent protein and carrying Δ24-RGD (hMSCs-Δ24) into the carotid artery of mice harboring orthotopic U87MG or U251-V121 xenografts. They found that there was an increase in accumulation of MSCs in the xenografts, and these MSCs released adenoviral vector infecting brain tumor cells. The tumor growth was suppressed, and some mice completely eradicated the tumor, and extended their survival from 42.4 days to 75.5 days, as compared to controls. This study proved the efficacy of MSCs-mediated recombinant oncolytic adenoviral delivery to the tumor site with improved eradication and animal survival [55]. A recent study utilized a mesenchymal stromal cell subpopulation (MO-MSCs), which displayed enhanced adhesiveness towards melanoma tumor xenografts. When these cells were loaded with oncolytic adenovirus and systemically administrated into mice harboring melanoma, the MO-MSCs suppressed tumor growth, and overcame the natural resistance of the tumor to the oncolytic adenovirus [56]. While not all studies have proved that MSCs exhibit tumor tropism, there is a definite improvement in the inhibition of tumor growth [57]. For example,

for hepatocellular carcinoma, active recruitment of MSCs into its xenografts has been confirmed by [124I]-PET imaging and immunohistochemistry [58]. Thus, it appears that MSCs tumor tropism depends on tumor type and the recognition of tumor surface markers by MSCs.

18.5.4 Delivery of TRAIL by genetically modified MSCs to induce apoptosis

In addition to being a carrier of oncolytic virus, genetically modified MSCs may work as a local factory producing therapeutic agents adjacent to tumor cells, and exert anti-tumoral effects *via* suppressing anti-angiogenesis, inducing apoptosis or intervening metastasis [59]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo Ligand 2 (ApoL2) is a pro-apoptotic protein that binds to cancer cells expressing death receptors 1 and 2. This protein is being extensively studied for its anticancer properties [53] and has been in clinical trials in late 2012 (www.clinicaltrials.gov). Despite these studies, the rapid clearance of TRAIL remains a challenge. TRAIL in a soluble form has a half-life of approximately 30 minutes [60], which is increased to approximately 15 hours when it is fused with carrier proteins such as human serum albumin. Moreover, the delivery of adenovirus encoding the TRAIL gene by MSCs was less immunogenic, and inhibited the growth of lung cancer xenografts in mice [61]. However, TRAIL may also have toxic effects on normal tissues such as the brain and liver. For these reasons, Reagan *et al.* aimed to introduce site-specific TRAIL expression by MSCs [45]. They designed an implant delivery system, and used a doxycycline-inducible promoter to control the expression of the TRAIL gene in the engineered MSCs [38, 46]. Tet-on and Tet-off systems may modulate gene expression of a cytotoxic protein in a controlled manner, and would be particularly useful in this case [62]. In other studies, the initiation of a particular gene expression by the tumor environment has been used to drive tumor-specific therapeutic expression. The promoter of the gene being turned on by the tumor environment is used to drive the expression of the therapeutic gene.

18.5.5 Tumor-specific promoter-driving thymidine kinase (TK) expression for prodrug conversion

Karnoub *et al.* demonstrated in a xenograft model that breast cancer cells actively recruit MSCs to the tumor environment. Once the MSCs are in relatively close proximity, the cancer cells will induce a potent upregulation of CCL5/RANTES gene expression [63]. CCL5/RANTES is a potent molecule stimulating migration and other metastatic mechanisms (see Fig. 18.6 and Section 18.9.3 for details). Bruns *et al.* took advantage of this property and utilized the CCL5/RANTES gene promoter to drive the expression of the suicide transgene HSV-TK in engineered MSCs with the use of ganciclovir,

Table 18.1: Selected summary of experimental studies of MSCs engineered for anticancer therapies.

Ref.	Source of MSCs	Type of MSCs-mediated therapeutic	Cancer Type	Observed Effects				
				Growth Inhibition	Reduced Tumor Size	Inhibition of Metastasis	Prolonged Survival	Other
Studeny [47]	BM	MSCs-transfected to express IFN-β	Melanoma	Yes	No	Suppression of pulmonary metastasis	Yes	
Loebinger [93]	BM	Tet promoter-driven MSCs-expression of TRAIL	Lung, breast, cervical	Yes	Yes	Clearance of pulmonary metastasis	Not reported	Clearance of micro-metastasis Some complete eradication
You [82]	BM	MSCs-transfected cytosine deaminase plus 5-fluorouracil prodrug treatment	Gastric	Yes	Yes		Not measured	Minimized side effects of 5-fluorouracil
Zischek [64]	BM	CCL5 promoter-driven HSV-TK suicide gene expression (with ganciclovir treatment)	Pancreatic	Yes	Yes	Reduced metastasis to peritoneum, spleen and liver	Not reported	
Conrad [66]	BM	Tie-2 promoter-driven HSV-TK suicide gene expression (with ganciclovir treatment)	Pancreatic, breast	Yes	Yes		Yes	
Reagan [45]	BM	Dox-induced TRAIL-expressing MSCs	Breast	Yes	Yes	Decreased bone, lung and liver metastasis	Not reported	
Zolochevska [94]	Adipose	MSCs-delivered pigment epithelial-derived factor (PEDF)	Prostate	Yes	Yes		Not reported	Prevented tumor establishment
Yong [55]	BM	MSCs-delivered oncolytic adenovirus	Brain	Yes	Yes		Yes	Some complete eradication

in a syngeneic model of pancreatic cancer [64]. Ganciclovir (GCV), a strong antiviral medication commonly used to treat and prevent cytomegalovirus (CMV) infection, is phosphorylated by HSV-TK resulting in an active deoxynucleotide analogue. As cell division occurs and DNA is synthesized, the incorporation of the ganciclovir/HSV-TK generated nucleotide terminates strand synthesis and arrests cell division [65]. Combining the selective migration of the engineered MSCs to tumors with the efficacy of the GCV/HSV-TK "suicide gene" system allows for highly selective tumor targeting using MSCs.

Another HSV-TK/GCV suicide gene therapy method being explored uses Tie-2 gene as a target, which is upregulated in tumor neoangiogenesis and is responsible for stimulating angiopoietin receptor tyrosine kinase activity (angiopoietin-TIE system), important in the tumor angiogenic switch. This system represents an alternative to VEGF-A in targeting tumor angiogenesis with several studies showing promising anticancer activity in early clinical trials. One study involving engineered MSCs demonstrated that in the MSCs differentiating towards tumor endothelial-associated cells, Tie-2 is upregulated, thus activating the HSV-TK/GCV suicide gene system. This resulted in reducing tumor volume in mice without the need for myeloablative therapy [66]. The use of Tie-2 promoter/enhancer elements to drive therapeutic gene expression in MSCs allows for the selective expression of these genes only after the MSCs have homed to the tumor bed and they have been stimulated to suppress tumor angiogenesis, and is therefore a very promising therapeutic use of MSCs for tumor targeting.

Table 18.1 summarizes several examples of MSCs-mediated delivery of therapeutics to xenografts in animal models of various tumor types. Most of the delivery methods used were *via* intratumoral injection, although genetically engineered MSCs have been demonstrated to exhibit tumor-homing property in these studies. However, it should be noted that intravenous administration of MSCs for lung cancer or metastatic sites yielded the first pass deposition of MSCs in pulmonary circulation [59]. Therefore, routes of MSCs administration remain to be carefully investigated in the translation of promising MSCs-mediated delivery of therapeutics for targeting tumor therapy.

18.6 Methods of therapeutic MSCs administration

While direct injection of therapeutically engineered MSCs may appear to be the best method, it is also obviously problematic for tumors located in tissue areas difficult to reach. Other less obvious reasons may also suggest that this may not be an advantageous approach. Over a decade ago, Ram *et al.* attempted to directly inject therapeutically engineered MSCs to the tumor. They injected MSCs expressing murine herpes simplex virus-thymidine kinase (HSV-TK) transgene intratumorally to patients with recurrent malignant brain tumors [67]. Unfortunately, the results were disappointing. Since then knowledge acquired in the field suggests that normal physical processes

associated with MSCs recruitment from the circulation may impart some imprinting through their passage which may be important to their physiology once within the tumor environment [68]. Most studies involving *in vivo* application of MSCs-engineered anticancer therapies now utilize intravenous injections to introduce the MSCs. This carries the advantages of being less invasive and allowing for intrinsic mechanisms of MSCs to guide them into tumor niches that may not necessarily be detectable or identified otherwise. The question remains whether the majority of injected MSCs will reach the tumor sites. In another words, what is the efficiency of MSCs-mediated delivery of drug, gene, virus or siRNA to the tumor surrounding or tumor parenchyma?

A preclinical study explored a delivery approach involving the implantation of silk scaffolding that provides a niche environment within which MSCs may be seeded [45]. Advantages for using silk scaffolding rather than a decellularized matrix is that silk is already used extensively in medical practice, and may be used in various forms with different sizing, mechanical strength, porosity, and may also be modified for degradation time from weeks to years [69]. Three different methods of administration were compared – co-injection of the MSCs expressing TRAIL under doxycycline control with breast cancer cells, tail vein injection of the MSCs, and implantation of the MSCs seeded on the silk scaffold. The study demonstrated that breast cancer cells recruited the engineered MSCs from the implanted silk scaffold to the tumor site [45, 70] and resulted in significant reductions in tumor growth. The study concluded that tail vein injection of the therapeutic MSCs resulted in decreased bone, lung and liver metastases, as did implantation of the therapeutic TRAIL-expressing MSCs on the biocompatible silk implant, with the exception of liver metastasis [45]. It appears therefore that such implants may be a valuable approach translatable for long-term treatment to inhibit tumor growth and help diminish at least some forms of metastasis.

18.7 The advantage of MSCs being immunoprivileged

Mesenchymal stem cells have a unique property of being immunoprivileged. This is an important and highly advantageous characteristic for the utilization of MSCs for allogeneic delivery of therapeutic genes and other molecules. This immunoprivileged nature is due to several different mechanisms acting on various different types of immune cells all in a coordinated fashion. MSCs are resistant to natural killer (NK) cell cytolysis, and inhibit NK cell proliferation and the generation of dendritic cells and macrophages [71]. MSCs inhibit proliferation and induce apoptosis of activated T cells [72], while also altering their migratory properties along with that of dendritic cells [73]. Another important component contributing to the immunoprivileged nature of MSCs is that these cells lack expression of MHC class II, as well as CD40, CD80, and CD86 costimulatory molecules. Importantly, the immunoprivileged nature of MSCs allows for the use and delivery of normal donor (allogeneic) MSCs without immuno-

modulation or subsequent immunosuppressive therapies to a wide patient population, made possible also by the highly proliferative nature of low passage MSCs *in vitro*. Thus, large batches of qualified, therapeutic MSCs may be prepared in good manufacturing practice (GMP) facilities and stored for future use in numerous cohorts of patients. This has already been demonstrated *in vitro*, *in vivo*, as well as in Phase I through Phase III clinical trials for the treatment of autoimmune diseases and in graft-versus-host disease (GvHD) for patients receiving hematopoietic cell transplantations [74]. Non-genetically-modified MSCs have been approved as drugs for GvHD in Canada, New Zealand, and Korea due to strong safety profiles. The use of MSCs as a carrier for antitumor therapeutics is an excellent example of potential personalized medicine that can be expanded to reach a large breadth of patients, and will be very valuable to oncology therapies.

18.8 Sources of acquiring MSCs for tumor therapy

The bone marrow is a primary source of nonhematopoietic and highly proliferative MSCs, holding differentiation ability. Standard isolation of MSCs from the mononuclear fraction of bone marrow aspirates involves the depletion of $CD45^+$ cells and adherence to plastic tissue culture dishes. Fibroblastic cells and macrophages are separated from MSCs in that they will adhere more strongly such that a standard enzymatic lift will leave these strongly adherent cells behind, releasing MSCs. Qualification of the MSCs populations to ensure that no macrophage or hematopoietic cell contamination remains in cultures must be done prior to use. Bone marrow MSCs are probably the most widely characterized and thus most widely used sources of MSCs in part due to their ready availability. However, MSCs can be isolated from adipose tissue, liver, lung, placenta, and even teeth [75]. Adipose tissue as a source of therapeutic MSCs is becoming more popular. Indeed, plastic-adherent adipose-derived stem cells appear not to solicit a T-cell response; and late-passage cells act to inhibit reactions of mixed populations of lymphocytes [76]. The umbilical cord can also be a rich resource for MSCs. MSCs are isolated from umbilical cord tissue that has been washed of any surrounding blood and striped of the umbilical cord veins. What remains is also known as Wharton's jelly and MSCs can readily be isolated from cultured explants. MSCs have also been isolated from amnion and subamniotic tissues [77, 78], as well as perivascular tissues surrounding the large umbilical cord veins.

Despite their origins from various sources, MSCs have a general fusiform shape and are able to actively move around. Their capacity for differentiation to adipose, bone, and cartilage lineages, as well as to pericytes and endothelial-associated cells, is part of the gold standard above the minimum criteria for characterizing MSCs [23]. Minimum criteria may include characterization by cell-surface markers which requires the use of a panel of antigens giving the signature $CD105^+$, $CD73^+$, $CD90^{hi}$, $CD14^-$, $CD34^-$, $CD19^-$, HLA-DR^-, $CD45^-$ [79]. As described in a previous section, differ-

ences in the antitumor actions of nonmanipulated MSCs are observed dependent on the tumor type but also on the source of MSCs. Therefore, further characterization of the differences between MSCs from various sources is essential for data interpretation and consistence.

18.9 Remaining challenges for the use of MSCs to deliver therapeutics

18.9.1 The immunoprivileged nature of MSCs

While the immunoprivileged nature of MSCs is clearly advantageous in the development and application of MSCs as a wide-ranging therapeutic biocarrier, some fear that their immunosuppressive properties may pose to be problematic in that they may further free cancer cells from immune surveillance and attack. In other words, therapeutic allogeneic MSCs, while clearly advantageous in being able to treat large numbers of patients, may also be immunosuppressive. Nonetheless, most aggressive tumors have already undergone immune escape in their early establishment, which allows them to continue to grow and expand. Thus, while important to keep in mind, the immunoprivileged nature of MSCs may turn out to not be a major cause for concern.

18.9.2 Varying responses to MSCs depending on cancer type, injection site, etc.

In the translational application of therapeutically engineered MSCs to various cancer types, caution should be taken as not all cancers may respond in a positive manner. While some researchers report MSCs aiding in tumor growth, for example like other stromal cells which may undergo autophagy to help feed the cancer cells [11], others have documented a reduction in tumor growth by MSCs. In fact, MSCs may participate in a balance of the two, and the discrepancies in published studies may stem from the timing of experimental MSCs administration [80]. A key example is given by gastrointestinal cancers, in which some conflicting results from studies with therapeutic MSCs have been observed. For example, tumor progression was observed in an esophageal cancer when MSCs were subcutaneously injected together with the cancer cells to nude mice, after the MSCs were shown to inhibit proliferation and invasion *in vitro* [81]. Nonetheless, profound tumor growth inhibition in a gastric cancer mouse model was observed when therapeutic MSCs engineered to express the suicide gene cytosine deaminase were administered in combination with the prodrug 5-fluorouracil (5-FU) [82]. Furthermore, Wang *et al.* showed that bone marrow-derived MSCs reduced tumor progression in a *Helicobacter felis*-induced gastric dysplasia model [83]. Taken together, these three studies implicate that (1) *in vitro* results may not always translate

to the *in vivo* model especially in oncogenic studies in which full understanding of the complicated milieu of signaling processes is still being elucidated; (2) nonengineered MSCs may have a beneficial anticancer progression effect depending on the type of cancer; and finally (3) therapeutically engineering the MSCs may have a greater antitumoral effect than unengineered MSCs.

18.9.3 Changes in MSCs induced by cancer cells within the tumor microenvironment

It is now clear that cancer cells stimulate or repress the expression of various genes in MSCs and other cells. For example, the discovery that CCL5, also known as RANTES (regulated upon activation, normal T-cell expressed and secreted) is specifically upregulated and secreted by MSCs in the presence of breast cancer cells was made by Karnoub *et al*, who described a role of CCL5 in the metastasis of breast cancer [63] (Fig. 18.6). The precise mechanism through which cancer cells initially stimulate the secretion of CCL5 from the MSCs is not fully understood. Recently, Mi *et al*. provided

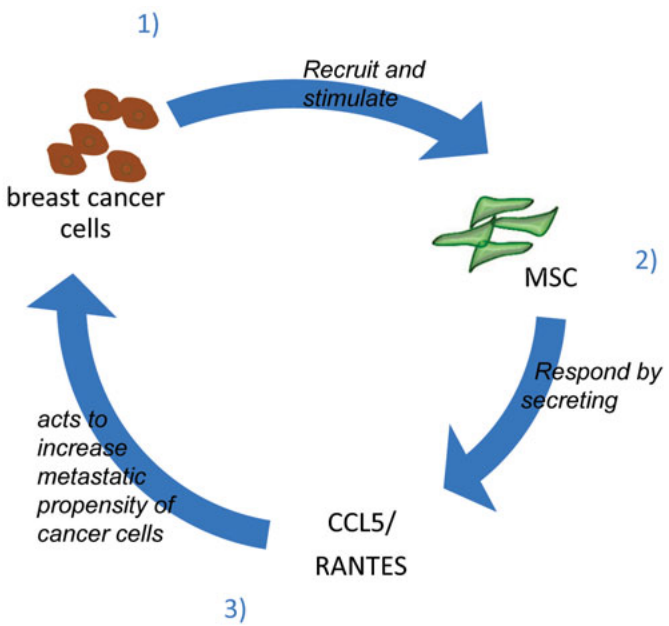

Fig. 18.6: Mesenchymal stem cells (MSCs) are recruited by breast cancer cells (MDA-MB-231). MSCs have been demonstrated to be stimulated by breast cancer cells to dramatically increase the expression and secretion of CCL5/RANTES with the cytokine subsequently acting in a paracrine manner upon the cancer cells to increase migration and other metastatic mechanisms.

initial evidence that tumor-derived osteopontin (OPN) may induce the production and secretion of CCL5 [84]. Osteopontin is highly expressed in tumors cells and, as illustrated earlier in Figures 18.4 and 18.6, may act on MSCs to cause the upregulation of CCL5 expression and secretion from MSCs. The chemokine CCL5 acts back on the cancer cells in a complimentary paracrine fashion as a chemoattractant interacting with receptors to increase mobility toward the MSCs [84, 85].

Increased HIF-1α expression and activation in cancer cells will result in increased expression of the receptor for CCL5, namely CCR5 [86]. However, it is very interesting that secreted CCL5 will additionally interact with CD44 [87, 88]. CCL5 binds with CD44 on the cancer cells and signals to enhance their mobility, invasive properties, and proliferation, resulting in an enrichment of tumor initiating cancer stem cells [85] likely through a CD44-intracytoplasmic domain response element. The CD44 intracytoplasmic domain (CD44-ICD) cleaves apart from the transmembrane protein, translocating itself within the nucleus [89]. Of note, cancer cells do not require a hypoxic environment to activate expression of HIF-1α genes. Here, CD44 is capable of activating HIF-1α responsive genes independent of a hypoxic environment, by binding to novel DNA consensus sequences that constitute a CD44-ICD response element in the promoter region of these genes. The expression of these genes results in an increase in cancer cell motility, increased cell survival, and tendency to undergo differentiation [89].

Thus, in this cross-talk, cancer cells may recruit MSCs *via* IL-6 and perhaps other pathways, stimulate them in part by secreting OPN (and possibly other molecules) thus causing MSCs to secrete CCL5 which may bind to CCR5 on cancer cells and/or CD44 on cancer stem cells. The CD44-ICD is cleaved from the membrane protein, traverses the cytosol to the nucleus binding to response elements in the promoter regions of HIF-1α responsive genes, and turns on or increases the expression of signaling pathways that aid cancer cell mobilization, proliferation, invasion, and ultimately metastasis. For these reasons, scientists believe that suppressing the communication between MSCs and cancer cells could have potential as a therapeutic target (*e.g.* zoledronic acid suppression of bone marrow MSCs decreases breast cancer cell migration) [90].

Importantly, however, the therapeutic potential is enormous if, instead of secreting tumor-promoting ligands when stimulated by cancer cells, MSCs can be engineered to secrete a deadly pharmaceutical molecule or gene product that will induce apoptosis in cancer cells, stop tumor growth, or halt metastatic spread. Through understanding the effects of the tumor environment on MSCs and how MSCs are attracted there, scientists may be able to best engineer "Mesenkillers" in fighting against cancers. Endless targeting possibilities arise if these properties of MSCs to migrate to and to be stimulated by the tumor microenvironment can be taken advantage of and utilized, such as with use of tumor-activated promoters.

18.10 Summary and prospective

Currently, radiation and chemotherapy are the standard of care for many types of cancers that are not suitable for surgical resection. A common side effect of such chemotherapy is suppression of the hematopoietic function of bone morrow along with effects on other systems. An early clinical trial using MSCs helped improve the hematopoietic recovery of patients having undergone chemotherapeutic treatment. Participants were breast cancer patients who had received myeloablative therapy, and the results of the trial demonstrate that MSCs improved hematopoietic recovery (without enhancing relapse) [91]. This was a clear demonstration of the enormous therapeutic potential of MSCs, in addition to genetically engineered MSCs for the delivery of therapeutics. The most promising prospects include MSCs-mediated oncolytic adenovirus for improved selective killing of tumor cells, prodrug delivery to the tumor site, thymidine kinase or TRAIL expression to induce apoptosis in a controllable fashion, generation of silencing molecules (*e.g.* antibody, siRNAs) at tumor sites for direct antiangiogenesis or specific inhibition of molecules critical for tumor growth, progression and metastatic pathways, such as Wnt signaling, EGFR signaling, and so on [92]. MSCs-mediated adenoviral delivery has been shown to not only reduce systemic toxicity of the recombinant adenovirus, but also enhances its cytotoxicity to tumor cells. The ability to track engineered MSCs, for example using biocompatible magnetic nanoparticles, will be a valuable tool to carefully evaluate the tumor-homing property and the longevity of MSCs after intravenous administration. Such tracking would be a highly advantageous and noninvasive modality to verify the therapeutic use of MSCs while confirming the selective delivery of therapeutics to tumor site. Despite this great potential, more research is needed to determine the tumor-suppressing benefits against possible tumor-promoting effects, the extent and the significance of immune suppression after MSCs administration, and the safety profiles of the therapeutics and the MSCs carrier. The translation of this "double-edged sword" yet potentially effective cell therapy approach, aimed at improving the current outcome of cancer treatments, to clinical application will take a reasonable time period. Nonetheless, this appears not too far in the future as the drug regulatory bodies of some countries, including Canada and New Zealand, have already approved the use of MSCs as a biologic therapy.

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Thomas Häupl

19 Systems biology approach to stem cells, tissues and inflammation

Abstract Systems biology is rapidly evolving since high-throughput technologies are providing a quickly growing amount of genome-wide information from various biological conditions. This stimulates the expectation that this comprehensive data collection may enable the inference of the gene regulatory network and the modeling of biological processes in cells, especially with respect to development and regeneration. This chapter gives a brief overview about biological aspects related to this systems biology approach, the technical challenges related to spatial and temporal resolution as well as simultaneous measurements of processes in a network and tries to outline the different qualities of modeling biology in gene regulatory networks. Finally, next steps to improve the current limitations are discussed and a brief example is provided that illustrates capabilities based on the magnitude of publicly available data.

19.1 Introduction

Systems biology aims to characterize and understand the molecular interactions in a biological system. This includes the development of models to describe the dependencies between individual molecular components and to calculate and predict the reaction patterns and changes in response to defined stimulation processes.

In the last century when enzyme kinetics or the process of DNA hybridization were described in mathematical models [1], data and calculation processes were much more limited than today and therefore modeling of molecular interactions was aiming to restrict or reduce to as few parameters as possible. Today "-omics" technologies for many different types of molecule class and exponentially-increasing computing power have generated a new hype for systems biology. Measuring, for example, the complete transcriptome of a defined tissue attracts the vision that knowing all components, which are active in this tissue, should enable to model this system.

Concepts to simulate such interactions are developing especially in network sciences. This field of science dates back to Leonard Eular, who laid an early basis for graph theory with the mathematical description of the problem of the Seven Bridges of Königsberg [2]. Other basic concepts include Boolean and Bayesian networks and strategies of network inference. However, when approaching the problem in its many facets and details, several questions arise with respect to the different mathematical concepts, the multitude of influencing factors, the experimental designs for data collection, the type of molecule classes, the concept how to integrate state-of-the-art literature knowledge and finally the phenomenon of individuality and disease. Although the calculation of reaction kinetics may in principle be possible [3], "there

is a need for methods that can handle large-scale data in a global fashion and that can analyse these large systems at some intermediate level, without going all the way down to the exact biochemical reactions" [4]. This may disillusion our enthusiasm and let us realize that any model based on computer algorithms will be rather fragmentary compared to biological reality.

Considering systems biology in a wider interpretation, it may involve first of all a descriptive approach of systematic and system-wide recording of information from a biological condition. This is currently the most expanding field in biology, generating -omics data for nearly all kinds of molecules and molecular changes. In parallel, technical advancements to collect more and more molecular information from single cell physiology to *in vivo* conditions with increasing spatial and temporal resolution of events and interactions on a multiparameter level enable the design of functional studies with sofar unreached insight into the mechanisms of molecular actions [5, 6]. Furthermore, computational sciences with technologies of parallel processing, networking and with warehousing of -omics data (*e.g.* GEO, ArrayExpress) [7, 8] provide new options for time-demanding iterative algorithms in automated analysis of high-throughput data as well as in screening for relationships between molecules across a large number of experiments. With this rapidly growing repertoire, functional annotations are constantly improved and contribute to the ongoing investigation of concepts that model the dynamics of the biological system.

19.2 Biological aspects

19.2.1 Cells are the regulatory units

Approaching the task from a biological perspective, the core unit of molecular regulatory processes is each single cell. With the full genetic information of the whole individual organism, each cell has in principle the potential to rebuild the complete body. That this can be achieved has been demonstrated not only by nuclear transfer when cloning the sheep Dolly [9] but also by the reprogramming of somatic cells and induction of pluripotency using ectopic expression of the transcription factors Oct4 (POU5F1), Sox2, cMyc and Klf4 [10, 11]. In both cases, the genome itself is not the only determinant but requires the appropriate environment. Even the steps from a single pluripotent cell to the blastocyst, embryo, fetus and finally newborn will not develop without appropriate signals from the environment, especially of the uterus and coordinated interactions between neighboring as well as distant cells of the developing offspring. The biological system constantly depends on and reacts with extracellular factors, which provide nutrition, signals, connections and anchors to other cells or tissue matrix *via* specific receptors. Intracellularly, such interactions induce protein modifications, processes of signal transduction and activation of transcription factors. Transcription of mRNA from genes in the cascade of such regulatory units depends on

the epigenetic constellation and may be influenced by regulatory miRNAs before being translated into proteins. There may be even transfer of RNA along with other molecules from one cell to another as a mode of intercellular communication [12]. Finally, the proteins may depend on modifications like phosphorylation or transportation to a compartment before exerting their function as activator, modulator or inhibitor of a biological process. Of these cascades of molecular interactions or pathways the Kyoto Encyclopedia of Genes and Genomes (KEGG) currently presents 267 for the human system. Other databases to retrieve information about components and products of biological pathways are for example DAVID [13], spike [14] and NCBI Biosystems [15].

19.2.2 Influence of cell number and phenotype

To understand and model the molecular interactions, we need to determine events, which we usually cannot observe with the naked eye and not without manipulation and amplification. Exemplified with transcriptome analysis, we need amplification by *in vitro* transcription and techniques for staining of RNA- or DNA-sequences. Although meanwhile possible even for single cells [16–18], we generally apply transcriptome analysis to many cells of the same type [19] or even to mixed populations of cells like whole blood [20] or tissues [21]. Using multiple cells reduces the skewing effect of *in vitro* transcription, which introduces disproportionate amplification of individual RNAs and thus differences to the native situation in cell biology [22, 23]. On the other hand, extracting and mixing RNA from many cells disrespects the individual conditions of each single cell. This is especially relevant when mixed populations are investigated and also when kinetics of changes is studied, for example during cell differentiation. That the transcriptome is not necessarily identical even for the same type of cells in the same culture system has been reported especially for stem cells, where gene expression oscillations seem to be necessary for stemness and potentiality [24]. In a similar way we may think of cell proliferation, which is not synchronous between the cells of a cultured population. Therefore, expression analysis will reveal average levels across all cell cycle stages for those transcripts that are altered during cell division. These limitations have to be considered when investigating with high-throughput technologies.

19.3 Technological aspects

19.3.1 Technology and type of molecules

The most comprehensive overview is currently provided by transcriptome analysis. Based on the decoded human genome in more than one thousand individuals (1000 genome project) [25] and the sequence-specific microarray hybridization technology,

activity of all known genes can be determined within few days. Although dating back to 2003, the Affymetrix HG-U133 Plus 2.0 GeneChip is the most frequently used technology with now more than 80,000 transcriptomes in the public repository of the NCBI (Gene Expression Omnibus – GEO). The previously published GeneChip HG-U133A (2002) with about 40 % of the HG-U133 Plus 2.0 probe sets has been applied to produce more than 33,000 public transcriptomes. Compared to other platforms provided by Illumina or Agilent, which were also used for a substantial number of public transcriptomes in GEO (≈12,400 and ≈8,600, respectively), the HG-U133 based information provides the largest biological variety that is comparable by technology and applicable for a systems biology approach based on transcript information. There are growing expectations that a next generation sequencing approach will provide more accurate transcript information. In fact, a more detailed picture arises but not necessarily by improving the quantitative assessment of the transcripts but by the identification of new or unexpected findings like somatic copy number mosaicism [26], canonical and noncanonical miRNA and endogenous siRNAs expression [27], alternative splicing, alternative 5'-exon usage, extended 5'UTRs and 3'-UTRs [28], or detection of macro-noncoding (nc)RNAs [29].

Protein and metabolite analysis are constantly expanding and provide important additional information [30] but screening has not yet achieved the genome-wide coverage comparable to transcriptomics. Although array technologies are supplied to screen for epigenetic changes, which are sequence specific and have the potential for genome-wide coverage [31], the knowledge is still very limited and far from a complete map that identifies the relevant CpG sites with regulatory impact on the existing transcriptome information. In the coming years, parallel application of the different -omics technologies will be of central importance to generate an overview of the relatedness between the different molecule classes and to provide a basis for more profound models of interaction [32]. With focus on single factors and their genome-wide interaction with DNA as gene regulators, CHiP-on-chip analysis already provides experimentally confirmed interaction of selected proteins with genes [33] and can serve as an essential experimental link for gene network discovery [34].

19.3.2 When "pictures start moving"

High-throughput screening is usually a snapshot of a biological condition. Beyond that as outlined above, the quantitative measure for each parameter is an average level derived from many cells supposed to be in a similar biological state. Such static data can be compared with a photograph, able to provide many details but lacking the information about the dynamics in the biological system. In fact, biology would cease to exist if dynamics would come to a stop. Energy consumption for maintenance of cellular integrity, stabilization of membrane potential, renewal because of molecular decay and disposal of waste requires constant replenishment of nutrients

and interaction with the environment. Widespread RNA and DNA sequence differences in the human transcriptome, which are also translated into protein sequences corresponding to the abnormal transcript [35], may further prime our awareness that not every logic inferred from an experiment is happening exactly as described. There is growing need to monitor the kinetics of cellular and molecular events to learn to understand how minor deviations can stepwise grow into new qualities of function. *In vitro* stimulation for example of mesenchymal stem cells to induce differentiation into bone [36], cartilage [37, 38] or adipose tissue [39] can provide kinetics of molecular events. Unfortunately, time points of measurement are usually days apart and the exact molecular events can be only assumed by interpolation. Furthermore, the conditions of stimulation *in vitro* even when applying three-dimensional culture conditions are usually far from the real physiologic process. Despite these limitations, there is constant progress towards the shift from photographs to motion pictures. Currently, we are only beginning to envision how this could become possible in the future when studying biology by *in vivo* imaging technology like Xiralite® for monitoring vascularization of inflamed joints [40] or by multiphoton *in vivo* microscopy, for example, to follow cell division or morphological reorganization of cells of the mesenchyme in the dermal papilla to test their requirement for hair growth [5]. Besides this increase in temporal resolution and the new possibility to monitor under physiologic conditions, the high-throughput technology of flow cytometry advances to now provide images in addition to previously only quantitative labels and thereby improves spatial resolution [41–43]. In addition, multiparameter analysis on the single cell level is also advancing with mass cytometry [6] and gives new insight into co-expression of molecules in individual cells as well as identification of transitions from one subtype to another when screening many cells in a heterogeneous population like bone marrow [44].

19.4 Mathematical aspects

19.4.1 Comparative statistics and interpretation

From a mathematical and modeling perspective, high-throughput experiments seem to be most attractive. Transcriptome comparison to controls or between two conditions generates a genome-wide overview of gene activity and enables to focus on the most relevant candidates. Many statistical tools and techniques have been developed or adopted from other research fields to cope with the huge amount of measurements. One of the most popular platforms is Bioconductor [45] with tools developed on the basis of the programming language R [46, 47]. But there are many more with similar or complementary functionality [48–51] as well as developing online platforms for convenient application by scientists with minor or no programming skills [52]. The subsequent questions arising from the discovery of gene lists concentrate on the func-

tion behind these genes. Such information can be either collected (1) from literature knowledge, (2) from existing high-throughput information, or (3) from strategies to model the dependencies between genes.

19.4.2 Interpretation based on pre-existing knowledge

Knowledge accumulating in the literature and organized and curated by experts in the field is developing in multiple facets. Linguistic analysis tools for mining of scientific literature are rapidly expanding this data collation process [53, 54]. Commercial platforms like Ingenuity Pathway Analysis [55] or MetaCore [56] provide methods to compare gene lists with a high content of information from various data collections united in a large data warehouses. Such data are extracted from the literature, pathway databases, genomic maps, motif databases or annotations like GeneOntology (GO). There are also open databases for mining of structured information like DAVID or COREMINE medical. The GeneOntology database contains multiple categories, to which genes are annotated. The VISTA database refers to the relatedness of genes based on common transcription factor binding motifs. The method of gene set enrichment analysis (GSEA) [57, 58] performs comparison with gene sets in the molecular signature database (MSigDB), containing not only genes curated by knowledge of function in defined pathways but also by GeneOntology terms, by common genomic position or regulatory motifs and finally by experimental data from microarray analysis especially in the field of oncology. Similar to all these approaches is the application of statistical methods to compare the gene list of own experiments with different predefined gene sets. These gene sets are associated with different biological functions. Comparing own experimentally defined genes with each gene set reveals a score or association with the gene set-related function. Ranking by score enables to prioritize functional processes in the list of genes identified by an experiment. These bioinformatics techniques are descriptive and compare to pre-existing knowledge about pathways or interactions.

19.4.3 Network models

Systems biology aims to decipher the qualitative and quantitative dependencies between genes and factors to understand and to model the dynamics of the molecular processes. As reviewed by Hecker *et al*. [59], there are several concepts towards the inference of gene regulatory networks.

As for pathways, a graph-based model is frequently applied. Genes are the nodes and information describing or calculating the dependencies between genes are depicted as connecting lines (edges). Undirected graphs can be used for a description of a network without knowing the direction of a molecular process. Such network

models are often applied to illustrate which genes were found to be associated with each other. This association can be retrieved by calculating the distances between genes across a series of data sets. Typical measures, which are also applied for example in hierarchical clustering, are Euclidean distance or Pearson's correlation coefficient [60]. Another estimate is mutual information, which can be seen as a probability measure to predict the expression of one gene based on the known expression of the other [61]. Several other algorithms have been developed like the algorithm for the reverse engineering of accurate cellular networks (ARACNE) [62], TimeDelay-ARACNE to investigate in time series experiments the dependencies between two genes at different time delay [63], maximum relevance/minimum redundancy network (MRNET) [64], context likelihood of relatedness (CLR) [65], relevance networks (RELNET) [66] or a variation of mutual information calculation to obtain directed networks [67]. Compared to the subsequently described dynamical networks, these networks are generated by the level of association.

Dynamical networks attempt to model effects between genes. Activity of one gene (node) has a defined effect on the activity of another gene (node). Boolean networks reduce this interaction to binary conditions. Genes are ON or OFF and turn other genes ON or OFF. Although this reduction seems to represent transcriptional information only insufficiently, development and differentiation are thought to be regulated in steps with molecular decisions and subsequent dynamical changes to new molecular "attractor states". Using the symbolic image of a landscape introduced by Conrad Waddington, the differentiating cell is behaving like a marble that is kicked out from its resting ground on the bottom of the bowl of pluripotency (attractor of stemness and self-renewal) and rolls down along one of the possible valleys of differentiation, from which it cannot escape until it enters into the new bowl of a specifically differentiated stable cell type, the new attractor [24, 68]. Therefore, modeling only with stable attractor conditions (defined cell types, differentiation stages, activation stages) may be achieved much more easily than the attempt to model the complexity of real interactions with many other regulatory components (epigenetics, miRNA, posttranslational modification/activation etc.). The particular advantage of simplicity and robustness of Boolean networks was also pointed out by Debashis Sahoo especially in the context of studying differentiation processes [69]. This may in part be explained by the underlying regulatory changes in epigenetics, which are like ON / OFF switches. In fact, Flöttmann *et al.* successfully applied a probabilistic Boolean network to model the epigenetic dynamics in somatic cell reprogramming [70].

To model in more detail the changes of gene expression as a mathematical function of the quantitative effects of multiple other genes / proteins / factors, differential equations or difference equations with discrete time points are needed. To apply such models, data from high-resolution kinetics are important. Equipped with this type of calculation, the NetGenerator V2.0 has been successfully applied to infer a network for chondrogenesis [71]. However, application of such a model required extensive prefiltering for significantly changed expression and also relied on previous knowledge

from GO annotations and known gene interactions from Pathway Studio. Given these limitations for a rather exact modeling approach, it is obvious that modeling of larger gene networks will need multiple steps.

Another dynamic model for gene regulation is the Bayesian network. The regulatory influence between genes is assumed to be a stochastic event. This strategy also requires a limited number of input genes/proteins. Woolf *et al*. applied the model to infer the factors involved in mouse ES cell self-renewal from proteomic signaling data [72]. The learning algorithm for such networks requires the selection of a model, the fitting of parameters and a rating to identify the model with the best score. Cyclic networks are not allowed in Bayesian networks. The dynamic process has to be resolved by constructing the model with different time points.

19.5 Systems biology of differentiation

The process of cell differentiation to regenerate organs as well as the inverse process of cellular reprogramming to induce directed differentiation into the lineage of choice are most desired capabilities for regenerative medicine. However, these steps are still insufficiently understood and inherit the risk of degenerative failure or even neoplasms. Therefore, the current knowledge about cellular reprogramming is carefully investigated on a gnome-wide scale with a systems biology orientation [73]. The basics required for understanding are the process of self-renewal and the steps involved in induction of differentiation. An important technology in this context is CHiP-on-chip analysis. With this approach, Gokul *et al*. demonstrated that epigenetic factors are the prominent differences in the odontogenic neural crest lineages, which seem to prevent dental follicle cells but not dental pulp cells from differentiation into odontoblast by H3K27me3 mediated repression of the odontoblast lineage genes DSPP and DMP1. Interestingly, further differences were also found in the pluripotency genes OCT4, NANOG and SOX2 being higher expressed in dental pulp cells [74]. Whether such differences are only related to these two phenotypes or whether additional phenotypes of cells are intermingled has to be clarified as outlined in the sections above. The complexity of the network around the pluripotency genes, which was modeled from data of several recent publications on CHiP-on-chip experiments was summarized and reconstructed by MacArthur *et al*. [73]. Their modeling revealed numerous regulatory loops between the factors of their network, which suggest a wide range of context-dependent dynamic behaviors. Additional complexity comes from studies by Luzzani *et al*. [75] investigating a number of genes previously identified by CHiP-on-chip analyses and now tested in human and murine embryonic stem (ES) cells as well as murine induced pluripotent stem cells (iPSCs). High modulation of some of these genes suggested a pivotal role in the undifferentiated state and during differentiation. Thus a deeper knowledge of the presence and role of these genes not only in stem cells but also in the various tissues is needed.

19.6 Important tasks

Although impressive and tempting, constructing a molecular model with detailed calculation of interactions is currently still limited to small networks. Furthermore, the need for a high temporal resolution of kinetics data during differentiation raises additional questions, which were also in the minds of Zoppoli *et al*. when applying TimeDelay-ARACNE [63]. If there is a time delay for the interaction of some of the molecules but not for others and if this delay is even variable, it will be impossible to infer dependencies without prior knowledge. Only detailed experiments can solve this problem.

Another problem may arise from impurities or unforeseen diversity. As long as differentiation steps are investigated with cells *in vitro*, possible deviations induced by different stages of individual cells may cause minor influence, which may be the underlying cause of the complex results in validation experiments with genes previously identified by CHiP-on-chip analysis [75]. However, investigating conditions under inflammation, completely different cell types will "contaminate" the profile with a broad pattern of transcripts specific for the infiltrated cells. In comparative analyses with noninflamed tissue this can add up to 80 % of all differentially expressed genes. If such profiles are modeled, associations will be identified between genes, which are certainly contributing to the inflammatory process but which are probably derived from different cell types.

Therefore, it will be important to extend current knowledge by identifying the gene associations for the majority of the stable "attractor" conditions. This will improve the filtering for those candidates that are relevant for subsequent modeling. Therefore, one of our main efforts is to identify samples which are needed to define attractor conditions. These consist of defined tissues like cartilage, bone, fat and muscle as well as stem cells. For dissecting immunological aspects, various immune cell types from healthy blood donors were separated by flow cytometry and profiled. To investigate potential triggers of inflammation in transcriptomes from chronic inflammatory diseases, defined signatures induced by LPS, TNF, IFNa and IFNg were produced and compared with transcriptomes from patients. This revealed significant differences between rheumatoid arthritis and systemic lupus erythematosus [76, 77].

In order to identify potential inflammatory networks we are currently selecting gene associations by correlation analysis [78]. With profiles from many different cell types and stimulation conditions, this correlation was performed across selected and very different biological conditions. It identifies networks of genes that are highly associated to each other. Figure 19.1a demonstrates this correlation matrix for all genes, which reached a correlation coefficient of $R>0.99$ for correlation with any of the other genes. Figure 19.1b includes the signals of the genes. This mapping indicates that all high correlations are specific for only one of the biological conditions. Human embryonic stem cells for example revealed a small but typical gene association including SOX2, NANOG and POU5F1. Interferon and TNF triggering in mono-

cytes also revealed a typical pattern as well as all major cell types included in this correlation matrix. Interestingly, bone marrow-derived samples as well as cancellous bone exhibited overlapping signatures, possibly related to minor impurities.

19.7 Conclusion

High-throughput technologies have enormously contributed to the growing knowledge in biology. Many different cells and tissues under various conditions have been profiled and data were deposited in public repositories. This treasure for future investigations is constantly explored to decipher molecular networks and pathways. Although the first promising concepts have been developed to model small networks

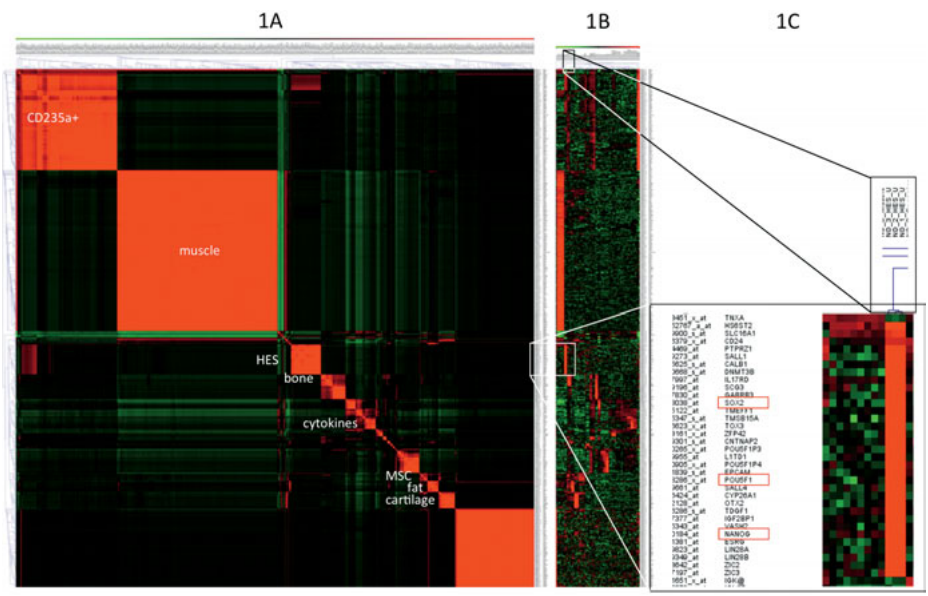

Fig. 19.1: Gene expression was determined by HG-U133 Plus 2.0 GeneChip hybridization of various cell types and tissues: Immune cells from the peripheral blood including granulocytes, monocytes, T-, NK- and B-cells, bone marrow derived CD34+, CD45+, CD11b+ and CD235a+ cells, stimulated monocytes with TNF, LPS, IFNa and IFNg, mesanchymal lineage tissue including cartilage, fat, and cancellous bone, as well as muscle cells and human embryonic stem cells. Pearson's correlation coefficient between all probe sets across these cell and tissue profiles was calculated and all probe sets/genes which revealed at least one correlation coefficient >0.99 were selected. The correlation matrix of these genes based on the selected cells and tissues was calculated and hierarchically clustered (1a). Signals were sorted in the same order of the genes, clustered by experiments and positioned next to the clustered correlation matrix (1b) to match expression data with correlation networks. Highly dominant correlation networks appear, which are related to specific cells and functions. Of interest are especially the genes related to human ES cells (1c).

of the biological system, dynamic modeling remains a challenge. Filtering relevant genes and exploring the dynamics of gene networks in a smaller scale will help to improve the models and to begin applying this knowledge for drug development and treatment strategies.

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